

ANALYTICAL METHODS FOR ELUCIDATING HARMFUL EXPOSURES RELATED TO VAPING

EDITED BY: Ben Blount, Jonathan Thornburg, David L. Ashley,
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ANALYTICAL METHODS FOR ELUCIDATING HARMFUL EXPOSURES RELATED TO VAPING

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Major Constituents of Cannabis Vape Oil Liquid, Vapor and Aerosol in California Vape Oil Cartridge Samples

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During the E-cigarette or Vaping product use Associated Lung Injury (EVALI) outbreak of August 2019 to February 2020, the California Department of Public Health, Food and Drug Laboratory Branch received numerous cannabis vape oil cartridge investigation samples from throughout the state. Many of these products were directly linked to patients; others were collected as part of investigations. We determined the major ingredients and additives in twelve unused cannabis vape oil cartridge samples obtained before ($n = 2$) and during the EVALI outbreak ($n = 10$) in California from September 2018 to December 2019. We tested for major constituents in vape oil liquid, vape oil vapor, and vape oil aerosol phases. A nontargeted Gas Chromatography Mass Spectrometry direct injection screening method was developed for vape oils, a headspace heating module used for vape oil vapors and a solid-phase microextraction (SPME) vaping rig for aerosols generated by vaping. We have identified more than 100 terpenes and natural extracts, 19 cannabinoids, and other potential toxic additives such as Vitamin E Acetate, Polyethylene Glycols, and Medium Chain Triglycerides. We determined more terpenes and minor cannabinoids can be produced via vaporizing and aerosolizing the vape oil. Delta9-THC and potential toxic additives were found at lower levels in the vapor and aerosol than in the vape liquid.

Keywords: vape oil, EVALI (e-cigarette or vaping product use-associated lung injury), aerosol, GC-MS, vapor, nontarget, toxin, delta-9 tetrahydrocannabinol

INTRODUCTION

Medical cannabis became legal in California since 1996 under Proposition 215—the Compassionate Use Act (CUA). In November 2016, 57% of voters passed Proposition 64—the Adult Use of Marijuana Act (AUMA), leading to recreational cannabis sales in California (CDPH Legislation). Since then, cannabis products are expanding into many innovative forms consumed by both medical patients and recreational cannabis users. A variety of cannabis products are available in California including joints, beverages (in different flavors), concentrates/distillate, vape cartridges (in different flavors), topicals, oral supplements, tinctures, capsules, and various infused edibles such as candies/chocolates, mint/chews, dried meat, crackers, dairy product and baked goods. Among these cannabis products, vape oil cartridges are particularly popular as they share the electronic nicotine delivery systems (ENDS). This method of consumption and delivery of tetrahydrocannabinol (THC) is claimed to be safer and more efficient than other products. However, ENDS use is not without short or long-term adverse effects due to additional chemicals generated in the system and the strength of active contents (Rehan et al., 2018; Livingston et al., 2019).

ENDS were first invented by Lik Hon in Hong Kong in 2003 and was entered in Europe and the United States in 2006 (PRLOG, Hon Lik, 2010). It became popular in 2012 after tobacco manufacturers joined the market (Hajek et al., 2014). There are many terms used to describe ENDS such as vapes, vaporizers, vape pens, hookah pens, electronic cigarettes, etc. (USFDA, 2020a). It consists of an atomizer as the heating element, a wick, a battery power source, and a cartridge or tank container. Instead of containing nicotine, cannabis vape cartridges typically contain a mixture of cannabinoids, terpenes, various solvents used as thinning agents, and flavoring additives. By pressing the power button, the vape oil is heated to create an aerosol that the user inhales. Overall, e-liquid aerosol contains fewer types and lower levels of toxicants than smoke from combustible tobacco cigarettes (Hajek et al., 2014; The National Academies of Sciences, E et al., 2018). However, the recent outbreak of EVALI has triggered health concerns in the vaping community.

The EVALI outbreak was first identified in August 2019 and peaked in September 2019 followed by a gradual, but persistent decline (CDC Update, 2019; Heinzerling et al., 2020). As of February 18, 2020, a total of 2,807 hospitalized EVALI cases or deaths have been reported to CDC from all 50 states. National and state data from patient reports show THC containing e-cigarette or vaping products, particularly from informal sources, online dealers, and illicit market are linked to most EVALI cases. Vitamin E acetate has been found in these product samples tested by FDA and state laboratories. It was also found in patient lung fluid samples collected from various states and tested by CDC (Heinzerling et al., 2020; Blount et al., 2020; Duffy et al., 2020). The surge of the EVALI outbreak strongly shows the need of routine investigation of cannabis products on the markets and in-depth research for the safe use of e-cigarettes or vaping products.

Numerous studies have been conducted on e-liquids containing nicotine using propylene glycol (PG) and Vegetable Glycerin (VG) as solvent thinning agents (also called cutting agents for the ease of vaporizing) with added flavonoids (The National Academies of Sciences, E, 2017; The National Academies of Sciences, E et al., 2018; LeBouf et al., 2018; Strongin, 2019). These studies revealed the concerns of Harmful and Potentially Harmful Constituents (HPHCs) formation during heating and aerosolization of the e-liquids. Such studies cannot be directly applied to cannabis products as they have different major constituents such as THC (50–80% concentration) and terpenes. From limited studies on cannabis vape cartridges, ketene as an exceptionally toxic gas may be a potential byproduct in the aerosol of vape cartridges containing Vitamin E acetate (Attfield et al., 2020; Strongin, 2020; Wu and O'Shea, 2020). Poklis and Peace et al. also found synthetic cannabinoids in vape liquids (Peace et al., 2017; Poklis et al., 2019). In addition, residual solvents, pesticides, heavy metals and other toxic chemicals can be concentrated during the cannabis extraction process and remain in the vape oil (Raber et al., 2015; Cannabis.net., 2020). Many vape pens have poor temperature control and the vape cartridge content may be heated to beyond the optimum temperatures, or even to the point of combustion (Wagner et al., 2020). Consequentially, users may inhale cannabis

smoke containing carbon-monoxide, tar, ammonia, heavy metals and other by-products that are harmful to the lungs and respiratory health (King, 2020). Therefore, to expand the understanding and collect more knowledge to ensure product safety for consumers, the National Academies of Sciences research group suggested studies focus on cannabis products containing cannabis, cannabinoids, or THC (The National Academies of Sciences, E, 2017).

In the current study, we investigated twelve cannabis cartridge samples obtained from various dispensaries in California from September 2018 to December 2019. Among these twelve samples, two were prior to the EVALI outbreak and ten were during the EVALI outbreak. We analyzed the composition of the vape oils focusing on volatile and semivolatile chemicals. By using a nontargeted gas chromatography mass spectrometry (GC-MS) screen method, we were able to detect and identify unknown and suspicious compounds in addition to cannabinoids, terpenes and other known major additives. We hypothesized there were different constituents in the cartridges collected before and during the EVALI outbreak. As manufacturers drive to improve profits, they may alter product formulations by using cheaper ingredients in their products, and these new ingredients may pose health risks to consumers. The new ingredients should have a safety assessment by following U.S. Department of Health and Human Services, Food and Drug Administration (USFDA) guidance prior adding them into the products [USFDA, 2020b. Nonclinical, 2020b]. A nonclinical toxicity assessment can also help to address the potential toxicity of chemicals, especially novel chemicals and impurities generated from heating in product delivery systems.

In this study, we also tested vape oil composition in its vapor and aerosol phases using headspace heating and solid-phase microextraction (SPME) GC-MS analyses. We hypothesized that there were some differences among original vape oil liquid, vapor and aerosol. By heating and aerosolizing the vape oil, we simulated the battery powered vape pen conditions used by consumers. This may help to determine the major constituents and their amount in vapor or aerosol that get into user's lungs. To the best of our knowledge, this is the first study that compared the major constituents in unused vape oil to those of its vaporized and aerosolized forms.

METHOD AND MATERIALS

Cannabis Vape Cartridge Samples

In this study, we investigated twelve cannabis vape cartridges obtained through the California state surveillance program from September 2018 to December 2019. Sample details are listed in **Table 1**. Each of the twelve cannabis vape cartridges went through analyses in its oil liquid, vapor, and aerosol phases for the determination of the major constituents.

Nontargeted GC-MS Screen for Cannabis Cartridge Vape Oil

The nontargeted GC-MS screen method uses a full scan mode in MS to tentatively identify known and unknown/nontargeted

TABLE 1 | New and unused vape cartridge samples obtained from September 2018 to December 2019 in California dispensaries.

Sample ID	Weight (g)	Condition	Labeled THC/CBD	Flavor name	Collection Date	Origin
F18CTS035	1.0	Full, high viscosity	N/A	Sour diesel	September 2018	San Diego
F18CTS046	0.5	Full, high viscosity	THC 86.6%	Lemon tree	December 2018	Oakland, CA
F1909018-005	1.1	Full, medium viscosity	THC 92.23%	Purple punch	September 2019	Lake Forest, CA
F1909018-008	1.1	Full, low viscosity	THC 90.99%	Lemon berry	September 2019	Los Angeles, CA
F1910011-001	0.5	Half full, dryness	N/A	Jack herer	October 2019	Rancho Cordova, CA
F1910013-001	0.5	Half full, high viscosity	N/A	Citron OG	October 2019	Rancho Cordova, CA
F1912005-002	1.0	Full, high viscosity	N/A	Blue dream	December 2019	Los Angeles, CA
F1912005-003	0.5	Half full, high viscosity	THC 65.21%, CBD 0.67%	Pure organics sativa	December 2019	Los Angeles, CA
F1912005-004	1.0	Full, high viscosity	N/A	Tangie sativa	December 2019	Los Angeles, CA
F1912012-001	1.0	Full, dryness	THC 84.35%, CBD 0.23%	Purple punch indica	December 2019	Los Angeles, CA
F1912011-001	0.5	Full, low viscosity	N/A	Gorilla glue	December 2019	Los Angeles, CA
F1912013-004	0.5	Half full, high viscosity	THC 80–85%	Topanga canyon OG	December 2019	Los Angeles, CA

chemical substances in a sample based on a match to an established mass spectral library. This method has been used for toxin screen in United States Department of Agriculture Food Safety and was validated in our laboratory for cannabis vape oil samples with modifications (United States Department of Agriculture Food Safety and Inspection Service, 2013). In general, minimum of 0.5 g of cannabis vape oil was taken out from cartridge device by centrifugation to a 15 ml tube. Approximately 10 mg of sample was aliquoted and accurately weighed into a 1.5 ml Eppendorf vial after homogenization by gently stirring with a pipette tip. Aliquoted samples were diluted in methanol by one thousand times (1000 x) and spiked with an internal standard mix (Triphenylphosphate and Phenanthrene- d_{10} , Sigma-Aldrich), before being injected on Agilent GC7890B coupled with MS5977B (Agilent, Santa Clara, CA). Quality control samples containing toxin standards (Nicotine, Parathion, Codeine, and Strychnine, from Sigma-Aldrich and AccuStandard) were included in each sample batch.

An injection of 1 μ l of each sample was injected on the GC injection port using splitless mode. Chromatographic separation was achieved in a 30 min run time using a DB-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m, Agilent) with 1 ml/min helium flow. The oven temperature program was set at 60°C at 1 min, followed by a 12°C/min ramp to 320°C and hold for 7.3 min. The transfer line temperature was set at 280°C, the ion source temperature at 250°C, and EI ionization energy at 70 eV. Mass spectral data was acquired in the scan mode from 25 to 550 m/z at a speed of 2.8 scan/s. Tentative compound identifications are based on a comparison of electron impact mass spectra with the Wiley11/NIST 2017 Mass Spectral libraries and Cayman Spectral library. The match criteria from the compound mass spectra to the database must have a fit of greater than 90% match ratio and visually verified by the analyst. All major cannabinoids identified by the library match were confirmed using the cannabinoid standards purchased from Cerillant and Cayman Chemical. Nineteen major terpenes available in cannabis terpene mix and additives such as VEA, MCTs, PEGs identified in samples were confirmed with the standards purchased from Sigma-Aldrich, and Emerald Scientific.

Headspace GC-MS Screen for Cannabis Vape Oil Vapor

The headspace GC-MS method uses a headspace PAL3 autosampler with a heating agitator to simulate the heating effects of vaping on vape oils and introduces the sample vapor to a GC-MS (Agilent 7890B/5977B). According to Chen et al., the heating temperatures were influenced by power settings, coil wetness conditions (the fullness or amount of vape oil in the cartridge), and nicotine e-liquid compositions (Chen et al., 2018). Propylene Glycol (PG) is one of the major solvents in e-liquids (Prochaska, 2019). Under the test conditions using a PG e-liquid, coil temperatures ranged from 322 to 1008°C for dry cartridge conditions, 145–334°C for wet-through-wick conditions, and 110–185°C for full-wet conditions (Chen et al., 2018). Based on the fullness of tested vape cartridges in the current study and user's practical consumption scenario, the wet-through-wick conditions are the most common. In addition, most terpenes and cannabinoids boiling points are from 150–200°C and around 200°C has the most desirable medical effects for users (Post, 2020). Therefore, the temperature of 200°C was set on the heating agitator to generate cannabis oil vapor.

Approximately 10 mg of sample was accurately weighed into a 20 ml headspace vial after homogenization by gently stirring with a pipette tip. Aliquoted samples were spiked with an internal standard mix (Triphenylphosphate and Phenanthrene- d_{10} , Sigma-Aldrich). Quality control samples were included in each sample batch run. Each sample was heated at 200°C in the agitator block for 15 min and 1 ml of vapor was injected into the GC-MS for analysis using the same GC-MS screen method for cannabis vape oil.

SPME GC-MS Screen for Cannabis Vape Oil Aerosol

SPME is an innovative, solvent-free sample preparation technology that uses a coated fiber to extract volatiles and non-volatiles from different sample matrices. During the process, the SPME fiber concentrates the analytes from the sample to the fiber. By injecting the SPME fiber directly into the GC port, the analytes on the fiber are thermally desorbed in the GC injector and then rapidly flushed to the GC column

(Sigma, 2020). This technique was used to collect the analytes in vape oil aerosols.

To determine the chemical compounds inhaled by the consumers when vaping cannabis oils using battery powered vape pens, a common vape pen was purchased (Brilliant) which has three different voltage settings (3.7, 3.9 and 4.2 V) and the 3.9 V was used for all the samples. Nicotine cartridge samples were used as the quality control samples throughout the SPME GC-MS analysis.

An e-liquid aerosol trap system was set up according to Peace et al. (2016) and Peace et al. (2018), with modifications. In brief, two Erlenmeyer flasks were connected in tandem to a vacuum with an air flow rate of 1457 ml/min. Deionized water was added to each trap flask and a gas dispersion tube bubbles the aerosol into the water. Glass wool was placed in between the two traps to contain the aerosol in the first trap. A 100 μ m polydimethylsiloxane (PDME) coated SPME fiber injector (fused silica fiber core in red hub, Supelco) was inserted through a septum in the first trap to absorb the aerosol cloud. The fiber inside the injector was exposed into the trap while the vape cartridge is activated by the battery power and the aerosol fills the trap. The SPME fiber was held in the trap for about 2 min while the vape pen is activated for 5 times (5 puffs). The fiber was retracted after the aerosol clouds disperse from the last puff. It was then manually inserted into the injection port with a 15 min thermal desorption time on a GC-MS (Agilent 7890B/5977B) and analyzed using the same GC-MS screen method for cannabis vape oil.

RESULTS AND DISCUSSION

Nontargeted GC-MS Screen for Cannabis Cartridge Vape Oil

Twelve vape oil liquid samples were tested for constituents. Terpenes, terpenoids, flavor and fragrance agents, cannabinoids, and many other additives were detected (Tables 2 and 3). We observed that the flavor names and cannabinoids listed on their respective package did not necessarily correspond with the terpene types and cannabinoids found in samples. For example, product type Blue Dream is high in myrcene, known for its relaxing and sedative effects and Sour Diesel is high in both myrcene and limonene, a combination known for its energizing and stress-relieving effects (Erickson, 2019), but we did not find the stated terpene types in those samples. Therefore, the package descriptions may merely serve as a marketing tool to attract consumers who seek for those added benefits.

Vape oil liquid samples consisted of small amounts of terpenes from below 1–7% based on the peak areas of the total compounds found in one sample (Figure 1). The most common terpenes and natural extracts found were Caryophyllene (12 samples), Alpha-Bisabolol (11 samples), Linalool (10 samples), Alpha-Humulene (9 samples), Caryophyllene oxide (8 samples), D-Limonene (8 samples), Phytol (8 samples), Fenchol (6 samples), Nerolidol (6 samples), Selina-3,7(11)-diene (6 samples), Squalene (6 samples), Vitamin E (6 samples), Beta-Myrcene (5 samples), and Gamma-Selinene (5 samples). Among these commonly found terpenes,

Caryophyllene, D-Limonene, Alpha-Humulene were found at higher percentage compared to other terpenes. These major terpenes we found are consistent with the ones described in USP from cannabis plants (Sarma et al., 2020). In general, typical Cannabis plants can have as many as 140 different terpenes (containing carbon and hydrogen) and terpenoids (containing carbon, hydrogen and oxygen) including monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), and triterpenoids (C30) (Brenneisen and ElSohly, 2007). In this study we found around 60 various terpenes, terpenoids, flavor and fragrance agents in the twelve tested samples. Although most of these 60 terpenes maybe natural substances carried over through extraction process from cannabis plants, it's possible that some of the terpenes, especially some flavor and fragrance agents such as Valencene, Menthone, Benzyl Alcohol, D-Carvone, and Triacetin were purposely added into the extracted vape oil to enhance the flavor. Adding flavors into e-liquids for nicotine vaping is a common practice despite the possible health implications (Erythropel et al., 2019) and it is an increasing trend that various terpenes, flavor and fragrance agents are being added into cannabis vape products (Erickson, 2019).

The most common cannabinoids found in this study were Delta9-Tetrahydrocannabinol (Delta9-THC) (12 samples), Cannabinol (CBN) (12 samples), Cannabicitran (CBT) (12 samples), Cannabigerol (CBG) (11 samples), Tetrahydrocannabivarin (THCV) (11 samples), Cannabichromene (CBC) (10 samples), (6aR,9R)-delta10-THC (7 samples), and Cannabifuran (5 samples). Delta9-THC was the most dominant cannabinoid in most of the samples with average around 50–60% peak areas of the total compounds found in one sample and with a maximum of 73% peak area (Figure 1). CBN as an oxidation and degradation byproduct of the Delta9-THC was consistently found in all twelve samples ranging from 1 to 20% peak areas of the total compounds. This reveals that the quality of tested vape products has great variations depending on the age of the product, original packing and storage conditions. The amount of CBC, CBG, THCV, CBT and Delta10-THCs also varied from sample to sample.

In two samples (F18CTS035 and F18CTS046) obtained in September and December of 2018, respectively (one year before the EVALI outbreak), the major content was Delta9-THC with greater than 70% peak areas of the total compounds found. CBN as the second major constituent had similar levels of around 5–6% peak areas in both samples and this indicates that vape products may have longer than 12 months of shelf life, especially stored at lower temperatures. Major terpenes and other additives were at much lower levels than cannabinoids with around 7% peak area in one sample and 4% peak area in the second sample (Figure 1).

In the ten samples collected in 2019 during the EVALI outbreak period, Delta9-THC varied significantly ranging from 4.5 to 70% based on the peak areas of the total compounds found in one sample. Five of the samples contained approximately 40–50% of Delta9-THC peak areas in those samples. In the samples with lower levels of cannabinoids, three samples contained more than 30% Vitamin E Acetate (VEA) peak areas of the total compounds with small amount of Vitamin E; two samples contained more than 25% of Medium Chain

TABLE 2 | Terpenes detected in vape liquid, vapor and aerosol samples.

Compound names	CAS#	Formula	MW	Functions	Liquid ^a	Vapor ^a	Aerosol ^a
Caryophyllene	000087-44-5	C15H24	204.4	Terpene	12	12	10
Alpha-Bisabolol	000515-69-5	C15H26O	222.4	Terpene	11	11	8
Linalool	000078-70-6	C10H18O	154.3	Terpene	10	10	9
Alpha-Humulene/Humulene	006753-98-6	C15H24	204.4	Terpene/Flavor	9	12	10
D-limonene	005989-27-5	C10H16	136.2	Terpene/Flavor	8	9	10
Phytol	000150-86-7	C20H40O	296.5	Terpene	8	9	x
Caryophyllene oxide	001139-30-6	C15H24O	220.4	Terpene	8	7	6
Eudesma-3,7(11)-diene/Selina-3,7(11)-diene	006813-21-4	C15H24	204.4	Terpene	6	11	10
Fenchol	001632-73-1	C10H18O	154.3	Terpene	6	7	9
Nerolidol/(+)-Nerolidol	007212-44-4/000142-50-7	C15H26O	222.4	Terpene/Flavor/Fragrance	6	7	3
Squalene	000111-02-4	C30H50	410.7	Natural extract	6	4	1
Gamma-Selinene	000515-17-3	C15H24	204.4	Terpene	5	7	5
Beta-Myrcene	000123-35-3	C10H16	136.2	Terpene	5	5	8
Terpinolene	000586-62-9	C10H16	136.2	Terpene/Flavor/Fragrance	4	6	10
Alpha-Terpineol/(+)-Alpha-Terpineol	000098-55-5/007785-53-7	C10H18O	154.3	Terpene/Flavor/Fragrance	4	2	1
2(10)-Pinene/Beta-Pinene	000127-91-3	C10H16	136.2	Terpene	3	8	9
Alpha-Selinene/(+)-Alpha-Selinene	000473-13-2	C15H24	204.4	Terpene	3	5	5
Alpha-Eudesmol	000473-16-5	C15H26O	222.4	Terpene	3	4	3
Guaiol	000489-86-1	C15H26O	222.4	Terpene	3	4	3
Valencene	004630-07-3	C15H24	204.4	Flavor/Fragrance	3	4	3
Beta-Maaliene	000489-29-2	C15H24	204.4	Terpene	3	2	2
Copaene	003856-25-5	C15H24	204.4	Terpene	2	7	8
Borneol/Endo-borneol	000507-70-0	C10H18O	154.3	Terpene	2	5	3
Neophytadiene	000504-96-1	C20H38	278.5	Terpene	2	5	1
Bulnesol	022451-73-6	C15H26O	222.4	Terpene	2	4	3
m-Camphorene	020016-73-3	C20H32	272.5	Terpene	2	3	x
Delta-Guaiene/Alpha-Bulnesene	003691-11-0	C15H24	204.4	Terpene	2	2	5
Gamma-Eudesmol	001209-71-8	C15H26O	222.4	Terpene	2	2	x
Junipercamphor	000473-04-1	C15H26O	222.4	Terpene	2	2	1
Alloaromadendrene	025246-27-9	C15H24	204.4	Terpene	2	1	2
Beta-Cadinene/(-)-beta-Cadinene	003858-53-5/523-47-7	C15H24	204.4	Terpene/Flavor/Fragrance	2	1	x
Isoledene	095910-36-4	C15H24	204.4	Terpene	2	x	1
2-Pinene	000080-56-8	C10H16	136.2	Terpene	1	9	9
3-Carene/Delta-3-carene	013466-78-9	C10H16	136.2	Terpene/Flavor/Fragrance	1	3	4
Isoborneol	000124-76-5	C10H18O	154.3	Terpene	1	3	3
(E)-Beta-Farnesene	018794-84-8	C15H24	204.4	Flavor/Fragrance	1	2	2
Aromandendrene	000489-39-4	C15H24	204.4	Terpene	1	2	2
Butylated hydroxytoluene	000128-37-0	C15H24O	220.4	Precervative	1	2	2
Citronellol	000106-22-9	C10H20O	156.3	Flavor/Fragrance	1	2	1
Eudesma-4(14),11-diene	058893-88-2/17066-67-0	C15H24	204.4	Terpene	1	2	3
Farnesol	004602-84-0	C15H26O	222.4	Terpene/Flavor/Fragrance/ Cosmetic	1	2	x
p-Camphorene	020016-72-2/000532-87-6	C20H32	272.5	Terpene	1	2	x
l-Menthone	000089-80-5	C10H18O	154.3	Flavor/Fragrance	1	1	1
(E,Z)-Alloocimene	007216-56-0	C10H16	136.2	Flavor/Fragrance	1	1	3
Alpha-Cubebene/(-)-Alpha-Cubebene	017699-14-8	C15H24	204.4	Terpene	1	1	3
Alpha-Gurjunene	000489-40-7	C15H24	204.4	Fragrance	1	1	3
Benzyl alcohol	000100-51-6	C7H8O	108.1	Flavor enhancer/ Precervative	1	1	x
Beta-Panasiene	997220-91-1/56684-97-0	C15H24	204.4	Terpene	1	1	1
D-Carvone	002244-16-8	C10H14O	150.2	Terpene/Flavor/Fragrance	1	1	1
Trans-Alpha-Bergamotene	013474-59-4	C15H24	204.4	Terpene	1	1	1
Beta-Amyrin	000559-70-6	C30H50O	426.7	Terpene	1	x	x
Calamenene/Cis-Calamenene	072937-55-4	C15H22	202.3	Terpene	1	x	1
Cetene	000629-73-2	C16H32	224.4	Terpene	1	x	x
Gamma-Cadinene/Gamma-Murolene	030021-74-0	C15H24	204.4	Terpene	1	x	3
Methyl salicylate	000119-36-8	C8H8O3	152.1	Terpene/Flavor/Fragrance	1	x	x
Trans-Verbenol	001820-09-3	C10H16O	152.2	Flavor/Fragrance	1	x	x
Triacetin	000102-76-1	C9H14O6	218.2	Flavor/Biocide	1	x	x
Viridiflorene	021747-46-6	C15H24	204.4	Terpene	1	x	x
3-Methylcyclopentyl acetate	024070-70-0/035897-13-3	C8H14O2	142.2	Terpene	x	6	x
Epi-γ-Eudesmol	117066-77-0	C15H26O	222.4	Terpene	x	6	2
Piperitenone	000491-09-8	C10H14O	150.2	Terpene/Flavor/Fragrance	x	6	3
Caryophylla-4(12),8(13)-dien-5.beta.-ol	019431-80-2	C15H24O	220.4	Flavoring agents	x	5	1

(Continued on following page)

TABLE 2 | (Continued) Terpenes detected in vape liquid, vapor and aerosol samples.

Compound names	CAS#	Formula	MW	Functions	Liquid ^a	Vapor ^a	Aerosol ^a
Perhydrofarnesyl Acetone/Hexahydrofarnesyl Acetone	000502-69-2	C18H36O	268.5	Flavor/Fragrance	x	5	x
Humulene oxide II	019888-34-7	C15H24O	220.4	Terpene	x	5	1
Geraniol	000106-24-1	C10H18O	154.3	Terpene	x	4	x
Rotundifolone/Piperitenone oxide	003564-96-3	C10H14O2	166.2	Natural extract??	x	4	2
Trans-Calamenene	073209-42-4	C15H22	202.3	Terpene	x	4	x
Beta-selinene	017066-67-0	C15H24	204.4	Terpene	x	3	3
Camphene	000079-92-5	C10H16	136.2	Terpene/Flavor/Fragrance	x	3	5
Methyl heptenone	000110-93-0	C8H14O	126.2	Flavor/Fragrance	x	3	x
p-Mentha-1,5,8-triene	021195-59-5	C10H14	134.2	Terpene	x	3	1
Alpha-Bisabolene/(E)-Alpha-Bisabolene	025532-79-0	C15H24	204.4	Flavor/Fragrance	x	2	x
Alpha-Guaiene	003691-12-1	C15H24	204.4	Terpene/Flavor/Fragrance	x	2	2
Calarene/(+)-Calarene	017334-55-3	C15H24	204.4	Terpene	x	2	x
Bicyclo[7.2.0]undec-3-ene, 4,11,11-trimethyl-8-methylene-	889360-49-0	C15H24	204.4	Terpene	x	2	2
Cherry propanol	001197-01-9	C10H14O	150.2	Flavor/Fragrance	x	2	2
Cis-Beta-Farnesene	028973-97-9	C15H24	204.4	Terpene	x	2	1
Bicyclo[7.2.0]undecane, 10,10-dimethyl-2,6-bis(methylene)-	357414-37-0	C15H24	204.4	Terpene	x	x	6
O-Cymene	000527-84-4	C10H14	134.2	Terpene/Flavor/Fragrance	x	x	4
Eudesma-4,6-diene	028624-28-4	C15H24	204.4	Terpene	x	2	x
Gamma-Terpinene	000099-85-4	C10H16	136.2	Terpene/Flavor/Fragrance	x	2	3
Gamma-Terpineol	000586-81-2	C10H18O	154.3	Terpene	x	2	1
Humulenol-II	019888-00-7	C15H24O	220.4	Terpene	x	2	1
Isocaryophyllene	000118-65-0	C15H24	204.4	Terpene	x	2	x
Tricyclene	000508-32-7	C10H16	136.2	Flavor/Fragrance	x	2	1
Ylangene	014912-44-8	C15H24	204.4	Terpene	x	2	2
1,3,8-p-Menthatriene	018368-95-1	C10H14	134.2	Flavoring	x	1	3
1,8-menthadien-4-ol	997077-81-3	C10H16O	152.2	Terpene??	x	1	x
3-Thujene	002867-05-2	C10H16	136.2	Terpene	x	1	x
6,9-Guaiadene	036577-33-0	C15H24	204.4	Flavor/Fragrance	x	1	x
Alpha-Curcumene	000644-30-4	C15H22	202.3	Terpene	x	1	x
Alpha-Himachalene	003853-83-6	C15H24	204.4	Terpene	x	1	x
Alpha-Panasinsen/(-)-Alpha-Panasinsen	056633-28-4	C15H24	204.4	Terpene	x	1	x
Alpha-Terpinene	000099-86-5	C10H16	136.2	Terpene/Flavor/Fragrance	x	1	1
aR-Himachalene	019419-67-1	C15H22	202.3	Terpene	x	1	1
Benzophenone	000119-61-9	C13H10O	182.2	Flavor/Fragrance	x	1	x
Beta-Fenchol/(-)-Beta-Fenchol	000470-08-6	C10H18O	154.3	Terpene	x	1	x
Myrtenol/(-)-Myrtenol	019894-97-4	C10H16O	152.2	Terpene/Flavor/Fragrance	x	1	x
Beta-Patchoulene	000514-51-2	C15H24	204.4	Fragrance/Perfuming	x	1	1
Beta-Vetivenene	027840-40-0	C15H24	204.4	Terpene	x	1	x
Cubenene	029837-12-5	C15H24	204.4	Terpene	x	1	1
Caryophyllene-(I1)	136296-37-2	C15H24	204.4	Terpene	x	1	x
Caryophyllenyl alcohol	913176-41-7	C14H24O	222.4	Flavor/Fragrance	x	1	x
Cis-8-Methylbicyclo[4.3.0]non-7-ene	057497-08-2	C10H16	136.2	Terpene	x	1	x
Clovanediol	002649-64-1	C15H26O2	238.4	Terpene	x	1	x
Delta-Cadinene	000483-76-1	C15H24	204.4	Terpene	x	1	3
Dihydrocarvyl acetate	020777-49-5	C12H20O2	196.3	Flavor/Fragrance	x	1	1
Eugenol	000097-53-0	C10H12O2	164.2	Cosmetic/Flavor/Fragrance	x	1	1
Gamma-Gurjunene	022567-17-5	C15H24	204.4	Terpene	x	1	x
Gamma-Maalinene	020071-49-2	C15H24	204.4	Terpene	x	1	1
Geranyl Acetone	003796-70-1	C13H22O	194.3	Flavor/Fragrance	x	1	x
Humulene epoxide I	019888-33-6	C15H24O	220.4	Terpene	x	1	1
Isobornyl Acrylate	005888-33-5	C13H20O2	208.3	Film forming	x	1	2
Lavandulyl propionate	059550-34-4	C13H22O2	210.3	Terpene	x	1	x
Menthyl acetate	000089-48-5/002623-23-6	C12H22O2	198.3	Flavor/Fragrance	x	1	1
Methyl salicylate	000119-36-8	C8H8O3	152.1	Terpene/Flavor/Fragrance	x	1	1
Olivetol	000500-66-3	C11H16O2	180.2	Natural extract	x	1	x
Phytol acetate	076337-16-1/010236-16-5	C22H42O2	338.6	Flavor/Fragrance	x	1	x
Pseudolimonene	000499-97-8	C10H16	136.2	Terpene	x	1	1
Schyobunol	035727-45-8	C15H26O	222.4	Terpene	x	1	x
Selin-6-en-4.alpha.-ol	118173-08-3	C15H26O	222.4	Terpene	x	1	x
Tetrahydrogeranyl Acetone	001604-34-8	C13H26O	198.3	Flavor/Fragrance	x	1	x

(Continued on following page)

TABLE 2 | (Continued) Terpenes detected in vape liquid, vapor and aerosol samples.

Compound names	CAS#	Formula	MW	Functions	Liquid ^a	Vapor ^a	Aerosol ^a
Trans-Farnesol	000106-28-5	C15H26O	222.4	Terpene/Flavor/Fragrance	x	1	x
Zonarene	041929-05-9	C15H24	204.4	Terpene	x	1	x
O-Cymene	000527-84-4	C10H14	134.2	Terpene/Flavor/Fragrance	x	x	4
Trans-Beta-Ocimene	003779-61-1	C10H16	136.2	Terpene/Flavor/Fragrance	x	x	2
Beta-Bisabolene	000495-61-4	C15H24	204.4	Flavor/Fragrance	x	x	2
Cis-2,6-Dimethyl-2,6-octadiene	002492-22-0	C10H18	138.3	Terpene	x	x	2
Beta-Bourbonene/(-)-Beta-Bourbonene	005208-59-3	C15H24	204.4	Flavor/Fragrance	x	x	1
Myrtenol/(-)-Myrtenol	019894-97-4	C10H16O	152.2	Terpene/Flavor/Fragrance	x	x	1
Selina-5,11-diene/(-)-Selina-5,11-diene	997220-96-1	C15H24	204.4	Terpene	x	x	1
Carvomenthene/(+)-Carvomenthene	001195-31-9	C10H18	138.3	Terpene	x	x	1
Menthol/(±)-menthol	001490-04-6	C10H22O	156.3	Terpene/Flavor/Fragrance	x	x	1
4-Terpinenol	000562-74-3	C10H18O	154.3	Flavor/Fragrance	x	x	1
8,9-Dehydrothymol	018612-99-2	C10H12O	148.2	Terpene	x	x	1
Alpha-Bergamotene	017699-05-7	C15H24	204.4	Terpene	x	x	1
Alpha-Elemene	005951-67-7	C15H24	204.4	Terpene	x	x	1
Alpha-Phellandrene	000099-83-2	C10H16	136.2	Terpene/Flavor/Fragrance	x	x	1
Beta-Cubebene	013744-15-5	C15H24	204.4	Flavoring	x	x	1
Beta-Gurjunene	997220-72-3/73464-47-8	C15H24	204.4	Terpene	x	x	1
Cadina-1(6),4-diene	016729-00-3	C15H24	204.4	Terpene	x	x	1
Cyperene	002387-78-2	C15H24	204.4	Terpene	x	x	1
Eremophilene	010219-75-7	C15H24	204.4	Terpene	x	x	1
(R)-Gamma-Cadinene	039029-41-9	C15H24	204.4	Terpene	x	x	1
Isopinocampone	018358-53-7	C10H16O	152.2	Flavor/Fragrance	x	x	1
Isoterpinolene	000586-63-0	C10H16	136.2	Terpene	x	x	1
Laevo-Bornyl acetate	005655-61-8	C12H20O2	196.3	Flavor/Fragrance	x	x	1
L-Alpha-Terpineol	010482-56-1	C10H18O	154.2	Terpene	x	x	1
Trans-Isolimonene	005113-87-1	C10H16	136.2	Terpene	x	x	1

^aNumber of samples where each compound was detected.

Glycerides (MCTs) peak areas; three samples contained Polyethylene Glycols (PEGs) with two of them greater than 10% peak areas of the total compounds found; one sample had more than 20% CBN peak area; and one sample had more than 3.5% caryophyllene peak area (**Figure 1**). In these variety of samples, minor cannabinoids such as Delta8-THC, CBT, (6aR, 9R)-delta10-THC/(6aR,9S)-delta10-THC, 9(R)-delta6a,10a-THC/9(S)-delta6a,10a-THC, Hexahydrocannabinol, Cannabidivanol (CBDV), Delta8-Tetrahydrocannabivarin, and Exo-THC were detected. Usually these minor cannabinoids are seen in the degradation of Delta9-THC or as byproducts of the extraction process in very small amounts (Hudalla, 2020).

Delta10-THCs ((6aR, 9R)-delta10-THC, (6aR,9S)-delta10-THC) have no pharmacological effects and 9(R)-delta6a,10a-THC and 9(S)-delta6a,10a-THC have very low or very limited psychoactivity. These byproducts are not well studied for their efficacy and toxicity and therefore, the long-term health effects in cannabis products are unknown to consumers (Hudalla, 2020; Williams, 2020). Delta8-THC exists in small amounts in cannabis plants (<1%). The identified Delta8-THC isomers may have resulted from raw cannabis plant material from the extraction or post extraction processing using ethanol, hydrocarbons, or CO₂ to extract/purifying cannabis oil, and remove waxes and chlorophyll (Wilhelm; Hudalla, 2020). Delta8-THC has become increasingly popular and drawn attention in recent days when CBD became nationally legalized. CBD can be easily converted to Delta 8-THC with addition of catalysts (e.g., p-toluenesulfonic

acid) in a solvent mixture (Barrie Webster and Leonard Sarna, 2004). However, the conversion process is unpredictable in producing other byproducts such as other minor cannabinoids that are not found in natural cannabis plants. Delta 8-THC is not currently covered under the current California cannabis regulations and it has almost two thirds of the psychoactivity compared to Delta9-THC (Inverse.com, 2020). In one sample (F1912013-004), in addition to VEA, more than 30% Delta8-THC and 10% 9(S)-delta6a,10a-THC peak areas were detected, indicating likely adulteration with the synthetic form of Delta8-THC. In these ten vape cartridges, the CBN ranged from 1–20% and most of the samples had 3–5% peak areas. The sample with 20% CBN peak area was half full in an unused cartridge and had sign of drying. This was likely due to a poor sample seal or storage problem.

Vitamin E and VEA were found in six and three of the samples, respectively. Vitamin E, also known as tocopherols, is well known for its antioxidant properties. The most active form of alpha-tocopherol is commonly found in plant material, especially in plants with high oil content and hemp seed (Callaway, 2004). It is essential for plant development and help to provide the major antioxidant function for free radical damages (Muñoz and Munné-Bosch, 2019). Therefore, it is possible to have small amount of Vitamin E in cannabis plant that is coextracted and carried into the vape oil product. However, according to Brenneisen et al., Vitamin K was the only Vitamin found in cannabis plants (Brenneisen and ElSohly, 2007). Large amount of

TABLE 3 | Cannabinoids and other constituents detected in vape liquid, vapor and aerosol samples.

Compound names	CAS#	Formula	MW	Functions	Liquid ^a	Vapor ^a	Aerosol ^a
Delta9-Tetrahydrocannabinol (delta9-THC)	001972-08-3	C21H30O2	314.5	Cannabinoid	12	10	10
Cannabinol (CBN)	000521-35-7	C21H26O2	310.4	Cannabinoid	12	12	10
Cannabicitran (CBT)	031508-71-1	C21H30O2	314.5	Cannabinoid	12	12	7
Cannabigerol (CBG)	025654-31-3	C21H32O2	316.5	Cannabinoid	11	4	9
Delta9-Tetrahydrocannabivarin (THCV)	031262-37-0	C19H26O2	286.4	Cannabinoid	11	6	8
Cannabichromene (CBC)	020675-51-8	C21H30O2	314.5	Cannabinoid	10	10	10
(6aR,9R)-delta10-THC/(6aR,9S)-delta10-THC	095543-62-7	C21H30O2	314.5	Cannabinoid	9	5	5
Cannabifuran	056154-58-6	C21H26O2	310.4	Cannabinoid	5	x	2
Cannabidiol (CBD)	013956-29-1	C21H30O2	314.5	Cannabinoid	4	x	2
Delta8-Tetrahydrocannabinol (delta8-THC)	005957-75-5	C21H30O2	314.5	Cannabinoid	3	9	4
Cannabicyclic acid	070474-97-4	C21H28O3	328.4	Cannabinoid	2	10	5
Hexahydrocannabinol	006692-85-9	C21H32O2	316.5	Cannabinoid	2	1	x
9(R)-delta6a,10a-THC/9(S)-delta6a,10a-THC	095720-01-7	C21H30O2	314.5	Cannabinoid	2	4	2
Cannabidivarin (CBDV)	024274-48-4	C19H26O2	286.4	Cannabinoid	1	x	x
Delta8-Tetrahydrocannabivarin	031262-38-1	C19H26O2	286.4	Cannabinoid	1	1	x
Exo-THC	027179-28-8	C21H30O2	314.5	Cannabinoid	1	2	x
Cannabivarin (CBV)	033745-21-0	C19H22O2	282.4	Cannabinoid	x	7	1
Olivetol	000500-66-3	C11H16O2	180.2	Natural extract	1	x	x
Linolenic acid, ethyl ester/9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	001191-41-9	C20H34O2	306.5	Fatty acid	3	2	1
Decanoic acid, methyl ester	000110-42-9	C11H22O2	186.3	Fatty acid	2	x	x
Hexadecanoic acid, ethyl ester	000628-97-7	C18H36O2	284.5	Fatty acid	2	4	x
Linoleic acid	000060-33-3	C18H32O2	280.4	Fatty acid	2	x	x
Linoleic acid, ethyl ester	000544-35-4	C20H36O2	308.5	Fatty acid	2	3	1
Linolenic acid	000463-40-1	C18H30O2	278.4	Fatty acid	2	4	x
Octanoic acid, methyl ester	000111-11-5	C9H18O2	158.2	Fatty acid	2	x	x
Linoleic acid, methyl ester/9,12-Octadecadienoic acid, methyl ester	000112-63-0	C19H34O2	294.5	Fatty acid	1	x	x
Oleic acid, methyl ester	000112-62-9	C19H36O2	296.5	Fatty acid	1	x	x
n-Hexadecanoic acid	000057-10-3	C16H32O2	256.4	Fatty acid	x	3	x
Butanoic acid, ethyl ester	000105-54-4	C6H12O2	116.2	Fatty acid	x	1	x
Oleic acid, ethyl ester	000111-62-6	C20H38O2	310.5	Fatty acid	x	1	x
Hexadecane	000544-76-3	C16H34	226.4	Fatty acid	x	1	x
Hexadecanoic acid, methyl ester	000112-39-0	C17H34O2	270.5	Fatty acid	x	1	x
Linoleic acid	000060-33-3	C18H32O2	280.4	Fatty acid	x	1	x
Decanoic acid	000334-48-5	C10H20O2	172.3	Fatty acid	x	1	x
Octadecanoic acid, ethyl ester	000111-61-5	C20H40O2	312.5	Fatty acid	x	1	x
Octanoic acid	000124-07-2	C8H16O2	144.2	Fatty acid	x	1	x
1-Octadecene	000112-88-9	C18H36	252.5	Fatty acid	x	1	x
1,2-Dioctanoin	001069-87-0	C19H36O5	344.5	MCT	2	x	x
1,3-Dioctanoin	001429-66-9	C19H36O5	344.5	MCT	2	2	x
2-(Decanoyloxy)propane-1,3-diyl dioctanoate	033368-87-5	C29H54O6	498.7	MCT	2	2	2
2-(Octanoyloxy)propane-1,3-diyl bis(decanoate)	033368-86-4	C31H58O6	526.8	MCT	2	2	2
Decanoic acid, 1,2,3-propanetriyl ester	000621-71-6	C33H62O6	554.8	MCT	2	x	x
Decanoic acid, 2-hydroxy-3-[(1-oxooctyl)oxy]propyl ester	093980-84-8	C21H40O5	372.5	MCT	2	x	x
Glycerol triacrylate	000538-23-8	C27H50O6	470.7	MCT	2	2	2
Glycerol 1,2-diacetate	000102-62-5	C7H12O5	176.2	MCT Derivative	x	1	1
Decaethylene glycol	005579-66-8	C20H42O11	458.5	Solvent	3	x	x
Undecaethylene glycol	006809-70-7	C22H46O12	502.6	Solvent	3	x	3
Pentaethylene glycol	004792-15-8	C10H22O6	238.3	Solvent	2	3	2
Octaethylene glycol	005117-19-1	C16H34O9	370.4	Solvent	2	1	3
Heptaethylene glycol	005617-32-3	C14H30O8	326.4	Solvent	1	x	1
Hexaethylene glycol	002615-15-8	C12H26O7	282.3	Solvent	1	3	1
Nonaethylene glycol	003386-18-3	C18H38O10	141.5	Solvent	1	x	2
Tetraethylene glycol	000112-60-7	C8H18O5	194.2	Solvent	1	1	x
Triethylene glycol	000112-27-6	C6H14O4	150.2	Solvent	x	2	x
Propylene glycol	000057-55-6	C3H8O2	76.1	Solvent	x	x	1
Vitamin E	000059-02-9	C29H50O2	430.7	Food additive	6	x	x
Vitamin E acetate	000058-95-7	C31H52O3	472.8	Food additive	3	3	2

^aNumber of samples where each compound was detected.

VEA were found in three cartridge samples. VEA is also called α -tocopheryl acetate and is a synthetic form of Vitamin E [EFSA, 2016]. It has a similar appearance to cannabis vape oil. It is commonly added to THC vaping liquids to dissolve/dilute or

thicken them as a cutting agent to cut down the cost. Recent studies by the Centers for Disease Control and Prevention (CDC), the US Food and Drug Administration (USFDA), state investigators and research institutes have concluded that VEA

is strongly associated with EVALI (Chand et al., 2019; Blount et al., 2020; Matsumoto et al., 2020; Muthumalage et al., 2020). After the EVALI outbreak, VEA is currently banned from cannabis products in many states (Boudi et al., 2019; Gibbons, 2021). In the samples containing large amounts of VEA, Vitamin E was also found and at higher amount than the other three samples with Vitamin E alone. They are probably the byproduct of VEA as VEA is sensitive to hydrolysis and breaks down to free Vitamin E and acetic acid [EFSA, 2016].

Medium-chain triglycerides (MCTs) are getting more attention in recent years for their health benefits as quick energy sources which are less likely to be stored as fat. They are also used as the supplement among athletes and bodybuilders as well as to aid weight loss (Mary Jane Brown, 2020). Triglyceride is simply the technical term for fat and has two main purposes either the body will burn it for energy or store as body fat. MCTs contain two or three fatty acids that have a chain length of 6–12 carbon atoms and they include caproic acid or hexanoic acid (C6), caprylic acid or octanoic acid (C8), capric acid or decanoic acid (C10), and lauric acid or dodecanoic acid (C12). Food sources rich for commercial extraction of MCTs include palm kernel oil and coconut oil (Mary Jane Brown, 2020). Like VEA, MCTs are added to THC e-liquid as dilute or thickening agent based on their appearance and claimed health benefits, especially in counterfeit products (Chand et al., 2019; Muthumalage et al., 2020). MCTs are generally regarded safe by the FDA as food additives under certain limitations (USFDA, 1938a). However, little is known about how they affect the respiratory tract and its local immune-inflammatory functions when used in vape products. MCTs were also found in bronchoalveolar lavage fluid in EVALI patients from CDC's study (Blount et al., 2019; Blount et al., 2020). In the current study, we found two samples containing around 25 and 39% MCT peak areas with the major compounds as 2-(Decanoyloxy)propane-1,3-diyl dioctanoate, 2-(Octanoyloxy)propane-1,3-diyl bis(decanoate), and Glycerol Triacrylate.

Polyethylene Glycols (PEGs) were also found in three vape cartridge samples and they contained 2–15% total peak areas including Tetraethylene Glycol, Pentaethylene Glycol, Hexaethylene Glycol, Heptaethylene glycol, Nonaethylene Glycol, Octaethylene Glycol, Decaethylene Glycol, and Undecaethylene Glycol. Propylene glycol (PG) and vegetable glycerin (VG) are two of the primary solvents heavily used in nicotine e-liquid as the thinning agents. PEGs have also been found in vape products (Traboulsi et al., 2020). PEG 400 is a low molecular weight grade of PEG that is widely used in cosmetics and pharmaceutical formulations as a solvent/lubricant due to its low oral and dermal toxicity. Even though they are safe as food additives (USFDA, 1938b), studies have shown that during vaping, PG and PEG 400 produced high levels of toxic compounds-acetaldehyde and formaldehyde when heated to 230°C. In addition, PEG 400 produced significantly higher levels of acetaldehyde and formaldehyde than PG, MCT, and VG (Troutt and DiDonato, 2017). Samples containing PEGs were also found in EVALI patients (USFDA, 2020c).

In these vape oil samples, we also found a set of fatty acids, mainly unsaturated fatty acids including linoleic acid, linolenic acid, oleic acid, and their methyl ester or ethyl ester derivatives (Table 3). These acids are commonly found in cannabis, and are especially rich in cannabis seeds (Callaway, 2004; Brenneisen and ElSohly, 2007). They have some terpene functions and offer flavors such as green, fruity, waxy, citrus, aldehydic soapy, creamy, and coconut (The Good Scents Company I). Other compounds we found were Benzyl Alcohol (1 sample) and Butylated Hydroxytoluene (1 sample) used as preservatives; and Triacetin (1 samples) reported to function as a cosmetic biocide, plasticizer, and solvent in cosmetic formulations. Finally, a small portion of the compounds in each sample could not be identified using Wiley11/NIST 2017 Mass Spectral libraries and Cayman library.

Headspace GC-MS Screen for Cannabis Vape Oil Vapor

Twelve cannabis vape oil samples were heated to 200°C to simulate how users consume vape oil. We found that many more terpenes were generated, and the major terpenes were at much higher concentrations in the vapor. The cannabinoid levels were much lower in vapor content compared to the liquid vape oil (Figure 2). In general, terpenes have smaller molecular weight and lower boiling point than cannabinoids. Therefore, heating increased the terpene compositions in headspace vials, resulting in more terpene types and higher amount observed in vape oil vapor samples.

The most common terpenes and natural extracts found were Caryophyllene (12 samples), Alpha-Humulene (12 samples), Alpha-Bisabolol (11 samples), Eudesma-3,7(11)-diene/Selina-3,7(11)-diene (11 samples), Linalool (10 samples), 2-Pinene (9 samples), D-Limonene (9 samples), Phytol (9 samples), Beta-Pinene (2(10)-Pinene) (8 samples), Caryophyllene Oxide (7 samples), Copaene (7 samples), Fenchol (7 samples), Gamma-Selinene (7 samples), Nerolidol (7 samples), 3-Methylcyclopentyl acetate (6 samples), Epi-γ-Eudesmol (6 samples), Piperitenone (6 samples), Terpinolene (6 samples), Alpha-Selinene (5 samples), Beta-Myrcene (5 samples), Caryophylla-4(12),8(13)-dien-5.beta.-ol (5 samples), Endo-Borneol (5 samples), Humulene oxide II (5 samples), Neophytadiene (5 samples). Among these commonly found terpenes, Caryophyllene, D-Limonene, Alpha-Humulene, Linalool, and Terpinolene were dominant. More than 100 terpenes and related compounds were released in vapor samples and they are shown in Tables 2. After heating, total terpenes can comprise of more than 60% peak areas of the total compounds and some major terpenes such as caryophyllene or 2-Pinene can have more than 20% peak area alone (Figure 1).

The most common cannabinoids found were CBT (12 samples), CBN (12 samples), Delta9-THC (10 samples), CBC (10 samples), Cannabicooumaronone (10 samples), Delta8-THC (9 samples), Cannabivarin (CBV) (7 samples), THCV (6 samples), (6aR,9R)-delta10-THC or (6aR,9S)-delta10-THC (5 samples) (Table 3). Delta9-THC dropped significantly to below 15% peak areas in most of the vapor samples (Figure 1).

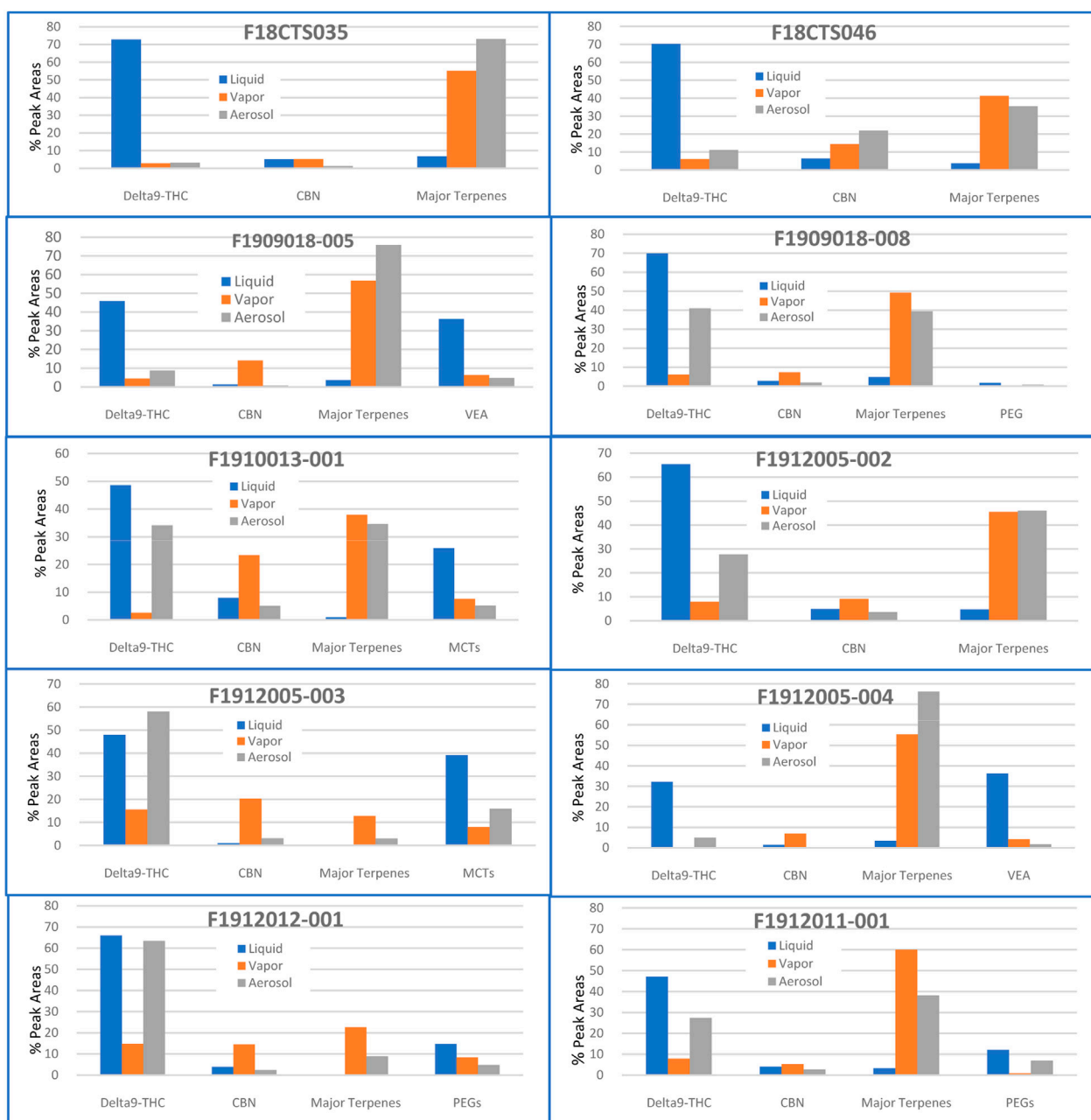


FIGURE 1 | Major terpenes, Delta9-THC, and major additives in vape oil liquid, vapor and aerosol of each vape oil cartridge sample. % Peak Area, area percentage of each peak or compound found in instrument analysis. It roughly represents the composition or amounts present in the sample.

We found minor cannabinoids such as Delta8-THC, Cannabichromene, CBV, and 9(S)-delta6a,10a-THC present in more samples, and CBG, and THCV in fewer samples compared to vape oil liquid. This minor cannabinoid profile change is probably due to the heating process and a study has shown that CBV is an oxidized product of THCV likely due to heating (Bailey and Gagné, 1975). In most of the samples, CBN as a degradation byproduct of Delta9-THC became more dominant than Delta9-THC after heating. This can be

beneficial effect as CBN is non-psychoactive with some therapeutic benefit/potential to treat disease. CBN acts as a sedative, anticonvulsant in animal and human studies, and has demonstrated significant properties related to anti-inflammatory and antibiotic activities (Brenneisen and ElSohly, 2007; EthanRusso, 2017).

The potential toxins or additives including VEA, PEGs and MCTs found in liquid injection were also found in vapor samples but at much lower levels (**Figure 1**). This is likely

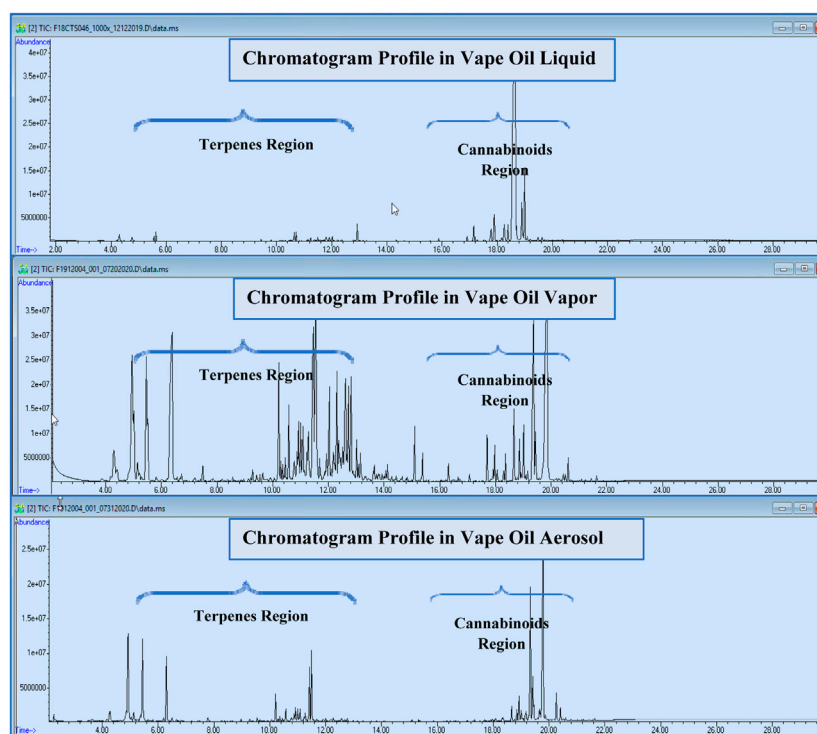


FIGURE 2 | Total ion chromatograms of liquid, vapor, and aerosol phases in one of tested samples.

due to their higher molecular weight and higher boiling points compared to certain terpenes and cannabinoids. VEA and MCTs dropped to below 7 and 9% peak areas in vapors compared to over 30 and 25% peak areas in vape liquid samples, respectively. PEG levels also dropped significantly. Even with smaller amounts released, these additives may pose toxic effects in EVALI patients addressed in previous section. We also found a similar set of fatty acids and they had similar behaviors as terpenes producing higher levels in vapor samples after heating. Vitamin E was not observed in vapor samples as it is unstable at high temperatures and may decay or break down to other unidentifiable compounds (Kuppithayanant, 2014).

SPME GC-MS Screen for Cannabis Vape Oil Aerosol

Ten out of twelve planned vape oil cartridges were able to be tested for constituents in vape oil aerosol samples. The total aerosol amounts generated from five puffs ranged from 15 to 31 mg. We noticed that some cartridges were much easier to light and generate aerosol while some generated aerosols very slowly. We found that easy lit and aerosolized cartridges typically had lower vape oil viscosities. Two vape cartridges were unable to generate aerosols at all using the same vape device, indicating their poor quality and short shelf life. We also noticed that different puffs generated from the same cartridge can be very different for the amounts of terpenes and

cannabinoids released. The lower amounts of the vape oil used to generate aerosols, the higher amounts of the terpenes and the lower amounts of cannabinoids were released, and vice versa. In general, more terpenes were released than cannabinoids in aerosol samples, similar to vapor samples (Figure 2).

In tested aerosol samples, the most common terpenes found were Alpha-Humulene (10 samples), Caryophyllene (10 samples), D-Limonene (10 samples), Eudesma-3,7(11)-diene or Selina-3,7(11)-diene (10 samples), Terpinolene (10 samples), 2-Pinene (9 samples), Beta-Pinene (9 samples), Fenchol (9 samples), Linalool (9 samples), Alpha-Bisabolol (8 samples), Beta-Myrcene (8 samples), Copaene (8 samples), Caryophyllene oxide (6 samples), Bicyclo[7.2.0]undecane, 10,10-dimethyl-2,6-bis(methylene)- (6 samples). Alpha-Selinene/(+)-Alpha-Selinene (5 samples), Camphene (5 samples), Delta-Guaiene (5 samples), Gamma-Selinene (5 samples). Among the most commonly found terpenes, Caryophyllene, D-Limonene, Alpha-Humulene, Linalool, and Terpinolene, and 2-Pinene were the most abundant. More than 100 terpenes and natural extracts were generated through vaping and they are listed in Tables 2. After heating, the major terpenes can have more than 75% of total peak area, more than those of in vapor samples. Some samples contained lower levels of terpenes compared to vapors (Figure 1).

The most common cannabinoids found were Delta9-Tetrahydrocannabinol (Delta9-THC) (10 samples), Cannabinol (CBN) (10 samples), Cannabichromene (CBC) (10 samples),

Cannabigerol (CBG) (9 samples), Delta9-Tetrahydrocannabivarin (THCV) (8 samples), Cannabicitran (CBT) (7 samples), (6aR,9R)-delta10-THC or (6aR,9S)-delta10-THC (5 samples), and Cannabicaunaronone (5 samples) (**Table 3**). Similar to the cannabis vapor, we found much higher amounts of terpenes and lower amounts of cannabinoids than vape oil liquid. However, Delta9-THC was at higher levels compared to the vapor and it was still the most dominant cannabinoid in six of the 10 aerosol samples ranging from 27 to 63% peak areas. This indicates that the vape pen used may generate temperatures higher than 200°C. Studies have shown that lower vape temperatures usually provide more terpene flavors, and higher vape temperatures give stronger psychoactive effects (more Delta9-THC content) (vaping360.com, 2020; zamnesia.com, 2020). This is also confirmed from our experiment by heating vape oil in headspace vials at 150°C and 200°C. We observed higher amounts of terpenes and lower amounts of cannabinoids in 150°C vapor samples. Both aerosol and vapor samples produced more terpene types than vape liquid samples, but aerosol produced less terpene types compared to vapor due to higher heating temperatures.

In aerosols, we also found minor cannabinoids such as Delta8-Tetrahydrocannabinol, (6aR,9R)-delta10-THC, (6aR,9S)-delta10-THC, 9(S)-delta6a,10a-THC, and Cannabifuran. This indicates that at the vaping temperature used, minor cannabinoids may be formed from major cannabinoid such as Delta9-THC. The potential toxins or additives including VEA, PEGs and MCTs found in vape liquid and vapor were also found in aerosol samples. Their levels were similar as vapor (**Figure 1**). Interestingly, Vitamin E was not found and only a few fatty acids were found in aerosol samples probably due to their decaying at high temperatures. The possible breakdown product of toxic gas ketene from VEA during vaping can be directly attributed to the illness in EVALI patients (Attfield et al., 2020; Strongin, 2020; Wu and O'Shea, 2020). However, ketene was not found in tested vapor or aerosol samples.

Some specific terpenes such as Bicyclo[7.2.0]undec-3-ene, 4,11,11-trimethyl-8-methylene-, Bicyclo[7.2.0]undec-3-ene, 4,11,11-trimethyl-8-methylene-, 1,3,8-p-Menthatriene; and Isobornyl Acrylate, and some flavor additives such as Cherry propanol, Citronellol; and Lavandulyl Propionate were observed in both vapor and aerosol samples. More isomers (e.g., alpha, beta, gamma) of some terpenes were found in vapor and aerosols likely due to heat transformation. Some compounds were degradation or derivative products from major terpenes after heating, for example Humulene epoxide I, Humulene oxide II, and Humulenol-II were likely produced from Alpha-Humulene. Caryophylla-4(12),8(13)-dien-5.beta.-ol, Caryophyllene oxide, Caryophyllene-(II), Caryophyllenyl alcohol, Caryophylla-4(12),8(13)-dien-5.beta.-ol, Isocaryophyllene were likely generated from Caryophyllene. Eudesma-3,7(11)-diene, Eudesma-4(14),11-diene, and Eudesma-4,6-diene were likely generated from Eudesma.

Strength and Limitations

To our knowledge, this was the first study to provide a comprehensive list of the terpenes, cannabinoids, and additives found in vape oil samples, especially in heated vapor and

aerosols. This small study compared the major constituents and potential toxic additives such as VEA, PEG, MCTs in vape cartridges before and after EVALI outbreak and in three different sample forms (liquid, vapor and aerosol). The data generated can aid to assess the types and amounts of the constituents inhaled through vaping by consumers. In this study, only 12 vape oil cartridges were tested. A larger number of samples should be investigated to confirm the current findings. The aerosol experiment was designed to simulate vaping, but it was not identical to vaping by users. The tested conditions used that may differ are: 1) vacuum flow used to generate aerosol can be different from inhaling by a person's breath; 2) SPME fiber used can only absorb limited amount of volatile and semivolatile compounds; 3) exact vape temperature cannot be measured and it can be different as used by consumers; 4) the same vape device with the same voltage was used which may be different from consumers that use various vape devices with different voltage settings. Finally, various constituents and additives were not accurately quantified in this study.

CONCLUSIONS AND FUTURE STUDIES

In the current study, we have detected over 100 terpenes and natural extracts, 19 cannabinoids including some minor cannabinoids such as cannabicitran (CBT), cannabivarin (CBV), cannabicaunaronone, (6aR,9R)-delta10-THC, (6aR,9S)-delta10-THC, 9(S)-delta6a,10a-THC, and Cannabifuran, exo-THC, and Hexahydrocannabinol, as well as other potential toxic additive such as VEA, PEGs, and MCTs in tested vape cartridges. Our study has shown that more terpenes and minor cannabinoids can be produced *via* vaporizing and aerosolizing the vape oil. Delta9-THC and potential toxic additives were found at lower levels in vapor and aerosol samples. Currently, the interactions among high amount of the terpenes released through heating, major and minor cannabinoids, and additives including VEA, MCTs or PEGs, as well as the potential interaction byproducts are not studied. The amounts of cannabinoids inhaled can vary from puff to puff and depend on the quality of the vape oil and devices. Due to the study limitations, we cannot detect other toxins such as ketene that may have direct toxic impact to lung injuries. Even though EVALI outbreak patients have significantly decreased in 2020, they still exist in California during COVID19 pandemic period (Sternlicht, 2020). Therefore, it's crucial to monitor for potential toxic additives through continuous testing of vape oil products from surveillance and investigations. We are also conducting an experiment to examine cannabis flower aerosol constituents using vaping devices. This study will shed light in discovering potential toxic chemicals formed during dried flower vaping. Furthermore, we have developed a liquid chromatography mass spectrometry (LCMS) toxin screen method to target for nonvolatile constituents and additives to expand the toxin screen capability in our future studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because Data can only be released through CDPH Public information officer. Requests to access the datasets should be directed to Gordon.Vrdoljak@cdph.ca.gov.

AUTHOR CONTRIBUTIONS

WG is the primary author and main scientist performing the research. GV is the manager, director of the research and author. V-CL is a scientific contributor to research and author. BM is the reviewer and author.

REFERENCES

- Attfield, K. R., Chen, W., Cummings, K. J., Jacob, P., O'Shea, D. F., Wagner, J., et al. (2020). Potential of Ethenone (Ketene) to Contribute to E-Cigarette, or Vaping, Product Use-Associated Lung Injury. *Am. J. Respir. Crit. Care Med.* 202 (8), 1187–1189. doi:10.1164/rccm.202003-0654le
- Bailey, K., and Gagné, D. (1975). Distinction of Synthetic Cannabidiol, Cannabichromene, and Cannabivarin by GLC Using On-Column Methylation. *J. Pharm. Sci.* 64 (10), 1719–1720. doi:10.1002/jps.2600641033
- Barrie Webster, G. R. M., and Leonard Sarna, P. (2004). *Raphael Mechoulam, Jerusalem*, Conversion Of CBD to Delta8-THC and Delta9-THC. United States Patent Application Publication. US 2004/0143126A1.
- Best Temperature for Vaping Weed. Available at: <https://vaping360.com/learn/optimal-temperature-to-vape-weed/>, (Accessed on 12/15/2020).
- Blount, B. C., Karwowski, M. P., Morel-Espinosa, M., Rees, J., Sosnoff, C., Cowan, E., et al. (2019). Evaluation of Bronchoalveolar Lavage Fluid from Patients in an Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - 10 States, August-October 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68 (45), 1040–1041. doi:10.15585/mmwr.mm6845e2
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382 (8), 697–705. doi:10.1056/NEJMoa1916433
- Boudi, F. B., Patel, S., Boudi, A., and Chan, C. (2019). Vitamin E Acetate as a Plausible Cause of Acute Vaping-Related Illness. *Cureus* 11 (12), e6350. doi:10.7759/cureus.6350
- Brenneisen, R. (2007). in *Marijuana and the Cannabinoids*. Editor M. A. ElSohly (Totowa, NJ: Humana Press) Available at: <https://www.calgarymmc.com/Ebooks%20%20i%20j%20k%20l%20m%20n%20o%20p%20q%20r%20s%20t%20u%20v%20w%20x%20y%20z%20%20Marijuana%20and%20the%20Cannabinoids.pdf>.
- Callaway, J. C. (2004). Hempseed as a Nutritional Resource: An Overview. *Euphytica* 140, 65–72. doi:10.1007/s10681-004-4811-6
- Cannabis.net. (2020). Contaminants in Your Cannabis Concentrates - A Cause for Real Concern? Available at: <https://cannabis.net/blog/opinion/contaminants-in-your-cannabis-concentrates-a-cause-for-real-concern>, Aug 26, 2020.
- CDC Update (2019). Characteristics of a Nationwide Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury — United States. *Weekly/J January* 24 (3), 90–94.
- CDPH Legislation, Available at: <https://www.cdph.ca.gov/Programs/CEH/DFDCS/MCSB/Pages/Legislation.aspx>. Last Updated: January 29, 2020.
- Chand, H. S., Muthumalage, T., Maziak, W., and Rahman, I. (2019). Pulmonary Toxicity and the Pathophysiology of Electronic Cigarette, or Vaping Product, Use Associated Lung Injury. *Front. Pharmacol.* 10, 1619. doi:10.3389/fphar.2019.01619
- Chen, W., Wang, P., Ito, K., Fowles, J., Shusterman, D., Jaques, P. A., et al. (2018). Measurement of Heating Coil Temperature for e-Cigarettes With a "Top-Coil" Clearomizer. *PLoS One* 13 (4), e0195925.
- DELTA-8-THC. *DELTA-8-THC: Scientists Explain the Cannabis Industry's "Latest Hype"*. Available at: <https://www.inverse.com/mind-body/delta-8-thc-science-explained>, (Accessed on Dec 8, 2020).
- Duffy, B., Li, L., Lu, S., Durocher, L., Dittmar, M., Delaney-Baldwin, E., et al. (2020). Analysis of Cannabinoid-Containing Fluids in Illicit Vaping Cartridges Recovered from Pulmonary Injury Patients: Identification of Vitamin E Acetate as a Major Diluent. *Toxics* 8 (1), 8. doi:10.3390/toxics8010008
- EFSA (2016). *Safety Assessment of the Substance α -tocopherol Acetate for Use in Food Contact Materials*. Available at: <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2016.4412> (Accessed April 13, 2021).
- Erickson, B. E. (2019). *Cannabis Industry Gets Crafty with Terpenes*. Available at: <https://cen.acs.org/biological-chemistry/natural-products/Cannabis-industry-crafty-terpenes/97/i29> (Accessed April 13, 2021).
- Erythropel, H. C., Jabba, S. V., DeWinter, T. M., Mendizabal, M., Anastas, P. T., Jordt, S. E., et al. (2019). Formation of Flavorant-Propylene Glycol Adducts with Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids. *Nicotine Tob. Res.* 21 (9), 1248–1258. doi:10.1093/ntr/nty192
- Ethan Russo, B. J. M. (2017). "Cannabinoid Pharmacology," in *Advances in Pharmacology*. 2017. Available at: <https://www.sciencedirect.com/topics/neuroscience/cannabinol>.
- Gibbons, L. (2021). *Michigan House OKs ban on vitamin E acetate in vaping products*. Available at: <https://www.mlive.com/public-interest/2020/02/michigan-house-oks-ban-on-vitamin-e-acetate-in-vaping-products.html> Feb 06, 2020).
- Hajek, P., Etter, J.-F., Benowitz, N., Eissenberg, T., and McRobbie, H. (2014). Electronic Cigarettes: Review of Use, Content, Safety, Effects on Smokers and Potential for Harm and Benefit. *Addiction* 109 (11), 1801–1810. doi:10.1111/add.12659
- Heinzerling, A., Armatas, C., Karmarkar, E., Attfield, K., Guo, W., Wang, Y., et al. (2020). Severe Lung Injury Associated with Use of E-Cigarette, or Vaping, Products-California 2019. *JAMA Intern. Med.* 180 (6), 861–869. doi:10.1001/jamainternmed.2020.0664
- Hudalla, C. (2020). Rumpelstiltskin's Modern Day Fairytale: The Allure (And Hazards) of Delta-8-THC. Available online: <https://www.labtube.tv/video/MTA3NjQz> (Accessed April 13, 2021).
- King, M. (2020). *What's in that Vape?* Cannabis & vaping PUBLIC HEALTH INSIDER.
- Kupipathanant, N. (2014). The Effect of Heating on Vitamin E Decomposition in Edible Palm. *Oil Int. J. Environ. Rural Develop.* 5 (2), 5–2. Available at: <http://iserd.net/ijerd52/IJERD%205-2-23.pdf>.
- LeBouf, R. F., Burns, D. A., Ranpara, A., Attfield, K., Zwack, L., and Stefaniak, A. B. (2018). Headspace Analysis for Screening of Volatile Organic Compound Profiles of Electronic Juice Bulk Material. *Anal. Bioanal. Chem.* 410 (23), 5951–5960. doi:10.1007/s00216-018-1215-3
- Livingston, C. J., Freeman, R. J., Costales, V. C., Westhoff, J. L., Caplan, L. S., Sherin, K. M., et al. (2019). Electronic Nicotine Delivery Systems or E-Cigarettes: American College of Preventive Medicine's Practice Statement. *Am. J. Prev. Med.* 56 (1), 167–178. doi:10.1016/j.amepre.2018.09.010
- Mary Jane Brown, R. L. *Adrienne Seitz (Reviewed)*, MCT Oil 101: A Review Of Medium-Chain Triglycerides. Available at: <https://www.healthline.com/nutrition/mct-oil-101#other-potential-benefits> (May 17, 2020).
- Matsumoto, S., Fang, X., Traber, M. G., Jones, K. D., Langelier, C., Hayakawa Serpa, P., et al. (2020). Dose-Dependent Pulmonary Toxicity of Aerosolized Vitamin E Acetate. *Am. J. Respir. Cel Mol Biol* 63 (6), 748–757. doi:10.1165/rcmb.2020-0209oc

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- Muñoz, P., and Munné-Bosch, S. (2019). Vitamin E in Plants: Biosynthesis, Transport, and Function. *Trends Plant Sci.* 24 (11), 1040–1051. doi:10.1016/j.tplants.2019.08.006
- Muthumalage, T., Lucas, J. H., Wang, Q., Lamb, T., McGraw, M. D., and Rahman, I. (2020). Pulmonary Toxicity and Inflammatory Response of E-Cigarette Vape Cartridges Containing Medium-Chain Triglycerides Oil and Vitamin E Acetate: Implications in the Pathogenesis of EVALI. *Toxics* 8 (3), 46. doi:10.3390/toxics8030046
- Peace, M. R., Butler, K. E., Wolf, C. E., Poklis, J. L., and Poklis, A. (2016). Evaluation of Two Commercially Available Cannabidiol Formulations for Use in Electronic Cigarettes. *Front. Pharmacol.* 7, 279. doi:10.3389/fphar.2016.00279
- Peace, M. R., Krakowiak, R. I., Wolf, C. E., Poklis, A., and Poklis, J. L. (2017). Identification of MDMB-FUBINACA in Commercially Available E-Liquid Formulations Sold for Use in Electronic Cigarettes. *Forensic Sci. Int.* 271, 92–97. doi:10.1016/j.forsciint.2016.12.031
- Peace, M. R., Mulder, H. A., Baird, T. R., Butler, K. E., Friedrich, A. K., Stone, J. W., et al. (2018). Evaluation of Nicotine and the Components of E-Liquids Generated from E-Cigarette Aerosols. *J. Anal. Toxicol.* 42 (8), 537–543. doi:10.1093/jat/bky056
- Poklis, J. L., Mulder, H. A., and Peace, M. R. (2019). The Unexpected Identification of the Cannabimimetic, 5F-ADB, and Dextromethorphan in Commercially Available Cannabidiol E-Liquids. *Forensic Sci. Int.* 294, e25–e27. doi:10.1016/j.forsciint.2018.10.019
- Post, W. What Do Different Vape Temperatures Mean for Cannabinoids and Terpenes? Available at: <https://www.siranaturals.org/blog/what-do-different-vape-temperatures-mean-for-cannabinoids-and-terpenes> (Accessed on 12/9/2020).
- PRLOG, *Hon Lik* – (2010). *Kancer Hero Nominee*. Available at: <https://www.prlog.org/10588774-hon-lik-2010-kancer-hero-nominee.html> (Accessed April 13, 2021).
- Prochaska, J. J. (2019). The Public Health Consequences of E-cigarettes: a Review by the National Academies of Sciences. A Call for More Research, a Need for Regulatory Action. *Addiction* 114 (4), 587–589. doi:10.1111/add.14478
- Raber, J. C., Elzinga, S., and Kaplan, C. (2015). Understanding Dabs: Contamination Concerns of Cannabis Concentrates and Cannabinoid Transfer during the Act of Dabbing. *J. Toxicol. Sci.* 40 (6), 797–803. doi:10.2131/jts.40.797
- Rehan, H. S., Maini, J., and Hungin, A. P. S. (2018). Vaping versus Smoking: A Quest for Efficacy and Safety of E-Cigarette. *Cds* 13 (2), 92–101. doi:10.2174/1574886313666180227110556
- Sarma, N. D., Waye, A., ElSohly, M. A., Brown, P. N., Elzinga, S., Johnson, H. E., et al. (2020). Cannabis Inflorescence for Medical Purposes: USP Considerations for Quality Attributes. *J. Nat. Prod.* 83 (4), 1334–1351. doi:10.1021/acs.jnatprod.9b01200
- Sigma, M. (2020). *SPME for GC Analysis, Getting Started with Solid Phase Microextraction*. Available at: https://www.sigmaldrich.com/analytical-chromatography/sample-preparation/spme.html?utm_source=redirect&utm_medium=promotional&utm_campaign=spme (Accessed April 13, 2021).
- Sternlicht, A. (2020). *Remember Vaping Injuries? They're Climbing Again in Some Places, but Covid-19 Makes Them Harder to Diagnose*. Forbes News. Available at: <https://www.forbes.com/sites/alexandernsternlicht/2020/07/24/remember-vaping-injuries-theyre-climbing-again-in-some-places-but-covid-19-makes-them-harder-to-diagnose/?sh=50f99ce2550a> (June 24, 2020).
- Strongin, R. M. (2019). E-cigarette Chemistry and Analytical Detection. *Annu. Rev. Anal. Chem.* 12 (1), 23–39. doi:10.1146/annurev-anchem-061318-115329
- Strongin, R. M. (2020). Toxic Ketene Gas Forms on Vaping Vitamin E Acetate Prompting Interest in its Possible Role in the EVALI Outbreak. *Proc. Natl. Acad. Sci. USA* 117 (14), 7553–7554. doi:10.1073/pnas.2003384117
- The Good Scents Company Information System (2020). Available at: <http://www.thegoodscentscompany.com/index.html> (Accessed April 13, 2021).
- The National Academies of Sciences, E (2018). in *Medicine, Public Health Consequences Of E-Cigarettes*. Editors D. L. Eaton, L. Y. Kwan, and K. Stratton (Washington (DC): National Academies Press (US)).
- The National Academies of Sciences, E (2017). *Medicine, the Health Effects Of Cannabis And Cannabinoids: The Current State Of Evidence And Recommendations For Research*. (Washington (DC): National Academies Press (US)).
- The Ultimate Guide To Vap, 1657. *The Ultimate Guide to Vaping Temperatures*. Available at: <https://www.zamnesia.com/blog-the-ultimate-vaping-temperature-guide-n1657>, (Accessed on 12/15/2020).
- Traboulsi, H., Cherian, M., Above Rjeili, M., Preteroti, M., Bourbeau, J., Smith, B. M., et al. (2020). Inhalation Toxicology of Vaping Products and Implications for Pulmonary Health. *Int. J. Mol. Sci.* 21 (10), 3495. doi:10.3390/ijms21103495
- Trout, W. D., and DiDonato, M. D. (2017). Carbonyl Compounds Produced by Vaporizing Cannabis Oil Thinning Agents. *J. Altern. Complement. Med.* 23 (11), 879–884. doi:10.1089/acm.2016.0337
- United States Department of Agriculture Food Safety and Inspection Service (2013). *CLG-TOX1.01: Identification of Poisons and Toxins by GC/MS*. Available at: <https://www.fsis.usda.gov/horses/CLG-TOX1.pdf>.
- USFDA (1938a). CFR - Code of Federal Regulations Title 21, CHAPTER I-FOOD AND DRUG ADMINISTRATION, Sec. 184.1025 Caprylic Acid. Available at: <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1025> (Revised as of April 1, 2019).
- USFDA (1938b). CFR - Code of Federal Regulations Title 21, CHAPTER I-FOOD AND DRUG ADMINISTRATION, Sec. 178.3750 Polyethylene Glycol (Mean Molecular Weight 200-9,500). Available at: <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=178.3750> (Revised as of April 1, 2019).
- USFDA (2020c). *Lung Illnesses Associated with Use of Vaping Products*. Available online: <https://www.fda.gov/news-events/public-health-focus/lung-illnesses-associated-use-vaping-products#Analysis> (Accessed on Dec 8, 2020).
- USFDA (2020b). *Nonclinical Testing of Orally Inhaled Nicotine-Containing Drug Products, Guidance for Industry*. Available at: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/nonclinical-testing-orally-inhaled-nicotine-containing-drug-products-guidance-industry> (Accessed April 13, 2021).
- USFDA (2020a). *Vaporizers, E-Cigarettes, and Other Electronic Nicotine Delivery Systems (ENDS)*. Available at: <https://www.fda.gov/tobacco-products/products-ingredients-components/vaporizers-e-cigarettes-and-other-electronic-nicotine-delivery-systems-ends> (Accessed April 13, 2021).
- Wagner, J., Chen, W., and Vrdoljak, G. (2020). Vaping Cartridge Heating Element Compositions and Evidence of High Temperatures. *PLoS One* 15 (10), e0240613.
- Wilhelm, J. (2018). What Is Delta-8-THC and How Is it Different From Delta-9? *Leafly*. Available at: <https://www.leafly.com/news/science-tech/what-is-delta8-thc> (March 8, 2018).
- Williams, J. (2020). "Phytocannabinoid Known Unknowns," in *Science of Cannabis 2020 Online Symposium*. Available at: <https://www.caymanchem.com/literature/phytocannabinoid-known-unknowns> (Accessed April 13, 2021).
- Wu, D., and O'Shea, D. F. (2020). Potential for Release of Pulmonary Toxic Ketene from Vaping Pyrolysis of Vitamin E Acetate. *Proc. Natl. Acad. Sci. USA* 117 (12), 6349–6355. doi:10.1073/pnas.1920925117

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Gas Chromatography-Tandem Mass Spectrometry Method for the Selective Detection of Glycols and Glycerol in the Liquids and Aerosols of E-Cigarette, or Vaping, Products

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The long-term health effects of using e-cigarette, or vaping, products (EVPs; also known as e-cigarettes, electronic nicotine delivery systems, and vape pens) remain largely unknown. The inhalation of excipients, such as propylene glycol (PG) and glycerin (GLY), may have long-term health effects. In addition to the direct health effects of PG and GLY, glycerin-containing products can be contaminated with toxic ethylene glycol (EG) and diethylene glycol (DEG). To assess this issue, we developed a simple, versatile, high-throughput isotope dilution gas chromatography-tandem mass spectrometry method for quantifying these common excipients and contaminants. The method is applicable to both the liquid contents and machine-generated aerosols of EVPs. Our rigorous method validation demonstrates that the new method is specific, precise, accurate, and rugged/robust. The calibration range is linear from 0.1–7 mg for the excipients and 2.5–1,000 µg for the contaminants. These ranges encompass expected excipients levels in EVP e-liquids and their machine-generated aerosols and the relevant maximum residue safety limit of 1 mg/g, or 0.1% (w/w), for the contaminants. The calculated limits of detection for PG, GLY, EG, and DEG were determined as 0.0109 mg, 0.0132 mg, 0.250 µg, and 0.100 µg, respectively. The method was applied to the aerosol emissions analysis of 141 EVPs associated with the 2019 lung injury outbreak, and found typical levels of PG (120.28–689.35 mg/g of aerosol) and GLY (116.83–845.96 mg/g of aerosol) in all nicotine-containing products; PG (81.58–491.92 mg/g of aerosol) and GLY (303.86–823.47 mg/g of aerosol) in 13% of cannabidiol (CBD) products; PG (74.02–220.18 mg/g of aerosol) and GLY (596.43–859.81 mg/g of aerosol) in products with neither nicotine nor CBD; and none detected in tetrahydrocannabinol (THC) products. No products contained glycol contaminants above the recommended maximum residue safety limit.

Keywords: e-cigarettes, ethylene glycol, diethylene glycol, propylene glycol, glycerin, gas chromatography-tandem mass spectrometry

INTRODUCTION

Since entering the United States marketplace in 2007, the product landscape and popularity of e-cigarette, or vaping, products (EVPs) has expanded considerably (Olfson et al., 2019). Having evolved from their original cigarette-like appearance, a multitude of EVPs that differ in design and function are sold commercially and have gained widespread market acceptance (Gentzke et al., 2019; Huang et al., 2019). Despite their differences in design, nearly all EVPs operate based on the same basic principle of generating an aerosol from a liquid mixture of dissolved flavors and active ingredients (e.g., nicotine, THC, CBD). This solution, often referred to as the e-liquid, is resistively heated, and rapidly condensed into an aerosol as the user inhales air (puffs) through the device. Many e-liquids consist of mixtures with varying concentrations of propylene glycol (PG) and glycerin (GLY; also known as glycerol) diluents. The PG/GLY mixture serves as the excipient for efficient aerosolization and transfer of the active ingredient and flavor constituents from the EVP liquid to the user *via* the inhaled aerosol. Both PG and GLY are substances considered “generally recognized as safe” for human oral consumption by the United States Food and Drug Administration (USFDA) (21 C.F.R. § 182, 2020; 21 C.F.R. § 184, 2020). Both are used in a wide variety of consumer products including foods, medicines, cosmetics, and many types of personal care products. Although PG and GLY exposure *via* oral and dermal routes appear to be innocuous, little is known of the long-term health consequences of inhaled PG and GLY from sources such as EVPs (Callahan-Lyon, 2014). Although the EVP aerosol contains fewer known carcinogens than tobacco smoke, more data is needed to characterize their long-term health effects (National Academies of Sciences et al., 2018; Gotts et al., 2019).

A potentially compounding health risk associated with PG- and GLY-containing e-liquids is the possible contamination of raw materials with the toxic glycols, ethylene glycol (EG) and diethylene glycol (DEG) (Molever, 2010; Famele et al., 2015; Kavvalakis et al., 2015; Varlet et al., 2015) — known nephrotoxins and hepatotoxins which cause acute renal failure. Historically, many poisonings have occurred because of DEG contamination of GLY-containing products (Schep et al., 2009). The first documented case (1937) resulted in the deaths of more than 100 Americans across 15 states and prompted the enactment of the United States Food, Drug, and Cosmetic Act (FD&C Act) in 1938. More recent poisonings from DEG-contaminated products prompted the USFDA and the United States Pharmacopeia (USP) to issue guidance to manufacturers by recommending defined screening methods to ensure EG and DEG concentrations do not exceed the specified maximum residue safety limit of 1 mg/g, or 0.1% (w/w), of either substance in PG/GLY-containing products (USFDA Center for Drug Evaluation and Research, 2007; USP/NF, 2009). However, prior to 2016, the FD&C Act, and the guidelines and limits recommended and set by the USFDA and the USP, did not give the USFDA regulatory authority over EVPs and their components (including e-liquids); exposing the market to a wide array of products, including counterfeit and potentially contaminated products (Gottlieb, 2019). For example, in 2013,

the USFDA issued alerts concerning possible DEG-contaminated EVPs and e-liquids imported from China entering the United States market (Peace et al., 2016). The USFDA ultimately gained regulatory authority over EVPs and their components in 2016; enabling scrutiny like that of other tobacco products, as well as the guidance laid out by the USFDA/USP for contaminants screening.

Despite the USFDA’s regulatory authority, no guarantees fully prevent the intentional or unintentional adulteration of do-it-yourself (DIY) (Cox et al., 2019), at-home e-liquid recipes prepared by persons attempting to make their own e-liquids, and/or the illegal introduction of counterfeit products. It is, therefore, important to remain vigilant in monitoring for these excipients and contaminants using accurate and reliable methodology that are fit for purpose. The USFDA/USP guidance describes a gas chromatography-flame ionization detector (GC-FID) method and offers an alternative procedure using a thin-layer chromatography (TLC) method (Kenyon et al., 1998). Other methods employ the use of derivatization (Molever, 2010; Kavvalakis et al., 2015) and/or do not target all four analytes mentioned thus far (Holloway et al., 2010; Varlet et al., 2015). Other methods have been developed to rapidly pre-screen samples prior to more thorough quantitative analyses by the GC-FID method, requiring two analytical runs for a possibly contaminated sample (Self, 2013; Peace et al., 2016). We describe here the development, validation, and application of a new, simple, sensitive, and selective isotope dilution gas chromatography-tandem mass spectrometry (ID-GC-MS/MS) method for the simultaneous quantitation and characterization of PG, GLY, EG, and DEG in the e-liquids and machine-generated aerosol emissions of EVP devices. This method was applied to the aerosol emissions analysis of EVPs associated with the 2019 outbreak of e-cigarette, or vaping, product use-associated lung injury (EVALI) (Blount et al., 2019; Centers for Disease Control and Prevention Office on Smoking and Health, 2019a; Blount et al., 2020; U.S. Food and Drug Administration, 2020).

MATERIALS AND METHODS

Chemicals and Materials

Propylene glycol (PG; CAS# 57-55-6; ≥99.5%; meets USP testing specifications), glycerin/glycerol (GLY; CAS# 56-81-5; ≥99.5%), ethylene glycol (EG; CAS# 107-21-1; ≥99.9%; analytical standard), diethylene glycol (DEG; CAS# 111-46-6; 99.8%; pharmaceutical secondary standard; certified reference material), and isotopically labeled propylene glycol-*d*₈ (PG-*d*₈; CAS# 80156-55-4; isotopic purity: 98 atom % D, 99% chemical purity), glycerin/glycerol-*d*₈ (GLY-*d*₈; CAS# 7325-17-9; isotopic purity: ≥98 atom % D, ≥98% chemical purity), and ethylene glycol-*d*₄ (EG-*d*₄; CAS# 107-21-1; isotopic purity: 98 atom % D, 99% chemical purity) were obtained from Sigma-Aldrich (St. Louis, MO, United States). Isotopically labeled diethylene glycol-*d*₈ (DEG-*d*₈; CAS# 102867-56-1) was obtained from Toronto Research Chemicals (North York, ON, Canada). A second set of alternate-source, unlabeled PG, GLY, DEG, and EG standards



FIGURE 1 | Experimental vaping setup of various EVPs analyzed. The two on the left demonstrate the custom-built holders and “lips” used for vaping products with mouthpieces of differing shape than those to the right which fit standard holders.

were purchased from the U.S. Pharmacopeia (Rockville, MD, United States).

Methanol (MeOH; CAS# 67-56-1; HPLC grade; $\geq 99.9\%$) was obtained from Thermo Fisher Scientific (Waltham, MA, United States). Research grade helium (He) and ultra-high purity grade nitrogen (N_2) gases were obtained from Airgas, Inc. (Hapeville, GA, United States). Deionized water ($dI-H_2O$) was generated in-house using an Aqua Solutions model RODI-C-11BL ultrapure water (18 M Ω) purifications system (Jasper, GA, United States).

Cambridge filter holders used for collecting aerosol were purchased from Cerulean (Molins PLC, Milton Keynes, United Kingdom). Cambridge filter pads (CFPs; 44 mm) were purchased from Thermo Fisher Scientific (Waltham, MA, United States). Custom-made adapters (“lips”) used for vaping uniquely shaped device mouthpieces were fabricated in-house (see **Figure 1**).

Standard and Quality Control Material Preparation

A Rainin AutoRep E Repeating Dispenser with corresponding syringe tips (Mettler-Toledo, Columbus, OH, United States) was used for positive displacement pipetting in the preparation of the following standard and quality control solutions. All solutions were stable and stored at room temperature.

Isotopically Labeled Internal Standards

Individual labeled internal standard (ISTD) stock solutions for each analyte were prepared in MeOH and combined to yield a single ISTD spiking solution with concentrations of 10 mg/ml (PG and GLY) and 500 μ g/ml (EG and DEG). A 100 μ l aliquot of this ISTD spiking solution was spiked into calibration standard

solutions (described below) and all blanks, unknowns, and quality control (QC) samples.

Native Standards

Individual stock solutions for each unlabeled (native) analyte were prepared in MeOH and combined to prepare four stock solutions. Individual PG and GLY stock solutions were combined to prepare two stock solutions (A and B) with concentrations of 0.5 and 50 mg/ml, respectively. Individual EG and DEG stock solutions were combined to prepare two other stock solutions (C and D) with concentrations of 12.5 and 500 μ g/ml, respectively. Stock solutions A–D were then used to prepare nine calibration standard solutions with concentration ranges of 0.01–7 mg/ml (PG and GLY) and 0.25–100 μ g/ml (EG and DEG). Calibration standard solutions were all spiked with ISTD spiking solution upon preparation.

QC Materials

Additional mixtures were prepared to serve as matrix-based QC materials with low and high analyte levels spanning the calibration ranges of both analyte groups. The first QC pool mixture, QC_a, was prepared by combining 32 g PG, 4 g GLY, 1 mg DEG, 1 mg EG, and 4 g $dI-H_2O$ to give 800 mg/g PG, 100 mg/g GLY, and 25 μ g/g EG and DEG [density-adjusted PG, GLY, and $dI-H_2O$ composition corresponded to 80/10/10 PG/GLY/ $dI-H_2O$ (v/v/v)]. A second QC pool, QC_b, was prepared by combining 4 g PG, 32 g GLY, 240 mg DEG, 240 mg EG, and 4 g $dI-H_2O$ to give 100 mg/g PG, 800 mg/g GLY, and 6,000 μ g/g EG and DEG [density-adjusted PG, GLY, and $dI-H_2O$ composition corresponded to 10/80/10 PG/GLY/ $dI-H_2O$ (v/v/v)]. The PG and GLY levels between QC_a and QC_b pools were intended to account for possible variations of PG/GLY compositions in commercial products available on the market. The QC

concentration levels were characterized to determine the mean concentrations and the 95th (1.96 σ) and 99th (2.96 σ) control limits by duplicate analysis of 20 samples of each QC level over at least 20 days. A 150 mg aliquot of each QC pool was extracted and analyzed concurrently with sample unknowns and the resulting QC data were compared to the established control limits to evaluate the validity of analyses using modified Westgard rules (Westgard et al., 1981; Caudill et al., 2008).

Aerosols/Vaping

For aerosol analyses, samples were generated using a Cerulean CETI-8 e-cigarette vaping machine equipped with button activation switches (Cerulean, Richmond, VA) as shown in **Figure 1**. A soap bubble meter was used to calibrate and verify the vaping machine puff volume prior to use. Samples were vaped according to the standard conditions described in CORESTA Recommended Method No. 81 (CORESTA, 2015) (i.e., 55 ± 0.3 ml puff volume, 3 ± 0.1 s puff duration, 30 ± 0.5 s puff interval, with a square wave puff profile). The aerosol from 15 puffs (no clearing puffs) taken from vaped EVPs was collected on individual CFPs and gravimetrically determined ($d = 0.00001$ g) by mass difference of pre- and post-vaping CFPs for a given sample [i.e., trapped total particulate matter (TPM)]. The puff number could be varied, when necessary or appropriate, up to 50 puffs. Post-vaped CFPs were carefully removed from CFP holders and placed into 16 ml vials for extraction.

EVP e-Liquids Sampling

EVP products vary significantly in their physical designs and, therefore, product-specific means were used to disassemble, when necessary, remove, and transfer approximately 100–150 mg liquid from a given product to a 16 ml vial for extraction. Sample masses of the liquids removed were recorded.

Sample Preparation

Sample vials containing blanks, QCs, and post-vaped CFP and/or liquid unknowns were spiked with isotopically labeled ISTD spiking solution. Ten milliliters (10 ml) of MeOH was then added to each vial and all samples placed on an orbital shaker for 10 min at 160 rpm. An undiluted aliquot of extract was transferred to GC autosampler vials for EG and DEG analysis, whereas, for PG and GLY, a 10-fold dilution of sample extracts was done prior to analysis.

Instrumental Analysis

Instrument parameters were optimized for chromatographic performance (i.e., injection, separation, peak shape, run time) and sensitive detection (i.e., collision energies, gain) of each analyte. An Agilent 7890B GC equipped with an Agilent GC Injector 80 autosampler and interfaced to an Agilent 7000C triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, United States) was used for GC-MS/MS analysis. Two separate injections were made for each sample: one for PG and GLY and one for EG and DEG analyses. A 1 μ l aliquot of sample extract was injected onto a 15 m Agilent J&W DB-WAX capillary column with a 0.25 mm I.D. and 0.50- μ m film

thickness using a 400:1 (PG and GLY) and 10:1 (EG and DEG) split injection. Helium carrier gas was used at a constant flow of 1 ml/min. The injector and transfer line temperatures were set isothermally at 230 and 240°C, respectively. The initial column temperature, 100°C, was held for 1 min, increased to 180°C at 60°C/min, held for 0.5 min, and then increased to 240°C at 60°C/min and held for 3 min. The mass spectrometer was operated in positive electron ionization (+EI) mode and the resulting ions mass analyzed *via* selected-reaction monitoring (SRM). MS/MS parameters were as follows: electron energy –70 eV, source temperature 230°C, MS1 and MS2 quadrupole temperatures 150°C, electron multiplier voltage gain factor 10 (PG, DEG, EG) and 1 (GLY), mass resolution wide (MS1) and unit (MS2), collision cell quench gas (He) 2.25 ml/min, collision cell collision gas (N₂) 1.5 ml/min.

Quantitation and confirmation ion transitions were monitored for each analyte and an isotopically labeled ISTD ion transition was monitored for the corresponding quantitation ion transition. The SRM transitions monitored, collision energies, dwell times, and transition type used for analysis are summarized in **Table 1**. Data acquisition and analysis were conducted using Agilent MassHunter Workstation software.

Quantitation

Calibration curves were constructed from the linear regression of the calibration standards' analyte-to-ISTD response ratios versus known standard concentrations, x , with $1/x$ weighting. The broad calibration concentration ranges used required weighting to improve the accuracy of the lower calibrators. For aerosol analysis, results (output in mg PG/GLY and μ g EG/DEG) were normalized by TPM mass and/or puff count to determine analyte yields per gram of TPM (mg/g TPM for PG and GLY; μ g/g TPM for EG and DEG) and/or per puff (mg/puff for PG and GLY; μ g/puff for EG and DEG). For e-liquid analysis, results were normalized by e-liquid sample mass to determine analyte levels per gram of sample (mg/g for PG and GLY; μ g/g for EG and DEG). A GLY-concentration-dependent correction factor for final calculation of EG measurements was also necessarily imposed (discussed below) according to the following equation:

$$EG_{corrected} = EG_{measured} - (GLY_{measured} \times 0.0145) \quad (1)$$

where:

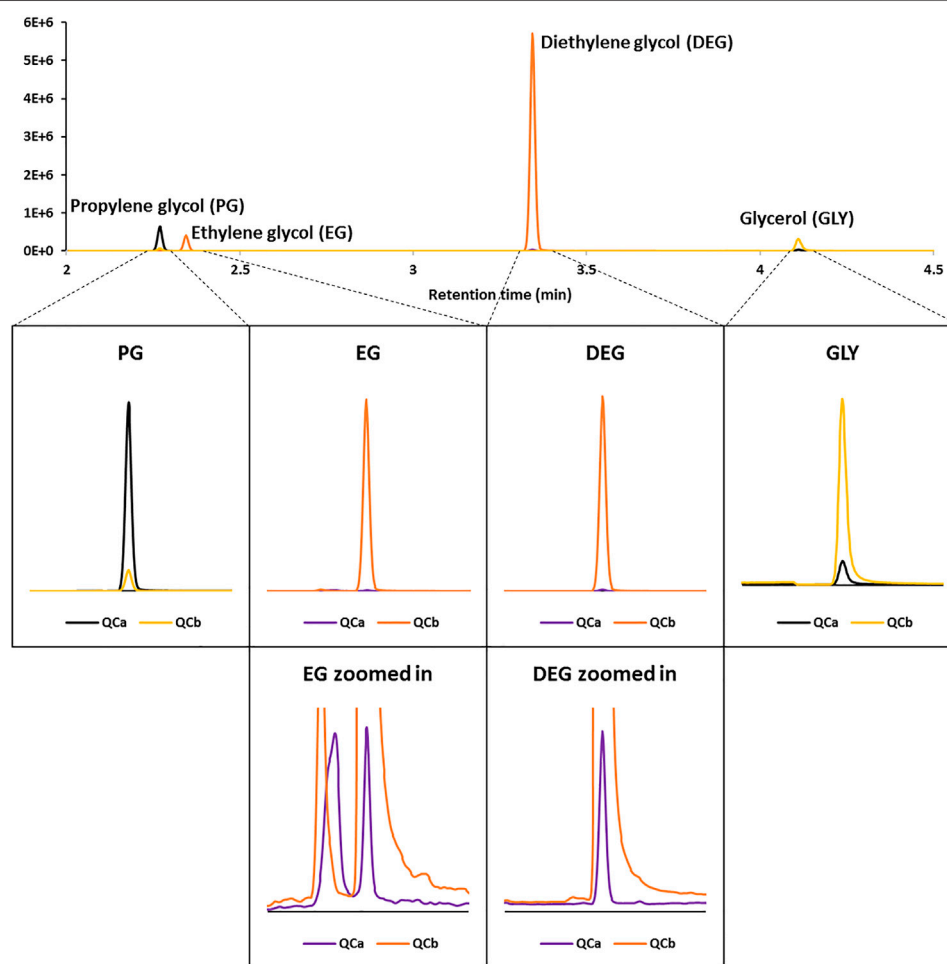
- $EG_{corrected}$ is the corrected amount of EG in μ g
- $EG_{measured}$ is the amount of EG in μ g measured by the instrument
- $GLY_{measured}$ is the amount of GLY in mg measured by the instrument
- 0.0145 is the average amount of EG in μ g produced per mg of GLY within a given sample

Method Validation

A full method validation was performed to confirm that the performance characteristics of the methods were accurate and fit-for-purpose. Figures of merit included analytical

TABLE 1 | SRM method specifications.

Analyte	Ion transitions	Collision energy (V)	Dwell time (msec)	Transition type
Propylene glycol (PG)	61.0→43.2	3	30	Quantitation
	45.1→43.2	15		Confirmation
	64.1→46.1	3		ISTD
Glycerol (GLY)	61.0→43.2	5	50	Quantitation
	61.0→61.0	1		Confirmation
	64.1→46.2	5		ISTD
Ethylene glycol (EG)	62.0→33.3	1	30	Quantitation
	62.0→31.3	5		Confirmation
	66.1→36.3	1		ISTD
Diethylene glycol (DEG)	76.1→45.2	8	30	Quantitation
	75.1→45.2	3		Confirmation
	82.1→49.1	8		ISTD

**FIGURE 2** | Overlaid selected-reaction monitoring (SRM) chromatograms of the two QC levels.

specificity, accuracy, dynamic range, linearity, limits of detection, matrix effects, precision, and ruggedness/robustness. A description of experiments and presentation of their results are described below in corresponding sections of the Results and Discussion section.

Application: Products Associated With the 2019 EVALI Outbreak

The method was applied to measure glycols and glycerol in the aerosol emissions of 141 EVP products associated with the EVALI outbreak. These products were categorized as tetrahydrocannabinol

(THC) containing products if THC >0.3% (w/w), nicotine containing products if nicotine >0.2% (w/w), and cannabidiol (CBD) products if CBD >1% (w/w) and THC <0.3%. A total of 194 samples was received; however, only 141 of these products were analyzed, as 35 products did not contain enough volume for testing and 18 products did not generate sufficient aerosol TPM deliveries (products that generated aerosols of less than 6.5 mg TPM per 15 puffs were considered inoperative and their data excluded). Samples were machine-vaped as described in *Aerosols/Vaping* and 15 puffs collected per product. All samples were handled following proper guidelines for the handling and analysis of potentially illicit drugs. Sample chain-of-custody was maintained and documented.

RESULTS AND DISCUSSION

Figures of Merit

Analytical Specificity

Specificity was demonstrated by baseline-resolved chromatograms and the absence of interfering matrix components in representative EVP samples. **Figure 2** shows overlaid SRM chromatograms of the two QC levels. The use of isotopically labeled ISTDs also provided an additional level of retention time specificity. Despite their relatively simple structures and small masses, each analyte produced MS/MS spectra which allowed for the monitoring of distinct quantitation and confirmation ion transitions (**Table 1**). The only exception, GLY, had no additional product ion that could be used for confirmation. In this case, a pseudo-MS/MS ion transition was used by monitoring m/z 61 for both precursor and product ions with a low collision energy voltage. MS/MS response ratios between quantitation and confirmation ion transitions further increased method specificity.

Dynamic Range, Linearity, and Limits of Detection

LODs determined based on the method described by Taylor (1987) yielded calculated analyte LODs well below the intended purpose of the described method. Therefore, calibration (dynamic) ranges were selected such that the lowest reportable limit (lowest calibrator) was health relevant and application appropriate. The calibration range chosen for PG and GLY (0.1–70 mg) encompassed a full range of e-liquid compositions that may be possible in EVPs. The calibration range for EG and DEG (2.5–1,000 μ g) was primarily chosen to encompass the maximum residue limits set by the USFDA (1 mg/g or 0.1%) and US Pharmacopeia Convention (620 μ g/g or 0.062%). The concentration range implemented also encompassed potential EG and DEG levels below and above that of the specified limits for additional screening capabilities. Calibration curve linearity was confirmed by residuals analysis of the linear regression of seven separately prepared calibration curves with a coefficient of determination (R^2) >0.98. Individual calibration curves yielded R^2 > 0.99. A summary of method dynamic ranges, linearity, and calculated limits of detection can be found in **Supplementary Table S1** of the Supplementary Material.

Accuracy and Matrix Effects

An accuracy study was conducted to evaluate potential concentration or matrix-based effects (i.e., PG/GLY content, samples with and without CFPs, and sample size) to ensure

compatibility with: 1) the analysis of the diverse and dynamic market of EVPs; 2) both e-liquid (no CFP) and aerosol (with CFP) samples; and 3) sample sizes of varying degrees stemming from variable aerosol deliveries between products, product types, and/or machine vaping regimes.

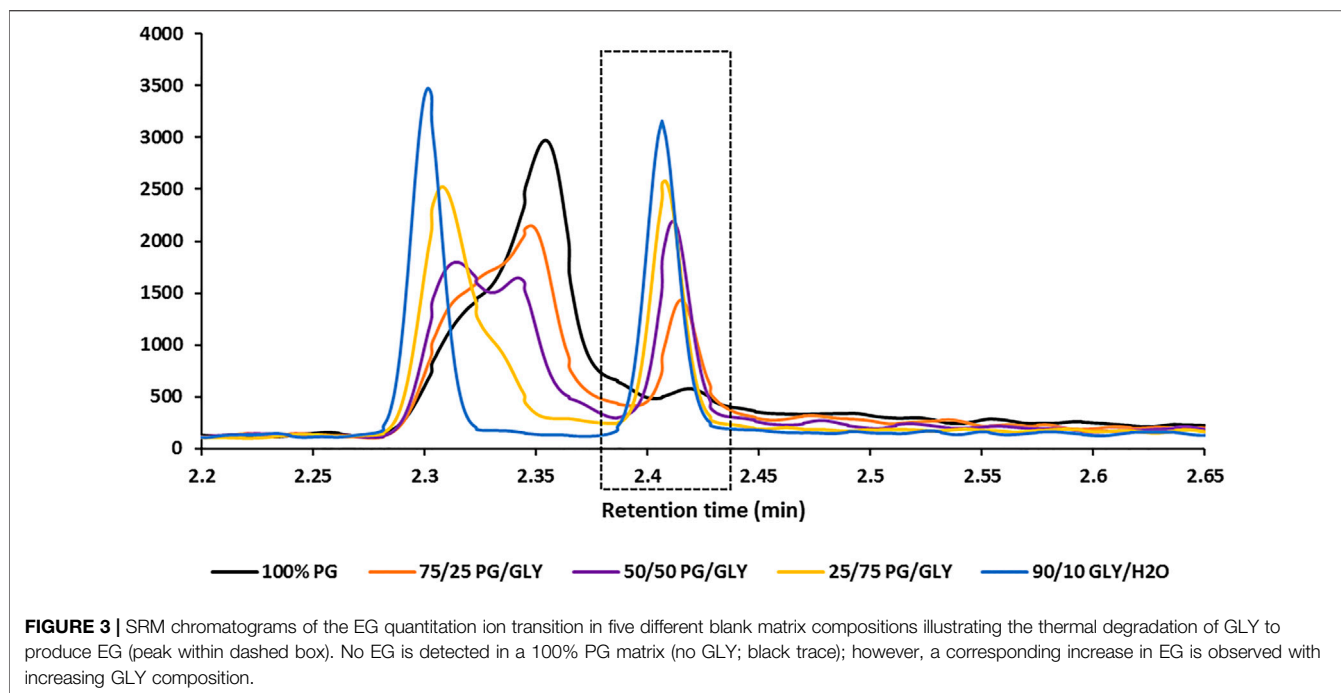
Solutions with known concentrations of varying PG/GLY composition [100% PG, 75/25 PG/GLY, 50/50 PG/GLY, 25/75 PG/GLY, and 90/10 GLY/H₂O (v/v)], each spiked with low, mid, and high concentrations of EG and DEG, were used as “matrix-matched” samples to assess accuracy. Accuracy results were acceptable at all concentrations and variations tested. All results were within 15% of their respective known concentrations, with most being within 5%. The accurate quantitation of matrix-based samples using a solvent-based calibration curve also indicated the absence of any matrix effects that could negatively affect measurements. These results show the applicability of the described method for accurate measurements in EVP e-liquid and aerosol samples of varying sample makeup. This recovery-based accuracy approach was necessary as no certified reference materials were available. A detailed summary of results can be found in **Supplementary Table S2** (excipients) and **Supplementary Table S3** (contaminants) of the Supplementary Material.

Precision

Method precision—evaluated as repeatability and intermediate precision—was assessed from the duplicate analysis of 20 samples of the QC materials (100 and 800 mg/g for PG and GLY; 25 and 6,000 μ g/g for EG and DEG) over at least 20 different days (**Supplementary Table S1**). Repeatability was calculated as within-run variation of duplicates, while intermediate precision was calculated as the among-run, or total, variation. PG and GLY repeatability and intermediate precision ranged between 0.41 and 1.39% relative standard deviations (%RSDs) and 5.34–6.55% RSD, respectively. For EG and DEG, repeatability and intermediate precision ranged between 0.62 and 7.28% RSD and 5.33–13.3% RSD, respectively, with greater reproducibility at the higher concentration. Overall, the method precision was deemed acceptable with %RSDs <15%.

Ruggedness/Robustness Testing

Method ruggedness/robustness was tested by evaluating critical method parameters [i.e., matrix/excipients composition, CFP (aerosol) vs. no CFP (e-liquid), sample mass, extraction time, and extraction volume] that could potentially affect method performance and applicability. The PG-to-GLY composition ratio, the presence/absence of a CFP in sample, and the sample amount/mass were evaluated as part of the previously described accuracy experiments and showed no influence on the accuracy of results (**Supplementary Tables S2, S3**). Increasing extraction volumes and extraction times also showed no appreciable differences. For extraction volume, a $\pm$ 20% change from the method-set parameter (10 ml MeOH) was tested and resulted in <3% difference in analytical results. Similarly, extraction time was also varied (15 and 30 min) from the method-set time of 10 min, and results showed <3% difference. Also tested was the vortexing (e-liquid) and repeat-inversion (10–15 $\times$) of samples (aerosol; CFPs fall apart if vortexed) rather than a defined extraction time at the defined



160 rpm. These results were also within 3% of results obtained under the prescribed method settings. These results indicate the efficient extraction/homogeneity of sample extracts prior to GC-MS/MS analysis.

Ethylene Glycol Correction Factor

An EG moiety is found within the chemical structure of GLY. Because GLY is otherwise chemically and structurally stable (i.e., no decomposition or equilibrium), it is presumed that the thermal degradation of GLY within the heated GC injection port produced small, but detectable levels of EG; artificially elevating measured EG levels. **Figure 3** illustrates the presence of an EG peak with the absence and increasing concentration of GLY from these mixtures. Measured EG levels from the analysis of blank PG/GLY mixtures [100% PG, 75/25 PG/GLY, 50/50 PG/GLY, 25/75 PG/GLY, and 90/10 GLY/H₂O (v/v)] was used to determine the GLY-generated concentration of EG per milligrams of GLY (μg EG/mg GLY). A corresponding increased production and detection of EG was observed with increasing GLY concentration. A 0.0145 ± 0.0012 (SD) μg EG/mg GLY correction factor was determined and used for final calculation of measured EG measurements.

Application: 2019 U.S. EVALI Outbreak

The method performed well in both hydrophilic (PG/GLY-based) and hydrophobic (oil-based medium chain triglycerides, vitamin E acetate, etc.) e-liquids obtained for the 2019 United States EVALI response, demonstrating its versatility. PG and GLY were detected at typical levels in nearly 43% (60 of 141) of the EVP aerosol samples analyzed, with no single product found to have had only PG or GLY alone. **Table 2** summarizes measured concentration ranges (mg/g of aerosol) for these excipients in

TABLE 2 | Measured propylene glycol (PG) and glycerin (GLY) excipient concentration ranges by product type in EVALI-associated aerosol samples.

Product type	<i>n</i>	PG (mg/g)	GLY (mg/g)
Hydrophilic (PG/GLY)			
Nicotine	39	120.28–689.35	116.83–823.47
CBD	2	106.26–491.92	322.67–635.07
no Active ingredient	19	74.02–443.72	491.33–859.81
Hydrophobic (oil-based) ^a	81	<LOD	<LOD

^aincludes products containing THC and products with no active ingredient

nicotine products (39 of the 60), CBD products (2 of the 60), and products with neither nicotine nor CBD (19 of the 60). PG (120.28–689.35 mg/g) and GLY (116.83–823.47 mg/g) were measured in all nicotine products. Although CBD products may be produced as either PG/GLY-based (hydrophilic) or oil-based (hydrophobic) liquids, only two CBD products were identified according to the criteria defined above (CBD >1%; THC <0.3%) and were both found to have PG (106.26 mg/g and 491.92 mg/g) and GLY (322.67 mg/g and 635.07 mg/g) as the excipients. Products void of either nicotine or CBD (i.e., no active ingredient) were also among the products analyzed with significant PG (74.02–443.72 mg/g) and GLY (491.33–859.81 mg/g) concentrations. Neither PG nor GLY were detected above LOD in any of the remaining 81 oil-based (hydrophobic), or THC-containing EVP samples [81 of 141 (57%)]. The absence of any detectable PG or GLY in the THC EVPs is consistent with the purportedly ubiquitous use of oil-based diluents [e.g., medium chain triglycerides (MCT) oil, coconut oil, vitamin E acetate, etc.]. Specifically, inhaled vitamin E acetate has been strongly linked with the EVALI outbreak (Blount et al., 2019; Centers for Disease Control and Prevention Office on Smoking and Health, 2019a;

U.S. Food and Drug Administration, 2020) and shown to cause lung injury in mice (Bhat et al., 2020).

For the glycol contaminants, no samples yielded results above the USFDA/USP specified relevant maximum residue safety limit of 1 mg/g (0.1% [w/w]). Trace signals of EG were detected in some samples (5%) but is most likely an analytical artifact of the previously discussed thermal degradation of GLY, as these samples contained GLY.

CONCLUSION

The described dual-purpose ID-GC-MS/MS method provides accurate and precise quantitation of EVP excipients (PG and GLY) concentrations in e-liquids and their machine-generated aerosols, as well as screening and quantitation capabilities of the contaminants, EG and DEG, from a single sample. The method can be used to ensure EVPs containing PG/GLY mixtures comply with USFDA and USP standards. Application of the method toward an array of EVPs associated with the 2019 EVALI outbreak showed that the method is fit for its intended purpose and demonstrated its versatility by extended applicability to oil-based EVPs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

REFERENCES

- 21 C.F.R. § 182 (2020). *Substances Generally Recognized as Safe*. U.S. Code of Federal Regulations.
- 21 C.F.R. § 184 (2020). *Direct Food Substances Affirmed as Generally Recognized as Safe*. U.S. Code of Federal Regulations.
- Bhat, T. A., Kalathil, S. G., Bogner, P. N., Blount, B. C., Goniewicz, M. L., and Thanavala, Y. M. (2020). An Animal Model of Inhaled Vitamin E Acetate and EVALI-like Lung Injury. *N. Engl. J. Med.* 382, 1175–1177. doi:10.1056/nejmc2000231
- Blount, B. C., Karwowski, M. P., Morel-Espinosa, M., Rees, J., Sosnoff, C., Cowan, E., et al. (2019). Evaluation of Bronchoalveolar Lavage Fluid from Patients in an Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - 10 States, August-October 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 1040–1041. doi:10.15585/mmwr.mm6845e2
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382, 697–705. doi:10.1056/nejmoa1916433
- Callahan-Lyon, P. (2014). Electronic Cigarettes: Human Health Effects. *Tob. Control.* 23 (Suppl. 2), ii36–ii40. doi:10.1136/tobaccocontrol-2013-051470
- Caudill, S. P., Schleicher, R. L., and Pirkle, J. L. (2008). Multi-rule Quality Control for the Age-Related Eye Disease Study. *Statist. Med.* 27, 4094–4106. doi:10.1002/sim.3222
- Centers for Disease Control and Prevention Office on Smoking and Health (2019a). Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products. Available at: <https://www.cdc.gov/lunginjury> [Accessed 12/25/2020].
- CORESTA (2015). *Recommended Method Number 81: Routine Analytical Machine for E-Cigarette Aerosol Generation and Collection – Definitions and Standard Conditions*. Paris, France: Cooperation Centre for Scientific Research Relative to Tobacco.

AUTHOR CONTRIBUTIONS

JP: methodology, validation, formal analysis, investigation, writing - original draft. CW: conceptualization, writing - review and editing, oversight. BB: conceptualization, visualization, funding acquisition, supervision, writing - review and editing. LV-B: visualization, supervision, project administration, funding acquisition, formal analysis, writing - review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.709495/full#supplementary-material>

- Cox, S., Leigh, N. J., Vanderbush, T. S., Choo, E., Goniewicz, M. L., and Dawkins, L. (2019). An Exploration into "Do-it-yourself" (DIY) E-Liquid Mixing: Users' Motivations, Practices and Product Laboratory Analysis. *Addict. Behaviors Rep.* 9, 100151. doi:10.1016/j.abrep.2018.100151
- National Academies of Sciences, Engineering, Medicine, Health, Medicine Division, Board on Population Health, Public Health Practice, and Committee on the Review of the Health Effects of Electronic Nicotine Delivery System (2018). *Public Health Consequences Of E-Cigarettes*. Editors D.L. Eaton, L.Y. Kwan, and K. Stratton (Washington (DC): National Academies Press (US)).
- Famele, M., Ferranti, C., Abenavoli, C., Palleschi, L., Mancinelli, R., and Draisci, R. (2015). The Chemical Components of Electronic Cigarette Cartridges and Refill Fluids: Review of Analytical Methods. *Nicotine Tob. Res.* 17, 271–279. doi:10.1093/ntr/ntu197
- Gentzke, A. S., Creamer, M., Cullen, K. A., Ambrose, B. K., Willis, G., Jamal, A., et al. (2019). Vital Signs: Tobacco Product Use Among Middle and High School Students - United States, 2011–2018. *MMWR Morb. Mortal. Wkly. Rep.* 68, 157–164. doi:10.15585/mmwr.mm6806e1
- Gottlieb, M. A. (2019). Regulation of E-Cigarettes in the United States and its Role in a Youth Epidemic. *Children (Basel)* 6, 40. doi:10.3390/children6030040
- Gotts, J. E., Jordt, S.-E., McConnell, R., and Tarran, R. (2019). What Are the Respiratory Effects of E-Cigarettes? *Bmj* 366, l5275. doi:10.1136/bmj.l5275
- Holloway, G., Maheswaran, R., Leeks, A., Bradby, S., and Wahab, S. (2010). Screening Method for Ethylene Glycol and Diethylene Glycol in Glycerin-Containing Products. *J. Pharm. Biomed. Anal.* 51, 507–511. doi:10.1016/j.jpba.2009.08.025
- Huang, J., Duan, Z., Kwok, J., Binns, S., Vera, L. E., Kim, Y., et al. (2019). Vaping versus JUULing: How the Extraordinary Growth and Marketing of JUUL Transformed the US Retail E-Cigarette Market. *Tob. Control.* 28, 146–151. doi:10.1136/tobaccocontrol-2018-054382
- Kavvalakis, M. P., Stivaktakis, P. D., Tzatzarakis, M. N., Kouretas, D., Liesivuori, J., Alegakis, A. K., et al. (2015). Multicomponent Analysis of Replacement Liquids

- of Electronic Cigarettes Using Chromatographic Techniques. *J. Anal. Toxicol.* 39, 262–269. doi:10.1093/jat/bkv002
- Kenyon, A. S., Xiaoye, S., Yan, W., Wai Har, N., Prestridge, R., and Sharp, K. (1998). Simple, At-Site Detection of Diethylene Glycol/ethylene Glycol Contamination of Glycerin and Glycerin-Based Raw Materials by Thin-Layer Chromatography. *J. AOAC Int.* 81, 44–50. doi:10.1093/jaoac/81.1.44
- Molever, K. (2010). Simplified Assay of Diethylene Glycol and Ethylene Glycol in Various Raw Materials by Capillary Gas Chromatography. *J. Cosmet. Sci.* 61, 225–234. doi:10.1111/j.1468-2494.2010.00619_2.x
- Olson, M., Wall, M. M., Liu, S.-M., Sultan, R. S., and Blanco, C. (2019). E-cigarette Use Among Young Adults in the U.S. *Am. J. Prev. Med.* 56, 655–663. doi:10.1016/j.amepre.2018.12.004
- Peace, M. R., Baird, T. R., Smith, N., Wolf, C. E., Poklis, J. L., and Poklis, A. (2016). Concentration of Nicotine and Glycols in 27 Electronic Cigarette Formulations. *J. Anal. Toxicol.* 40, 403–407. doi:10.1093/jat/bkw037
- Schep, L. J., Slaughter, R. J., Temple, W. A., and Beasley, D. M. G. (2009). Diethylene Glycol Poisoning. *Clin. Toxicol.* 47, 525–535. doi:10.1080/15563650903086444
- Self, R. L. (2013). Direct Analysis in Real Time-Mass Spectrometry (DART-MS) for Rapid Qualitative Screening of Toxic Glycols in Glycerin-Containing Products. *J. Pharm. Biomed. Anal.* 80, 155–158. doi:10.1016/j.jpba.2013.02.037
- Taylor, J. K. (1987). *Quality Assurance of Chemical Measurements*. Boca Raton, FL, United States: Taylor & Francis.
- USFDA Center for Drug Evaluation and Research (2007). *Guidance for Industry: Testing of Glycerin for Diethylene Glycol*. Rockville, MD: FDA Guidance Documents.
- U.S. Food and Drug Administration (2020). Lung Injuries Associated with Use of Vaping Products. Available at: <https://www.fda.gov/news-events/public-health-focus/lung-injuries-associated-use-vaping-products> [Accessed 10/31/2020].
- USP/NF (2009). *United States Pharmacopeia/National Formulary*. 35th/30th ed. Rockville, MD: USP-NF.
- Varlet, V., Farsalinos, K., Augsburger, M., Thomas, A., and Etter, J.-F. (2015). Toxicity Assessment of Refill Liquids for Electronic Cigarettes. *Ijerph* 12, 4796–4815. doi:10.3390/ijerph120504796
- Westgard, J. O., Barry, P. L., Hunt, M. R., and Groth, T. (1981). A Multi-Rule Shewhart Chart for Quality Control in Clinical Chemistry. *Clin. Chem.* 27, 493–501. doi:10.1093/clinchem/27.3.493

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Development, Validation, and Application of a Novel Method for the Analysis of Vitamin E Acetate and Other Tocopherols in Aerosol Emissions of E-Cigarettes, or Vaping Products Associated With Lung Injury

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E-cigarette, or vaping, product (EVP) use has increased dramatically in the United States over the last 4 years, particularly in youth and young adults. Little information is available on the chemical contents of these products. Typically, EVPs contain an active ingredient such as nicotine, CBD, or THC dissolved in a suitable solvent that facilitates aerosol generation. One EVP solvent, vitamin E acetate (VEA), has been measured in EVP liquids associated with lung injury. However, no validated analytical methods for measuring VEA in the aerosol from these devices was previously available. Therefore, we developed a high throughput isotope dilution LC-MS/MS method to simultaneously measure VEA and three other related tocopherols in aerosolized EVP samples. The assay was precise, with VEA repeatability ranging from 4.0 to 8.3% and intermediate precision ranging from 2.5 to 6.7%. Similar precision was obtained for the three other tocopherols measured. The LODs for the four analytes ranged from 8.85×10^{-6} to 2.28×10^{-5} μg analyte per mL of aerosol puff volume, and calibration curves were linear ($R^2 > 0.99$). This method was used to analyze aerosol emissions of 147 EVPs associated with EVALI case patients. We detected VEA in 46% of the case-associated EVPs with a range of 1.87×10^{-4} –74.1 μg per mL of aerosol puff volume and mean of 25.1 μg per mL of aerosol puff volume. Macro-levels of VEA ($>0.1\%$ w/w total aerosol particulate matter) were not detected in nicotine or cannabidiol (CBD) products; conversely 71% of the EVALI associated tetrahydrocannabinol (THC) products contained macro-levels of VEA. Trace levels of other tocopherol isoforms were detected at lower rates and concentrations (α -tocopherol: 41% detected, mean 0.095 μg analyte per mL of aerosol puff volume; γ -tocopherol: 5% detected, mean 0.0193 μg analyte per mL of aerosol puff volume; δ -tocopherol: not detected). Our results indicate that VEA can be efficiently transferred to aerosol by EVALI-associated EVPs vaped using a standardized protocol.

Keywords: vitamin E acetate, tocopherols, EVP aerosol, LC-MS/MS, EVALI

INTRODUCTION

In recent years the use of e-cigarettes, or vaping products (EVPs) have dramatically increased¹. More than eight million U.S. adults reported using these products on a regular basis¹ (Creamer MR, 2019). The use of nicotine containing EVPs could potentially benefit adult smokers if used as a complete substitute for traditional cigarettes and other combusted tobacco products rather than dual use (Shahab et al., 2017). However, EVP use increases disease risk for those who are not already using tobacco products (Orzabal and Ramadoss, 2019; Wang et al., 2020). Research is needed to better understand potential long-term health effects of inhaling EVP aerosols, including solvents, additives, and diluents (Ghosh and Drummond, 2017; Bhatnagar et al., 2019; Hajek et al., 2019). One of the main challenges for assessing the potential health impacts of EVP use is the accessibility to e-liquid components and formulations through “informal” and individualized marketing (Zhu et al., 2014). Decriminalization of cannabinoids for medical and non-medical purposes by some states facilitated a surge in use of EVPs for vaping cannabis as these products are more available in some markets² (Pacula and Smart, 2017; Mcnamara, 2020).

From August 2019 to February 2020 an outbreak of e-cigarette, or vaping, product use associated lung injury (EVALI) occurred across the United States: at least 2,807 people were hospitalized, and 68 deaths reported in 29 states. Most EVALI cases were <35 years of age and previously healthy (Lozier et al., 2019; Moritz et al., 2019). Most patients that were hospitalized reported using e-liquid products containing THC (Butt et al., 2019; Lozier et al., 2019; Siegel et al., 2019). These lung injury cases have been associated to the use of vitamin E acetate (VEA)-containing EVPs (Duffy et al., 2020). Analysis of bronchoalveolar lavage fluid from EVALI patients identified VEA accumulation and thus implicated inhaled VEA as the likely cause of the 2019 EVALI lung injury outbreak (Blount et al., 2019; Blount et al., 2020). Multiple trade websites report the use of VEA and medium-chain triglycerides in THC products to enhance quality, appearance, and aroma, as well as a way to lower production cost (Downs, 2019a; Downs, 2019b; Zachary Eisenberg, 2019).

VEA is the shelf-stable synthetic form of vitamin E often used in skin care products and dietary supplements. Oral and topical administration of VEA has been used for years without significant adverse health effects. Because of the 2019 EVALI outbreak, the effects of inhaled VEA are starting to be evaluated. In fact, two recent studies find that mice exposed to VEA emissions develop lung injury and other pathologies similar to EVALI patients (Bhat et al., 2020; Matsumoto et al., 2020). Traditional analysis of VEA and other tocopherols are mainly performed through HPLC-UV for cosmetics and foods products intended for dermal application or ingestion (Cunha et al., 2006; Bustamante-Rangel et al., 2007; Almeida et al., 2009; Nada et al., 2010; Şeker et al., 2012; Cortés-Herrera et al., 2019; Sadrykia et al., 2019). Recently, the analysis of

VEA in EVP liquids was reported using screening and targeted GC-MS and LC-MS/MS assays³ (Health, 2019). No methods existed for the analysis of VEA and other tocopherols in aerosol emissions of e-liquid products samples, thus here we report on the development and validation of an analytical method for VEA and other tocopherols in aerosol emissions samples. The efficacy of the method is subsequently demonstrated by analysis of aerosol emissions from EVPs associated with EVALI case patients.

EXPERIMENTAL

Materials and Methods

Methanol (CAS# 67-56-1; LC-MS/MS grade) purchased from Fischer Scientific was mixed with deionized water from an ultrapure water purifications system (Aqua Solutions model RODI-C-11BL, Jasper, GA, United States) and formic acid (CAS# 64-18-6; chemical purity: $\geq 98\%$; ACS reagent) to form a 90% methanol: 10% water and 0.1% formic acid mobile phase.

VEA, DL-alpha Tocopherol acetate (CAS# 7695-91-2, $\geq 99\%$ purity), (+)-alpha Tocopherol (CAS# 59-02-9, $\geq 99\%$ purity), (+)-gamma Tocopherol (CAS# 54-28-4, $\geq 96\%$ purity), delta-Tocopherol (CAS# 119-13-1, $\geq 99\%$ purity), alpha-Tocopherol-(phenyl-¹³C₆) ($\geq 99\%$ atom purity, $\geq 96\%$ compound purity), labeled vitamin E acetate - (trimethyl-d₉) ($\geq 98\%$ atom %; chemical purity: $\geq 98\%$) were all purchased from Sigma Aldrich (St. Louis, Missouri, United States).

Stock solutions and calibrators of unlabeled VEA and other tocopherols were prepared by individually weighing neat compounds using a calibrated analytical balance and dissolving each in methanol. Multianalyte working solutions were prepared from individual stocks and stored at -20°C until use. The internal standard stock solutions were prepared in a similar manner using labeled alpha-tocopherol-(phenyl-¹³C₆) and labeled VEA-(trimethyl-d₉) in methanol. A working solution containing both labeled tocopherols was prepared in methanol and stored at -20°C .

Sample Preparation

Aerosol emissions were generated by vaping the EVP liquid samples on a Cerulean CETI-8 e-cigarette vaping machine equipped with button activation switches (Cerulean, Richmond, VA). The vaping machine puff volume was calibrated and verified using a soap bubble flow meter prior to use. Vaping conditions were adopted from the CORESTA Recommended Method No. 81 (CORESTA, Recommended Method Number 81: Routine analytical machine for e-cigarette aerosol generation and collection-definition and standard conditions). Freshly charged batteries were used in an EVP to vape each provided cartridge or liquid. Pre-filled cartridges provided without an EVP were vaped using a Honeystick (Fort Lauderdale, FL, United States) 510 Twist Vape Pen with

¹https://www.cdc.gov/tobacco/basic_information/e-cigarettes

²<https://www.ncsl.org/research/health/state-medical-marijuana-laws.aspx>

³<https://www.fda.gov/news-events/public-health-focus/lung-injuries-associated-use-vaping-products>

TABLE 1 | MRM transitions and parameters for VEA and tocopherols at a dwell time of 250 ms for all analytes.

Analyte	Transition type	Ion transition	DP (V)	CE (V)	CXP (V)
VEA	Quantitation	473.1→207.1	206	25	16
	Confirmation	473.1→165.1	206	51	12
α-tocopherol	Quantitation	431.2→165.1	61	33	12
	Confirmation	431.2→137.0	61	57	10
δ-tocopherol	Quantitation	403.2→137.0	46	35	10
	Confirmation	403.2→81.0	46	65	10
γ - Tocopherol	Quantitation	417.2→151.1	76	27	12
	Confirmation	417.2→123.0	76	55	14
α-tocopherol-(phenyl- ¹³ C ₆)	Internal standard	437.2→171.1	56	27	14
VEA-(trimethyl-d ₉)	Internal standard	482.3→216.1	21	25	14

the battery set at the highest voltage (4 V). When case-associated products included a compatible battery, the provided battery was charged and used at the highest voltage setting for vaping that case-associated cartridge. The aerosol from 15 consecutive puffs from vaped EVPs liquids was trapped on individual pre-conditioned Cambridge filter pads (CFPs; 44 mm) that were purchased from Thermo Fisher Scientific (Waltham, MA, United States) and housed in filter pad holders from Cerulean (Molins PLC, MK, United Kingdom). Custom-made connectors (“mouth pieces”) were fabricated in-house *via* 3D printing technology as needed for non-circular mouth piece geometries. The total particulate matter (TPM) was gravimetrically determined by mass difference of pre- and post-vaping CFP for each sample. EVPs that produced less than 6.5 mg TPM/15 puffs were considered unacceptably low, flagged as a QC failure, and not reportable. Post-vaped CFPs were individually placed into 16 ml amber vials for extraction. CFPs were extracted with 10 ml of methanol on an orbital shaker for 10 min at 160 rpm. Sample extracts were diluted 100-fold prior to tocopherol analysis. Prior to analysis, 100 µL of the dilute solution was spiked with labeled internal standard and diluted with methanol to 1 ml in an autosampler vial.

Instrumentation

A high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, United States) coupled with electrospray tandem mass spectrometry (SCIEX 5500 Triple Quad Applied Biosystems, Foster City, CA, United States) was used to quantitatively measure vitamin E acetate and other tocopherols in trapped aerosol emissions of e-liquids. Chromatographic separation was achieved using isocratic elution at a flow rate of 0.75 ml/min on an XTerra MS C18 column 3.5 µm × 50 mm × 150 mm (Waters Corporation Milford, MA United States) with methanol, water, and formic acid (89.9:10:0.1) as the mobile phase. The eluent from the column was ionized using an electrospray interface to generate and transmit positive ions into the mass spectrometer for selective, quantitative analysis. Analyst software version 1.6.2 (Applied Biosystems, Foster City, CA, United States) was used to operate the HPLC and the 5500 Triple Quad. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode for positive ions. The ion source temperature was set at 350°C and the electrospray ion voltage at 5,500 V.

Table 1 presents the optimized MRM transitions used for quantification, confirmation, and internal standard.

Quantification

Analyst software 1.6.2 was used for peak integration, calibration, and quantification. Analyte quantification was achieved using the ratio of relative peak area of the analyte to that of the labeled internal standard. Aerosol emissions samples results (instrument output in ng/mL) were normalized by aerosol puff volume to determine analyte yields per puff following the equation below.

$$\mu\text{g per mL aerosol puff volume} = \text{tocopherol}_{\text{measured}} \left(\frac{\text{ng}}{\text{mL}} \right) \times \frac{10 \text{ mL}}{1000 \frac{\text{ng}}{\mu\text{g}} \times 15 \text{ puff} \times 55 \frac{\text{mL}}{\text{puff}}}$$

where tocopherol measured is the instrument calculated analyte concentration in ng/mL multiplied by the result of the division of the total sample extraction volume of 10 ml by the total puff aerosol volume defined as 15 puffs/pad × 55 ml/puff and a factor of 1,000 to convert ng to µg.

Quality Control Samples

EVPs are chemically diverse; therefore, we created a diverse surrogate matrix for preparing calibration curves, QC pools and blanks. The surrogate matrix was created by combining the aerosol extract from four different vape liquids, a commercial product (VUSE Solo Menthol), and three custom mixtures (25% squalene/25% squalane/50% mineral oil, 100% CDB oil, and 25% vitamin E acetate/25% medium chain triglycerides/50% hemp oils). The surrogate matrix was vaped using a Vaporin Presidential device and the aerosol trapped using the same technique as for unknown samples. Each pad was extracted with methanol for 10 min in an orbital shaker and combined to produce an 80 ml mixture. The vaped surrogate matrix extract was stored at −20°C. QC samples were prepared daily by individually spiking diluted vaped surrogate matrix extract with known amounts of mixed VEA and tocopherols. Two replicates of a low (QCL) and a high (QCH) level were analyzed per analytical batch. Characterization of each QC level was performed using 20 independent analyses to establish control limits. This QC characterization was subsequently used to evaluate assay performance for each analytical batch based on

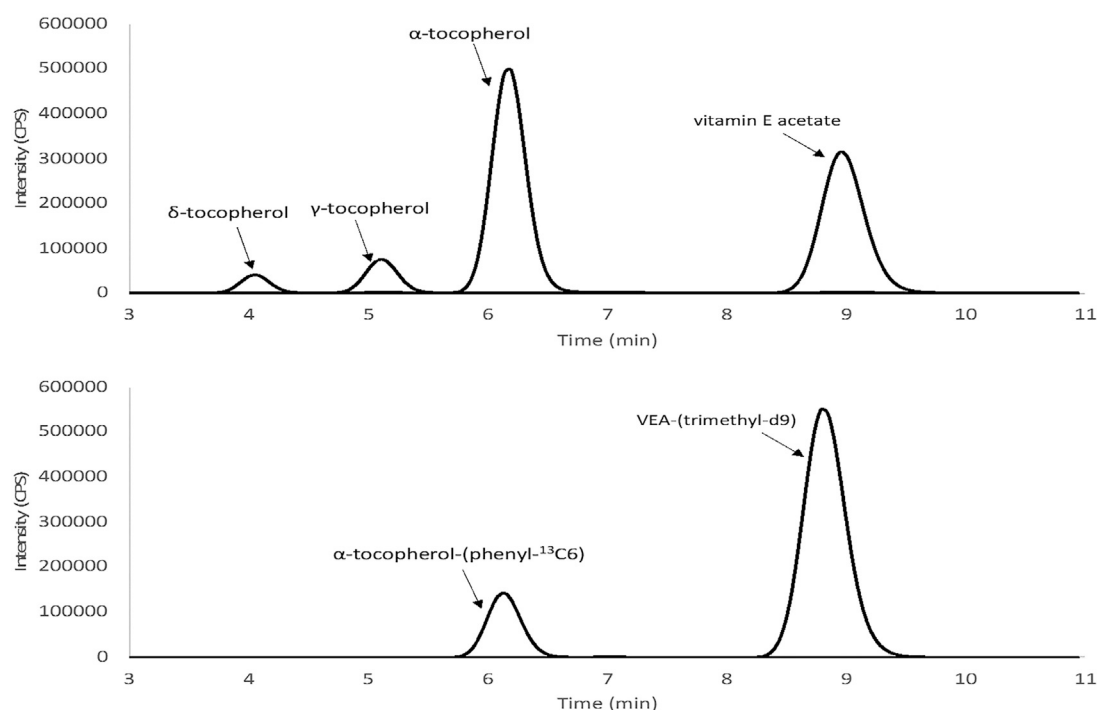


FIGURE 1 | Representative chromatograms of vitamin E acetate (VEA) and other tocopherols spiked into emissions of simulated EVP liquid: **(Top)** VEA and other tocopherols combined quantitation transitions (500 ng/ml) **(bottom)** labeled α -tocopherol (200 ng/ml) and deuterated VEA (200 ng/ml).

modified Westgard Rules as described by Caudill et al. (2008). If an analyte failed QC, then none of the results for that analyte in that analysis batch was reportable.

Calibration

Each analytical batch consisted of a set of seven calibration standards prepared in vaped surrogate matrix extract. The calibration was fit to a weighted $1/x$ least square model for all analytes generating linear curves with $r^2 > 0.9988$. The limit of detection was defined as three times the standard deviation at zero concentration derived from the analysis of six replicates of the three lowest calibration standards (Taylor, 1987). Data are only reported that fall within the calibrated range. Samples exceeding the highest calibration point are diluted and reanalyzed.

Accuracy, Dynamic Range, Linearity, and Precision

Method accuracy was assessed by spiking the vaped surrogate matrix extract at three different levels of VEA and tocopherols. Six replicates of each level, 200, 400, and 600 ng/ml were used to calculate the analyte recovery. The dynamic range selected covers two orders of magnitude (10–1,000 ng/ml equivalent to 1.21×10^{-4} – $0.0121 \mu\text{g}$ per mL aerosol emission) to expand the screening capabilities of the assay. Linearity of the dynamic range was evaluated by residual analysis of seven independent curves. Method precision was evaluated as repeatability and intermediate precision of 20 independent QC samples results.

Method Application

EVP liquid samples were transferred to CDC by FDA and various state health departments for aerosols analysis. Samples that did not contain adequate liquid volume for the assay were not analyzed and the contents saved for liquid analyses. Strict chain of custody was maintained throughout the duration of the study. We applied the validated method to analyze aerosol emissions from 147 EVPs associated with the 2019 U.S. EVALI outbreak. EVP liquid samples were transferred to CDC by FDA and various state health departments for aerosols analysis. Strict chain of custody was maintained throughout the duration of the study. Of those 147 samples, a subset of 138 had reportable corresponding nicotine, CBD, and THC levels. These products were categorized as tetrahydrocannabinol (THC) products if $\text{THC} \geq 0.3\%$ (w/w), nicotine products if nicotine $> 0.2\%$ (w/w), and cannabidiol (CBD) products if $\text{CBD} > 1\%$ (w/w) and $\text{THC} < 0.3\%$.

RESULTS AND DISCUSSION

Method Validation

We developed a sensitive and quantitative method using LC-MS/MS to detect VEA and other tocopherols in aerosol emissions of EVALI case-associated EVPs. Complete chromatographic separation was achieved for the tocopherols without any presence of potential matrix interferences as shown in **Figure 1**. Method specificity was attained by using isotopically

TABLE 2 | Analyte recovery in EVP vaped surrogate matrix extract at three spike levels.

Analyte	Spike concentration (ng/ml)	Spike recovery (%)	CV, %	Mean recovery (%)	Overall CV, %
VEA	200	102	4.11	100	4.30
	400	98	3.92		
	600	100	4.86		
α -tocopherol	200	111	10.8	107	7.33
	400	109	3.66		
	600	102	7.55		
δ -tocopherol	200	116	11.2	115	11.2
	400	113	11.9		
	600	116	10.5		
γ -tocopherol	200	120	13.3	115	9.88
	400	110	6.82		
	600	114	9.49		

(based on six replicates for each spike level).

TABLE 3 | Method precision, range, and linearity for VEA and other tocopherols.

Analyte	LOD ng/mL	Dynamic range ng/mL	Linearity (R^2 ; n = 7)	Precision (%RSD; n = 20)			
				Repeatability		Intermediate precision	
				QCL	QCH	QCL	QCH
VEA	1.53 (1.85×10^{-5}) ^a	10–1,000 (1.21×10^{-4} –0.0121) ^a	0.99	4.72	6.14	4.90	6.66
α -tocopherol	0.73 (8.85×10^{-6}) ^a	10–1,000 (1.21×10^{-4} –0.0121) ^a	0.99	4.36	4.96	6.73	6.00
δ -tocopherol	1.88 (2.28×10^{-5}) ^a	10–1,000 (1.21×10^{-4} –0.0121) ^a	0.99	8.32	6.06	2.98	4.56
γ -tocopherol	1.77 (2.15×10^{-5}) ^a	10–1,000 (1.21×10^{-4} –0.0121) ^a	0.99	4.86	3.96	2.47	4.16

^aUnits of μg analyte per mL aerosol puff volume.

labeled tocopherols to establish the presence of unlabeled tocopherols using both the LC retention time and MS/MS mass selection of the triple quad platform.

Method accuracy was analyzed based on six replicates each of un-spiked and spiked vaped surrogate matrix extract at three different levels of VEA and other tocopherols. Spike recoveries (comparison of spiked calculated result to target concentration) and coefficients of variation (CV) were calculated for each spike level. The mean recoveries for VEA and other tocopherols ranged from 100 to 115% with overall CVs of 4–11% as shown in **Table 2**. We also evaluated matrix effects by analyzing calibration standards in both methanol and vaped surrogate matrix extract. The average slope of six independent calibration curves in solvent and vaped surrogate matrix extract showed a difference of less than 5% for all analytes. These measures document that the method accurately measures VEA and other tocopherols in aerosol emissions of simulated EVP liquids.

Method precision was evaluated as repeatability and intermediate precision from the analysis of 20 independent results for each of two QC levels, QC low (150 ng/mL) and QC high (800 ng/mL), over 10 days (**Table 3**). Repeatability of both QC levels ranged from 3.96 to 8.32% for all the analytes. Intermediate precision ranged from 2.47 to 6.73% among all analytes for both QC levels. These data document the excellent precision of the method and the characterization of QCs to allow for evaluation of assay accuracy and precision for each analytical batch analyzed.

TABLE 4 | VEA and other tocopherols concentrations and detection frequency in aerosol emissions of EVALI case-associated EVPs (μg per mL aerosol puff volume).

Analyte	N	% Detected	Mean $\pm$ Std Dev ^a
VEA	147	46	25.1 $\pm$ 22.4
α -tocopherol	126	41	0.095 $\pm$ 0.150
γ -tocopherol	112	5	0.0193 $\pm$ 0.0073
δ -tocopherol	112	0	NA

^aDescriptive Statistics for detects only.

The method demonstrates excellent linearity $R^2 \geq 0.99$ for VEA and other tocopherols within the selected dynamic range of 10–1,000 ng/mL (**Table 3**). The sensitivity of the method is adequate to measure background levels of VEA (LOD = 1.85×10^{-5} μg per mL of aerosol puff volume (1.53 ng/mL methanol extract)) and other tocopherols (LODs: 8.85×10^{-6} – 2.28×10^{-5} μg per mL of aerosol puff volume (0.73–1.88 ng/mL methanol extract)) in aerosol emissions of EVPs liquid samples. The sensitivity for VEA detection was significantly better than previously published methods (LC-UV used to achieve an LOD of 580 ng/mL) (Brabcová et al., 2013). Our method was also 3–90 fold more sensitivity for α -tocopherol, δ -tocopherol, and γ -tocopherol compared with previously published methods (Bustamante-Rangel et al., 2007; Lanina et al., 2007; Cortés-Herrera et al., 2019).

Method Application

The analytical method was applied to aerosol emissions from 147 EVPs associated with EVALI cases (Table 4). VEA and α -tocopherol had the highest detection rates of 46 and 41% respectively. VEA content in aerosol emissions ranged from 1.87×10^{-4} to $74.1 \mu\text{g}$ per mL of aerosol puff volume followed by α -tocopherol with a range of 1.47×10^{-2} – $0.908 \mu\text{g}$ per mL of aerosol puff volume. VEA levels were 264 times higher than α -tocopherol with a mean of $25.1 \mu\text{g}$ per mL of aerosol puff volume compared to mean of $0.095 \mu\text{g}$ per mL of aerosol puff volume for α -tocopherol. Further quantification of VEA and α -tocopherol in e-liquid and in vaped aerosol will help provide insight about possible VEA degradation to form reactive byproducts such as ethenone (Wu and O'Shea, 2020). Gamma-tocopherol was detected in five EVPs while δ -tocopherol was not present in any of the analyzed products.

A subset of 139 products were stratified by active ingredient to further investigate the presence of macro-levels of VEA (>0.1%) in different product types. We evaluated macro-levels of VEA because VEA accumulation in the lungs could physically disrupt the tertiary structure of the alveolus, cause alveolar collapse, and subsequently lead to EVALI pathologies (Casals and Cañadas, 2012; Kamal and Raghunathan, 2012; Blount et al., 2020; Jonas and Raj, 2020). Products with higher VEA in aerosol emissions (>0.1% TPM) would deliver significant amounts of VEA to the lungs of people using the products. We show here that no nicotine or CBD products contain these high levels of VEA, and that 71% of case-associated THC products contained VEA as a macro-component (mean $32.0 \mu\text{g}$ per mL of aerosol puff volume). The high prevalence of VEA in THC products is consistent with the solubility of THC in VEA and the absence of VEA in nicotine products is consistent with the insolubility of nicotine in VEA. This result is also aligned with reported use of VEA as a diluent in the formulation of THC products (Downs, 2019a; Downs, 2019b; Zachary Eisenberg, 2019). VEA was also detected in two products with no THC, CBD, or nicotine with a mean level of $31.7 \mu\text{g}$ per mL of aerosol puff volume. One of these products was marketed as a THC-containing product by Dank Vapes but contained no detectable THC by our analysis. The high prevalence of macrolevel VEA in EVALI case-associated THC products further implicates VEA as a potential cause of vaping-associated lung injury (Cunha et al., 2006; Cilla et al., 2014).

REFERENCES

- Almeida, M. M., Alves, J. M. P., Patto, D. C. S., Lima, C. R. R. C., Quenca-Guillen, J. S., Santoro, M. I. R. M., et al. (2009). Determination of Tocopheryl Acetate and Ascorbyl Tetraisopalmitate in Cosmetic Formulations by HPLC. *HPLC* 31, 445–450. doi:10.1111/j.1468-2494.2009.00514.x
- Bhat, T. A., Kalathil, S. G., Bogner, P. N., Blount, B. C., Goniewicz, M. L., and Thanavala, Y. M. (2020). An Animal Model of Inhaled Vitamin E Acetate and EVALI-like Lung Injury. *N. Engl. J. Med.* 382, 1175–1177. doi:10.1056/nejmc2000231
- Bhatnagar, A., Payne, T. J., and Robertson, R. M. (2019). Is There A Role for Electronic Cigarettes in Tobacco Cessation?, *J Am Heart Assoc.* 8. e012742 doi:10.1161/jaha.119.012742
- Blount, B. C., Karwowski, M. P., Morel-Espinosa, M., Rees, J., Sosnoff, C., Cowan, E., et al. (2019). Evaluation of Bronchoalveolar Lavage Fluid from Patients in an

CONCLUSION

A rapid, isotope dilution LC-MS/MS method was developed for the simultaneous analysis of VEA and other tocopherols in EVP aerosol emissions. The method demonstrated high accuracy, precision, and sensitivity. VEA and other tocopherols, except for δ -tocopherol, were detected in aerosol emissions from EVALI case-associated EVPs; the mean VEA concentration was several orders of magnitude higher than the mean α -tocopherol concentration. VEA was predominantly found in THC products, consistent with the reported use of VEA as a diluent in the formulation of these products. Our results also indicate that VEA can be efficiently transferred to aerosol by EVALI-associated devices vaped using a standardized protocol. This method can serve as a valuable tool to improve surveillance for the potentially harmful additive VEA in EVPs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AP and MM contributed to the development and validation of the analytical method. AP wrote the first draft of the manuscript. CW, BB, and LV contributed to conception and design of the study. All authors contributed to manuscript revision, read, and approves the submitted version.

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Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - 10 States, August-October 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 1040–1041. doi:10.15585/mmwr.mm6845e2

- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382, 697–705. doi:10.1056/nejmoa1916433
- Brabcová, I., Kovářová, L., Satinsky, D., Havlíková, L., and Solich, P. (2013). A Fast HPLC Method for Determination of Vitamin E Acetate in Dietary Supplements Using Monolithic Column. *Food Anal. Methods* 6, 380–385. doi:10.1007/s12161-012-9452-0
- Bustamante-Rangel, M., Delgado-Zamarreño, M. M., Sánchez-Pérez, A., and Carabias-Martínez, R. (2007). Determination of Tocopherols and Tocotrienols in Cereals by Pressurized Liquid Extraction-Liquid Chromatography-Mass Spectrometry. *Analytica Chim. Acta* 587, 216–221. doi:10.1016/j.aca.2007.01.049

- Butt, Y. M., Smith, M. L., Tazelaar, H. D., Vaszar, L. T., Swanson, K. L., Cecchini, M. J., et al. (2019). Pathology of Vaping-Associated Lung Injury. *N. Engl. J. Med.* 381, 1780–1781. doi:10.1056/nejmc1913069
- Casals, C., and Cañadas, O. (2012). Role of Lipid Ordered/disordered Phase Coexistence in Pulmonary Surfactant Function. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1818, 2550–2562. doi:10.1016/j.bbame.2012.05.024
- Caudill, S. P., Schleicher, R. L., and Pirkle, J. L. (2008). Multi-rule Quality Control for the Age-Related Eye Disease Study. *Statist. Med.* 27, 4094–4106. doi:10.1002/sim.3222
- Centers for Disease Control and Prevention. *Electronic Cigarettes (E-cigarettes)* [Online]. Available: https://www.cdc.gov/tobacco/basic_information/e-cigarettes. [Accessed].
- Cilla, A., Zanirato, V., Rodriguez-Estrada, M. T., and Garcia-Llatas, G. (2014). “Nutritional Hazards: Micronutrients: Vitamins and Minerals,” in *Encyclopedia of Food Safety*. Editor Y. Motarjemi (Waltham: Academic Press), 86–94. doi:10.1016/b978-0-12-378612-8.00431-5
- Coresta Recommended Method Number 81: Routine analytical machine for e-cigarette aerosol generation and collection-definition and standard conditions.
- Cortés-Herrera, C., Chacón, A., Artavia, G., and Granados-Chinchilla, F. (2019). Simultaneous LC/MS Analysis of Carotenoids and Fat-Soluble Vitamins in Costa Rican Avocados (Persea Americana Mill.), *Molecules* 24, 4517. doi:10.3390/molecules24244517
- Creamer, M. R., Wang, T. W., Babb, S., Cullen, K. A., Day, H., Willis, G., et al. (2019). Tobacco Product Use and Cessation Indicators Among Adults - United States, 2018. *MMWR Morb. Mortal. Wkly. Rep.* 68, 1013–1019. doi:10.15585/mmwr.mm6845a2
- Cunha, S. C., Amaral, J. S., Fernandes, J. O., and Oliveira, M. B. P. P. (2006). Quantification of Tocopherols and Tocotrienols in Portuguese Olive Oils Using HPLC with Three Different Detection Systems. *J. Agric. Food Chem.* 54, 3351–3356. doi:10.1021/jf053102n
- Downs, D., David, H., and Barcott, Bruce. (2019b). *Journey of a Tainted Cartridge; From China's Labs to Your Lungs* [Online]. Leafly. Available: <https://www.leafly.com/news/politics/vape-pen-injury-supply-chain-investigation-leafly>. [Accessed].
- Downs, D. (2019a). Vape Pen Lung Injury: Here's what You Need to Know. [Online]. Leafly. Available: <https://www.leafly.com/news/health/vape-pen-lung-disease-advice-consumers>. [Accessed].
- Duffy, B., Li, L., Lu, S., Durocher, L., Dittmar, M., Delaney-Baldwin, E., et al. (2020). Analysis of Cannabinoid-Containing Fluids in Illicit Vaping Cartridges Recovered from Pulmonary Injury Patients: Identification of Vitamin E Acetate as a Major Diluent. *Toxics* 8, 8, doi:10.3390/toxics8010008
- Ghosh, S., and Drummond, M. B. (2017). Electronic Cigarettes as Smoking Cessation Tool 23, 111–116. doi:10.1097/mcp.0000000000000348
- Hajek, P., Phillips-Waller, A., Przulj, D., Pesola, F., Myers Smith, K., Bisal, N., et al. (2019). A Randomized Trial of E-Cigarettes versus Nicotine-Replacement Therapy. *N. Engl. J. Med.* 380, 629–637. doi:10.1056/nejmoa1808779
- Health, N. Y. S. D. O. (2019). New York State Department of Health announces update on investigation into vaping-associated pulmonary illnesses: department warns against use of black-market vaping products: lab test results show high levels of vitamin E acetate, now focus of investigation [Online]. Available: https://www.health.ny.gov/press/releases/2019/2019-09-05_vaping.htm. [Accessed].
- Jonas, A. M., and Raj, R. (2020). Vaping-Related Acute Parenchymal Lung Injury. *Chest* 158, 1555–1565. doi:10.1016/j.chest.2020.03.085
- Kamal, M. A., and Raghunathan, V. A. (2012). Modulated Phases of Phospholipid Bilayers Induced by Tocopherols. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1818, 2486–2493. doi:10.1016/j.bbame.2012.06.016
- Linana, S. A., Toledo, P., Sampels, S., Kamal-Eldin, A., and Jastrebova, J. A. (2007). Comparison of Reversed-phase Liquid Chromatography-Mass Spectrometry with Electrospray and Atmospheric Pressure Chemical Ionization for Analysis of Dietary Tocopherols. *J. Chromatogr. A* 1157, 159–170. doi:10.1016/j.chroma.2007.04.058
- Lozier, M. J., Wallace, B., Anderson, K., Ellington, S., Jones, C. M., Rose, D., et al. (2019). Update: Demographic, Product, and Substance-Use Characteristics of Hospitalized Patients in a Nationwide Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injuries - United States, December 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 1142–1148. doi:10.15585/mmwr.mm6849e1
- Lung Injuries Associated with Use of Vaping Products. Food and Drug Administration. Available: <https://www.fda.gov/news-events/public-health-focus/lung-injuries-associated-use-vaping-products>. [Accessed].
- Matsumoto, S., Fang, X., Traber, M. G., Jones, K. D., Langelier, C., Hayakawa Serpa, P., et al. (2020). Dose-Dependent Pulmonary Toxicity of Aerosolized Vitamin E Acetate. *Am. J. Respir. Cel Mol Biol* 63, 748–757. doi:10.1165/rcmb.2020-0209oc
- Mcnamara, A. (2020). *CBS News, These states now have legal weed, and which states could follow suit in 2020* [Online]. Available: <https://www.cbsnews.com/news/where-is-marijuana-legal-in-2020-illinois-joins-10-other-states-legalizing-recreational-pot-2020-01-01/>. [Accessed 2020].
- Moritz, E. D., Zapata, L. B., Lekichvili, A., Glidden, E., Annor, F. B., Werner, A. K., et al. (2019). Update: Characteristics of Patients in a National Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injuries - United States, October 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 985–989. doi:10.15585/mmwr.mm6843e1
- Nada, A., Krishnaiah, Y. S., Zaghloul, A. A., and Khatib, I. (2010). Analysis of Vitamin E in Commercial Cosmetic Preparations by HPLC. *J. Cosmet. Sci.* 61, 353–365.
- National Conference of States Legislature State Medical Marijuana Laws [Online]. Available: <https://www.ncsl.org/research/health/state-medical-marijuana-laws.aspx>. [Accessed October 12, 2020].
- Orzabal, M., and Ramadoss, J. (2019). Impact of Electronic Cigarette Aerosols on Pregnancy and Early Development. *Curr. Opin. Toxicol.* 14, 14–20. doi:10.1016/j.cotox.2019.05.001
- Pacula, R. L., and Smart, R. (2017). Medical Marijuana and Marijuana Legalization. *Annu. Rev. Clin. Psychol.* 13, 397–419. doi:10.1146/annurev-clinpsy-032816-045128
- Sadrykia, F., Shayanfar, A., Valizadeh, H., and Nemati, M. (2019). A Fast and Simple Method for Determination of Vitamin E in Infant Formula by Dispersive Liquid-Liquid Microextraction Combined with HPLC-UV. *Food Anal. Methods* 12, 23–31. doi:10.1007/s12161-018-1331-x
- Seker, M. E., Çelik, A., and Dost, K. (2012). Determination of Vitamin E Isomers of Grape Seeds by High-Performance Liquid Chromatography-UV Detection. *J. Chromatogr. Sci.* 50, 97–101. doi:10.1093/chromsci/bmr032
- Shahab, L., Goniewicz, M. L., Blount, B. C., Brown, J., McNeill, A., Alwis, K. U., et al. (2017). Nicotine, Carcinogen, and Toxin Exposure in Long-Term E-Cigarette and Nicotine Replacement Therapy Users. *Ann. Intern. Med.* 166, 390–400. doi:10.7326/m16-1107
- Siegel, D. A., Jatlaoui, T. C., Koumans, E. H., Kiernan, E. A., Layer, M., Cates, J. E., et al. (2019). Update: Interim Guidance for Health Care Providers Evaluating and Caring for Patients with Suspected E-cigarette, or Vaping, Product Use Associated Lung Injury - United States, October 2019. *Am. J. Transpl.* 19, 3420–3428. doi:10.1111/ajt.15690
- Taylor, J. K. (1987). *Quality Assurance of Chemical Measurements*. New York: Lewis Publishers.
- Wang, T. W., Neff, L. J., Park-Lee, E., Ren, C., Cullen, K. A., and King, B. A. (2020). E-cigarette Use Among Middle and High School Students - United States, 2020. *MMWR Morb. Mortal. Wkly. Rep.* 69, 1310–1312. doi:10.15585/mmwr.mm6937e1
- Wu, D., and O'Shea, D. F. (2020). Potential for Release of Pulmonary Toxic Ketene from Vaping Pyrolysis of Vitamin E Acetate. *Proc. Natl. Acad. Sci. USA* 117, 6349–6355. doi:10.1073/pnas.1920925117
- Zachary Eisenberg, D. M., Lam, Vu., Cheng, Cathy., Richard, Josh., and Burack, Benjamin. (2019). Contaminant Analysis of Illicit vs Regulated Market Extracts. [Online]. anresco Laboratories. Available: <https://cannabis.anresco.com/analysis-of-illicit-vs-regulated-market-extracts/>. [Accessed 2020].

Zhu, Sh., Bonnevie, E., Sun, J. Y., Cummins, S. E., Gamst, A., Yin, L., et al. (2014). Four Hundred and Sixty Brands of E-Cigarettes and Counting: Implications for Product Regulation. *Tob. Control.* 23, iii3-iii9. doi:10.1136/tobaccocontrol-2014-051670

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Simultaneous Temperature Measurements and Aerosol Collection During Vaping for the Analysis of Δ^9 -Tetrahydrocannabinol and Vitamin E Acetate Mixtures in Ceramic Coil Style Cartridges

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Incidence of e-cigarette, or vaping, product use-associated lung injury (EVALI) has been linked to the vaping of tetrahydrocannabinol (THC) products to which vitamin E acetate (VEA) has been added. In this work we vaped THC/VEA mixtures at elevated power levels using a variety of ceramic coil vaping cartridges and a commercially available vaping device, while simultaneously measuring temperature and collecting the vaporized condensate. The collected vapor condensate was analyzed for evidence of VEA decomposition by GC/MS, GC/FT-IR/MS, and LC-APCI-HRMS/MS. Mean temperature maxima for all examined cartridges at the selected power exceeded 430°C, with a range of 375–569°C, well beyond that required for thermal decomposition of VEA. The percent recovery of VEA and Δ^9 -THC from the vaporized mixture in six cartridges ranged from 71.5 to 101% and from 56.4 to 88.0%, respectively. Analysis of the condensed vaporized material identified VEA decomposition products duroquinone (DQ), 1-pristene, and durohydroquinone monoacetate (DHQMA); a compound consistent with 4-acetoxy-2,3,5-trimethyl-6-methylene-2,4-cyclohexadienone (ATMMC) was also detected. The concentration of DQ produced from vaporization of the THC/VEA mixture in one cartridge was found to be 4.16 ± 0.07 μ g per mg of vapor condensate.

Keywords: EVALI, vaping, temperature, vitamin E acetate, Δ^9 -tetrahydrocannabinol, ceramic coil

INTRODUCTION

In August of 2019, reports of an increasing number of hospitalizations for respiratory difficulties, and in some cases acute respiratory failure, among users of e-cigarettes and other vaping products started to emerge. The outbreak of e-cigarette, or vaping, product use-associated lung injury (EVALI) peaked in September of 2019. As of February 2020, 2,807 people had been hospitalized across the U.S., including Puerto Rico and the U.S. Virgin Islands, with 64 deaths. (U.S. Centers for Disease Control and Prevention, 2020). Investigations into the cause of EVALI were initiated at state and federal laboratories, including the CDC, and the U.S. Food and Drug Administration (FDA). These

efforts mainly focused on whether components of the e-liquids being vaped were responsible for the injuries observed.

According to statistics compiled by the CDC, of the 2,022 patients for whom data were available, 82% (as of January 14th, 2020) reported using tetrahydrocannabinol (THC)-containing products, including 33% who claimed to use THC-containing products exclusively. (U.S. Centers for Disease Control and Prevention, 2020). In September of 2019, the New York State Department of Health announced that in an examination of 34 EVALI patients who used THC-containing products, all used at least one product that also contained vitamin E acetate (α -tocopherol acetate, VEA), a compound which was not an approved additive for New York State Medical Marijuana Program-authorized vape products. (Department of Health, 2019). The FDA reported that, in a sample of 93 verified EVALI patients, 73% were linked to at least one THC-containing product, and 81% of those THC-containing products also contained VEA. (U.S. Food and Drug Administration, 2020). The FDA also found that in a broader analysis of 511 THC-containing products, 50% contained VEA as a diluent, with concentrations ranging from 23 to 88%. (U.S. Food and Drug Administration, 2020). In a study of bronchoalveolar-lavage (BAL) fluid from 51 confirmed or probable EVALI patients, 47 (92%) were linked to the use of THC-containing products, and the BAL fluid of 48 (94%) contained VEA. (U.S. Centers for Disease Control and Prevention, 2020). VEA was not found in the BAL fluid of a comparison group of 99 healthy individuals. (U.S. Food and Drug Administration, 2020). Subsequently, an animal study found that mice exposed to an 88% VEA aerosol showed histological markers consistent with EVALI. (Bhat et al., 2020). In addition to VEA, studies of EVALI-associated THC products have revealed the presence of medium-chain triglycerides (also sometimes used as a cutting agent) in some products, as well as residual organic solvents, silica compounds, and pesticides. (Raber et al., 2015; Muthumalage et al., 2020). The CDC has issued guidance to avoid the use of any e-cigarette or vaping product containing VEA, and any product obtained from informal sources. Both the CDC and FDA caution, however, that although there appears to be a strong link between VEA and EVALI, the evidence is insufficient to rule out other contributing factors, and the precise mechanism by which VEA may cause injury is unknown. (U.S. Centers for Disease Control and Prevention, 2020; U.S. Food and Drug Administration, 2020; Blount et al., 2019; Lanzarotta et al., 2020).

While the types of products associated with EVALI have been well characterized, one variable that requires deeper consideration is the impact of vaping temperature on these products. Vaping, whether of nicotine or THC products, can be done over a range of power levels, determined by the resistance of the heating element and the voltage applied by the vaping device. Many devices allow the user to set the voltage or temperature used to vape, with varying accuracy. (Dibaji et al., 2018). Identifying a common or uniform practice in vaping power levels is difficult. However, it has been shown that vaping at higher power levels results in higher temperatures during vapor generation, and differences in temperature can affect many features of the vapor generated, including volume,

particle size and particle size distribution, appearance, and flavor. (Varlet et al., 2016; Talih et al., 2017; Soulet et al., 2018).

Differences in temperature, especially at elevated power, may also result in thermal decomposition of one or more components of the e-liquid. (Geiss et al., 2016; Bitzer et al., 2018; Meehan-Atrash et al., 2019). The potential for components of THC and VEA-containing products to be converted to more toxic species at higher temperatures merits further investigation, and may be a factor in the causation of EVALI by vaping mixtures that include VEA. (Blount et al., 2019; Bhat et al., 2020). A thermogravimetric study found that VEA decomposition begins at around 200°C in air, and accelerates rapidly above 300°C. (Ushikusa et al., 1991). Another study catalogued a number of products of the thermal degradation of VEA between 180 and 300°C, including formic acid, acetic acid, and 2-hexanone. (Riordan-Short et al., 2019). Both of these studies examined the decomposition of VEA over temperature exposures lasting several minutes, well in excess of the few seconds of exposure produced during vaping.

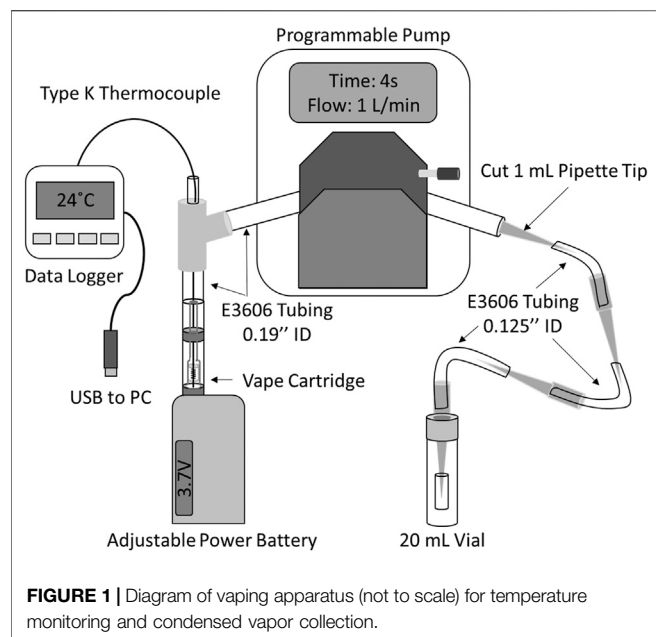
An examination of VEA aerosolized by vaping demonstrated the potential for the generation of ketene gas (C_2H_2O), a toxic pulmonary irritant (National Research Council, 2014), from thermal decomposition of the aryl acetate moiety of VEA. This study was performed using a device designed for nicotine vaping, producing very high power using a sub-Ohm (0.25 Ω) resistance coil (Wu and O'Shea, 2020), in contrast to the ceramic wick cartridges commonly used for vaping of THC concentrates, with typical coil resistances in the 1.3–2.2 Ω range. One study has identified and quantitated the VEA decomposition products duroquinone and durohydroquinone in a vaporized VEA solution in one 1.4 Ω ceramic wick cartridge operated at 3.6 V (Jiang et al., 2020). Another study on VEA aerosolization monitored the temperature of a cartridge with a stainless steel coil by IR temperature measurement through a custom designed cell following the removal of the tank and found temperatures from 500–600°C, though no temperature data on ceramic coil cartridges was provided. (Mikheev et al., 2020). The operating temperature and the effects of elevated temperature, regarding the thermal decomposition of VEA in ceramic coil cartridges designed for the vaping of THC concentrates have not been well characterized.

In this work, we use a variety of THC concentrate compatible cartridges and a commercially available vaping device with an adjustable voltage setting to monitor the temperature at multiple power levels and examine the effects of sustained vaping at elevated power levels on a mixture of THC concentrate and VEA (THC/VEA). The THC/VEA concentrations and cartridges used were representative of products associated with EVALI. We then collected and compared the chemical composition of the vapor condensate produced with the unvaped mixture to identify any thermal decomposition products created from vaping the THC/VEA mixture.

EXPERIMENTAL

Samples

A 50% THC-concentrate, 50% VEA mixture (w/w, THC/VEA) was prepared by combining approximately equal weights of a



THC concentrate collected during an investigation into the alleged manufacturing and distribution of illicit vaping cartridges (75% Δ^9 -tetrahydrocannabinol, verified by HPLC-UV, also contained cannabitol, cannabigerol, cannabidiol, and Δ^9 -tetrahydrocannabivarin identified by GC/MS) and commercially available diluting agent (100% VEA, verified by GC-FID). The mixture was then heated to 90°C for 45 min and vortexed. Prior to dispensing into cartridges, the THC/VEA mixture was again heated to 90°C for at least 30 min to decrease viscosity. Between 0.5 and 0.9 g of the mixture was dispensed into empty 510 threaded vaping cartridges identified C1-C6 (various ceramic coil type cartridges are shown in **Supplementary Figure S1**) using a 3 ml Luer-Lok syringe with a 16G needle (BD Precision Glide Needle, Becton, Dickinson and Company, Franklin Lakes, NJ).

Cartridges C1, C2, C3, C4, and C6 were ceramic coil type cartridges purchased online to represent the types of THC containing cartridges commonly seen during the EVALI investigation and C5 was a ceramic coil type cartridge locally purchased. Additionally, one black market and one authentic (commercially available in a jurisdiction where such products are legal according to state and local law) cartridge containing Δ^9 -tetrahydrocannabinol received by the FCC as part of the EVALI investigation were used. The resistance of each cartridge shown in **Supplementary Figure S1** was measured prior to use with a multimeter (FLUKE 79 Series II Multimeter, Fluke Corp., Everett, WA).

An unvaped portion of the THC/VEA mixture was dispensed into a section of tygon tubing (0.19" internal diameter (ID), Masterflex L/S 15 E-3606, Cole-Parmer, Vernon Hills, IL, United States), allowed to rest 15 min, and then collected for use as a control.

Vaping Apparatus for Aerosol Production and Collection

A direct method for aerosol generation and collection was adapted from previous work (Olmedo et al., 2016; Lanzarotta et al., 2020) to allow for the insertion of a thermocouple for temperature measurements as shown in **Figure 1**. Aerosol was generated using a peristaltic pump (drive no. 07522-20 and head no. 77200-62, Cole-Parmer, Vernon Hills, IL, United States), which pulls the aerosol from a cartridge attached to a vaping device (Eleaf iStick 30W, locally purchased). The cartridge was connected to a y-junction by a variable length of 0.19" ID tygon tubing (approximately 5–8 cm) fit snugly over the cartridge mouthpiece. From the y-junction, a 20 cm piece of 0.19" ID tygon tubing was run through the peristaltic pump. Downstream of the pump, the 20 cm tube was connected to a series of four 1 ml pipette tips (one cut to fit inside the 0.19 ID tubing and the rest uncut to fit over the 1/8" ID tubing), three 6 cm sections of tygon tubing (E-3606 tygon, 1/8" ID tubing, Cole-Parmer, Vernon Hills, IL, United States), and one 2 cm section of 1/8" ID tygon tubing. The last pipette tip and 2 cm of tubing was inserted loosely into a hole in the cap of a 20 ml glass vial, to allow for ventilation. The tubing and pipette tips were used as received and all tubing and pipette tips were replaced following vaporization of each cartridge.

For all vaping experiments the peristaltic pump was programmed to operate at a flow rate of 1.0 L/min and a puff topography of 4 s puffs every 30 s, with manual activation of the vaping device starting 3 s prior to puff start at a voltage set according to experimental design. This puff topography is similar to that of an average experienced e-cigarette slow user as described by Talih et al. (2015). (Talih et al., 2017). Condensate was collected by centrifuging (Eppendorf 5810 centrifuge, Eppendorf, Hauppauge, NY) each portion of the tubing system containing condensed material at 4,000 rpm for 2 min into a glass vial following each experiment.

Measuring Temperature

Temperature was measured during vaping by threading a Type-K thermocouple probe (Models TJ36-CASS-020G-6 and TJ36-CASS-020U-6, Omega Engineering Inc., Norwalk, CT) through a sealed junction in the vaping apparatus tubing and down through the mouthpiece and airway to the coil/wick of each cartridge (**Figure 1**). Temperature measurements were very sensitive to the position of the probe tip, with the highest temperature measurements obtained when the probe tip was located between the top and bottom of the heating coil. Except where otherwise stated, probes were positioned to read the maximum temperature during vaping. Temperature data was logged at 1 s intervals throughout each experiment with a portable data logger (OM-74, Omega Engineering Inc., Norwalk, CT). The thermocouple probes were cleaned following vaporization of each cartridge by rinsing with methanol (Fisher Scientific, St. Louis, MO, United States) and water (18 M Ω purity, Millipore, Massachusetts, United States) while gently wiping the exterior with Kimwipes to remove any

excess material. The probes were replaced if they appeared damaged or the material could not be sufficiently removed.

GC-FID Analysis

An internal standard solution was prepared by weighing a portion of squalane, purchased from USP (Rockville, MD, United States), into a volumetric flask that yielded a 2.5 mg/ml solution when diluted to volume with cyclohexane, purchased from Fisher Scientific (St. Louis, MO, United States). The solution was sonicated for approximately 5 min. All samples and vitamin E acetate standards were diluted using the internal standard solution. The vitamin E acetate standard was purchased from USP.

Adapting previously developed methods (Ciolino et al., 2018a), triplicate preparations of approximately 10 mg from cartridges C1-C6 and one unvaped portion of sample were combined with 250 μ l of internal standard solution. Vials were vortexed to mix and sonicated for approximately 10 min. An additional 1:40 dilution was prepared from the initial dilution. A portion of C6 was spiked with vitamin E acetate at a level 480 mg/g.

The vitamin E acetate stock standard was prepared at approximately 10 mg/ml. A calibration curve was constructed from dilutions of the stock standard. The vitamin E acetate curve was linear 10 μ g/ml to 1 mg/ml, with $r = 0.9999$ for duplicate injections of the high and low standard and five injections of the mid standard. Separation and detection of VEA was conducted using a GC-FID. Chromatography was performed on an Agilent Technologies (Santa Clara, California, United States) GC 7890B Series outfitted with a G4567A Series autosampler and an Agilent HP 5% phenyl column with length, I.D., and film thickness dimensions of 30 m, 0.32 mm, and 0.25 μ m, respectively. Helium carrier gas was employed in constant flow mode using a flow rate of 1.3 ml/min. Injections were performed in a 50:1 split mode with an injection volume of 1.0 μ l and an injector temperature of 290°C. The method included a starting temperature of 60°C with a hold time of 0.5 min then a ramp rate of 25°C/min until a temperature of 220°C was reached and held for 10.0 min. A second ramp rate was performed of 10°C/min until a final temperature of 300°C was reached and held for 9.0 min, which resulted in a total run time of 33.9 min.

Flame ionization detection was accomplished using an Agilent Technologies FID. A H₂ flow rate of 30 ml/min, an airflow rate at 400 ml/min, and 25 ml/min helium makeup gas flow were used. The temperature of the detector was set at 300°C. Data were acquired and analyzed using Agilent OpenLab CDS software version C.01.07 SR2 [255].

HPLC-UV

Adapting previously developed methods (Ciolino et al., 2018b), the material from cartridges C1-C6 and one unvaped portion of sample was heated to 90°C for approximately 5 min to ensure ease of sample transfer. Triplicate preparations of approximately 25 mg of material from each were weighed and placed into 4 ml glass vials. 1.0 ml of 95% ethanol ACS USP Grade (Fisher Scientific, St. Louis, MO, United States) was added to each vial, capped and vortexed. A 0.1 ml aliquot of each initial dilution and

10.0 ml of 95% ethanol were added to a scintillation vial, capped, vortexed and then filtered using 0.45 μ m nylon membrane filter. A Δ^9 -THC standard, purchased from Cerilliant (Round Rock, TX, United States), was used to prepare an external calibration curve.

Analysis was performed on an Agilent HPLC system with UV detection (1,260 Infinity) with an ACE 5 C18-AR, 250 $\times$ 4.6 mm, 5 μ m HPLC column. Column compartment temperature was maintained at 30°C. Using isocratic conditions, the mobile phase consisting of 0.5% acetic acid and acetonitrile (34:66). The total run time was 60 min with a flow rate of 1.0 ml/min, UV detection at 240 nm, spectral collection, 190–400 nm, and injection volume of 25 μ l.

GC/MS Analysis

Adapting previously developed methods (Ciolino et al., 2018a), duplicate preparations of approximately 25 mg from cartridges C1-C6 and one unvaped portion of sample were combined with 1 ml of 95% ethanol. Vials were vortexed to mix. 25 μ l of the sample extract was added to 1 ml of acetonitrile and vortexed to mix. 200 μ l of sample extract was derivatized using pyridine and BSTFA + 1% TMCS (bis(trimethylsilyl)trifluoroacetamide +1% Trimethylchlorosilane).

Separation and detection for the underivatized and derivatized sample preparations were conducted using a GC/MS. Chromatography was performed on an Agilent Technologies GC 7890B Series outfitted with a 7963 Series autosampler and an Agilent 5% phenyl column for the underivatized and a Restek 35% silphenylene column for the derivatized, both with length, I.D., and film thickness dimensions of 30 m, 0.25 mm, and 0.25 μ m, respectively. Helium carrier gas was employed in constant flow mode using a flow rate of 0.8 ml/min. Injections were performed in a splitless mode with an injection volume of 1.0 μ l and an injector temperature of 250°C. The method included a starting temperature of 60°C with a hold time of 0.5 min then a ramp rate of 25°C/min until a temperature of 220°C was reached and held for 10.0 min. A second ramp rate was performed of 10°C/min until a final temperature of 300°C was reached and held for 15.0 min, which resulted in a total run time of 39.9 min.

Mass spectrometric detection was performed using an Agilent 5977B series mass selective detector. Data were collected with a mass range of 40–600 Da using full scan mode, a 3.5 min solvent delay for the underivatized and a 7.0 min solvent delay for the derivatized, a threshold of 150, quadrupole temperature of 150°C, a source temperature of 230°C, and electron ionization energy of 70 eV. Data analysis was performed using Agilent Mass Hunter Version B.07.06.2704.

GC/FT-IR/MS Analysis

Sample extracts from the GC/MS preparation were examined. 50 μ l of acetonitrile was added to 50 μ l of the C5 cartridge sample extract. A duroquinone standard was purchased from Sigma (St. Louis, MO, United States), and prepared and analyzed at a concentration of 0.5 mg/ml.

Separation and detection of the C5 cartridge extract was conducted using a fully integrated GC/FT-IR/MS instrument. Chromatography was conducted using an Agilent 7890B Series

GC outfitted with a G4567A Series autosampler and a Phenomenex Zebron ZB-5MSi column with length, I.D. and film thickness dimensions of 30 m, 0.25 mm, and 0.25 μm , respectively. Helium carrier gas was employed in constant flow mode using a flow rate of 2 ml/min. Injections were performed in a splitless mode with an injection volume of 1.0 μl and an injector temperature of 250°C. The method included a starting temperature of 75°C with a hold time of 1.0 min and a ramp rate of 30°C/min until a final temperature of 330°C was reached. The final temperature was held for 10.5 min, which resulted in a total run time of 20 min. The terminus of the column was inserted into an inert capillary tee that splits approximately 66% of the GC effluent to a transfer line connected to the IR interface and approximately 34% of the GC effluent to a transfer line connected to the MS interface. The transfer line temperatures from the GC to the mass-selective detector and from the GC to the IR detector were 280 and 300°C, respectively.

Infrared detection was accomplished using a Dani Instruments DiscovIR FT-IR spectrometer. The terminus of one transfer line exiting the inert capillary tee from the GC was inserted into the IR interface and positioned directly above the ZnSe disk. Data were collected using a 100 $\mu\text{m} \times 100 \mu\text{m}$ MCT detector, 4,000–700 cm^{-1} spectral range, 4 cm^{-1} resolution, 10 mm/min disk speed, 5.0 min solvent delay, 300°C restrictor temperature, 300°C oven temperature, 35°C dewar cap temperature and –40°C disk temperature. Instrument operations and data analysis were conducted using workbooks designed in Grams software version 9.2 by Dani Instruments.

Mass spectrometric detection was performed using an Agilent 5977A series mass selective detector. The terminus of the second transfer line exiting the inert capillary tee from the GC was inserted into the MS and positioned directly in front of the electron ionization (EI) source. Data were collected with a mass range of 50–550 Da using full scan mode, a 5.0 min solvent delay, detector turned off at 8 min, –125 relative voltage, a threshold of 150, quadrupole temperature of 150°C, a source temperature of 230°C, and electron ionization energy of 70 eV. Data analysis was performed using Agilent MSD Chemstation software version F.01.03.2357.

LC-APCI-HRMS/MS Qualitative and Quantitative Analysis

The samples prepared for GC/MS analysis were further diluted by a factor of 10 in 50/50 $\text{H}_2\text{O}/\text{MeOH}$ for qualitative analysis. A 42.0 mg portion of sample C1 was dissolved in 1 ml of acetonitrile for quantitative analysis; five 50.0 μl aliquots of this solution were each combined with 950 μl of methanol. Four of these diluted solutions were spiked with appropriate amounts of a 1.00 mg/ml solution of duroquinone reference standard to measure the level of duroquinone by standard additions.

Qualitative analysis was performed on a Thermo Scientific Dionex UltiMate 3000 LC equipped with a Phenomenex Luna Phenyl-Hexyl, 3.0 μm , 2.0 $\times$ 150 mm column held at 40°C, coupled to a Thermo Scientific Q Exactive high-resolution mass spectrometer (HRMS) equipped with an atmospheric pressure chemical ionization (APCI) source. Data were

acquired using Xcalibur 4.0 software from Thermo Scientific. Mobile phase flowed at a constant rate of 0.250 ml/min. Following a 5-min pre-injection equilibration at 90% A (18.2 M Ω -cm H_2O) and 10% B (HPLC-grade MeOH), gradient elution was performed by linearly ramping to 95% B in 20 min and holding for 12 min. Eluant was directed to the HRMS inlet from 2.5 to 21 min and diverted to waste at all other times to avoid saturation of the HRMS with high levels of THC and VEA. The injection volume was 5.0 μl .

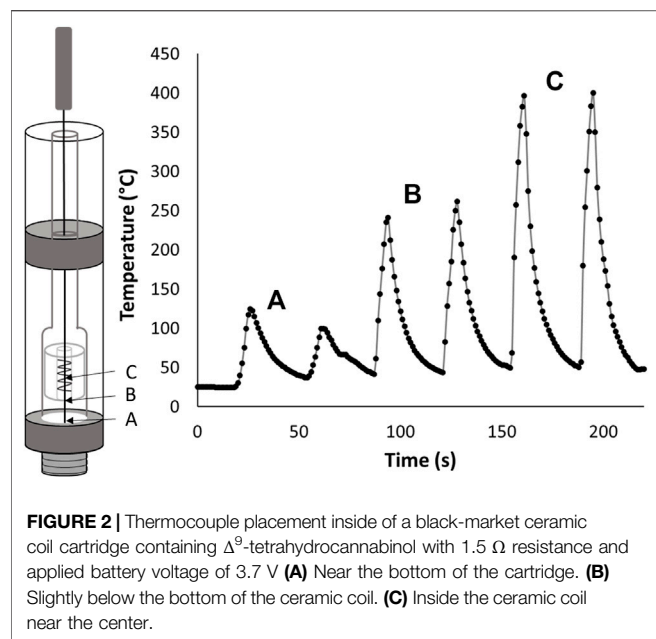
The instrument parameters for the mass spectrometer were as follows: positive polarity; sheath gas flow = 25 arbitrary units; auxiliary gas flow = 5 arbitrary units; sweep gas flow = 2 arbitrary units; APCI temperature = 250°C; discharge current = 5.0 μA ; capillary temperature = 275°C; S-lens RF level = 60; resolution = 140,000 (full scan), 35,000 (MS/MS); automatic gain control (AGC) target = 1×10^6 ; scan range for full scan data collection = m/z 130–1,200. Data-dependent MS/MS spectra were collected on either the two most abundant ions in the preceding full scan spectrum, or from an inclusion list that included only duroquinone (DQ) and 4-acetoxy-2,3,5-trimethyl-6-methylene-2,4-cyclohexadienone (ATMMC), employing a 1.0 s dynamic exclusion window, ± 0.5 Da isolation window, and collision energy of 30 eV. The instrument was calibrated according to manufacturer's specifications.

Quantitative duroquinone analysis was performed using the same LC, column, mobile phase, and flow rate, but using a 4-min pre-injection equilibration at 40% A and 60% B, followed by isocratic elution for 14 min, followed by a step to 90% B and holding for 10 min. Eluant was directed to the HRMS inlet from 2 to 13.5 min and diverted to waste at all other times. The injection volume was 2.0 μl and each solution was injected in triplicate. The HRMS ion source parameters were identical, but data acquisition was limited to MS/MS spectra of m/z 165.0910 $\pm$ 0.5 at a collision energy of 30 eV. Extracted ion chromatograms (EICs) of m/z 107.0495 (± 0.0005) were used for measuring duroquinone peak areas.

RESULTS AND DISCUSSION

Temperature Measurements and Aerosol Collection

To allow for temperature measurements that are the most representative of a typical ceramic coil cartridge user experience, the cartridges were left completely intact. While this is advantageous for mimicking the end user experience it makes exact positioning of the probe in the cartridge difficult because the coil cannot be seen from the outside of the cartridge. To examine the effect of probe placement in the cartridge, the temperature was monitored at multiple sites from the bottom of the cartridge to the estimated center of the ceramic coil as shown in **Figure 2**. For the cartridges tested in this work there was generally approximately 3–5 mm of space between the bottom of the coil and the bottom of the cartridge. The total coil lengths for the cartridges used in this work were approximately 5 mm. This gives an approximate 8–10 mm of space from the bottom of the

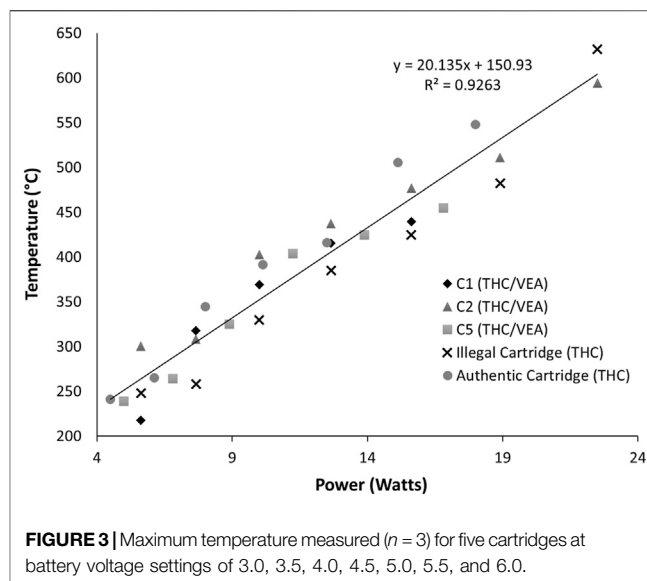


cartridge to the top of the coil. Based on our experience, these dimensions were similar for all ceramic coil type cartridges.

Figure 2 shows extreme differences in temperature measurements are seen based on probe placement. At or near the bottom of the cartridge, temperatures in the 100°C range were seen while movement to the estimated center of the cartridge only approximately 5.5–7.5 mm away, produced temperatures of 400°C. Moving only about 3 mm away from the center of the coil to at or just below the bottom of the coil results in temperatures of 250°C.

Similar results were seen but not shown for movement of the probe above the coil. The clear temperature difference within the cartridge makes probe positioning an essential part of accurately measuring the maximum temperature. To ensure proper probe placement for all vaping experiments performed in this work, the top of the coil was identified by inserting the probe gently until resistance from the top of the coil was felt then slowly feeding the probe an additional 2.5 mm into the cartridge. During the initial vaping for each cartridge the probe was moved slightly up or down until the placement resulting in the highest temperature was found. The probe was kept at this position for the remainder of the vaping process.

To determine the temperature associated with different voltage settings and the maximum power level at which the THC/VEA mixture could be sustainably vaped, we examined the temperature response of five different cartridges during vaping at increasingly higher power levels (**Figure 3**). Three cartridges (C1, C2, and C5) contained the THC/VEA mixture prepared by the laboratory. Two cartridges (Illegal and Authentic) contained unknown mixtures which included Δ^9 -tetrahydrocannabinol. Each cartridge was vaped for three puffs at 3.0 V, then the set voltage was increased by 0.5 V. These steps were repeated until the heating element failed prior to the completion of the three puffs, or until the voltage increase would result in power exceeding the device limit of 30 W.



Heating element failure was determined by a failure to produce vapor and a subsequent failure state reading by the device. Temperature was recorded throughout each experiment.

Figure 3 shows the average maximum temperature at each power setting. Power (P in watts) was calculated from the applied voltage (V in volts) and the resistance (R in ohms) of each cartridge by combining Ohm's and Joule's Law.

$$P = \frac{V^2}{R}$$

As anticipated, temperature increased with power in all cases. In all five cartridges ample vapor was produced with each puff at applied voltages between 3.0 and 5.0 V, corresponding to calculated powers ranging from 4.5 to 16.8 W, with temperatures ranges of 217–300°C at 3.0 V and 415–476°C at 5.0 V. Four cartridges out of six (C2, C5, Illegal, Authentic) produced vapor at 5.5 V, at calculated powers from 15.1 to 18.9 W and a temperature range of 454–510°C. Only three cartridges (C2, Illegal, Authentic) were successfully vaped at 6.0 V, at powers of 18–22.5 W and temperatures of 548–632°C. No vaping was done above 6.0 V due to the power limit of the battery. The temperature response to power of these five cartridges was fit to a linear model with a coefficient of determination (R^2) of 0.926.

The effects of sustained vaping at elevated power levels on the composition of the THC/VEA mixture, were evaluated by collecting vapor condensate from six cartridges after extended vaping runs (25+ puffs) for analysis (**Table 1**). Based on the temperature responses observed in the previous experiment, we selected an approximate power target of 16 W (representing applied voltages of 4.7–5.3 V) for vaping of five of the six cartridges. Previous experience with a cartridge similar to C1 suggested that 16 W was beyond the capacity of that cartridge's heating coil, therefore a power of 12.7 W (4.5 V applied) was selected for C1.

Vapor condensate was collected with yields ranging from 55.5 to 87.6% of the calculated total weight of THC/VEA mixture

TABLE 1 | Average vaped per puff determined by change in mass of the cartridge before and after vaporization/total puffs. %Recovery of vaped material determined from the mass of condensate collected/calculated total weight of THC/VEA mixture consumed. The average temperature excludes the first ten vapes to optimize thermocouple placement.

Cartridge	Applied Voltage (V)	Calculated Power (Watts)	Average Vaped per puff (mg)	% Recovery of Vaped Material	Average Temperature $\pm$ SD and Range $^{\circ}$ C	VEA % Recovery $\pm$ SD	THC % Recovery $\pm$ SD
C1	4.5	12.7	12.1	75.3	439 $\pm$ 22 (387–466)	78.9 $\pm$ 1.6	69.1 $\pm$ 1.5
C2	4.7	15.8	15.1	83.3	441 $\pm$ 8.0 (429–461)	100 $\pm$ 2.7	88.0 $\pm$ 1.3
C3	5.0	15.6	10.7	73.2	456 $\pm$ 37 (378–569)	82.0 $\pm$ 0.7	62.4 $\pm$ 1.6
C4	4.7	15.8	9.04	69.3	445 $\pm$ 17 (421–477)	91.4 $\pm$ 2.2	73.2 $\pm$ 0.1
C5	5.3	15.6	9.05	55.5	503 $\pm$ 23 (469–567)	71.5 $\pm$ 0.1	56.4 $\pm$ 0.2
C6	4.9	16.0	13.9	87.6	448 $\pm$ 22 (375–493)	101 $\pm$ 3.2	85.0 $\pm$ 0.7

consumed. The likely determinant of recovery yields are the proportions of vapor that escaped from below the cartridge during the initial 3 s of battery operation prior to activation of the pump. In some instances, the amount of vapor seen escaping from the bottom cartridge inlets was significant especially at higher temperatures. The recovery reported here is significantly higher than previously reported for nicotine based products. (Olmedo et al., 2016). Differences in the calculated material consumed per puff, which ranged from 9.04 to 15.1 mg, are presumed to be due in part to variations in cartridge geometry.

The reported temperature in **Table 1** is the average maximum temperature recorded per puff, with the first ten measurements excluded from the average because of the time it takes to find the center and hottest location in the coil. Excluding the first ten measurements of each run results in a measured average maximum temperature range of 439 $\pm$ 22 (C1) to 503 $\pm$ 23 $^{\circ}$ C (C5), with individual minimum and maximum measurements of 375 and 569 $^{\circ}$ C, respectively. Excluding C1, the average maximum temperature (excluding the first 10 measurements) of the five cartridges vaped at a target power of approximately 16 W was 459 $^{\circ}$ C. The temperatures measured in this work are higher than those commonly reported for e-cigarette products intended for use with nicotine based e-liquids, but similar to the temperature reported for a stainless steel coil saturated with VEA. (Geiss et al., 2016; Mikheev et al., 2020).

In order to determine the effect these temperatures have on the major components of cartridges associated with EVALI the concentration of both Δ^9 -THC and VEA were compared to the initial unvaped material. Four of the six cartridges showed reduced recovery for VEA in the range of 71.5–91.4%. Two cartridges exhibited no change though all were vaped at similar temperatures. To further examine this difference two additional sample mixtures were vaped in cartridge C2. This resulted in VEA recoveries of 103 and 109% of the initial VEA concentration. Differences in the recovery of VEA between cartridges is believed to be due in part to variations in cartridge geometry and air flow within the cartridges. All six cartridges showed reduced recoveries for Δ^9 -THC with a range of 56.4–88.0% recovery compared to the unvaped portion. In general, the trend for recovery of Δ^9 -THC correspond to that of VEA for all vaped cartridges. For quality control purposes the material collected from one cartridge, C6 was fortified with Δ^9 -THC and VEA resulting in a 100 and 90% recovery, respectively.

Analysis of Collected Aerosol

GC/MS analysis was performed on the unvaped sample (blank) and the material from the six vaped cartridges (C1, C2, C3, C4, C5, and C6) to determine if additional compounds were produced during vaping. As shown in **Figure 4** and **Supplementary Figure S2**, all samples yielded peaks at 19.8 and 27.1 min that exhibited mass spectra corresponding to Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and vitamin E acetate (VEA), respectively. A comparative analysis of the unvaped sample and the six vapor condensates indicated significant differences in the 5–8 min range. Based on a GC/MS library match, one of the peaks observed was determined to be duroquinone (DQ). Two additional peaks in this range were suspected to be 1-pristene and durohydroquinone monoacetate (DHQMA) based on mass spectral interpretation of the ions that were exhibited in these peaks. This range was further examined using GC/FT-IR/MS to verify the composition of these additional compounds. Additionally, a peak in the derivatized data was observed that supports the presence of DHQMA.

GC/FT-IR/MS analysis was performed on the collected material from cartridge C5. The total ion chromatogram (TIC) and absorbance chromatogram (AC) of C5 in the 5–8 min region are shown in **Figure 5**. The C5 extract yielded multiple peaks that were not present in the blank, unvaped sample; the TIC peaks at 5.30, 6.86, and 6.92 min correlate with the AC peaks at 5.43, 6.93, and 7.05 min, respectively. The retention times of the TIC peak at 5.30 min and corresponding AC peak at 5.43 min were consistent with those of the duroquinone standard. Mass and IR spectra corresponding to these peaks in the C5 extract are shown in **Figures 6A,B**, respectively, and are each consistent with spectra of the duroquinone standard, which are shown in **Figures 6C,D**. Both compounds exhibited significant ions at m/z 164 (molecular ion), 136, 121, 108 and 93 and both compounds exhibited characteristic infrared absorptions at 1,641, 1,448, 1,380, and 1,026 cm^{-1} .

The mass spectrum of the peak at 6.86 min in the C5 TIC is shown in **Supplementary Figure S3A** and the corresponding IR spectrum of the peak at 6.93 min in the C5 AC is shown in **Supplementary Figure S3B**. The mass spectrum exhibited significant ions at m/z 266, 196, 140, 126, and 111; this pattern of loss indicates that the compound is likely a hydrocarbon chain. The IR spectrum indicates a CH_2 chain-containing hydrocarbon that contains branching CH_3 groups and

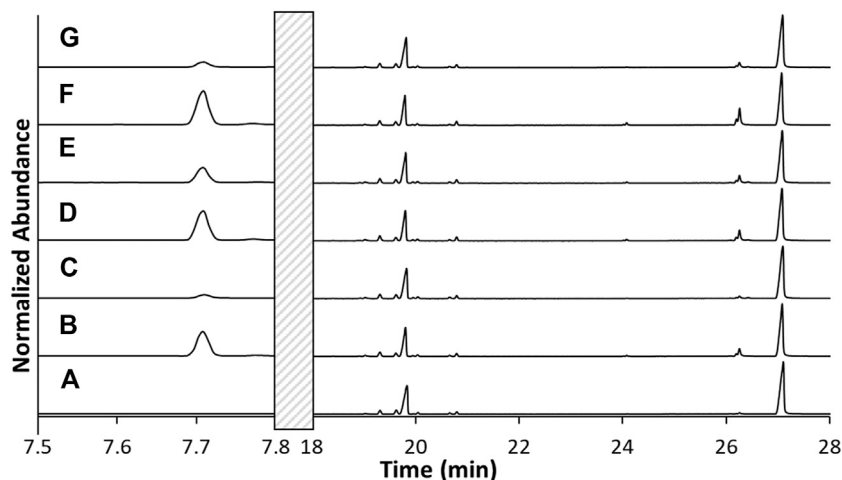


FIGURE 4 | TIC of the unvaped THC and VEA-containing E-liquid (A) compared to TICs of the post-vaped E-liquids from cartridges C1-C6 (B-G), respectively. No peaks from 7.8 to 18 min.

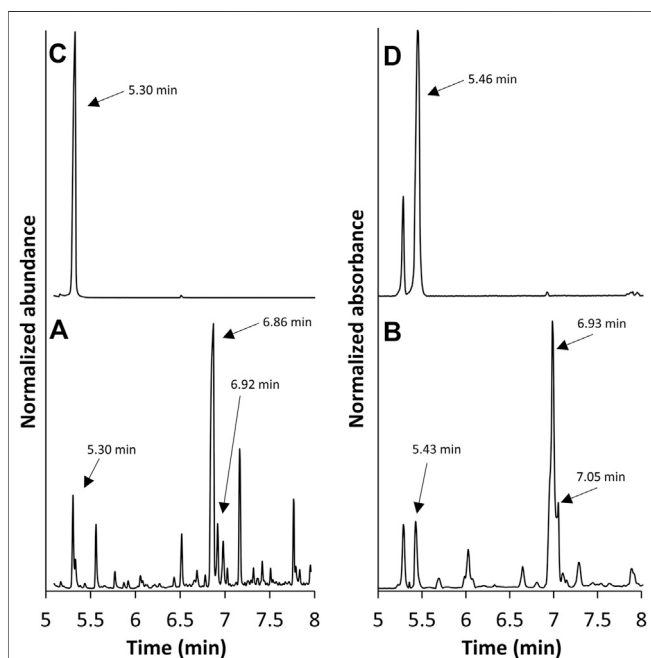


FIGURE 5 | GC/FT-IR/MS TIC (A) and AC (B) of the post-vape E-liquid from cartridge C5 along with the TIC (C) and AC (D) of a duroquinone standard.

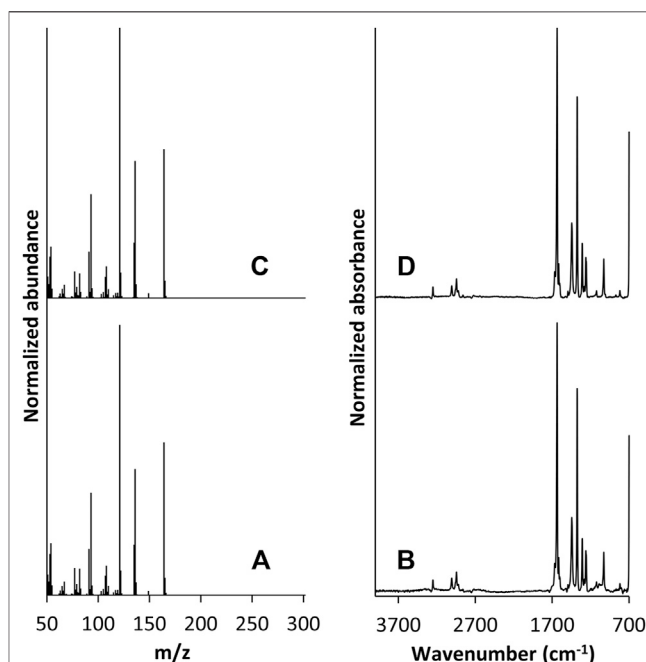


FIGURE 6 | GC/FT-IR/MS mass spectrum of the peak at 5.30 min in the suspect chromatogram from cartridge C5 (A) and corresponding IR spectrum (B). Mass spectrum of the peak at 5.30 min in the duroquinone chromatogram (C) and corresponding IR spectrum (D).

a terminal C=C group (vinylidene) in a *cis* configuration. Specific peak assignments are provided in **Supplementary Table S1**. Both IR and mass spectra support the structure of 1-pristene.

The mass spectrum of the peak at 6.92 min in the C5 TIC is shown in **Supplementary Figure S3C** and the corresponding IR spectrum of the peak at 7.05 min in the C5 AC is shown **Supplementary Figure S3D**. The TIC peak at 6.92 min exhibited significant ions at m/z 208, 166, 151, and 43; the ion at m/z 166 represents a loss of 42 Da that could be due to a loss of

an acetyl moiety and the addition of a hydrogen, an ion at m/z 43 indicates the presence of the acetyl moiety. The ion at m/z 151 represents a loss of 15 Da from the ion at m/z 166, which could be due to loss of a methyl group.^{19,21} The IR spectrum from the AC peak at 7.05 min exhibited many of the same absorptions observed in the IR spectrum from the AC peak at 6.93 min. However, since the AC peak at 7.05 min is not baseline resolved from the peak at 6.93 min, it is unknown if these shared

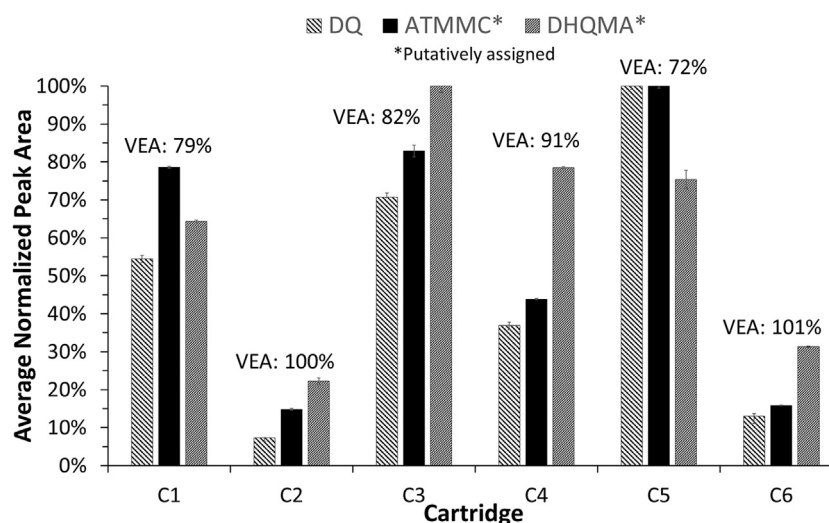


FIGURE 7 | Peak areas, normalized to the cartridge with the largest peak area for each compound, measured in the vaped samples for duroquinone (using the LC-MS EIC of m/z 165.0910), putatively assigned ATMMC (using the LC-MS EIC of m/z 207.1016), and putatively assigned DHQMA (using the LC-MS EIC of m/z 149.0962), reported as the average of three injections. Error bars represent two standard deviations. The measured recovery of VEA for each cartridge is shown above each cluster in the graph for reference.

absorptions can be assigned to the peak at 7.05 min or if they are simply carryover from the peak at 6.93 min. Nevertheless, the IR spectrum of the AC peak at 7.05 min clearly exhibited additional absorptions at 3470, 1740, 1,229, and 1,069 cm^{-1} that were not observed in the IR spectrum of the AC peak at 6.93 min. The peak at 3470 cm^{-1} may be assigned to OH stretching vibration, the peak at 1740 cm^{-1} may be due to an acetate ester C=O stretching vibration and the peak at 1,229 cm^{-1} may be due to an acetate ester C-C-O stretching vibration. Both IR and mass spectra support the structure of durohydroquinone monoacetate (DHQMA).

LC-APCI-HRMS/MS Analysis

The acquired data were interrogated for the presence of the predicted VEA degradants (Wu and O'Shea, 2020; Mikheev et al., 2020) ATMMC, DHQMA, and DQ; the other expected VEA degradants, 1-pristene, and ketene, are not detectable by this analysis. Features consistent with ATMMC, DHQMA, and DQ based on accurate mass measurement were observed in each of the vaped condensates, but not observed in the unvaped sample (see **Supplementary Figures S4–S9**). DQ was further identified by comparison to a reference standard (**Supplementary Figures S8, S9**) and, as described above, a compound consistent with DHQMA was also detected by GC/MS analysis of both the derivatized and non-derivatized vaped condensates, as well as by GC/FT-IR/MS analysis; no compounds consistent with ATMMC were observed by GC/MS or GC/FT-IR/MS, perhaps due to lower abundance, which would be consistent with its proposed role as a reactive degradation intermediary. (Mikheev et al., 2020).

From their respective EICs, the peak areas were measured for these three compounds and compared to the recoveries of VEA measured for each cartridge. If these compounds are formed from

the degradation of VEA, it is expected that larger peak areas would be observed for those samples that exhibited the lowest VEA recoveries, and this is indeed the case as shown in **Figure 7**. As shown in **Table 1**, cartridge C5 yielded the lowest VEA recovery, 71.5%, and the largest peak areas for DQ, putatively assigned ATMMC, and putatively assigned DHQMA. Similarly, cartridges C1 and C3 yielded the next lowest VEA recoveries, approximately 80%, and the next largest peak areas for DQ and putatively assigned ATMMC. Cartridges C2 and C6 exhibited the highest VEA recoveries, approximately 100%, and the smallest peak areas for DQ and putatively assigned ATMMC. The peak areas for putatively assigned DHQMA exhibited a similar pattern of relative abundances, with a couple of exceptions: cartridges C3 and C4 yielded a higher abundance than expected, and cartridge C5 a lower abundance than expected, relative to the other cartridges based on the VEA recoveries.

Although the presence of ketene could not be detected directly, the proposed reaction schemes that explain its formation indicate that one molecule of ketene is formed for each molecule of DQ that is formed. (Wu and O'Shea, 2020; Mikheev et al., 2020). Therefore, measurement of the amount of DQ can be used as an indirect measurement of the amount of ketene generated. Standard additions were used to measure the level of DQ in the vaped liquid collected from cartridge C1. The EIC of the product ion m/z 107.0495 was used to obtain the peak areas since it had the lowest background in the region of the DQ peak (**Supplementary Figure S10**). The calibration curve obtained is shown in **Supplementary Figure S11**, in which the dots represent the average peak areas, measured from triplicate injections. The calculated concentration of DQ in the analyzed solution of the vaped condensate collected from cartridge C1 was $8.74 \pm 0.14 \mu\text{g/ml}$, which corresponds to $4.16 \pm 0.07 \mu\text{g}$ DQ per mg of vaped condensate, or $0.416 \pm 0.007\%$ by mass (the uncertainties

represent two standard deviations). This results in a VEA to DQ conversion rate of approximately 1%, which is significantly less than that shown using cartridges with lower resistance and higher power. (Wu and O'Shea, 2020). Using the information from **Table 1** that 12.1 mg of sample were vaped per puff for cartridge C1, the measured DQ level corresponds to an average of 50.4 μ g DQ generated per puff and, by extension, an estimated maximum of 12.9 μ g ketene generated per puff.

The qualitative LC-APCI-HRMS/MS data were also evaluated for the presence of additional compounds that were present either only in the vaped condensates or at visibly higher levels in the vaped condensates than the unvaped liquid. The chromatograms of the vaped condensates contained many features that were not observed in the unvaped liquid. The major features were tentatively assigned as isomerization or degradation products of Δ^9 -THC and/or other cannabinoids present in the sample based on molecular formulae generated from HRMS data that corresponded to compounds previously observed in thermal decomposition studies of cannabinoids. (Mechoulam, 1970; Küppers et al., 1975; Salemin, 1976; Spronck et al., 1978; Tjeerdema, 1987). An in-depth investigation of the many features present in the LC-MS and derivatized GC/MS data is beyond the scope of this study and will be the focus of future work.

CONCLUSION

In this study we establish that vaping using a commercially available device and ceramic coil cartridges can produce coil temperatures sufficient for the thermal decomposition of VEA in THC/VEA mixtures. The initial VEA concentration in the THC/VEA mixture was reduced following vaporization in four of the six cartridges, though all cartridges achieved similar maximum temperatures under the experimental conditions in this work. The initial concentration of THC in the THC/VEA mixture was reduced following vaporization in all six cartridges. These results were generated using elevated power settings, and the congruence of this method with typical vaping practices among consumers is presently unexamined. Additionally, we identified compounds consistent with the production of ketene gas by proposed

pathways for VEA decomposition, (Wu and O'Shea, 2020) including detection of a substance that, based on exact mass measurement, was consistent with the previously undetected quinone methide ATMMC. Although ketene itself was not detected by the methods employed in this study, detection of the previously identified VEA degradants (Jiang et al., 2020; Mikheev et al., 2020; Wu and O'Shea, 2020) in the vaped condensates collected from the THC/VEA mixtures indicates that, at least under some conditions that consumers may encounter, pulmonary toxins may be produced from vaping of mixtures containing VEA in ceramic coil cartridges.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RW conceived, designed, and supervised the study. JL and RW performed temperature measurements and condensate collection. LL, JB, and AL performed the GC/MS, HPLC-UV, and/or GC/FT-IR/MS analysis. TF performed the LC-APCI-HRMS/MS analysis. All authors assisted in the preparation and approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.734793/full#supplementary-material>

REFERENCES

- Bhat, T. A., Kalathil, S. G., Bogner, P. N., Blount, B. C., Goniewicz, M. L., and Thanavala, Y. M. (2020). An Animal Model of Inhaled Vitamin E Acetate and EVALI-Like Lung Injury. *N. Engl. J. Med.* 382, 1175–1177. doi:10.1056/nejmc2000231
- Bitzer, Z. T., Goel, R., Reilly, S. M., Foulds, J., Muscat, J., Elias, R. J., et al. (2018). Effects of Solvent and Temperature on Free Radical Formation in Electronic Cigarette Aerosols. *Chem. Res. Toxicol.* 31 (1), 4–12. doi:10.1021/acs.chemrestox.7b00116
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2019). Vitamin E Acetate in Bronchoalveolar Lavage Fluid Associated with EVALI. *New Engl. J. Med.* 382, 697–705. doi:10.1056/NEJMoa1916433
- Ciolino, L. A., Ranieri, T. L., and Taylor, A. M. (2018). Commercial Cannabis Consumer Products Part 1: GC-MS Qualitative Analysis of Cannabis Cannabinoids. *Forensic Sci. Int.* 289, 429–437. doi:10.1016/j.forsciint.2018.05.032
- Ciolino, L. A., Ranieri, T. L., and Taylor, A. M. (2018). Commercial Cannabis Consumer Products Part 2: HPLC-DAD Quantitative Analysis of Cannabis Cannabinoids. *Forensic Sci. Int.* 289, 438–447. doi:10.1016/j.forsciint.2018.05.033
- Department of Health (2019). New York State Department of Health Announces Update on Investigation into Vaping-Associated Pulmonary Illnesses. Available at: https://www.health.ny.gov/press/releases/2019/2019-09-05_vaping.htm (accessed Feb 27, 2020).
- Dibaji, S. A. R., Guha, S., Arab, A., Murray, B. T., and Myers, M. R. (2018). Accuracy of Commercial Electronic Nicotine Delivery Systems (ENDS) Temperature Control Technology. *PLOS ONE* 13 (11), e0206937. doi:10.1371/journal.pone.0206937
- Geiss, O., Bianchi, I., and Barrero-Moreno, J. (2016). Correlation of Volatile Carbonyl Yields Emitted by E-Cigarettes with the Temperature of the Heating Coil and the Perceived Sensorial Quality of the Generated Vapours. *Int. J. Hyg. Environ. Health* 219 (3), 268–277. doi:10.1016/j.ijheh.2016.01.004

- Jiang, H., Ahmed, C. M. S., Martin, T. J., Canchola, A., Oswald, I. W. H., Garcia, J. A., et al. (2020). Chemical and Toxicological Characterization of Vaping Emission Products from Commonly Used Vape Juice Diluents. *Chem. Res. Toxicol.* 33, 2157–2163. doi:10.1021/acs.chemrestox.0c00174
- Küppers, F. J. E. M., Bercht, C. A. L., Salemink, C. A., Lousberg, R. J. J. C., Terlouw, J. K., and Heerma, W. (1975). Cannabis XIV. Pyrolysis of Cannabidiol—Analysis of the Volatile Constituents. *J. Chromatogr. A* 108, 375–379. doi:10.1016/s0021-9673(00)84684-1
- Lanzarotta, A., Falconer, T. M., Flurer, R., and Wilson, R. A. (2020). Hydrogen Bonding between Tetrahydrocannabinol and Vitamin E Acetate in Unvaped, Aerosolized, and Condensed Aerosol E-Liquids. *Anal. Chem.* 92 (3), 2374–2378. doi:10.1021/acs.analchem.9b05536
- Mechoulam, R. (1970). Marijuana Chemistry. *Science* 168, 1159–1165. doi:10.1126/science.168.3936.1159
- Meehan-Atrash, J., Luo, W., McWhirter, K. J., and Strongin, R. M. (2019). Aerosol Gas-phase Components from Cannabis E-Cigarettes and Dabbing: Mechanistic Insight and Quantitative Risk Analysis. *ACS Omega* 4 (14), 16111–16120. doi:10.1021/acsomega.9b02301
- Mikheev, V. B., Klupinski, T. P., Ivanov, A., Lucas, E. A., Strozier, E. D., and Fix, C. (2020). Particle Size Distribution and Chemical Composition of Aerosolized Vitamin E Acetate. *Aerosol Sci. Technol.* 54, 1–8. doi:10.1080/02786826.2020.1783431
- Muthumalage, T., Friedman, M. R., McGraw, M. D., Ginsberg, G., Friedman, A. E., and Rahman, I. (2020). Chemical Constituents Involved in E-Cigarette, or Vaping Product Use-Associated Lung Injury (EVALI). *Toxics* 8, 25. doi:10.3390/toxics8020025
- National Research Council (2014). *Acute Exposure Guideline Levels for Selected Airborne Chemicals: Volume 16*. Washington, DC: The National Academies Press, 398.
- Olmedo, P., Navas-Acien, A., Hess, C., Jarmul, S., and Rule, A. (2016). A Direct Method for E-Cigarette Aerosol Sample Collection. *Environ. Res.* 149, 151–156. doi:10.1016/j.envres.2016.05.008
- Raber, J. C., Elzinga, S., and Kaplan, C. (2015). Understanding Dabs: Contamination Concerns of Cannabis Concentrates and Cannabinoid Transfer during the Act of Dabbing. *J. Toxicol. Sci.* 40 (6), 797–803. doi:10.2131/jts.40.797
- Riordan-Short, S., Nguyen, T. D., Sagar, N., Noestheden, M., and O'Brian, R. (2019). Supra Research and Development Inc. Available at: <https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/can-73327-thermal-decomposition-vitamin-e-acetate-can73327-en.pdf/> (Accessed August 2, 2021).
- Salemink, C. A. (1976). *Marihuana: Chemistry, Biochemistry, and Cellular Effects*. New York: Springer-Verlag, 31–38. doi:10.1007/978-3-642-51624-5_3
- Soulet, S., Duquesne, M., Toutain, J., Pairaud, C., and Lalo, H. (2018). Influence of Coil Power Ranges on the E-Liquid Consumption in Vaping Devices. *Int. J. Environ. Res. Public Health* 15 (9), 1853. doi:10.3390/ijerph15091853
- Spronck, H. J. W., Luteijn, J. M., Salemink, C. A., and Nugteren, D. H. (1978). Inhibition of Prostaglandin Biosynthesis by Derivatives of Olivetol Formed under Pyrolysis of Cannabidiol. *Biochem. Pharmacol.* 27, 607–608. doi:10.1016/0006-2952(78)90403-3
- Talih, S., Balhas, Z., Salman, R., El-Hage, R., Karaoghlanian, N., El-Hellani, A., et al. (2017). Transport Phenomena Governing Nicotine Emissions from Electronic Cigarettes: Model Formulation and Experimental Investigation. *Aerosol Sci. Technol.* 51 (1), 1–11. doi:10.1080/02786826.2016.1257853
- Tjeerdema, R. S. (1987). *Reviews of Environmental Contamination and Toxicology*. New York, NY: Springer, 61–81. doi:10.1007/978-1-4613-8719-0_3
- U.S. Centers for Disease Control and Prevention (2020). Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products. Available at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html#investigation-details (Accessed 12 11, 2020).
- U.S. Food and Drug Administration (2020). Lung Injuries Associated with Use of Vaping Products. Available at: <https://www.fda.gov/news-events/public-health-focus/lung-injuries-associated-use-vaping-products> (Accessed 2 27, 2020).
- Ushikusa, T., Maruyama, T., and Niiya, I. (1991). Pyrolysis Behavior and Thermostability of Tocopherols. *J. Jpn. Oil. Chem. Soc.* 40 (12), 1073–1079. doi:10.5650/jos1956.40.1073
- Varlet, V., Concha-Lozano, N., Berthet, A., Plateel, G., Favrat, B., De Cesare, M., et al. (2016). Drug Vaping Applied to Cannabis: Is “Cannavaping” a Therapeutic Alternative to Marijuana? *Scientific Rep.* 6 (1), 25599. doi:10.1038/srep25599
- Wu, D., and O'Shea, D. F. (2020). Potential for Release of Pulmonary Toxic Ketene from Vaping Pyrolysis of Vitamin E Acetate. *Proc. Natl. Acad. Sci. USA* 117 (12), 6349–6355. doi:10.1073/pnas.1920925117

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A Novel UHPLC-MS/MS Method for Measuring 8-iso-Prostaglandin F_{2α} in Bronchoalveolar Lavage Fluid

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In August 2019, the Centers for Disease Control and Prevention (CDC) received the first reports of lung injuries that were eventually termed e-cigarette, or vaping, product use-associated lung injury (EVALI). As part of the investigation, CDC laboratories rapidly developed assays for analyzing substances in bronchoalveolar lavage (BAL) fluid collected from EVALI case patients. This report describes the development and validation of a high-throughput isotope dilution UHPLC-MS/MS method for measuring a major oxidative stress biomarker, 8-iso-prostaglandin F_{2α} (8-isoprostane), in BAL fluid samples. The method showed good sensitivity, 17.6 pg/ml LOD, and requires only 50 μl of sample volume. The method had high throughput with an analytical run time of 11 min. The within-day and between-day coefficient of variation (CV) were below 2%. Accuracy, calculated from spiked recovery, at three spiking levels, ranged from 95.5–101.8%. This novel UHPLC-MS/MS method characterizes oxidative stress in lung epithelial tissue and thus helps to elucidate potential pathologic processes.

Keywords: EVALI (e-cigarette or vaping product use-associated lung injury), 8-isoprostane, UHPLC-MS/MS, BAL fluid, oxidative stress

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) received initial case reports of a range of pulmonary illnesses requiring hospitalization in otherwise healthy users of e-cigarette, or vaping, products (EVPs) in August 2019. The number of hospitalized cases eventually rose to 2,807 with 68 confirmed deaths. (C (2020). Outbreak of L, 2020) The disease was initially attributed to an “unknown chemical exposure” and was eventually found to be strongly linked to inhaled Vitamin E acetate in vaping products. (Schier et al., 2019) EVALI patients were generally healthy before onset of symptoms, which included inflammation. (Krishnasamy et al., 2020) Inflammation can lead to oxidative stress (OS) which could contribute to the acute lung injury observed in EVALI case patients. (Imai et al., 2008) OS is characterized as an imbalance between reactive oxygen species (ROS) and anti-oxidants. ROS are unstable chemical compounds generated endogenously by immune responses, mitochondrial metabolism, and exposure to environmental toxicants such as tobacco smoke. (Ray et al., 2012) Elevated 8-iso-prostaglandin F_{2α} (8-isoprostane) concentrations are indicative of OS and have been linked to the pathophysiology of many diseases including neurodegenerative diseases, lung diseases, and cancers. (Montuschi et al., 1998) (Malli et al., 2013) (Cracowski et al., 2002) (Baraldi et al., 2003) (Montuschi et al., 2000) To analyze 8-isoprostane, bronchoalveolar lavage (BAL) fluid was obtained via bronchoscopy by spraying normal

saline onto the lung epithelial lining and then applying mild suction to retrieve a fraction of that saline along with components from the lung epithelial lining fluid. This report highlights the rapid development and validation of a novel isotope dilution UHPLC-MS/MS method measuring 8-isoprostane in BAL fluid. Application of this method allows for a useful measure of OS in the lung epithelial lining and thus provides insights about the pathophysiology of EVALI. (Blount et al., 2020).

MATERIALS AND METHODS

Chemicals

Acetonitrile (HPLC grade), methanol (HPLC grade), formic acid ($\geq 99.5\%$), ammonium hydroxide (certified ACS plus), and phosphate-buffered saline (PBS) without calcium and magnesium were purchased from Fisher Scientific (NJ, United States). Water (HPLC grade) was purchased from JT Baker (NJ, United States). Synthetic 8-*Iso*-prostaglandin $F_{2\alpha}$ (8-isoprostane; CAS# 27415-26-5), 8-*iso*-prostaglandin $F_{2\alpha}-d_4$ ($>99\%$) (8-isoprostane d_4 ; CAS# 211105-40-7), ent-8-iso-15(S)-PGF $_{2\alpha}$ ($\geq 98\%$) (CAS# 214748-66-0); ent-8-iso-PGF $_{2\alpha}$ ($\geq 98\%$) (CAS# 159812-83-6), 8-iso-PGE $_1$ ($\geq 98\%$) (CAS# 21003-46-3); PGE $_1$ ($\geq 98\%$) (CAS# 745-65-3); 8-iso-15(R)-PGF $_{2\alpha}$ ($\geq 98\%$) (CAS# 214748-65-9); PGF $_{2\alpha}$ ($\geq 98\%$) (CAS# 551-11-1); and 15(R)-PGF $_{2\alpha}$ ($\geq 98\%$) (CAS# 37658-84-7) were obtained from Cayman Chemical Company (MI, United States). Potassium phosphate monobasic crystals (Reagent ACS) was obtained from Acros (NJ, United States). Potassium phosphate buffer with a pH of 6.1 was prepared using a Mettler Toledo S220 pH meter (Greifensee, Switzerland).

Standard Solutions

The initial 8-isoprostane stock solution was prepared by adding 7.05 mg of the dry powder (99% purity) into a 200 ml volumetric flask and diluting with methanol in HPLC water (v/v 1:1) to obtain a native spiking solution concentration of 34.9 $\mu\text{g/ml}$. Working solutions were prepared for standards from serial dilutions of initial native and ISTD stock solutions with methanol and HPLC water (v/v 1:1). Standards were prepared at 10 concentrations ranging from 0 to 1,410 pg/ml by serial dilution of working solutions with methanol and HPLC water (v/v 1:9) and stored in 1.5 ml amber glass vials at -70°C . The materials for the standard solutions were all prepared gravimetrically, and mass results were reported on the conventional basis for weighing in air. These standard solutions were only used as external calibration standards for each analytical run.

Internal Standard Solutions

The isotopically labeled internal standard (ISTD), 8-isoprostane d_4 , was dissolved in methyl acetate (100 $\mu\text{g/ml}$), then 0.5 ml of the initial solution was added to a 100-ml volumetric flask and diluted with methanol in HPLC water (v/v 1:1) resulting in a working solution with a concentration of 500 ng/ml. The final ISTD solution was made by adding 60 ml of the ISTD working solution to a 2,000 ml volumetric flask and diluting with

methanol in HPLC water (v/v 1:9), bringing the final concentration to 15 ng/ml. The ISTD solution was dispensed into 2 ml cryovials, stored at -70°C , and thawed before sample preparation.

Anonymous Bronchoalveolar Lavage Fluid Collection

Non-EVALI BAL fluids used in this study were acquired from Discovery Life Sciences (Huntsville, AL, United States). They were shipped frozen on dry ice and then stored in -70°C freezers until analyzed.

Sample Stability

Individual BAL fluids were screened and three BAL fluid samples with no detectable 8-isoprostane levels were selected to make a blank pool for accuracy, precision, and stability testing. Native 8-isoprostane was dissolved into methanol and water (v/v 1:9) to make spiking solutions. These solutions were spiked into the pooled BAL fluid to achieve six final pools with concentrations ranging from 0–2,000 pg/ml. The spiked pools were used for all method validation experiments. One of the BAL fluid samples with no detectable 8-isoprostane was spiked to create a series of four individual BAL fluid spiked levels with concentrations from 0–2,000 pg/ml. This spiked individual sample was used for LOD testing.

Saline Quality Controls

Because the available quantity of BAL fluid was not sufficient to perform all experiments, blanks and QC pools were prepared using phosphate-buffered saline (PBS). The PBS solution was spiked with native 8-isoprostane to form low, medium, and high QC concentrations of 200, 500, and 2,000 pg/ml, respectively. The saline QCs were processed in every analytical run and monitored for accuracy and precision.

Sample Preparation

An analytical run consisted of a blank, three quality controls (low, medium, and high), and up to 44 unknown BAL fluid samples, and each 96-well plate could hold two analytical runs. Ten external calibration standards were run in duplicate for each analytical run.

BAL fluid was thawed and centrifuged at 1,500 rpm for 12 min at 4°C , and the supernatant was transferred to 2 ml Nalgene cryovials. Prior to aliquoting, each BAL fluid sample was vortexed for approximately 10 s to homogenize the sample. A Hamilton Starlet system was utilized for the automated liquid transfer of internal standard, phosphate buffer, water, and methanol. Liquid transfers were performed using 50, 300, and 1,000 μl black conductive pipette tips from Hamilton in which 40 μl of the isotopically labeled internal standard working solution (15 ng/ml), 160 μl of buffer solution (0.5 M phosphate buffer, pH 6.1), and 1,150 μl of HPLC water, respectively, were dispensed into glass test tubes (12 $\times$ 75 mm). Due to variations in sample consistency, a manual transfer of 50 μl of BAL fluid was performed using 250 μl Ranin Precision Tips. A sample volume of 50 μl represents a 20 fold dilution and an

TABLE 1 | Accuracy and spike recovery of 8-isoprostane in BAL fluid (pg/ml).

Replicate		BAL Fluid 1					BAL Fluid 2				
		Spike concentration	Measured concentration			Recovery ^a (%)	Spike concentration	Measured concentration			Recovery ^a (%)
			Day 1	Day 2	Mean			Day 1	Day 2	Mean	
Initial BAL	1	0	32.2	29.4	35.6	N/A	0	0	0	0.0	N/A
	2		37.2	37.9				0	0		
	3		39.9	36.9				0	0.0		
BAL + spike 1	1	200	230	228	226.8	95.6	200	192	215	203.5	101.8
	2		230	244				207	192		
	3		218	211				212	203		
BAL + spike 2	1	500	494	539	531.8	99.3	500	473	482	492.5	98.5
	2		532	553				505	501		
	3		529	544				476	518		
BAL + spike 3	1	2000	1900	2000	1953	95.9	2000	1950	1900	1910	95.5
	2		1910	1970				1930	1920		
	3		1930	2010				1850	1910		

^aRecovery % = [(mean of measured concentration with spike—mean of measured concentration without spike)/spike concentration].

appropriate correction factor was applied to the measured concentration. Finally, 400 µl of methanol was added to each sample tube. The entire contents in the glass tube were transferred to a 96-well weak anion exchange SPE plate using the Hamilton Starlet system. SPE cleanup was done using the Strata-X-AW 33 µm Polymeric Weak Anion, 60 mg/ 96-well plate from Phenomenex (Torrance, CA, United States). A Biotage Pressure + 96 positive pressure manifold (Biotage, Charlotte, NC, United States) using nitrogen gas generated in-house with a NM20ZA Peak Generator was used to apply positive pressure to the SPE plate. The SPE plate was washed with 1.8 ml of HPLC water, followed by a 1.8 ml solution of methanol in HPLC water (v/v 1:3), and finally 1.8 ml of acetonitrile. Samples were then eluted using methanol and collected in an Advantage Series SiliGuard coated 2 ml 96 deep square well collection plate with a tapered V-bottom (Analytical Sales and Services Inc., Flanders, NJ, United States), evaporated under nitrogen flow at 37°C, reconstituted with 50 µl of 25% methanol in water, vortexed lightly for approximately 2 min, and subsequently injected into the LC-MS/MS.

UHPLC-MS/MS

The LC-MS/MS instrument parameters were kept the same as our previously published CLIA urinary assay. (Holder et al., 2020) In brief, chromatographic separation was achieved using a Waters ACQUITY reversed-phase column (150 mm × 2.1 mm, particle size 1.8 µm, C18) and a Waters ACQUITY reversed-phase pre-column (5 mm × 1 mm, particle size 1.7 µm, C18) (Milford, MA, United States) on an ultra high performance liquid chromatographic system from Shimadzu Corp. (Columbia, MD, United States) A gradient program was performed with a combination of 0.15% formic acid in water (mobile phase A) and acetonitrile in 0.15% formic acid in water (v/v 1:1) (mobile phase B). The combined chromatographic flow rate was 0.65 ml/min, and acetonitrile was infused, post-column, at 0.15 ml/min. Tandem mass spectrometry analysis was performed using an AB SCIEX 6500 triple quadrupole with a Turbo IonSpray source (Foster City, CA) with a Peak

Scientific (Scotland, United Kingdom) Table-N₂ gas generator. Quantitation was achieved by monitoring the native compound transition, 353.3 to 193 m/z (quantitative) and 353.3 to 291 m/z (qualitative), with the corresponding isotopically labelled internal standard transition, 357.3 to 197 m/z. The total cycle time for this method was 11 min.

Method Validation

Accuracy for this assay was assessed through spike-and-recovery analyses of blank and spiked BAL fluid with known concentrations. For determining accuracy of both the pools and the individual samples, A pool of BAL fluid samples and an individual BAL fluid sample (BAL fluid 1 and BAL fluid 2, respectively, in **Table 1**) was spiked with three different concentration levels of native 8-isoprostane (200, 500, and 2,000 pg/ml) and compared the spiked concentrations to the initial measurement. Each sample was prepared in triplicate and measured using two different runs spanning 2 days, resulting in a total of 12 samples per spiking level. The concentration from each triplicate sample was then averaged to get the mean concentration of 8-isoprostane for that spiking level. The mean concentration values were used to calculate percent recovery (equation shown in **Table 1**).

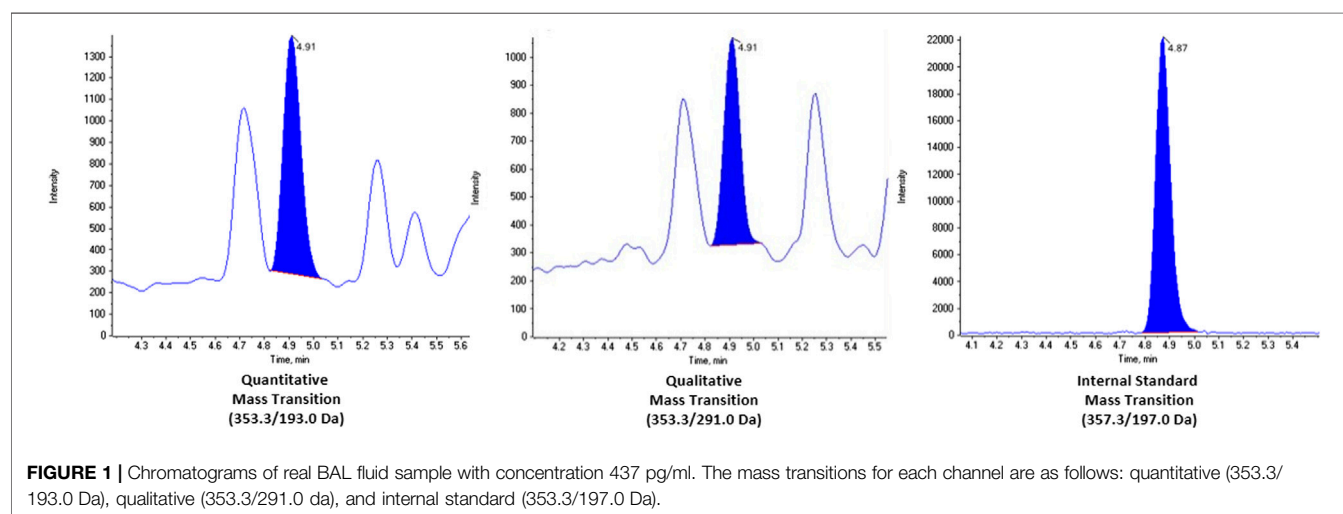
Precision within a run and between runs was determined by using duplicate samples from two BAL fluid pools spiked with native 8-isoprostane at concentrations 200 and 2,000 pg/ml. These results were obtained from five analytical runs over the span of 3 days. The coefficient of variation (CV) was calculated to evaluate both within-run and between-run variation (**Table 2**).

Stability testing was done with two spiked BAL fluid pools of concentrations 500 and 2,000 pg/ml. Six samples from each pool were aliquoted and tested for their initial concentrations. The three test conditions were designed to simulate common sample preparation scenarios, freeze-thaw stability, benchtop stability, and processed sample stability. To test freeze-thaw stability, two samples from each pool were frozen at −70°C and then thawed a

TABLE 2 | Precision of quantitation of 8-isoprostane in BAL fluid (pg/ml).

Quality material 1				Quality material 2			
Run	Result 1	Result 2	Mean	Run	Result 1	Result 2	Mean
1	230	230	230	1	1930	1910	1920
2	234	237	235	2	1940	2010	1975
3	228	244	236	3	1970	2000	1985
4	229	232	230	4	1960	2030	1995
5	220	230	225	5	1950	1950	1950

Quality material 1		Quality material 2	
	%CV		%CV
Within run	1.95	Within run	1.36
Between run	1.92	Between run	1.54



room temperature three times each. Testing benchtop stability was done by leaving two samples from each pool out at room temperature for 24 h. To test processed sample stability, samples were left in a collection plate for 24 h in an autosampler set to 4°C, before being reinjected. Results from the initial measurements were then compared to the measurements following the stability testing.

We looked at potential chromatographic interferences in 18 individual BAL fluid samples and none were observed. Representative chromatograms of a real BAL fluid sample, with a calculated concentration of 437 pg/ml, are shown in **Figure 1**. Additional chromatograms of extracted saline blanks, saline spikes and spiked BAL fluid pools are shown in the **Supplemental Figure S1**. Multiple ion transitions (quantitative, qualitative, and isotopically labeled internal standard) were monitored to ensure the method was selective to a single compound, 8-isoprostane.

Limit of detection (LOD) was determined by analyzing four BAL fluid pools with known 8-isoprostane concentrations (0–2,000 pg/ml) in triplicate on 5 separate runs spanning 3 days. The standard deviation of each pool was plotted against the mean concentrations, and found the Y-intercept, which represents the standard deviation at zero-spike (S_0). The LOD was defined as 3 times S_0 . (Taylor, 1987)

RESULTS AND DISCUSSION

Accuracy and Linearity

Accuracy was calculated by comparing the obtained mean concentration with the native 8-isoprostane spiking level, with values that ranged from 95.5–101.8%. The mean recovery was determined to be 97.8% with a standard deviation of 2.5% (**Table 1**). Our previous assay of 8-isoprostane in urine resulted in recoveries ranging from 92.7 to 106.7% with a mean recovery of 99.7%, indicating that the results from our BAL fluid assay are consistent from analysis of a different physiological matrix. (Holder et al., 2020) We compared the ISTD responses between unextracted calibration samples and extracted samples to evaluate sample recovery and calculated an average recovery of 55% for all extracted samples.

As outlined in our previous assay, the calibration curve was prepared by spiking 10 known standard levels of 8-isoprostane in water, with concentrations ranging from 8.8 to 1,410 pg/ml. (Holder et al., 2020) Each standard level was run in duplicate for each analytical run. Strong linearity was observed with an R^2 of 0.9999.

TABLE 3 | 8-isoprostane stability in BAL fluid (pg/ml).

	Initial measurement	3 freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	529	543	540	522
Replicate 2	532	510	539	535
Mean	530.5	526.5	539.5	528.5
% Difference from initial measurement	--	-0.8	1.7	-0.4

	Initial measurement	3 freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	1910	1950	1970	1940
Replicate 2	1930	1920	1950	1940
Mean	1920	1935	1960	1940
% Difference from initial measurement	--	0.8	2.1	1.0

Precision

The within run precision ranged from 1.36–1.95%, and the between run precision ranged from 1.54–1.92% (Table 2). Thus our BAL fluid method was more precise than our previously published urine assay, possibly because BAL fluid is a cleaner matrix. Furthermore, our BAL fluid method had substantially better precision than a typical EIA assay (e.g., Cayman EIA within run: 9.5%, between run: 20.2%). (Cayman Chemical 8-Isopros, 1635)

Selectivity

Fully resolving 8-isoprostane from all interfering peaks is critical to achieving a reliable and repeatable measurement since this analyte belongs to a class of compounds, F2-isoprostanes, consisting of 64 isomers. Immunoassays are known to suffer from cross-reactivity which could explain the reported poor agreement between LC-MS and EIA for 8-isoprostane, and while GC-MS can be extremely sensitive, extensive sample preparation using harsh derivatizing agents is a necessity, making the GC-MS approach less desirable. (Klawitter et al., 2011; Smith et al., 2011) It is important to note that some published LC-MS/MS methods are not highly selective and measure a sum of F2-isoprostanes and not 8-isoprostane specifically. (Taylor et al., 2008) To evaluate possible interference with 8-isoprostane, we examined the following eicosanoids with similar mass transitions: ent-8-iso-15(S)-PGF_{2α}; ent-8-iso-PGF_{2α}; 8-iso-PGE₁; PGE₁; 8-iso-15(R)-PGF_{2α}; PGF_{2α}; and 15(R)-PGF_{2α}. Our UHPLC-MS/MS method fully resolves the analyte from all interfering peaks and can reliably be used to quantify 8-isoprostane in BAL fluid and other matrixes.

Stability

Percent differences of the initial measurements and post-test measurements ranged from -0.8–2.1% across all three test conditions and both pools (Table 3). These results indicate that 8-isoprostane is stable under all three conditions tested. Long term stability was not assessed due to the rapid method development timeline dictated by the emergency response.

While working in a high throughput laboratory, samples may undergo many freeze-thaw cycles and or be left in an

autosampler over the weekend. The results of these tests show that 8-isoprostane levels did not change in samples that underwent the tested conditions, and thus can be used for future analyses. Furthermore, being a stable and robust analyte further supports the use of 8-Isoprostane as a key biomarker of oxidative stress usable in high throughput studies.

Limit of Detection and Calibration Range

The method detection limit for 8-isoprostane (8.8 pg/ml) was calculated as 3 times S_0 . (Taylor, 1987) We ultimately set the LOD to 17.6 pg/ml for the EVALI samples as we used half the sample volume for analysis due to limited supply. Applications of this method that require an LOQ can use 10 S_0 (29.3 pg/ml). The calibration range for this method is 8.8 pg/ml to 1,410 pg/ml; our lowest calibrator is lower than our set LOD and a typical deviation is less than 5% of the target concentration. However, Malli et al. measured 8-isoprostane, using EIA, in serum and BAL fluids of patients with either sarcoidosis or idiopathic pulmonary fibrosis (IPF) and found similar concentrations in serum and BAL. They reported median (25–75% interquartile range) concentrations of 8-isoprostane in sarcoidosis patients [serum: 132.8 (92.27–194.9) pg/ml; BAL: 220.6 (133.6–403.3) pg/ml] and IPF patients [serum: 77.25 (52.42–162.5) pg/ml; BAL: 74.87 (62.23–115.1) pg/ml]. (Malli et al., 2013) Bastani and others applied their LC-MS/MS method measuring 8-isoprostane in plasma, urine, full blood, and erythrocytes. (Bastani et al., 2009) To our knowledge there are no other LC-MS methods for measuring 8-isoprostane in BAL fluid, however, our calibration curve is appropriate for reported BAL fluid 8-isoprostane concentrations using EIA. It is difficult to compare the results of LC-MS methods to EIA methods due to the differences in selectivity, so LODs between the two methods cannot be compared. (Klawitter et al., 2011; Smith et al., 2011) Additionally, the volume of BAL fluid obtained from a patient can vary according to technique used to obtain it. Thus, we only report qualitative results for the BAL fluid samples in this study. (De Jesús et al., 2020; Morel Espinosa et al., 2021) Of the 18 samples that were tested using this method, 14 of them had a concentration above the 17.6 pg/ml LOD.

CONCLUSION

We have developed and validated a partially automated, selective, and robust UHPLC-MS/MS method for quantifying 8-isoprostane in bronchoalveolar lavage (BAL) fluid. This method is easily adaptable for high-throughput work flow and will be applied to BAL fluid samples collected from EVALI case patients in support of CDC's 2019 EVALI response. Although EVALI has been strongly linked to inhaled vitamin E acetate from EVPs (Blount et al., 2020), the pathology of how vitamin E acetate causes lung injury remains uncharacterized and may involve OS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

CH and LW designed the experiments. CH, AA, CA, OC, and RL conducted experiments. CH, LW, and BB reviewed data. BB lead

CDC's 2019 Lung Injury Laboratory Task Force and secured funding. All authors contributed to drafting and editing this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.695940/full#supplementary-material>

REFERENCES

- Baraldi, E., Ghironi, L., Piovani, V., Carraro, S., Ciabattini, G., Barnes, P. J., et al. (2003). Increased Exhaled 8-Isoprostane in Childhood Asthma. *CHEST* 124 (1), 25–31. doi:10.1378/chest.124.1.25
- Bastani, N. E., Gundersen, T. E., and Blomhoff, R. (2009). Determination of 8-epi PGF₂α concentrations as a biomarker of oxidative stress using triple-stage liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass. Spectrom.* 23, 2885–2890. doi:10.1002/rcm.4197
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382 (8), 697–705. . Epub 2019 Dec 20. PMID: 31860793; PMCID: PMC7032996. doi:10.1056/NEJMoa1916433
- Cayman Chemical 8-Isoprostane ELISA Kit. Item No. 516351. 2016. Cayman Chemicals. Available at: <https://www.caymanchem.com/pdfs/516351.pdf> (Accessed April 14, 2021).
- CDC (2020). *Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products*. Atlanta, Georgia: Centers for Disease Control and Prevention. Available at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html#what-we-know (Accessed April 14, 2021).
- Cracowski, J.-L., Durand, T., and Bessard, G. (2002). Isoprostanes as a biomarker of lipid peroxidation in humans: physiology, pharmacology and clinical implications. *Trends Pharmacol. Sci.* 23 (8), 360–366. doi:10.1016/s0165-6147(02)02053-9
- De Jesús, V. R., Silva, L., Newman, C. A., and Blount, B. C. (2020). Novel methods for the analysis of toxicants in bronchoalveolar lavage fluid samples from e-cigarette, or vaping, product use associated lung injury (EVALI) cases: Terpenes. *Rapid Commun. Mass. Spectrom.* 34 (19), 0951–4198. doi:10.1002/rcm.8879
- Holder, C., Adams, A., McGahee, E., Xia, B., Blount, B. C., and Wang, L. (2020). High-Throughput and Sensitive Analysis of Free and Total 8-Isoprostane in Urine with Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry. *ACS Omega* 5 (19), 10919–10926. PMID: 32455212; PMCID: PMC7241033. doi:10.1021/acsomega.0c00661
- Imai, Y., Kuba, K., Neely, G. G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., et al. (2008). Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* 133 (2), 235–249. PMID: 18423196; PMCID: PMC7112336. doi:10.1016/j.cell.2008.02.043
- Klawitter, J., Haschke, M., Shokati, T., Klawitter, J., and Christians, U. (2011). Quantification of 15-F_{2t}-isoprostane in human plasma and urine: results from enzyme-linked immunoassay and liquid chromatography/tandem mass spectrometry cannot be compared. *Rapid Commun. Mass. Spectrom.* 25 (4), 463–468. doi:10.1002/rcm.4871
- Krishnasamy, V. P., Halliwell, B. D., Ko, J. Y., Board, A., Hartnett, K. P., Salvatore, P. P., et al. Update: Characteristics of a Nationwide Outbreak of E-cigarette, or Vaping, Product Use-Associated Lung Injury - United States, August 2019-January 2020. *MMWR Morb. Mortal. Wkly. Rep.* 2020;69:90–94. doi:10.15585/mmwr.mm6903e2
- Malli, F., Bardaka, F., Tsilioni, I., Karetsi, E., Gourgoulis, K. I., and Daniil, Z. (2013). 8-isoprostane levels in serum and bronchoalveolar lavage in idiopathic pulmonary fibrosis and sarcoidosis. *Food Chem. Toxicol.* 61, 160–163. doi:10.1016/j.fct.2013.05.016
- Montuschi, P., Collins, J. V., Ciabattini, G., Lazzeri, N., Corradi, M., Kharitonov, S. A., et al. (2000). Exhaled 8-Isoprostane as an In Vivo Biomarker of Lung Oxidative Stress in Patients with COPD and Healthy Smokers. *Am. J. Respir. Crit. Care Med.* 162 (3), 1175–1177. doi:10.1164/ajrccm.162.3.2001063
- Montuschi, P., Toni, G. C., Paredi, P., Pantelidis, P., du Bois, R. M., Kharitonov, S. A., et al. (1998). 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am. J. Respir. Crit. Care Med.* 158 (5 Pt 1), 1524–1527. doi:10.1164/ajrccm.158.5.9803102
- Morel Espinosa, M., Blount, B. C., and Valentin-Blasini, L. (2021). Liquid chromatography-tandem mass spectrometry method for measuring vitamin E acetate in bronchoalveolar lavage fluid. *J. Chromatogr. B* 1171, 122607. doi:10.1016/j.jchromb.2021.122607
- Ray, P. D., Huang, B.-W., and Tsuiji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal.* 24 (5), 981–990. doi:10.1016/j.cellsig.2012.01.008
- Schier, J. G., Meiman, J. G., Layden, J., Mikos, C. A., VanFrank, B., King, B. A., et al. Severe Pulmonary Disease Associated with Electronic-Cigarette-Product

- Use - Interim Guidance. *MMWR Morb Mortal Wkly Rep.* 2019;68:787–790. doi:10.15585/mmwr.mm6836e2
- Smith, K. A., Shepard, J., Wakil, A., and Kilpatrick, E. S. (2011). A comparison of methods for the measurement of 8-isoPGF₂α: a marker of oxidative stress. *Ann. Clin. Biochem.* 48 (Pt 2), 147, 2017. PMID: 21292864. doi:10.1258/acb.2010.010151
- Taylor, A. W., Bruno, R. S., and Traber, M. G. (2008). Women and Smokers Have Elevated Urinary F2-Isoprostane Metabolites: A Novel Extraction and LC-MS Methodology. *Lipids* 43, 925–936. doi:10.1007/s11745-008-3222-1
- Taylor, J. K. (1987). *Quality assurance of chemical measurements*. 1st ed. Routledge. doi:10.1201/9780203741610

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Nominal Operating Envelope of Pod and Pen Style Electronic Cigarettes

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Many Electronic Nicotine Delivery Systems (ENDS) employ integrated sensors to detect user puffing behavior and activate the heating coil to initiate aerosol generation. The minimum puff flow rate and duration at which the ENDS device begins to generate aerosol are important parameters in quantifying the viable operating envelope of the device and are essential to formulating a design of experiments for comprehensive emissions characterization. An accurate and unbiased method for quantifying the flow condition operating envelope of ENDS is needed to quantify product characteristics across research laboratories. This study reports an accurate, unbiased method for measuring the minimum and maximum aerosolization puff flow rate and duration of seven pod-style, four pen-style and two disposable ENDS. The minimum aerosolization flow rate ranged from 2.5 to 23 (mL/s) and the minimum aerosolization duration ranged from 0.5 to 1.0 (s) across the ENDS studied. The maximum aerosolization flow rate was defined to be when the onset of liquid aspiration was evident, at flow rates ranging from 50 to 88 (mL/s). Results are presented which provide preliminary estimates for the effective maximum aerosolization flow rate and duration envelope of each ENDS. The variation in operating envelope observed between ENDS products of differing design by various manufacturers has implications for development of standardized emissions testing protocols and data reporting required for regulatory approval of new products.

Keywords: operating envelope, E-cigarette, electronic nicotine delivery system, pod-style, pen-style

INTRODUCTION

There is little consistency in puffing regimes being used for ENDS emission studies; studies have used 15 ml/s, 4 s puffs (1), 27 ml/s, 3 s puffs (49), 39 ml/s, 1.8 s puffs (2), 27.5 ml/s, 2 s puffs (3, 4), 17.5 ml/s 2 s puffs (5–7), 10 ml/s, 4 s puffs (8), and in some articles the puffing protocol is unclear (9, 10). It remains unclear how the puffing regimes used relate to the normal range of the device permitted by the manufacturer, or how the puffing regimes correlated with user behavior. Prior work shows that emissions are a strong function of puff flow rate (11), and that puff flow rate and other topography behavior varies widely with individual users and devices (12, 13). To date, no standard emissions outcome measures have been agreed upon, while a wide variety of metrics have been reported. Emissions have frequently been reported as the total condensed aerosol,

commonly referred to as the Total Particulate Matter (TPM) yield per puff [Y_{TPM} , (mg/puff)] and the Harmful and Potentially Harmful Constituents (HPHC) Yield [Y_{HPHC} , (mg/puff), i.e., the mass of selected HPHCs per number of puffs] (2, 3, 14–32). Some studies have reported emissions in terms of the HPHC mass ratio, f_{HPHC} (mg/mg) (i.e., the mass of selected HPHCs per unit mass of TPM) (17, 26, 30, 31, 33). Previously proposed smoking machine standards such as (34) provided a basis for product comparisons but did not reflect the range of user behaviors observed (35), thus limiting their utility for public health assessments. Similarly, recent vaping machine standards (36) provide some basis for product comparisons, but also do not reflect the range of use behavior anticipated in the natural environment. Yield terms, such as Y_{TPM} , are normalized “per puff,” while mass concentration terms, such as C_{TPM} , are normalized by the puff volume expressed in (mg/mL). The functional dependence of outcome measures (C_{HPHC} , f_{HPHC} , C_{TPM} , and Y_{TPM}) on the combined factors of user topography behavior and product characteristics has not been mechanistically studied and warrants further investigation. The variety of test protocols and outcome measures reported in the literature may simply reflect various laboratory capabilities. Nonetheless, the lack of standardization has made it difficult to compare products across studies or make inferences about the impact of product characteristics on emissions.

The FDA 2016 draft guidance for Pre-Market Tobacco Application, PMTA, for ENDS (81 FR 28781) suggests manufacturers consider the chemical and physical identity and quantitative levels of aerosol emissions under the range of operating conditions and use patterns within which consumers are likely to use the new tobacco product. Previous protocols for combustible cigarettes, influenced by the tobacco industry, resulted in inaccurate emissions that did not represent exposure under actual use conditions (37). The FDA recognizes the influence of topography on emissions, and suggests that topography be considered when assessing substantial equivalence of tobacco products (76 FR 789). Yet product-specific topography data are still lacking and no systematic study has been done to determine appropriate puffing protocols to generate accurate emissions for subsequent chemical constituent analysis. In the absence of studies which characterize the range of user behavior associated with various products, emissions characteristics must be determined for the full range of operating conditions (flow rates and puff durations). Though many studies have investigated aerosol emissions from ENDS over a variety of conditions, none have presented a comparative evaluation of the effective operating envelope of ENDS. This paper addresses this gap by introducing a robust method for empirically quantifying

the operating envelope and presents data for thirteen pod- and pen-style ENDS with both button and flow-activated power.

The operating envelope of an ENDS is bounded by the Minimum and Maximum Aerosolization Flowrate (MinAF, MaxAF), and the Minimum and Maximum Aerosolization Duration (MinAD, MaxAD). The MinAF is the puff flow rate above which the ENDS coil consistently activates on every puff and generates TPM yield per puff above the limit of quantitation measurable by the analytical balance. The MaxAF is the puff flow rate below which the ENDS aerosolizes E-Liquid and above which E-Liquid aspiration onset is observed. The MinAD is the puff duration above which the ENDS coil consistently activates on every puff and generates TPM yield per puff above the limit of quantitation. The MaxAD is the puff duration above which no incremental TPM is generated, most likely because the coil has been deactivated by the ENDS power control unit. In the absence of natural environment topography to inform protocols for machine-generated puffing profiles, characterizing products over the entire operating envelope describes the full range of exposure possible for a given ENDS.

Selection of devices to study was based on their relevance in the current ENDS market, product attributes, operating parameters of devices, and manufacturers of ENDS products. We chose to study popular products on the US ENDS market, with a focus on popular products with ohm/sub-ohm coils and “tobacco” flavor e-liquid options. Priority was given to devices with disposable (non-refillable) tanks and fixed (non-user adjustable) power. We selected products from a variety of manufacturers.

In general, the market has trended toward more widespread use of “pod style” devices, an increase in customizability in “mod style” devices, and a trend away from “pen style” ENDS for nicotine use. Additionally, disposable devices that visually resemble pod style products, sometimes referred to as “smoke bars,” have become popular throughout 2020. Meanwhile, pen style ENDS are still relevant for users of Cannabidiol (CBD) and tetrahydrocannabinol (THC) containing e-liquids and oils.

The majority of ENDS product literature refers to the power control unit (PCU) with the benign title “battery,” obfuscating the fact that the PCU often contains sophisticated power management and control logic. Some e-cigarettes have incorporated 510 threads (10 male threads at 0.5 mm pitch with a diameter of 7 mm, aka M7 × 0.5) onto their PCUs to make them compatible with 510 reservoirs. These 510 reservoirs seem to be a product of choice, alongside pods, for mid-chain triglyceride (MCT) solvent e-liquids containing CBD and THC. Therefore, priority was put on choosing pen style products that use a 510 thread. Keyword searches such as “510 batteries” and “510 cartridges” returned ENDS-relevant results for “pen style” devices. While pen style products are not fully customizable, it was observed that some PCUs often offer approximately three discrete voltage settings which users can vary by pushing the activation button in a specific way (e.g., triple click). Devices enabling user adjustable power were not selected for this study. Some ENDS reservoirs are sold with the heating coil integrated into the reservoir, often marketed as with the name “pods” or “cartridges,” while other ENDS reservoirs permit the user to

Abbreviations: C, Mass concentration (mg/mL) of a constituent; ENDS, Electronic Nicotine Delivery System; F, Ratio of mass of a constituent to the mass of total particulate matter; HPHC, Hazardous or Potentially Hazardous Compound; MinAF, Minimum Aerosolization Flowrate; MaxAF, Maximum Aerosolization Flowrate; MinAD, Minimum Aerosolization Duration; MaxAD, Maximum Aerosolization Duration; PCU, Power Control Unit; PMTA, Pre-market tobacco product application, U.S. Food and Drug Administration; TPM, Total Particulate Matter; Y, Mass yield (mg per puff) of a constituent.

interchange the heating coil. This study focuses primarily on products with reservoirs having integrated heating coils. The ENDS PCU, reservoir and heating coil may each contribute in novel ways to the emissions generated by the device. Thus, it is important to accurately characterize each ENDS product tested to permit meaningful comparisons between products.

The primary objective of this study was to demonstrate an accurate, unbiased method for quantifying the effective operating envelope of ENDS in terms of four parameters: the minimum and maximum aerosolization puff flow rate and duration, denoted MinAF, MaxAF, MinAd, and MaxAd, respectively. The second objective is to report ENDS packaging and product characteristics and descriptive statistics of the nominal coil resistance, R_{coil} , and nicotine mass ratio, f_{Nic} , expressed as mg of nicotine per mg of aerosolized TPM and un-puffed E-Liquid for each ENDS studied. These product characteristics are proposed for PMTA reporting under 81 FR 28781.

MATERIALS AND METHODS

Test Specimens

A summary of the ENDS assessed herein is presented in **Table 1**. Thirteen unique ENDS products were chosen for this study, including seven popular pod style, four pen-style and two disposable devices as illustrated in **Figure 1**. Test specimens of each product were obtained from a variety of sources, including the manufacturer's website (M), on-line third party distributors (O), and local retail stores and vape/smoke shops (R). For devices that used pre-filled pods or tanks, the reservoirs were generally purchased at the same time as the PCUs. When additional pre-filled reservoirs were required, they were purchased via the same channel as the original ENDS purchase. For ENDS devices that employed refillable reservoirs, a common e-liquid from the same lot was used, which was purchased over the internet from an e-liquid distributor. All products were purchased between July, 2018 and December, 2020. A New York State law prohibiting on-line and mail-order sales of electronic cigarettes and e-liquid went into effect on July 1, 2020, and all product purchases were made from retail establishments after that date. The source(s) used to purchase the products are provided, along with the country of manufacture. Investigation of company websites and marketing materials was used to identify associated parent companies and/or tobacco-company affiliates of the ENDS manufacturer.

Each ENDS PCU was documented as it was unpackaged, to observe all labeling on the outer and inner packaging as received from the vendor. The product manufacturer was noted for each device, and ENDS "Product Model" is used as a legend key for subsequent presentation of results. Several attributes of each device were noted, including whether the PCU was rechargeable, the general geometric form factor (shape) of the PCU, and if its e-liquid reservoir was refillable. Most devices studied herein had a coil integrated with the reservoir, while three products permitted user replacement of the coil in the reservoir. The packaging and user instructions were evaluated to determine if each device was flow rate activated, "puff," or manually activated, "button." None of the products tested permitted the user to make adjustments

to the power, except for the Aspire Breeze 2, SMOK Novo 2, and SMOK Stick which permit users to replace the coil in the reservoir and thereby influence the power dissipated in the coil.

We measured the assembled dimensions of the ENDS PCU plus reservoir in their nominal configuration as intended for use. The volumetric capacity of the reservoir as stated by the manufacturer was recorded, along with the name of the manufacturer of the e-liquid used for each product test. In those cases when the manufacturer provided non-refillable reservoirs, we elected to use e-liquid product from the same manufacturer, marketed for sale with the PCU, and chose the "tobacco" flavored e-liquid. All products which provided refillable reservoirs were operated with a common lot of bulk "classic tobacco" e-liquid manufactured by Mad Hatter Juice. We observed non-uniformity in the units employed to report nicotine concentration of e-liquid, and report the values as observed on the product packaging.

We observed the majority of product packages did not report the battery chemistry, battery capacity, coil resistance, or operating power of the product, and there was non-uniformity in reporting these characteristics between product manufacturers and models. Any data not reported on the external or internal packaging is indicated as "Not Reported (NR)" in **Table 1**.

Each ENDS Power Control Unit (PCU) and ENDS Reservoir (pod or tank with integrated coil and wick) to be tested was marked with a unique identification number and QR code assigned by the lab. These unique identifiers were scanned prior to each measurement and emissions trial and recorded by data logging scripts. All data measurements and analysis results are traceable by these unique identifiers.

Gravimetric Test Method

The analytical balance used for this study was a Mettler Toledo Model Number AE240-1 S/N J65956 with a manufacturer reported readability of 0.1 (mg), approximate accuracy of 0.4 (mg) and full scale range of 200 (grams) with a linearity of ± 0.02 (mg) mounted on a heavy work bench to minimize vibration effects. The analytical balance was used to measure the mass of each ENDS reservoir and filter pad "before" and "after" each trial. The decrease in the mass of the ENDS reservoir is one measure of the Total Particulate Matter (TPM) generated by the ENDS via aerosolization while the increase in the mass of the filter pad is a measure of the TPM delivered to the user over the same time interval. When the ENDS Yield (mass decrease) is nearly equal to the Pad Yield (mass increase) there is high confidence that minimal TPM deposition has occurred between the ENDS device and the filter pad in the flow path of the emissions test system.

Sample loading and unloading was done carefully to avoid disturbing the balance, and each sample was positioned near the center of the sample pan, with gentle opening and closing of the balance doors. The analytical balance was maintained at room temperature in the lab, and located in a corner away from drafts and room air ducting. The analytical balance was routinely turned on and allowed to warm up for at least 1 h before taking measurements. Prior to each test series, the analytical balance was confirmed to be level using the bubble level built into the instrument. The accuracy of the balance was verified with its

TABLE 1 | Test specimens used in screening trials for this study.

ENDS manufacturer	JUUL Labs	VUSE	SMOK	Blu	NJOY	Uwell	Aspire	Vapor4Life	Logic Vapes	Loontech	VUSE	SMOK	Puff bar
ENDS Model	JUUL	Alto	Novo 2	myblu	Ace	Caliburn	Breeze 2	Titan	Logic Pro	Hyde	Vibe	Smok Stick	Puff Bar
ENDS Style	Pod	Pod	Pod	Pod	Pod	Pod	Pod	Pen	Pen	Single use	Pen	Pen	Single use
Features													
Rechargeable PCU	X	X	X	X	X	X	X	X	X	X	X	X	
Refillable reservoir			X			X	X					X	
Replaceable coil			X				X					X	
Adjustable power			By Coil				By Coil					By Coil	
Actuation type	Puff	Puff	Puff	Puff	Puff	Button	Button and Puff	Puff	Button	Puff	Puff	Button	Puff
Size and shape characteristics													
PCU form factor	Rect.	Rect.	Rect.	Rect.	Ovoid	Rect.	Rect.	Cyl.	Cyl.	Rect.	Cyl.	Cyl.	Rect.
Reservoir capacity (mL)	0.7	1.8	2	1.5	1.9	2	3	1	1.5	1.8	1.9	8	1.3
Assembled dimensions (mm)	94.7 × 15.1 × 6.9	104.6 × 19.1 × 10.6	88.5 × 24.2 × 14.5	106.7 × 18 × 9.5	88.3 × 29.8 OD × 13.5 OD	109.9 × 21.2 × 11.8	94.5 × 35 × 20	121.7 × 9.3 OD	136.6 × 14.1 OD	79.6 × 24.7 × 7.6	136.8 × 13 OD	146 × 24.4 OD	96.6 × 15.6 × 6.5
Axial × lateral × vertical													
Manufacturer reported ENDS characteristics													
Mfg stated battery chemistry	NR	LiPo	NR	NR	NR	NR	NR	NR	LiOn	LiOn	LiOn	NR	NR
Mfg stated battery capacity (mAh)	NR	350	800	350	NR	520	1,000	300	650	380	600	3,000	350
Mfg stated coil resistance (ohm)	NR	NR	1.0, 1.4	1.3	NR	1.4	0.6, 1.0	2.3	2.3	NR	NR	0.17	NR
Mfg stated power (watt)	NR	NR	6–25 W	10.5 W	NR	11 W Max	NR	NR	3.70 V and 2.3 Ohms	NR	NR	30–70 W	NR
Manufacturer reported eliquid characteristics													
Eliquid manufacturer	JUUL Labs	VUSE	Mad Hatter Juice	Blu	NJOY	Mad Hatter Juice	Mad Hatter Juice	Vapor4Life	Logic	Hyde	VUSE	Mad Hatter Juice	Puff Bar
Eliquid branded flavor name	Virginia Tobacco	Original	Classic Tobacco	Classic Tobacco	Classic Tobacco	Classic Tobacco	Classic Tobacco	Wowbacco	Tobacco	Spear Mint	Original	Classic Tobacco	Tobacco
Eliquid branded nicotine concentration	5%	5%	50 mg/5% by vol.	2.40%	5% by wt.	50 mg / 5% by vol.	50 mg / 5% by vol.	3.60%	20 mg/ml	50 mg	3%	50 mg / 5% by vol.	5%
Purchasing information													
Place of purchase	M, R	O, R	M	M	M	O	M	M	O	O	R	O	M
Country of manufacturer	China (PCU) USA (Res)	China (PCU) USA (Res)	China	China	China (PCU) USA (Res)	China	China	NR	China	China	China (PCU) USA (Res)	China	China
Parent company	JUUL Labs; Altria	RJRVC; Reynolds America	Shenzhen IVPS Technology Co.	Imperial Brands; Fontem US	NJOY LLC	Shenzhen Uwell Technology Co.	Shenzhen Eigate Technology Co.	Vapor4Life	Logic Technology Development, LLC	Loontech	RJRVC; Reynolds America	Shenzhen IVPS Technology Co.	NR

M, Manufacturer's website; O, Online distributor; R, Local retail shop; NR, Not Reported; LiPo, Lithium Polymer; LiOn, Lithium Ion; Rect, Rectilinear; Cyl, Cylindrical.



FIGURE 1 | Image of the thirteen ENDS products tested for this investigation.

internal standard prior to the beginning of each measurement session. The balance was tared to assure a reading of 0.0000 (gram) prior to placing a sample on the measurement pan.

The limit of detection (LoD) was assumed equal to the gravimetric instrument accuracy at the low end of the measurement range, $\text{LoD} = 0.4$ (mg). The limit of quantitation (LoQ) (38) was set to five times the detection limit, $\text{LoQ} = 5 \text{ LoD} = 2.0$ (mg). The LoQ was then divided by the number of puffs per each trial (typically 50 puffs) to establish the $Y_{\text{TPM}} = 0.04$ (mg/puff) limit. The relative mass error, ΔM (%), was computed for each observation in the “variable flow rate” series of trials, as the relative difference between the decrease in the mass of the ENDS device, ΔENDS (mg) and the increase in the mass of the filter pad, ΔPad (mg). A large relative mass error is indicative of deposition of aerosol between the exit plane of the ENDS and the surface of the filter pad.

Coil Resistance Test Method

The effective resistance of the coil and reservoir to PCU connection was measured using a four-wire constant current resistance measurement method as introduced in (39, 40). Custom fixtures were developed for several ENDS products, while hand held measurements were made for the remainder. The two single use ENDS, Hyde and Puff Bar, were destructively opened in order to access their coils’ terminals for resistance readings. The coil resistance of each reservoir was measured using four-wire leads connected to a Keysight Model 34465A digital multimeter connected to a data logging computer running a data sampling script. The script was used to read and report the mean and standard deviation of 10 sequential readings of the same reservoir and coil/heating element taken at ~ 1 s intervals, to monitor stability of the resistance readings. The mean value for each measured reservoir/coil assembly is recorded and assessed to describe the inter-coil variation observed by product.

Emissions Screening Protocol

Emissions were machine-generated using two sets of puffing profiles: “variable flow rate set” and “variable duration set.” The number of different durations and flow rates run in each set depended on the individual behavior of the product. There were at least 10 profiles run in the variable flow rate set, and each profile had nominally 50 homogeneous square-wave puffs of 3.5 (s) and flow rates ranging from nominally 10 (mL/s) up to 100 (mL/s) for the different profiles in the set. Similarly, there were at least 10 profiles run in the variable duration set, and each profile had nominally 50 homogeneous square-wave puffs of 30 (mL/s), and puff durations ranging from nominally 0.5 to 10 s for the different profiles in the set. All profiles had a nominal puff period of 30 s.

At shorter puff durations or lower flow rates, some products did not activate, and the operator had discretion to conduct additional trials to narrow in on the minimum puff duration or minimum flow rate at which the ENDS began to generate measurable TPM. At higher puff durations or higher flow rates, the operator would limit the number of puffs in the profile to < 50 puffs, to ensure the coil remained supplied with e-liquid throughout. For example, one high powered ENDS, when operated for long duration puffs, consumed liquid in the reservoir over only 20 puffs. In those cases, the operator adjusted the series of trials to achieve nominally 50 puffs per flow condition while ensuring that no single emissions profile exhausted the liquid supply or over-loaded the filter pad. For some products, particularly with higher puff flow rates, the operator would need to adjust the range of flow rates studied when significant deposition was visually observed in the tubing of the puffing machine between the exit plane of the ENDS and the entrance to the filter pad.

All emissions tests were conducted using the PES-1 system, previously described (41), which is a computer-controlled

programmable puffing and emissions capture machine, consisting of a flow controller connected to a vacuum tank. Puff flow rate was controlled using a proportioning valve with command signals from a closed-loop feedback controller. For button activated devices, an actuator commanded by the computer pushed the PCU activation button of button-activated PCUs at the start of each puff, and released the button at the end of a puff. Flow rate measurements were made using an Alicat Scientific M-50SLPM-D-30PSIA/5M calibrated flow meter sampled by the computer at a rate of 100 Hz. Tank vacuum pressure was fixed at 37.4 (kPa) and was maintained by a vacuum pump and a proportioning valve. The PES-1 system can generate puffs between 5 and 150 (mL/s) with puff duration and inter-puff gap as small as 0.2 (s) (41). There is no maximum limit for puff duration and inter-puff gap. The command profile was specified to the system as a flow rate time series. All topography parameters (puff flow rate, duration, interval) were reported “as measured” in order to ensure that any inaccuracies in the ability of the emissions system to follow any particular command profile do not introduce error (41). Time-stamped flow rate measurements were stored in a csv file along with information of the product used and the experimental setup parameters. Vapor phase emissions were collected on Cambridge filter pads.

Analytical Chemistry Methods

NMR analysis was conducted on un-puffed E-Liquid samples taken from each product tested to determine the proportion of propylene glycol to the glycerin which formed the solvent base for the liquid. The instrument used for NMR was a Bruker Advance III 500 MHz NMR (Billerica MA). Approximately 10 mg of an e-liquid was added to an NMR tube followed by 600 μ L of D₂O and a typical NMR spectrum was obtained. After NMR spectra were obtained the spectra were processed using KnowItAll™ spectral processing software (Wiley). For each sample, the water peak was centered at 4.79 ppm and baseline was corrected to ensure proper integration. Peaks for propylene glycol (3.38–3.47 ppm, 1H) and glycerol (3.605–3.675 ppm, 2H) were integrated and molar ratios of each were determined. The integration ranged varied for e-liquids with acid added (known as salted e-liquids) as pH will slightly shift NMR peaks. Volume and mass ratios were calculated for each solution using known density and molar mass of each. To confirm the validity of the method, mixtures of propylene glycol and glycerol (mass ratios: 0:100, 25:75, 50:50, 75:25, 100:0) were prepared and analyzed using the described method. In all cases, correct ratios were confirmed for each test mixture.

GC-MS analysis was conducted to determine the mass ratio of nicotine to total particulate matter from the condensed aerosol captured on the filter pad during emissions trials. Emissions trials above the mass limit of detection were analyzed. Mass of nicotine in the aerosol was determined in the same manner as previously reported (42). In brief, pads used to collect aerosol were spiked with quinoline as an internal standard, submerged in methanol, shaken to break up the pad and filtered prior to quantification. Nicotine concentration was determined by GC-MS (Shimadzu QP2010 GCMS with an AOC-20s autosampler). Each sample was run in triplicate to ensure accurate results. The mass fraction of nicotine in the aerosol (f_{NIC}) was calculated as the ratio of the

mass of nicotine found on the pad to the total mass of particulate matter deposited.

Determination of Operating Envelope

Three figures were generated for each ENDS model and used collectively to assess the effective operating envelope of each product (not shown). The emissions testing of each model may require multiple devices (ENDS PCUs and Reservoirs) as reported in the Results. All devices associated with a particular ENDS model were analyzed together. The figures included a scatter plot of (1) pad yield per-puff Y_{TPM} (mg/puff) vs. mean puff flow rate q (mL/s) for the “variable flow rate set” of conditions, (2) Y_{TPM} (mg/puff) vs. mean puff duration d (s) for the “variable duration set” of conditions, and (3) relative gravimetric error ΔM (%) vs. q (mL/s) for the “variable flow rate set” of conditions.

Each figure was annotated with notes recorded by the operator during trials. For example, the operator recorded when an LED indicator behaved in a different manner. Some ENDS devices, for example, documented the LED would change color when the battery dropped below a predefined voltage or when the maximum puff duration was exceeded. Observations of the LED were then compared with other quantifiable characteristics, such as changes in TPM pad yield per puff. The operator noted if any bubbles appeared to be generated in the ENDS Reservoir, if droplets were evident in the connection between the exit plane of the ENDS device and the surface of the filter pad, or if discrete droplets or gravity distribution of deposition pattern were evident on the filter pad. Likewise, the operator noted if there was a significant increase in the coil resistance between the “before” and “after” resistance measurements when using the fixture 4 wire resistance measurement method (39, 40), and if so, would retire that reservoir from further testing to decrease the likelihood of using a failed coil in further trials. The operator noted whether each puff-activated ENDS appeared to consistently activate for every puff in the multi-puff sequence, or if the ENDS device operated unreliably.

After all three scatter plots were generated and annotated for each ENDS, the analyst interpreted the yield results in the context of the emission operator's notes. The analyst determined four parameters to characterize the effective operating envelope of each ENDS: (1) the minimum aerosolization flow rate (MinAF), (2) the maximum aerosolization flow rate (MaxAF), (3) the minimum aerosolization duration (MinAD), and (4) the maximum aerosolization duration (MaxAD).

The MinAF was defined as the lowest puff flow rate at which the ENDS device consistently activated and generated aerosol, while the puff duration was held fixed at nominally 3.5 sec. The MinAF simultaneously (1) generated yield above the per-puff LoQ, $Y_{\text{TPM}} \geq 0.04$ (mg/puff) for the “variable flow rate set” of conditions, (2) exhibited a relative gravimetric error, $\Delta M \approx \leq 10$ (%), and (3) appeared to consistently activate the ENDS coil based on operator observations. The error bound on the MinAF were taken to be the difference between trial conditions wherein constraints 1 through 3 were and were not consistently satisfied.

The MaxAF were defined as the lowest flow rate at which there was visual and gravimetric evidence of aspiration, while

the puff duration was held fixed at nominally 3.5 s. The MaxAF simultaneously (1) exhibited a sudden sharp increase in the slope of the Y_{TPM} vs. q curve, (2) exhibited a relative gravimetric error, $\Delta M > 10$ (%), and (3) exhibited evidence of liquid suction in addition to or in place of aerosolization as reflected by operator observations and photographs. The error bound on the MaxAF were taken to be the difference between flow conditions wherein constraints 1 through 3 were and were not consistently satisfied. If the MaxAF could not be determined definitively for the conditions tested, the maximum flow rate tested was recorded.

The MinAD were defined as the lowest puff duration at which the ENDS device consistently activated and generated aerosol while the nominal puff flow rate was held fixed at ~ 30 mL/s. The MinAD simultaneously (1) generated yield above the per-puff LoQ, $Y_{TPM} \geq 0.04$ (mg/puff) curve, (2) exhibited a relative gravimetric error, $\Delta M \leq 10$ (%), and (3) appeared to consistently activate the ENDS coil based on operator observations. The error bound on the MinAD were taken to be the difference between duration conditions wherein constraint 1 through 3 were and were not consistently satisfied.

The MaxAD were defined as the upper time limit above which the ENDS no longer provided power to the coil. Many, not all, ENDS manufacturers cut off the current provided to the coil after some manufacturer-determined time limit, and this feature is not reported by most manufacturers. The MaxAD simultaneously (1) exhibited a distinct flattening of the Y_{TPM} vs. d curve, (2) exhibited a relative gravimetric error, $\Delta M \leq 10$ (%), and (3) appeared consistent with operator visual and audible observations of ENDS behavior. The error bound on the MaxAD were taken to be the difference between conditions wherein constraints 1 through 3 were and were not consistently satisfied. If the MaxAD could not be determined definitively for the conditions tested, the maximum duration tested was recorded.

RESULTS

Figure 2 shows a scatter plot of TPM pad yield per puff deposited on the filter pad as a function of flow rate for five exemplar ENDS products: JUUL LABS Juul and BLU myBlu (both puff-activated non-refillable pod-style), NJOY Ace (button-activated non-refillable pod-style), SMOK Novo 2 (button-activated pod-style with refillable reservoir and user-replaceable coil), and VUSE Vibe (puff-activated non-refillable pen-style). Experimental results for all thirteen products are presented in **Supplementary Material**. The data are reported as a function of the actual mean flow rate achieved by the emissions system (41) across nominally 50 puffs per trial. The PES command puff duration was 3.5 (s) and the actual puff duration achieved by the emissions system had a mean of 3.29 (St. Dev. 0.37) (s). The LoQ Lower Y_{TPM} limit of 0.04 (mg/puff) is represented as a horizontal broken line. Lines are used to connect the markers as a visual aid and are not intended to be indicative of a curve. Selected operator and analyst annotations regarding the MinAF and MaxAF are presented on the figure.

Consider the button activated Ace device which generated low TPM at flow rates of 2.6 and 4.9 (mL/s) with a noticeable

increase at 7.3 (mL/s). The MinAF for the Ace was declared to be 6 ± 1.2 (mL/s). Similarly, the MinAF was declared to be 9.75 ± 3.5 , 12 ± 2.4 , and 23 ± 1.2 (mL/s) for the Vibe, Juul, and myBlu, respectively. The Novo 2 exhibited erratic behavior between 12 and 18 (mL/s). Operator notes indicated the device failed to “fire consistently” for every puff, and trials also exhibited relatively large gravimetric error, shown in a subsequent figure. The Novo 2 appeared to be fully activated at flow rates above 24 (mL/s) and essentially inactive at flow rates below 22 (mL/s). Thus, the MinAF for the Novo 2 was declared to be 21.3 ± 2.5 (mL/s) with the broad error bar indicating the observed variability in the ENDS performance. The maximum pad yield per puff was $Y_{TPM,Max} \approx 13$ (mg/puff) for the Novo 2 and the lowest was $Y_{TPM,Max} \approx 2$ (mg/puff) for the Juul, which was also the minimum value of $Y_{TPM,Max}$ across the 13 products studied. Conversely, the Stick Prince, presented in **Supplementary Material** exhibited the highest pad yield per puff of $Y_{TPM,Max} \approx 50$ (mg/puff) within the normal operating range of all devices tested (excluding cases where e-liquid was clearly aspirated into the flow path). **Figure 2** illustrates the relatively erratic yield response of the Novo 2, while the Vibe appeared quite stable until 45 (mL/s), with an unusual response at 60 (mL/s), and then evidence of aspiration was observed officially at 70 (mL/s). The myBlu product exhibited the most uniform TPM yield per puff across the range of flow rates of all thirteen products studied here. Both the emissions stability and value of $Y_{TPM,Max}$ are potentially significant regulatory outcome measures. Of the thirteen products tested here at a nominal puff duration of 3.5 (s), for example, the Stick Prince delivered the highest $Y_{TPM,Max} \approx 50$ (mg/puff) while the Juul delivered the lowest $Y_{TPM,Max} \approx 2$ (mg/puff) – a ratio of 25:1. Clearly, some ENDS are capable of delivering far more TPM to the mouth of a user within the product’s normal operating envelope.

Figure 3 shows a scatter plot of TPM yield per puff deposited on the filter pad as a function of duration for the same five ENDS presented in **Figure 2**. Each emissions trial result is represented by a single marker; lines are a visual aid only. The data are reported as a function of the actual mean puff duration achieved by the emissions system (41) across nominally 50 puffs per trial. The command puff flow rate was 30 (mL/s) for all conditions presented in this figure, while the measured puff flow rate had a mean of 28.5 (St. Dev. 1.5) (mL/s). The LoQ Lower Y_{TPM} limit of 0.04 (mg/puff) was first exceeded when the ENDS mean duration ranged between 0.5 and 0.65 (s) for the five ENDS illustrated, denoted as the range of MinAD.

The five PCUs are useful to illustrate unique features evident across the family of thirteen devices tested. First, we observe the myBlu response, which exhibits a linear increase in pad yield per puff (at this fixed flow rate) until a duration of 10 (s) is achieved, at which point the yield curve flattens out. This is consistent with operator observations that the PCU de-energized after approximately MaxAD ≈ 10 (s) puff duration. The Ace PCU curve exhibited similar response, with the PCU de-energizing at approximately MaxAD ≈ 5.5 (s) as supported by both operator observation and manufacturer documentation. The Vibe PCU exhibited similar response; the operator notes indicate a cut-off at approximately MaxAD ≈ 6 (s), with greater variability

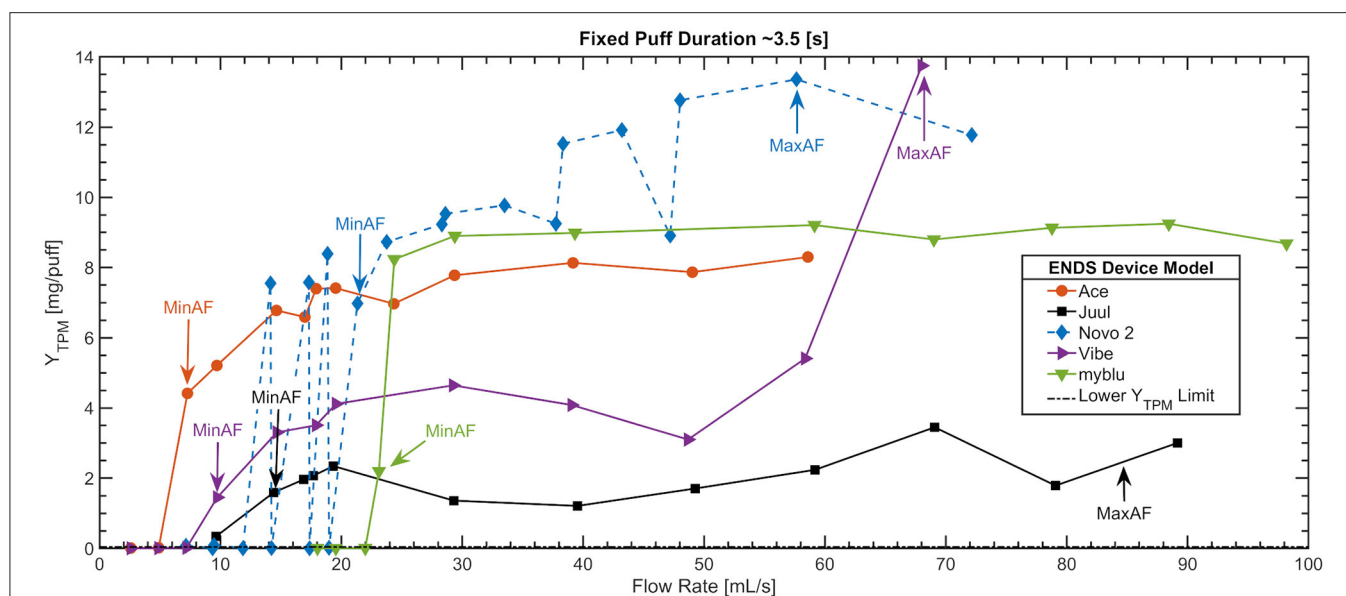


FIGURE 2 | Minimum and maximum activation flow rate, MinAF and MaxAF, for 5 of the 13 ENDS devices tested. Shown is the mean TPM yield per puff for each flow rate condition, based on nominally 50 puffs per flow rate conditions. Puffs were square-wave and nominally 3.5 s in duration, with 30 s puff period.

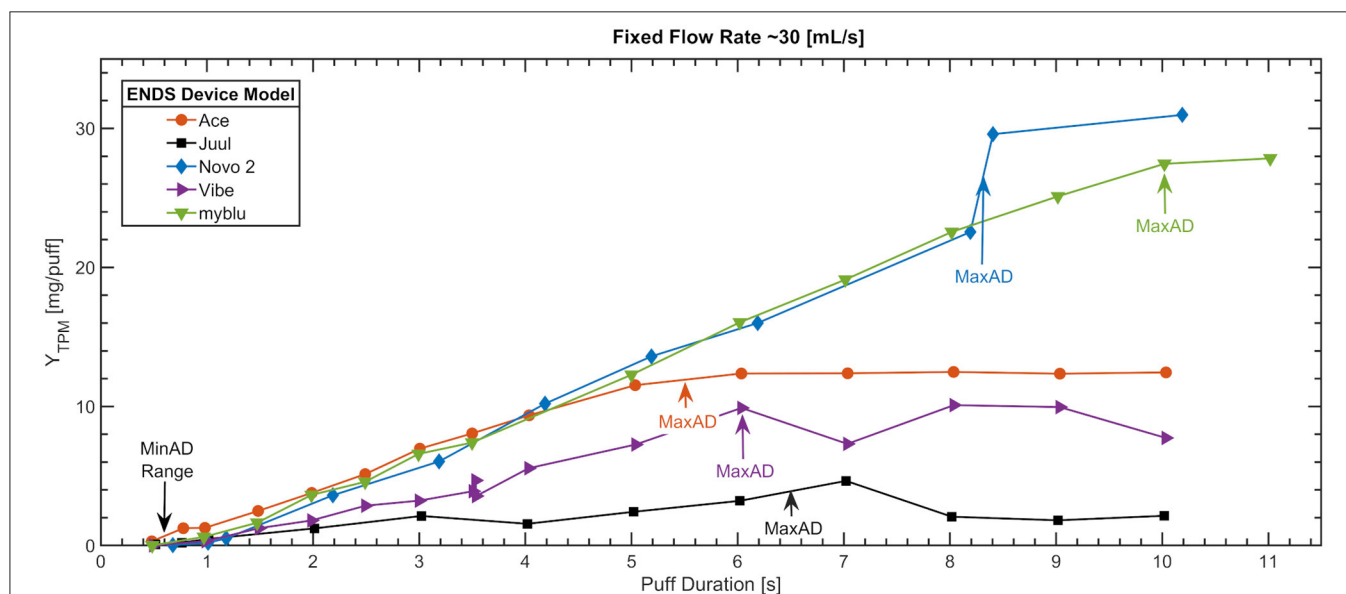


FIGURE 3 | Minimum and maximum activation duration, MinAD and MaxAD, for 5 of the 13 ENDS devices tested. Shown is the mean TPM yield per puff for each duration condition, based on nominally 50 puffs per flow rate conditions. Puffs were square-wave and nominally 30 ml/s, with 30 s period.

in emissions yield from Vibe relative to the other ENDS. The Novo 2 PCU exhibited a fundamentally different response. The expected linear increase of yield with duration (at fixed flow rate) was observed until 8 (s), but then a dramatic increase in yield was observed. Operator notes taken during the last two trials indicated visual evidence of liquid being suctioned from the ENDS reservoir, and deposited between the exit plane of the ENDS and the entrance to the filter pad surface [a distance of <1 (cm)], which we declare as the onset of aspiration. The Juul

ENDS exhibited a comparatively constant pad yield per puff as a function of duration (at fixed flow rate) though some linearly increasing trend is implied between 4 and 7 (s).

Figure 4 shows a scatter plot of the gravimetric measurement consistency as a function of flow rate arising from the same conditions presented in **Figure 2**. The vertical axis is relative percent error, ΔM , computed as the total mass decrease observed in the ENDS compared to the total mass increase observed on the filter PAD for each condition. The MinAF determined by

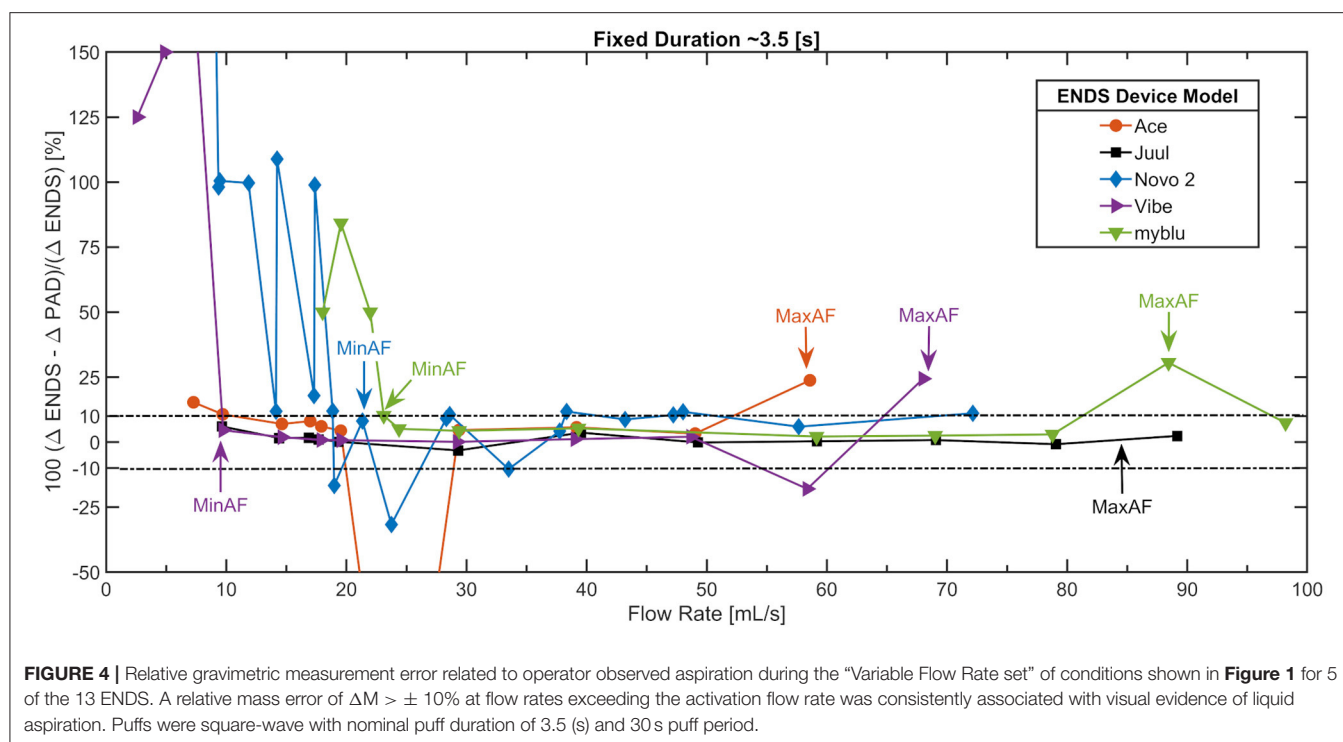


Figure 2 is repeated here for reference. Operator notes associated with onset of liquid aspiration are annotated as an aide to understand corresponding conditions between **Figures 2, 4**. The large relative errors at low flow rates are associated with the small magnitudes observed and reflect that the data is below the acceptable LoQ. The relative mass error reiterates the results presented in **Figure 1** for the Novo 2 which behaves erratically at flow rate below MinAF $\approx 21.3 \pm 2.5$ (mL/s). All five ENDS illustrated here exhibited visual and/or deposition evidence of liquid aspiration as the flow rate increased. Aspiration onset was evident at flow rates as low MaxAF ≈ 48 (mL/s) and as high as MaxAF ≈ 88 (mL/s) for the thirteen products studied. The Novo 2 exhibited potential aspiration and visibly large droplets even at moderate flow rates. The Juul in **Figure 4** exhibited the first visible signs of aspiration (MaxAF criteria 3) between 80 and 90 (mL/s).

The analysis described in the methods and illustrated by **Figures 2–4** was applied to experimental data from all 13 ENDS designs. A summary of findings is presented in **Table 2**. The mean and median f_{Nic} ratios are reported as the average across all flow conditions within the operating envelope of each device. There was insufficient evidence in the results from the screening trials to assess significant variation in f_{Nic} as a function of flow rate or duration. The primary outcome measures for the operating envelope (MinAF, MaxAF, MinAD, MaxAd) are shown in the upper portion of the table. The mean, median and standard deviation effective coil resistance using the four wire resistance measurement method (40), emissions nicotine mass ratio, unpuffed E-Liquid nicotine mass ratio, and solvent Propylene Glycol to Glycerin composition are reported for each product.

The number of PCUs and reservoirs used for each device model are presented in **Table 2**, and ranged from a low of 1 PCU and reservoir up to 8 PCU/Reservoir combinations for disposable ENDS. Comprehensive characterizations, well beyond the screening conditions described herein, require more PCUs and reservoirs; we used 5 PCUs and 41 reservoirs in assessment of the Vuse Alto.

DISCUSSION

Limitations and Scope

The results presented herein are for 13 ENDS products of either pen- or pod-style designs, and share a common attribute of no user-adjustable power options (other than swapping coils), and no user adjustable flow path (e.g., variable inlet restrictors). The study investigated both flow- and button-activated coil designs and disposable and refillable reservoirs. The numerical values presented in the results, discussion and conclusion may thus be limited to pen- and pod-style ENDS. The screening method and outcome measures may be broadly applied to a variety of inhaled tobacco products including ENDS, combustibles and heated tobacco products (also referred to as “Heat Not Burn”).

This article has presented a comparative evaluation of the effective operating envelope of thirteen popular ENDS (pod and pen-style) products. Concurrently, the article demonstrated a robust method for empirically quantifying the operating envelope of ENDS products. The method may be used to compare operating envelope and emissions characteristics between ENDS products and may enable data sharing and reproducibility studies between research laboratories.

TABLE 2 | Nominal operating envelope and emissions characteristics of test specimens used in this study.

Product: model	JUUL	Alto	Novo 2	myblu	Ace	Caliburn	Breeze 2	Titan	Logic Pro	Hyde	Vibe	Smok Stick	Puff Bar
Eliquid manufacturer	JUUL Labs	VUSE	Mad Hatter Juice	Blu	NJOY	Mad Hatter Juice	Mad Hatter Juice	Vapor4Life	Logic	Hyde	VUSE	Mad Hatter Juice	Puff Bar
Operating envelope													
Number of ENDS PCU's tested	1	5	1	1	1	1	1	2	1	8	1	1	5
Number of ENDS reservoirs tested	5	41	1	13	9	1	1	6	8	8	6	1	5
Min AF (mL/s)	12 ± 2.4	15.8 ± 1.1	21.3 ± 2.4	23 ± 1.2	6 ± 1.2	11.8 ± 2.25	2.5 ± 2	12.2 ± 2.5	2.6 ± 2.25	16 ± 1.6	9.75 ± 3.75	2.5 ± 2.5	14 ± 4
Max AF (mL/s)	>85 ± 5	50 ± 2	58 ± 10	88 ± 5	58 ± 5	> 88	~30 @ d > 8s	> 50	>48	>86	68 ± 5	ADR	>50
Min AD (s)	0.5 ± 0.25	0.85 ± 0.15	0.9 ± 0.15	1 ± 0.25	0.5 ± 0.25	0.5 ± 0.25	1 ± 0.52	1 ± 0.25	0.75 ± 0.25	0.5 ± 0.25	1 ± 0.25	1 ± 0.25	1 ± 0.25
Max AD (s)	6.5 ± 0.5	5.0 ± 0.1	8 ± 0.5	10 ± 0.5	5.5 ± 0.5	>10	10.5 ± 0.25	10 ± 0.25	>10	>10	6 ± 0.5	8 ± 0.25	>3.5
Emissions and E-liquid characteristics													
Number of f _{Nic} samples	21	135	13	17	25	27	24	15	27	20	11	38	11
f _{Nic} Mean (-)	0.046 ^a	0.0468	0.0503	0.0256	0.055	0.046	0.036	0.0305	0.0211	0.0596	0.0315	0.0496	0.0454
f _{Nic} Median (-)	0.046 ^a	0.0464	0.05097	0.025	0.055	0.046	0.039	0.0295	0.0197	0.0607	0.0304	0.0523	0.0486
f _{Nic} Std Dev (-)	0.003 ^a	0.0048	0.0038	0.0033	0.0046	0.0037	0.009	0.00296	0.007	0.0041	0.005	0.0058	0.0063
Eliquid nicotine mass ratio (-)	0.052	0.052	0.039	0.02	0.05	0.039	0.039	0.023	0.016	0.0503	0.030	0.039	0.048
Eliquid nicotine mass ratio StDev (-)	0.0005	0.0008	0.0019	0.0012	0.0002	0.0019	0.0019	0.0009	0.0009	0.0017	0.0004	0.0019	NR
Eliquid molar ratio PG:GL	33:67	NR	44:56	42:58	48:52	44:56	44:56	73:27	77:23	NR	23:77	44:56	54:56
Eliquid mass ratio PG:GL	29:71	NR	39:61	38:62	43:57	39:61	39:61	69:31	74:26	NR	20:80	39:61	50:50
Eliquid volume ratio PG:GL	33:67	NR	44:56	42:58	48:52	44:56	44:56	73:27	77:23	NR	23:77	44:56	54:56
Heating element/coil characteristics													
R measurement method	F4W	F4W	M4W	F4W	F4W	M4W	M4W	M4W	M4W	M4W	M4W	M4W	M4W
Number of coil R measured	16	17	3	18	8	3	1	4	8	1	6	2	1
Effective coil resistance mean (ohm)	1.633	1.063	1.463	1.416	1.034	1.405	0.631	2.258	2.443	1.61	2.693	0.174	1.688
Effective coil resistance StDev (ohm)	0.033	0.075	0.047	0.017	0.079	0.013	0	0.053	0.077	0	0.018	0.006	0

NR, Not Reported; F4W, Fixture 4 Wire; M4W, Manual 4 Wire.

^aThese nicotine mass ratios were computed without the use of the internal standard (IS) and simply from calibration curve of nicotine due to an error in the IS reference sample preparation.

ENDS products have the ability to limit the maximum coil activation time and hence TPM per puff.

The maximum aerosolization duration, MaxAD, after which the ENDS power control unit de-energizes the coil, varies widely between ENDS products. All thirteen ENDS products permitted puff durations of at least 5 s. Five products energized the coil for puff durations between 5 and 7.5 s, five products between 7.5 and 10 s, and three products continued to energize the coil for more than 10 s (the maximum duration studied in these experiments). There is no doubt that limiting the coil activation duration time, MaxAD, is feasible; this is a product characteristic which may be regulated and has a direct correlation with the per-puff TPM delivered to a user.

Aerosol Observed Nicotine Mass Ratio Is Comparable to That in Unpuffed E-Liquids

We observed a correlation between the nicotine mass ratio in the un-puffed e-liquid (denoted Eliquid nicotine mass ratio in **Table 2**) and the mass ratio present in the generated aerosols (denoted f_{Nic} in **Table 2**), for both the mean ($\beta = 1.068$, $r = 0.84$, $R^2 = 0.700$) and the median ($\beta = 1.085$, $r = 0.86$, $R^2 = 0.735$). We found the nicotine mass ratio to be largely independent of puff flow rate, duration, and volume by conducting a multi-variate linear regression analysis of f_{Nic} for all 13 products. There was insufficient evidence to support a flow condition dependence of f_{Nic} for eleven products ($p > 0.05$). The Novo 2 exhibited an f_{Nic} slightly dependent on flow rate ($\beta = 0.003$, $p = 0.045$) and puff duration ($\beta = 0.023$, $p = 0.049$), but not on puff volume ($\beta = -0.0009$, $p = 0.053$). The SMOK Stick value of f_{Nic} was slightly associated with puff volume ($\beta = 0.0002$, $p = 0.038$) but was not associated with either puff flow rate ($\beta = 0.0007$, $p = 0.073$) or duration ($\beta = -0.005$, $p = 0.097$). None of the ENDS products selected for this study permitted user-adjustable power settings. It remains an unanswered question, worthy of further investigation, to assess the dependence of f_{Nic} for higher power devices such as box-mod ENDS.

We conclude that, for moderately powered pen-style and pod-style devices, it is a reasonable first-order approximation to assume the mass ratio of nicotine present in the aerosol emissions is similar to the mass ratio of nicotine in the unpuffed E-Liquid. Pagano et al. (42) studied five brands of first generation cig-a-likes and reported the nicotine mass delivered to the pad ranged from 14 to 58% of the nicotine mass in the un-puffed cig-a-like ENDS. Pagano et al. defined this ratio as the nicotine transfer efficiency and suggested a significant mass of nicotine was retained in the cig-a-like wick at its end of life. In this study, we specifically avoided puffing the ENDS until the reservoir was empty. While the Pagano article demonstrated that cig-a-like ENDS inherently retained significant residual nicotine at end-of-product-life, modern pen- and pod-style ENDS can deliver virtually all of the nicotine from the reservoir to the user. A parallel study investigates the relationship between consumption of all E-Liquid in two pod-style ENDS reservoirs and its impact on coil lifetime (43). As power levels increase and coil temperature is permitted to rise, as anticipated for modern sub-ohm box-mod style ENDS, the variability in the saturation temperature of the E-Liquid constituents is likely to invalidate

this approximation, and caution should be taken if extrapolating this approximation to other devices and E-Liquids.

Most ENDS Devices Exhibit E-Liquid Aspiration in Addition to Aerosolization

ENDS are known to produce condensation aerosols which contain submicron particles suspended in vapor and inhalable by the user. However, we observed at high flow rates formation of droplets in the flow path of the emission system and in some cases formation of bubbles within the un-puffed E-Liquid in the ENDS Reservoir. We hypothesize this phenomena, which we have named E-Liquid aspiration, to result from excess suction pressure in the reservoir at high flow rates sufficient to overcome the surface tension of the solvent. This is analogous to sucking liquid droplets through a straw when the container is nearly empty and the distal end of the straw is not submerged. While our visual observations were consistent with fluid transport phenomena, further investigation is warranted. If indeed E-Liquid aspiration was occurring at user-achievable flow rates, this could be a potential poisoning hazard. Al-Delaimy and Sim (44) documented up to 4,000 cases annually of E-Liquid and ENDS poisoning in the USA since 2014. Even if users are not aspirating an entire bolus of E-Liquid, such mechanisms could dramatically increase the yield of TPM, nicotine and other E-Liquid additives in a single puff, as observed by the data, and alter patterns of lung deposition. Aspiration of MCT oils (commonly used in ENDS devices for delivery of CBD and THC active ingredients) in liquid form has potentially significant adverse health implications, particularly in light of public health concerns related to E-cigarette or Vaping Product Use-Associated Lung Injury (EVALI) (45).

Recommended Product Characteristics for ENDS Regulations

Traditional product characteristics considered for regulation include items such as E-Liquid nicotine concentration and coil resistance. However, such regulations may not achieve the desired public health outcomes. Even if ENDS manufacturers are constrained to a maximum E-Liquid nicotine mass concentration, they are able to adjust numerous product characteristics to achieve a high nicotine yield per puff including: increase the PCU Maximum Aerosolization Duration (MaxAD), decrease the coil resistance, increase the coil voltage or current, increase the coil power duty cycle, decrease the ENDS flow path resistance, or decrease the solvent saturation temperature. All of these adjusted product characteristics may result in potentially adverse unintended public health consequences. In fact, decreasing the nicotine concentration in the E-Liquid, while keeping all other product characteristics fixed, will result in a net increase in TPM exposure for a user who consumes a given mass of nicotine per day. We propose it is more effective to regulate the product characteristics of TPM (Y_{TPM}) and nicotine yield per puff ($Y_{\text{Nic}} = f_{\text{Nic}} \times Y_{\text{TPM}} = f_{\text{Nic}} \times C_{\text{TPM}} \times V_{\text{Puff}}$). In the proposed case, manufacturers have free reign to adjust numerous design parameters of their PCUs and E-Liquids, but the end-result outcome measure remains consistently regulated.

Recommended Standard Manufacturer Packaging Information for ENDS Devices and E-Liquids

We observed variability in descriptions provided to consumers on product packaging between manufacturers. We recommend manufacturers be required to prominently disclose several characteristics of ENDS Power Control Units including: battery chemistry and capacity (mAh), designed flow rate operating range, designed maximum coil activation puff duration, and operating range of root mean square (RMS) power (watts) dissipated in a coil of a stated nominal design coil resistance (ohms). We recommend manufacturers be required to prominently disclose several characteristics of ENDS Reservoirs including: all materials present in the reservoir, solder, wick, coil, and mouthpiece, the reservoir fill volume (mL), the nominal coil resistance (ohm) and coil manufacturing variability expressed as a standard deviation (ohm). We recommend that E-Liquid manufacturers (including E-Liquid sold in disposable reservoirs and refill liquid) be required to prominently display the composition of un-puffed E-Liquid in tabular format listing the solvent components and composition (i.e., Propylene Glycol, Glycerin, water, etc.) (22) and additives including nicotine, menthol, and all other additives on a mass fraction basis, such that the sum of all constituents is unity. This is similar to the nutrition labels familiar to many consumers on food products. These product characteristics (ENDS PCU, Reservoir and E-Liquid) collectively affect the mass concentration and composition of emissions generated by ENDS devices and consumables.

Recommended Standard Emissions Outcome Measures

No standard emissions outcome measures have previously been agreed upon. We recommend that ENDS manufacturers be required to conduct and report flow condition dependent emissions as an integral aspect of premarket regulatory approval processes. The emissions trials should be conducted over the entire range of operating envelope indicated on their product packaging and consumer information. Manufacturers should be required to report emissions outcome measures at each of several operating conditions (puff flow rate, puff duration and RMS coil power) spanning the product operating envelope. Emissions outcome measures should include at least: Total Particulate Matter (TPM) yield per puff [Y_{TPM} , (mg/puff)], TPM mass concentration [C_{TPM} , (mg/mL)], and aerosol mass ratio of every constituent listed in the un-puffed E-Liquid [$f_{\text{constituent}}$, (mg constituent/mg TPM)]. The nicotine mass ratio was demonstrated for eight different E-Liquids herein as one example of a constituent mass ratio. Use of the emissions outcome measures (Y, C, f) has been demonstrated previously for a variety of products (11, 46) and can be used as input characteristics to an experimentally validated behavior-based yield model (46–51). With addition of this article documenting thirteen ENDS, these outcome measures now provide a basis for future product comparisons.

Recommended Standard Information for PMTAs

Collectively, these labeled product characteristics and emissions pre-market data serve to document the effective operating range (envelope) of an ENDS product. We propose this documentation be mandated for PMTA reporting under 81 FR 28781. The product labeling information ensures that consumers are well-informed of potential chemical exposure arising from actual product use. The proposed emission outcome measures permit regulators to assess the likelihood of potentially hazardous decomposition products which may be present in the emissions. If the E-Liquid product manufacturers are required to document 100% of the product's mass ratio content and the Reservoir product manufacturers are required to document the materials present in the ENDS reservoir assembly, then the union of these two documents result in a relatively short list of $f_{\text{constituent}}$ outcome measures, which can be used to inform regulators of potential decomposition products. That is, if the summation of all $f_{\text{constituent}}$ reported in the emissions adds to <100% of the mass collected during emissions testing, there is a reasonable probability that other compounds may be present in the emissions, thus warranting further regulatory review prior to market approval.

CONCLUSIONS

A standard method for characterizing the operating envelope of ENDS products using four parameters (MinAF, MaxAF, MinAD, and MaxAD) has been presented and demonstrated. The study demonstrated good emissions study practices by thoroughly documenting the ENDS test specimen product characteristics, TPM yield per puff, Y_{TPM} , TPM mass concentration, C_{TPM} , and descriptive statistics of the nominal coil resistance, R_{coil} , and nicotine mass ratio, f_{Nic} , expressed as mg of nicotine per mg of TPM.

Three emissions outcome measures (Y, C, f) are recommended for adoption as standard quantities for emissions testing by manufacturers and research laboratories. Recommendations for minimum required product labeling have been proposed for ENDS power control units, reservoirs, coils and E-Liquids. Recommendations for required data to be included in premarket tobacco applications have been proposed.

Further investigation into mechanisms of E-Liquid aspiration is needed to inform potential regulations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EH, RR, and NE: conceptualization and funding acquisition. GD, NE, and EH: methodology. EH: software, formal analysis, writing—original draft preparation, and visualization. EH and RR: validation, resources, supervision, and project

administration. NE, GD, SS, SJ, QS, BM, EH, and RR: investigation. GD, SJ, SS, BM, and EH: data curation. RR and NE: writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

- Farsalinos KE, Yannovits N, Sarri T, Voudris V, Poulas K. Protocol proposal for, and evaluation of, consistency in nicotine delivery from the liquid to the aerosol of electronic cigarettes atomizers: regulatory implications. *Addiction*. (2016) 111:1069–76. doi: 10.1111/add.13299
- Goniewicz ML, Kuma T, Gawron M, Knysak J, Kosmider L. Nicotine levels in electronic cigarettes. *Nicotine Tobacco Res.* (2013) 15:158–66. doi: 10.1093/ntr/nts103
- Farsalinos KE, Kistler KA, Gillman G, Voudris V. Evaluation of electronic cigarette liquids and aerosol for the presence of selected inhalation toxins. *Nicotine Tobacco Res.* (2015) 17:168–74. doi: 10.1093/ntr/ntu176
- Misra M, Leverette RD, Cooper BT, Bennett MB, Brown SE. Comparative in vitro toxicity profile of electronic and tobacco cigarettes, smokeless tobacco and nicotine replacement therapy products: e-liquids, extracts and collected aerosols. *Int J Environ Res Public Health.* (2014) 11:11325–47. doi: 10.3390/ijerph111111325
- Fields W, Maione A, Keyser B, Bombick B. Characterization and application of the VITROCELL VC1 smoke exposure system and 3D EpiAirway models for toxicological and e-cigarette evaluations. *Appl In Vitro Toxicol.* (2017) 3:68–83. doi: 10.1089/aivt.2016.0035
- Geiss O, Bianchi I, Barahona F, Barrero-Moreno J. Characterisation of mainstream and passive vapours emitted by selected electronic cigarettes. *Int J Hygiene Environ Health.* (2015) 218:169–80. doi: 10.1016/j.ijheh.2014.10.001
- Zhao J, Pyrgiotakis G, Demokritou P. Development and characterization of electronic-cigarette exposure generation system (Ecig-EGS) for the physico-chemical and toxicological assessment of electronic cigarette emissions. *Inhalation Toxicol.* (2016) 28:658–69. doi: 10.1080/08958378.2016.1246628
- Herrington JS, Myers C. Electronic cigarette solutions and resultant aerosol profiles. *J Chromatogr A.* (2015) 1418:192–9. doi: 10.1016/j.chroma.2015.09.034
- Lee MS, LeBouf RF, Son YS, Koutrakis P, Christiani DC. Nicotine, aerosol particles, carbonyls and volatile organic compounds in tobacco- and menthol-flavored e-cigarettes. *Environ Health.* (2017) 16:42. doi: 10.1186/s12940-017-0249-x
- Farsalinos KE, Voudris V, Poulas K. E-cigarettes generate high levels of aldehydes only in 'dry puff' conditions. *Addiction.* (2015) 110:1352–6. doi: 10.1111/add.12942
- Robinson RJ, Eddingsaas NC, DiFrancesco AG, Jayasekera S, Hensel EC Jr. A framework to investigate the impact of topography and product characteristics on electronic cigarette emissions. *PLoS ONE.* (2018) 13:e0206341. doi: 10.1371/journal.pone.0206341
- Robinson RJ, Hensel EC, Morabito PN, Roundtree KA. Electronic cigarette topography in the natural environment. *PLoS ONE.* (2015) 10:e0129296. doi: 10.1371/journal.pone.0129296
- Robinson RJ, Hensel EC, Roundtree KA, DiFrancesco AG, Nonnemaker JM, Lee YO. Week long topography study of young adults using electronic cigarettes in their natural environment. *PLoS ONE.* (2016) 11:e0164038. doi: 10.1371/journal.pone.0164038
- Trehy ML, Ye W, Hadwiger ME, Moore TW, Allgire JF, Woodruff JT, et al. Analysis of electronic cigarettes cartridges, refill solutions, and smoke for nicotine and nicotine related impurities. *J Liquid Chromatogr Relat Technol.* (2011) 34:1442–58. doi: 10.1080/10826076.2011.572213
- Goniewicz ML, Hajek P, McRobbie H. Nicotine content of electronic cigarettes, its release in vapour and its consistency across batches: regulatory implications. *Addiction.* (2014) 109:500–7. doi: 10.1111/add.12410
- Kosmider L, Sobczak A, Fik M, Knysak J, Zaciera M, Kurek J, et al. Carbonyl compounds in electronic cigarette vapors: effects of nicotine solvent and battery output voltage. *Nicotine Tob Res.* (2014) 16:1319–26. doi: 10.1093/ntr/ntu078
- Tayyarah R, Long GA. Comparison of select analytes in aerosol from e-cigarettes with smoke from conventional cigarettes and with ambient air. *Regul Toxicol Pharmacol.* (2014) 70:704–10. doi: 10.1016/j.yrtph.2014.10.010
- El-Hellani A, El-Hage R, Baalbaki R, Salman R, Talih S, Shihadeh A, et al. Free-base and protonated nicotine in electronic cigarette liquids and aerosols. *Chem Res Toxicol.* (2015) 28:1532–7. doi: 10.1021/acs.chemrestox.5b00107
- Farsalinos K, Gillman G, Poulas K, Voudris V. Tobacco-specific nitrosamines in electronic cigarettes: comparison between liquid and aerosol levels. *Int J Environ Res Public Health.* (2015) 12:9046. doi: 10.3390/ijerph120809046
- Jensen RP, Luo W, Pankow JF, Strongin RM, Peyton DH. Hidden formaldehyde in E-cigarette aerosols. *New Engl J Med.* (2015) 372:392–4. doi: 10.1056/NEJMc1413069
- Talih S, Balhas Z, Eissenberg T, Salman R, Karaoghlanian N, El Hellani A, et al. Effects of user puff topography, device voltage, and liquid nicotine concentration on electronic cigarette nicotine yield: measurements and model predictions. *Nicotine Tob Res.* (2015) 17:150–7. doi: 10.1093/ntr/ntu174
- El-Hellani A, Salman R, El-Hage R, Talih S, Malek N, Baalbaki R, et al. Nicotine and carbonyl emissions from popular electronic cigarette products: correlation to liquid composition and design characteristics. *Nicotine Tob Res.* (2016) 20:215–23. doi: 10.1093/ntr/ntw280
- Kosmider L, Madej D, Gawron M, Sobczak A. Influence of electronic cigarettes puffing regimes on amount of vaporized liquid. *Przegl Lek.* (2016) 73:699–703.
- Kosmider L, Kimber CF, Kurek J, Corcoran O, Dawkins LE. Compensatory puffing with lower nicotine concentration E-liquids increases carbonyl exposure in e-cigarette aerosols. *Nicotine Tobacco Res.* (2017) 20:998–1003. doi: 10.1093/ntr/ntx162
- Margham J, McAdam K, Forster M, Liu C, Wright C, Mariner D, et al. Chemical composition of aerosol from an E-cigarette: a quantitative comparison with cigarette smoke. *Chem Res Toxicol.* (2016) 29:1662–78. doi: 10.1021/acs.chemrestox.6b00188
- Sleiman M, Logue JM, Montesinos VN, Russell ML, Litter MI, Gundel LA, et al. Emissions from electronic cigarettes: key parameters affecting the release of harmful chemicals. *Environ Sci Technol.* (2016) 50:9644–51. doi: 10.1021/acs.est.6b01741
- Farsalinos KE, Voudris V, Spyrou A, Poulas K. E-cigarettes emit very high formaldehyde levels only in conditions that are aversive to users: a replication study under verified realistic use conditions. *Food Chem Toxicol.* (2017) 109:90–4. doi: 10.1016/j.fct.2017.08.044
- Talih S, Salman R, Karaoghlanian N, El-Hellani A, Saliba N, Eissenberg T, et al. "Juice Monsters": Sub-Ohm vaping and toxic volatile aldehyde emissions. *Chem Res Toxicol.* (2017) 30:1791–3. doi: 10.1021/acs.chemrestox.7b00212
- Sala C, Medana C, Pellegrino R, Aigotti R, Bello FD, Bianchi G, et al. Dynamic measurement of newly formed carbonyl compounds in vapors from electronic cigarettes. *Eur J Mass Spectrom.* (2017) 23:64–9. doi: 10.1177/1469066717699078

30. Pankow JF, Kim K, McWhirter KJ, Luo W, Escobedo JO, Strongin RM, et al. Benzene formation in electronic cigarettes. *PLoS ONE*. (2017) 12:e0173055. doi: 10.1371/journal.pone.0173055
31. Korzun T, Lazurko M, Munhenzva I, Barsanti KC, Huang Y, Jensen RP, et al. E-cigarette airflow rate modulates toxicant profiles and can lead to concerning levels of solvent consumption. *ACS Omega*. (2018) 3:30–6. doi: 10.1021/acsomega.7b01521
32. Behar RZ, Luo W, McWhirter KJ, Pankow JF, Talbot P. Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids. *Sci Rep*. (2018) 8:8288. doi: 10.1038/s41598-018-25575-6
33. Malek N, Nakkash R, Talih S, Lotfi T, Salman R, Karaoghlanian N, et al. A transdisciplinary approach to understanding characteristics of electronic cigarettes. *Tobacco Regulat Sci*. (2018) 4:47–72. doi: 10.18001/TRS.4.3.5
34. ISO/TC126. 3308:2012 *Tobacco and Tobacco Products - Routine Analytical Cigarette-Smoking Machine-Definitions and Standard Conditions*. Geneva, Switzerland. (2012).
35. Robinson RJ, Sarles SE, Jayasekera S, al Olayan A, DiFrancesco AG, Eddingsaas NC, et al. A comparison between cigarette topography from a one-week natural environment study to FTC/ISO, Health Canada, and Massachusetts Department of Public Health Puff Profile Standards. *Int J Environ Res Public Health*. (2020) 17:3444. doi: 10.3390/ijerph17103444
36. ISO/T-126/SC-3. ISO 20768:2018 *Vapour Product - Routine Analytical Vaping Machine - Definitions and Standard Conditions*. Geneva, Switzerland: International Organization for Standardization (2018). p. 7.
37. (US) N. C. I. *Risks Associated With Smoking Cigarettes With Low Machine-Measured Yields of Tar and Nicotine*. Bethesda, MD: U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, National Cancer Institute (2001).
38. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem. Rev.* (2008) 29:S49–S52. doi: 10.19080/OMCIJ.2018.07.555722
39. Saleh Q, Hensel EC, Robinson R. *Coil Resistance Testing Apparatus for VUSE ALTO*. (2020). Available online at protocols.io (accessed July 8, 2021).
40. Saleh QM, Hensel EC, Robinson RJ. Method for quantifying variation in the resistance of electronic cigarette coils. *Int J Environ Res Public Health*. (2020) 17:7779. doi: 10.3390/ijerph17217779
41. Hensel EC, Jayasekera S, Robinson RJ. Accounting for effects of system dynamics to improve accuracy of emissions reported in e-cig vaping machines. *Inhal Toxicol*. (2018) 30:343–53. doi: 10.1080/08958378.2018.1526232
42. Pagano DT, DiFrancesco G, Smith DSB, George J, Wink G, Rahman DI, et al. Determination of nicotine content and delivery in disposable electronic cigarettes available in the USA by Gas chromatography-mass spectrometry. *Nicotine Tobacco Res*. (2015) 18:700–7. doi: 10.1093/ntr/ntv120
43. Saleh QM, Hensel EC, Eddingsaas NC, Robinson RJ. Effects of manufacturing variation in electronic cigarette coil resistance and initial pod mass on coil lifetime and aerosol generation. *Int J Environ Res Public Health*. (2021) 18:4380. doi: 10.3390/ijerph18084380
44. Al-Delaimy WK, Sim F. Electronic cigarettes and public health: a policy brief. *Int J Epidemiol*. (2021) 50:705–10. doi: 10.1093/ije/dyab017
45. Taylor J, Wiens T, Peterson J, Saravia S, Lunda M, Hanson K, et al. Characteristics of e-cigarette, or vaping, products used by patients with associated lung injury and products seized by law enforcement—Minnesota, 2018 and 2019. *Morb Mortal Wkly Rep*. (2019) 68:1096. doi: 10.15585/mmwr.mm6847e1
46. Hensel EC, Eddingsaas NC, O'Dea S, DiFrancesco AG, Robinson RJ. Framework to report total particulate matter and aldehyde emission characteristics from hookah, podium presentation 4: symposium 15 effects of waterpipe constituents and design on toxicity of waterpipe tobacco smoke and smoking behaviors. In: *Paper Presented at the Society for Research on Nicotine & Tobacco, San Francisco, CA* (2019).
47. Robinson RJ, Hensel EC. Behavior-based yield for electronic cigarette users of different strength e liquids based on natural environment topography. *Inhalation Toxicol*. (2019) 31:8. doi: 10.1080/08958378.2020.1718804
48. Robinson RJ, Hensel EC. A new approach to assess yield delivered to the mouth of ends users based on product emissions and natural environment topography, podium presentation 3: symposium 12 measurement of ENDS exposure. In: *Paper Presented at the Society for Research on Nicotine & Tobacco, San Francisco, CA* (2019).
49. Neilson L, Mankus C, Thorne D, Jackson G, DeBay J, Meredith C. Development of an in vitro cytotoxicity model for aerosol exposure using 3D reconstructed human airway tissue; application for assessment of e-cigarette aerosol. *Toxicol In Vitro*. (2015) 29:1952–62. doi: 10.1016/j.tiv.2015.05.018
50. Goniewicz ML, Knysak J, Gawron M, Kosmider L, Sobczak A, Kurek J, et al. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tob Control*. (2013) 23:133–9. doi: 10.1136/tobaccocontrol-2012-050859
51. Hensel EC, Eddingsaas NC, DiFrancesco AG, Jayasekera S, O'Dea S, Robinson RJ. Framework to estimate total particulate mass and nicotine delivered to E-cig users from natural environment monitoring data. *Sci Rep*. (2019) 9:8752. doi: 10.1038/s41598-019-44983-w

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EVALI Vaping Liquids Part 1: GC-MS Cannabinoids Profiles and Identification of Unnatural THC Isomers

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Tetrahydrocannabinol (THC)-containing products played a major role in the 2019 US nationwide outbreak of pulmonary lung illness associated with e-cigarettes or vaping liquids (EVALI). Due to the severity of the illness which resulted in 68 deaths, a comprehensive identification of the components in the vaping liquids was required. Our laboratory received over 1000 vaping liquid products for analysis including hundreds of vaping products from EVALI patients. In this work, we present the results for the GC-MS identification of the cannabinoids from a large subset of ca. 300 Cannabis-based vaping liquids, with emphasis on the identification of a series of unnatural THC isomers. GC-MS analysis was conducted using a validated, published method in which the cannabinoids were identified as the trimethylsilyl derivatives after separation on a commercial 35% silphenylene phase. Δ^9 -Tetrahydrocannabinol is the naturally occurring THC isomer found in the Cannabis plant, and was found in the majority of the vaping liquids. However, we also identified the presence of one or more additional THC isomers in many of the vaping liquids including Δ^8 -tetrahydrocannabinol, $\Delta^{6a,10a}$ -tetrahydrocannabinol, Δ^{10} -tetrahydrocannabinol, and exo-tetrahydrocannabinol. Significant or major amounts of unnatural THC isomers were found in over 10% of the THC vaping liquids, with lesser amounts found in another 60% of the vaping liquids. Exposure of the Cannabis source materials (such as marijuana concentrates or converted hemp materials) to chemical and thermal treatments during manufacturing, is proposed as the primary cause for the THC isomerizations.

Keywords: EVALI, vaping liquids, e-cigarettes, tetrahydrocannabinol, THC isomers, THC distillates, THC concentrates, GC-MS

INTRODUCTION

In 2019, there was a nationwide outbreak of pulmonary lung illness associated with the use of e-cigarettes or vaping products (EVALI). The outbreak resulted in over 2,800 hospitalizations and 68 deaths (US Centers for Disease Control Office on Smoking and Health National Center for Chronic Disease Prevention and Health Promotion, 2020). After months of investigation, the US Centers for Disease Control (CDC) concluded that tetrahydrocannabinol (THC)-containing products played a major role in the outbreak (Lozier et al., 2019), and that the presence of the additive vitamin E acetate in the products was also strongly linked to the outbreak (Blount et al., 2020). While vitamin E acetate

was strongly implicated in the outbreak, the CDC also concluded that the contribution of other chemicals in either THC or non-THC vaping liquid products could not be ruled out in some EVALI cases (US Centers for Disease Control Office on Smoking and Health National Center for Chronic Disease Prevention and Health Promotion, 2020).

In 2019 and 2020, our laboratory received more than 1000 Cannabis-based vaping products for analysis, including over 500 products from EVALI patients, as well as unused products from various sources. Our laboratory was charged with a comprehensive identification of the components in the vaping liquids which included the cannabinoids, and additives such as cutting agents and diluents. A full array of qualitative analysis was conducted on the vaping liquids including Fourier transform infrared (FT-IR) and Raman spectroscopy, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-high resolution mass spectrometry (LC-HRMS), and direct analysis in real time-high resolution mass spectrometry (DART-HRMS).

GC-MS is ideal for the analysis of vaping liquids because they are largely formulated using volatile or semi-volatile substances. In Part 1 of this work, we report on the GC-MS identification of the cannabinoids in the vaping fluids, with prominence given to the identification of a series of unnatural THC isomers. In Part 2¹, we report on the GC-MS identification of several vaping liquid additives, with confirmation of selected additives using LC-HRMS. The vaping liquids presented in this work represent a large subset (ca. 300) of the Cannabis vaping liquids which were analyzed by our laboratory, and include over 150 vaping liquids from EVALI patients. Given the scope and severity of the EVALI illnesses, this work provides valuable information towards a more complete understanding of vaping liquid compositions, and on analytical approaches for characterizing vaping liquids.

The cannabinoids discussed in this work will include Δ^9 -tetrahydrocannabinol (hereafter “d9THC”), Δ^8 -tetrahydrocannabinol (d8THC), 9R- $\Delta^{6a,10a}$ -tetrahydrocannabinol (9R-d6a,10aTHC), 9S- $\Delta^{6a,10a}$ -tetrahydrocannabinol (9S-d6a,10aTHC), 6aR,9R- Δ^{10} -tetrahydrocannabinol (6aR,9R-d10THC), 6aR,9S- Δ^{10} -tetrahydrocannabinol (6aR,9S-d10THC), exo- Δ^9 -tetrahydrocannabinol (exoTHC), Δ^9 -tetrahydrocannabinolic acid A (THCA), Δ^9 -tetrahydrocannabivarin (d9THCV), Δ^8 -tetrahydrocannabivarin (d8THCV), $\Delta^{6a,10a}$ -tetrahydrocannabivarin (d6a,10aTHCV), cannabidiol (CBD), cannabidivarin (CBDV), cannabinol (CBN), cannabigerol (CBG), and cannabichromene (CBC).

MATERIALS AND METHODS

Standards, Solvents, and Reagents

d9THC, d8THC, exoTHC, THCA, CBD, CBN, CBG, CBC, d9THCV, and CBDV were obtained as certified 1.0 mg/ml stock solutions (acetonitrile or methanol) from Cerilliant Corporation (Round Rock, TX). 9R-d6a,10aTHC, 9S-

d6a,10aTHC, 6aR,9R-d10THC, 6aR,9RS-d10THC, and d8THCV were obtained as 1.0 or 5.0 mg/ml stock solutions (acetonitrile) from Cayman Chemical Company (Ann Arbor, MI, all purities $\geq 95\%$). Olivetol (95%) was obtained from Sigma Aldrich (St. Louis, MO), and a stock solution was prepared in ethanol. For analysis, aliquots (10–200 μ L) of the standard stock solutions were taken for trimethylsilyl (TMS) derivatization in the same manner as the samples (see GC-MS Analysis Section).

Ethanol (200 proof, USP/ACS grade) was obtained from Sigma Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) and pyridine (certified ACS) were obtained from Fisher Scientific (Waltham, MA). BSTFA reagent [99:1 N,O-bis(trimethylsilyl) trifluoroacetamide: trimethylchlorosilane] was obtained from Regis Technologies (Morton Grove, IL). Deionized water (18 Mohm) was obtained from a Millipore filtration system fed by a service deionized water source.

Preparation of Sample Concentrates

Used and unused vaping cartridges were received. For used cartridges, the remaining vaping liquid amounts ranged from residues to almost full cartridges. Full cartridges contained up to 1 g or 1 ml of vaping liquid. Prior to sampling for analysis, the vaping liquid contents were transferred from the cartridges or vaping devices to 2 ml autosampler glass vials (Water Corp.) for storage as follows. The receiving vial was placed in the bottom of a 15 ml conical bottom centrifuge tube (Falcon brand). A 5 ml plastic disposable pipet tip (Rainin RC-L5000) was placed into the receiving vial with the pipet tip end pointed downward. The vaping cartridge or device was disassembled, and the open end was placed into the top end of the pipet tip so as to allow flow of the vaping liquid out of the device through the pipet tip and into the receiving vial. The entire assembly was then placed in a centrifuge and spun until transfer of the vaping liquid into the receiving vial was complete (3–5 min). An IEC clinical centrifuge (dial setting 3) or Thermo Sorvall Legend RT centrifuge (2000 rpm) was used. The amount of vaping liquid recovered from unused cartridges was in the range 0.7–1.0 g, and the amount of vaping liquid recovered from used cartridges was in the range 0.002–0.9 g. Based on visual observation of their flow behaviors, the vaping liquids we encountered typically consisted of medium to high viscosity liquids.

Due to the limited sample amounts for many of the vaping liquids, and the difficulty of sampling viscous liquids without considerable waste, an initial concentrated extract of the vaping liquid (referred to as the “sample concentrate”) was prepared in 95% ethanol. Sample concentrates were prepared in 1.0 ml or 4.0 ml glass sample vials, with vaping liquid sample weights typically in the range 10–100 mg. Solvent volumes were typically in the range 0.5–1.0 ml, resulting in finished sample concentrates generally in the range 20–100 mg vaping liquid per ml. After addition of solvent, the sample vial was capped and then briefly warmed on a hot plate as needed to speed dissolution of the vaping liquid (one or 2 min, $\leq 100^\circ\text{C}$). After dissolution of the vaping liquid, the sample vial was mixed on a vortexer to produce a homogeneous solution. Once prepared, aliquots of the sample concentrate were taken as described below for GC-MS qualitative analysis or HPLC-DAD quantitative analysis of the Cannabis cannabinoids. When sufficient vaping liquid was available,

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duplicate preparations of sample concentrates were made, and analyzed as described.

GC-MS Analysis

GC-MS analysis was conducted using a validated method (Ciolino et al., 2018a). The sample concentrates were mixed on a vortex mixer prior to sampling for GC-MS analysis. A dilution of the sample concentrate was made directly into a GC vial using acetonitrile as the diluent, with sample concentrate aliquot volumes generally in the range 25–100 μ L and a finished volume of ca. 1.0 ml after addition of acetonitrile. A portion (generally in range 50–200 μ L) of the diluted sample was transferred to a GC vial for derivatization. The solvent was evaporated under a stream of dry air on a Pierce Reacti-therm block (nominal block temperature 70–80°C). 200 μ L pyridine and 200 μ L BSTFA reagent were added to the vial, the vial was capped, mixed, and incubated for 30 min (70–80°C). Analysis was carried out using an Agilent 7890B 70 eV EI GC-MS system with 5977B MS detector. The column was a 30 m Restek Rxi-35Sil MS (35% silphenylene) with 0.25 mm internal diameter and 0.25 μ m film thickness. Injection volume was 1 μ L (splitless) with an injection port temperature of 250°C. The carrier gas was helium with a flow rate of 0.8 ml/min (constant flow mode). Oven program was as follows: initial temperature 60°C with 0.5 min hold, first ramp 25°C/min to 220°C, hold for 10 min, second ramp 10 °C/min to 300°C, with a final hold time of 15 min (run time 39.9 min). Transfer line temperature was 280°C. Solvent delay was 7.0 min, and MS acquisition used full scan mode with mass range 40–600 amu.

HPLC-DAD Analysis

HPLC-DAD analysis was conducted using a validated method (Ciolino et al., 2018b). The sample concentrates were mixed on a vortex mixer prior to sampling for HPLC-DAD analysis. Further dilutions of the vaping liquid sample concentrates were made using 95% ethanol either into volumetric flasks, or directly into LC vials to bring the final cannabinoid concentrations into the linear ranges (generally less than 500 μ g/ml). Because most vaping liquids were completely soluble in the 95% ethanol, the preparations were only filtered (using 0.45 micron nylon membrane filters) if there was sufficient volume, or in rare cases of seeing precipitates. Analysis was conducted using Agilent 1100, 1200, or 1260 HPLC-DAD systems. Separations were carried out using MacMod ACE 5 C18-AR analytical columns (5 μ m, 4.6 mm ID x 250 mm length). The mobile phase comprised 66:34 acetonitrile: 0.5% acetic acid (no pH adjustment, nominal pH 2.9). The injection volume was 25 μ L, flow rate 1.0 ml/min, and run time 60 min. Detection wavelength was 240 nm. Chromatographic peak spectra were obtained over the range 190–400 nm.

RESULTS AND DISCUSSION

The commercial production of highly concentrated marijuana extracts or resins intended for use in products such as vaping liquids has been reported (Finley et al., 2016; WHO Expert

Committee on Drug Dependence, 2018a; Finley and McKee, 2019; Ko and Hughes, 2019). Finished concentrates with d9THC purities in the range 80–99% w/w have been described (Finley et al., 2016; Finley and McKee, 2019; Ko and Hughes, 2019). Prior to use in a vaping liquid, the marijuana extracts are typically dewaxed (WHO Expert Committee on Drug Dependence, 2018a; Ko and Hughes, 2019), and may undergo additional processing steps including distillation, and decarboxylation of the acidic cannabinoids (Finley et al., 2016; Finley and McKee, 2019; Ko and Hughes, 2019). The processed marijuana extracts are frequently formulated with additives 1 to produce vaping liquids. Both high d9THC marijuana concentrates and finished vaping liquids are also produced on the black market or in clandestine labs (US Drug Enforcement Administration Diversion Control Division, 2020a; 2020b). Some end users may formulate their own vaping fluids.

The majority of the vaping liquids we analyzed contained substantial levels of d9THC in the liquids, or d9THC represented the predominant cannabinoid in the cannabinoids profile. Using the validated HPLC-UV method (Ciolino et al., 2018b), we determined levels of up to 80% w/w d9THC, and frequently above 50% w/w, in vaping liquids in which no additives were identified (unpublished data). Despite this commonality, we saw a variety of cannabinoids profiles for the vaping liquids, making it challenging to group them into simple categories. The most striking result was the occurrence of many high THC vaping liquids in which unnatural THC isomers were encountered, including d8THC, 9R- or 9S-d6a,10aTHC, 6aR,9R-d10THC, 6aR,9RS-d10THC, and exoTHC (see **Figure 1**, THC isomer structures). Among a tally of 214 high THC vaping liquids, we found 58 vaping liquids with unnatural levels of d8THC, 26 vaping liquids with significant or major levels of the d6a,10aTHC/d10THC isomers, 138 vaping liquids with some or minor levels of the d6a,10aTHC/d10THC isomers, and only 38 vaping liquids which contained d9THC as the only THC isomer.

The vaping liquids also contained other naturally occurring cannabinoids such as CBD, CBN, CBG, and CBC, as well as the varinol cannabinoids. The varinol cannabinoids, such as d9THCV and CBDV, are analogous in structure to the main cannabinoids except that the alkyl side chain on the resorcinol ring is a propyl chain instead of a pentyl chain (Hanuš et al., 2016, see also **Figure 1**, d9THCV structure). As discussed later, the presence of varinol cannabinoids in the vaping liquids argues against a synthetic source for the THC raw materials. Among another tally of 299 vaping liquids, only 18 contained both d9THC and THCA. A few of the vaping liquids contained CBD or CBN as the predominant cannabinoid. The remainder of the work presented here will focus on the details of the THC isomer profiles which were encountered in the vaping liquids.

Vaping Liquid THC Isomer Profiles

In the figures and discussion which follow, we have organized the vaping liquid examples according to the one or two most predominant cannabinoids or other distinctive features. All GC-MS chromatograms are for the “derivatized sample preparations”, in which the cannabinoids have been converted

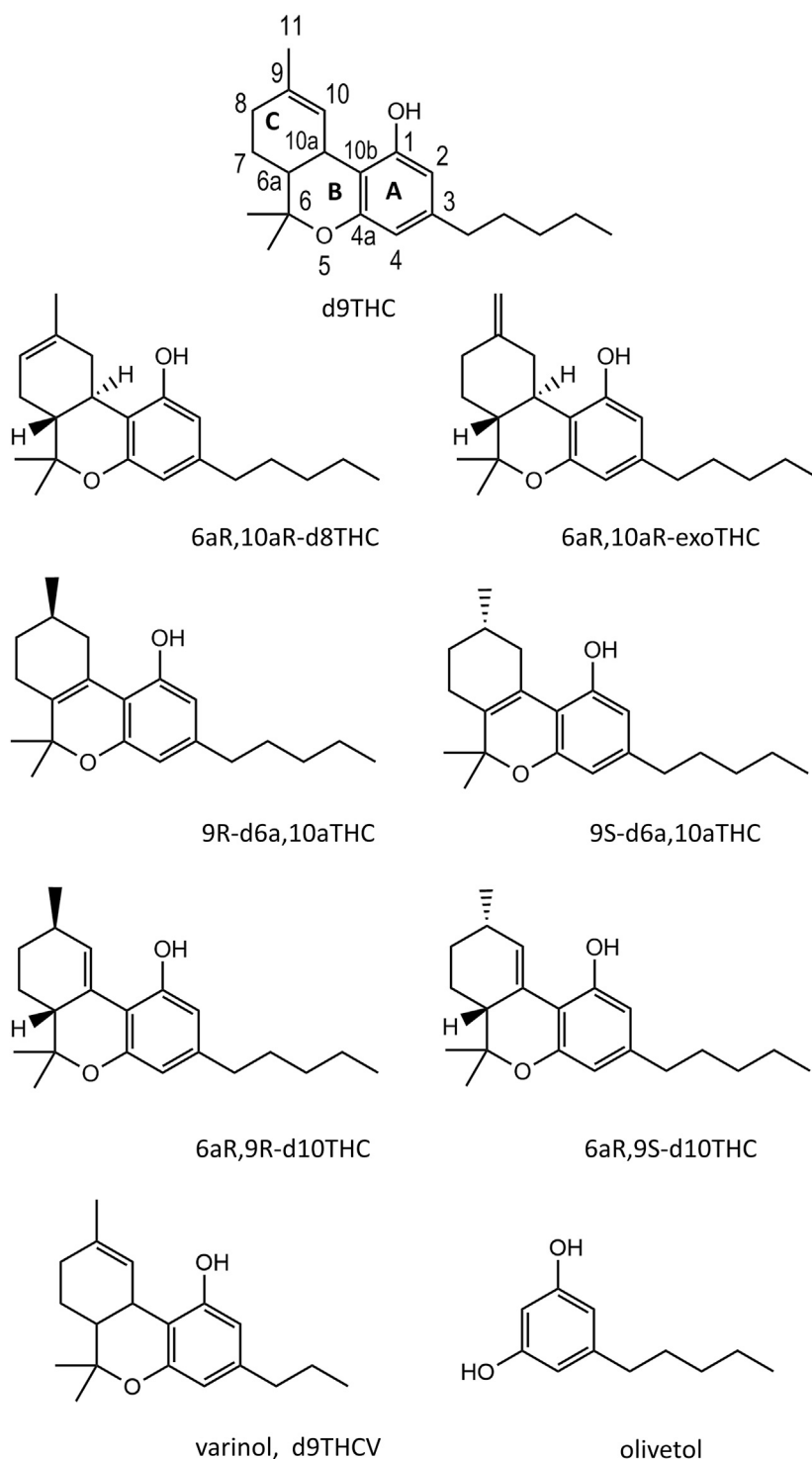


FIGURE 1 | Chemical structures for seven THC isomers, a representative varinol cannabinoid (d9THCV, lowest left structure), and olivetol (lowest right structure).

to their fully trimethylsilylated derivatives (d9THC monoTMS, CBD diTMS, THCA diTMS, etc.). However, for purposes of simplicity in the discussion, the cannabinoids will be referred to as the parent compounds. At the time of the EVALI crisis, our

validated HPLC-DAD method for cannabinoids quantitation addressed a total of 13 cannabinoids which included only two of the THC isomers, d9THC and d8THC. HPLC-DAD retention times were subsequently established for the exoTHC, d6a,

TABLE 1 | GC-MS peak area percentages (PAPs) for cannabinoids and olivetol in vaping liquids and bulk THC distillates.

Vaping Liquids	d9THC	d8THC	d6a,10a THC	6aR,9R-d10THC	6aR,9S-d10THC	exoTHC	CBN	CBD	Olivetol ^a	Amount Recov.(g) ^b	Additives ^c
VL#1	89	ND	ND	ND	ND	ND	7.0	3.8	ND	0.26	none ID
VL#2	94	ND	ND	ND	ND	ND	6.0	0.28	ND	0.028	none ID
VL#3	44	36	ND	ND	ND	trace	9.8	10	2.2	0.22	SAIB
VL#4	17	69	ND	ND	ND	1.0	4.1	9.6	6.6	0.044	MCT/SAIB
VL#5	16	66	ND	ND	ND	0.79	3.3	14	3.7	0.38	VEA
VL#6	66	11	2.5	0.83	0.39	ND	6.5	13	0.29	0.76	none ID
VL#7	66	16	7.2	0.97	0.58	ND	9.5	0.67	ND	0.42	none ID
VL#8	12	64	8.5	2.1	1.2	3.4	9.1	0.19	0.13	0.067	none ID
VL#9	9.5	69	9.4	1.6	0.98	2.6	7.1	ND	0.33	0.33	VEA
VL#10	6.1	72	13	2.0	1.1	2.2	3.8	ND	0.24	0.91	PEG
VL#11	1.4	79	7.8	1.3	0.75	2.9	7.0	ND	0.23	0.19	MCT/PEG
VL#12	33	1.4	38	10	4.7	ND	9.6	3.2	0.11	0.75	VEA/MCT
VL#13	51	3.0	21	11	4.6	ND	8.7	0.89	0.041	0.86	VEA
VL#14	43	0.4	33	7.51	2.7	ND	13	0.31	0.020	0.014	MCT/PEG
Distillates											
DST#1	76	0.68	9.6	6.2	2.1	ND	3.6	1.8	trace	NA	NA
DST#2	7.8	60	20	3.2	1.5	2.0	4.8	0.19	0.17	NA	NA

^aOlivetol peak areas not included in peak area percentage calculations for cannabinoids.

^bWeight of vaping liquid recovered from vaping cartridge or device.

^cAdditives identified in vaping liquids: SAIB-sucrose acetate isobutyrate; MCT-medium chain triglycerides oil; VEA-vitamin E acetate; PEG-polyethylene glycol; none ID-no additives identified per protocol (see Part 2).

ND-not detected; NA-not applicable.

10aTHC and d10THC isomers, and showed that these THC isomers did not coelute with either d9THC or d8THC. However, the d6a, 10aTHC and d10THC isomers were only sufficiently resolved from one another to allow for a qualitative assessment, and not for strict quantitation. Moreover, only qualitative not quantitative reference standards for the d6a, 10aTHC and d10THC isomers were commercially available. HPLC-DAD quantitation was also not conducted for exoTHC, as it was either not detected, or found in only minor amounts. Hence, in this work, quantitative results are provided only for d9THC, d8THC, and CBD. However, as will be demonstrated, the GC-MS peak area percentages (PAPs) for the THC isomers provide reasonable estimates of the relative amounts of all the THC isomers in the vaping liquids. We analyzed vaping liquids from both unused and used vaping product cartridges. Many labels for vaping cartridges declare fill weights or volumes of 1 g or 1 ml. In our work with emptying and weighing the vaping liquid contents from previously unused vaping cartridges, we obtained weights in the range 0.7–1 g. For the used cartridges, the remaining amount of vaping liquid recovered by our laboratory ranged from residues (less than 10 mg) to near the nominal fill weights. For each example we note the amount of vaping liquid recovered from the cartridge (see **Table 1**, second last column).

Figure 1 shows the structures for seven THC isomer reference standards which were used in the analysis of the vaping liquids. The structure for d9THC shows the ring letter and numbering scheme. The THC isomers vary both with respect to the position of the double bond in the terpenoid ring (C ring), and the stereoisomeric forms. ExoTHC ($\Delta^{9,11}$ -tetrahydrocannabinol) is the exception with the double bond being present outside the terpenoid ring. The diastereomeric form for the d9THC standard

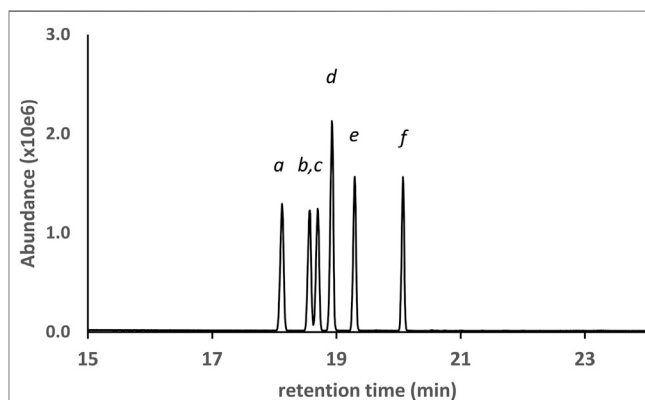


FIGURE 2 | GC-MS chromatogram for a 25 µg/ml standard mix of seven positional and stereoisomeric THC monoTMS isomers. Peak labels: 6aR,10aR-d8THC(a); 6aR,10aR-exoTHC(b); 6aR,10aR-d9THC(c); coeluted 9R- and 9S- d6a,10aTHC(d); 6aR,9S-d10THC(e); 6aR,9R-d10THC(f). See text for discussion.

is not depicted in the figure but was the 6aR,10aR-d9THC isomer, which corresponds to the natural form found in the Cannabis plant (Hanuš et al., 2016). The d8THC and exoTHC reference standards were also the 6aR, 10aR isomers. Standards of both enantiomers of d6a, 10aTHC (9R and 9S), and two diastereomers of d10THC (6aR, 9R and 6aR,9S), were used.

Figure 2 shows the GC-MS chromatogram for a 25 µg/ml standard mix of the monoTMS derivatives of the seven THC isomers. All of the positional isomers were resolved, as was the diastereomeric pair 6aR,9R- and 6aR,9S-d10THC. The only unresolved isomers were the enantiomeric pair 9R- and 9S-d6a,10aTHC, which showed complete coelution (peak label d).

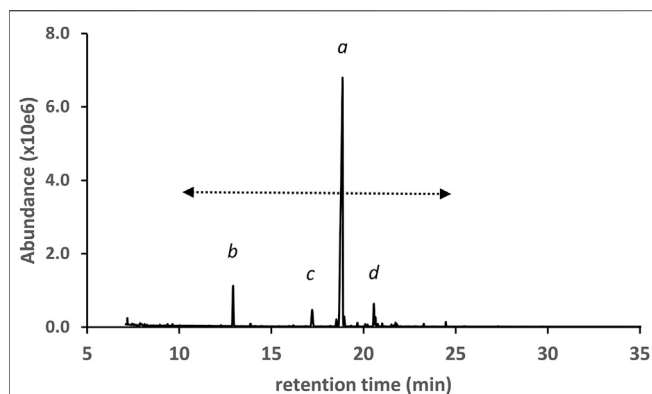


FIGURE 3 | GC-MS chromatogram for a cannabis vaping liquid in which the predominant cannabinoid was d9THC(a), and other cannabinoids include CBD, CBG, and CBN (peak labels b, c, and d, respectively). The cannabinoid TMS derivatives retention range (ca. 10–25 min) is indicated by the double arrow.

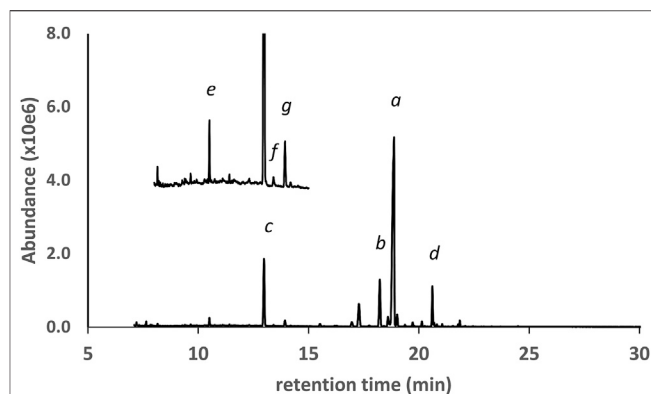


FIGURE 5 | GC-MS chromatogram for a cannabis vaping liquid in which the predominant cannabinoid was d9THC(a), and with similar levels of CBD(c) and d8THC(b). Minor levels of other THC isomers were identified (*not labeled*). CBN(d) and the varinols (expanded scale and offset): CBDV(e), d8THCV(f), d9THCV(g).

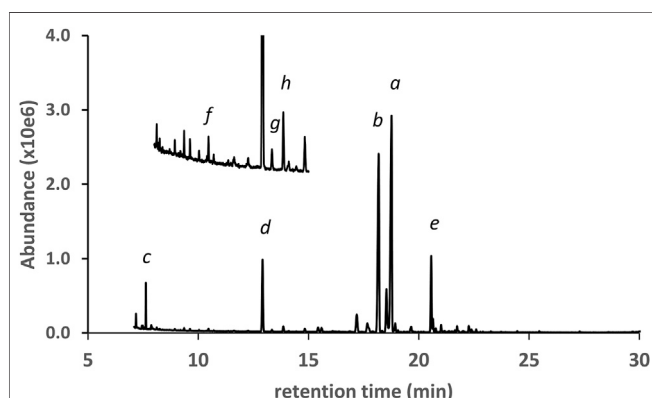


FIGURE 4 | GC-MS chromatogram for a cannabis vaping liquid in which the predominant cannabinoids were d9THC(a) and d8THC(b), with lower levels of CBD(d) and CBN(e). A minor level of exoTHC (*not labeled*, peak between peaks a and b), and an elevated level of olivetol(c), were observed. Three corresponding varinols were identified (expanded scale and offset): CBDV(f), d8THCV(g), d9THCV(h).

These results are typical for GC-MS analysis conducted under achiral conditions. Since the 9R- and 9S- enantiomers of d6a, 10aTHC were not resolved, further discussion will only refer to “d6a,10aTHC” for this THC isomer.

Figure 3 shows the GC-MS chromatogram for a high d9THC potency vaping liquid (VL#1, **Table 1**) in which the predominant cannabinoid was d9THC (peak label a), and no additives were detected. The d9THC level in the vaping liquid was determined at 78% w/w (HPLC-DAD). Other cannabinoids identified include CBD, CBG, and CBN (peak labels b, c, and d respectively), and very low levels of d9THCV and CBC (peaks not visible on current scale). No d8THC or other THC isomers were identified in this vaping liquid. The figure also shows the cannabinoids retention range (10–25 min) for the current method as indicated by the double arrow (<---->). While the presence of any of the minor

cannabinoids may indicate a natural Cannabis source, the presence of the varinol d9THCV in the vaping liquid is taken as stronger evidence for a plant source. The varinol series of cannabinoids are frequently found at much lower levels in Cannabis plants relative to the main cannabinoids (Hillig and Mahlberg, 2004). The presence of the varinol d9THCV, which contains a propyl side chain on the resorcinol ring (**Figure 1**, lowest left structure), would not be expected in any d9THC synthetic schemes, as d9THC contains a pentyl side chain in this position (**Figure 1**, highest center structure).

Figure 4 shows the GC-MS chromatogram for a vaping liquid (VL#3, **Table 1**) in which the predominant cannabinoids were d9THC and d8THC (peak labels a and b, respectively). The levels of d9THC, d8THC, and CBD (peak label d) were determined (HPLC-DAD, %w/w) as 17, 11, and 2.2%, respectively. A minor amount of exoTHC and an elevated level of olivetol (peak label c, see also **Figure 1**, lowest right structure) were observed. The olivetol level was considered elevated based our prior experience with THC vaping liquids in which we have not detected olivetol, or only observed trace amounts. Low levels of the varinols CBDV, d8THCV, and d9THCV (peak labels f, g, and h, respectively) were also found. Again, the presence of the varinols in this vaping liquid is evidence for a natural plant source with isomerization of d9THCV to d8THCV occurring in parallel to the isomerization of d9THC to d8THC. However, the high level of d8THC is unnatural and cannot be attributed to typical processing methods for Cannabis plants. Only minor levels of d8THC have been reported in some historical studies of processed hashish or marijuana materials, with the d8THC representing one percent or less of the combined d9THC and d8THC amounts (Mechoulam, 1970). It is unclear whether minor levels of d8THC are present in unharvested Cannabis plants or if d8THC forms during plant processing steps such as the extraction or isolation of other cannabinoids. Chemical mechanisms for the conversion of either d9THC or CBD into d8THC during isolation procedures have been proposed (Hanuš et al., 2016).

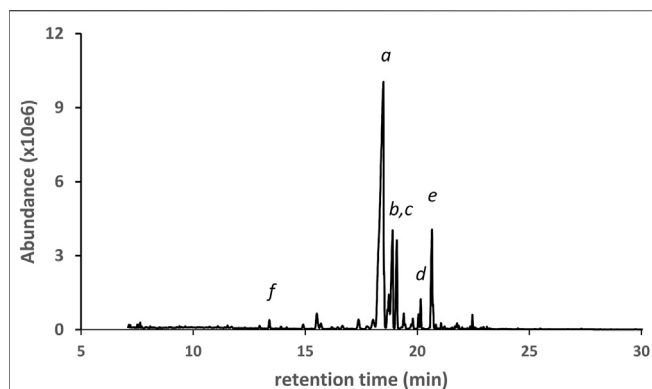


FIGURE 6 | GC-MS chromatogram for a cannabis vaping liquid in which the predominant cannabinoid was d8THC(a). d9THC(b), d6a,10aTHC(c), 6aR,9R-d10THC(d), 6aR,9S-d10THC(not labeled), and exoTHC(not labeled) were also found. Other cannabinoids: CBN(e) and d8THCV(f).

Duffy et al. (2020) also reported finding unnatural amounts of d8THC in vaping liquids associated with the EVALI outbreak. The unnatural level of d8THC shows that the Cannabis material was most likely subjected to chemical and/or thermal treatments to cause substantial conversion to d8THC. Long time Cannabis researcher Mechoulam reported an early procedure for the isomerization of d9THC to d8THC which was conducted in the presence of a strong organic acid such as p-toluenesulfonic acid (Mechoulam, 1970). Later, a group which also included Mechoulam, patented processes for conversion of CBD to either d8THC or d9THC (Webster et al., 2008). In the patented processes, conversion to d8THC was conducted using p-toluenesulfonic acid catalyst with a yield of 81% and high purity, and conversion to d9THC was conducted using boron trifluoride diethyl etherate with a yield of 57% and high purity (Webster et al., 2008).

The next three examples represent vaping liquids in which d9THC, d8THC, and several other THC isomers were found, but with different patterns for the overall profiles of the THC isomers. **Figure 5** shows the GC-MS chromatogram for a vaping liquid (VL#6, **Table 1**) in which the predominant THC isomer was d9THC (peak label a) and d8THC (peak label b) was second most predominant. The levels of d9THC, d8THC, and CBD (peak label c) were determined (HPLC-DAD, %w/w) as 49, 5.4, and 5.2% respectively. Minor levels of three other THC isomers (not labeled in figure) were also identified as follows: d6a,10aTHC, 6aR,9R-d10THC, and 6aR,9S-d10THC. Low levels of the varinols CBDV, d8THCV, and d9THCV (expanded scale and offset, peak labels e, f, and g, respectively), were also found. **Figure 6** shows the GC-MS chromatogram for a vaping liquid (VL#8, **Table 1**) in which the predominant cannabinoid was d8THC (peak label a), and substantial levels of both d9THC (peak label b) and d6a, 10aTHC (peak label c) were also observed. The levels of d8THC and d9THC were determined (HPLC-DAD, %w/w) as 51 and 9.8% respectively. Minor levels of other THC isomers were identified as follows: 6aR,9R-d10THC (peak label d), 6aR,9S-d10THC (not labeled in figure), and exoTHC (not labeled in figure).

Figure 7 shows the GC-MS chromatogram for a vaping liquid (VL#12, **Table 1**) in which the predominant THC isomers were d9THC (peak label a) and d6a, 10aTHC (peak label b). A low level of d8THC (peak label d), but marked levels of both 6aR,9S-d10THC (peak label e) and 6aR,9R-d10THC (peak label f), were also found. The level of d9THC was determined as 11% w/w, but the level of d8THC could not be determined due to a coeluting interferent. Interestingly, both varinols d9THCV and d6a, 10aTHCV were detected (expanded scale and offset, peak labels h and i, respectively). Parallel conversion of d9THCV to d6a, 10aTHCV would be expected to occur in whatever process caused the conversion of d9THC to d6a,10aTHC. No standard of d6a, 10aTHCV was available for comparison. The identification of d6a, 10aTHCV was based on comparison of its mass spectra with the standard mass spectra for d6a, 10aTHC (**Figure 8**, comparison of both parent compounds and monoTMS derivatives). The mass spectra for d6a, 10aTHC are unique among all of the THC isomers with fewer high mass ions and overall much less fragmentation both for the parent compound (unit mass 314) and monoTMS derivative (unit mass 386, see **Figures 8A,C**). The corresponding spectra for d6a, 10aTHCV show the analogous patterns, both for the parent compound (unit mass 286) and monoTMS derivative (unit mass 358, see **Figures 8B,D**). The elution of d6a, 10aTHCV just after d9THCV also mirrored the elution order for d6a, 10aTHC and d9THC (**Figure 7**).

The vaping liquids shown in **Figures 3-7** represent several different patterns of THC isomer/cannabinoid compositions we encountered. These patterns are evident in **Table 1**, in which the peak area percentages (PAPs) of selected cannabinoids from the GC-MS chromatograms are summarized. The PAPs represent a good estimate of the relative amounts of the cannabinoids in a given vaping liquid. The selected cannabinoids include all the THC isomers, CBD, and CBN. Both CBD and CBN were included in the peak area percentage (PAP) calculations because they represent possible precursor or degradation products for the THC isomers. CBD amounts may also represent the degree of THC enrichment in the source materials. PAPs for a total of 14 vaping liquids ("VL#") are listed in the table. Olivitol PAPs are also listed in the table (see last column); however, the olivitol peak areas were not included in the listed PAP calculations for the THC isomers, CBD, and CBN.

VL#1 and VL#2 are vaping liquids in which the only THC isomer detected was d9THC, and d9THC is also the predominant cannabinoid (PAPs near 90%). VL#3 - 5 are vaping liquids in which both d9THC and d8THC are the two predominant THC isomers, with the sum of the PAPs for d9THC and d8THC in the range 80–86%. Although the relative amounts of d9THC and d8THC vary in this group of vaping liquids, they show the same pattern with respect to the other THC isomers. Minor amounts (up to 1.0% PAP) of exoTHC were found. No d6a,10aTHC, nor either of the d10THC stereoisomers, were detected in these vaping liquids. The THC isomer profiles for VL#3 - 5 are similar to the isomer profiles disclosed in patented processes (Grondin and Ward., 2021a and 2021b) for the conversion of either CBD or d9THCA to mixtures of d9THC and d8THC. These processes use heat, and a concentrated or dilute

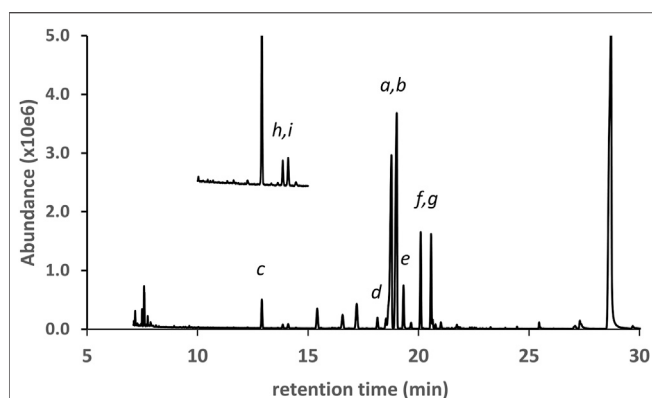


FIGURE 7 | GC-MS chromatogram for a cannabis vaping liquid in which the predominant THC isomers were d9THC(a) and d6a,10aTHC(b). Additional THC isomers were identified as follows: d8THC(d), 6aR,9S-d10THC(e), and 6aR,9R-d10THC(f). Other cannabinoids include CBD(c), CBN(g), and the varinols (expanded scale, offset): d9THCV(h) and d6a,10aTHCV(i).

hydrochloric acid catalyst, to effect the cannabinoid conversions on various source materials, which may include isolates, distillates, concentrates, plant extracts, or synthetic sources. The finished products show widely varying ratios of d9THC and d8THC, with minor amounts of exoTHC (Grondin and Ward, 2021b). One further note is that VL#3–5 all show the same trend of elevated olivetol levels, with olivetol PAPs (Table 1, third last column) substantially above those for all the other groupings of vaping liquids.

VL#6 and VL#7 are vaping liquids in which d9THC is the predominant cannabinoid (d9THC PAP 66%), with d8THC as the next most prominent (PAPs 11–16%). In these vaping liquids, minor amounts (up to 7% PAP) of the d6a, 10aTHC isomer, and both d10THC stereoisomers, were found. ExoTHC was not detected. VL#8–11 are vaping liquids in which d8THC is the predominant cannabinoid (d8THC PAPs in range 64–79%). In this group of vaping liquids, the next most prominent THC isomers are d9THC and d6a, 10aTHC (combined PAPs in range 9–21%). Minor amounts of both d10THC stereoisomers, and exoTHC were also found. Although the exoTHC isomer was present in minor amounts, this grouping of vaping liquids showed the highest levels of exoTHC. ExoTHC is known to be an impurity associated with dronabinol, a synthetic d9THC (United States Pharmacopoeia, 2008), but is also regarded as an impurity that may be found in Cannabis plant THC isolates (Cid and Van Houten, 2015). This is also the only grouping in which all six of the THC isomers listed in Table 1 were found.

VL#12–14 are vaping liquids in which d9THC and the d6a, 10aTHC are the two predominant THC isomers (combined PAPs all above 70%), with varying ratios of d9THC to d6a,10aTHC. These vaping liquids have the highest amounts of the two d10THC stereoisomers (sum of PAPs in range 10–16%) compared to the other vaping liquid groupings, and also have the lowest amount of d8THC (all PAPs less than 3%) relative to VL#3–11. ExoTHC was not detected. The cannabinoids profiles for VL#12–14 are similar to the profiles disclosed in a US patent (Siegel et al., 2021) for the conversion of d9THC to d10THC, d6a,10aTHC, and CBN. The conversion processes use intact plant

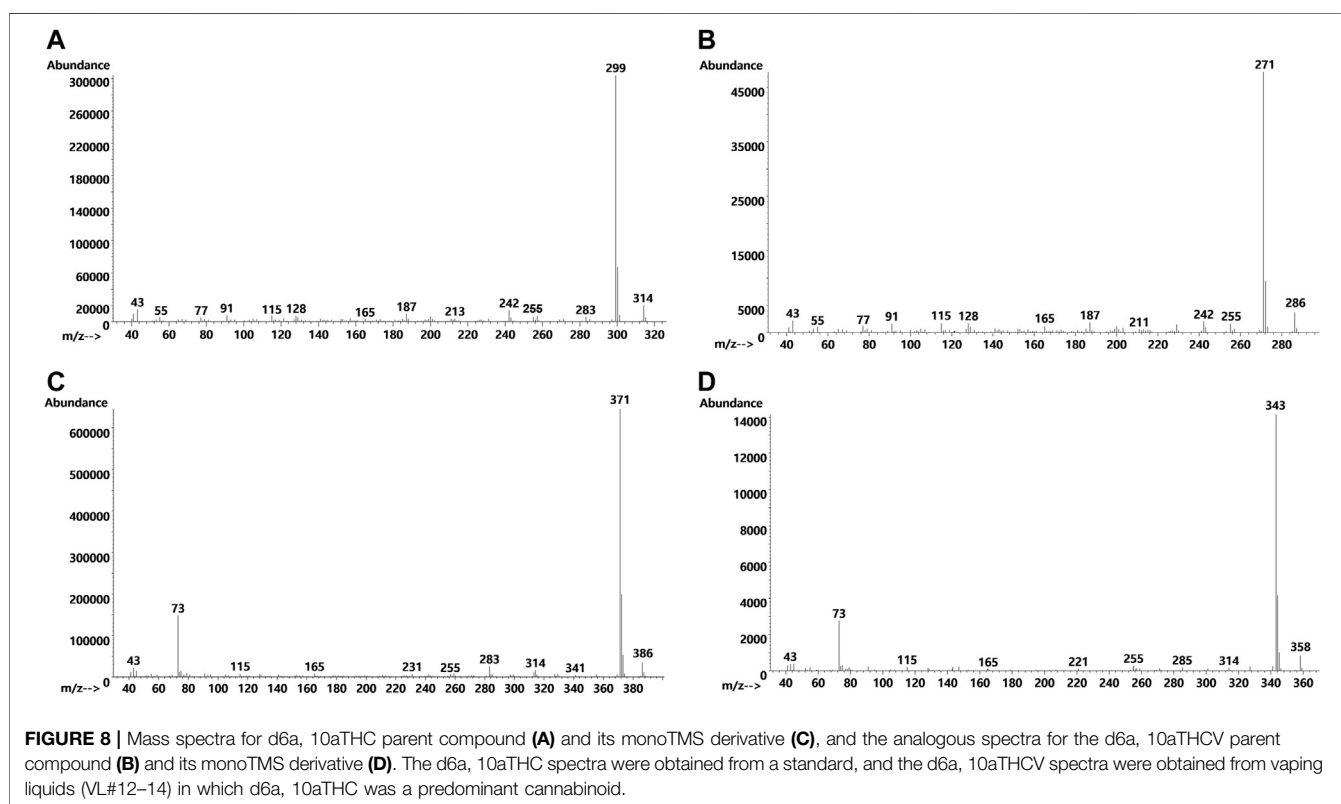


FIGURE 8 | Mass spectra for d6a, 10aTHC parent compound (A) and its monoTMS derivative (C), and the analogous spectra for the d6a, 10aTHCV parent compound (B) and its monoTMS derivative (D). The d6a, 10aTHC spectra were obtained from a standard, and the d6a, 10aTHCV spectra were obtained from vaping liquids (VL#12–14) in which d6a, 10aTHC was a predominant cannabinoid.

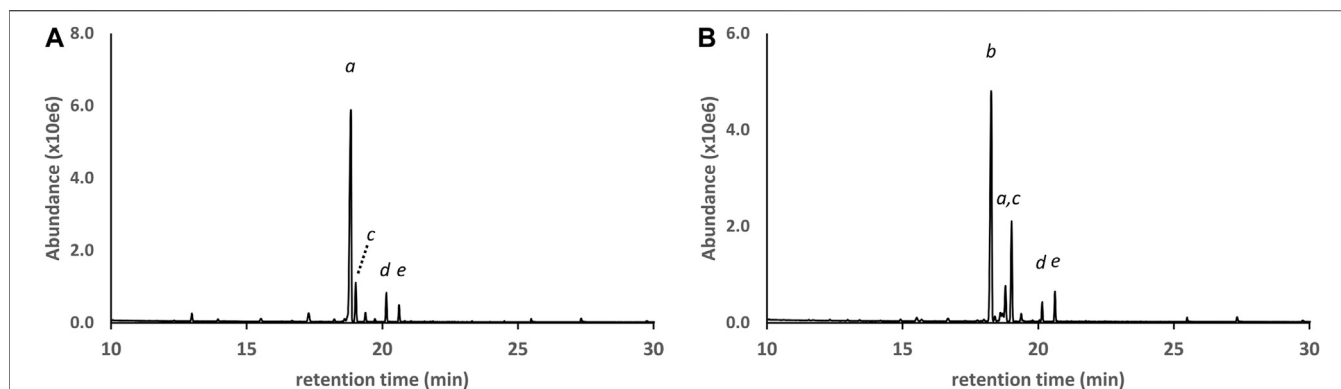


FIGURE 9 | GC-MS chromatograms for two different bulk THC distillates in which the primary cannabinoid was either d9THC (**A**) or d8THC (**B**). Peak labels d9THC(a); d8THC(b); d6a,10aTHC(c); 6aR,9R-d10THC(d); CBN (e). Lower levels of additional THC isomers (not labeled) were also identified in both distillates. See text for discussion.

materials, THC-sparse oils, or THC-rich oils as starting materials. The reaction conditions include heat and a Lewis acid catalyst such as elemental sulfur. The inventors provide a series of examples in which the finished reaction mixtures contain varying amounts of d9THC, d10THC, d6a,10aTHC, and CBN (Siegel et al., 2021). They further claim to be able to tailor the conditions to maximize or minimize residual d9THC content, and to affect the finished yield ratios of d10THC, d6a,10aTHC, and CBN. As for VL#12–14, the cited examples in the patent contain d9THC, d10THC, and d6a, 10aTHC as the three most predominant THC isomers. Interestingly, d8THC is not reported as present in the finished reaction mixtures, except for one example in which the relative level of d8THC was reported as 0.7% (Siegel et al., 2021). This is also similar to the relative levels of d8THC in VL#12–14, which ranged from 0.4–3.0% (Table 1, PAP results).

The second to last column in Table 1 shows the amount of vaping liquid recovered from each of the 14 vaping devices. Inspection of these results shows that the presence of the THC isomers, and the trends in THC isomer profiles, were not obviously correlated with the extent of vaping liquid consumed, and hence the thermal exposure of the liquid during the vaping process. For example, VL#2 represents a vaping liquid in which over 95% of the cartridge contents were consumed, yet no unnatural THC isomers were detected. The trends for both the VL#8–11, and VL#12–14 groupings, each include vaping liquids which came from unused or minimally used cartridges, as well as almost empty cartridges. This is not to conclude that the vaping process and exposure of the vaping liquid to the vaporizer heating elements cannot alter the vaping liquid composition. It is important to note that the vaping products in this work represent several different types of vaping devices. Different vaping devices may alter vaping liquid compositions to varying degrees, and this aspect is not studied here. The last column in Table 1 shows the additives identified in the vaping liquids according to the protocol described in Part 2¹. Again, there is no obvious correlation between the THC isomer profiles and the additive types.

As discussed above, the THC isomer profiles for the VL#3 – 5 grouping show similarities to the reported isomer profiles for THC source materials produced by one particular commercial process (Grondin and Ward, 2021a and 2021b). In addition, the THC isomer profiles for the VL#12–14 grouping are similar to the reported isomer profiles of THC source materials produced by yet another distinct commercial process (Siegel et al., 2021). The trends in THC isomer profiles suggest that the vaping liquids were likely formulated with THC source materials which already contained unnatural THC isomers. In the course of our work with the vaping liquids, we received two different bulk THC distillates (“DST#1” and “DST#2”). The THC distillates represent examples of high THC source materials prior to incorporation into a vaping liquid composition, and thus provide data on THC isomer profiles associated with the manufacturing process. The specifics of the processes used to manufacture these distillates are not known.

GC-MS analysis for DST#1 (Figure 9A and Table 1) showed that the primary cannabinoid was d9THC, with all of the other THC isomers present except for exoTHC. The d9THC level in DST#1 was 60% w/w (HPLC-DAD), but the level of d8THC could not be determined due to a coeluting interferent. The THC isomer profile for DST#1 resembles the profiles for the grouping of vaping liquids VL#12–14, and the Siegel process (Siegel et al., 2021). GC-MS analysis for DST#2 (Figure 9B and Table 1) showed that the primary cannabinoid was d8THC, with all of the other THC isomers present. The d8THC and d9THC levels in DST#2 were 53 and 6.2% w/w, respectively (HPLC-DAD). The THC isomer profile for DST#2 resembles the profiles for the grouping of vaping liquids VL#8–11. The results for these THC distillates confirm that some manufacturing processes produce unnatural THC isomers at significant levels. The similarities between the THC distillates and the vaping liquids provides further evidence that the vaping liquids were likely formulated with Cannabis source materials which already contained unnatural THC isomers, whether or not further changes in composition occurred during the vaping process.

Additional distinct commercial scale processes have been reported for the production of THC source materials which

contain unnatural THC isomers. Tegen and Cho have reported the use of p-toluenesulfonic acid catalyst to convert CBD to mixtures of d8THC and d9THC, or convert CBD to mainly d8THC or mainly d9THC (Tegen and Cho., 2019a; 2019b). The conversion processes may be conducted starting with pure CBD or hemp extracts. The inventors provided examples with crude product relative amounts of 1.1% CBD, 18.4% d8THC, 80.0% d9THC, or 96.5% d8THC, 2.5% d9THC, from pure CBD starting materials. A third example had crude product relative amounts of 2% d8THC and 98% d9THC from hemp extract starting materials.

A recent world patent application (Hartman, 2020) reports on commercial processes for the conversion of either CBD or d9THC to various combinations of d8THC, d10THC, and cannabinol. Cited starting materials include hemp isolates, marijuana isolates, CBD isolates, and hexane or butane extracts of hemp or marijuana. The isolates are obtained via CO₂ supercritical fluid extraction. Conversions are effected using heat, and may include halogen or free radical generator catalysts such as iodine. Specified reaction temperatures can range from 55–800°C. The disclosed reaction scheme shows conversion of CBD to d9THC, followed by conversion of d9THC to d8THC, d10THC, and cannabinol. Relative conversion yields of CBD to d8THC in the range 50–99% are claimed, but no conversion yields for d10THC were cited. The parallel conversions of some varinol cannabinoids such as conversion of CBDV to d9THCV, with subsequent conversion of d9THCV to d8THCV, are also disclosed.

For all of the vaping liquids and THC distillates, a similar pattern with respect to the relative isomer amounts of d6a,10aTHC, 6aR,9R-d10THC, and 6aR,9S-d10THC was observed. The amount of d6a, 10aTHC was always higher

than either of the two d10THC isomers, and the amount of the 6aR,9R-d10THC isomer was always higher than the amount of the 6aR,9S-d10THC isomer. These results suggest preferential formation within this set of isomers. The formation of 6aR,9R-d10THC and 6aR,9S-d10THC from d9THC and/or d8THC under base-catalyzed conditions was reported by Srebnik et al. (1984), with the 6aR,9R-d10THC isomer being thermodynamically favored. Further isomerization of the d10THC isomers to the d6a, 10aTHC isomers was carried out under acid-catalyzed conditions (Srebnik et al., 1984), with 6aR,9R-d10THC forming the 9R-d6a, 10aTHC enantiomer and 6aR,9S-d10THC forming the 9S-d6a, 10aTHC enantiomer.

It is also of note that olivetol, which represents a structural piece (see **Figure 1**, lowest right structure) of the THC molecule, was only detected in vaping liquids and THC distillates in which unnatural THC isomers were also present. Olivetolic acid (the carboxylated form of olivetol), is known to be a key compound in the plant biosynthesis of the cannabinoids (Hanuš et al., 2016), and olivetol is used as a starting material in the classical synthesis of d9THC (Bloemendal et al., 2020). The significance of the olivetol finding in the vaping liquids is not clear. However, the olivetol peak area percentages appear to vary among the different vaping liquid groupings, but are rather consistent within each vaping liquid grouping (**Table 1**). This suggests some relation to the THC source materials, such as varying degrees of concentration of the plant constituents during purification steps, or even varying degrees of degradation of the cannabinoids among distinct chemical and/or thermal treatments. However, we could find no relevant literature to address this question.

It is not our intention to present the GC-MS peak area percentage (PAP) results for the THC isomers in the vaping liquids and distillates (**Table 1**) as strict quantitative values.

TABLE 2 | Comparison of GC-MS peak area percentages (PAP) and HPLC-DAD quantitative analysis (%w/w) for d9THC and d8THC in vaping liquids and bulk THC distillates.

Vaping Liquids	GC-MS analysis			HPLC-DAD analysis		
	d9THC PAP	d8THC PAP	PAP ratio d9THC:d8THC	d9THC %w/w	d8THC %w/w	%w/w ratio d9THC:d8THC
VL#1	89.2	ND	NA	78.2	ND	NA
VL#2	93.7	ND	NA	77.1	ND	NA
VL#3	44.2	35.5	1.2	17.4	10.5	1.6
VL#4	16.9	69.2	0.24	5.40	16.9	0.32
VL#5	16.2	66.3	0.24	7.41	21.1	0.35
VL#6	65.8	10.4	6.3	48.8	5.36	9.1
VL#7	65.5	15.6	4.2	44.7	7.78	5.7
VL#8	11.8	63.8	0.18	9.80	51.2	0.19
VL#9	9.49	68.8	0.14	5.35	52.4	0.10
VL#10	6.06	72.0	0.084	3.77	35.2	0.11
VL#11	1.41	79.0	0.018	0.993	58.6	0.017
VL#12	33.0	1.42	23	10.9	^a	^a
VL#13	51.1	2.96	17	19.9	0.88	23
VL#14	43.1	0.439	98	11.5	^a	^a
DISTILLATES						
DST#1	76.0	0.683	111	60.4	^a	^a
DST#2	7.75	60.4	0.13	6.19	53.1	0.12

^aThe d8THC levels in these items was observed to be low in the HPLC-DAD analysis, but accurate quantitation was not possible due to a coeluting interferent.
ND-not detected; NA-not applicable.

Rather, the GC-MS PAP results are considered reasonable estimates of the relative amounts of the THC isomers, and are supported by the HPLC-DAD analysis. For reasons stated earlier, HPLC-DAD quantitative results were only obtained for the d9THC and d8THC isomers. **Table 2** shows a comparison of GC-MS PAPs and HPLC-DAD quantitative analysis (%w/w) for d9THC and d8THC in the vaping liquids and distillates from **Table 1**. To allow for a more direct comparison of the GC-MS and HPLC-DAD results, the ratios of d9THC:d8THC were calculated for both the GC-MS PAP data, and the HPLC-DAD % w/w data (**Table 2**, columns 4 and 7, respectively). Inspection of the d9THC:d8THC ratio data columns shows a strong correlation between the GC-MS and HPLC-DAD results. The d9THC:d8THC PAP ratios agree with the d9THC:d8THC % w/w ratios by factors which range from 0.7–1.4 (factors calculated as the ratio of the PAP and %w/w ratios). Although quantitation was not obtained for the other THC isomers identified in the GC-MS analysis, their presence was confirmed in the HPLC-DAD analysis of the vaping liquids and distillates by retention time and UV spectral matches with the qualitative reference standards; this applies to the exoTHC, d10THC, and d6a, 10aTHC isomers.

CONCLUDING REMARKS

While the grouping of vaping liquids presented in this work is somewhat subjective, this work provides strong evidence that different THC isomer patterns observed in the products represent different processes to produce high THC source materials. The vaping liquids reported in this work were obtained from multiple locations throughout the US, showing that the occurrence of unnatural THC isomers in these products was widespread. Given that these isomers are likely already present in high THC source materials, it is to be expected that unnatural THC isomers will be encountered in other THC containing products. We recently encountered some THC candies in which both d9THC and d6a, 10aTHC were predominant cannabinoids, and significant

amounts of d8THC and both d10THC stereoisomers were found (unpublished data). The presence of unnatural THC isomers in Cannabis products raises questions with respect to both their legality and potential safety concerns. All of the THC positional isomers and their stereochemical variants are listed as Schedule 1 in the 1971 Convention on Psychotropic Substances (WHO Expert Committee on Drug Dependence, 2018b), and by the US DEA (US Drug Enforcement Administration (DEA) Department of Justice, 2020c). Many of the state laws in the US only address the d9THC isomer, leaving much ambiguity. With regard to the pharmacological effects and safety of the other THC isomers, only the d8THC isomer has been studied to some extent (Hollister and Gillespie 1973; Thomas et al., 1990; Punyamurthula et al., 2017; Thapa et al., 2018), and we are not aware of any published reports for the other THC isomers.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data may be considered sensitive or require a special request for public disclosure. Requests to access the datasets should be directed to laura.ciolino@fda.hhs.gov.

AUTHOR CONTRIBUTIONS

LC and TR performed conception and design of the study. All authors conducted research and analysis of the subject vaping liquids and cannabinoids. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

- Bloemendal, V. R. L. J., van Hest, J. C. M., and Rutjes, F. P. J. T. (2020). Synthetic Pathways to Tetrahydrocannabinol(THC): an Overview. *Org. Biomol. Chem.* 18, 3203–3215. doi:10.1039/D0OB00464B
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382, 697–705. doi:10.1056/NEJMoa1916433
- Cid, M. V. F., and Van Houten, D. (2015). *Echo Pharmaceuticals BV, Assignee. Cannabis Plant Isolate Comprising delta-9-tetrahydrocannabinol and a Method for Preparing Such an Isolate*. Weesp, Netherlands: U.S. Patent Application. No 2015 0126754 A1. 2015 June 24.
- Ciolino, L. A., Ranieri, T. L., and Taylor, A. M. (2018a). Commercial Cannabis Consumer Products Part 1: GC-MS Qualitative Analysis of Cannabis Cannabinoids. *Forensic Sci. Int.* 289, 429–437. doi:10.1016/j.forsciint.2018.05.032
- Ciolino, L. A., Ranieri, T. L., and Taylor, A. M. (2018b). Commercial Cannabis Consumer Products Part 2: HPLC-DAD Quantitative Analysis of Cannabis Cannabinoids. *Forensic Sci. Int.* 289, 438–447. doi:10.1016/j.forsciint.2018.05.033
- Duffy, B., Li, L., Lu, S., Durocher, L., Dittmar, M., Delaney-Baldwin, E., Panawennage, D., LeMaster, D., Navarette, K., and Spink, D. (2020). Analysis of Cannabinoid-Containing Fluids in Illicit Vaping Cartridges Recovered from Pulmonary Injury Patients: Identification of Vitamin E Acetate as a Major Diluent. *Toxics* 8 (1), 1–19. doi:10.3390/toxics8010008
- Finley, C., and McKee, L., (2019). *Cannabinoid Composition and Products Including Alpha-Tocopherol* San Francisco, CA: Constance Therapeutics, Inc. U.S. Patent No 10,238,745 B2. 2019 Mar 26.
- Finley, C., McKee, L., Poole Bestwick, H., and Roethle, P., (2016). *Constance Therapeutics Inc, Assignee. Methods for Preparation of Cannabis Oil Extracts and Compositions* Richmond, CA. WO Patent Application No 2016 123475 A1. 2016 Aug 4.
- Grondin, R. A., and Ward, J. R., (2021a). *Pure Tonic Concentrates, LLC, Assignee. Conversion of Cannabidiol or delta-9 Tetrahydrocannabinolic Acid to delta-9 Tetrahydrocannabinol and delta-8 Tetrahydrocannabinol in Nontoxic Heterogeneous Mixtures* Reno, NV. U.S. Patent No 10,941,131.
- Grondin, R. A., and Ward, J. R. (2021b). *Pure Tonic Concentrates, LLC, Assignee. Conversion of Cannabidiol or delta-9 Tetrahydrocannabinolic Acid to delta-9 Tetrahydrocannabinol and delta-8 Tetrahydrocannabinol in Nontoxic Heterogeneous Mixtures* Reno, NV. WO Patent No 2021/086675 A2. 2021 May 6.

- Hanuš, L. O., Meyer, S. M., Muñoz, E., Tagliatela-Scafati, O., and Appendino, G. (2016). Phytocannabinoids: a Unified Critical Inventory. *Nat. Prod. Rep.* 33, 1357–1448. doi:10.1039/C6NP00074F
- Hartman, M. S. (2020). *Making Delta 8 THC, Delta 10 THC And/or Cannabinol from CBD* Burlingame, CA. inventor. Isomerz Inc., original assignee WO Patent No 2020/252277 A1. 2020 Dec 17.
- Hillig, K. W., and Mahlberg, P. G. (2004). A Chemotaxonomic Analysis of Cannabinoid Variation in Cannabis (Cannabaceae). *Am. J. Bot.* 91 (6), 966–975. doi:10.3732/ajb.91.6.966
- Hollister, L. E., and Gillespie, H. K. (1973). Delta-8- and delta-9-tetrahydrocannabinol; Comparison in Man by Oral and Intravenous Administration. *Clin. Pharmacol. Ther.* 14 (3), 353–357. doi:10.1002/cpt1973143353
- Ko, R. D., and Hughes, B., (2019). *Nextleaf Solutions Ltd, Assignee. Cannabinoid Extraction and Distillation* Vancouver, CA. U.S. Patent No 10,413,843 B2. 2019 Sept 17.
- Lozier, M. J., Wallace, B., Anderson, K., Ellington, S., Jones, C. M., Rose, D., Baldwin, G., King, B. A., Briss, P., Mikosz, C. A., Austin, C., Brown, S., Chandra, G., Coulliette-Salmond, A., Coy, K., Currie, D., Cyrus, A., Danielson, M., Fajardo, G., Gately, A., Goyal, S., Graves, S., Hamilton, J., Hayes, D., Hughes, D., Israel, M., Landen, M., Lynfield, R., Newton, S., Njai, R., Pollack, L., Ratto, J., Ritchey, M., Roguski, K., Salvatore, P., Soroka, S., Swedo, E., Thomas, K., and Thomas, S. (2019). Update: Demographic, Product, and Substance-Use Characteristics of Hospitalized Patients in a Nationwide Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injuries - United States, December 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 1142–1148. doi:10.15585/mmwr.mm6849e1
- Mechoulam, R. (1970). Marijuana Chemistry. *Science* 168 (3936), 1159–1165. doi:10.1126/science.168.3936.1159
- Punymurthula, N. S., Adelli, G. R., Gul, W., Repka, M. A., ElSohly, M. A., and Majumdar, S. (2017). Ocular Disposition of Δ^8 -Tetrahydrocannabinol from Various Topical Ophthalmic Formulations. *AAPS PharmSciTech* 18 (6), 1936–1945. doi:10.1208/s12249-016-0672-2
- Siegel, A. W., Valle, A. E., and Wall, D. J. (2021). Conversion of Δ^9 -THC to Δ^{10} -THC. U.S. Patent No 10,894,780. 2021 Jan 19.
- Srebnik, M., Lander, N., Breuer, A., and Mechoulam, R. (1984). Base-catalysed Double-Bond Isomerizations of Cannabinoids: Structural and Stereochemical Aspects. *J. Chem. Soc. Perkin Trans. 1*, 2881–2886. doi:10.1039/p19840002881
- Tegen, M. G., and Cho, J., (2019a). *Socati Technologies, Assignee. Continuous Isolation of Cannabidiol and Conversion of Cannabidiol to delta 8-tetrahydrocannabinol and delta 9- Tetrahydrocannabinol* Tumwater, WA. U.S. Patent Application No 2019 0276420 A1. 2019 Sept 12.
- Tegen, M. G., and Cho, J., (2019b). *Socati Technologies, Assignee. Continuous Isolation of Cannabidiol and Conversion of Cannabidiol to delta 8-tetrahydrocannabinol and delta 9- Tetrahydrocannabinol* Austin, TX. WO Patent Application No 2019 173582. 2019 Dec 9.
- Thapa, D., Cairns, E. A., Szczesniak, A.-M., Toguri, J. T., Caldwell, M. D., and Kelly, M. E. M. (2018). The Cannabinoids Δ^8 THC, CBD, and HU-308 Act via Distinct Receptors to Reduce Corneal Pain and Inflammation. *Cannabis Cannabinoid Res.* 3 (1), 11–20. doi:10.1089/can.2017.0041
- Thomas, B. F., Compton, D. R., and Martin, B. R. (1990). Characterization of the Lipophilicity of Natural and Synthetic Analogs of delta 9-tetrahydrocannabinol and its Relationship to Pharmacological Potency. *J. Pharmacol. Exp. Ther.* 255 (2), 624–630.
- United States Pharmacopoeia (2008). *United States Pharmacopeia [and] National Formulary Dronabinol*. USP32–NF27, 2217.
- US Centers for Disease Control Office on Smoking and Health National Center for Chronic Disease Prevention and Health Promotion (2020). Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products Rockville, MD. February 25, 2020 Update https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html (Accessed Sept 22, 2020).
- US Drug Enforcement Administration (DEA) Department of Justice (2020c). RIN 1117-AB53 Implementation of the Agriculture Improvement Act of 2018. *Fed. Reg.* 85 (163), 51639–51645.
- US Drug Enforcement Administration Diversion Control Division (2020b). *National Forensic Laboratory Information System: 2019 Annual Report*. Springfield, VA: US Drug Enforcement Administration.
- US Drug Enforcement Administration, Department of Justice (2020a). *Vaping & Marijuana Concentrates*. Springfield, VA: DOJ/DEA Drug Fact Sheet. April.
- Webster, G. R. B., Sarna, L. P., and Mechoulam, R., (2008). *Conversion of CBD to Δ^8 -THC and Δ^9 -THC*. U.S. Patent No 7,399,872 B2. 2008 July 15.
- WHO Expert Committee on Drug Dependence (2018a). *Extracts and Tinctures of Cannabis*. Geneva, Switzerland: World Health Organization. Critical review reports on cannabis - 41st ECDD 2018.
- WHO Expert Committee on Drug Dependence (2018b). *Isomers of THC*. Geneva, Switzerland: World Health Organization. Critical review reports on cannabis - 41st ECDD 2018.

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Diacetyl and Other Ketones in e-Cigarette Aerosols: Some Important Sources and Contributing Factors

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Background: Concerns over the presence of the diketones 2,4 butanedione (DA) and 2,3 pentanedione (AP) in e-cigarettes arise from their potential to cause respiratory diseases. Their presence in e-liquids is a primary source, but they may potentially be generated by glycerol (VG) and propylene glycol (PG) when heated to produce aerosols. Factors leading to the presence of AP, DA and acetoin (AC) in e-cigarette aerosols were investigated. We quantified direct transfer from e-liquids, examined thermal degradation of major e-liquid constituents VG, PG and 1,3 propanediol (1,3 PD) and the potential for AC, AP and DA production from sugars and flavor additives when heated in e-cigarettes.

Method: Transfers of AC, AP and DA from e-liquids to e-cigarette aerosols were quantified by comparing aerosol concentrations to e-liquid concentrations. Thermal generation from VG, PG or 1,3 PD e-liquids was investigated by measuring AC, AP and DA emissions as a function of temperature in an e-cigarette. Thermal generation of AC, AP and DA from sugars was examined by aerosolising e-liquids containing sucrose, fructose or glucose in an e-cigarette. Pyrolytic formation of AP and DA from a range of common flavors was assessed using flash pyrolysis techniques.

Results: AC transfer efficiency was >90%, while AP and DA were transferred less efficiently (65%) indicating losses during aerosolisation. Quantifiable levels of DA were generated from VG and PG, and to a lesser extent 1,3 PD at coil temperatures >300°C. Above 350°C AP was generated from VG and 1,3 PD but not PG. AC was not generated from major constituents, although low levels were generated by thermal reduction of DA. Aerosols from e-liquids containing sucrose contained quantifiable (>6 ng/puff) levels of DA at all sucrose concentrations tested, with DA emissions increasing with increasing device power and concentration. 1% glucose, fructose or sucrose e-liquids gave comparable DA emissions. Furanose ring compounds also generate DA and AP when heated to 250°C.

Abbreviations: DA, 2,3 butanedione, diacetyl; AP, 2,3 pentanedione, acetyl propionyl; AC, acetoin; VG, Vegetable glycerol, propane-1,2,3-triol; PG, 1,2-propylene glycol; 1,3 PD, 1,3-propylene diol; NIOSH, National Institute for Occupational Safety and Health; LOD, Limit of Detection; LOQ, Limit of Quantitation; TWA, Time weighted average; STEL, Short term exposure limit.

Conclusions: In addition to less than quantitative direct transfer from the e-liquid, DA and AP can be present in the e-cigarette aerosol due to thermal decomposition reactions of glycols, sugars and furanone ring flavors under e-cigarette operating conditions.

Keywords: e-cigarette, diacetyl, flavors, acetyl propionyl, acetoin, pyrolysis - gas chromatography

INTRODUCTION

Electronic nicotine delivery devices (ENDS), or e-cigarettes, have the potential for being less harmful alternatives to conventional combustion cigarettes (Shahab et al., 2017). They operate by heating e-liquids to produce an inhalable aerosol on puffing. E-liquids are composed of aerosol-formers (usually propane-1,2,3-triol or “vegetable” glycerol (VG), and/or propane-1,2-diol or propylene glycol (PG) and much less frequently 1,3-propylene diol (1,3-PD)), a viscosity regulator (water), nicotine and flavorings. When activated, the heating coil (or coils) used to generate the aerosol reaches temperatures in normal operation of between 145°C and 330°C, depending on the power supplied (Chen et al., 2018).

There are concerns about the use of e-liquid ingredients that may introduce unintended health risks to the consumer. One ingredient of particular concern is the flavor compound, diacetyl (2,3-butanedione, DA), which is a volatile α -diketone with the structure shown in **Figure 1**. It imparts a buttery/vanilla flavor and occurs naturally in a variety of foodstuffs such as dairy products, beer, coffee, honey and fruits (Clark and Winter, 2015). DA is also used widely in foods as a flavor additive and is

“generally recognised as safe” (GRAS) when used for this purpose. However, there is strong evidence, from both occupational exposure and animal studies, that inhalation of high levels of DA vapour can cause serious lung damage in humans (NIOSH, 2016). Compounds with flavors similar to DA (**Figure 1**) such as acetyl propionyl (2,3-pentanedione, AP), an α -diketone homolog of diacetyl, and acetoin (AC), a hydroxyl ketone, are also used in foodstuffs. Acetyl propionyl has also been shown to cause lung damage in exposed animals, while in contrast, AC does not have the reactive α -dicarbonyl group and is thought to be considerably less hazardous than DA (NIOSH, 2016). In 2016 the National Institute for Occupational Safety and Health (NIOSH) established recommended exposure limits (RELs) for DA and AP but not for AC.

As early as 2008 there were health concerns amongst vapers about the use of DA as a flavorant in e-liquids (Farsalinos et al., 2015; Vas et al., 2019). However, over the last decade a growing number of surveys have continued to identify the presence of DA, AC and AP in American, Canadian and European e-liquids (Farsalinos et al., 2015; Barhdadi et al., 2017; Moldoveanu et al., 2017; LeBouf et al., 2018; Vas et al., 2019; Czoli et al., 2019), (**Supplementary Table S1**), and aerosol emissions from commercial e-cigarettes (Allen et al., 2016; Margham et al., 2016; Sleiman et al., 2016; Klager et al., 2017; Moldoveanu et al., 2017; Melvin et al., 2020).

Given the volatile nature of these compounds it can be anticipated that they would volatilise and transfer from the e-liquid to the aerosol during puffing. However, despite the growing range of studies identifying these compounds in e-liquids or e-cigarette emissions, surprisingly no study has clearly evaluated emissions from e-cigarettes containing known e-liquid content at levels relevant to commercial e-liquids. The closest reported study was that of Farsalinos et al. (2015), who created three experimental e-liquids at very high DA and AP contents, and identified near-quantitative transfer to the aerosol even though the concentrations were significantly higher than measured in the great majority of commercial e-liquids. Moldoveanu et al. (2017) also measured both e-liquid and aerosol DA concentrations in their study. Both of these studies examined aerosol emissions from freshly prepared e-liquids. However, Vas et al. (2019) demonstrated that DA and particularly AP are chemically reactive in e-liquids, generating a range of reaction products over a period of weeks after e-liquid manufacture. It can be hypothesised therefore that the operation of such chemical reactions during product shelf-life might influence the efficiency with which these species are transferred from e-liquid to aerosol during puffing. Consistent with this, Pankow et al. (2018) in a study of gas/particle partitioning of e-cigarette flavors, commented on the

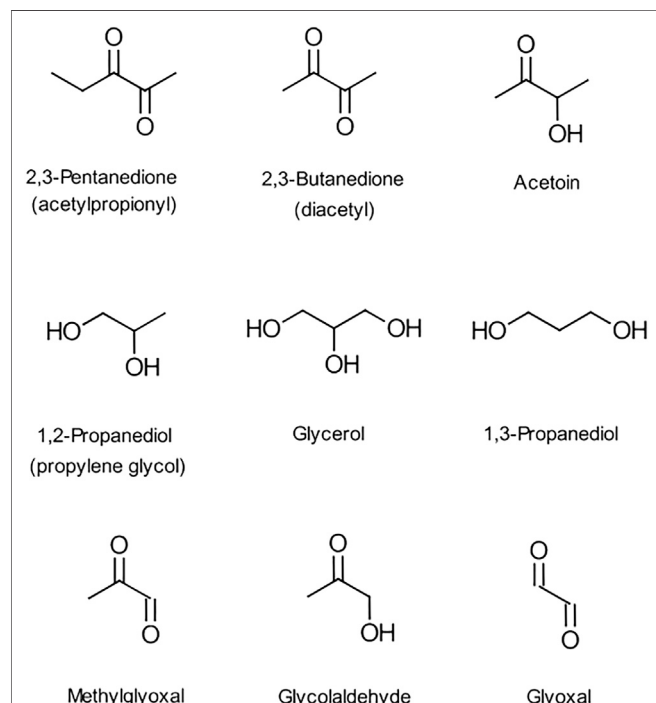


FIGURE 1 | Structures of the ketones, glycols, methylglyoxal, glycolaldehyde and glyoxal.

formation of significant amounts of reaction products from DA. Understanding of the hazards associated with DA and AP exposure during vaping, particularly dosimetry aspects, would therefore be advanced by insight into the efficacy with which these species transfer to the aerosol during vaping.

In addition, there are indications of other sources of these compounds in e-cigarette aerosols. For example, we have recently shown (Vas et al., 2019) that when AC is added to e-liquids, some of it is gradually oxidised to DA during storage at room temperature. Oxidation of AC is accelerated by higher pH conditions such as those obtained in nicotine-containing solutions. Thus, vapers were at risk of DA exposure without DA being initially present in the e-liquid formulation. Our findings were consistent with the relative concentrations of DA and AC measured in commercial e-cigarette aerosols by Allen et al. (2016) for all but one of the 51 e-liquids they analysed.

It is also plausible that these compounds may arise in e-cigarette aerosols from thermal degradation sources. At the higher temperatures experienced in e-cigarettes it has been clearly established (Uchiyama et al., 2020) that the aerosol formers - VG and PG - can undergo thermal degradation to a number of lower molecular weight carbonyls such as acrolein and formaldehyde. There is indirect evidence from gas phase catalytic dehydration of glycerol that DA can be formed via an addition reaction at temperatures of about 300°C. Consistent with this, two studies (Behar et al., 2016; Sleiman et al., 2016) have identified the presence of DA in the aerosol from e-cigarettes containing e-liquids free from DA, including neat PG and neat VG. Recent model studies using a microwave reactor heated to 180°C for several minutes have provided additional evidence for AP and DA production from VG and PG (Melvin et al., 2020). Together, these observations strongly suggest that thermal degradation of the main aerosol formers can produce DA, AC or AP during e-cigarette use. However, further information is needed on this possibility, particularly the threshold temperatures for ketone formation, the extent with which the ketones are generated, and the relative efficiency of generation by different aerosol former compounds.

Compounds besides PG and VG, such as flavors, may also potentially degrade thermally during aerosolization leading to the formation of DA, AC or AP in e-cigarette aerosols. One such class of additive, saccharides, have been used to create sweet flavored e-liquids (National Academies of Sciences Engineering, and Medicine, 2018), although prohibited under voluntary regulations in some jurisdictions (AFNOR 2016). Two studies (Kubica et al., 2014; Fagan et al., 2017) have shown both that a high proportion of e-liquids contain sucrose and quantified its presence in those e-liquids. In addition to intentional addition (Vape Club, 2020), sugars can also be introduced as natural components of flavor additives such as fruit extracts (Myeliquid, 2021; Soussy et al., 2016; and Fagan et al., 2017) demonstrated the operation of thermal breakdown reactions of sucrose, glucose and sorbitol under vaping conditions. We had concerns that DA or AP may also be formed from sugars during vaping since DA formation has been observed during caramelisation of sucrose under non-vaping conditions (Monte and Maga 1981). Finally, a

range of more volatile flavors were also investigated for their potential to form DA, AC and AP on heating in e-cigarettes.

The current paper therefore describes our investigations into potential sources of DA, AP and AC in e-cigarette aerosols. The paper reports results from four sets of experiments. The first was a study of the transfer efficiencies of DA, AP and AC from e-liquids to the e-cigarette aerosol during typical e-cigarette shelf-life times. The second experiment involved the analysis of aerosols generated at different power levels from model e-liquids containing only either PG, VG or 1,3 PD, plus sufficient water to ensure compatibility with the wicking characteristics of the e-cigarette device. The third series describes the analysis of aerosols from e-liquids containing sucrose concentrations in the range of 0–10%, as well as from 1% solutions of glucose and fructose. The final experiments were a pyrolysis screening exercise examining production of DA and AP from a range of common flavor compounds.

METHODS

Reagents

Pharmaceutical grade glycerol (99.9% purity) was obtained from Sigma Aldrich (Gillingham, United Kingdom. Product code 49779, lot number BCBQ6768V); pharmaceutical-grade propylene glycol (>99% purity) was obtained from Sigma Aldrich, Fluka (code 82281, lot number BCBQ0147V) and pharmaceutical-grade nicotine (99.4% purity by non-aqueous titrimetric determination) was obtained from Siegfried (Minden, Germany. Lot number 1517/024). The water used in the study was city water connected to a Millipore (Watford, United Kingdom) deionised ultra-filter (DIUF) and purified to a water resistivity value of 18.2 MΩ.cm at 25°C. 10 M sodium hydroxide solution was obtained from Sigma Aldrich. Acetoin was sourced from Sigma Aldrich (product code: A17951, lot number MKBQ2240V), with a declared purity of 99.3% by GC. Diacetyl (a mix of the monomer and dimer) was sourced from Sigma Aldrich (product Code: B85307, lot number BCBM5232V), with a declared purity of 97%. Acetyl propionyl was sourced from Sigma Aldrich (product Code 241962, lot number MKBB7504V), with a declared purity of 97.1%. Sucrose, glucose, and fructose were supplied by Sigma Aldrich. Compounds for the pyrolysis study were variously obtained from Sigma-Aldrich (St. Louis, MO, United States), Vigon International (East Stroudsburg, PA, USA), Tobacco Technology Inc. (Eldersburg, MD, USA), I.P. Callison and Sons (Lacey, WA, USA), Vantage Oleochemicals (Chicago, IL, USA), and Archer Daniels (Chicago, IL, USA).

AC, AP and DA Transfer Studies

An e-liquid formulation (2,500 ml) consisting of glycerol (48.76% w/w), 1,2 propylene glycol (25% w/w), water (25% w/w) and nicotine (1.24% w/w) was prepared at British American Tobacco's, (BAT) R&D laboratories. 1,3-PD was not used in this formulation due to its infrequent use in commercial e-liquids compared to PG and VG. The formulation was sent to Enthalpy Analytical (Durham, NC, United States), where it was

split into four e-liquid sub-samples. The first sub-sample was untreated and acted as a control. The second sub-sample was spiked with 1,000 µg/ml AP, the third was spiked with 1,000 µg/ml of AC and the fourth was spiked with 1,000 µg/ml of DA.

The four sub-samples were stored in clear volatile organic analyte vials at $20 \pm 2^\circ\text{C}$ and 60% RH for time periods up to 64 days, before being analysed in triplicate. Not all analyses were conducted at each time-point for each e-liquid, as reported below. The use of a time-course approach allowed us to examine the transfer of these species during a typical shelf-life period, as well as at time of e-liquid manufacture. Also, because concentrations of DA, AP and AC decline considerably in e-liquids over a 64 day period (Vas et al., 2019) the experimental design allowed for transfer efficiency to be examined across a range of compound concentrations that were consistent with previously reported ketone emissions from e-cigarettes (Farsalinos et al., 2015, **Supplementary Table S1**). The sub-samples were analysed in triplicate for AC (LOD: 2 µg/ml, LOQ: 20 µg/ml), AP (LOD: 1 µg/ml, LOQ: 10 µg/ml) and DA (LOD: 1 µg/ml, LOQ: 10 µg/ml) by GC/MS in accordance with Enthalpy SOP ENT-225.

In these experiments, aerosol testing was also conducted by Enthalpy Analytical (Durham, NC, United States). Using the same schedule as for the e-liquid analyses the aerosol emissions were analysed for AC, AP and DA, with three replicates at each time point. Aerosol and e-liquid concentrations were both determined at the same point and analysed as part of the same analytical batch to minimise interference from time-based reactions of the investigated species (Vas et al., 2019). Aerosols of the e-liquids were generated using a Vype eTank clearomiser. Two ml of the e-liquid samples were placed inside the tank and left to “wick” for 5 minutes. The tank, battery and mouthpiece were assembled. The device was operated at an angle of 45° (battery side down) and the voltage was set at 3.8 V. Aerosols were collected for 100 puffs with a puff volume of 80 ml, a puff duration of 3 s, a puff interval of 30 s and a square wave puff profile. The device button was turned on manually 1 s prior to each puff. Device weight loss was recorded as a measure of aerosol mass generated during puffing. Methods used by Enthalpy Analytical for the analyses of AC, AP and DA have previously been reported by Vas et al. (2019).

Expected aerosol per puff yields of the analytes (assuming 100% transfer from e-liquid to aerosol) were calculated from the e-liquid concentrations of the analytes and the mass loss per puff as follows:

$$\text{Expected yield } (\mu\text{g/puff}) = (W \times C) / (10^3 \times D)$$

Where W is the weight loss of the e-cigarette per puff (mg), D is the density of the e-liquid (g/ml) and C is the e-liquid concentration of the component (µg/ml). The e-liquid density, D , was calculated as 1.123 g/ml from the densities of the individual components and the proportions of the un-spiked e-liquid components (PubChem 2018).

The transfer efficiency of these compounds from the e-liquid to the aerosol was estimated by comparing the expected and measured aerosol yields on a percentage basis. The expected yields were based on the total weight of e-liquid lost during

aerosol generation and the concentration of the component in the e-liquid. Thus:

$$\text{Transfer Efficiency } (\%) = \frac{Y \times D \times 10^5}{W \times C}$$

where: Y = measured aerosol yield of the component (µg/puff).

Thermal Generation of AC, AP and DA by e-Liquid Solutions of VG, PG and 1,3-PD

The potential for major e-liquid components to generate DA, AP and AC was tested by creating three model e-liquid formulations comprising only one of the aerosol formers, plus a level of water appropriate to ensure a suitable viscosity to operate effectively with the test e-cigarette. These model e-liquid formulations were heated (separately) in an atomiser, with operating power levels increased systematically from 10 to 35 W in order to create a range of temperatures in the atomiser covering normal e-cigarette operating temperatures as well as the higher temperatures that might be encountered in dry wicking or over-powered e-cigarette scenarios. Parameters for the study were defined by published data on the generation of thermal decomposition products from e-liquids where power levels up to 85 W have been applied to an e-cigarette (Uchiyama et al., 2020), as well as reported e-cigarette operating temperatures. Schripp et al. (2013) measured heating coil temperatures of around 350°C , Geiss et al. (2016) reported temperatures $>300^\circ\text{C}$, (Zhao et al., 2016) reported coil temperatures up to 300°C , (Chen et al., 2018) reported coil temperatures of $110\text{--}185^\circ\text{C}$ operating with a PG e-liquid under fully wet conditions, $145\text{--}334^\circ\text{C}$ with a wet-through-wick condition, and $322\text{--}1,008^\circ\text{C}$ under dry wick conditions. In the present study, screening experiments showed e-cigarette power levels up to 35 W could generate the coil temperatures described above.

The model e-liquid formulations were prepared at British American Tobacco (Southampton, United Kingdom) and comprised (on a % w/w basis) a) 75% VG + 25% water, b) 91% PG + 9% water and c) 91% 1,3-PD + 9% water. While these % water levels are seen in some commercial e-cigarette e-liquids, their inclusion in this experiment was driven primarily by the need to ensure effective wicking of the e-liquids with the e-cigarette used.

Commercially available modular e-cigarettes were used for the generation of the emissions. The e-cigarette comprised an Aspire Nautilus mini 2 ml tank, an Aspire 1.8 Ω BVC atomiser with a cotton wick and a bottom vertical coil (Aspire 2016). For powers up to 30 W, a 30 W eLeaf iStick battery/power supply was used. This has a 2,200 mAh battery with variable voltage (2.0–8.0V) and power (5–30 W) settings (Eleaf 2016). For powers greater than 30 W a 40 W eLeaf iStick battery/power supply was used.

Analytical testing, test-piece assembly, machine puffing, and aerosol collection were conducted by Labstat International (Kitchener, Ontario, Canada). The Aspire tank was pre-filled with 2 ml of test e-liquid and allowed to “wick” for 5 minutes. The filled tanks were then connected to fully charged batteries. Aerosols were generated with a puffing regime of 80 ml puff

volume, 3 s puff duration and 30 s interval. Five replicate collections of 25 puffs were obtained at power settings of 10, 12, 14, 16, 18, 20, 25, 26.5, 27.5, 28.5, 30, 32 and 35 W for each of the three e-liquids. Three replicate air blanks were also obtained. The device was weighed before and after aerosol collection to record the device mass loss, which is a measure of the amount of aerosol generated.

The coil temperatures operating in the Aspire device during puffing were determined using a RS Pro Type K thermocouple (RS Number: 131–4,749) that had a calibrated temperature range of 0°C to +700°C. The thermocouple was attached to the atomiser wicking material as close as possible to, but not touching, the coil using thermal insulation tape. A second RS Pro Type K thermocouple (as a control) was used to monitor the ambient air temperatures of the laboratory. Temperatures were recorded using a thermocouple data logger and software, manufactured by Pico Technology Limited. Temperatures were measured under identical conditions to the chemical analyses described above. For temperature measurements conducted at different power settings, a fresh atomiser was used for each power level, with the tank filled to 2 ml before temperature measurements commenced. A Borgwaldt A14 single port smoke machine engine was used with identical puffing conditions to the aerosol measurements conducted by Labstat; temperature measurements were conducted for all 25 puffs.

Labstat method TMS-00155 (Carbonyls and Dicarbonyls) was used for analysis of aerosol carbonyl and dicarbonyl compounds in these studies (Bao 2015). Aerosols and blanks were collected using a 44 mm Cambridge filter pad (CFP) and a cryogenic impinger containing 20 ml of acetonitrile at –35°C. The CFP was extracted using the same impinger solution. The carbonyls captured in the impinger solution were derivatised using pentafluorobenzyl-hydroxylamine (PFBHA) prior to analysis by gas chromatography mass spectrometry (GC-MS). The column used was a Rtx-5ms (30 m × 0.25 mm × 0.25 µm) with an injector volume of 1 µl and a flow of 1 ml/min. The injector temperature was 260°C with an oven temperature regime of 70°C for 30 min, followed by 5°C/min to 220°C and then 30°C/min to 280°C and hold for 2 min. The mass spectrometer (MS) transfer temperature was 260°C, MS source temperature 250°C and MS quad temperature 150°C using a SIM scan mode. LOD and LOQ values for these analyses are provided in **Supplementary Table S2**.

Generation of AC, AP and DA From e-Liquids Containing Sugars

The investigation of sugars as potential sources of AC, AP and DA in e-cigarettes was carried out in two parts. The first part focused on sucrose at concentrations of up to 1% in the e-liquids, to reflect levels potentially present in e-liquids. Also included were e-liquid formulations containing 1% fructose and 1% glucose to determine if the monosaccharides also produced the aerosol ketones to the same extent as sucrose. In these formulations, increasing sugar levels were incorporated by reducing water content. A second stage of this experiment involved analysis of aerosols from e-liquids with higher

sucrose levels (up to 10%, assembled by increasing sucrose and reducing glycerol) in order to compare the efficiency of diketone production from sucrose and VG, and to provide greater clarity on trends in AP emissions.

E-liquids used in the first part of this experiment comprised sucrose (%w/w) at 0, 0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.0%, together with 1.86% nicotine, 89.14% VG, and water ranging from 9% for 0% sucrose to 8% for 1.0% sucrose. E-liquids containing 1% glucose or fructose in place of the sucrose were prepared with the same formulation as the 1% sucrose solution. E-liquids used in the second part of the experiment comprised sucrose at concentrations (w/w) of 0, 1, 2.5, 5, 7.5 and 10%. The solutions contained 9% water, 1.86% nicotine and levels of VG ranging from 79.14% for the 10% sucrose solution to 89.14% for the 0% sucrose solution.

E-liquids containing 0–10% sucrose were analysed by Labstat International (Kitchener, Ontario, Canada) for sucrose and carbonyls and dicarbonyls that might potentially contribute to ketone presence in the aerosol. For aerosol analysis a 30W eLeaf iStick e-cigarette was used to generate the aerosol. The puffing parameters were a 80 ml puff volume, 3 s puff duration and 30 s interval. The device was tested at two power settings (10 and 20 W) to observe whether increased power influenced yields. Blocks of 50 puffs were collected for analysis. For both series of experiments, machine puffing, aerosol collection and analytical testing were conducted by Labstat International (Kitchener, Ontario, Canada). Labstat method TMS-00155 (Carbonyls and Dicarbonyls) were used for these studies (Bao 2015) as described in the previous section.

Pyrolysis Screening Experiments for AC, AP and DA From Flavor Compounds

A series of five- and six-membered ring flavor compounds were investigated in the pyrolysis experiments. Some samples consisted of “neat” material and some were solutions in 3:1 VG:PG (by weight). PG and VG were also examined in the pyrolysis study to provide comparability with the other compounds of this study, and also to provide baseline levels of DA or AP for those experiments in which they were used as carriers. The flavor compounds investigated were furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone) tested as both a 5% solution in 3:1 VG:PG and 15% in PG; 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone tested as both a 5% solution in 3:1 VG:PG and as the neat compound; mesifurane (2,5-dimethyl-4-methoxy-3(2H)-furanone) tested as the neat compound; furaneol acetate (2,5-dimethyl-4-acetoxy-3(2H)-furanone) tested as the neat compound; ethyl maltol (3-hydroxy-2-ethyl-4-pyranone) tested as the neat compound and as a 5% solution in VG; cyclotene (3-methyl-2-cyclopenten-2-ol-1-one) tested both as a 5% solution in VG and neat; 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane) tested as the pure compound; vanillin (4-hydroxy-3-methoxybenzaldehyde) tested neat; ethyl vanillin (4-hydroxy-3-ethoxybenzaldehyde) tested neat; 4-ketoisophorone (2,6,6-trimethylcyclohex-2-ene-1,4-dione) tested neat; β-damascone ((E)-1-(2,6,6-trimethyl-1-cyclohexenyl)but-2-en-1-one) tested neat, and peppermint oil

(mix of menthol (7–48%), menthone (20–46%), menthyl acetate (3–10%), menthofuran (1–17%), 1,8-cineole (3–6%), etc.) tested neat.

The samples were pyrolyzed using a filament pyrolyzer Pyroprobe 5000 Model 520 equipped with autosampling capability from CDS Analytical INC. (Oxford, PA 19363, United States). All materials were loaded on a fiberglass bed at a specific amount (around 2 mg material precisely weighed). The pyrolysate was directly transferred to a 6890/5973 GC/MS from Agilent (Wilmington, DE 19808, United States). This system was used in flash mode with pyrolysis performed in the carrier gas (helium). Except for the pyrolysis temperature, other conditions for the pyrolysis were kept the same in all experiments: purge time $t = 0.0$ s, equilibration time, $t = 0.0$ s, pyrolysis time 40 s, post pyrolysis time $t = 12$ s, valve temperature 250°C, transfer line temperature 250°C. The temperatures for each sample were set at specific values such as 250°C, 350°C, 450°C and in a few cases at 550°C. The higher temperatures were not expected to be attained in e-cigarettes and the pyrolysis at these temperatures was performed only for verifying an expected increase in the level of pyrolytic products. The GC/MS parameters for the separation are described in **Supplemental Table S3**. During data analysis, peak identification used the Wiley275 and NIST14 mass spectral libraries. Retention times and spectra for DA and AP were identified using 2% solutions in acetone.

Statistical Testing and LOQ/LOD Results

Statistical analysis of the data from all the studies was performed using Minitab version 16 (Minitab Inc, PA, United States) and Minitab version 20. Tests of significance were carried out using one-way analysis of variance at a confidence level of 95%. Comparisons were made with Tukey's method. For graphical presentation, where results were LOD, values of LOD/2 were used, and where results were LOQ, values were assigned as LOD+(LOQ-LOD)/2. Regression analyses were conducted using the Minitab v20 regression assistant.

RESULTS

Transfer of AC, AP and DA From e-Liquids to Aerosols

Results of the experiments to determine the effects of storage time on the concentrations of AC, AP and DA in e-liquids spiked with 1,000 µg/ml of these chemicals, and their corresponding aerosol emissions are shown in **Tables 1–3**. The e-liquid results (but not the aerosol emission data) have been reported previously (Vas et al., 2019).

Table 1 shows that e-liquid AC concentrations fell significantly ($p < 0.005$) with increasing time, with an $[AC]_{\text{liquid}}$ 35% lower after 64 days than the day 0 value. In contrast, aerosol AC emissions from the e-cigarettes did not appear to change significantly over time. However, allowing for variation in device mass loss, i.e. total amount of aerosol generated by the e-cigarettes over the course of the experiment, showed the DML-normalized $[AC]_{\text{aerosol}}$ values did decline

significantly ($p = 0.005$) by around 16% over the 64 day time course of the experiment. As reported previously (Vas et al., 2019) the AC-containing e-liquid generated DA. Levels of DA in both e-liquid and aerosol increased significantly ($p < 0.001$) over time. Transfer efficiency of AC from e-liquid to aerosol was near-quantitative, at $92.5 \pm 8.2\%$, while the transfer efficiency of DA generated from AC e-liquid was quantitative, albeit highly variable, at $101 \pm 34.9\%$.

The data in **Table 2** show that DA levels in e-liquids and aerosol fell significantly over time ($p < 0.05$). $[AC]_{\text{liquid}}$ fell by 85% over the 36 day experiment, and $[AC]_{\text{aerosol}}$ fell by 80% (90% when normalized to DML). These changes were substantially greater than found with AC. DA transfer efficiency was $63.4 \pm 12.3\%$, with the first time point providing a % transfer efficiency significantly higher than found at the other time points ($p < 0.05$). Interestingly, for half of the time points relatively low-level AC emissions were detected in the aerosol of the DA e-liquid, despite their absence from the e-liquid at any time point. The aerosol AC levels were greater than the e-liquid detection limit, and these levels of AC would have been detected in the e-liquid if present.

Table 3 shows that $[AP]_{\text{liquid}}$ fell over the 64-day experiment to approximately 4% of the amount added to the e-liquid. Aerosol AP levels fell in a very similar way, reaching non-quantifiable levels after 64 days ($<7\%$ of the day 0 value). Transfer efficiency of AP from e-liquid to aerosol was similar to the value found with DA, at $67.1 \pm 16.6\%$. Small quantities of DA were found in the AP e-liquid but transfer of the DA to the aerosol was too low to quantify.

Regression analysis of aerosol emissions against e-liquid content showed significant correlations ($p < 0.001$) for both AP and DA, with 98.2% of the variation in the AP data and 82.4% of the variation in the measured DA emissions accounted for by the e-liquid contents of these compounds. Multiple regression of the DA emissions against both the DA e-liquid content and the DML raised the r^2 value to 98.6%, but the corresponding analysis for the AP data did not change the r^2 value from the 98.2% provided by the simple regression against e-liquids AP concentration. In contrast, the aerosol AC emissions were not significantly correlated with the e-liquid AC levels ($r^2 = 12.2\%$, $p = 0.07$). However, multiple regression of aerosol AC emissions against both e-liquid $[AC]$ and DML showed significant ($r^2 = 87.2\%$, $p < 0.001$) correlation.

The Potential of e-Liquid VG and PG to Generate AC, AP and DA in e-Cigarette Aerosols

Results from experiments examining the potential thermal formation of AC, AP and DA from VG, PG and 1,3 PD are shown in **Table 4**. The table shows the power setting, resulting coil temperature, per-puff aerosol yields of AC, AP and DA, and e-cigarette total mass loss for the three glycol solutions tested. **Supplementary Table S2** shows the LODs and LOQs for the method.

The device mass loss of the e-cigarette (per 25 puffs), i.e. the weight of aerosolized e-liquid, increased as the power to the coil

TABLE 1 | Transfer of AC from e-liquid to e-cigarette aerosol.

Time (Days)	Device weight loss (mg/100 puffs)		E-liquid [AC] (µg/ml)		Aerosol AC emissions µg/100 puffs		AC transfer efficiency (%)	E-liquid [DA] (µg/ml)		Aerosol DA emissions µg/100 puffs		DA transfer efficiency (%)
	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Control	303	40	<1.87	—	<2.08	—	—	<1.13	—	<1.79	—	—
0	323	61	1,169	46	281	50	83.6	2.36	0.06	<1.79	—	—
3	339	41	1,054	8	286	36	89.9	13.7	0.2	2.88	1.88	69.6
6	374	22	1,034	14	329	26	95.5	17.1	0.3	3.15	0.56	55.3
9	364	39	963	13	295	44	94.5	19.6	0.3	4.70	1.2	74.0
12	371	9	963	10	302	10	94.9	21.2	0.8	11.0	0.5	157.1
15	358	29	1,059	14	270	25	80.0	26.5	0.9	11.1	2.2	131.4
18	504	26	981	20	396	6.93	89.9	29.3	0.7	15.0	0.074	114.1
21	453	12	905	14	347	28	95.1	29.6	0.1	14.1	1.0	118.1
64	460	13	760	6	339	16.1	108.9	46.4	0.4	16.5	2.38	86.8
Mean % Transfer	—	—	—	—	—	—	92.5 ± 8.2	—	—	—	—	101 ± 34.9

Control samples run at days 0 and 36 showed no detectable levels of AC, AP or DA in the control e-liquid or corresponding aerosol sample. AP was not found at quantifiable levels in any e-liquid or aerosol sample, other than aerosol emissions of 5 µg/100 puffs at T = 0 days. ± values presented are ±1 standard deviation.

TABLE 2 | Transfer of diacetyl from e-liquid to e-cigarette aerosol.

Time (Days)	Device weight loss (mg/100 puffs)		E-liquid [AC] (µg/ml)		Aerosol AC emissions µg/100 puffs		E-liquid [DA] (µg/ml)	Aerosol DA emissions µg/100 puffs	DA transfer efficiency (%)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Control	303	40	<1.87	—	<2.08	—	<1.13	—	<1.79
0	280	95	<1.87	—	8.56	11.2	1,114	15	245
6	333	62	<1.87	—	18.6	8.3	603	3	115
12	360	30	<0.751	—	2.08	0.00	348	18	68.3
18	483	69	<0.751	—	28.1	12.7	366	2	76.3
24	556	2	<1.87	—	<2.08	—	240	3	66.1
30	552	33	<1.87	—	<1.72	—	190	1	59.4
36	524	43	<1.87	—	<1.72	—	164	3	47.6
Mean % Transfer	—	—	—	—	—	—	—	—	63.4 ± 12.3

Control samples run at days 0 and 36 showed no detectable levels of AC, AP or DA in the control e-liquid or corresponding aerosol sample. AP was not found at quantifiable levels in any e-liquid or aerosol sample. ± values presented are ±1 standard deviation.

TABLE 3 | Transfer of acetyl propionyl from e-liquid to e-cigarette aerosol.

Time (Days)	Device weight loss (mg/100 puffs)		E-liquid [AP] (µg/mL)		Aerosol AP emissions µg/100 puffs		AP transfer efficiency (%)	E-liquid [DA] (µg/ml)		Aerosol DA emissions µg/100 puffs		DA transfer efficiency (%)
	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Control	303	40	<1.87	—	<2.08	—	—	<1.13	—	<1.79	—	—
0	386	6	522	9	158	24	88.1	4.38	0.09	<1.79	—	—
3	356	19	135	1	27.6	1.7	64.5	3.49	0.09	<1.79	—	—
6	342	39	92.3	2.8	20.8	1.8	74.0	2.94	0.14	<1.79	—	—
9	286	114	64.2	4.6	14.3	4.8	87.5	1.40	0.08	<1.79	—	—
12	369	9	60.5	2.8	12.3	0.4	61.9	1.26	0.05	<1.79	—	—
15	405	170	55.0	0.8	14.0	5.1	70.6	0.869	0.070	<1.79	—	—
18	491	104	70.3	0.7	13.8	1.7	44.9	0.861	0.011	<1.79	—	—
21	558	7	60.5	1.4	13.6	0.5	45.2	0.581	0.070	<1.79	—	—
64	416	149	38.5	0.8	<10.3	—	—	0.977	0.017	<4.23	—	—
Mean % Transfer	—	—	—	—	—	—	67.1 ± 16.6	—	—	—	—	—

Control samples run at days 0 and 36 showed no detectable levels of AC, AP or DA in the control e-liquid or corresponding aerosol sample. AC was not found at quantifiable levels in any e-liquid or aerosol sample. ± values presented are ±1 standard deviation.

TABLE 4 | Effect of power setting on aerosol yields of acetoin, acetyl propionyl and diacetyl from e-cigarettes with e-liquids consisting of mixtures of humectants and water.

Formulation	Setting	Temperature	Aerosol yield [ng/puff]			Mass loss
	(W)	(°C)	Acetoin (Mean ± SD)	Acetyl propionyl (Mean ± SD)	Diacetyl (Mean ± SD)	(mg/collection)
VG 75% Water 25%	10	248	<LOD ⁰	<LOD ⁰	<LOD ⁰	198
	12	259	<LOD ⁰	<LOD ⁰	<LOD ⁰	225
	14	254	<LOD ⁰	<LOD ⁰	<LOD ⁰	300
	16	254	<LOD ⁰	<LOD ⁰	<LOD ⁰	403
	18	262	<LOD ⁰	<LOD ¹	<LOD ¹	446
	20	257	<LOD ⁰	<LOD ⁰	<LOD ⁰	493
	25	247	<LOD ⁰	<LOD ⁰	<LOD ⁰	661
	26.5	265	<LOD ⁰	<LOD ⁰	53.6 ± 97.3	727
	27.5	278	<LOD ⁰	<LOD ⁰	<LOQ ¹	783
	28.5	293	<LOD ⁰	<LOD ⁰	26.3 ± 33.3	801
	30	302	<LOD ¹	<LOD ¹	31.1 ± 42.1	842
	32	359	<LOD ⁰	45.2 ± 20.3	204.7 ± 72.7	1,057
	35	394	83.6 ± 122.4	87.8 ± 55.6	369.4 ± 218.6	883
1,3 PD 91% Water 9%	10	204	<LOD ⁰	<LOD ⁰	<LOD ⁰	309
	12	215	<LOD ⁰	<LOD ⁰	<LOD ⁰	368
	14	219	<LOD ⁰	<LOD ⁰	<LOD ⁰	436
	16	220	<LOD ⁰	<LOD ⁰	<LOD ¹	565
	18	225	<LOD ⁰	<LOD ⁰	<LOD ⁰	622
	20	238	<LOD ⁰	<LOD ⁰	<LOD ⁰	624
	25	335	<LOD ⁰	<LOD ⁰	<LOD ⁰	764
	26.5	382	<LOD ⁰	<LOD ⁰	<LOD ¹	1,048
	27.5	398	<LOD ⁰	<LOD ⁰	<LOD ¹	1,083
	28.5	387	<LOD ⁰	<LOD ⁰	<LOD ¹	1,113
	30	363	<LOD ⁰	<LOD ⁰	12.4 ± 12.1	976
	32	404	<LOD ⁰	29.4 ± 47.3	<LOQ ³	1,331
	35	441	<LOD ⁰	45.7 ± 77.0	14.1 ± 13.9	1,177
PG 91% Water 9%	10	185	<LOD ⁰	<LOD ⁰	<LOD ⁰	327
	12	182	<LOD ⁰	<LOD ⁰	<LOD ⁰	368
	14	181	<LOD ⁰	<LOD ⁰	<LOD ⁰	515
	16	180	<LOD ⁰	<LOD ⁰	<LOQ ¹	594
	18	180	<LOD ⁰	<LOQ ²	17.1 ± 18.8	650
	20	179	<LOD ⁰	<LOD ⁰	<LOD ⁰	564
	25	233	<LOD ⁰	<LOD ⁰	<LOD ⁰	725
	26.5	244	<LOD ⁰	<LOD ⁰	<LOD ²	816
	27.5	234	<LOD ⁰	<LOD ⁰	12.1 ± 12.7	828
	28.5	ND	<LOD ⁰	<LOD ⁰	22.2 ± 33.1	969
	30	325	<LOD ⁰	<LOQ ¹	55.4 ± 46.1	810
	32	417	<LOD ⁰	<LOQ ²	87.9 ± 94.0	825
	35	452	<LOD ⁰	<LOQ ²	125.0 ± 61.7	1,254

ND, not determined. ⁰, ¹, ², ³ Numbers of replicates (out of 5) with values >LOD. ± values presented are ±1 standard deviation.

increased. This is illustrated in **Supplementary Figure S1**. The relationship between power and mass loss appears linear for all three of the e-liquids studied, with correlation coefficients (r^2) of 0.862, 0.928 and 0.956 and for PG, 1,3-PD and VG containing e-liquids, respectively. The mass loss/unit power is highest for 1,3 PD, lowest for VG and intermediate for PG.

Coil temperatures, as measured by thermocouple, generally increased with power for the three e-liquids studied but the relationship was not linear and each of the three e-liquids gave a different pattern, as shown in **Supplementary Figure S2**. As power was increased from 10 to 20 W, the coil temperatures remained fairly constant for both VG (at about $255 \pm 7^\circ\text{C}$) and PG (at about $182 \pm 3^\circ\text{C}$). For 1,3 PD, coil temperatures increased gradually from about 200 to 240°C as power was increased. At 25 W there were sharp increases in coil temperature for both PG (up to 233°C) and 1,3-PD (up to 335°C).

As power was increased to a maximum of 35 W, coil temperatures increased up to 452°C for PG and 441°C for 1,3 PD. However, there were shoulders in the power/temperature curves at 26.5 W/ 244°C for PG and at 27.5 W/ 398°C for 1,3 PD. For VG the coil temperature remained at about 250°C for power inputs of up to about 25 W and thereafter rose monotonically up to a maximum of 394°C at 35 W.

Table 4 shows the effect of coil temperature on AC emissions (**Supplementary Figure S3**), AP emissions (**Supplementary Figure S4**), and DA emissions (**Figure 2**).

Levels of aerosol AC produced by the PG and 1,3 PD e-liquids were all below the LOD ($0.0134 \mu\text{g/puff}$) for coil temperatures up to around 450°C . The VG-containing e-liquid produced only one value for AC above the LOQ ($0.045 \mu\text{g/puff}$) and that was $0.084 \mu\text{g/puff}$ at the maximum coil temperature of 394°C . However, this value was not significantly different ($p > 0.05$).

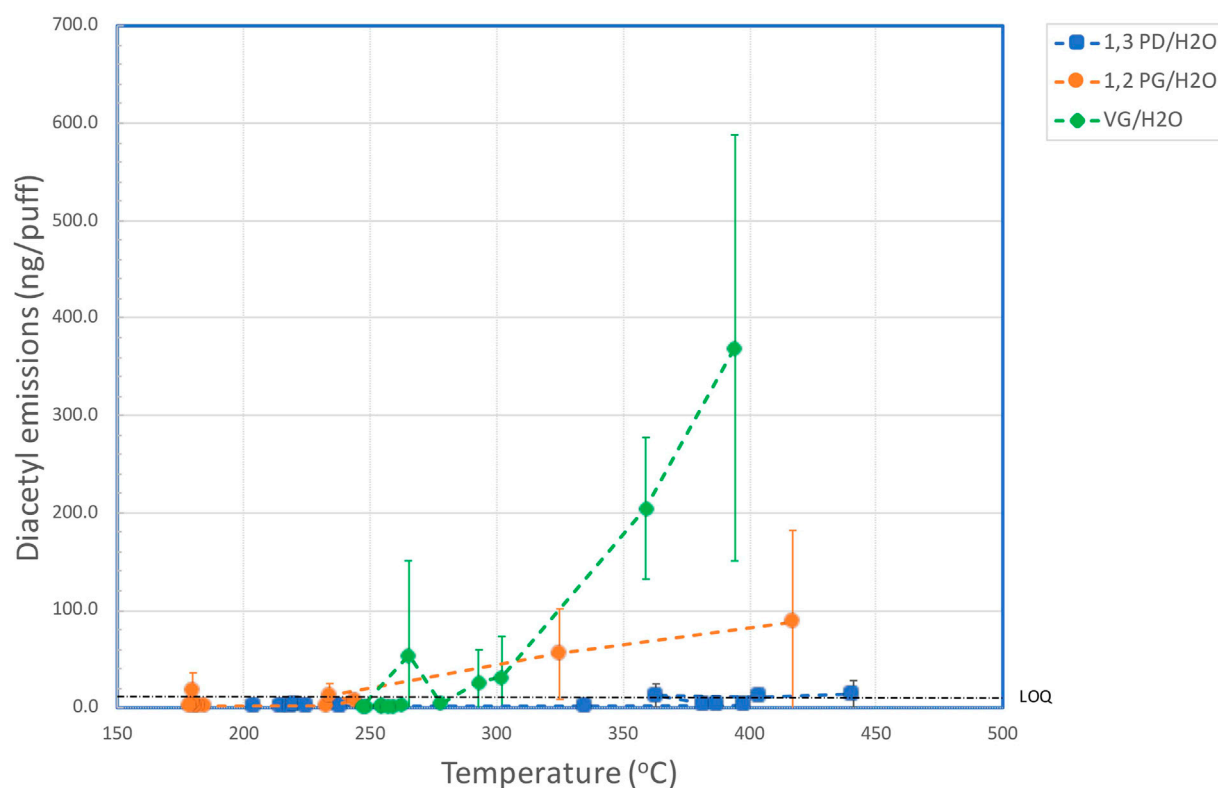


FIGURE 2 | Aerosol yields of diacetyl versus coil temperature for aqueous solutions of VG, PG and 1,3-PD.

to the results obtained at lower temperatures, with only two of the five replicates showing values of AC > LOQ.

AP was detected in the aerosol from PG at various temperatures but levels never exceeded the LOQ (0.0235 µg/puff) even at the highest coil temperature (452°C). For 1,3-PD, levels of AP in the aerosol remained below the LOD (0.007 µg/puff) until coil temperatures exceeded 400°C. Quantifiable levels of AP were observed at the two highest coil temperatures achieved: 0.029 µg/puff at 404°C and 0.046 µg/puff at 441°C. These yields were not significantly greater (at 95%) than those generated at lower temperatures. For VG, levels of AP were below LOD up to 302°C, but quantifiable levels of AP were measured at the two higher coil temperatures: 359°C (0.045 µg/puff) and 394°C (0.088 µg/puff), with the latter yield significantly greater (at 95%) than that at 302°C.

The yields of DA as a function of coil temperature for the three e-liquids are shown in **Figure 2**. For 1,3 PD, levels of DA in the aerosol were <LOD for coil temperatures up to 335°C, but quantifiable levels of DA were observed for the aerosol generated at 363°C and 441°C. These emissions were not significantly different (at 95%) to those at lower temperatures. With PG quantifiable levels of DA were observed with one measurement at 180°C and for coil temperatures above 234°C. Levels of DA increased with coil temperature with the highest level of DA (0.125 µg/puff) observed at the maximum coil temperature of 452°C. With VG, levels of DA increased rapidly at coil temperatures above 293°C. The level of DA in

the aerosol at the highest coil temperature (394°C) was 0.369 µg/puff, which was significantly greater than the yield at 302°C.

Potential of Sugars in e-Liquids to Generate Aerosol AC, AP and DA

E-liquid analysis showed that apart from sucrose there were detectable levels of formaldehyde (mean 1.56 µg/g), glycolaldehyde (mean 1.153 µg/g), AC (mean 0.565 µg/g), glyoxal (mean 0.991 µg/g) and methylglyoxal (mean 2.61 µg/g). These levels were all greater than the laboratory reagent blank (LRB) and the concentrations were similar for all the formulations. Acetone was also detected in all the formulations (mean 0.657 µg/g) but the levels in the LRB were not significantly different to the test liquids which indicates acetone was introduced via the analytical procedure. There were no detectable levels of the other analytes included in the assay: acetaldehyde, propionaldehyde, acrolein, isobutyraldehyde, methyl ethyl ketone, 3-buten-2-one, n-butyraldehyde, crotonaldehyde, acetol, DA, AP, 2,3-hexanedione or 2,3-heptanedione.

The results showing the concentrations of DA and AP in the aerosols of the e-liquids are shown in **Table 5** and DA emissions illustrated in **Figure 3**. AC concentrations were all below the LOD and are therefore not shown.

Aerosol DA emissions from the sucrose-free e-liquids were < LOQ (0.0058 µg/puff) at 10 W, but quantifiable (0.064–0.093 µg/

TABLE 5 | Device mass losses and aerosol yields of AC^a, AP and DA per puff at power settings of 10 and 20 W, from 0 to 10% sucrose solutions.

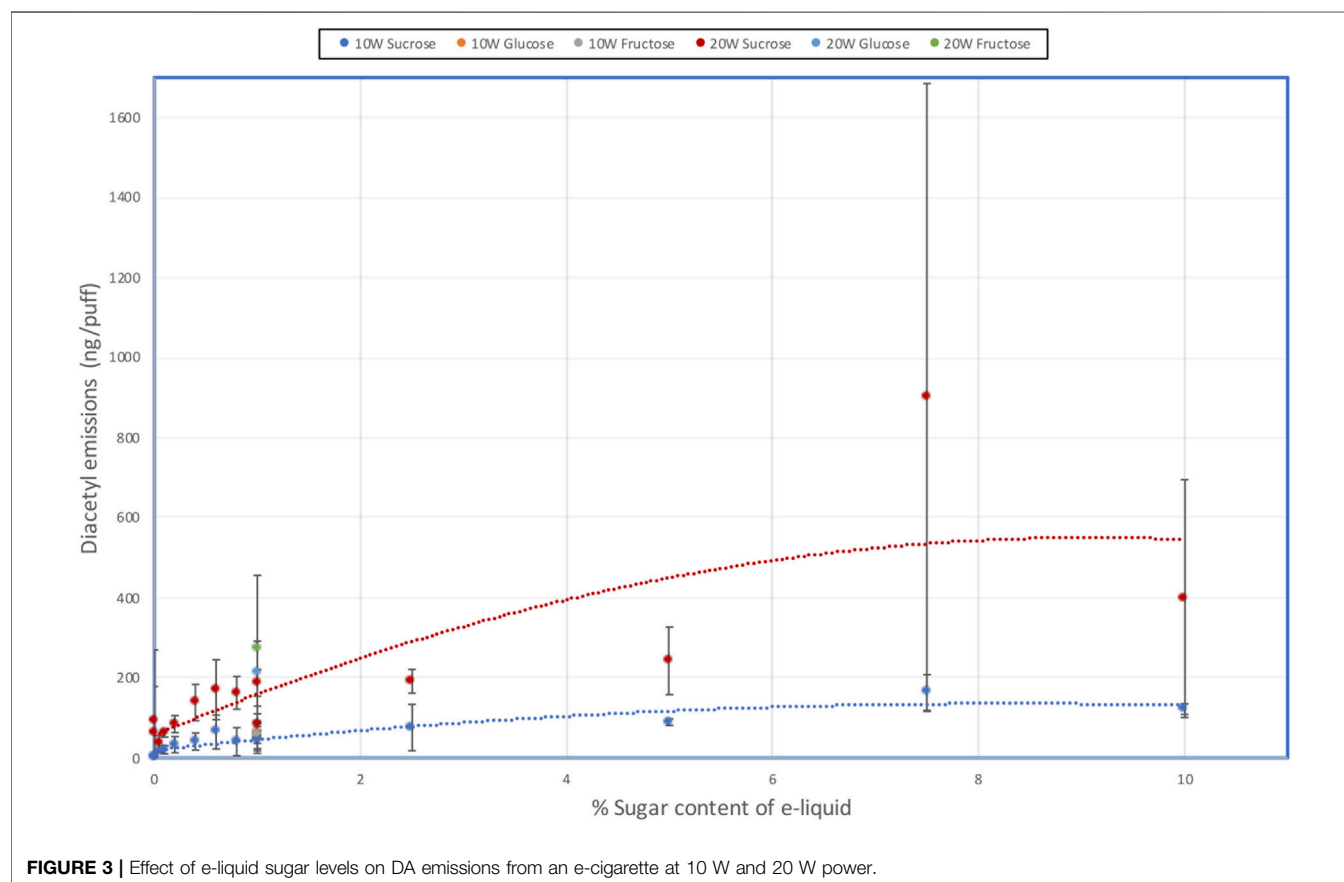
Formulation (%)						DML @10 W mg/puff	DA emissions @10 W (ng/puff)		AP emissions @10 W (ng/puff)		DML @20 W mg/puff	DA emissions @20 W (ng/puff)		AP emissions @20 W (ng/puff)	
Sucrose	Fructose	Glucose	Glycerol	Water	Nicotine	Average	Average	St Dev	Average	St Dev	Average	Average	St Dev	Average	St Dev
0.00 ^b			89.14	9.00	1.86	6.76	NQ	NQ	BDL	BDL	24.40	64.00	114.80	15.16	29.98
0.05 ^b			89.14	8.95	1.86	8.18	15.04	2.22	BDL	BDL	25.40	37.20	18.40	NQ	NQ
0.10 ^b			89.14	8.90	1.86	7.66	20.60	10.02	BDL	BDL	23.70	60.60	9.00	NQ	NQ
0.20 ^b			89.14	8.80	1.86	10.80	33.00	20.40	NQ	NQ	23.70	84.40	21.20	NQ	NQ
0.40 ^b			89.14	8.60	1.86	10.50	41.00	21.40	NQ	NQ	24.70	138.60	45.20	13.52	8.58
0.60 ^b			89.14	8.40	1.86	9.66	64.60	42.60	NQ	NQ	23.90	170.60	75.00	22.20	10.40
0.80 ^b			89.14	8.20	1.86	7.05	40.40	34.80	NQ	NQ	24.70	162.80	41.00	19.28	8.58
1.00 ^b			89.14	8.00	1.86	9.93	45.20	33.60	BDL	BDL	24.10	187.60	33.40	21.20	8.00
	1.00 ^b		89.14	8.00	1.86	8.18	57.40	34.40	NQ	NQ	25.20	272.00	184.00	32.00	29.80
		1.00 ^b	89.14	8.00	1.86	7.70	61.20	25.00	NQ	NQ	24.90	210.00	82.00	14.84	11.30
0.00 ^c			89.14	9.00	1.86	7.69	NQ	NQ	BDL	BDL	26.24	92.64	177.22	NQ	NQ
1.00 ^c			89.14	8.00	1.86	6.20	43.53	25.77	NQ	NQ	25.10	83.20	26.96	BDL	BDL
2.50 ^c			86.64	9.00	1.86	5.58	75.68	57.96	BDL	BDL	24.82	191.52	29.86	17.50	17.43
5.00 ^c			84.14	9.00	1.86	6.74	89.44	8.28	11.71	8.80	23.58	242.56	84.45	19.29	24.18
7.50 ^c			81.64	9.00	1.86	6.17	163.36	44.78	25.69	33.02	28.14	900.80	784.38	16.76	33.56
10.0 ^c			79.14	9.00	1.86	5.47	121.76	13.31	17.53	21.61	25.72	398.09	296.98	33.31	20.79

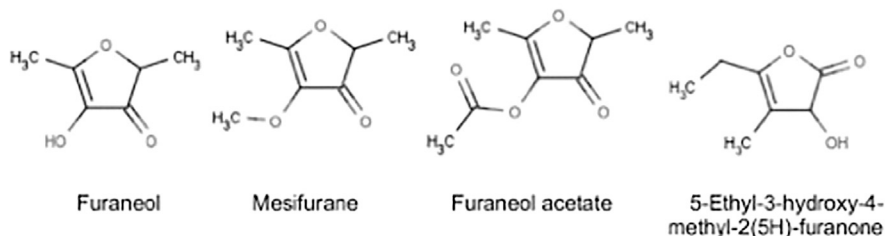
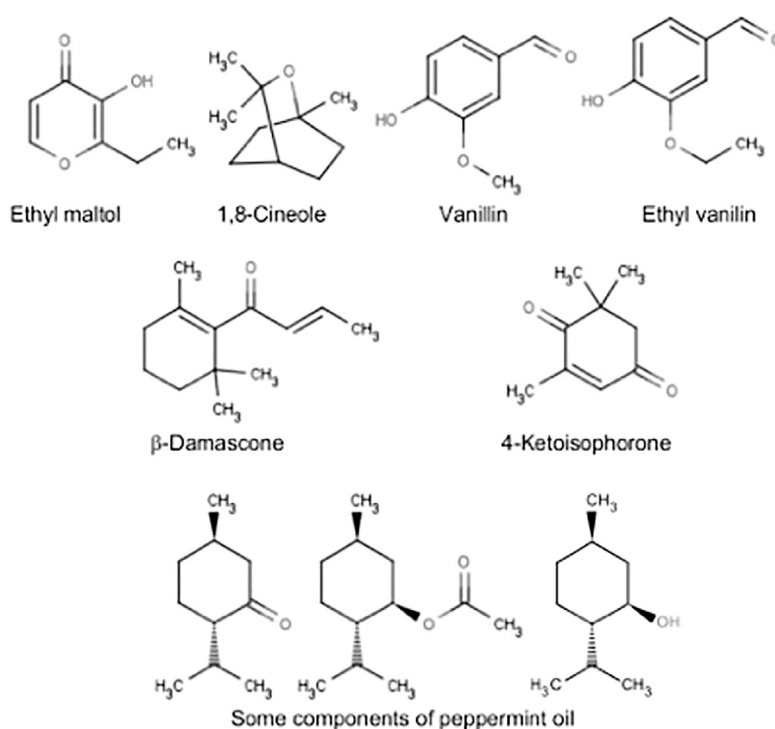
DML, Device Mass Loss; NQ, not quantifiable; BDL, below detection limit.

^aAC emissions were BDL for all samples, and therefore not shown.

^bData for the 0–1% sugar experiment.

^cData for the 0–10% sugar experiment.

**FIGURE 3** | Effect of e-liquid sugar levels on DA emissions from an e-cigarette at 10 W and 20 W power.

Furanone ring compounds:**6 membered ring flavour compounds:****FIGURE 4 |** Flavour compounds examined in the pyrolysis screening study.

puff) at 20 W; AP emissions were <LOD or <LOQ. With the e-liquids containing sugars there were increases in DA emissions as both the sucrose concentration and heating coil power increased, although there was some scatter in the data. At 10 W, DA yields increased up to 0.16 µg/puff at 7.5% sucrose and then tapered off to 0.12 µg/puff at 10% sucrose. At 20 W DA yields reached 0.90 µg/puff at 7.5% sucrose and then dropped to 0.40 µg/puff at 10% sucrose. DA emissions from the 1% fructose and 1% glucose e-liquids at both power levels were quantifiable and not significantly different to those from the corresponding 1% sucrose containing e-liquid.

AP yields were found to be at significantly lower levels than the DA emissions, but AP emissions also increased as sucrose concentration and power increased. At 10 W, AP emissions were only quantifiable at and above 5% sucrose. At 20 W coil power, quantifiable yields of AP were observed for sucrose

concentrations of 0.4% and above, other than one of the 1% sucrose solution measurements. AP emissions from the 1% fructose and 1% glucose e-liquids at 20 W power levels were quantifiable and not significantly different to those from the corresponding 1% sucrose containing e-liquid.

When the e-cigarette was disassembled after analysis, considerable char formation was observed on the coil and wick. The observable char level increased with increasing sugar level but was variable from device to device. This may have contributed to the scatter in the results for both DA and AP analyses.

Pyrolysis Screening Study

The structures of the compounds investigated in the pyrolysis experiments are presented in **Figure 4**, and the results of these experiments are shown in **Table 6** for both DA and AP. **Table 6** is

TABLE 6 | Presence or absence of acetyl propionyl or diacetyl in pyrolysis screening experiments on flavor compounds.

Compound	250°C		350°C		450°C		550°C	
	DA	AP	DA	AP	DA	AP	DA	AP
VG	N	N	N	N	Y	T	Y	Y
PG	N	N	N	N	T	N	T	T
Furaneol in PG + VG	Y	Y	Y	Y	Y	Y	Y	Y
Furaneol 15% in PG	Y	Y	Y	Y	Y	Y	Y	Y
EHM-furanone in PG + VG	N	T	N	Y	Y	Y	—	—
EHM-furanone (neat)	T	Y	Y	Y	—	—	—	—
Mesifurane (neat)	Y	Y	Y	Y	—	—	—	—
Furaneol acetate (neat)	Y	Y	Y	Y	—	—	—	—
Ethyl maltol (neat)	—	—	N	N	N	N	—	—
Ethyl maltol + VG	—	—	N	N	T	T	—	—
Cyclotene in PG + VG	N	N	N	N	T	T	—	—
Cyclotene (neat)	—	—	N	N	N	N	—	—
1,8-Cineole (neat)	—	—	N	N	N	N	—	—
Vanillin (neat)	—	—	N	N	N	N	—	—
Ethyl vanillin (neat)	—	—	N	N	N	N	—	—
Vanillin PG acetal (neat)	—	—	N	N	N	N	—	—
Ethyl vanillin PG acetal (neat)	—	—	N	N	N	N	—	—
4-Ketoisophorone (neat)	—	—	N	N	N	N	Y	Y
β -Damascone (neat)	—	—	N	N	—	—	—	—
Peppermint oil (neat)	—	—	N	N	N	N	T	T

Y, present; N, not found; T, trace present in chromatogram

constructed so as to indicate the presence or absence of the compounds in a specific pyrolysis experiment at a specific temperature. When the presence of DA or AP was detected in the pyrolyzates, it was at an extremely low level, and the diketones were not, by far, the major decomposition products of the pyrolyzed compound. The levels of DA and AP were generally 10^6 – 10^9 times less than the parent pyrolyzed compound. The results from **Table 6** describe specific behavior upon heating as follows:

Aerosol Formers

VG did not generate either DA or AP in the pyrolysis experiments when heated at temperatures up to 350°C. However, when heated at higher temperatures traces of the two compounds were observed, with greater levels of formation at higher temperatures, consistent with **Figure 2**. PG was more stable than VG to heating, however, traces of DA and AP were also found at temperatures starting at 450°C.

Furanose Ring Compounds

Furaneol was found to form DA and AP when pyrolyzed at temperatures as low as 250°C. Mesifurane was even more unstable to heating than furaneol, and both DA and AP formation was observed at 250°C. Furaneol acetate was also more unstable to heating than furaneol and formed DA and AP starting at 250°C. 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone (EHM-furanone in **Table 6**) was more stable compared to furaneol and formed DA only when heated to about 450°C. However, the formation of AP from 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone started as low as 250°C (in traces) and the AP yield increased at higher temperatures.

Six-Membered Ring Flavor Compounds

Ethyl maltol, cyclotene, 1,8-cineole, vanillin, ethyl vanillin, vanillin PG acetal, ethyl vanillin, and PG acetal did not form DA or AP (when pyrolyzed at temperatures up to 450°C), and β -damascone did not form DA or AP (at least up to 350°C). Similarly, 4-ketoisophorone formed DA and AP at 550°C but not lower temperatures. The sample of 4-ketoisophorone evaluated in this study also showed a trace of 2,4-pentanedione in the GC trace. Traces of DA and AP were detected in the pyrolyzate of peppermint oil at 550°C but not at lower temperatures.

DISCUSSION - SOURCES OF AC, AP AND DA IN E-CIGARETTE AEROSOLS

Direct Transfer From e-Liquids

Transfer of these species from e-liquids in which they are present to e-cigarette aerosols were found to be less than 100%, other than the case of DA formed by AC in e-liquids. Transfer of the hydroxyketone AC was greater (92.5%) than found with the di-ketones DA (63.4%) and AP (67.1%). This is a similar finding to the relative stabilities of the three species found when they were stored in nicotine-containing e-liquids (Vas et al., 2019). The only other study to report transfer levels of DA and AP from e-liquids to e-cigarette aerosols was that of Farsalinos et al. (2015), who conducted limited transfer experiments (three data points) without the extended e-liquid storage times of the present study. Farsalinos et al. (2015) reported near-quantitative transfer of AP and DA to the aerosol. Using the regression equations reported by the authors and the 1,000 $\mu\text{g}/\text{ml}$ initial e-liquid concentrations of the present study, suggests transfer efficiencies of 83% for AP and 86% for DA. These estimates are higher than the values reported in the present study, but the test e-liquid used by Farsalinos et al. (2015) did not contain nicotine. The reactivity of AP and DA in e-liquids has been shown to be strongly enhanced by basic materials such as nicotine (Vas et al., 2019), and it is highly plausible that the presence of nicotine in the current study e-liquids would have led to the lower stabilities of AP and DA found here.

Thermal Generation From Major e-Liquid Constituents

In the present study, increasing e-cigarette power from 10 to 20 W was found to produce a fairly constant coil temperature of about 255°C for VG/water (VG B.Pt. 290°C). The coil temperature was also relatively constant at about 182°C with PG/water (PG B.Pt. 188°C). For 1,3 PD/water (1,3 PD B.Pt. 213°C) coil temperatures increased from about 200 to 240°C as power was increased. The relatively steady temperatures at these lower power settings, at around or just below the boiling point of the pure polyol, indicates that sufficient liquid is reaching the coil for stable aerosolisation. The sharp increases in temperature observed at higher power settings probably indicate that the e-liquids can no longer stabilize the coil

temperature and overheating is occurring within the atomizer (Geiss et al., 2016).

The levels of AC in the aerosol did not increase significantly for any of the e-liquids up to the maximum coil temperatures achieved, although a single quantifiable value was recorded with VG/water at 394°C. Thermal production of acetoin from these three aerosol formers does not therefore appear to be a viable process in e-cigarettes.

In contrast, emissions of AP were quantifiable from the VG-containing and 1,3-PD-containing e-liquids at the highest coil temperatures, although only the emissions from the VG/water e-liquid showed significant increases over the lower temperature values. VG also had a greater potential to generate AP (and DA) compared to PG and 1,3 PD even though there was a lower concentration of VG (75%) in the e-liquid tested, compared with the concentrations of 91% for both PG and 1,3 PD. DA was generated at coil temperatures over 293°C for VG/water and over 234°C for PG/water. However, DA emissions from 1,3-PD/water were not significantly different from baseline values at up to 441°C.

In the experiments with sugar containing e-liquids, quantifiable yields of DA and AP were observed at 20 W (but not 10 W) from the control e-liquids with no sugar content. One possibility for this observation is that one or more of the contaminants in the solution may have contributed to the DA in the aerosol. DA and AP were not detected in the e-liquid, thereby removing the possibility of direct transfer as the source of these compounds. Alternatively, AC might be oxidised to DA during aerosol formation, however the concentration of AC in the e-liquid (0.565 µg/g) would contribute less than 0.0015 µg/puff even with all the AC oxidised to DA and with 100% transfer. The other contaminants in the e-liquid have shorter carbon chains than DA and AP and would require an associative reaction to form diketones. Martinuzzi et al., (2014) proposed that methylglyoxal was an intermediate in the formation of DA during gas-phase catalytic dehydration of VG, but the e-liquid methylglyoxal concentration in the present study was too low to account for the DA yield in the aerosol. It is therefore more likely that thermal decomposition of VG itself is the source of DA and AP in the sucrose-free liquids.

Three studies have examined the thermal generation of DA (and one examined AP) from VG or PG (Behar et al., 2016; Sleiman et al., 2016; Melvin et al., 2020) in e-cigarettes. Sleiman et al. collected between one and five puffs, sampled both “early” i.e. between the 1st and 5th puffs, or “late” i.e. between the 30th and 40th puff in their experiment. They identified DA in aerosols generated from neat VG and PG as well as from commercial, flavored e-liquids. VG generated 45 and 179 ng/puff respectively from the “early” and “late” puffs. PG generated 113 and 586 ng/puff. Behar et al., (2016) also identified the presence of DA in the aerosol from e-cigarettes containing DA-free e-liquids. Melvin et al., (2020) conducted model studies using a microwave heater to heat an e-liquid at 180°C for 1–15 min, as well as using the same system to examine temperatures over the range 80–220°C with a 3 min heating time. They found that both VG and PG could generate DA under these conditions, via a thermal degradation mechanism involving hydroxyacetone. The

production of DA was accelerated by the presence of nicotine. The authors also compared DA and AP e-liquid contents and aerosol emissions from eight cigalike e-cigarettes, and found increased levels of DA in the aerosol samples over and above the e-liquid levels, but little evidence of increased levels of AP. The authors suggested that thermal generation of DA was occurring, and it followed a different or faster mechanistic pathway to that required to thermally generate AP. Our study results support the findings of Melvin et al., (2020), but also provides more realistic temperature and time conditions to establish the thermal conditions required in an e-cigarette to generate these ketones from the major aerosol formers.

Other authors have noted the formation of DA from the aerosol carriers. For example, studies of gas-phase glycerol dehydration using acid-catalysts have shown that DA can be formed at temperatures of about 300°C. Lauriol-Garbay et al. (2011) vaporised a 20% aqueous glycerol solution with an inert gas flow and passed it over Zr/Nb mixed oxide catalysts at 280–300°C. The major product was AC, but increased concentrations of DA were observed as the temperature increased. Selectivity for production of DA increased with temperature from 0% at 280°C, to 0.3% at 290°C and 0.8% at 300°C. Similarly, Martinuzzi et al. (2014) studied glycerol dehydration at 270–308°C over a solid acid catalyst, with up to 6% oxygen in the gas stream. DA was found as a reaction product with a selectivity ranging from 0.015 to 0.061%. By passing a number of potential intermediates and fragmentation products through the catalytic system, they found that methylglyoxal, an intermediate in the thermal breakdown of glycerol, was a major precursor for DA with a product selectivity of 5%. However, the precise pathway from methylglyoxal to DA was not elaborated.

Together these data confirm that thermal generation of DA and AP from the most common e-cigarette aerosol carriers can occur, providing threshold temperatures are reached. Given the similar temperature profiles noted for formation of DA in the present study and for the catalytic studies described above, we think that the possibility of DA formation from glycerol through a surface reaction on the coil, possibly via methylglyoxal or hydroxyacetone is a hypothesis that is worthwhile investigating in future studies.

Thermal Generation From Minor e-Liquid Constituents (Flavor Compounds and Sugars)

The transfer experiments of the present study showed the presence of AC in the aerosol from an e-liquid containing DA, despite AC not being detected in the e-liquid. Aerosol AC levels were sufficiently high that they would have been detectable in the e-liquid if they had been present. Data reported by Vas et al., (2019) did not find any conversion of DA to AC at room temperature storage conditions. The simplest explanation for the observation from the present study is to hypothesise thermal reduction of DA to AC at the temperatures found in the e-cigarette atomizer. Further work is needed to investigate this potential mechanism more fully.

Our pyrolysis experiments showed results with PG and VG that were consistent with the findings from our e-cigarette thermal generation studies, despite the differing temperature and time conditions operating in the two studies. A clear finding from the pyrolysis experiments was the relative ease of formation of DA and AP from compounds containing a furanone ring structure, compared to various flavor compounds based on 6-membered ring structures. The importance of ring size identified in this experiment is highlighted by the comparison of furaneol and ethyl maltol, both of which have heterocyclic ring structures with ketone side groups. With furaneol, DA and AP were identified at a pyrolysis temperature of 250°C, whereas pure ethyl maltol did not show evidence for DA and AP formation even at a pyrolysis temperature of 450°C. Five-membered ring compounds are generally regarded as being under greater internal strain than six-membered rings, and it is likely that ring opening can occur at lower temperatures with the furanone ring compounds than with the six-membered ring compounds. The possibility of DA and AP generation from other flavor compounds has received little attention to date, although Behar et al., 2016 found DA was formed as a secondary reaction product of aerosolized e-liquids containing cinnamaldehyde, benzyl alcohol and triacetin. However, the authors did not distinguish between possible formation from flavor compounds or aerosol formers.

Our data also demonstrated that DA and to a lesser extent AP were generated from e-liquids containing sucrose, glucose and fructose. Thermal decomposition of sucrose is well characterised (Monte and Maga 1981) and can take place at temperatures as low as 150–200°C via fragmentation to glucose and fructose with the loss of water. As the temperature increases, caramelisation occurs with the monosaccharides either decomposing to a large variety of smaller molecules including the diketones, DA and AP (Monte and Maga 1981), or oligomerizing to larger molecules with further loss of water. Continued loss of hydrogen and oxygen (as water) eventually leads to the formation of char. There is some debate as to whether the diketones are primarily formed from the backbones of the monosaccharides or from recombination of smaller fragments. A recent study of diketone formation from coffee beans infused with ¹³C-labeled sucrose and roasted at 200°C showed that diacetyl was mostly formed from recombined sucrose C₂ fragments while AP was formed from the sucrose skeleton (Poisson et al., 2018). Interestingly, glucose is a six-membered ring molecule, and fructose exists in solution as a mixture of the five-membered ring compound β-D-fructofuranose, and the six membered ring compound β-D-fructopyranose. Sucrose, fructose and glucose provided similar DA and AP emissions, in contrast to the findings from our pyrolysis experiments of significantly easier production of DA and AP from five-membered ring compounds. The most likely explanation of this is that the flavor compounds examined in our pyrolysis experiments are volatile and can evaporate away from hot metal surfaces, whereas the involatile sugars are unable to leave the heated coil area and thermally decompose. Exposure of sugars to the temperatures of e-cigarette coils at certain power settings appear sufficient to generate diketones. The similarity of diketone emissions from sucrose, fructose and glucose e-liquids

supports the mechanism proposed by Monte and Maga (1981) of sucrose thermal degradation proceeding via fructose and glucose production prior to DA/AP generation.

Assessing the likely contribution of sugars to DA and AP emissions from e-cigarettes requires an understanding of the sugar levels found in e-liquids. Kubica et al., (2014) determined sucrose levels in 37 e-liquid samples from seven manufacturers. With a detection limit of 0.73 µg/g, sucrose was found in all the samples with concentrations ranging from 0.76 to 72.93 µg/g. Most (78%) of the samples had less than 20 µg/g of sucrose. The liquids were also analysed for the disaccharides, maltose and lactose, and the monosaccharides, glucose and fructose, but none of the samples contained sugars other than sucrose. Fagan et al., (2017) analysed 66 e-liquids for sugars and aldehydes. With LOQs of 6 µg/ml for glucose and fructose, and 12 µg/ml for sucrose, glucose was quantified in 22% of samples (range: 6.4–88.9 µg/ml, median: <6 µg/ml), fructose in 53% of samples (range: 8.8–331.2 µg/ml, median: 9.7 µg/ml) and sucrose in 53% of samples (range: 9.3–620 µg/ml, median: 18.9 µg/ml).

These two reports show that commercial e-liquids have considerably lower sugar levels than were used in our present study. The lowest level used in our study was 0.05% sucrose, which is equivalent to 600 µg/ml. The highest sugar level found in the Fagan et al. study was 620 µg/ml, which is similar to the lowest level in our study, but the highest level reported by Kubica et al. was 72.9 µg/g sucrose, which is significantly below the lowest level used in our study (0.05%). At the lowest sugar level of our study, DA emissions were 15–37 ng/puff and AP emission were <LOD/<LOQ. Therefore, it appears that while sugar levels reported to be in commercial e-liquids may generate very low levels of DA, AP is unlikely to be generated at measurable levels.

Supplementary Table S4 collates published aerosol emission measurements of AC, AP and DA from commercial e-cigarettes. When available as a per-puff value, the published data is very consistent with the levels found in the experiments of the current study. This suggests that our study findings on the sources of these ketones in e-cigarette aerosols are very relevant to the levels of diketones found with commercial e-cigarettes, and may well point to reasons for the presence of AC, AP and DA in these published studies.

A limitation of the present study is that it was not possible to comprehensively characterize in a single exercise the ketone production potential of all ingredients added to e-cigarettes. While our study identifies some key sources and conditions that lead to ketone production, other potential sources such as other flavour compounds, ethanol and surface reactions at metal coils should also be examined in future studies for their relevance to ketone production during vaping.

CONCLUSION

Our results show that AC added to e-liquids is transferred efficiently (>90%) to the aerosol, while transfer efficiencies of AP and DA from e-liquids are lower (ca. 65%), indicating

some losses during the thermal processes leading to aerosolisation.

Although thermal degradation of VG can potentially contribute to DA in the aerosol, DA was not detected in aerosols generated at less than 16 W in the e-cigarette used. Significant quantities are only produced at coil temperatures which are much higher than normally achieved during vaping. Quantifiable levels of DA from PG were only found in the aerosol at even higher coil temperatures, while 1,3 PD produced very little DA under any of the conditions studied.

Sucrose, glucose and fructose were also found to generate DA in the aerosols. Quantifiable amounts of DA were found in the aerosol generated at 10W from e-liquids containing sucrose at levels of 0.05%, the lowest concentration studied. DA emissions generally increased with the concentration of the sugar in the e-liquid and with the power supplied to the coil. Our experiments indicated that glucose and fructose have a similar potential to sucrose in generating DA when heated. These observations are important since in addition to direct sugar addition to e-liquids, they may be present in e-liquids if manufacturers incorporate natural fruit and plant extracts. Pyrolysis experiments demonstrated that compounds containing five-membered furanose rings can easily generate AP and DA on heating. Our findings should be considered by manufacturers selecting flavor compounds for use in e-liquids, in order to minimize diketone exposure amongst e-cigarette users.

REFERENCES

- AFNOR (2016). *XP D90-300-3 – (2016); Cigarettes électroniques et e-liquides – Partie 3 : exigences et méthodes d'essais relatives aux émissions*. Association Française de Normalisation (AFNOR), Saint-Denis, France.
- Allen, J. G., Flanagan, S. S., LeBlanc, M., Vallarino, J., MacNaughton, P., Stewart, J. H., et al. (2016). Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes. *Environ. Health Perspect.* 124, 733–739. doi:10.1289/ehp.1510185
- Aspire (2016). Nautilus Mini. [Online]. Accessed 02.11.16. available at: <http://www.aspirecig.com/products/tank-series/tank156.html>.
- Bao, M. (2015). *Analysis of Selected Carbonyl Compounds in E-Liquids and E-Aerosols by Using Pentafluorobenzylhydroxylamine Derivatization and Gas Chromatography-Mass Spectrometry*. Juju, South Korea: CORESTA SSPT Meeting.
- Barhaddi, S., Canfyn, M., Courselle, P., Rogiers, V., Vanhaecke, T., and Deconinck, E. (2017). Development and Validation of a HS/GC-MS Method for the Simultaneous Analysis of Diacetyl and Acetylpropionyl in Electronic Cigarette Refills. *J. Pharm. Biomed. Anal.* 142, 218–224. doi:10.1016/j.jpba.2017.04.050
- Behar, R. Z., Luo, W., Lin, S. C., Wang, Y., Valle, J., Pankow, J. F., et al. (2016). Distribution, Quantification and Toxicity of Cinnamaldehyde in Electronic Cigarette Refill Fluids and Aerosols. *Tob. Control.* 25, ii94–ii102. doi:10.1136/tobaccocontrol-2016-053224
- Chen, W., Wang, P., Ito, K., Fowles, J., Shusterman, D., Jaques, P. A., et al. (2018). Measurement of Heating Coil Temperature for E-Cigarettes with a "Top-Coil" Clearomizer. *PLOS One* 13 (4), e0195925. doi:10.1371/journal.pone.0195925
- Clark, S., and Winter, C. K. (2015). Diacetyl in Foods: a Review of Safety and Sensory Characteristics. *Compr. Rev. Food Sci. Food Saf.* 14, 634–643. doi:10.1111/1541-4337.12150
- Czoli, C. D., Goniewicz, M. L., Palumbo, M., Leigh, N., White, C. M., and Hammond, D. (2019). Identification of Flavouring Chemicals and Potential

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KM and AP co-wrote the publication. GW, JM, SM, and CV managed the individual elements of the experimental programme. AC, KM, and HD directed the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.742538/full#supplementary-material>

- Toxicants in E-Cigarette Products in Ontario, Canada. *Can. J. Public Health* 110, 542–550. doi:10.17269/s41997-019-00208-1
- ELeaf (2016). eLeaf iStick 30W Product. [Online] Available at: <https://www.eleafworld.co.uk/istick-30w-battery> (Accessed 08 31, 21).
- Fagan, P., Pokhrel, P., Herzog, T. A., Moolchan, E. T., Cassel, K. D., Franke, A. A., et al. (2017). Sugar and Aldehyde Content in Flavored Electronic Cigarette Liquids. *Nicotine Tobacco Research*, 985–992. doi:10.1093/ntr/ntx234
- Farsalinos, K. E., Kistler, K. A., Gillman, G., and Voudris, V. (2015). Evaluation of Electronic Cigarette Liquids and Aerosol for the Presence of Selected Inhalation Toxins. *Nicotine Tob. Res.* 17 (2), 168–174. doi:10.1093/ntr/ntu176
- Geiss, O., Bianchi, I., and Barrero-Moreno, J. (2016). Correlation of Volatile Carbonyl Yields Emitted by E-Cigarettes with the Temperature of the Heating Coil and the Perceived Sensorial Quality of the Generated Vapours. *Int. J. Hyg. Environ. Health* 219, 268–277. doi:10.1016/j.ijheh.2016.01.004
- Klager, S., Vallarino, J., MacNaughton, P., Christiani, D. C., Lu, Q., and Allen, J. G. (2017). Flavoring Chemicals and Aldehydes in E-Cigarette Emissions. *Environ. Sci. Technol.* 51 (18), 10806–10813. doi:10.1021/acs.est.7b02205
- Kubica, P., Wasik, A., Kot-Wasik, A., and Namieśnik, J. (2014). An Evaluation of Sucrose as a Possible Contaminant in E-Liquids for Electronic Cigarettes by Hydrophilic Interaction Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Bioanal. Chem.* 406, 3013–3018. doi:10.1007/s00216-014-7690-2
- Lauriol-Garbay, P., Millet, J. M. M., Lorient, S., Bellière-Baca, V., and Rey, P. (2011). New Efficient and Long-Life Catalyst for Gas-phase Glycerol Dehydration to Acrolein. *J. Catal.* 280, 68–76. doi:10.1016/j.jcat.2011.03.005
- LeBouf, R. F., Burns, D. A., Ranpara, A., Attfield, K., Zwack, L., and Stefaniak, A. B. (2018). Headspace Analysis for Screening of Volatile Organic Compound Profiles of Electronic Juice Bulk Material. *Anal. Bioanal. Chem.* 410, 5951–5960. doi:10.1007/s00216-018-1215-3
- Margham, J., McAdam, K., Forster, M., Liu, C., Wright, C., Mariner, D., et al. (2016). Chemical Composition of Aerosol from an E-Cigarette: a Quantitative Comparison with Cigarette Smoke. *Chem. Res. Toxicol.* 29, 1662–1678. doi:10.1021/acs.chemrestox.6b00188

- Martinuzzi, I., Azizi, Y., Devaux, J.-F., Tretjak, S., Zahraa, O., and Leclerc, J.-P. (2014). Reaction Mechanism for Glycerol Dehydration in the Gas Phase over a Solid Acid Catalyst Determined with On-Line Gas Chromatography. *Chem. Eng. Sci.* 116, 118–127. doi:10.1016/j.ces.2014.04.030
- Melvin, M. S., Avery, K. C., Ballentine, R. M., Flora, J. W., Gardner, W., Karles, G. D., et al. (2020). Formation of Diacetyl and Other α -Dicarbonyl Compounds during the Generation of E-Vapor Product Aerosols. *ACS Omega* 5 (28), 17565–17575. doi:10.1021/acsomega.0c02018
- Moldoveanu, S. C., Hudson, A. G., and Harrison, A. (2017). The Determination of Diacetyl and Acetylpropionyl in Aerosols from Electronic Smoking Devices Using Gas Chromatography Triple Quad Mass Spectrometry. *Beiträge zur Tabakforschung Int.* 27 (7), 145–153. doi:10.1515/cttr-2017-0015
- Monte, W. C., and Maga, J. A. (1981). Flavor Chemistry of Sucrose. *Sugar Technol. Rev.* 8, 181–204.
- Myeliquid (2021). *Is there sugar in e-liquid?* Available at: <https://www.myliquid.ca/blogs/myeliquid/is-there-sugar-in-e-liquid> (Accessed September 10, 2021).
- National Academies of Sciences Engineering, and Medicine (2018). *Public Health Consequences of E-Cigarettes*. Washington, DC: The National Academies Press. doi:10.17226/24952
- NIOSH (2016). Criteria for a recommended standard: occupational exposure to diacetyl and 2,3-pentanedione DHHS (NIOSH) Publication Number 2016-111. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. Available at: <https://www.cdc.gov/niosh/docs/2016-111/default.html> (Accessed May 16, 2018).
- Pankow, J. F., Kim, K., Luo, W., and McWhirter, K. J. (2018). Gas/Particle Partitioning Constants of Nicotine, Selected Toxicants, and Flavor Chemicals in Solutions of 50/50 Propylene Glycol/glycerol as Used in Electronic Cigarettes. *Chem. Res. Toxicol.* 31 (9), 985–990. doi:10.1021/acs.chemrestox.8b00178
- Poisson, L., Auzanneau, N., Mestdag, F., Blank, I., and Davidek, T. (2018). New Insight into the Role of Sucrose in the Generation of α -Diketones upon Coffee Roasting. *J. Agric. Food Chem.* 66 (10), 2422–2431. doi:10.1021/acs.jafc.6b04849
- PubChem (2018). *National Center for Biotechnology Information*. Bethesda MD, 20894 USA: U.S. National Library of Medicine, 8600 Rockville Pike. Available at: <https://pubchem.ncbi.nlm.nih.gov/compound/962#section=Top> (Accessed May 17, 2018).
- Schripp, T., Markewitz, D., Uhde, E., and Salthammer, T. (2013). Does E-Cigarette Consumption Cause Passive Vaping?. *Indoor Air* 23, 25–31. doi:10.1111/j.1600-0668.2012.00792.x
- Shahab, L., Goniewicz, M. L., Blount, B. C., Brown, J., McNeill, A., Alwis, K. U., et al. (2017). Nicotine, Carcinogen, and Toxin Exposure in Long-Term E-Cigarette and Nicotine Replacement Therapy Users. *Ann. Intern. Med.* 166 (6), 390–400. doi:10.7326/M16-1107
- Sleiman, M., Logue, J. M., Montesinos, V. N., Russell, M. L., Litter, M. I., Gundel, L. A., et al. (2016). Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. *Environ. Sci. Technol.* 50 (17), 9644–9651. doi:10.1021/acs.est.6b01741
- Soussy, S., El-Hellani, A., Baalbaki, R., Salman, R., Shihadeh, A., and Saliba, N. A. (2016). *Tob Control* 25, ii88–ii93.
- Uchiyama, S., Noguchi, M., Sato, A., Ishitsuka, M., Inaba, Y., and Kunugita, N. (2020). Determination of Thermal Decomposition Products Generated from E-Cigarettes. *Chem. Res. Toxicol.* doi:10.1021/acs.chemrestox.9b00410
- Vape Club (2020). Sugar E-liquid. Available at: <https://www.vapeclub.co.uk/e-liquids/sugar> (Accessed February 6th, 2020).
- Vas, C. A., Porter, A., and McAdam, K. (2019). Acetoin Is a Precursor to Diacetyl in E-Cigarette Liquids. *Food Chem. Toxicol.* 133, 110727. doi:10.1016/j.fct.2019.110727
- Zhao, T., Shu, S., Guo, Q., and Zhu, Y. (2016). Effects of Design Parameters and Puff Topography on Heating Coil Temperature and Mainstream Aerosols in Electronic Cigarettes. *Atmos. Environ.* 134, 61–69. doi:10.1016/j.atmosenv.2016.03.027

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Isotope-Dilution Gas Chromatography-Mass Spectrometry Method for the Selective Detection of Nicotine and Menthol in E-Cigarette, or Vaping, Product Liquids and Aerosols

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We developed a quantitative method for analyzing nicotine and menthol in e-cigarette, or vaping, products (EVPs). These products may adversely impact health through inhalational exposure to addictive and harmful chemicals. The presence of unknown substances in do-it-yourself e-liquids, counterfeits, or unregulated products may increase exposure to harmful chemicals, as underscored by the 2019 EVP use-associated lung injury (EVALI) outbreak. To minimize these risks, it is important to accurately quantify nicotine and menthol in e-liquids and aerosol emissions to evaluate EVP authenticity, verify product label accuracy, and identify potentially hazardous products. We developed a simple, versatile, high-throughput method using isotope-dilution gas chromatography-mass spectrometry for quantifying nicotine and menthol concentrations in both e-liquid contents and machine-generated aerosol emissions of EVPs. Rigorous validation has demonstrated that the method is specific, precise (CV<2.71%), accurate (percent error ≤7.0%), and robust. Linear calibration ranges from 0.01 to 1.00 mg/ml for both analytes was achieved, corresponding to expected analyte levels in e-liquids and machine-generated EVP aerosols. Limits of detection (LODs) in the final 10-ml sample extract were 0.4 µg/ml for nicotine and 0.2 µg/ml for menthol. The method was used to analyze aerosol emissions of 141 EVPs associated with the 2019 EVALI outbreak; detectable levels of nicotine (2.19–59.5 mg/g of aerosol) and menthol (1.09–10.69 mg/g of aerosol) were observed in 28 and 11%, respectively, of the samples analyzed. Nicotine was not detected in any of the tetrahydrocannabinol (THC), cannabidiol (CBD), or oil-based products, while menthol (2.95 mg/g of aerosol) was only detected in one of these products (THC-labeled). The analytical method can be used to quantify nicotine and menthol concentrations in the e-liquids and aerosols from a range of EVPs, and these findings highlight a difference between e-cigarette and other vaping products.

Keywords: e-cigarettes, vapes, nicotine, menthol, gas chromatography-mass spectrometry

INTRODUCTION

E-cigarette, or vaping, product (EVP) use has increased substantially in the United States over the last few years, both for traditional atomizer/cartomizer e-cigarettes designed to deliver nicotine (Wang et al., 2020) and for more recent ceramic-cell vape products designed to deliver cannabinoids (Knapp et al., 2019). These increases are likely driven by a number of factors and are primarily impacting youth and young adults (King et al., 2020). The 2019 U.S. e-cigarette, or vaping, product use-associated lung injury (EVALI) outbreak (Centers for Disease Control and Prevention Office on Smoking and Health, 2019) underscored the diversity of products used by many EVALI case patients (Trivers et al., 2021). Furthermore, many young EVP users tend to experiment with products from unregulated/online vendors as well as do-it-yourself (DIY) liquids (Cox et al., 2019). These unregulated products have unknown health consequences and their chemical ingredients—including their origin, quality, and safety—may also be unknown. For example, the EVALI outbreak was strongly associated with vitamin E acetate which was being used as a diluent in EVPs (Blount et al., 2020; Krishnasamy et al., 2020; Puetz et al., 2021). Other chemical constituents in these unregulated products may also contribute to adverse health outcomes.

Analytical methods are needed to measure nicotine and menthol accurately and precisely in different types of e-liquids, vape liquids, and EVP aerosols. Nicotine is the primary addictive chemical in tobacco products and its accurate quantitation is critical for establishing product authenticity, verifying product-label accuracy, and assessing addiction potential of a product. Menthol, which is used as a tobacco product flavor additive (U.S. Food and Drug Administration, 2021a), may contribute to adverse health outcomes by altering users' smoking behavior (Watson et al., 2017) and the U.S. Food and Drug Administration has declared its intention to ban its use in cigarettes (U.S. Food and Drug Administration, 2021b). Various methods have been described to measure nicotine concentrations in traditional atomizer/cartomizer devices and in hydrophilic solvents such as propylene glycol (PG) and glycerol (GLY) (Trehly et al., 2011; Goniewicz et al., 2013; Famele et al., 2015; Lisko et al., 2015; Ogunwale et al., 2017; Gholap et al., 2018; Reilly et al., 2020). However, these existing methods have not been validated for measuring nicotine and menthol in hydrophobic, oil-based liquids and aerosols that can result from DIY mixing of ingredients and from using different types of liquids in the same device. We describe the development, validation, and application of a new, simple, sensitive, high-throughput, and selective isotope-dilution gas chromatography-mass spectrometry (ID-GC-MS) method for the simultaneous quantitation and characterization of nicotine and menthol in e-liquids and machine-generated aerosol emissions of EVPs. This method was used to quantitatively analyze aerosol emissions from 141 EVPs associated with the 2019 EVALI outbreak. To enable analysis of a broader range of EVPs with differing chemical compositions, the new method was validated for quantitative analysis in both hydrophilic and hydrophobic

e-liquid matrices. This is the first report of these analytes quantitatively measured in samples from the EVALI response.

MATERIALS AND METHODS

Chemicals and Materials

(-)-Nicotine [CAS# 54-11-5; ≥99% (GC), liquid] and isotopically labeled ($\pm$)-nicotine-(pyridine- d_4) internal standard (ISTD; CAS# 350818-69-8; isotopic purity: ≥98 atom% D, ≥98% chemical purity) were purchased from Sigma-Aldrich (St. Louis, MO, United States). L (-)-menthol (CAS# 2216-51-5; 99.5%; category 1 standard), PG (CAS# 57-55-6; ≥99.5%; USP/FCC), GLY (CAS# 56-81-5; ≥99.5%; certified ACS), and methanol (MeOH; CAS# 67-56-1; ≥99.9%; HPLC grade) were purchased from Thermo Fisher Scientific (Waltham, MA, United States). The isotopically labeled (-)-menthol-(1,2,6,6- d_4) ISTD (98%) was obtained from Cambridge Isotope Laboratories (Andover, MA, United States). Research grade helium was purchased from Airgas Inc. (Hapeville, GA, United States).

Cambridge filter pads (CFPs; 44-mm) for collecting machine-generated EVP aerosol emissions were purchased from Thermo Fisher Scientific (Waltham, MA, United States). CFP holders were purchased from Cerulean (Molins PLC, Milton Keynes, United Kingdom). Custom-made adapters ("lips") used for vaping uniquely shaped device mouthpieces were fabricated in-house.

Standard and Quality Control Material Preparation

Isotopically Labeled ISTD

A combined nicotine- d_4 and menthol- d_4 ISTD spiking solution was prepared in MeOH with concentrations of 10 mg/ml for each isotopically labeled standard. A 100- μ L aliquot of this ISTD spiking solution was added to calibration standards, blanks, unknowns, and quality control (QC) samples.

Native Standards

A 200-mg/ml menthol stock solution in MeOH was prepared and used to make a combined nicotine and menthol stock solution with a concentration of 4 mg/ml for each analyte. We used this combined nicotine/menthol solution to prepare seven calibration standards with a concentration range of 0.010–1.000 mg/ml for both analytes and prepared with 100 μ L of the ISTD spiking solution.

QC Materials

We prepared PG/GLY mixtures spiked with low (QCL; 2.5 mg/g) and high (QCH; 80 mg/g) nicotine and menthol concentrations to serve as matrix-based QC materials that spanned the calibration range. First, a 70/30 (v/v) PG/GLY mixture was prepared. QCL was then prepared by combining approximately 62.5 mg nicotine, 62.5 mg menthol, and 25 g of the 70/30 (v/v) PG/GLY mixture. QCH was prepared by combining approximately 2 g nicotine, 2 g menthol, and 21 g

of the 70/30 (v/v) PG/GLY mixture. The QC concentrations were characterized to determine the mean concentrations and the 95th (1.96 σ) and 99th (2.96 σ) control limits by duplicate analysis of 19 samples of each QC level over at least 19 days. A 100-mg aliquot of each QC pool was extracted and analyzed concurrently with sample unknowns and the resulting QC data were compared to the established control limits to evaluate the validity of analyses using a set of modified Westgard rules (Westgard et al., 1981; Caudill et al., 2008).

Aerosols/Vaping

Aerosol samples were generated according to the standard conditions described in CORESTA Recommended Method No. 81 (CORESTA, 2015) (i.e., 55 ± 0.3 ml puff volume, 3 ± 0.1 s puff duration, 30 ± 0.5 s puff interval, with a square wave puff profile) using a Cerulean CETI-8 e-cigarette vaping machine equipped with button activation switches (Cerulean, Richmond, VA, United States). We calibrated/verified the vaping machine puff volume before each use using a soap-bubble meter. Fifteen (15) puffs were taken from EVPs and the resulting aerosol was collected on individual sample CFPs; mass differences of pre- and post-vaping CFPs for a given sample [i.e., trapped total particulate matter (TPM)] were then determined gravimetrically ($d = 0.00001$ g). Post-vaped CFPs were removed from CFP holders and placed into 16-ml vials for extraction.

EVP e-Liquids Sampling

For the routine analysis of e-liquids, a 100- μ L sample was transferred from a given product cartridge or refill container to a 16-ml extraction vial. The masses of the liquid sample aliquots were recorded.

Sample Preparation

Sample vials containing blanks, QCs, and post-vaped CFPs and/or liquid unknowns were spiked with 100- μ L of the MeOH-based nicotine- d_4 and menthol- d_4 isotopically labeled ISTD spiking solution. Ten milliliters (10 ml) of MeOH were then added to each vial and all samples were placed on an orbital shaker for 10 min at 160 rpm. Aliquots of extract were transferred to GC autosampler vials for analysis.

Instrumental Analysis

For ID-GC-MS analysis, we used an Agilent 7890A GC interfaced to an Agilent 5975C mass selective detector (MSD) MS (Agilent Technologies, Santa Clara, CA, United States) and equipped with a CTC PAL autosampler (LEAP Technologies, Carrboro, NC, United States). A 2- μ L aliquot of sample extract was injected onto a 30-m Agilent J&W DB-5MS capillary column with a 0.32-mm I.D. and 1.0- μ m film thickness using a 40:1 split injection. Helium was used as the carrier gas at a constant pressure of 10 psi. The injector and transfer line temperatures were set isothermally at 250 and 300°C, respectively. The initial column temperature, 150°C, was held for 1 min and then increased to 300°C at 30°C/min and held for 3 min. The MS was operated in positive electron ionization (+EI) mode and

TABLE 1 | SIM method parameters.

Analyte	Ion (m/z)	Dwell time (msec)	Ion type
Menthol	95.1	40	Quantitation
	123.1		Confirmation 1
	138.2		Confirmation 2
	99.1		ISTD
Nicotine	162.2	40	Quantitation
	133.1		Confirmation 1
	161.1		Confirmation 2
	166.2		ISTD

the resulting ions were analyzed using selected-ion monitoring (SIM). MS parameters were as follows: electron energy -70 eV, source temperature 230°C, quadrupole temperature 150°C, electron multiplier mode gain factor 1, mass resolution high.

We monitored one quantitation ion and two confirmation ions for each analyte and monitored an analogous isotopically labeled ISTD ion for the corresponding quantitation ion. **Table 1** summarizes the SIM ions monitored, dwell times, and ion type. Data acquisition was conducted using Agilent GC/MSD ChemStation software. ChemStation data files were converted for data processing using Thermo Fisher Scientific Xcalibur™ 2.2 software.

Quantitation

Calibration curves were constructed from the linear regression of the calibration standards' analyte-to-ISTD relative response ratios versus known standard concentrations, x , with $1/x$ weighting. The broad calibration concentration ranges used required weighting to improve the accuracy of the lower calibrators. For aerosol analysis, results were normalized by mass of TPM, puff count, and/or total puff volume to determine analyte yields per gram of TPM (mg/g TPM), per puff (mg/puff), or per unit volume (μ g/mL or mg/L), respectively. For e-liquid analysis, results were normalized by e-liquid sample mass to determine analyte levels per gram of sample (mg/g). Because the use of isotopically labeled ISTDs at the high nicotine and menthol concentrations typical of EVPs produces MS signals with increased potential for isotope contributions between native and isotopically labeled (ISTD) analogue ion channels, we implemented a correction factor (Colby and McCaman, 1979) (entered within the Xcalibur™ software quantitation method) to account for these contributions.

Method Validation

A method validation procedure was conducted to adequately assess method performance across a broad range of EVP matrices including both hydrophilic and hydrophobic e-liquids (PG/GLY and oil-based e-liquids, respectively). The figures of merit evaluated included analytical specificity, accuracy, dynamic range, LODs, matrix effects, and precision. A description of experiments and presentation of their results are described below.

TABLE 2 | Method accuracy (% error) in hydrophilic (PG/GLY) and hydrophobic (oil-based) matrix-based spiked solutions.

Analyte	Hydrophilic (PG/GLY; <i>n</i> = 5)		Hydrophobic (oil-based; <i>n</i> = 6)		
	2.5 mg/g	80 mg/g	0.025 mg/ml	0.125 mg/ml	0.750 mg/ml
Menthol	−3.0%	−1.9%	−2.5%	0.0%	−1.6%
Nicotine	4.8%	7.0%	3.6%	5.4%	5.6%

Application: Aerosol Analysis of Products Associated With the 2019 U.S. EVALI Outbreak

The described method was used to measure nicotine and menthol in aerosol emissions from a set of EVPs associated with the 2019 U.S. EVALI outbreak. We conducted aerosol emissions testing on 141 EVP samples, including various products containing nicotine, CBD, and THC. Corresponding e-liquid analysis was not performed on this sample set. A total of 194 EVALI-related samples were received; however, 35 samples did not contain sufficient volume for analysis, and 18 samples did not produce appreciable aerosol TPM deliveries. Products that generated aerosols of less than 6.5 mg TPM per 15 puffs were considered inoperative and their data excluded. Samples were machine-vaped as described in *Aerosols/Vaping*. Due to limited sample availability, only 15 puffs per product were collected. All samples were handled following proper guidelines for the handling and analysis of potentially illicit drugs. Sample chain-of-custody was maintained and documented.

RESULTS AND DISCUSSION

Figures of Merit

Analytical Specificity

The chromatographic specificity of the method was excellent based on baseline-resolved peaks in EVP chromatograms, the absence of interfering matrix components in representative EVP samples, and the use of isotopically labeled ISTDs. Specificity was further improved through selective detection *via* + EI and monitoring of three distinct ions (one quantitation ion and two confirmation ions) for each analyte. Retention-time monitoring and response ratios between quantitation and confirmation ions further contributed to method specificity.

LODs and Dynamic Range

Instrument LODs were determined based on the method described by Taylor (1987) and resulted in calculated LODs (based on final 10-ml extract concentrations) of 0.4 and 0.2 µg/ml for nicotine and menthol, respectively. For EVP liquids, these LODs correspond to 0.04 mg/ml for nicotine and 0.02 mg/ml for menthol in a 100-µL sample. For aerosols, however, TPM deliveries and nicotine/menthol concentrations vary from product-to-product, resulting in variable aerosol LODs that are based on product analyte concentrations and their respective deliveries. Despite this sample-to-sample variability,

the calculated LODs are well below expected EVP nicotine and menthol concentrations and aerosol deliveries, and we thus set the limit of quantitation as the lowest calibrator and used a calibration range of 0.01–1.00 mg/ml for both analytes. Despite their addition to e-liquids at considerably high concentrations (≥ 0.1 –5% w/w), lower analyte levels may be expected in products with low aerosol delivery (i.e., smaller sample size), as well as potential low-level products, particularly for menthol in products not obviously identified as menthol-containing.

Accuracy and Matrix Effects

Because the new method is intended for analyzing a variety of EVPs with e-liquids comprising PG/GLY (hydrophilic) and various hydrophobic solvents (e.g., medium chain triglycerides, vitamin E acetate, and others), we evaluated method accuracy by analyzing spiked matrix-matched samples [both hydrophilic (PG/GLY-based) and hydrophobic (oil-based products)] prepared with known concentrations of nicotine and menthol. **Table 2** shows the matrix-spiked accuracy of measurements for 1) the previously described PG/GLY-based QCs, and 2) matrix-based spiked solutions with low, mid, and high analyte concentrations diluted with a pooled MeOH extract of machine-generated aerosols from commercial oil-based e-liquids. Unfortunately, no certified reference materials are available for nicotine and menthol in either hydrophobic- or hydrophilic-based products. However, our spiked matrix experiments all yielded results within 7% of their respective known concentrations across the matrix compositions and analyte concentrations tested. The accurate quantitation of these matrix-based samples using a solvent-based calibration curve demonstrated the absence of matrix effects in both hydrophilic and hydrophobic matrices (**Table 2**). These results confirm the applicability and use of the described method for accurate measurements of nicotine and menthol in EVP liquids and/or aerosol samples of varying matrix composition.

Precision

Method precision was assessed as repeatability and intermediate precision in terms of percent relative standard deviations (% RSDs). Precision was assessed from the duplicate analysis of 19 sample sets of the QC materials (2.5 and 80 mg/g) over 19 different days. Repeatability was calculated as within-run variation of duplicates, while intermediate precision was calculated as the between-run, or total, variation. We observed excellent method repeatability (<0.5%) and intermediate precision (<3%) at both low and high concentrations for both analytes.

Application: Aerosol Analysis of Products Associated With the 2019 U.S. EVALI Outbreak

To demonstrate “fit for purpose,” we applied the described method to analyze aerosol samples from the 2019 U.S. EVALI outbreak (Blount et al., 2020). The method performed well for both hydrophilic (PG/GLY-based) and hydrophobic (e.g., oil-based medium chain triglycerides, vitamin E acetate, and others) e-liquids, demonstrating its versatility. Nicotine and menthol were detected at quantifiable concentrations in 28% (39 samples) and 11% (16 samples) of the 141 samples analyzed, respectively. Nicotine was only detected in PG/GLY-based products at concentrations ranging between 2.19 and 59.5 mg/g of aerosol TPM (0.2–6.0% nicotine) and was not detected in any of the THC or oil-based products. Of the 39 nicotine product samples, 15 also contained menthol with concentrations that ranged between 1.09 and 10.69 mg/g of aerosol TPM (0.1–1.1% menthol). Menthol was detected in only one THC-containing oil-based product at a concentration of 2.95 mg/g of aerosol (0.3% menthol). No nicotine was detected above LOD in any samples containing vitamin E acetate (the likely causal toxicant); thus, the EVALI outbreak is more closely associated with THC products than nicotine products (Blount et al., 2020).

CONCLUSION

The described ID-GC-MS method provides accurate and precise quantitation of nicotine and menthol concentrations in hydrophilic (PG/GLY-based) and hydrophobic (oil-based) e-liquids and machine-generated aerosols of EVPs. Application of the method for EVPs associated with the 2019 EVALI outbreak

and the detection of nicotine and menthol in a portion of these products demonstrate that the method is fit-for-purpose.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JP: methodology, validation, formal analysis, investigation, writing – original draft. CW: conceptualization, writing – review and editing, oversight. BB: conceptualization, visualization, funding acquisition, supervision, writing – review and editing. LV-B: visualization, supervision, project administration, funding acquisition, formal analysis, writing – review and editing.

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REFERENCES

- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382, 697–705. doi:10.1056/nejmoa1916433
- Caudill, S. P., Schleicher, R. L., and Pirkle, J. L. (2008). Multi-rule Quality Control for the Age-Related Eye Disease Study. *Statist. Med.* 27, 4094–4106. doi:10.1002/sim.3222
- Centers for Disease Control and Prevention Office on Smoking and Health (2019). Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products. [Online]. Available at: <https://www.cdc.gov/lunginjury> (Accessed 10 15, 2020).
- Colby, B. N., and McCaman, M. W. (1979). A Comparison of Calculation Procedures for Isotope Dilution Determinations Using Gas Chromatography Mass Spectrometry. *Biol. Mass. Spectrom.* 6, 225–230. doi:10.1002/bms.1200060602
- CORESTA (2015). *Recommended Method Number 81: Routine Analytical Machine for E-Cigarette Aerosol Generation and Collection – Definitions and Standard Conditions*. Paris, France: Cooperation Centre for Scientific Research Relative to Tobacco.
- Cox, S., Leigh, N. J., Vanderbush, T. S., Choo, E., Goniewicz, M. L., and Dawkins, L. (2019). An Exploration into “Do-it-yourself” (DIY) E-Liquid Mixing: Users’ Motivations, Practices and Product Laboratory Analysis. *Addict. Behav. Rep.* 9, 100151. doi:10.1016/j.abrep.2018.100151
- Famele, M., Ferranti, C., Abenavoli, C., Palleschi, L., Mancinelli, R., and Draisci, R. (2015). The Chemical Components of Electronic Cigarette Cartridges and Refill Fluids: Review of Analytical Methods. *Nicotine Tob. Res.* 17, 271–279. doi:10.1093/ntr/ntu197
- Gholap, V. V., Kosmider, L., and Halquist, M. S. (2018). A Standardized Approach to Quantitative Analysis of Nicotine in E-Liquids Based on Peak Purity Criteria Using High-Performance Liquid Chromatography. *J. Anal. Methods Chem.* 2018, 1720375. doi:10.1155/2018/1720375
- Goniewicz, M. L., Kuma, T., Gawron, M., Knysak, J., and Kosmider, L. (2013). Nicotine Levels in Electronic Cigarettes. *Nicotine Tob. Res.* 15, 158–166. doi:10.1093/ntr/nts103
- King, B. A., Jones, C. M., Baldwin, G. T., and Briss, P. A. (2020). The EVALI and Youth Vaping Epidemics - Implications for Public Health. *N. Engl. J. Med.* 382, 689–691. doi:10.1056/nejmp1916171
- Knapp, A. A., Lee, D. C., Borodovsky, J. T., Auty, S. G., Gabrielli, J., and Budney, A. J. (2019). Emerging Trends in Cannabis Administration Among Adolescent Cannabis Users. *J. Adolesc. Health* 64, 487–493. doi:10.1016/j.jadohealth.2018.07.012
- Krishnasamy, V. P., Hallowell, B. D., Ko, J. Y., Board, A., Hartnett, K. P., Salvatore, P. P., et al. (2020). Update: Characteristics of a Nationwide Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - United States, August 2019-January 2020. *Morb. Mortal. Wkly. Rep.* 69, 90–94. doi:10.15585/mmwr.mm6903e2
- Lisko, J. G., Tran, H., Stanfill, S. B., Blount, B. C., and Watson, C. H. (2015). Chemical Composition and Evaluation of Nicotine, Tobacco Alkaloids, pH, and Selected Flavors in E-Cigarette Cartridges and Refill Solutions. *Nicotine Tob. Res.* 17, 1270–1278. doi:10.1093/ntr/ntu279

- Ogunwale, M. A., Chen, Y., Theis, W. S., Nantz, M. H., Conklin, D. J., and Fu, X.-A. (2017). A Novel Method of Nicotine Quantification in Electronic Cigarette Liquids and Aerosols. *Anal. Methods* 9, 4261–4266. doi:10.1039/c7ay00501f
- Puetz, A., Morel-Espinosa, M., Watson, C., Blount, B. C., and Valentin-Blasini, L. (2021). Development, Validation, and Application of a Novel Method for the Analysis of Vitamin E Acetate and Other Tocopherols in Aerosol Emissions of E-Cigarettes, or Vaping Products Associated with Lung Injury. *Front. Chem.* 9, 730954. doi:10.3389/fchem.2021.730954
- Reilly, S. M., Cheng, T., and Dumond, J. (2020). Method Validation Approaches for Analysis of Constituents in ENDS. *Tob. Regul. Sci.* 6, 242–265. doi:10.18001/trs.6.4.3
- Taylor, J. K. (1987). *Quality Assurance of Chemical Measurements*. Boca Raton, FL: Taylor & Francis.
- Trehy, M. L., Ye, W., Hadwiger, M. E., Moore, T. W., Allgire, J. F., Woodruff, J. T., et al. (2011). Analysis of Electronic Cigarette Cartridges, Refill Solutions, and Smoke for Nicotine and Nicotine Related Impurities. *J. Liquid Chromatogr. Relat. Tech.* 34, 1442–1458. doi:10.1080/10826076.2011.572213
- Trivers, K. F., Watson, C. V., Neff, L. J., Jones, C. M., and Hacker, K. (2021). Tetrahydrocannabinol (THC)-containing E-Cigarette, or Vaping, Product Use Behaviors Among Adults after the Onset of the 2019 Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury (EVALI). *Addict. Behav.* 121, 106990. doi:10.1016/j.addbeh.2021.106990
- U.S Food and Drug Administration (2021b). FDA Commits to Evidence-Based Actions Aimed at Saving Lives and Preventing Future Generations of Smokers. [Online]. Available at: <https://www.fda.gov/news-events/press-announcements/fda-commits-evidence-based-actions-aimed-saving-lives-and-preventing-future-generations-smokers> (Accessed 4 29, 2021).
- U.S Food and Drug Administration (2021a). Menthol and Other Flavors in Tobacco Products. [Online]. Available at: <https://www.fda.gov/tobacco-products/products-ingredients-components/menthol-and-other-flavors-tobacco-products> (Accessed 3 29, 2021).
- Wang, T. W., Neff, L. J., Park-Lee, E., Ren, C., Cullen, K. A., and King, B. A. (2020). E-cigarette Use Among Middle and High School Students - United States, 2020. *Morb. Mortal. Wkly. Rep.* 69, 1310–1312. doi:10.15585/mmwr.mm6937e1
- Watson, C. V., Richter, P., De Castro, B. R., Sosnoff, C., Potts, J., Clark, P., et al. (2017). Smoking Behavior and Exposure: Results of a Menthol Cigarette Cross-Over Study. *Am. J. Health Behav.* 41, 309–319. doi:10.5993/ajhb.41.3.10
- Westgard, J. O., Barry, P. L., Hunt, M. R., and Groth, T. (1981). A Multi-Rule Shewhart Chart for Quality Control in Clinical Chemistry. *Clin. Chem.* 27, 493–501. doi:10.1093/clinchem/27.3.493

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Non-Targeted Analysis Using Gas Chromatography-Mass Spectrometry for Evaluation of Chemical Composition of E-Vapor Products

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The Premarket Tobacco Product Applications (PMTA) guidance issued by the Food and Drug Administration for electronic nicotine delivery systems (ENDSs) recommends that in addition to reporting harmful and potentially harmful constituents (HPHCs), manufacturers should evaluate these products for other chemicals that could form during use and over time. Although e-vapor product aerosols are considerably less complex than mainstream smoke from cigarettes and heated tobacco product (HTP) aerosols, there are challenges with performing a comprehensive chemical characterization. Some of these challenges include the complexity of the e-liquid chemical compositions, the variety of flavors used, and the aerosol collection efficiency of volatile and semi-volatile compounds generated from aerosols. In this study, a non-targeted analysis method was developed using gas chromatography-mass spectrometry (GC-MS) that allows evaluation of volatile and semi-volatile compounds in e-liquids and aerosols of e-vapor products. The method employed an automated data analysis workflow using Agilent MassHunter Unknowns Analysis software for mass spectral deconvolution, peak detection, and library searching and reporting. The automated process ensured data integrity and consistency of compound identification with >99% of known compounds being identified using an in-house custom mass spectral library. The custom library was created to aid in compound identifications and includes over 1,100 unique mass spectral entries, of which 600 have been confirmed from reference standard comparisons. The method validation included accuracy, precision, repeatability, limit of detection (LOD), and selectivity. The validation also demonstrated that this semi-quantitative method provides estimated concentrations with an accuracy ranging between 0.5- and 2.0-fold as compared to the actual values. The LOD threshold of 0.7 ppm was established based on instrument sensitivity and accuracy of the compounds identified. To demonstrate the application of this method, we share results from the comprehensive chemical profile of e-liquids and aerosols collected from a marketed e-vapor product. Applying the data processing workflow developed here, 46 compounds were detected in the e-liquid formulation and 55 compounds in the aerosol sample. More than 50% of compounds reported have been confirmed with reference

standards. The profiling approach described in this publication is applicable to evaluating volatile and semi-volatile compounds in e-vapor products.

Keywords: non-targeted analysis, electronic vapor products, ENDS, aerosol, e-liquids, semi-quantitative analysis, GC-MS, e-cigarettes

INTRODUCTION

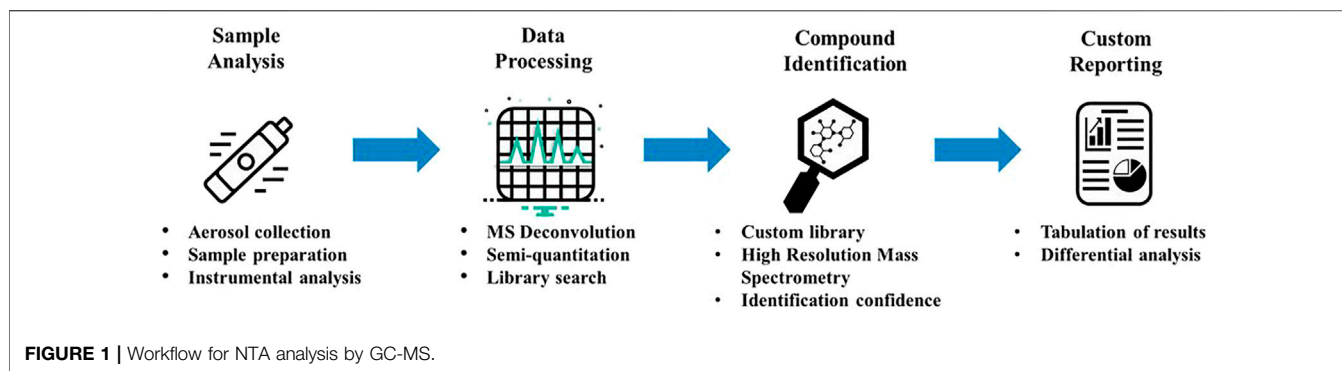
Electronic nicotine delivery system (ENDS) product usage has increased in popularity over the past decade as a potential alternative to combustible cigarettes for the adult tobacco consumer (Ayers et al., 2011; Adkison et al., 2013; Delnevo et al., 2016), and usage continues to increase (McMillen et al., 2015; Rigotti et al., 2015; Rawlinson et al., 2017). ENDSs are non-combustible tobacco products and are also referred to as electronic cigarettes (e-cigs), vapes, vaporizers, vape pens, or e-vapor products. Their designs have evolved from the first-generation devices (“cig-a-likes”) to devices with disposable, prefilled cartridges or “pods” and “mods” with user-controllable settings, such as wattage, voltage, and temperature control (Zhu et al., 2014; Talih et al., 2017; FDA, 2019c). ENDS products aerosolize the e-liquid that is typically composed of a mixture containing propylene glycol (PG), vegetable glycerol (VG), nicotine, and flavors (Hahn et al., 2014; Flora et al., 2016; FDA, 2019c; Cunningham et al., 2020).

In June 2019, the Food and Drug Administration (FDA) provided final guidance to the industry for submitting a premarket tobacco product application (PMTA) for ENDS products (FDA, 2019b). This guidance recommended that all ENDS products, including e-liquids and devices, be evaluated in order to ensure that these new products would be appropriate for the protection of public health. These recommendations included the evaluation of both chemical and physical characterization of the product and product performance across the lifespan of the device under both intense and non-intense use conditions (FDA, 2019b). The guidance also recommends the characterization of these product attributes for inclusion in stability studies used for determining product shelf-life. For chemical characterization, the guidance recommends reporting a specific list of harmful and potentially harmful constituents (HPHCs), as well as other constituents of toxicological concern, contained in the product or delivered by the product (FDA, 2019b). The list of 33 HPHCs includes combustion-related compounds (Wagner et al., 2018), thermal degradation products from humectants and other compounds specific to the product category such as flavorants, and potential impurities from raw materials (Flora et al., 2016; Uchiyama et al., 2020; Li et al., 2021). Due to the significantly low temperatures (i.e., < 350°C) typically used to generate the aerosol within ENDS products, the combustion (~900 °C)-related HPHCs formed by conventional cigarettes are not typically formed or are produced at significantly lower levels in aerosols of ENDS products compared to cigarette smoke (Goniewicz et al., 2014; Tayyarah and Long, 2014; Margham et al., 2016; Wagner et al., 2018). Thermal degradation of the e-liquid has been reported to occur at temperatures typically required for the aerosol formation process (Flora et al., 2017; Uchiyama et al.,

2020; Li et al., 2021). Other compounds specific to the product category are observed in the aerosol through direct transfer from the e-liquid to the aerosol. In order to accurately quantitate these HPHCs, analytical methods that target the specific analytes are typically developed and validated according to International Council of Harmonization (ICH) guidelines to generate data that are used for regulatory reporting. The characterization of ENDS products for constituents of toxicological concern contained in the product or delivered by the product may require an additional type of analysis to complement the targeted analysis for HPHCs as described above. This type of analysis requires performing chemical characterization that is non-selective and provides the detection of constituents across a wide range of chemical classes, often referred to as non-targeted analysis (NTA).

NTA techniques are widely used in the environmental, food, and plastic industries and for the evaluation of biological samples to identify impurities, contaminants, or compounds of concern (Andra et al., 2017; Keppler et al., 2018; Sobus et al., 2018; Martínez-Bueno et al., 2019). Coupling chromatographic separation with mass spectrometry detection and custom internal mass spectral libraries improves the peak identification of compounds within these mixtures (Rawlinson et al., 2017). Current analytical methods used for NTA span a broad range of techniques, including unit-mass resolution gas chromatography-mass spectrometry (GC-MS), two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOF MS), ultra-high-resolution Fourier transform ion cyclotron resonance mass spectrometry, and liquid chromatography coupled with high-resolution Orbitrap or time-of-flight mass spectrometry (LC-MS) (Reichenbach et al., 2012; Herrington and Myers, 2015; Andra et al., 2017; Junot and Witting, 2017; Sobus et al., 2018; Knorr et al., 2019; Martínez-Bueno et al., 2019; Savareear et al., 2019). The data obtained from the analysis are utilized to determine the chemical structure of the detected compounds. Some NTA methods can provide semi-quantitative concentrations of all compounds detected in the analysis. Compound structural identification and semi-quantitative concentration provided by NTA for each compound can then be used by toxicologists to perform risk assessments.

To fully characterize e-vapor products, additional screening methods capable of evaluating the chemical composition of e-liquids and aerosols must be developed. NTA screening methods can prove to be useful tools for the evaluation of e-vapor products for the presence of compounds that may potentially be of toxicological concern in addition to HPHCs. There are some NTA methods reported in the literature for characterization of e-vapor products (Reichenbach et al., 2012; Herrington and Myers, 2015; Rawlinson et al., 2017). Herrington



and Myers described non-targeted GC-MS analysis of e-liquids where the sample was collected manually on a thermal desorption tube prior to desorption and qualitative analysis on a quadrupole mass analyzer. Their analysis resulted in detectable levels of more than 115 volatile organic compounds (VOCs) and semi-volatile organic compounds in one 40 ml puff. Several compounds, including formaldehyde, acrolein, acetaldehyde, and siloxanes, were detected in the e-vapor aerosol and not in the original e-liquid, which suggested that these compounds were formed during aerosolization (Herrington and Myers, 2015). The sensitivity of their method was impacted for several analytes due to overloading of PG and VG in the system. Rawlinson et al. (2017) described a non-targeted method for e-vapor products using thermal desorption gas chromatography time-of-flight mass spectrometry with a 5 ng/puff limit of detection (LOD). The method employed a heart-cutting process with a Deans Switch to avoid saturation of the mass analyzer by high-abundance ingredients (e.g., PG, VG, and nicotine). However, this process eliminates the identification of compounds that co-elute with these high-concentration analytes. The LOD for their method was established based on a toxicologically relevant threshold and the ability to identify compounds. The method by Rawlinson et al. (2017) was generally compatible for analysis of volatile organic and nitrogen-containing compounds but was not applicable for identification of very low-molecular-mass compounds, some organic acids, and high-boiling-point compounds. The method did employ an automated workflow that increased data throughput significantly; however, it was challenged by partial deconvolution of some chromatographic peaks due to low abundance and co-elution of multiple compounds within one peak. Chemical identification is a major challenge with NTA due to the composition of the matrix, including flavor ingredients, and high-abundance ingredients present in the e-liquid's carrier system. Additional identification challenges arise particularly for unknowns due to insufficient compound libraries, mass spectral ions that match with different chemical structures within the mixture, and co-elution of multiple compounds due to complexity of the matrices (Reichenbach et al., 2012). In addition, the National Institute of Standards and Technology (NIST) mass spectral library is largely based upon known compounds analyzed with unit-mass-resolution GC-MS instruments, and the library in select cases may include multiple mass spectra associated with a single

chemical compound. A comprehensive chemical characterization of the aerosol generated by a heated tobacco product (HTP) and mainstream smoke from a reference cigarette was reported using two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-TOFMS) and liquid chromatography coupled to high-resolution accurate mass spectrometry (LC-HRAM-MS) (Knorr et al., 2019; Arndt et al., 2020; Bentley et al., 2020). This comprehensive non-targeted analysis workflow used two parallel platforms utilizing multiple analytical methods to maximize the chemical space coverage in order to fully characterize mainstream cigarette smoke and heated tobacco aerosols. Although this technique was required for characterization of these products, due to the highly complex matrix, the instrumentation is expensive and requires specialized expertise to operate. In addition, two-dimensional GC is not required for the e-vapor matrix since it is relatively less complex and has been reported to contain an order of magnitude fewer compounds than HTPs (Rawlinson et al., 2017).

Here, we describe an approach to non-targeted analysis using unit-mass-resolution GC-MS with the electron impact ionization mode (EI) and a new automated data analysis process workflow. The method uses relatively inexpensive GC-MS instruments and software, making it a practical method for many analytical testing laboratories. The Agilent MassHunter Unknowns Analysis software assisted in peak detection and deconvolution, followed by identification of compounds using the NIST library and a custom in-house library. A traditional validation of an e-vapor NTA method was not possible due to the absence of a standard method or guidance document. Because of this and unique challenges associated with any non-targeted analysis, it was necessary to modify validation experiments to demonstrate that our method was fit for its intended purpose. Most validation experiments were modified to include the analysis of known compounds for fortification, which included degradation products from nicotine, impurities from PG/VG, and flavor-related compounds. Since the fortification compounds were prepared and added to the blank matrices at known concentrations, comparisons could be made between actual and estimated concentrations. Critical validation parameters (i.e., accuracy, precision, selectivity, and LOD) were established to demonstrate that the method is appropriate for analysis of e-liquids and aerosols of e-vapor products. The

modified experiments conducted for method validation in this article could aid in defining best practices leading to standardized guidance for the validation of semi-quantitative NTA methods. The technique described here was used for stability evaluation of a commercial e-vapor product using differential analysis to determine new compounds that formed between an initial assessment ($T = 0$) and after 6 months ($T = 6$). The method and complete workflow were integrated to an internal Laboratory Information Management System (LIMS) and was accredited under the ISO 17025 scope of accreditation.

MATERIALS AND METHODS

Overall Non-Targeted Analysis Workflow

We developed an NTA workflow that includes four major steps: sample analysis, data processing, compound identification, and custom reporting. Illustrated in **Figure 1** is an overview of each step of the workflow. Sample analysis and data processing were two steps in the workflow that required optimization of parameters to ensure that the method was sensitive, selective, and reproducible. An automated data processing approach was developed and employed to minimize the subjectivity and time needed for data processing and review. Our NTA workflow leverages MassHunter Unknowns Analysis, which is part of the Agilent MassHunter Quantitative software package (Agilent Technologies, Santa Clara, CA), and was specifically designed for non-targeted screening and incorporates mass spectral deconvolution, compound identification, and quantitation capabilities. This data processing software was a critical step to the workflow, and development of the processing method required optimization of processing parameters to ensure that repeated analyses provide reproducible compound identification and quantitation in samples. The peak deconvolution algorithm extracts ions from background noise and reconstructs spectra of the individual components from retention time and peak shape information. Compound identification was achieved using both the NIST mass spectral library and a custom in-house library that contains compounds that have been confirmed with reference standards. Using this custom library allowed us to track compounds based on retention times and relative retention times that resulted in improved consistency of compound identifications.

Unknown identification can be extremely challenging and may require highly experienced subject matter experts and tools for compound identification and structural characterization [e.g., high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR)]. In instances where compounds could not be identified based on our custom library or the NIST library, a secondary analysis was required. The secondary analysis was performed using a Thermo Scientific GC-Orbitrap system that provides high-resolution accurate mass spectra to aid in the identification of unknown compounds. Using the GC-Orbitrap in both electron ionization (EI) and chemical ionization (CI) modes allowed for the determination of the molecular formula from the molecular ion, base peak, and other peak fragments for mass spectral

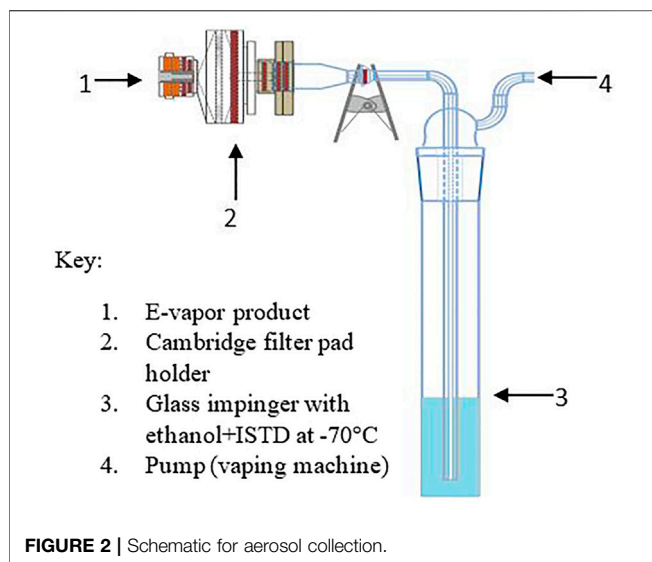
interpretation and structural elucidation. Based on the tentative chemical structure identification, unknowns were subsequently confirmed by custom synthesis or acquiring reference standards. Once the data were processed and compounds had been identified, the results were imported into the laboratory information management system (LIMS) data application to ensure data integrity and reporting. The results were then exported from LIMS into the Tableau software application (Tableau Software, Seattle, WA) for final reporting. Tableau was used to create the custom report templates and perform blank subtractions using specified criteria. The software was also used for tracking individual compounds over multiple time points to evaluate any trends and changes in the composition of the e-liquid or aerosol from a product over time.

Sample Generation

The aerosol was collected on a linear aerosol collection machine (Borgwaldt LX20; Hamburg, Germany) that was exclusively used for e-vapor collection to avoid any potential contamination from prior use with conventional cigarette products. The e-vapor aerosols were collected on a 55 mm Cambridge filter pad (CFP) with a trailing impinger containing 10 ml of the extraction solvent [absolute ethanol containing 10 $\mu\text{g/ml}$ of the internal standard (ISTD) 6-methyl coumarin] chilled at -70°C using a dry ice/isopropanol slurry (see **Figure 2**). The puffing regime parameters consisted of a 55 ml puff volume, a 5 s puff duration, a 30 s puff interval, and a square wave puff profile. A total of 140 puffs were collected. The aerosol trapping efficiency was evaluated prior to the final collection process. The trapping efficiency experiment results indicated that the aerosol sample collection using the Cambridge filter pad attached to a single trailing impinger was acceptable for the machine smoking regime used.

Once the designated number of puffs was collected, the pad and extraction solvent from the impinger were both combined in a 20 ml glass vial, followed by mixing for 30–60 min on an inversion-type rotator (~ 15 rpm). After the extraction was complete, an aliquot of the sample was transferred to an autosampler vial and analyzed by GC-MS.

The corresponding e-liquid(s) used to generate the aerosol using the above-described methodology was also analyzed according to the procedure outlined below. The target extraction weight of each batch of e-liquid samples was determined from the average aerosol mass collected for all replicates of the same product analyzed for aerosols. E-liquid samples were generated by removing the e-liquid from a product cartridge or from the bulk formulation container. To collect the e-liquid from a product, e-vapor sample cartridges were centrifuged for 2–6 min at 1,000–6,000 rpm. The e-liquid ($\sim 0.600\text{--}0.800 \pm 0.050$ g as determined based on the aerosol mass for the corresponding e-vapor product analyzed) was weighed into a 20 ml amber screw cap vial and combined with 10 ml of the extraction solvent containing the internal standard. The samples were then mixed on an inversion-type rotator (~ 15 rpm) for 30–60 min. The last steps were to transfer an aliquot into an autosampler vial and analyze by GC-MS.



Gas chromatography-Mass Spectrometry Method Conditions

Aerosol and e-liquid samples were analyzed on an Agilent technologies (Santa Clara, CA) single quadrupole GC-MS system (7890 B with 5977 A) equipped with an EI source at 230°C, a quadrupole mass spectrometer at 150°C, and the transfer line temperature at 260°C. Mass spectra were recorded in the full scan mode with a mass range of 35–450 amu and 3.5 scan/sec. The instrument was operated in constant flow at 1.2 ml/min with an inlet temperature of 260°C and an injection volume of 1 µl (split 5:1). Chromatographic separation was achieved using a Restek Stabilwax® (Restek Corporation; Bellefonte, PA) GC column (30 m × 0.25 mm × 0.25 µm) with an infused 5 m Restek Integra guard column. The GC oven was initially held at 60°C for 1.25 min, followed by a 15°C/min ramp to 210°C with a 2 min hold time, followed by a 30°C/min ramp to 260°C with a 9 min hold time. The total run time was 23.92 min. A typical total ion chromatogram for the e-vapor aerosol extract using the sample preparation and GC-MS method conditions described above is represented in **Figure 3**.

Data Processing and Reporting

The instrument raw data files were processed using a data analysis workflow through Agilent MassHunter Unknowns Analysis software version B.08.00 (Agilent Technologies; Santa Clara, CA). The automated data processing workflow included automatic peak detection, deconvolution, library searches for compound identifications, and calculation of the estimated concentrations. The method used for this data processing contained predetermined parameters and thresholds, such as signal-to-noise ratio (S/N), peak detection limits, deconvolution, library search criteria, and compound identification and target match criteria. These parameters were optimized during method development and represent a balance between the software's ability to correctly identify compounds and the maximum number of peaks detected while maintaining acceptable mass spectral quality. More details on

the MassHunter Unknowns Analysis parameters used for the data processing method can be found in the **Supplementary Material**. Semi-quantitation of the detected compounds was achieved using a manual response factor that was calculated from an analog internal standard. The manual response factor was calculated using the peak area of the internal standard (ISTD) and included the extraction volume and average aerosol masses of the samples analyzed as described by following equation:

$$\text{Manual Response Factor} = \frac{(\text{ISTD Area}/\text{ISTD Conc}) * (\text{Sample Wt.})}{\text{Volume}}$$

where, ISTD Area is the base peak area for the internal standard 6-methylcoumarin, ISTD Conc is the known concentration of ISTD (µg/ml), sample Wt. refers to the weight of the e-liquid or aerosol mass (mg), and volume refers to the extraction solution volume used for the extraction of each sample (ml). The calculated response factor was entered into the data processing method to determine the estimated concentration for each analyte. During data processing, compound identifications for peaks in the study samples were obtained by comparing the mass spectra from the samples to the NIST 2017 mass spectral library in addition to the in-house custom mass spectral library. The in-house custom library contained known peak identifications with their corresponding peak retention times and mass spectra. Agilent MassHunter Unknowns Analysis software compared the mass spectra and retention times of the library compounds to those in the samples, resulting in a tentative identification based on criteria specified by method parameters.

Compound identifications were separated into five classifications (i.e., confirmed, high, medium, low, and NA) based on identification confidence and the NIST MS library match factor score (**Table 1**). Confirmed, high, medium, and low identification confidence classifications were based on mass spectrum match factor scores from an Automated Mass spectral Deconvolution and Identification System (AMDIS; NIST, Gaithersburg, MD), a secondary deconvolution software available with the NIST MS library that was used to investigate or confirm peak identifications and to assign identification confidence levels. It was observed that a high match factor score did not always represent an acceptable mass spectral library match. Identification confidence classification assigned to every chemical component was determined by visual inspection of the mass spectrum by an experienced analyst, in addition to the match factor score. Compounds labeled with a confirmed identification confidence classification were positively identified by comparing the compound's mass spectra and relative retention time (RRT) to a reference standard. Compounds that did not have an acceptable mass spectral library match (i.e., match factors lower than 500) were classified as unknowns (NA).

Chemicals

The following compounds were purchased from Sigma-Aldrich (St. Louis, MO): piperonal (99.0%), 2,3,5-trimethylpyrazine (99.0%), menthone (99.0%), (E)-beta-damascone (99.0%), cinnamic acid methyl ester (99.0%), myosmine (99.0%),

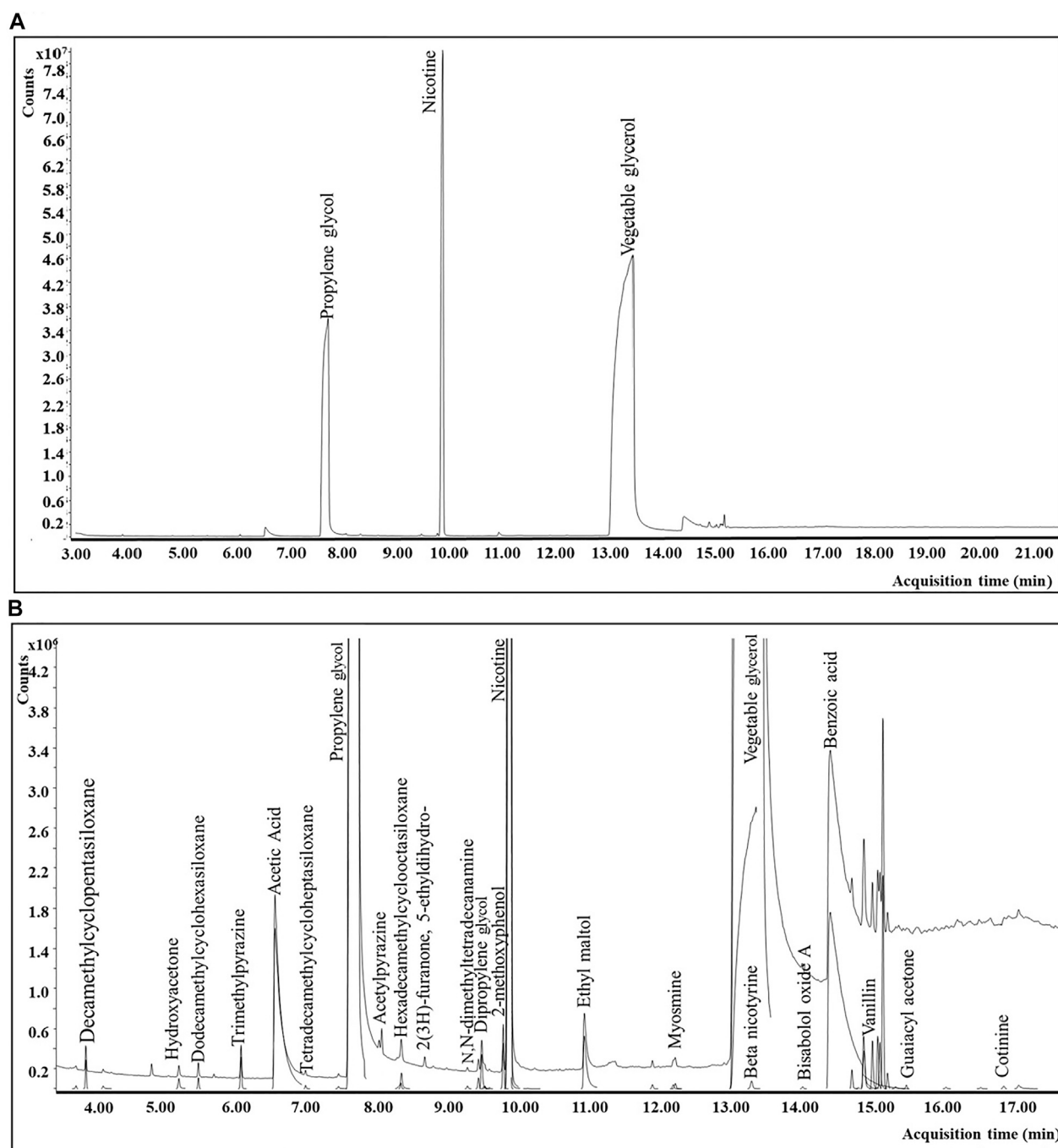


FIGURE 3 | GC-MS chromatograms for a commercial e-vapor product: **(A)** total ion chromatogram (TIC) for the e-vapor aerosol sample. **(B)** Magnified version (approximately $\times 10$) of **Figure 3A** including peak deconvolution.

cotinine (98.5%), and the internal standard (ISTD) 6-methyl coumarin (99.0%). Hydroxyacetone (96.0%) was purchased from Santacruz Biotechnology (Dallas, TX). 200 proof ethanol was purchased from Pharmco-Aaper. Propylene glycol (PG) and vegetable glycerol (VG) were purchased from Spectrum (New Brunswick, NJ).

Test Samples for Method Validation

For method validation, a mixture of eight compounds (validation fortification standards)—hydroxyacetone, piperonal, 2,3,5-trimethylpyrazine, menthone, (E)-beta-damascone, cinnamic acid methyl ester, myosmine, and cotinine—were selected by classification (e.g., flavors, degradation products, etc.) and

TABLE 1 | Peak identification confidence criteria.

Identification confidence	NIST MS match factor score criteria
Confirmed	Identification confirmed by comparison of the compound mass spectrum and relative retention time (RRT), to a reference standard
High	850–1,000
Medium	700–849
Low	500–699
NA	Unknown compound

retention time to ensure that the separation of compounds was well distributed across the entire chromatographic method run time. All matrices used for the method validation experiments were fortified using the eight compounds. Test samples (F1–F5) for method assessment listed in **Table 2** were prepared with a mixture of nicotine, water, PG, and VG to cover the wide range of PG/VG concentrations available in commercial e-vapor products. The samples contained 0% or 2.5% nicotine by weight (NBW) and 0% or 15% water. PG/VG is the ratio of the percent remainder, minus the sum of % H₂O and % NBW: [(100-(% H₂O+ %NBW))]. The ratio of PG/VG is based on the amount remaining such that %PG+%VG = 100%. (see **Table 2**). In addition to the method assessment samples, the e-liquid and aerosol of two e-vapor product prototypes aged to approximately 2 years under controlled ambient storage conditions [25 °C ± 2 °C/60% relative humidity (RH) ± 5%RH] representing tobacco flavor containing 4.0% NBW + PG/VG (30:70) + 15% H₂O (product A) and menthol flavor containing 3.5% NBW + PG/VG (60:40) + 10% H₂O (product B) were also used to demonstrate the method's ability to identify the eight validation fortification compounds correctly in the presence of the complex matrix of aged e-vapor products. These e-vapor prototype products, designated as product A and product B, were only used for evaluation of selectivity during method validation and were fortified at a 10 ppm level with the mixture of validation fortification standards.

Method Validation Characteristics

The primary purpose of this method performance evaluation was to demonstrate that the method could accurately detect and identify compounds in the aerosol and e-liquid of e-vapor products. The most critical parameters for the method validation are detailed below and include accuracy, repeatability, intermediate precision, selectivity, LOD, and evaluation of false negatives. Other validation elements were evaluated but are not discussed in detail, including aerosol trapping efficiency, instrument precision, robustness, stability of sample extracts, and system suitability. The linearity as evaluated in the typical targeted analysis method validation was not evaluated as this semi-quantitative method uses a single-point calibration curve and no regression analysis was performed, including generation of coefficients of variance (r^2), as is typically done for targeted quantitative analysis techniques.

The method validation experiments were conducted using both fortified and unfortified matrix samples listed in **Table 2**.

TABLE 2 | Method assessment sample information.

Sample ID	%Base (PG:VG)	%H ₂ O	%NBW
F1	82.5 (50:50)	15	2.5
F2	97.5 (50:50)	0	2.5
F3	85 (50:50)	15	0
F4	85 (80:20)	15	0
F5	85 (20:80)	15	0

Samples were fortified with known amounts of the following eight compounds to evaluate method performance: hydroxyacetone, piperonal, 2,3,5-trimethylpyrazine, menthone, (E)-beta-damascone, cinnamic acid methyl ester, myosmine, and cotinine. Accuracy of this semi-quantitative method was evaluated by comparing the fortified concentrations to the measured concentrations for the eight compounds described above. Blank non-flavored e-liquid matrices listed in **Table 2** (F1–F5) were fortified at three concentration levels (2 ppm, 5 ppm, and 10 ppm) with the eight compounds. Additionally, an unfortified sample for each matrix type was extracted and analyzed to identify any potential interferences to evaluate selectivity. Repeatability and intermediate precision were evaluated for each of the matrices (F1–F5) fortified at the mid-level (5 ppm) by analyzing three replicates over 3 days. The semi-quantitative concentration data for all individual replicates, mean, standard deviation, and % RSD were calculated for each day (repeatability) and over a 3 day period (intermediate precision). Selectivity was evaluated based upon the ability of the MassHunter Unknowns Analysis data processing method to detect and accurately identify compounds in fortified and unfortified samples. Peak identification included matching retention times and mass spectra from the sample to the in-house custom and the NIST mass spectral libraries. The sensitivity of the method was evaluated by determining the LOD of the method. For this, the F1 blank e-liquid matrix (**Table 2**) was fortified with the validation fortification standards at 0.50, 0.70, 1.0, 2.0, and 5.0 ppm. Evaluation of match factor scores and S/N threshold was used to establish the LOD. Additional validation experiments were conducted to evaluate for potential false negatives and to assess a threshold of the method to detect changes in samples to allow for differential analysis. A threshold for significant change to perform differential analysis was established to allow comparison between two samples. A limitation of the Agilent MassHunter Unknowns analysis software is that it is not possible to track the reason(s) for peak identification failure due to method parameters. For example, if any given peak is misidentified or not identified, there is no mechanism for identifying exactly which parameter(s) failed the acceptance criteria, such as match factor score, signal-to-noise ratio, or retention time window results. Also, a probability of detection curve could not be generated due to the match factor score parameters not having an impact on compound identifications at lower concentration levels. Additionally, adjustment of the S/N ratio method parameters to a lower setting caused some fortification compounds to be non-detectable (false negatives), resulting from background and instrument noise interferences, which both increased at the lower setting.

Comprehensive Non-Targeted Analysis Profile of a Commercial Product

To demonstrate the application of the method for characterizing aerosol and e-liquid formulations from e-vapor products, we applied our workflow to perform a comprehensive chemical profile for a commercially available e-vapor product (tobacco flavor—product C), which was available at the time of analysis at local convenience stores in the Richmond, Virginia area. The analysis included e-liquid and aerosol samples at an initial time point ($T = 0$) and product aged to 6 months ($T = 6$) stored in environmental chambers under ambient storage conditions ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$). The aerosol samples were generated using an intense puffing regime (a 5 s puff duration, a 55 cc puff volume, and a 30 s puff interval) for the aerosol collection and analyzed in triplicate ($n = 3$). The e-liquid samples were analyzed using an equivalent amount of e-liquid to the collected aerosol mass as the test samples and extracted with the same extraction solvent. Data processing was conducted with MassHunter Unknowns Analysis software to provide identification and quantitation of all detected peaks. All peaks identified by the software were confirmed for accuracy.

RESULTS AND DISCUSSION

Accuracy

This method is a semi-quantitative method that provides estimated concentrations for the analytes detected in the samples. The estimated concentration is based upon the response factor of a single internal standard as described above. Estimation of the concentration using the response of a single internal standard is inherently less accurate than targeted methods that quantitate the concentration of an analyte using multi-level calibration curves prepared with reference standards, followed by regression analysis. In addition, the response for analytes in GC-MS with EI varies based on compound fragmentation, which is different for all compounds. To evaluate accuracy, the blank matrix samples in **Table 2** (F1–F5) were fortified with the eight validation fortification standards at 2 ppm, 5 ppm, and 10 ppm. All samples were background-subtracted based upon analysis of the corresponding unfortified matrix sample (F1–F5). Accuracy was determined using the following equation:

$$\% \text{ Accuracy} = \frac{\text{Measured Amount (ppm)}}{\text{Background Amount (ppm)} + \text{Fortified Amount (ppm)}} \times 100\%.$$

The results of these accuracy studies are presented in **Table 3**. The combined average recovery for each of the levels evaluated for all analytes ranged from 82.6 to 90%. The accuracy for most matrices and fortification levels for six out of eight analytes tested fell between 70 and 120% recovery, with the exception of 2 ppm fortification in the F1 matrix for (E)-beta-damascone and the cotinine fortification in the F3 matrix, resulting in 67 and 193% accuracy, respectively. Hydroxyacetone and menthone had accuracy with values between 43 and 116%, and 47 and 52%, respectively. The results indicate that the accuracy of the method

varies from approximately 43 to 193% across all analytes and matrices evaluated, **Table 3**. The variability for hydroxyacetone for accuracy was high in comparison to other analytes due to the inconsistency of the amount of this compound detected in the unfortified samples (see the **Supplementary Material** for %RSD). The lowest recovery was observed with menthone due to the difference in response factor compared to the internal standard. Cotinine's large deviation for the F3 matrix at 2 ppm was determined to be related to matrix interferences, which resulted in issues with the peak deconvolution. In this case, the incorrect base peak was selected by the processing software, resulting in a different response for quantitation. Cotinine's observed deviations were due to a limitation of the automated data processing software, which does not allow users to edit or change the base peak used for quantitation. These accuracy results demonstrate compound specific variability; however, data support that our NTA semi-quantitative method is fit for the intended purpose.

Repeatability and Intermediate Precision

Repeatability, a measure of a method's ability to generate equivalent results from multiple preparations of the same sample within a single laboratory, along with intermediate precision (over 3 days of analysis), was evaluated using the 5.0 ppm fortification level for all eight analytes in all the sample matrices listed in **Table 2**. A summary of the percent relative standard deviation (%RSD) for repeatability and intermediate precision, which is representative of the average of three replicates, is provided in **Tables 4** and **5**, respectively. For all analytes, the repeatability was between 0.2 and 14.1% with an average %RSD of 5.6%. The intermediate precision data were 22.1% RSD or lower for all eight compounds in each of the matrices. The overall average %RSD for all compounds and matrices evaluated for intermediate precision was 10.1%. The results demonstrated that the sample analysis and data processing were reproducible across multiple days and sample types.

Selectivity

This method is inherently selective due to the use of mass spectrometry detection. Method selectivity was evaluated using MassHunter Unknowns Analysis software based upon the ability of the processing method to detect and correctly identify compounds in aged e-vapor prototype aerosol and e-liquid samples. Product A and B samples were stored under ambient conditions ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$) for approximately 2 years prior to analysis. Aerosol and e-liquid samples were fortified with the mix of eight compounds, resulting in an analyte concentration of 10 ppm of each compound. These fortified samples were analyzed in triplicate ($n = 3$) in conjunction with their corresponding unfortified samples and were treated independently during data processing. The average estimated concentrations obtained from the evaluation of these samples for fortified and unfortified samples showed an increase in the concentration for each compound in fortified samples, and 99.4% were identified correctly by MassHunter Unknowns Analysis workflow (see details in the **Supplementary Material**). Additionally, the frequency of correct/incorrect

TABLE 3 | Summary of accuracy results.

Matrix	Hydroxyacetone	2,3,5-Trimethylpyrazine	Menthone	(E)-beta-damascone	Cinnamic acid methyl ester	Myosmine	Piperonal	Cotinine
% accuracy at 2 ppm								
F1	87	98	48	67	102	93	106	97
F2	116	103	50	83	107	90	116	100
F3	81	103	52	84	110	84	120	193
F4	69	99	51	78	107	79	111	99
F5	93	104	50	80	105	79	116	99
% accuracy at 5 ppm								
F1	66	108	52	83	111	94	119	108
F2	62	101	49	75	99	81	111	96
F3	63	104	50	79	107	81	116	99
F4	52	97	48	75	102	74	104	97
F5	87	108	51	79	109	77	114	102
% accuracy at 10 ppm								
F1	44	100	47	75	103	84	110	98
F2	49	102	50	77	106	81	113	99
F3	50	100	48	74	100	74	109	94
F4	43	96	48	72	98	72	105	94
F5	68	104	50	78	107	78	109	94

TABLE 4 | Repeatability results summary—%RSD (*n* = 3).

Matrix	Hydroxyacetone	2,3,5-Trimethylpyrazine	Menthone	(E)-beta-damascone	Cinnamic acid methyl ester	Myosmine	Piperonal	Cotinine
F1	7.4	7.6	9.6	6.6	6.9	5.8	9.0	4.7
F2	8.7	0.2	2.4	2.9	2.1	2.1	4.1	2.5
F3	9.4	7.5	8.4	11.7	14.1	10.6	13.5	5.3
F4	4.2	1.3	3.1	2.7	1.8	2.3	2.6	5.8
F5	1.9	3.7	6.0	3.4	6.7	3.5	8.7	2.5

TABLE 5 | Intermediate precision summary—%RSD (*n* = 9).

Matrix	Hydroxyacetone	2,3,5-Trimethylpyrazine	Menthone	(E)-beta-damascone	Cinnamic acid methyl ester	Myosmine	Piperonal	Cotinine
F1	13.3	9.2	9.6	7.1	11.2	7.6	7.1	11.5
F2	11.2	9.5	10.1	5.6	9.5	8.8	10.2	14.5
F3	15.0	7.3	9.8	8.9	10.7	9.2	9.6	9.9
F4	8.1	7.1	9.1	10.0	9.7	8.6	9.5	11.7
F5	10.1	7.8	11.7	9.1	11.6	11.5	9.9	22.1

chemical identifications from the data processing software was evaluated for the fortified sample extracts based on the library mass spectrum match factor scores. The frequency of correct chemical identification for the fortified sample extract for each product and sample type (aerosol and e-liquid) was 99.0% (see the **Supplementary Material**). Thus, the experiments for method selectivity successfully demonstrated the method's ability to perform compound identifications correctly in the presence of a complex matrix.

Limit of Detection

The LOD of this screening method is dependent on the analyte response, and these responses must be sufficient to produce detailed mass spectra with fragmentation patterns to accurately identify compounds based on comparison of the spectra to those in the in-house and/or NIST libraries. The automated data processing by MassHunter Unknowns Analysis software generates a mass spectrum match factor score for each possible identification, which ranges between 0

and 100 (with a score of 100 being the best possible match). During the optimization of the parameters for data processing, if a peak is detected with a match factor score less than 55, then the response is insufficient to provide a reliable mass spectrum for compound identification. To determine the LOD, we evaluated the match factor score and S/N for each of the eight compounds fortified in the F1 blank e-liquid matrix samples at 0.5, 0.7, 1.0, 2.0, and 5.0 ppm. A S/N threshold of 8:1 was chosen based upon the minimum response needed for acceptable mass spectral deconvolution. We set the LOD such that at least 50% of the validation fortification compounds were identified correctly and provided a match factor score greater than 55. Out of the eight fortification compounds, the total number of compounds with confirmed identification for fortification levels of 0.5, 0.7, 1.0, 2.0, and 5.0 ppm was 3, 4, 6, 7, and 8, respectively. Samples fortified at 0.7 ppm provided acceptable identifications for four out of the eight target compounds. Piperonal, menthone, myosmine, and cotinine were correctly identified. Based on this information, the method's LOD was determined to be 0.7 ppm.

Evaluation of False Negatives

It is critical to evaluate the reporting limit of a screening method and understand the potential for false negative occurrences. A limit test is commonly used for the semi-quantitative screening method to set a "cutoff" or threshold value such that a false negative rate is less than 5% of the analytical results (FDA, 2019a). This is based upon the lowest concentration that would provide a response where compounds would be detected 95% of the time. Due to the complexity of e-vapor samples, it is not always feasible to evaluate the threshold value for every analyte. Therefore, we used the data from the LOD determination where 0.7 ppm was established as the lowest concentration that provided correct identification for four of the eight compounds in the F1 e-liquid matrix. The calculated concentrations for four analytes that were correctly identified in samples at the LOD provided a range of concentrations due to their differences in response factors. Myosmine and Cotinine were present in the unfortified F1 blank e-liquid samples; therefore, we conducted a blank subtraction to ensure that the threshold value was based upon the fortified concentration. After blank subtraction, the following equation was used to determine the threshold value:

$$\text{Threshold Value} = [\text{Mean concentration} - (t \times \text{Standard Deviation})],$$

where t = one-tailed Student's t value for $(n-1)$ degrees of freedom at the 95% confidence level. **Table 6** contains the calculated concentration (ppm) for each of the four analytes used to determine the LOD with and without (corrected) blank subtraction and calculation of the threshold limit of the method.

The threshold value ranged from 0.142 to 0.883 ppm for these four analytes. Using the average for all analytes, we determined a threshold of 0.5 ppm. Compound concentrations above 0.5 ppm should have a sufficient response to be detected 95% of the time.

Threshold of Significant Concentration Change

Differential analysis is a technique that allows for comparison between two samples to determine if the differences in semi-quantitative results are of statistical significance. Differential analysis is conducted by establishing a threshold of change that could be detected by the method, such as detecting increases in analyte concentration in different samples. We used a statistical approach similar to Bonferroni correction to determine the threshold for detecting differences, which takes in to account the variability associated with the replicate analysis. This was accomplished by using the data from intermediate precision F1–F5 samples fortified at 5 ppm to determine the variability observed for the estimated concentration results for each analyte. The variability attributed to intermediate precision provides an indication of what may be expected during a study and therefore represents the variability associated with the measured concentrations for different compounds.

Our approach used the method variation σ_m assuming an estimated variation based on 16 degrees of freedom. The value of 16 was derived from using the values for all eight identified compounds with two degrees of freedom each. The criterion $|X_t - X_c| > k \cdot \sigma_m$ was used to determine whether the test product concentration (X_t) is different from the control concentration (X_c). The concentration of a test product that is different from the control concentration represents the calculated value (k) that is multiplied by method variation (i.e., standard deviation, S.D.).

The following equation was used to derive a reasonable value for the calculated value, k :

$$P(|\bar{x}_t - \bar{x}_c| > k\sigma_m) = P\left(\frac{|\bar{x}_t - \bar{x}_c|}{\sqrt{2}\sigma_m} > \frac{k}{\sqrt{2}}\right) \Rightarrow k = \sqrt{2} t$$

In the equation, t represents the t -critical value, which is the inverse of the two-tailed student's t -distribution (a continuous probability distribution for testing on a small data set). In order to minimize the number of false positives, the probability (P) associated with the t -critical value would be inversely proportional to the number of comparisons being made. For example, assuming a scenario of 60 comparisons, the probability would be 0.05/60. In this case, t is equal to 4.10 and k is equal to 5.80 (rounded to 6.0 for simplicity). Therefore, a reasonable level that can be considered an increase relative to the control would be 6.0 multiplied by S.D. measured using the intermediate precision studies. Listed in **Table 5** of the **Supplementary Material** are the determinations of fold increase used for identification of changes for all matrices. Data in the table show the days 1–3 intermediate precision means, the mean control concentration X_c for all intermediate precision results ($n = 9$), and the standard deviations for each of the five matrices evaluated for intermediate precision, along with the calculated value ($X_t = \text{mean estimated concentration} + 6 \text{ S.D.}$) for a measurable increase. Fold increase for each

TABLE 6 | Calculated threshold values for fortification compounds.

Sample	Menthone	Myosmine	Piperonal	Cotinine
Blank (ppm)	NA	3.91	NA	4.97
Replicate-1 (ppm)	0.41	4.54	0.99	5.36
Replicate-1 (ppm) Corrected	0.41	0.63	0.99	0.40
Replicate-2 (ppm)	0.45	5.03	1.02	6.03
Replicate-2 (ppm) Corrected	0.45	1.12	1.02	1.07
Replicate-3 (ppm)	0.43	4.68	0.91	6.26
Replicate-3 (ppm) Corrected	0.43	0.77	0.91	1.29
Replicate-4 (ppm)	0.38	4.97	0.92	5.43
Replicate-4 (ppm) Corrected	0.38	1.06	0.92	0.46
Replicate-5 (ppm)	0.41	4.98	1.00	5.87
Replicate-5 (ppm) Corrected	0.41	1.07	1.00	0.90
Replicate-6 (ppm)	0.43	5.07	1.00	6.01
Replicate-6 (ppm) Corrected	0.43	1.16	1.00	1.05
Average (ppm)	0.42	0.969	0.974	0.86
S.D.	0.022	0.216	0.045	0.357
%RSD	5.4	22.3	4.6	41.4
Student <i>t</i> -test Value (<i>n</i> -1)	2.015	2.015	2.015	2.015
Threshold value (ppm)	0.371	0.534	0.883	0.142

compound is the ratio of test product concentration to control concentration (X_t/X_c). The average fold increases for F1, F2, F3, F4, and F5 are 1.37, 1.40, 1.36, 1.37, and 1.58, respectively, with an overall average of 1.42. The data from the intermediate precision were consistent; however, the data may not accurately represent the overall variation we may see over the course of a long-term stability study. Therefore, we would expect that using this data set would provide a good estimation of the minimum fold change that could be detected. Based on these data, it was determined that the method can be used to report a 1.4-fold change when comparing two samples. Individual replicate data for each analyte fortified in each matrix (F1–F5) are included in the **Supplementary Material**.

Application to Commercial e-Vapor Products

To demonstrate the application of the method to characterize aerosol and e-liquid formulations from e-vapor products, we conducted comprehensive chemical profiling for a commercially available e-vapor product (tobacco flavor, 3.5% NBW—product C). The analysis included e-liquid and aerosol samples at an initial time point ($T = 0$) and product aged to 6 months ($T = 6$), stored under ambient conditions. The set of samples was analyzed in triplicate ($n = 3$), resulting in an average aerosol mass of 0.652 g for the $T = 0$ sample and 0.606 g for the $T = 6$ sample, using an intense puffing regime for the aerosol collection of over 140 puffs. The liquid samples were analyzed using an equivalent amount of the e-liquid as the average of aerosol mass for the test samples and were prepared with the same extraction solvent. Data were processed using MassHunter Unknowns Analysis to generate a list of compounds with tentative identifications. The data were then manually verified, and AMDIS software was used to confirm peak identifications and assigned identification confidence based on the NIST MS library

match factor scores. **Tables 7** and **8** include the results of all the compounds that were identified at $T = 0$, excluding the major ingredients in the e-vapor formulation (i.e., PG, VG, water, and nicotine), and compounds identified in the blanks. Our analysis detected 46 compounds in the e-liquid formulation and 55 compounds in the aerosols, with approximately 50% of these compounds having a confirmed identification confidence. We observed 19 peaks with an unknown identification confidence classification in the aerosol and 13 peaks in the e-liquid. Unknown compound classification was given to compounds that did not meet the acceptable match factor score criteria with the NIST library or in-house library. The process of aerosolization of the e-liquid resulted in 12 new unknown compounds, 3 of which were designated as nicotine-related compounds based on the similarity of their mass spectral fragmentation patterns to that of nicotine.

Differential analysis between the $T = 0$ and $T = 6$ samples was conducted for both aerosol and e-liquid samples. There were 14 additional compounds in the e-liquid and 19 additional compounds detected in the aerosol generated from the aged sample compared to the corresponding initial ($T = 0$) profiles of the e-liquid and aerosol (see the **Supplementary Material** for details). These additional compounds found at the $T = 6$ time point included various chemical classes related to flavors (e.g., beta-citronellol, delta-decalactone, ethanone,1(-3-pyridinyl), hexanal, etc.), nicotine degradation products (e.g., n-methylnicotinamide, 3,4-dipyridyl ketone), leachable compounds (e.g., a siloxane, diethoxydimethylsilane), and nine unidentified compounds. The tentatively identified compounds that were not confirmed with a reference standard and unknown compounds would require additional characterization of the peaks, such as HRMS, NMR, and expert evaluation, for structure elucidation. The additional peak characterization was outside of the scope of this article.

TABLE 7 | Analysis of product C, e-liquids, and average concentration ($T = 0$, $n = 3$).

Retention time (min)	Compound	CAS#	Identification confidence	Avg ($\mu\text{g/gm}$)	Count (# of times identified)
4.08	Pyridine	110-86-1	CONFIRMED	5.29	1
4.75	Dimethoxydimethylsilane	1112-39-6	CONFIRMED	6.33	3
5.38	Ethyl lactate	97-64-3	CONFIRMED	2.85	1
6.00	Trimethylpyrazine	14667-55-1	CONFIRMED	44.80	3
6.50	Acetic acid	64-19-7	CONFIRMED	487.54	3
7.72	1-Dodecanamine, N,N-dimethyl-	112-18-5	CONFIRMED	7.28	3
7.95	Menthol	89-78-1	CONFIRMED	5.06	3
8.14	Acetylpyrazine	22047-25-2	CONFIRMED	9.36	3
8.55	2(3H)-Furanone, 5-ethylidihydro-	695-06-7	CONFIRMED	15.79	3
8.66	Unknown	0-00-0	NA	1.87	3
9.31	gamma-Heptalactone	105-21-5	CONFIRMED	20.43	3
9.36	Dipropylene glycol	110-98-5	CONFIRMED	20.01	3
9.40	beta-damascenone	23726-93-4	CONFIRMED	2.52	3
9.46	Geraniol	106-24-1	CONFIRMED	1.05	1
9.66	2-Methoxyphenol	90-05-1	CONFIRMED	46.55	3
10.11	Unknown	0-00-0	NA	0.97	3
11.08	Ethyl maltol	4940-11-8	HIGH	138.83	3
11.10	Unknown	0-00-0	NA	2.64	3
11.20	Propanoic acid, 2-hydroxy, 2-hydroxypropyl ester	14396-73-7	CONFIRMED	22.18	3
12.00	Eugenol	97-53-0	CONFIRMED	16.86	3
12.04	delta-Octalactone	698-76-0	HIGH	2.24	3
12.04	Myosmine	532-12-7	CONFIRMED	2.23	3
12.10	Unknown	0-00-0	NA	2.95	3
13.10	Beta nicotyrine	487-19-4	CONFIRMED	1.39	2
13.90	Bisabolol oxide A	22567-36-8	MEDIUM	2.25	3
14.05	Benzoic acid	65-85-0	CONFIRMED	1588.47	3
14.10	Ethanone, 1-(4-methylphenyl)-	122-00-9	CONFIRMED	2.71	1
14.60	Unknown	0-00-0	NA	1.28	2
14.75	p-Dioxane-2,5-dimethanol	14236-12-5	CONFIRMED	14.47	3
14.86	Vanillin	121-33-5	CONFIRMED	52.95	3
14.92	Bis(2,6-hydroxymethyl) dioxane	54120-69-3	CONFIRMED	51.93	3
15.14	Bis(2,6-hydroxymethyl) dioxane—Iso2	54120-69-3	HIGH	6.42	2
15.15	Bis(2,6-hydroxymethyl) dioxane—Iso3	54120-69-3	HIGH	24.60	3
15.19	Unknown	0-00-0	NA	1.80	1
15.26	Bis(2,6-hydroxymethyl) dioxane—Iso4	54120-69-3	HIGH	35.78	3
15.30	Guaiacyl acetone	2503-46-0	CONFIRMED	1.16	3
15.40	Bis(2,6-hydroxymethyl) dioxane—Iso5	54120-69-3	HIGH	10.47	3
16.29	Unknown long-chain alkane	0-00-0	NA	1.13	1
16.70	Cotinine	486-56-6	CONFIRMED	2.18	3
16.96	Unknown long-chain alkane	0-00-0	NA	4.15	1
17.60	Unknown long-chain alkane	0-00-0	NA	6.67	1
18.64	Unknown long-chain alkane	0-00-0	NA	8.48	1
19.58	Unknown long-chain alkane	0-00-0	NA	10.33	1
22.45	Unknown	0-00-0	NA	7.83	1
22.46	Unknown	0-00-0	NA	6.62	1

NA is not applicable; Iso—Isoomer.

CONCLUSIONS

As demonstrated by our chemical characterization, e-vapor products are a complex mixture that contains a variety of chemicals including flavor-related compounds in addition to the typical primary formulation ingredients PG, VG, and nicotine. We have provided a novel non-targeted analysis approach for chemical characterization of aerosols and e-liquids in e-vapor products using an automated data processing workflow. The GC-MS profiling method performance was validated, and criteria were established

for precision, accuracy, selectivity, and LOD. In addition, other unique validation elements deemed necessary for an NTA method, such as evaluating potential for occurrence of false negatives and threshold of significant concentration change, were evaluated. MassHunter Unknowns Analysis method parameters were optimized to ensure the method's ability to perform the automated peak picking, deconvolution, and compound identifications with an appropriate match factor from the available library and provide semi-quantitative concentration for each compound. The validation parameters of precision and

TABLE 8 | Analysis of product C, aerosols, and average concentration ($T = 0$, $n = 3$).

Retention time (min)	Compound	CAS#	Identification confidence	Avg ($\mu\text{g/gm}$)	Count (# of times identified)
3.72	Decamethylcyclopentasiloxane	541-02-6	CONFIRMED	13.48	3
4.75	Dimethoxydimethylsilane	1112-39-6	CONFIRMED	5.45	3
5.04	Hydroxyacetone	116-09-6	CONFIRMED	8.59	3
5.28	Dodecamethylcyclohexasiloxane	540-97-6	CONFIRMED	8.98	3
6.00	Trimethylpyrazine	14667-55-1	CONFIRMED	37.41	3
6.50	Acetic acid	64-19-7	CONFIRMED	446.47	3
6.80	Tetradecamethylcycloheptasiloxane	107-50-6	CONFIRMED	2.48	3
7.72	1-Dodecanamine, N,N-dimethyl-	112-18-5	CONFIRMED	5.12	1
7.95	Menthol	89-78-1	CONFIRMED	5.20	3
8.14	Acetylpyrazine	22047-25-2	CONFIRMED	8.10	3
8.31	Hexadecamethylcyclooctasiloxane	556-68-3	HIGH	0.84	3
8.55	2(3H)-Furanone, 5-ethylidihydro-	695-06-7	CONFIRMED	14.96	3
8.66	Unknown	0-00-0	NA	3.85	3
9.13	Unknown	0-00-0	NA	0.74	1
9.16	N,N-Dimethyltetradecanamine	112-75-4	CONFIRMED	4.22	1
9.31	gamma-Heptalactone	105-21-5	CONFIRMED	19.94	3
9.36	Dipropylene glycol	110-98-5	CONFIRMED	19.58	3
9.40	beta-Damascenone	23726-93-4	CONFIRMED	3.08	3
9.46	Geraniol	106-24-1	CONFIRMED	0.94	2
9.66	2-Methoxyphenol	90-05-1	CONFIRMED	41.72	3
10.11	Unknown	0-00-0	NA	0.94	3
11.08	Ethyl maltol	4940-11-8	HIGH	129.63	3
11.10	Unknown	0-00-0	NA	1.53	3
11.20	Propanoic acid, 2-hydroxy, 2-hydroxypropyl ester	14396-73-7	CONFIRMED	27.92	3
11.30	Unknown	0-00-0	NA	0.80	1
12.00	Eugenol	97-53-0	CONFIRMED	15.42	3
12.04	delta-Octalactone	698-76-0	HIGH	3.34	3
12.04	Myosmine	532-12-7	CONFIRMED	2.83	3
12.10	Unknown	0-00-0	NA	3.08	3
12.30	Unknown	0-00-0	NA	0.82	1
12.70	Unknown nicotine-related compound	0-00-0	NA	0.87	3
13.10	Beta nicotyrine	487-19-4	CONFIRMED	4.09	3
13.90	Bisabolol oxide A	22567-36-8	MEDIUM	2.16	3
13.90	Unknown nicotine-related compound	0-00-0	NA	1.41	3
14.05	Benzoic acid	65-85-0	CONFIRMED	1617.05	3
14.40	Unknown	0-00-0	NA	0.75	2
14.50	Unknown	0-00-0	NA	2.51	3
14.60	Unknown	0-00-0	NA	1.27	2
14.75	p-Dioxane-2,5-dimethanol	14236-12-5	CONFIRMED	15.52	3
14.86	Vanillin	121-33-5	CONFIRMED	51.70	3
14.87	1-Octadecanol	112-92-5	CONFIRMED	0.75	2
14.92	Bis(2,6-hydroxymethyl) dioxane	54120-69-3	CONFIRMED	58.49	3
15.14	Bis(2,6-hydroxymethyl) dioxane—Iso2	54120-69-3	HIGH	12.21	1
15.15	Bis(2,6-hydroxymethyl) dioxane—Iso3	54120-69-3	HIGH	24.98	3
15.20	Unknown	0-00-0	NA	2.13	3
15.26	Bis(2,6-hydroxymethyl) dioxane—Iso4	54120-69-3	HIGH	37.37	3
15.30	Guaiacyl acetone	2503-46-0	CONFIRMED	1.47	3
15.40	Bis(2,6-hydroxymethyl) dioxane—Iso5	54120-69-3	HIGH	11.51	3
16.70	Cotinine	486-56-6	CONFIRMED	2.76	3
17.00	Unknown nicotine-related compound	0-00-0	NA	1.34	2
17.60	Unknown long-chain alkane	0-00-0	NA	2.48	1
18.50	Unknown	0-00-0	NA	1.02	2
18.60	Unknown	0-00-0	NA	2.10	2
18.70	Unknown	0-00-0	NA	0.77	1
19.58	Unknown long-chain alkane	0-00-0	NA	1.60	1

NA is not applicable; Iso—Isoomer.

accuracy had a %RSD of less than or equal to 8.5% for all matrices and concentration levels. Estimated concentrations ranged from 0.5 to 2 times the actual value, as calculated based on the manual

response factor of the internal standard. This method was able to detect a 1.4-fold change in a compound level when comparing two samples. The LOD of this method was determined to be 0.7 ppm.

In the absence of guidance documents for validation of non-targeted methods, the semi-quantitative NTA method validation described here is an example of potential best practices and was successful in determining the method to be fit for the purpose of comprehensive screening of e-vapor products. Evaluation of the commercial e-vapor, product e-liquid, and aerosol demonstrates the ability of the automated data processing method to identify compounds consistently across time and to detect new compounds that may form during aging. Overall, this approach is applicable for the chemical characterization of volatile and semi-volatile compounds in the e-liquids and aerosols of e-vapor products to support the assessment of the products, including toxicological risk assessments, for the FDA's PMTA authorization pathway.

DATA AVAILABILITY STATEMENT

The original contribution presented in the study is included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding author.

REFERENCES

- Adkison, S. E., O'Connor, R. J., Bansal-Travers, M., Hyland, A., Borland, R., Yong, H.-H., et al. (2013). Electronic Nicotine Delivery Systems. *Am. J. Prev. Med.* 44 (3), 207–215. doi:10.1016/j.amepre.2012.10.018
- Andra, S. S., Austin, C., Patel, D., Dolios, G., Awawda, M., and Arora, M. (2017). Trends in the Application of High-Resolution Mass Spectrometry for Human Biomonitoring: An Analytical Primer to Studying the Environmental Chemical Space of the Human Exposome. *Environ. Int.* 100, 32–61. doi:10.1016/j.envint.2016.11.026
- Arndt, D., Wachsmuth, C., Buchholz, C., and Bentley, M. (2020). A Complex Matrix Characterization Approach, Applied to Cigarette Smoke, that Integrates Multiple Analytical Methods and Compound Identification Strategies for Non-targeted Liquid Chromatography with High-resolution Mass Spectrometry. *Rapid Commun. Mass. Spectrom.* 34 (2), e8571. doi:10.1002/rcm.8571
- Ayers, J. W., Ribisl, K. M., and Brownstein, J. S. (2011). Tracking the Rise in Popularity of Electronic Nicotine Delivery Systems (Electronic Cigarettes) Using Search Query Surveillance. *Am. J. Prev. Med.* 40 (4), 448–453. doi:10.1016/j.amepre.2010.12.007
- Bentley, M. C., Almstetter, M., Arndt, D., Knorr, A., Martin, E., Pospisil, P., et al. (2020). Comprehensive Chemical Characterization of the Aerosol Generated by a Heated Tobacco Product by Untargeted Screening. *Anal. Bioanal. Chem.* 412 (11), 2675–2685. doi:10.1007/s00216-020-02502-1
- Cunningham, A., McAdam, K., Thissen, J., and Digard, H. (2020). The Evolving E-Cigarette: Comparative Chemical Analyses of E-Cigarette Vapor and Cigarette Smoke. *Front. Toxicol.* 2 (7). doi:10.3389/ftox.2020.586674
- Delnevo, C. D., Giovenco, D. P., Steinberg, M. B., Villanti, A. C., Pearson, J. L., Niaura, R. S., et al. (2016). Patterns of Electronic Cigarette Use Among Adults in the United States. *Nictob* 18 (5), 715–719. doi:10.1093/ntr/ntv237
- FDA (2019a). *Guidelines for the Validation of Chemical Methods for the FDA FVM Program*. section 3.4. 3rd edition, 11–12. Available at: <https://www.fda.gov/media/81810/download> (accessed July 15, 2021).
- FDA (2019b). *Premarket Tobacco Product Applications for Electronic Nicotine Delivery Systems: Guidance for Industry*. Finalized June 2019. Available at: <https://www.fda.gov/media/127853/download> (accessed July 4, 2019).
- FDA (2019c). *Vaporizers, E-Cigarettes, and Other Electronic Nicotine Delivery Systems (ENDS)*. Updated June 19, 2019. Available at: <https://www.fda.gov/tobacco-products/products-ingredients-components/vaporizers-e-cigarettes-and-other-electronic-nicotine-delivery-systems-ends> (accessed July 5, 2019).
- Flora, J. W., Meruva, N., Huang, C. B., Wilkinson, C. T., Ballentine, R., Smith, D. C., et al. (2016). Characterization of Potential Impurities and Degradation Products in Electronic Cigarette Formulations and Aerosols. *Regul. Toxicol. Pharmacol.* 74, 1–11. doi:10.1016/j.yrtph.2015.11.009
- Flora, J. W., Wilkinson, C. T., Wilkinson, J. W., Lipowicz, P. J., Skapars, J. A., Anderson, A., et al. (2017). Method for the Determination of Carbonyl Compounds in E-Cigarette Aerosols. *J. Chromatogr. Sci.* 55 (2), 142–148. doi:10.1093/chromsci/bmw157
- Goniewicz, M. L., Knysak, J., Gawron, M., Kosmider, L., Sobczak, A., Kurek, J., et al. (2014). Levels of Selected Carcinogens and Toxicants in Vapour from Electronic Cigarettes. *Tob. Control.* 23 (2), 133–139. doi:10.1136/tobaccocontrol-2012-050859
- Hahn, J., Monakhova, Y. B., Hengen, J., Kohl-Himmelseher, M., Schüssler, J., Hahn, H., et al. (2014). Electronic Cigarettes: Overview of Chemical Composition and Exposure Estimation. *Tob. Induced Dis.* 12 (1), 23. doi:10.1186/s12971-014-0023-6
- Herrington, J. S., and Myers, C. (2015). Electronic Cigarette Solutions and Resultant Aerosol Profiles. *J. Chromatogr. A* 1418, 192–199. doi:10.1016/j.chroma.2015.09.034
- Higgins Keppler, E. A., Jenkins, C. L., Davis, T. J., and Bean, H. D. (2018). Advances in the Application of Comprehensive Two-Dimensional Gas Chromatography in Metabolomics. *Trac Trends Anal. Chem.* 109, 275–286. doi:10.1016/j.trac.2018.10.015
- Junot, C., and Witting, M. (2017). Identification of Molecules from Non-targeted Analysis. *J. Chromatogr. B* 1071, 1–2. doi:10.1016/j.jchromb.2017.11.030
- Knorr, A., Almstetter, M., Martin, E., Castellon, A., Pospisil, P., and Bentley, M. C. (2019). Performance Evaluation of a Nontargeted Platform Using Two-Dimensional Gas Chromatography Time-Of-Flight Mass Spectrometry Integrating Computer-Assisted Structure Identification and Automated Semiquantification for the Comprehensive Chemical Characterization of a Complex Matrix. *Anal. Chem.* 91 (14), 9129–9137. doi:10.1021/acs.analchem.9b01659
- Li, Y., Burns, A. E., Tran, L. N., Abellar, K. A., Poindexter, M., Li, X., et al. (2021). Impact of E-Liquid Composition, Coil Temperature, and Puff Topography on the Aerosol Chemistry of Electronic Cigarettes. *Chem. Res. Toxicol.* 34, 1640–1654. doi:10.1021/acs.chemrestox.1c00070
- Margham, J., McAdam, K., Forster, M., Liu, C., Wright, C., Mariner, D., et al. (2016). Chemical Composition of Aerosol from an E-Cigarette: A Quantitative Comparison with Cigarette Smoke. *Chem. Res. Toxicol.* 29 (10), 1662–1678. doi:10.1021/acs.chemrestox.6b00188
- Martínez-Bueno, M. J., Gómez Ramos, M. J., Bauer, A., and Fernández-Alba, A. R. (2019). An Overview of Non-targeted Screening Strategies Based on High

AUTHOR CONTRIBUTIONS

NS, MN, KA, YP, WG, and JM contributed to the conception and design of the study. JM and NS managed the study program. MN performed the method validation, automated data processing and analysis with support from NS. Aerosol was collected by NM and additional data analysis and review was done by HG. NS, MN, SC, and TH supported the creation of the in-house custom library. NS is the primary author, and JM and MSM assisted in writing sections of the manuscript. MJM provided statistical support. All authors contributed to the revision, review and approved the submitted version of the manuscript.

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- Resolution Accurate Mass Spectrometry for the Identification of Migrants Coming from Plastic Food Packaging Materials. *Trac Trends Anal. Chem.* 110, 191–203. doi:10.1016/j.trac.2018.10.035
- McMillen, R. C., Gottlieb, M. A., Shaefer, R. M. W., Winickoff, J. P., and Klein, J. D. (2015). Trends in Electronic Cigarette Use Among U.S. Adults: Use Is Increasing in Both Smokers and Nonsmokers. *Nicotine Tob.* 17 (10), 1195–1202. doi:10.1093/ntr/ntu213
- Rawlinson, C., Martin, S., Frosina, J., and Wright, C. (2017). Chemical Characterisation of Aerosols Emitted by Electronic Cigarettes Using thermal Desorption-Gas Chromatography-Time of Flight Mass Spectrometry. *J. Chromatogr. A* 1497, 144–154. doi:10.1016/j.chroma.2017.02.050
- Reichenbach, S. E., Tian, X., Cordero, C., and Tao, Q. (2012). Features for Non-targeted Cross-Sample Analysis with Comprehensive Two-Dimensional Chromatography. *J. Chromatogr. A* 1226, 140–148. doi:10.1016/j.chroma.2011.07.046
- Rigotti, N. A., Harrington, K. F., Richter, K., Fellows, J. L., Sherman, S. E., Grossman, E., et al. (2015). Increasing Prevalence of Electronic Cigarette Use Among Smokers Hospitalized in 5 US Cities, 2010–2013. *Nicotine Tob. Res.* 17 (2), 236–244. doi:10.1093/ntr/ntu138
- Savareear, B., Escobar-Arnanz, J., Brokl, M., Saxton, M. J., Wright, C., Liu, C., et al. (2019). Non-targeted Analysis of the Particulate Phase of Heated Tobacco Product Aerosol and Cigarette Mainstream Tobacco Smoke by thermal Desorption Comprehensive Two-Dimensional Gas Chromatography with Dual Flame Ionisation and Mass Spectrometric Detection. *J. Chromatogr. A* 1603, 327–337. doi:10.1016/j.chroma.2019.06.057
- Sobus, J. R., Wambaugh, J. F., Isaacs, K. K., Williams, A. J., McEachran, A. D., Richard, A. M., et al. (2018). Integrating Tools for Non-targeted Analysis Research and Chemical Safety Evaluations at the US EPA. *J. Expo. Sci. Environ. Epidemiol.* 28 (5), 411–426. doi:10.1038/s41370-017-0012-y
- Talih, S., Balhas, Z., Salman, R., El-Hage, R., Karaoghlanian, N., El-Hellani, A., et al. (2017). Transport Phenomena Governing Nicotine Emissions from Electronic Cigarettes: Model Formulation and Experimental Investigation. *Aerosol Sci. Technology* 51 (1), 1–11. doi:10.1080/02786826.2016.1257853
- Tayyarah, R., and Long, G. A. (2014). Comparison of Select Analytes in Aerosol from E-Cigarettes with Smoke from Conventional Cigarettes and with Ambient Air. *Regul. Toxicol. Pharmacol.* 70 (3), 704–710. doi:10.1016/j.yrtph.2014.10.010
- Uchiyama, S., Noguchi, M., Sato, A., Ishitsuka, M., Inaba, Y., and Kunugita, N. (2020). Determination of Thermal Decomposition Products Generated from E-Cigarettes. *Chem. Res. Toxicol.* 33 (2), 576–583. doi:10.1021/acs.chemrestox.9b00410
- Wagner, K. A., Flora, J. W., Melvin, M. S., Avery, K. C., Ballentine, R. M., Brown, A. P., et al. (2018). An Evaluation of Electronic Cigarette Formulations and Aerosols for Harmful and Potentially Harmful Constituents (HPHCs) Typically Derived from Combustion. *Regul. Toxicol. Pharmacol.* 95, 153–160. doi:10.1016/j.yrtph.2018.03.012
- Zhu, S.-H., Sun, J. Y., Bonnevie, E., Cummins, S. E., Gamst, A., Yin, L., et al. (2014). Four Hundred and Sixty Brands of E-Cigarettes and Counting: Implications for Product Regulation. *Tob. Control.* 23 (Suppl. 3), iii3–iii9. doi:10.1136/tobaccocontrol-2014-051670

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The Chemical Complexity of e-Cigarette Aerosols Compared With the Smoke From a Tobacco Burning Cigarette

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Background: As e-cigarette popularity has increased, there is growing evidence to suggest that while they are highly likely to be considerably less harmful than cigarettes, their use is not free of risk to the user. There is therefore an ongoing need to characterise the chemical composition of e-cigarette aerosols, as a starting point in characterising risks associated with their use. This study examined the chemical complexity of aerosols generated by an e-cigarette containing one unflavored and three flavored e-liquids. A combination of targeted and untargeted chemical analysis approaches was used to examine the number of compounds comprising the aerosol. Contributions of e-liquid flavors to aerosol complexity were investigated, and the sources of other aerosol constituents sought. Emissions of 98 aerosol toxicants were quantified and compared to those in smoke from a reference tobacco cigarette generated under two different smoking regimes.

Results: Combined untargeted and targeted aerosol analyses identified between 94 and 139 compounds in the flavored aerosols, compared with an estimated 72–79 in the unflavored aerosol. This is significantly less complex (by 1–2 orders of magnitude) than the reported composition of cigarette smoke. Combining both types of analysis identified 5–12 compounds over and above those found by untargeted analysis alone. Gravimetrically, 89–99% of the e-cigarette aerosol composition was composed of glycerol, propylene glycol, water and nicotine, and around 3% comprised other, more minor, constituents. Comparable data for the Ky3R4F reference tobacco cigarette pointed to 58–76% of

Abbreviations: ACM, Aerosol Collected Mass (total weight of aerosol particulate matter from an e-cigarette, equivalent to TPM from a tobacco cigarette); AMB, Air/method blank; ANOVA, Analysis of variance; CM/ePen2CM, “Crisp Mint” flavored variant of the e-cigarette; CORESTA, Cooperation Centre for Scientific Research Relative to Tobacco; DC/ePen2DC, “Dark Cherry” flavored variant of the e-cigarette; FDA, US Food and Drug Administration; FID, Flame ionisation detector; GC/MS, Gas chromatography-mass spectroscopy; GT/ePen2GT, “Golden Tobacco” flavored variant of the e-cigarette; HPHC, Harmful and potentially harmful constituents; ICP-MS, Inductively coupled plasma-mass spectrometer; ISO, International organization for standardization; ISO-INTENSE (ISO-I), More intensive cigarette smoking regime than traditional ISO puffing parameters; LOD, Limit of detection; LOQ, Limit of quantification; MF, Match factor; NF, Non-flavored - an unflavored variant of the e-cigarette; PAH, Polycyclic Aromatic Hydrocarbon; TOFMS, Time-of-flight mass spectrometer; TPM, Total Particulate Matter (total weight of mainstream smoke from a cigarette); TSNAs, Tobacco specific nitrosamines.

cigarette smoke “tar” being composed of minor constituents. Levels of the targeted toxicants in the e-cigarette aerosols were significantly lower than those in cigarette smoke, with 68.5–>99% reductions under ISO 3308 puffing conditions and 88.4–>99% reductions under ISO 20778 (intense) conditions; reductions against the WHO TobReg 9 priority list were around 99%.

Conclusion: These analyses showed that the e-cigarette aerosols contain fewer compounds and at significantly lower concentrations than cigarette smoke. The chemical diversity of an e-cigarette aerosol is strongly impacted by the choice of e-liquid ingredients.

Keywords: e-cigarette, flavor, aerosol chemistry, targeted, untargeted

INTRODUCTION

Since their emergence in the early 2000s, e-cigarettes have emerged as popular alternatives to tobacco cigarettes. Reviews of the e-cigarette science base suggest that while the absolute risks of vaping cannot yet be determined unambiguously, e-cigarette use appears to be associated with reduced exposure to many cigarette smoke toxicants (National Academies of Sciences, Engineering, and Medicine, Health and Medicine Division, Board on Population Health and Public Health Practice, Committee on the Review of the Health Effects of Electronic Nicotine Delivery Systems, 2018; PHE 2019]. However, e-cigarette use is not free from risk, with reports of adverse events in the pulmonary, oral, gastrointestinal and other bodily systems following exposure to e-cigarette aerosol (Seiler-Ramadas et al., 2020).

Historically, an established starting point in characterising the risks of using nicotine inhalation products has been the thorough chemical characterisation of the matrix inhaled by the user. For example, in the case of cigarettes, over 60 years of detailed scientific work undertaken to elucidate the chemical composition of both tobacco and smoke have highlighted their extreme chemical complexity at over 8,000 identified compounds in tobacco and over 6,500 identified compounds in cigarette smoke (Rodgman and Perfetti 2013). Clarity over the numbers, identities and concentrations of these compounds has enabled scientists, non-governmental agencies and regulators to compile priority lists of smoke constituents that are considered to contribute to the toxicity of cigarette smoke (Hoffmann and Hoffmann, 1988; Health Canada 1999a; Liu et al., 2011). These constituents have been referred to variously as “biologically active agents,” toxicants, and Harmful or Potentially Harmful Constituents (HPHC). In 2008 the WHO’s Tobacco Product Regulation (TobReg) working group proposed 9 toxicants for mandated lowering of levels in cigarette smoke (Burns et al., 2008). More recently, the US Food and Drug Administration (FDA 2012) published a list of 93 cigarette smoke HPHCs, with reporting requirements for a subset.

The growth in e-cigarette use as an alternative to cigarette smoking has led to significant efforts to understand the chemical composition of both e-liquids and e-cigarette aerosols. In contrast to the chemically diverse composition of tobacco, e-liquids are in

principle compositionally simple, being composed of four main constituents: vegetable glycerol (VG), propylene glycol (PG), nicotine and water. However, there are also many thousands of flavored e-liquids available for sale, (Zhu et al., 2014; Krüsemann et al., 2021), whose flavor character is made up of synthetic flavor compounds, extracts of natural materials, or combinations of these. In addition to flavor compounds, minor components of ingredients and device materials, and potential reaction products of flavor compounds with major e-liquid components, and device materials, can extend the chemical complexity of the e-liquid. There is growing interest in the chemical composition of e-liquids, particularly the number, identities, quantities and toxicological impacts of flavorants used in them (Erythropel et al., 2020; Krüsemann et al., 2021; Omaiye et al., 2020).

E-cigarette aerosols are more chemically complex than e-liquids, due to formation of reaction and degradation products when the e-liquid is heated during aerosolisation. E-cigarette aerosol studies tend to use targeted HPHC analyses (National Academies of Sciences, Engineering, and Medicine, Health and Medicine Division, Board on Population Health and Public Health Practice, Committee on the Review of the Health Effects of Electronic Nicotine Delivery Systems, 2018), such as, carbonyls (Farsalinos and Gillman, 2018), metals (Williams et al., 2013) and major e-liquid component thermal decomposition products (Uchiyama et al., 2020). A few e-cigarette studies have targeted broader ranges of HPHC emissions, Lauterbach and Laugesen (2012), Tayyarah and Long 2014, Cunningham et al. (2020) with up to 150 measurands examined (Margham et al., 2016). These studies have shown considerable differences between e-cigarette aerosols and cigarette smoke, detecting fewer HPHCs in e-cigarette aerosols, and lower concentrations of those that are present. Such “targeted analyses” are powerfully informative in that they use analytical methods appropriate to the analyte being investigated, offer clear identification of the species present, and can quantify their concentration. However, a drawback of targeted analyses for specific, compounds (particularly cigarette smoke HPHCs) is that even the broadest study is unlikely to cover all of the constituents or toxicants present in e-cigarette aerosols. For example, a number of flavor-related chemicals of toxicological concern have been identified in e-cigarette aerosols, but were not prioritised in historic cigarette

smoke toxicant lists, e.g., diacetyl (Allen et al., 2016; Vas et al., 2019), cinnamaldehyde (Behar et al., 2016), furfurals (Soussy et al., 2016), benzaldehyde (Kosmider et al., 2016), and vitamin E acetate (Boudi et al., 2019).

An alternative approach for examining the breadth of compounds present in a matrix is to conduct an “untargeted” analysis. Untargeted GC-MS approaches have shown greater capability for this type of analysis than their HPLC-MS counterparts. GC approaches use thermal conditions that are very consistent with the operating temperatures of e-cigarettes, suggesting analytical compatibility with the aerosol species present; also GC-MS libraries currently offer greater capability than HPLC-MS libraries. Two studies have reported successful application of untargeted GC-MS analysis to e-cigarette aerosols and have identified similar numbers of compounds. Using thermal desorption GC-MS (TD-GC-MS) Herrington and Myers (2015) identified 85 aerosol compounds. Using TD-GC-TOFMS Rawlinson et al. (2017) identified 33 compounds in an unflavored commercial e-cigarette product, and 69–87 compounds in flavored e-cigarettes. GC-MS approaches are not universal in their analytical capability, mainly due to limitations associated with chromatographic performance including “blind-spots” in the analysis for compounds eluting closely to major constituents (Herrington and Myers 2015). They do not easily identify very low molecular weight compounds, high boiling point species, nitro compounds, metals, most organic acids, tobacco-specific nitrosamines and compounds that require derivatisation prior to analysis (Rawlinson et al., 2017). Using a non-chromatographic approach that sampled e-cigarette puffs directly into a secondary electrospray ionisation (ESI) high resolution mass spectrometer (SESI-HRMS) García-Gómez et al. (2016) identified 142 compounds in an e-cigarette aerosol. SESI-HRMS has challenges with detection of low molecular weight species and compounds that are not easily ionised by ESI (such as PAHs), compound identification, and quantification. All of the techniques employed to date have limitations, and none are capable by themselves of fully characterising the chemical composition of an e-cigarette aerosol.

Therefore, despite it being a fundamental step in understanding e-cigarette science, and central to current public health concerns over vaping risks, there remains ongoing uncertainty concerning the chemical composition of e-cigarette aerosols. This is a basic characteristic defining e-cigarette aerosol properties, serving as a gateway to more complete studies of their chemical toxicity. Our study seeks to address this gap by combining untargeted and targeted analytical methods to more completely characterise the chemical composition of e-cigarette aerosols. We used the untargeted GC-MS method described by Rawlinson et al. (2017) and 18 additional targeted validated chemical assays for 98 specific compounds. The targeted methods covered many compounds that are poorly dealt with by the untargeted scan, including metals, nitrosamines, permanent gases, low molecular weight compounds, compounds requiring derivatisation and high boiling point aromatic species such as PAHs and aromatic amines. We examined aerosols from three common examples of e-cigarette flavors, tobacco, mint and a fruit flavor, in the same e-cigarette.

This analytical strategy provided some insights into the impact of flavor complexity on aerosol composition. We further used quantitative data to conduct a mass-balance of the aerosol composition, providing some insights into the proportion of the aerosol made up by constituents other than the main e-liquid ingredients. Finally, these measurements were conducted in comparison to the mainstream smoke from a reference tobacco cigarette.

EXPERIMENTAL PROCEDURES

Products

The e-cigarette used in this study, Vype ePen2 (Nicoventures Trading Ltd., Blackburn, United Kingdom), was an updated version of Vype ePen (as tested by Margham et al., 2016). Like the earlier version, it consisted of a rechargeable battery section and a disposable e-liquid containing cartridge (eCap). The battery section comprised a USB-rechargeable battery and an integrated circuit power controller with two voltage settings selectable by the consumer *via* an external twin-setting surface mounted switch. Device operation commences when the user presses either setting of the power switch, usually in advance of the puff starting, with power operating within the device as long as the button remained pressed. The liquid contained in the eCap was fed to the atomizer through a sintered porous ceramic disk in contact with a silica transport wick. The atomizer comprised a 2.85 Ω nichrome (80% Ni/20% Cr) wire coil heater wrapped around the wick. The updates incorporated into the ePen2 model included physical alterations to the dimensions and appearance of the device and cartridge, and also changes to the electronic features such as micro-USB charging and a reduction in the voltage settings from 3.6–4.0 to 3.5–3.7 V range for the low and high power settings, respectively.

The e-liquids studied were contained in Vype eCaps, that are disposable e-liquid cartridges containing 1.58 ml of e-liquid. In this study, we tested Golden Tobacco (ePen2GT), Dark Cherry (ePen2DC), and Crisp Mint (ePen2CM) flavored e-liquids. All the liquids contained VG, PG, water, flavors, and nicotine. The ingredient specifications for the e-liquids used in this study are shown in **Table 1**. The ingredients used in the three flavors were toxicologically assessed using the approach of Costigan and Meredith (2015). Also shown in **Table 1** are the specifications for a non-commercial, unflavored e-liquid (referred to as “ePen2NF”) that we included in our untargeted aerosol emissions investigations. All the e-liquids had similar specifications (w/w) for water (25.00%) and nicotine (1.86%). The DC, GT, and unflavored e-liquids had the same specifications for VG (48.14%) and similar specifications for PG (23.86–25.00%), while the specifications for the CM e-liquid were 37.64% for VG and 34.73% for PG. The total percentage incorporation of all flavor compounds (the sum of all individual compounds) in the e-liquids was 0.77% for CM, 1.14% for DC, and 0.03% for GT. Commercial manufactured e-cigarettes from a single batch were tested (for both device and eCaps). Quality control checks were conducted on physical characteristics of all products against their manufacturing specifications before conducting the chemical analyses. This e-cigarette has an operating life of over 200 puffs per

TABLE 1 | Specified composition of the e-liquids used in this study.

eCap flavor	Product code	Proportions of ingredients (% w/w)				
		VG	PG	Water	Nicotine	Other (flavors)
CM 18 mg/ml	ePen2CM	37.64	34.73	25.00	1.86	0.77
DC 18 mg/ml	ePen2DC	48.14	23.86	25.00	1.86	1.14
GT 18 mg/ml	ePen2GT	48.14	24.97	25.00	1.86	0.03
Unflavored	ePen2NF	48.14	25.00	25.00	1.86	0.00

cartridge, depending on usage patterns, and we conducted these tests at the “High Power” setting (3.7 V).

The tobacco cigarette used for comparison in the current work was the Ky3R4F Kentucky Reference Cigarette, a US-blended king-sized product that has been widely used as a standard test-piece for scientific studies. It has a cellulose acetate filter and a tar yield under ISO 3308 puffing conditions International Organization for Standardization, (2012) of 9.4 mg/cigarette in 9 puffs. Main technical specifications are available on the website of the Kentucky Tobacco Research and Development Center, (2017). The mainstream smoke HPHC yields of the Ky3R4F under both ISO 3308 and ISO-Intense smoking conditions (ISO 20778:2018) have been reported previously (Roemer et al., 2012).

Aerosol and Smoke Generation

Untargeted analyses were conducted at British American Tobacco R&D laboratories (Southampton, United Kingdom). Targeted analyses were conducted by Labstat International ULC (Kitchener, ON, Canada) using established methods developed, validated, and operated according to ISO17025. In all cases, aerosol, cigarette smoke, or Air/Method Blanks (AMBs) were generated using commercial puffing machines, adapted where necessary to accommodate e-cigarette button activation as part of the puffing cycle.

For untargeted analyses 80 ml puffs, over 3 s, taken twice per minute were produced using a Borgwaldt LX1 automated syringe unit (Borgwaldt KC, Hamburg, Germany), and the generated aerosol collected on conditioned Tenax TA/Sulficarb thermal desorption tubes (Markes International, Llantrisant, United Kingdom). For targeted emission testing, the e-cigarette puffing regime used was the Recommended Method 81 developed by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) for machine puffing of e-cigarettes.

Both of these methods reflect the longer puff duration commonly observed (McAdam et al., 2019) for e-cigarette users as compared with cigarette smokers (CORESTA 2015). The e-cigarette puffing regime specifies a puff volume of 55 ml, and a puff duration of 3 s, taken twice per minute (ISO 20768, 2018a). For products that require button activation to initiate aerosol generation, CORESTA specifies the activation timing parameters. In the current study, the activation button was pressed 1 s before each puff and held down for the duration of the puff (4 s in total for each puff). For this study, we activated the button using robotic, programmed devices synchronized to the puffing engines. Ky3R4F cigarettes were prepared for smoking according to ISO 3402 (International Organization for Standardization, 1999) and smoked under two different

smoking regimes, the ISO 3308 International Organization for Standardization, (2012) and the ISO-Intense regimes (Health Canada 1999, ISO 20778, 2018b). The ISO 3308 regime specifies a 35 ml puff volume and 2 s puff duration taken once per minute. The ISO-Intense regime is an internationally standardized version of the smoking regime introduced in 1999 by Health Canada to compensate for potential blocking of the filter ventilation holes during smoking and to reflect the larger puff volumes taken by many smokers. The regime specifies a 55 ml puff volume and a 2 s puff duration taken twice per minute, with all filter ventilation holes blocked. The ISO-Intense smoking regime results in higher smoke yields than the ISO regime. When cigarettes are machine-smoked, the butt length and hence tobacco rod length smoked are predetermined. Under ISO 20768 puffing conditions, the e-cigarette cartridge provides more than 200 puffs of aerosol before the e-liquid becomes exhausted, and the Ky3R4F cigarette yields about 9 puffs under ISO 3308 conditions and 9–12 puffs under ISO-Intense parameters (ISO 20778:2018).

For targeted analyses, emissions data were collected on a per-cigarette basis for Ky3R4F, with the puff number recorded. For the e-cigarettes, the analyses were conducted on the cumulative emissions collected over 100 puffs. In the earlier paper by Margham et al. (2016), emissions were collected and analyzed from two successive 100 puff blocks. Since no significant differences were found between the first and second 100-puff block, it was decided to analyze only the first 100 puffs in the present study. The reported data for the Ky3R4F and the e-cigarette variants each comprises five independent replicates of products sampled at one point in time.

E-cigarette puffing and cigarette smoking were conducted in different dedicated laboratories, to minimise the potential for atmospheric contamination from cigarette smoke on e-cigarette measurements. AMB measurements were conducted to control for potential laboratory background levels of the target analytes (Margham et al., 2016; Wagner et al., 2018). For the e-cigarettes, AMBs were generated by drawing 100 puffs of laboratory air through empty ports of the puffing machine, and samples were analyzed in the same way as aerosol samples. For reference cigarettes, AMB measurements were also taken by drawing puffs through empty ports of the smoking machine under both ISO 3308 and ISO-Intense conditions.

Chemical Analysis of Emissions From the E-Cigarette Aerosol and Cigarette Smoke

The untargeted aerosol scan was conducted using the semi-quantitative screening method described by Rawlinson et al.

(2017). This method detects volatile and semi-volatile organic compounds with volatilities in the range from C_3 hydrocarbons up to C_{28} hydrocarbons. PAHs with 5 or more rings, as well as other high molecular weight species with low volatilities at 250°C , are not detected by this method. The analysis is semi-quantitative, with compound concentrations estimated in comparison to known quantities of internal standard compounds. The method has been described in detail by Rawlinson et al. (2017) and is summarised in **Supplementary File S1**.

The emissions of major components (total aerosol and smoke masses, VG, PG, nicotine and water) from tobacco and electronic cigarettes were measured by trapping the generated smoke and aerosol on Cambridge Filter Pads (CFP). The total mass gained by the CFP during cigarette smoke experiments is defined as the TPM. With e-cigarettes, the same approach provides the gravimetric determination of ACM. Chemical analysis of TPM for nicotine and water allows for the calculation of the quantity ("nicotine-free dry particulate matter") known as cigarette smoke tar. Chemical analysis of the ACM in this study for the major aerosol components PG, VG, water, and nicotine allows estimation of the mass of other unmeasured aerosol components. In both cases the quantity "Balance" was used, which was defined as the difference between either TPM or ACM and the sum of the major measured components.

A total of 98 individual compounds were measured in the emissions from the three flavor variants of the e-cigarette, the Ky3R4F reference cigarette, and respective AMBs. Many of these compounds are on one or more of the regulatory lists of harmful or potentially harmful cigarette smoke components. These include the Health Canada list of 42 toxicants in cigarette smoke (not including tar, nicotine and carbon monoxide) that are required to be measured and reported for cigarettes on the Canadian market (Health Canada 1999a), the FDA's established list of 93 HPHCs in tobacco products and tobacco smoke (not including nicotine and carbon monoxide) of which 18 have to be reported currently (FDA 2012), and the World Health Organization list of 9 cigarette smoke components for which maximum levels have been proposed (Burns et al., 2008).

In analysing the 98 compounds of regulatory interest 18 different analytical methods were employed. Generally, groups of analytes belonging to the same chemical class were analyzed together in each method. Fewer compounds were tested than in the earlier paper by Margham et al. (2016) as that study showed no evidence for the presence of radioactive elements, polychlorinated dibenzodioxins or dibenzofurans in the e-cigarette aerosol. The concentrations of all these HPHC compounds were previously below the LODs of their measurement methods. The analytical methods used have been reported previously Margham et al. (2016), are summarised in **Supplementary File S1** and described briefly here. Nicotine, propylene glycol, menthol, ethylene glycol, diethylene glycol, glycidol, and glycerol were analysed using GC/FID. Nicotine related alkaloids were analyzed by LC-MS/MS. Volatile carbonyls and dicarbonyls were determined by O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) derivatization of trapped aerosol, followed by GC-MS analysis. Carbon monoxide was analyzed by non-dispersive infrared analysis. Nitrogen oxides were analyzed using chemiluminescent techniques following reaction with ozone.

Volatile and semi-volatile organic compounds were analyzed using GC-MS methods. Ammonia was analyzed by HPLC and conductivity detection. Hydrogen cyanide was quantified using continuous flow analysis. Phenolic compounds were analyzed by HPLC/FLD analysis. Aromatic amines were analyzed by GC-MS following derivatization by pentafluoropropionic acid anhydride. PAHs were quantified using GC-MS. Tobacco-specific nitrosamines were analyzed by LC-MS/MS. Volatile nitrosamines were analyzed by LC-MS. Metals were determined using inductively coupled plasma-mass spectrometry (ICP-MS) with a H_2/He collision reaction interface.

Data Analysis

Estimating Numbers of Aerosol Compounds

The chromatograms were interrogated using the following sequence to estimate the numbers of compounds detected in the aerosol:

- A. The peaks in both aerosol samples and AMBs were attributed to specific compounds, the library Match Factor (MF) recorded, and numbers of peaks counted.
- B. Duplicate compounds that eluted closely together in a chromatogram were counted as one peak, and the total peak number reduced accordingly.
- C. Compounds that were present in the AMBs, at comparable levels to the e-cigarette aerosol levels, i.e., contaminants, were removed from the aerosol list.
- D. The remainder constituted the total peaks in the non-targeted scan provided exclusively by the e-cigarette.
- E. The number of compounds detected in the targeted analysis suite were counted; with the e-cigarette aerosols only those compounds present at levels > those in the AMB sample were counted.
- F. Numbers of compounds detected in both untargeted (D) and targeted analyses (E), i.e. counted twice, were identified.
- G. The totals from (F) were subtracted from the numbers counted in the targeted suite of analyses (E) to establish the numbers of compounds detected only in the targeted suite.
- H. The totals of compounds found in the untargeted analysis (D) and only in the targeted analyses (G) were summed to provide the total number of compounds detected for each sample in this study.

Once the list of identified compounds had been assembled, we attempted to assign sources of these compounds in the e-cigarette aerosols, by categorising them into the following groups using knowledge of in-going materials and plausible reaction chemistries:

- i. Known ingoing ingredients,
- ii. Ingredient or device related minor constituents,
- iii. Ingredient reaction products,
- iv. Thermal decomposition products and
- v. Compounds for which specific sources could not be assigned

With the unflavored ePen2 sample, which was not analysed for targeted analytes, typical targeted analytes found with the flavored samples were used for guidance purposes in this analysis.

TABLE 2 | Numbers of the 98 targeted analytes undetected ($\leq$ LOD), detected but not quantified ($>$ LOD but $\leq$ LOQ) and quantified ($>$ LOQ) in the test articles and AMB.

Product	Number of targeted analytes reported			Total detected
	$\leq$ LOD	$>$ LOD but $\leq$ LOQ	$>$ LOQ	
ePen2CM	60	16	22	38
ePen2DC	55	14	29	43
ePen2GT	63	10	25	35
AMB	75	13	10	23
3R4F (ISO)	16	6	76	82

Differences in Magnitude of Emissions From e-Cigarettes and Cigarette Smoke

Due to the substantial differences in puff numbers obtained from machine-smoking a tobacco cigarette (approximately 9–12 puffs) compared with an e-cigarette cartridge (up to several hundred puffs), the data are presented both “as measured” (i.e., per stick for Ky3R4F or per 100 puff block for the e-cigarette) and also on a per puff basis by dividing the reported values by the number of puffs taken during the measurement. The calculated per-puff values allow a direct comparison of emissions between products. Percentage differences between the emissions from the e-cigarettes and the Ky3R4F are calculated on a per puff basis.

We followed the same procedure as Margham et al. (2016) for calculating percentage differences in analyte concentrations from the e-cigarette aerosol that were below the limit of detection (LOD) or limit of quantification (LOQ) compared with smoke from the Ky3R4F cigarette results. For data $<$ LOD, the value was calculated as one-half of the analytical method’s reported LOD. For data $<$ LOQ but $>$ LOD, the value was calculated as the midpoint between the reported LOD and LOQ of the analytical method:

$$\text{Imputed value} = \text{reported LOD} + 0.5 \times (\text{reported LOQ} - \text{reported LOD})$$

In cases where the e-cigarette and reference tobacco cigarette emissions were both $<$ LOD or $<$ LOQ, the measurand was omitted from the percentage difference calculations. In addition, the analysing laboratory provided “machine read values” when the test article measurement was $>$ LOD but $<$ LOQ. These enabled the comparisons to be conducted statistically in cases where a minimum of 3 replicates were reported $>$ LOD but $<$ LOQ.

Reductions in e-cigarette yields were calculated for each toxicant of regulatory interest (i.e., that appears on one or more lists) except where the yields were $<$ LOD for both the e-cigarette and Ky3R4F cigarette. Where nicotine was on the toxicant list, composite yield reductions were calculated both with and without nicotine. Toxicant yields from the e-cigarettes were compared to yields from the Ky3R4F cigarette determined using the ISO-Intense smoking regime. The composite percentage reductions were calculated as the average reductions for all the toxicants on each list. Calculations were conducted without subtraction of AMB values.

Differences in results between products were tested for statistical significance (at the 95% level) using one-way analysis of variance (ANOVA) with a Tukey’s pairwise comparison, or two-way T-tests, where appropriate. Comparisons included toxicant concentrations in the e-cigarette aerosols vs. the AMB measurements, across the different flavored aerosols and between the e-cigarette aerosols and the smoke from the Ky3R4F cigarettes obtained under different smoking regimes. Data analyses were performed using SAS software (SAS System for Windows Version 9.4, SAS Institute Inc., Cary, NC, United States). Some additional analyses were carried out with the Minitab 16 statistical software package (State College, PA, United States).

RESULTS

Aerosol Complexity

Numbers of peaks quantified, detected but at levels too low to quantify, or undetected in the targeted analyses are presented in Table 2. These data are also illustrated graphically in Figure 1. Of the 98 compounds analysed, a total of 23 compounds were detected in the e-cigarette AMB. For the e-cigarette aerosols 35 (ePen2GT), 38 (ePen2CM), and 43 (ePen2DC) analytes were detected. For Ky3R4F smoke, under ISO 3308 smoking conditions, 82 were detected. In terms of quantifiable levels of the detected compounds, for the AMB 10 compounds could be quantified. For the e-cigarettes 22 (ePen2CM), 25 (ePen2GT) and 29 (ePen2DC) analytes were quantifiable. 31 of the targeted analytes were measured across all the samples (plus ACM) that could be quantified for at least one of the e-cigarettes. For Ky3R4F smoke 76 analytes were quantifiable.

Findings from the untargeted analyses are summarised in Table 3. The AMB chromatogram contained 22 detected components (row B2), the unflavored e-cigarette (ePen2NF) aerosol had 68, and greater numbers of

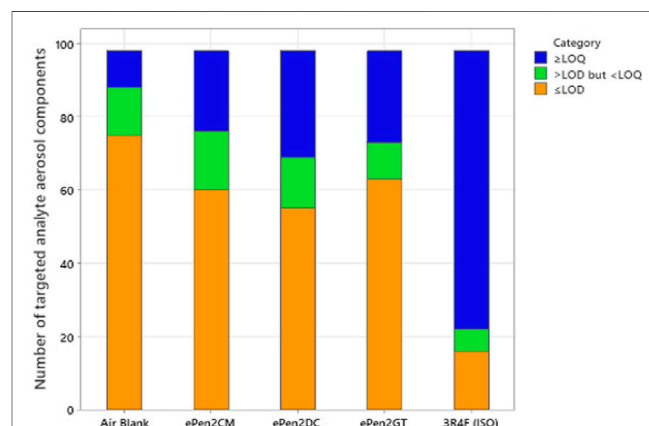


FIGURE 1 | Number of targeted aerosol compounds in the three e-cigarette flavor variants, the air blank and the Ky3R4F cigarette (smoked under ISO conditions). The components are categorised according to whether their concentrations were quantifiable ($>$ LOQ), detectable but not quantifiable ($>$ LOD but $<$ LOQ) or not detectable ($<$ LOD).

TABLE 3 | Numbers of peaks detected in untargeted and targeted analyses.

	Air method blank (AMB)	ePen2NF	ePen2GT	ePen2CM	ePen2DC
A. Detected compounds in untargeted scan	22	72	91	99	131
B1. Number of duplicate peaks	0	4	2	5	3
B2. Detected compounds after subtraction of duplicates	22	68	89	94	128
C. Compounds in AMB also present at comparable levels to analysed ePen aerosol scan	-	1	0	3	1
D. Number of untargeted compounds generated exclusively by e-cigarette	-	67	89	91	127
E. Number of detected targeted analysis peaks (for the ePen samples only those > AMB were counted)	22	n/a	13	22	23
F. Compounds identified in both untargeted (D) and targeted analyses (E)	1	n/a	8	12	11
G. Compounds uniquely identified in targeted analyses	21	n/a (5–12) ^a	5	10	12
H. Total peaks detected (D + G)	43	>67 (72–79)	94	101	139

^aFigure in parenthesis is an estimate for the number of compounds in the targeted scan of the unflavored e-cigarette aerosol; the estimate used was the range of targeted analytes detected with the flavored e-cigarettes.

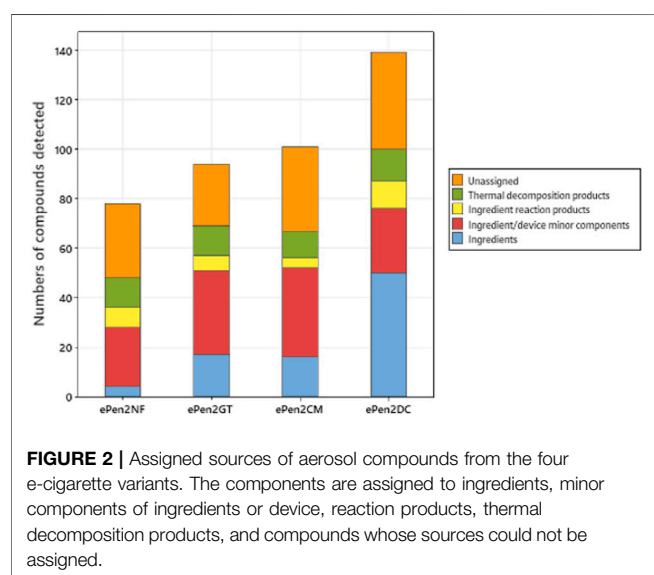


FIGURE 2 | Assigned sources of aerosol compounds from the four e-cigarette variants. The components are assigned to ingredients, minor components of ingredients or device, reaction products, thermal decomposition products, and compounds whose sources could not be assigned.

compounds were detected in the flavored e-cigarette aerosols: 89 for ePen2GT, 94 for ePen2CM, and 128 for ePen2DC. In each case a small number (0–3) were also detected in the AMB at comparable levels to the e-cigarette samples (although greater numbers of compounds were also detectable in the AMB at significantly lower levels than in the e-cigarette samples).

Table 3 also shows further analysis of aerosol constituent numbers. The total numbers of aerosol constituents measured in this study were calculated for each aerosol (**Table 3**, row H); the total number of compounds from the untargeted analysis were added to those detected (whether quantifiable or not) in the targeted analyses. Those compounds identified in both targeted and untargeted analyses (8–12 for the flavored e-cigarettes) were counted once only. **Table 3**, row H shows that in total 43 compounds were detected in the AMBs. For the unflavored e-cigarette a total count was estimated because the targeted scans were not conducted on the unflavored variant; the range of values for compounds uniquely found in the targeted analyses of the

flavored e-cigarettes suggested a range of 72–79 compounds in the unflavored e-cigarette aerosol. In contrast, greater numbers of compounds were observed in the three flavored e-cigarette aerosols, from 94 to 139, using the combined analytical techniques.

The detected compounds were assigned to estimated sources as shown in **Figure 2**. Three e-liquid ingredients were detected in the aerosol of the unflavored e-cigarette. Had the targeted analyses been conducted on this sample water would have been detected, and hence number of ingredients would be four for this sample. With the flavored aerosols between 17 and 50 peaks were assigned as ingredients (with DC > CM > GT). Other sources of detected compounds in the aerosol included between 4 and 11 ingredient reaction products, such as acetals/hemiacetals/ketals formed by reaction of carbonyls with PG or VG. Compounds consistent with minor components of ingredients (e.g., minor components of flavors or solvent residues) or deriving from device materials (such as monomer residues) comprised 24 compounds (unflavored) and 26–36 compounds for the flavored aerosols. We also detected 11–13 compounds that were regarded as thermal decomposition products of ingoing ingredients. Subtraction of all these assigned compounds from the total numbers of compounds resulted in totals of 30 compounds for the unflavored aerosol and 25–39 compounds for the flavored aerosols that could not be assigned to a source. The inability to assign a source for these compounds was either because they could not be identified with sufficient confidence (e.g., low library match factor) or there was no clear explanation for their presence in the aerosol.

Quantitative Analyses

In the targeted analyses the emissions of 98 aerosol components were quantified. The yields of these components for the three e-cigarette variants, the Ky3R4F cigarette (under both ISO and ISO-Intense smoking conditions), and the matching AMB samples are shown in **Supplementary Table S1**. As discussed above, 30 analytes were found in one or more of the e-cigarette aerosols and the emissions of these compounds are shown in **Supplementary Table S2**. These emission values are summarised in the following paragraphs:

TABLE 4 | Comparison of total quantities of cigarette smoke (3R4F) and aerosol (ePen2), and their major smoke/aerosol components.

Components (mg/puff)	ePen2CM	ePen2DC	ePen2GT	3R4F (ISO)	3R4F (ISO-I)
ACM/TPM	3.77 ± 0.41	3.69 ± 0.42	3.54 ± 0.52	1.14 ± 0.12	4.29 ± 0.25
Water	0.99 ± 0.11	0.98 ± 0.07	0.92 ± 0.10	0.086 ± 0.015	1.38 ± 0.13
Nicotine	0.052 ± 0.006	0.049 ± 0.006	0.047 ± 0.08	0.085 ± 0.014	0.185 ± 0.008
VG	1.47 ± 0.16	1.90 ± 0.09	1.52 ± 0.17	0.10 ± 0.005	0.22 ± 0.005
PG	1.05 ± 0.11	0.73 ± 0.05	0.67 ± 0.10	<LOQ ^a	0.002 ± 0.005
Balance	0.21	0.03	0.39	0.87	2.50
Balance (% of ACM/TPM)	5.6	0.8	11	76	58

<LOQ—below limit of quantification, i.e. not quantifiable, ACM—aerosol collected mass from the e-cigarettes; TPM—total particulate matter from the tobacco cigarette; Balance—TPM less water, nicotine, glycerol and propylene glycol; ISO-I—Iso-Intense; VG—Glycerol; PG—propylene glycol.

^aLOQ = 0.001 mg/puff.

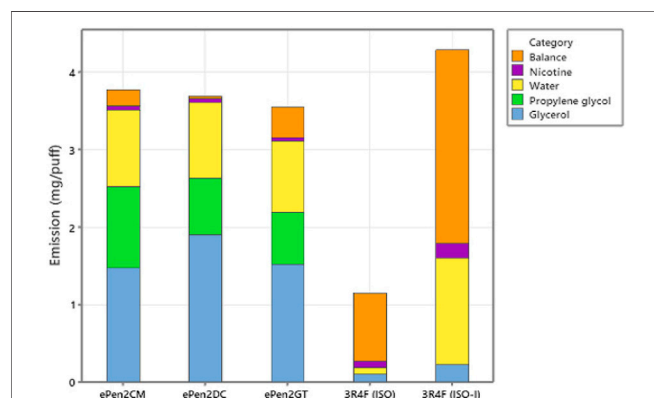


FIGURE 3 | Major components of the aerosol masses collected from the three e-cigarette variants and the mainstream smoke from the Ky3R4F reference cigarette smoked under ISO and ISO-Intense regimes. The balance is the difference between the total collected mass and yields of glycerol, propylene glycol, nicotine and water. Emissions are in mg/puff.

Major Aerosol Components

ACM comprises the total mass of aerosol particles collected from the e-cigarettes generated during the puffing block. **Table 4** and **Figure 3** shows the major ACM components were the e-liquid compounds VG, PG, water, and nicotine. ACM values for the three e-cigarettes did not differ significantly. We note that the measured aerosol VG emission for GT was lower than expected by 0.3 mg/puff (by comparison to the other two products, factoring in their initial VG compositions and ACM/puff). Repeat analysis provided a higher per-puff VG in emissions, but higher than expected. We therefore reported the original measured value but note our concern over its robustness. There were differences in aerosol VG and PG concentrations between the e-cigarette variants consistent with the specified e-liquid compositions shown in **Table 1**. There were also differences in the values for “Balance” between the e-cigarette variants, with the GT variant having a higher value (11% of the ACM value—much of which can be explained by the VG measurement issue) than the CM (5.6% of the ACM) and DC (0.8% of the ACM) variants. For the Ky3R4F reference cigarette, the major components of the ISO 3308 TPM were VG, water, and nicotine, and under ISO-Intense conditions were water, VG, nicotine, and PG. The Balance after subtracting these components from the ISO 3308 TPM was

0.87 mg/puff (76.4% of the TPM), and 2.5 mg/puff (58% of the TPM) under ISO-Intense.

Carbonyls

Ten of the eighteen carbonyls analyzed were not detected in the aerosols of the three e-cigarettes: isobutyraldehyde, methyl ethyl ketone, 3-buten-2-one, n-butyraldehyde, crotonaldehyde, acetoin, 2,3-butanedione (diacetyl), 2,3-pentanedione (acetyl propionyl), 2,3-hexanedione and 2,3-heptanedione (**Supplementary Table S1**). The remaining eight carbonyls had quantifiable concentrations in the aerosols of all three flavor variants of the e-cigarette (**Supplementary Table S2**). These were formaldehyde, acetaldehyde, acetone, propionaldehyde, acrolein, glycolaldehyde, glyoxal and methylglyoxal. There were no significant differences (at 95%) in per puff yields with any of the quantified carbonyls between the three different flavor versions, other than propionaldehyde and acetone (**Table 5**) despite the differences of the in-going ingredients between the three variants (**Table 1**). Two of the carbonyls analyzed—formaldehyde and acetone—had quantifiable levels in the e-cigarette AMB. The AMB levels of formaldehyde represented 5.6–19% of the yields measured for the e-cigarette products, while the levels of acetone were 34–50% of the e-cigarette yields. On a per puff basis, quantified carbonyl emissions from the e-cigarette were, depending on the carbonyl, 68.6–>99.9% lower than ISO 3308 smoke yields from the Ky3R4F cigarette and 88.4–>99.9% lower than ISO-Intense Ky3R4F smoke yields (**Supplementary Table S1** and **Supplementary Table S2**). For the Ky3R4F AMBs, six carbonyls were quantifiable under ISO 3308 and three under ISO-Intense puffing conditions. Formaldehyde (AMB value 12–24% of the mainstream emission values), acetaldehyde (<1%) and 2,3-butanedione (<5%) were identified in both puffing regimes AMBs. Acetone (3% of mainstream smoke levels), methyl ethyl ketone (4%), and crotonaldehyde (16%) were quantified under ISO 3308 puffing conditions.

Phenolics

None of the phenolic toxicants measured were quantified in any of the e-cigarettes or the AMB. In contrast, all of these compounds were quantifiable (except resorcinol under ISO 3308 conditions) in mainstream smoke from Ky3R4F under both puffing regimes. None of the compounds were detected

TABLE 5 | Means (per collection) and ANOVA results for analytes with at least one quantifiable result.

Analyte	CM		DC		GT		AMB		P
ACM, mg	377	A	369	A	354	A	0.00	B	<0.001
"Tar," mg	273	A	266	A	258	A	0.18	B	<0.001
VG, mg	147	B	190	A	152	B	0.13	C	<0.001
PG mg	105	A	73.1	B	66.9	B	0.13	C	<0.001
Water, mg	98.5	A	97.6	A	91.7	A	0.30	B	<0.001
Nicotine, mg	5.18	A	4.88	A	4.65	A	0.015	B	<0.001
Menthol, mg	0.613	A	0.121	B	0.014	C	0.006	C	<0.001
Allyl alcohol, µg	1.06	B	4.08	A	0.64	B	0.03	B	<0.001
Formaldehyde, µg	59.0	A	17.5	A B	39.8	A B	3.32	B	0.018
Acetaldehyde, µg	18.0	A	7.71	A B	10.3	A B	0.91	B	0.026
Acetone, µg	6.15	A B	6.94	A	4.84	B	2.40	C	<0.001
Propionaldehyde, µg	6.01	A	4.94	A B	2.30	B C	0.12	C	<0.001
Acrolein, µg	19.2	A	12.3	A	12.4	A	0.230	B	0.002
Glycolaldehyde, µg	9.95	A	2.43	AB	8.16	AB	0.187	B	0.02
Glyoxal, µg	4.78	A	1.71	AB	2.97	AB	0.063	B	0.013
Methylglyoxal, µg	6.46	A	6.26	A	5.24	A	0.039	B	0.005
Nicotine-N-oxide, µg	1.89	B	4.37	A	1.60	B	0.44	C	<0.001
Cotinine, µg	0.855	B	1.37	B	5.64	A	0.14	B	<0.001
Myosmine, µg	0.815	B	1.95	B	6.23	A	0.22	B	<0.001
Normicotine, µg	2.57	B	7.39	A	2.52	B	0.12	C	<0.001
β-Nicotyrine, µg	0.658	B	2.20	A	0.095	C	0.095	C	<0.001
Chromium, ng	37.6	A	41.8	A	33.3	A	42.3	A	0.227
Iron, ng	26.5	A	95.6	A	14.3	A	22.6	A	0.222
Zinc, ng	52.9	A	96.5	A	44.1	A	38.1	A	0.119
Naphthalene, ng	16.9	A	15.4	A	19.6	A	15.9	A	0.596
Benzo(a)anthracene, ng	0.92	A	0.79	A	1.23	A	1.00	A	0.333
Chrysene, ng	2.40	A	2.38	A	2.98	A	2.75	A	0.445
Propylene oxide, µg	338	B	808	A	182	B C	78	C	<0.001
2-aminonaphthalene, ng	0.106	A	0.187	A	0.201	A	0.153	A	0.625
3-aminobiphenyl, ng	0.035	A	0.059	A	0.070	A	0.043	A	0.471
4-aminobiphenyl, ng	0.028	A	0.031	A	0.067	A	0.030	A	0.300
o-toluidine, ng	0.923	A	0.995	A	1.037	A	0.823	A	0.733
Ammonia, µg	9.30	A	9.25	A	7.70	A	6.02	A	0.241

The use of the letters A, B, C and D in the table indicates whether the differences in the mean values between products or AMB are statistically significant or not. For a particular analyte, results for products that share the same letter are not significantly different, and where the letters differ the means are significantly different.

in the cigarette smoke AMBs. Emissions from the e-cigarettes were >99% lower than in cigarette smoke.

Gases and Volatiles

Of the 12 gases and volatiles analyzed, only ammonia was quantified in all three of the e-cigarette variants and was the only gas quantified in the e-cigarette AMB. There were no significant differences in yields between any of the e-cigarette products or corresponding AMB (Table 5), and its presence was attributed to AMB contamination. In contrast, the cigarette AMB did not have quantifiable levels of ammonia. E-cigarette ammonia emissions were 91–93% lower (ISO 3308) and 97% lower (ISO-Intense) than from Ky3R4F. Propylene oxide was quantified in the DC e-cigarette and was <LOQ from the other 2 variants. Propylene oxide was not detected in either of the cigarette or e-cigarette AMBs. Compared with yields from the Ky3R4F cigarette, e-cigarette PO emissions were >73% lower (ISO 3308) and >91% lower (ISO-Intense). Nitric oxide and hydrogen cyanide were each detected in one of the three e-cigarettes but not in the others. None of the other gases and volatiles - CO, NO_x, 1,3-butadiene, isoprene, acrylonitrile, benzene, toluene, and ethylene oxide were detected in the aerosol or AMB, other than toluene being detected but not

quantified in the AMB. The tobacco cigarette AMB had quantifiable levels of several compounds: 1,3-butadiene, isoprene, acrylonitrile, benzene, and toluene. The measured toluene in the AMB reached 8% of the mainstream smoke emissions, but levels of the other compounds were lower, at 3% and less of the respective mainstream smoke emissions.

Polycyclic Aromatic Hydrocarbons (PAHs)

All three of the e-cigarettes and the e-cigarette AMB had quantifiable levels of naphthalene and chrysene. AMB levels were not significantly different from the e-cigarette emissions (Table 5). The cigarette smoke samples contained substantially higher levels of these compounds, with the AMB values at up to 6% of the cigarette smoke value. Benzo(a)anthracene was quantified in the aerosol of GT and was detected but not quantified in the other two variants and AMB; e-cigarettes and AMB values were not significantly different. Emissions from Ky3R4F were substantially higher. The matching cigarette AMB level was not quantifiable under ISO 3308 conditions, but quantified at 6% of the smoke yield under ISO-Intense conditions. Benzo(a)pyrene was <LOQ in two of the e-cigarette aerosols, but not detected in the other e-cigarette aerosol or the AMB. Benzo(b)fluoranthene was <LOQ in two

of the e-cigarettes and the AMB, but undetectable in the other variant. These two compounds were quantified in the AMB for the reference cigarette at up to 5% of the mainstream smoke emissions. Indeno(1,2,3-cd)pyrene and benzo(k)fluoranthene were not detectable for any of the e-cigarettes or the AMB. Where quantifiable, e-cigarette PAHs emissions were 98.4–99.6% (ISO 3308) and 99.1–99.8% (ISO-Intense) lower than from the Ky3R4F cigarette.

Aromatic Amines

Four of the eight aromatic amines analyzed were quantified in some of the e-cigarettes: 2-aminonaphthalene, 3- and 4-aminobiphenyls, and o-toluidine. Of these, all but 4-aminobiphenyl were also quantified in the e-cigarette AMB. Levels measured for e-cigarettes and AMB were not significantly different (Table 5). Ky3R4F mainstream smoke contained quantifiable levels of all of the measured aromatic amines except benzidine, which was not detected. Five of the aromatic amines were quantified in the reference cigarette AMB, but not consistently across puffing regimes. Levels were <1% of the mainstream smoke emissions, other than o-anisidine, which gave levels up to 13% of the Ky3R4F mainstream emissions. Reductions in the e-cigarette aromatic amine emissions compared with the ISO 3308 and ISO-Intense Ky3R4F yields were >99.6% in all cases.

Nicotine-Related Tobacco Alkaloids

Anatabine and anabasine were not detected in the e-cigarette aerosols or the AMB. Nornicotine, cotinine, and β -nicotyrine (apart from GT) were quantified in the e-cigarette aerosols. Myosmine was quantified in two of the e-cigarette aerosols (DC and GT) and detected in CM. Nicotine-N-oxide was quantified in one e-cigarette aerosol and detected in the other two variants. Other than nicotine and nicotine-N-oxide, levels of these compounds in aerosols from the e-cigarettes were >90% lower than in mainstream smoke from the reference cigarette. The e-cigarette AMB contained detectable levels of nicotine and cotinine but none of the other nicotine related alkaloids. None of these compounds were detected in the Ky3R4F AMB, other than nicotine, which was present at non-quantifiable levels.

Nitrosamines

There were no detectable levels of the four tobacco-specific nitrosamines (TSNA) or ten volatile nitrosamines in the e-cigarette aerosols or the AMB. All four of the TSNA were quantified in the Ky3R4F smoke under both ISO 3308 and ISO-Intense conditions. Compared with the Ky3R4F smoke, reductions in the e-cigarette aerosol yields of TSNA were >99.9%. Two of the volatile nitrosamines (N-nitrosopiperidine and N-nitrosopyrrolidine) were detected in Ky3R4F smoke (ISO regime) and AMBs. Their levels in the AMBs were a substantial proportion (50–66%) of the mainstream smoke emissions.

Metals

Mercury, cadmium, nickel, cobalt, beryllium, and tin were not detected in emissions from any of the e-cigarettes nor in the corresponding AMB. Ky3R4F emissions did not contain

detectable levels of cobalt, beryllium, or tin. Nickel was detected (<LOQ) under both ISO 3308 conditions and ISO-Intense, but not in the corresponding AMBs. Both mercury and cadmium were quantified in Ky3R4F smoke but not detected in the corresponding AMBs. E-cigarette emissions of mercury and cadmium were 97–>99% lower than in cigarette smoke.

Lead was detected but not quantified in all the e-cigarette emissions and the AMB. Lead was quantified in Ky3R4F mainstream smoke but not detected in the corresponding AMBs. E-cigarette emissions were >98% and >99% lower than from Ky3R4F smoked under the ISO 3308 and ISO-Intense regimes, respectively. Arsenic was detected but not quantified in all e-cigarette variants but was not detected in the AMB. Arsenic was quantified in cigarette smoke but not detected in the corresponding AMB. E-cigarette levels were therefore >85% and >93.8% lower than those from the Ky3R4F smoked under ISO 3308 and ISO-Intense conditions, respectively. Selenium and copper were detected but not quantified in the e-cigarette CM and DC variants and were not detected in the GT variant or in the AMB. Selenium was not quantifiable in Ky3R4F emissions, copper was quantified in cigarette smoke but not quantified in the corresponding AMB.

Iron and zinc were quantified in the DC variant but not quantified in the other e-cigarettes nor the AMB. Compared to Ky3R4F the levels from DC were 79% (iron) and 93% (zinc) lower (ISO 3308) and 74 and 96% lower respectively under ISO-Intense conditions. A comparison of Ky3R4F and its AMB data showed that substantial quantities of the measured iron and zinc in Ky3R4F mainstream smoke were found in the AMB (56–78% and 43–66%, respectively). Chromium was quantified in all the e-cigarette aerosols and the corresponding AMB, with e-cigarette values not significantly different to the AMB value. Emissions from Ky3R4F and corresponding AMBs were not quantifiable. Levels measured in both the e-cigarette aerosols and the corresponding AMB were 79–125% higher than the Ky3R4F ISO 3308 yield and 17–47% higher than the ISO-Intense Ky3R4F yield.

Semi-volatiles

Quinoline was not detected in the e-cigarette aerosols or the AMB. Pyridine and styrene were detected but not quantified in the DC aerosol and were not detected in the other e-cigarette aerosols or the AMB. All three compounds were quantified in Ky3R4F smoke and detected inconsistently at lower levels in the corresponding AMBs. E-cigarette levels of these compounds were >99% lower than from cigarette smoke.

Polyols and Alcohols

As noted previously, the humectants, VG and PG were the major constituents of the aerosols of the e-cigarettes. Allyl alcohol, a possible decomposition product of VG, was quantified in the aerosols of all three e-cigarettes. Ethylene glycol and diethylene glycol were not detected in any aerosol. Glycidol was <LOQ in one of the e-cigarette aerosols, and not detected in two variants and the AMB. Menthol, a flavor component, was quantified in the aerosols of the CM and DC variants of the e-cigarette, but was not

TABLE 6 | Composite percentage reductions in yields from the ePen2 variants vs. the 3R4F smoked under ISO-I for toxicants listed by WHO TobReg and Health Canada.

Toxicant list	Number of toxicants on the list	ePen2CM	ePen2DC	ePen2GT
		Composite average reduction per puff vs. 3R4F ISO-I (%)		
WHO TobReg Mandated list ^a	9	98.5	99.5	99.0
FDA abbreviated list	18	97.5 (99.0 excluding nicotine)	98.1 (99.5 excluding nicotine)	97.9 (99.3 excluding nicotine)

^a9 toxicants proposed by WHO TobReg to be mandated for lowering.

quantifiable from GT. The Ky3R4F VG emissions were significantly lower than the yields from the e-cigarettes. None of the polyols or alcohols were quantified in the e-cigarette AMB except for PG. In the Ky3R4F cigarette AMB, allyl alcohol was detected under both puffing regimes at 4–8% of the cigarette mainstream smoke emissions.

Semi-Quantitative Analyses

It is also of interest to understand the quantities of compounds present in the untargeted analysis. However, full quantitation was not achievable in the untargeted analysis because compound identities were not verified, and moreover where library match factors were low it is possible that the assigned identities were incorrect. Uncertainty over compound identity meant that it was not possible to conduct MS calibrations, which renders the concentration data semi-quantitative at best, providing “order of magnitude” information only. The estimated concentrations of detected compounds in aerosols of the e-cigarette variants that were not common to the AMB and disclosed ingredients were calculated. Of the aerosol components with Mass Spectral Library match factors (MF) ≥ 75 , 65% had concentrations in the estimated range 0–20 ng/puff, and 36% had estimated levels of 0–5 ng/puff. Of the aerosol components with $50 \leq \text{MF} < 75$, 79% had estimated levels in the range 0–20 ng/puff, and 21% had estimated concentrations of 0–5 ng/puff. The highest estimated value of around 450 ng/puff was found for a compound eluting at 7.14 min in the chromatogram of the unflavored e-cigarette aerosol. The peak had a poor match factor to the MS library (70%); the best match was a silane–benzeneacetic acid, alpha, 4-bis [(trimethylsilyl)oxy-, trimethylsilyl] ester. However, the poor match factor means that its identification should be regarded as tentative at best.

Composite Reductions in Toxicants Compared to Cigarette Smoke

We calculated the composite average percent reductions in toxicant yields from the e-cigarettes compared with those from the Ky3R4F cigarette for different regulatory interest “lists” shown in **Supplementary Table S3**. **Table 6** shows that the WHO TobReg 9 constituents were reduced by 98.5–99.5% in the emissions from all three flavor variants of the e-cigarette when compared on a puff-by-puff basis with smoke from a Ky3R4F cigarette smoked under the ISO-Intense regime. Although not statistically significant, the slightly lower percentage reduction for formaldehyde in CM (>88%) compared with the other flavor variants (96.6 and 92.2%) caused a slightly lower composite percentage reduction for the CM flavor variant. Toxicants on

the FDA abbreviated list of 18 compounds generated under ISO-Intense were reduced on average by >97% compared with the Ky3R4F; the % reductions increased to >99% when nicotine is removed from the list.

DISCUSSION

Chemical Complexity of e-Cigarette Aerosols in Comparison to Cigarette Smoke

Chemical Complexity of e-Cigarette Aerosols

In this study, between 94 and 139 compounds were detected in the flavored e-cigarette aerosols, and an estimated 72–79 compounds in the aerosol from the unflavored e-cigarette aerosol. The differences between flavored and unflavored e-cigarettes reported here (**Figure 2**) demonstrate the contribution of flavor ingredients to the overall composition of e-cigarette aerosols. Havermans et al. (2021) reported the identification of an average of 10 ± 15 flavor compounds in their analysis of more than 100 e-liquids, whilst studies by a range of authors (Aszyk et al., 2018; Behar et al., 2018; Bitzer et al., 2018; Czoli et al., 2019; Hua et al., 2019; Hutzler et al., 2014; Lisko et al., 2015; Omaiye et al., 2019; Tierney et al., 2016) reported the detection of between 1 and 47 flavoring chemicals in individual e-liquids. Our findings of 15–67 additional compounds in the aerosols of flavored e-cigarettes compared to an unflavored sample are consistent with published values for flavor complexity of e-liquids, particularly when the potential for the presence of additional reaction products (such as acetals) between flavor compounds and PG is considered, as well contributions (**Figure 2**) to compound counts from minor components of ingoing ingredients (Bitzer et al., 2018).

Combining targeted and untargeted analyses clearly provides a more complete picture of aerosol complexity than untargeted analyses alone. In the present study a further 5–12 compounds were detected through use of the targeted analyses over and above those detected in the untargeted GC-MS analysis alone. However, our approach cannot be viewed as a complete characterisation of aerosol complexity, as the scope of the analyses was subject to three main limitations. First, the untargeted TD-GC-MS method used in this study adopted chromatographic heart-cutting to avoid detector overload by PG and VG; it is possible that aerosol components with similar retention times to the major constituents could be missed. Second, a particular weakness of the present study was a relatively limited examination of elemental species. In our study we analysed for thirteen metals but a broader range of elemental species can also be tested for, as demonstrated by Williams et al. (2013). In their study Williams et al. examined

e-cigarette aerosols for the presence of 24 additional elements to those examined in this study. Four elements (Bi, Ir, Pd and Ti) were not detected from any e-cigarette, six were detected in only one sample (In, La, Mn, Rb, Ag and W), and several (Al, Ba, Ca, Mg, K, Na, Si, Sr and Zr) were found in most of the samples examined. In principle, the results of Williams et al. (2013) suggests that 6–20 elements may be present in the aerosols studied in the present work, in addition to the 72–139 compounds detected in this study. A third potential limitation of our study is that other compounds may exist within the aerosol that are incompatible with the untargeted GC-MS and targeted analytical methods. The combination of these three limitations means that our study is likely to have underestimated the total aerosol complexity of these e-cigarettes, but possibly not to a substantial degree.

The numbers of compounds reported here are generally higher than reported from studies using untargeted GC-MS analyses alone. For example, Rawlinson et al. (2017) detected 51–87 compounds in the aerosols from flavored second-generation modular e-cigarette devices, while Herrington et al. (2015) (Herrington and Myers 2015) detected 85 compounds in the aerosol from a flavored first-generation e-cigarette, 8 of which were common to the AMB. The other study in which untargeted e-cigarette aerosol analysis was reported (García-Gómez et al., 2016) did not use GC-MS, but rather employed direct sampling secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS) and detected comparable numbers of compounds (142) to one of the e-cigarettes detected in this study. The higher compound count reported by (García-Gómez et al., 2016) than Herrington et al. (2015) (Herrington and Myers 2015) and Rawlinson et al. (2017) may possibly reflect differences in the complexities of the e-cigarette aerosols examined by the various studies, alternatively it may reflect superiority of non-chromatographic ESI-HRMS for these purposes.

It is of interest to further understand the sources of detected compounds in the aerosols tested in this study. Assignment of sources is heavily dependent upon correct identification of compounds, and compound identification in the untargeted analysis used in the present study should be regarded as indicative, as they relied upon MS library matches. Further confirmatory steps, such as retention time matching, would be required to render the identities definitive. However, focusing on compounds with the highest MS library match factors suggested that the detected compounds were present in the aerosols due to a number of different sources.

Figure 2 shows that many of the compounds were ingredient related, whether aerosol former, nicotinic or flavor compounds. Reaction products of PG and VG such as acetals/hemiacetals and ketals further increased the contribution of ingredients to the compound count. Flavor compounds in particular had a significant impact on the numbers of detected compounds. The dependence of detected aerosol compound count on such ingredients, means that there is no simple fixed value for the numbers of compounds in an e-cigarette aerosol. Across manufacturers and products, flavor formulations can differ significantly in their compositional complexity, and the incorporation of natural flavor extracts (as opposed to synthetic flavor chemicals) will further drive complexity as extracts can offer

substantial intrinsic compositional complexity. Furthermore, differences in device operating conditions across different products, notably power/temperature/time, could also be expected to impact the degree of e-liquid reaction or breakdown, thereby influencing aerosol complexity.

Comparison With Cigarette Smoke

Studies characterising the complexity of cigarette smoke indicate a substantially more diverse chemical environment than found with e-cigarette aerosols. Rodgman & Perfetti's monograph on the composition of tobacco and tobacco smoke (Rodgman and Perfetti 2013) lists a total of over 6,500 identified tobacco smoke components. These include, of course, many compounds that would not be detectable with the analytical techniques used in this study. In the present work we were unable to conduct untargeted analysis on cigarette smoke with the available method due to the low capacity of the thermal desorption tube used in the analyses. However, a greater number of the targeted analytes (81) were found with cigarette smoke than with the flavored e-cigarettes (35–42).

Two studies have reported untargeted analysis of cigarette smoke. Brokl et al. (2014) conducted a scan of cigarette smoke's particulate phase (but not the vapour phase) using headspace solid-phase microextraction coupled with 2-D GC-TOFMS, and detected >2000 GC-amenable compounds. Their findings point to cigarette smoke being 1–2 orders of magnitude more complex than the e-cigarette aerosols in this current study. A less sensitive scanning approach was reported by RJReynolds in a semi-quantitative gas chromatography study of smoke from a Kentucky Reference 1R4F cigarette (Reynolds, 1988). Their method, which was designed to detect compounds at >50 ng/puff, identified more than 660 compounds in cigarette smoke. In the current study between 12 and 19 compounds with yields ≥ 50 ng/puff, were found with the flavored e-cigarettes using both quantitative data from targeted analyses and semi-quantitative estimates of aerosol yield from the untargeted analysis. These counts were, again, 1–2 orders of magnitude lower than those found with in cigarette smoke.

Quantified Emissions

Figure 3 shows that the sum of measured major e-liquid and aerosol constituents (VG, PG, water and nicotine) accounted on average for 94.2% of the ACM, (rising to 97% using the expected VG value for GT). The calculated difference between ACM and the sum of the major components ("balance") is clearly sensitive to errors in the determination of the major species. Accurate quantification of water in aerosol streams has traditionally presented significant challenges and may also be associated with relatively large quantification errors in these measurements. In contrast, the comparable balance for cigarette smoke lay between 58 and 76% of the trapped particulate mass. These data also suggest a much more diverse composition of cigarette smoke compared to e-cigarette aerosols.

The carbonyl yields measured in the present study were not significantly different from those found previously by Margham et al. (2016), but the AMB values for formaldehyde, acetone, and MEK were significantly lower in the present study. Such compounds (formaldehyde, acetaldehyde, acetone,

propionaldehyde, acrolein, glycolaldehyde, glyoxal, and methylglyoxal), and the compounds propylene oxide and allyl alcohol are thermal decomposition products of the humectants, PG and VG, used as aerosol generators (Stein et al., 1983; Laino et al., 2011; Laino et al., 2012; Sleiman et al., 2016).

Higher levels of aerosol nicotine and tobacco alkaloids were reported in the present study than by Margham et al. (2016) even though the e-liquid nicotine levels were the same in the two studies and ACM emissions were comparable. The tobacco alkaloids other than nicotine are present as low-level constituents of the pharmaceutical nicotine used in e-liquid formulations. Those quantified in the aerosol–nicotine, myosmine, nicotine-N-oxide, cotinine, and β -nicotyrine—are naturally present in the tobacco leaf used to produce the pharmaceutical grade nicotine used in e-liquids and some may also be formed through nicotine oxidation in e-liquids (Marion 1950; Kiskaki et al., 1978; Martinez et al., 2014).

A number of metals were measured in the e-cigarette emissions. In an earlier paper (Margham et al., 2016) describing the aerosol chemistry of a similar product to that reported in the present study, chromium was quantified in the first 100 of 200 puffs at an average of 0.50 ng/puff but was not quantified (<0.45 ng/puff) in the second 100 puffs. The AMB contained detectable but not quantifiable levels of chromium (>0.13 but <0.45 ng/puff). Some levels of chromium generated by the e-cigarette could therefore not be ruled out. In the present study, levels of iron, zinc, and chromium found in the e-cigarette aerosols and the AMB were not significantly different (Table 5), and we can conclude that the presence of these metals likely arise as artifacts from aerosol collection or other analytical processes. For iron and zinc, Margham et al. (2016) also concluded that their presence in the aerosols was due to laboratory contamination. Williams et al. (2013) using non-standard smoking parameters analyzed 20 metals in the aerosol of a single brand of e-cigarette, including chromium (0.7 ng/puff), iron (52 μ g/puff) and zinc (5.8 ng/puff), but none were detected in an AMB. Their results are significantly higher than those of the present study. Tayyarah and Long (2014) reported detectable but non-quantifiable levels of chromium (1–4 ng/puff) in three products they tested as well as the AMB. However, other studies, such as that of Goniewicz et al. (2014) have not detected chromium in e-cigarette emissions.

In the present study, consistent with the findings of Margham et al. (2016), emissions of four aromatic amines were quantified in the e-cigarette aerosols and AMB, with levels not significantly different between the background and aerosol samples. Three PAHs and ammonia were quantified in the e-cigarette emissions and AMB; levels were not significantly different between samples. Emissions of ammonia, chrysene, and naphthalene were higher in the present study for all the e-cigarettes as well as the AMB than found previously by Margham et al. (2016). TSNA were not detected in any of the e-cigarette samples, even with picogram per puff LODs.

Contribution From Laboratory Air, Analytical Equipment or Analytical Reagents

Given the very low levels of many of the toxicants that are now measured in e-cigarette aerosols, combined with the relatively

large numbers of puffs taken on e-cigarettes in comparison to tobacco cigarettes, it is essential to understand the contribution to measured values from environmental factors. These include toxicants that may already be present in the laboratory air, in reagents, or that may be introduced by operators or equipment (such as puffing machines) used to generate and collect the aerosol. Hence the importance of the AMB as a means of minimising the possibility of false-positives and overestimates (Tayyarah and Long, 2014; Margham et al., 2016; Wagner et al., 2018; Belushkin et al., 2020). AMB control measurements are widely used in different e-cigarette research areas, such as chemical analysis and indoor air quality studies (Goniewicz et al., 2014; Herrington and Myers, 2015; Marco and Grimault, 2015; Mikheev et al., 2016; Palazzolo et al., 2016; Aherrera et al., 2017; Beauval et al., 2017; Moldoveanu et al., 2017; Olmedo et al., 2018; Halstead et al., 2019).

Table 5 shows that in the present study, there were eight components where AMB values were numerically higher than one or more of the e-cigarette aerosol samples. These were chromium (higher than all 3 e-cigarette samples), iron (higher than 1 sample), naphthalene (1), benzo(a)anthracene (2), chrysene (2), 2-aminonaphthalene (1), 3- and 4-aminobiphenyls (1 sample each). However, ANOVA testing showed that none of these differences were significant at the 95% confidence level. Therefore, in none of these cases was there a significant difference between AMB and the e-cigarette samples. In addition, the ANOVA tests showed that zinc, o-toluidine and ammonia emissions were not significantly different from those found in the AMB (Table 5).

Further insights into the potential presence or concentrations of these toxicants will require greater reductions of chemical background than are currently achievable using established methods. Gaseous and volatile contaminants could be excluded during e-cigarette experiments by use of air-tight assemblies fed by high purity air, as reported by García-Gómez et al. (2016). However, not all of the contamination arises from the laboratory air. Metals such as chromium, iron and zinc appear to arise at least in part from the puffing machines used to generate aerosols and the associated trapping matrices (data not shown); reducing the impact of metal contamination from these sources may represent a way to minimise AMB contamination with these compounds. These approaches represent valuable avenues for future investigation.

AMB experiments for the Ky3R4F cigarette were conducted under two puffing regimes, and contributions to the measured cigarette smoke emissions were found with 29 of the 98 analytes measured in this study. Levels per puff were generally much higher than found with the e-cigarette AMB, due in the main to some elements of environmental tobacco smoke around the smoking engine (generated by the cigarette sidestream smoke as it leaves the burning cigarettes) being pulled into the empty port of the smoking engine during the puffing steps of the AMB experiment. This source does not exist for the e-cigarette AMB experiment. Despite the higher absolute levels measured with the Ky3R4F AMB, their contribution to the measured levels in smoke was generally less due to the relatively high concentrations of toxicants in mainstream cigarette smoke.

CONCLUSION

This study has demonstrated that e-cigarette aerosols contain significantly fewer toxic components and at lower concentrations than a reference cigarette. In contrast to the thousands of identified compounds in cigarette smoke, between 94 and 139 aerosol compounds were detected from flavored e-cigarettes when data from both targeted and untargeted analytical methods were combined. Using a combined approach provided greater compositional insights than either targeted or untargeted approaches alone. Identities of the detected e-cigarette aerosol constituents were attributed to sources including ingredients such as flavor compounds, reaction products of those ingredients, minor components of device and ingredients, thermal decomposition products, and compounds that could not be accurately identified.

Toxicant yields per puff from the e-cigarettes were 68–>99.9% lower than those from the reference cigarette under both ISO and ISO-Intense puffing conditions. Overall, the levels of the 9 WHO TobReg prioritized toxicants were around 99% lower than measured from the reference cigarette under ISO-Intense puffing conditions. Our results agree with the emerging scientific literature in that the e-cigarette aerosols are chemically much simpler than cigarette smoke, and contain fewer toxicants at lower concentrations.

REFERENCES

- Aherrera, A., Olmedo, P., Grau-Perez, M., Tanda, S., Goessler, W., Jarmul, S., et al. (2017). The Association of e-Cigarette Use With Exposure to Nickel and Chromium: A Preliminary Study of Non-Invasive Biomarkers. *Environ. Res.* 159, 313–320. doi:10.1016/j.envres.2017.08.014
- Allen, J. G., Flanagan, S. S., LeBlanc, M., Vallarino, J., MacNaughton, P., Stewart, J. H., et al. (2016). Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes. *Environ. Health Perspect.* 124, 733–739. doi:10.1289/ehp.1510185
- Aszyk, J., Kubica, P., Woźniak, M. K., Namieśnik, J., Wasik, A., and Kot-Wasik, A. (2018). Evaluation of Flavour Profiles in E-Cigarette Refill Solutions Using Gas Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. A* 1547, 86–98. doi:10.1016/j.chroma.2018.03.009
- Bao, M. (2015). Analysis of Selected Carbonyl Compounds in E-Liquids and E-Aerosols by Using Pentafluorobenzylhydroxylamine Derivatization and Gas Chromatography-Mass Spectrometry. CORESTA SSPT Meeting.
- Beauval, N., Antherieu, S., Soyez, M., Gengler, N., Howsam, M., Hardy, E. M., et al. (2017). Chemical Evaluation of Electronic Cigarettes: Multicomponent Analysis of Liquid Refills and their Corresponding Aerosols. *J. Anal. Toxicol.* 41, 670–678. doi:10.1093/jat/bkx054
- Behar, R. Z., Luo, W., Lin, S. C., Wang, Y., Valle, J., Pankow, J. F., et al. (2016). Distribution, Quantification and Toxicity of Cinnamaldehyde in Electronic Cigarette Refill Fluids and Aerosols. *Tob. Control.* 25, ii94. doi:10.1136/tobaccocontrol-2016-053224
- Behar, R. Z., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2018). Analytical and Toxicological Evaluation of Flavor Chemicals in Electronic Cigarette Refill Fluids. *Sci. Rep.* 8, 8288. doi:10.1038/s41598-018-25575-6
- Belushkin, M., Djoko, D. T., Esposito, M., Korneliou, A., Jeannet, C., Lazzarini, M., et al. (2020). Selected Harmful and Potentially Harmful Constituents Levels in Commercial e-Cigarettes. *Chem. Res. Toxicol.* 33, 657–668. doi:10.1021/acs.chemrestox.9b00470

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JM was the study manager. SF conducted statistical analyses on the data. KM co-directed the study and co-wrote the manuscript. AP co-wrote the manuscript. CP co-directed the study. DM oversaw analytical measurements. AC and HD managed manuscript writing and quality control steps. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.743060/full#supplementary-material>

- Bitzer, Z. T., Goel, R., Reilly, S. M., Elias, R. J., Silakov, A., Foulds, J., et al. (2018). Effect of Flavoring Chemicals on Free Radical Formation in Electronic Cigarette Aerosols. *Free Radic. Biol. Med.* 120, 72–79. doi:10.1016/j.freeradbiomed.2018.03.020
- Boudi, F. B., Patel, S., Boudi, A., and Chan, C. (2019). Vitamin E Acetate as a Plausible Cause of Acute Vaping-Related Illness. *Cureus* 11 (12), e6350. doi:10.7759/cureus.6350
- Brokl, M., Bishop, L., Wright, C. G., Liu, C., McAdam, K., and Focant, J.-F. (2014). Multivariate Analysis of Mainstream Tobacco Smoke Particulate Phase by Headspace Solid-phase Micro Extraction Coupled with Comprehensive Two-Dimensional Gas Chromatography-Time-Of-Flight Mass Spectrometry. *J. Chromatogr. A* 1370, 216–229. doi:10.1016/j.chroma.2014.10.057
- Burns, D. M., Dybing, E., Gray, N., Hecht, S., Anderson, C., Sanner, T., et al. (2008). Mandated Lowering of Toxicants in Cigarette Smoke: a Description of the World Health Organization TobReg Proposal. *Tob. Control.* 17, 132–141. doi:10.1136/tc.2007.024158
- CORESTA (2015). CORESTA Recommended Method No 81. Routine Analytical Machine for E-Cigarette Aerosol Generation and Collection – Definitions and Standard Conditions. June 2015. Available at: https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf (Accessed April 29, 2017).
- Costigan, S., and Meredith, C. (2015). An Approach to Ingredient Screening and Toxicological Risk Assessment of Flavours in E-Liquids. *Regul. Toxicol. Pharmacol.* 72, 361–369. doi:10.1016/j.yrtph.2015.05.018
- Cunningham, A., McAdam, K., Thissen, J., and Digard, H. (2020). The Evolving E-Cigarette: Comparative Chemical Analyses of E-Cigarette Vapor and Cigarette Smoke. *Front. Toxicol.* 2, 586674. doi:10.3389/ftox.2020.586674
- Czoli, C. D., Goniewicz, M. L., Palumbo, M., Leigh, N., White, C. M., and Hammond, D. (2019). Identification of Flavouring Chemicals and Potential Toxicants in E-Cigarette Products in Ontario, Canada. *Can. J. Public Health* 110, 542–550. doi:10.17269/s41997-019-00208-1
- Erythropel, H. C., Anastas, P. T., Krishnan-Sarin, S., O'Malley, S. S., Jordt, S. E., and Zimmerman, J. B. (2020). Differences in Flavourant Levels and Synthetic

- Coolant Use between USA, EU and Canadian Juul Products. *Tob. Control.* 30, 453. doi:10.1136/tobaccocontrol-2019-055500
- Farsalinos, K. E., and Gillman, G. (2018). Carbonyl Emissions in E-Cigarette Aerosol: A Systematic Review and Methodological Considerations. *Front. Physiol.* 8, 1119. doi:10.3389/fphys.2017.01119
- FDA (2012). FDA: Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke: Established List. Available at: <http://www.fda.gov/TobaccoProducts/GuidanceComplianceRegulatoryInformation/ucm297786.htm>.
- García-Gómez, D., Gaisl, T., Barrios-Collado, C., Vidal-de-Miguel, G., Kohler, M., and Zenobi, R. (2016). Real-time Chemical Analysis of E-Cigarette Aerosols by Means of Secondary Electrospray Ionization Mass Spectrometry. *Chem. Eur. J.* 22, 1–7. doi:10.1002/chem.201504450
- Goniewicz, M. L., Knysak, J., Gawron, M., Kosmider, L., Sobczak, A., Kurek, J., et al. (2014). Levels of Selected Carcinogens and Toxicants in Vapour from Electronic Cigarettes. *Tob. Control.* 23, 133–139. doi:10.1136/tobaccocontrol-2012-050859
- Halstead, M., Gray, N., Gonzalez-Jimenez, N., Fresquez, M., Valentin-Blasini, L., Watson, C., et al. (2019). Analysis of Toxic Metals in Electronic Cigarette Aerosols Using a Novel Trap Design. *J. Anal. Toxicol.*, 1–7. doi:10.1093/jat/bkz078
- Havermans, A., Krüsemann, E. J. Z., Pennings, J., de Graaf, K., Boesveldt, S., and Talhout, R. (2021). Nearly 20 000 E-Liquids and 250 Unique Flavour Descriptions: an Overview of the Dutch Market Based on Information from Manufacturers. *Tob. Control.* 30, 57–62. doi:10.1136/tobaccocontrol-2019-055303
- Health Canada (1999). *Official Method T-115. Determination of “tar”, Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke*. Ottawa, Canada.
- Health Canada (1999a). Tobacco Reporting Regulations. Available at: <http://www.hc-sc.gc.ca/hc-ps/tobac-tabac/legislation/reg/indust/method/index-eng.php#main> (Accessed Aug 1, 2017).
- Herrington, J. S., Myers, C., and Rigdon, A. (2015). Analysis of Nicotine and Impurities in Electronic Cigarette Solutions and Vapor. *Restek Pure Chromatogr.* Available at: http://www.restek.com/Technical-Resources/Technical-Library/Foods-Flavors-Fragrances/fff_FFAN2127-UNV.
- Herrington, J. S., and Myers, C. (2015). Electronic Cigarette Solutions and Resultant Aerosol Profiles. *J. Chromatogr. A* 1418, 192–199. doi:10.1016/j.chroma.2015.09.034
- Hoffmann, D., and Hoffmann, I. (1998). Letters to the Editor - Tobacco Smoke Components. *Beitrag Zur Tabakforschung Int.* 18, 49–52. doi:10.2478/cttr-2013-0668 Available at: <https://www.degruyter.com/downloadpdf/j/cttr.1998.18.issue-1/cttr-2013-0668/cttr-2013-0668.pdf>
- Hua, M., Omaiye, E. E., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2019). Identification of Cytotoxic Flavor Chemicals in Top-Selling Electronic Cigarette Refill Fluids. *Sci. Rep.* 9, 2782. doi:10.1038/s41598-019-38978-w
- Hutzler, C., Paschke, M., Kruschinski, S., Henkler, F., Hahn, J., and Luch, A. (2014). Chemical Hazards Present in Liquids and Vapors of Electronic Cigarettes. *Arch. Toxicol.* 88, 1295–1308. doi:10.1007/s00204-014-1294-7
- International Organization for Standardization (2012). *Routine Analytical Cigarette-Smoking Machine – Definitions and Standard Conditions*. ISO 3308:2012 (Geneva).
- International Organization for Standardization (1999). *Tobacco and Tobacco Products – Atmosphere for Conditioning and Testing*. ISO 3402:1999 (Geneva).
- ISO 20768 (2018a). Vapour Products–Routine Analytical Vaping Machine–Definitions and Standard Conditions. Geneva: International Organization for Standardization.
- ISO 20778 (2018b). Cigarettes–Routine Analytical Cigarette Smoking Machine–Definitions and Standard Conditions with an Intense Smoking Regime. Geneva: International Organization for Standardization.
- Kentucky Tobacco Research and Development Center (2017). University of Kentucky Reference Cigarette Ky3R4F Preliminary Analysis. Available at: <https://cttr.uky.edu/resources/pdf/webdocs/3R4F%20Preliminary%20Analysis.pdf> (Accessed April 29, 2017).
- Kisaki, T., Maeda, S., Koiwai, A., Mikami, Y., Sasaki, T., and Matsushita, H. (1978). Transformation of Tobacco Alkaloids. *Beitrag Zur Tabakforschung Int.* 9, 308–316. doi:10.2478/cttr-2013-0461
- Kosmider, L., Sobczak, A., Prokopowicz, A., Kurek, J., Zaciera, M., Knysak, J., et al. (2016). Cherry-flavoured Electronic Cigarettes Expose Users to the Inhalation Irritant, Benzaldehyde. *Thorax* 71, 376–377.
- Krüsemann, E. J. Z., Havermans, A., Pennings, J. L. A., de Graaf, K., Boesveldt, S., Talhout, R., et al. (2021). Comprehensive Overview of Common E-Liquid Ingredients and How They Can Be Used to Predict an E-Liquid’s Flavour Category. *Tob. Control.* 30, 185. doi:10.1136/tobaccocontrol-2019-055447
- Laino, T., Tuma, C., Curioni, A., Jochnowitz, E., and Stolz, S. (2011). A Revisited Picture of the Mechanism of Glycerol Dehydration. *J. Phys. Chem. A* 115, 3592–3595. doi:10.1021/jp201078e
- Laino, T., Tuma, C., Moor, P., Martin, E., Stolz, S., and Curioni, A. (2012). Mechanisms of Propylene Glycol and Triacetin Pyrolysis. *J. Phys. Chem. A* 116, 4602–4609. doi:10.1021/jp300997d
- Lisko, J. G., Tran, H., Stanfill, S. B., Blount, B. C., and Watson, C. H. (2015). Chemical Composition and Evaluation of Nicotine, Tobacco Alkaloids, pH, and Selected Flavors in E-Cigarette Cartridges and Refill Solutions. *Nictob* 17, 1270–1278. doi:10.1093/ntr/ntu279
- Liu, C., G. McAdam, K., and A. Perfetti, T. (2011). Some Recent Topics in Cigarette Smoke Science. *Mini-Reviews Org. Chem.* 8, 349–359. doi:10.2174/157019311797440272 Available at: <http://www.ingentaconnect.com/content/ben/mroc/2011/00000008/00000004/art00002?crawler=true>
- Marco, E., and Grimalt, J. O. (2015). A Rapid Method for the Chromatographic Analysis of Volatile Organic Compounds in Exhaled Breath of Tobacco Cigarette and Electronic Cigarette Smokers. *J. Chromatogr. A* 1410, 51–59. doi:10.1016/j.chroma.2015.07.094
- Margham, J., McAdam, K., Forster, M., Liu, C., Wright, C., Mariner, D., et al. (2016). Chemical Composition of Aerosol from an E-Cigarette: A Quantitative Comparison with Cigarette Smoke. *Chem. Res. Toxicol.* 29, 1662–1678. doi:10.1021/acs.chemrestox.6b00188
- Marion, L. (1950). *The Alkaloids*. Editors R. H. F. Manske and H. L. Homes (Cambridge: Academic Press NY), Vol. 1.
- Martinez, R. E., Dhawan, S., Sumner, W., and Williams, B. J. (2014). On-line Chemical Composition Analysis of Refillable Electronic Cigarette Aerosol-Measurement of Nicotine and Nicotyrine. *Nicotine Tob. Res.* 17, 1263. doi:10.1093/ntr/ntu334
- McAdam, K., Warrington, A., Hughes, A., Adams, D., Margham, J., Vas, C., et al. (2019). Use of Social Media to Establish Vapers Puffing Behaviour: Findings and Implications for Laboratory Evaluation of e-Cigarette Emissions. *Regul. Toxicol. Pharmacol.* 107, 104423. doi:10.1016/j.yrtph.2019.104423
- Mikheev, V. B., Brinkman, M. C., Granville, C. A., Gordon, S. M., and Clark, P. I. (2016). Real-Time Measurement of Electronic Cigarette Aerosol Size Distribution and Metals Content Analysis. *Nicotine Tob. Res.* 18, 1895–1902. doi:10.1093/ntr/ntw128
- Moldoveanu, S. C., Zhu, J., and Qian, N. (2017). Analysis of Traces of Tobacco-specific Nitrosamines (TSNAs) in USP Grade Nicotine, E-Liquids, and Particulate Phase Generated by the Electronic Smoking Devices. *Beiträge Zur Tabak Intl* 27 (6), 86–96. doi:10.1515/cttr-2017-0009
- National Academies of Sciences, Engineering, and Medicine; Health and Medicine Division; Board on Population Health and Public Health Practice; Committee on the Review of the Health Effects of Electronic Nicotine Delivery Systems (2018). *Public Health Consequences of E-Cigarettes*. Editors D. L. Eaton, L. Y. Kwan, and K. Stratton (Washington (DC): National Academies Press (US)). doi:10.17226/24952
- Olmedo, P., Navas-Acien, A., Hess, C., Jarmul, S., and Rule, A. (2018). A Direct Method for e-Cigarette Aerosol Sample Collection. *Environ. Health Perspect.* [Epub ahead of print]. doi:10.1289/EHP2175
- Omaiye, E. E., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2020). Electronic Cigarette Refill Fluids Sold Worldwide: Flavor Chemical Composition, Toxicity and hazard Analysis. *Chem. Res. Tox.* 33, 2972. doi:10.1021/acs.chemrestox.0c00266
- Omaiye, E. E., McWhirter, K. J., Luo, W., Tierney, P. A., Pankow, J. F., and Talbot, P. (2019). High Concentrations of Flavor Chemicals Are Present in Electronic Cigarette Refill Fluids. *Sci. Rep.* 9, 2468. doi:10.1038/s41598-019-39550-2
- Palazzo, D. L., Crow, A. P., Nelson, J. M., and Johnson, R. A. (2016). Trace Metals Derived from Electronic Cigarette (ECIG) Generated Aerosol: Potential Problem of ECIG Devices That Contain Nickel. *Front. Physiol.* 7, 663. doi:10.3389/fphys.2016.00663

- Public Health England (2019). Vaping and Lung Disease in the US: PHE's Advice. Available at: <https://publichealthmatters.blog.gov.uk/2019/10/29/vaping-and-lung-disease-in-the-us-phes-advice/> (Accessed September 21, 2021).
- Rawlinson, C., Martin, S., Frosina, J., and Wright, C. (2017). Chemical Characterisation of Aerosols Emitted by Electronic Cigarettes Using thermal Desorption-Gas Chromatography-Time of Flight Mass Spectrometry. *J. Chromatogr. A* 1497, 144–154. doi:10.1016/j.chroma.2017.02.050
- Reynolds, R. J. (1988). Chemical and Biological Studies on New Cigarette Prototypes that Heat Instead of Burn Tobacco. R.J. Reynolds Tobacco Company, Winston-Salem, North Carolina. Available at: <http://legacy.library.ucsf.edu/>.
- Rodgman, A., and Perfetti, T. A. (2013). *The Chemical Components of Tobacco and Tobacco Smoke*. 2nd ed. Boca Raton, FL: CRC Press.
- Roemer, E., Schramke, H., Weiler, H., Buettner, A., Kausche, S., Weber, S., et al. (2012). Mainstream Smoke Chemistry and *In Vitro* and *In Vivo* Toxicity of the Reference Cigarettes 3R4F and 2R4F. *Beitr. Tabakforsch. Int.* 25, 316–335. doi:10.2478/cttr-2013-0912
- Seiler-Ramadas, R., Sandner, I., Haider, S., Grabovac, I., and Dorner, T. E. (2020). Health Effects of Electronic Cigarette (e-Cigarette) Use on Organ Systems and Its Implications for Public Health. *Wien. Klin. Wochenschr.* [Epub ahead of print]. doi:10.1007/s00508-020-01711-z
- Sleiman, M., Logue, J. M., Montesinos, V. N., Russell, M. L., Litter, M. I., Gundel, L. A., et al. (2016). Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. *Environ. Sci. Technol.* 50, 9644–9651. doi:10.1021/acs.est.6b01741
- Soussy, S., El-Hellani, A., Baalbaki, R., Salman, R., Shihadeh, A., and Saliba, N. A. (2016). Detection of 5-hydroxymethylfurfural and Furfural in the Aerosol of Electronic Cigarettes. *Tob. Control.* 25, ii88–ii93. doi:10.1136/tobaccocontrol-2016-053220
- Stein, Y. S., Antal, M. J., Jr, and Jones, M., Jr (1983). A Study of the Gas-phase Pyrolysis of Glycerol. *J. Anal. Appl. Pyrolysis* 4, 283–296. doi:10.1016/0165-2370(83)80003-5
- Tayyarah, R., and Long, G. A. (2014). Comparison of Select Analytes in Aerosol from E-Cigarettes with Smoke from Conventional Cigarettes and with Ambient Air. *Regul. Toxicol. Pharmacol.* 70, 704–710. doi:10.1016/j.yrtph.2014.10.010
- Tierney, P. A., Karpinski, C. D., Brown, J. E., Luo, W., and Pankow, J. F. (2016). Flavour Chemicals in Electronic Cigarette Fluids. *Tob. Control.* 25, e10–e15. doi:10.1136/tobaccocontrol-2014-052175
- Vas, C. A., Porter, A., and McAdam, K. (2019). Acetoin is a Precursor to Diacetyl in E-Cigarette Liquids. *Food Chem. Toxicol.* 133, 110727. doi:10.1016/j.fct.2019.110727
- Uchiyama, S., Noguchi, M., Sato, A., Ishitsuka, M., Inaba, Y., and Kunugita, N. (2020). Determination of Thermal Decomposition Products Generated from E-Cigarettes. *Chem. Res. Toxicol.* 33 (2), 576–583. doi:10.1021/acs.chemrestox.9b00410
- Wagner, K. A., Flora, J. W., Melvin, M. S., Avery, K. C., Ballentine, R. M., Brown, A. P., et al. (2018). An Evaluation of Electronic Cigarette Formulations and Aerosols for Harmful and Potentially Harmful Constituents (HPHCs) Typically Derived from Combustion. *Regul. Toxicol. Pharmacol.* 95, 153–160. doi:10.1016/j.yrtph.2018.03.012
- Williams, M., Villarreal, A., Bozhilov, K., Lin, S., and Talbot, P. (2013). Metal and Silicate Particles Including Nanoparticles Are Present in Electronic Cigarette Cartomizer Fluid and Aerosol. *PLoS One* 8 (3), e57987. doi:10.1371/journal.pone.0057987
- Zhu, S.-H., Sun, J. Y., Bonnevie, E., Cummins, S. E., Gamst, A., Yin, L., et al. (2014). Four Hundred and Sixty Brands of E-Cigarettes and Counting: Implications for Product Regulation. *Tob. Control.* 23 (Suppl. 3), iii3–iii9. doi:10.1136/tobaccocontrol-2014-051670

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KM was also employed by McAdam Scientific Ltd. DM was also employed by Mariner Science Ltd. CP was also employed by DoctorProctorScience Ltd.

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Open Characterization of Vaping Liquids in Canada: Chemical Profiles and Trends

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Currently, there is a lack of comprehensive data on the diversity of chemicals present in vaping liquids. To address this gap, a non-targeted analysis of 825 vaping liquids collected between 2017 and 2019 from Canadian retailers was conducted. Prior to mass spectrometry analysis, samples were diluted 1:500 v/v with methanol or acetonitrile. Chemical compound separation and analysis was carried out using gas chromatography and triple quadrupole mass spectrometry (GC-MS/MS) systems operated in the full scan mode and mass range of 35–450 m/z. Mass spectrum for each sample was obtained in electron ionization at 70 eV and processed. Non-targeted identification workflow included use of automated mass spectral deconvolution and identification system (AMDIS), where required, as well as a number of commercially available spectral libraries. In order to validate identities, an in-house database of expected compounds previously detected in vaping liquids was used along with genuine analytical standards for compounds of interest. This resulted in a dataset of over 1,500 unique detected chemicals. Approximately half of these chemical compounds were detected only once in a single product and not in multiple products analyzed. For any sample analyzed, on average, 40% of the chemical constituents appeared to have flavouring properties. The remainder were nicotine and related alkaloids, processing, degradation or indirect additives, natural extractives and compounds with unknown roles. Data published here from the project on the Open Characterization of vaping liquids is unique as it offers a detailed understanding of products' flavour chemical profiles, the presence and frequency of chemicals of potential health concern, as well as trends and changes in products' chemical complexity over a three-year period. Non-targeted chemical surveillance such as this present valuable tools to public health officials and researchers in responding to emergent issues such as vaping associated lung injury or informing chemical based strategies which may be aimed at addressing product safety or appeal.

Keywords: vaping, nicotine, non-targeted analysis, E-cigarettes, gc ms, flavours, pods, vaping liquids

1 INTRODUCTION

Nicotine containing vaping products are a less harmful source of nicotine for people who smoke and are unable to cease the use of traditional tobacco products such as combustible cigarettes (Government of Canada, 2020a). Vaping products are not free from harm, in fact, for people who do not smoke, inhalation of vaping aerosol represents an unnecessary source of exposure to chemicals of potential health concern. Vaping products are a highly varied (Office of the Surgeon General, 2016) class of consumer products that continue to rapidly evolve and exhibit dynamic changes in product design and performance. This lack of product homogeneity as well as high variability in product use behaviors are thought to be one of the main reasons for not more fully understanding the harms and benefits of vaping products. The chemical exposure profile depends on vaping device parameters and design, user behavior and vaping liquid chemical composition. Elucidating the chemical composition of vaping liquids informs not only on the product's safety and health risks relative to smoking, it can also provide information on aspects of product appeal and addiction liability among the products studied.

Nearly all vaping products intended for use with nicotine contain a liquid made up of approximately 90% carrier solvents (humectants-propylene glycol and glycerol), 0–6% nicotine with the remainder comprised of flavouring agents, processing aids, contaminants and water. The chemical heterogeneity of the vaping products originates from the variability among flavouring and processing agents used and presence of contaminants and post-formulation chemical transformations due to product storage and ageing. The traditional approach to analyzing chemicals in products is through targeted chemical analysis, wherein known chemicals are examined using optimized laboratory methods. Data generated using these methods offer an important support for decisions and actions but are limited to the known chemical space for which reference standards exist. In comparison to traditional chemical analytical methods, non-targeted analysis (NTA) methods aim to discover and prioritize total chemical exposures from as many as possible sources of chemicals present in the products. These methods use advanced analytical equipment, chemical libraries, and software based workflows to handle large datasets and detect as many chemicals as possible, including those previously unknown or understudied. The main aim of our study is to create a foundational library of chemicals present in Canadian vaping products using data collected from an analysis of 825 vaping liquids. This work can be used to better understand health risks, appeal and addiction associated with vaping products. In the current report we outline the study design, details of the non-targeted approach applied, large dataset organization and preliminary data analysis.

2 MATERIALS AND METHODS

2.1 Chemicals and Reagents

99.7% pure propylene glycol and 99.2% pure glycerol were purchased from Sigma-Aldrich (Oakville, ON, Canada). HPLC grade methanol

and acetonitrile were purchased from Fisher Scientific (Ottawa, ON, Canada). For a full list of individual compounds used to detect select chemicals refer to **Supplementary Table S1**.

2.2 Samples

A diverse sample of 825 vaping liquids were collected from vaping stores and physical retailers in seven cities across Canada and from online Canadian retailers, between 2017 and 2019. The samples included liquids of various nicotine concentrations (0–59 mg/ml) as well as varying proportions of propylene glycol (PG) and vegetable glycerine (VG) (0/100% to 100/0% PG/VG). Overall, samples represented 182 different brands. While 8% of samples had no declared product origin, a majority of products were formulated in Canada (82.5%), followed by United States (7.3%), and elsewhere (2.2%). Ninety-seven percent of products collected were packaged in refillable bottle format (30 or 60 ml, glass or plastic), while the rest were in plastic pod based format.

2.2.1 Vaping Liquid Flavour Classification

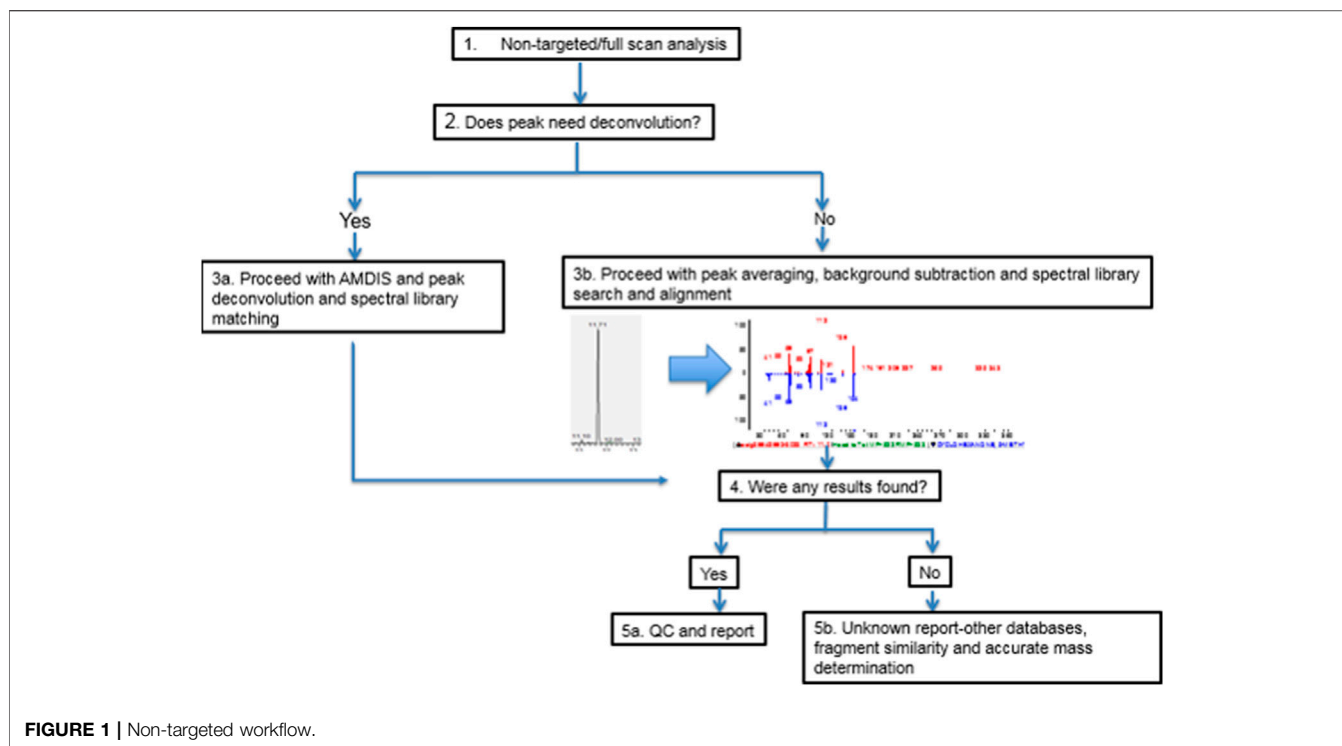
Flavour-related information from product packaging and from product descriptions on manufacturer websites were used to inform the primary, intended flavour of the vaping liquid and systematically classify each sample into one of 18 flavour categories in a modified vaping liquid flavour wheel (Krüsemann et al., 2019), adapted for vaping liquid flavours available in the Canadian market. The following 18 flavour categories were used for product classification: Fruit ($N = 108$), Desserts ($N = 76$), Tobacco ($N = 134$), Mint/menthol ($N = 97$), Coffee ($N = 33$), Tea ($N = 35$), Energy Drinks ($N = 19$), Confectionary ($N = 49$), Savoury ($N = 24$), Spices ($N = 19$), Herbal/floral ($N = 7$), Nuts ($N = 21$), Alcohol ($N = 34$), Breakfast cereals ($N = 33$), Soft drinks ($N = 29$), Milk/cream/yogurt ($N = 26$), Unflavoured ($N = 26$), and Other ($N = 55$).

2.3 Sample Preparation

Following thorough sample mixing, 40 μ l of each vaping liquid was diluted to 20 ml with methanol (Quantum TSQ GC MS/MS methodology) or acetonitrile (7000C GC MS/MS methodology). Diluted samples were vortex mixed and 1 μ l was injected and analyzed using gas chromatography mass spectrometry. Solvent blank (methanol or acetonitrile) was injected after each sample to ensure no carryover between samples. Matrix blank consisting of propylene glycol and glycerol was used during the method development process to assess possibility of PG/VG thermal degradation during GC analysis.

2.4 GC MS/MS Analysis

Two instruments (Quantum TSQ and 7000C GC MS/MS) were used to acquire data, as such, two different methods were optimized. The acquisition mode for the both instruments was full-scan acquisition mode. The Quantum TSQ MS/MS instrument was coupled to a Trace GC Ultra gas chromatograph (Thermo Electron Corp.). The oven ramp for this instrument was set as followed: 65°C hold for 1 min, followed by an increase of 5°C/min to 280°C and held for 3 min thereafter. The source temperature and interface were held at 200°C and



250°C, respectively. The MS was operated in Electron Ionization, full-scan mode with scan range 35–450 m/z and emission current set at 100 μA . Source temperature was set to 200°C, while GC interface temperature was 250°C. The second instrument was a 6890N gas chromatograph coupled to a 7000C MSMS detector (Agilent Technologies Inc.). The GC oven programming was started at 50°C and held for 2 min, followed by a ramp at 5°C/min to 240°C where it was held for 3 min. Both source and the interface temperature were held at 280°C. The MS was operated in a full-scan acquisition mode and scan range 30–450 m/z . GC analyte separation was performed using the Zebron ZB-5HT GC capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) from Phenomenex (CA, United States) on both instruments. The injector temperature was set at 280°C for both GCs with splitless injection mode for GC Ultra and pulsed splitless mode for 6890N GC. In both cases GC carrier gas was helium operated in constant flow mode at 1 ml/min rate.

2.5 Non-Targeted Workflow

Immediately following the sample analysis the chromatograms were processed as described in **Figure 1**. In some instances, where peak separation was poor, automated mass spectral deconvolution and identification system (AMDIS) (NIST, National Institute of Standards and Technology, 2019) was used for peak deconvolution. In general, the spectrum of individual compounds was matched against spectra from the National Institute of Standards and Technology (NIST 17) library reference peaks. In addition, the Agilent GC MS/MS instrument was also equipped with Wiley's library of Mass Spectra of Flavors and Fragrances of Natural and Synthetic Compounds (FFNSC), 3rd Edition, while the Quantum GC also, used the Wiley Registry of Mass Spectral Data, 11th Edition for improved

detection and confirmation. The peaks at signal intensity higher than signal to noise 3:1 are at first tentatively identified. In general, the compounds which score higher when matched against spectral libraries (>70 Agilent, >700 Quantum) and have an appropriate Retention Index, where available, are considered to be a good fit. In order to improve the analyte identification "starting confidence" or "prior probability" was utilized as previously described (Stein 2012). A database of previously detected and reported chemical compounds in vaping liquids from other published sources ($N = 151$, **Supplementary Table S2**) was used to develop categories of expected chemical compounds in vaping liquids (**Table 1**). Moreover, the same expected chemical compounds list served as the basis to set up an internal mass spectral database using genuine analytical standards of individual chemical compounds.

The chemical compounds with poor matching were compiled and a follow up analysis (e.g., accurate mass determination) will be performed in the future, if required.

2.6 Data Processing and Chemical Roles

Each identified chemical was assigned one or more roles in order to have a better understanding of the function they may have within a vaping liquid formulation. A literature synthesis was conducted which involved drawing from a variety of sources including published literature, open source websites and databases (e.g. PubChem (NIH, National Institutes of Health, 2021a), Chem Spider (Royal Society of Chemistry, 2021), The Human Metabolome Database (HMDB) (Wishart et al., 2018), Flavor DB (Garg et al., 2018), FooDB (Harrington et al., 2019)), manufacturer specifications, patents, Safety Data Sheets (SDS) and others, in order to aid in data processing and assignment of roles. Each chemical was classified into at least one of the six (6)

TABLE 1 | Identification of detected chemicals.

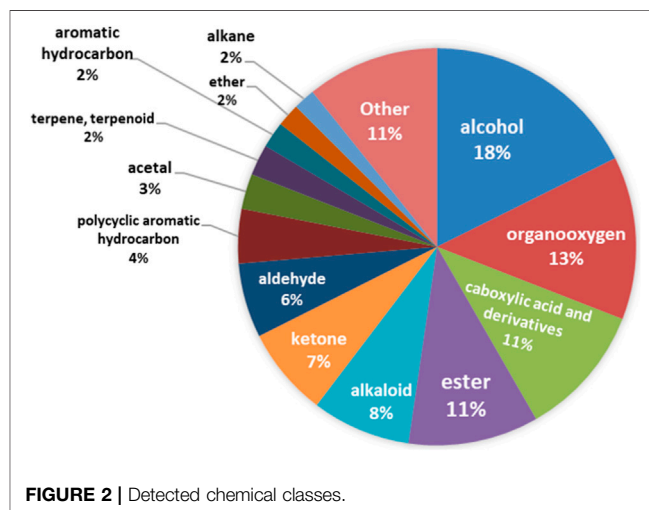
Spectral library matching	Previously reported in vaping liquids (database)	
	Known (expected)	Unknown (unexpected)
Known	Known Known (e.g., nicotine)	Known Unknown (e.g., cinnamaldehyde propylene glycol acetal)
Unknown	Unknown Known (e.g., n-nitrosomonicotine-NNN)	Unknown Unknown

roles: nicotine and related alkaloids, processing chemicals, natural extracts, flavours or fragrances, indirect additives and chemicals with unknown role. Supplementary information provides more information on specific functional role categories.

3 RESULTS AND DISCUSSION

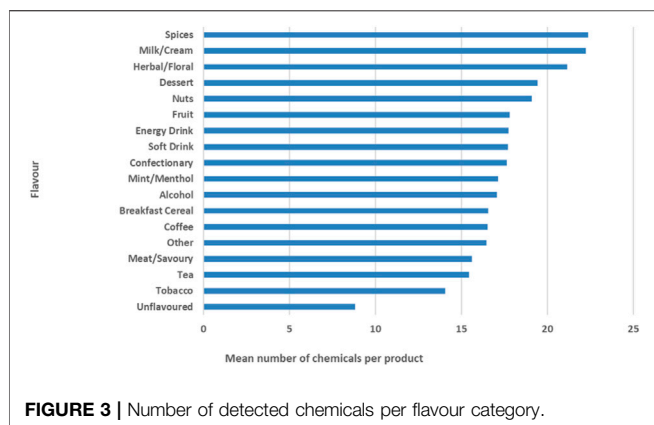
3.1 Workflow and Method Challenges

A number of challenges, which were successfully resolved, were encountered during this project. During the method development stages significant amount of time was invested in optimizing methodology as to minimize any compounds that may form during chemical analysis and degradation of product carrier solvents. More details and discussion are provided on method validation in **Supplementary Section S2**. Simple matrix blanks of PG and VG were put through dilution and analysis and no detected chemical compounds were formed during the analysis run time. Of note is that there was no carryover between samples analyzed as observed through testing of analytical blank samples between each injected sample. Simple dilution prior to mass spectrometry analysis did not result in any background contamination either. The 500 times solvent dilution often resulted in a broad glycerol peak and challenging chromatographic separation that, at times, would overlap with a signal for another chemical compound. In those instances, AMDIS was applied successfully, **Supplementary Section S3**. Processing of the resulting chromatograms was time consuming task, but was simplified using genuine analytical standards and established retention times for the group of chemical compounds previously reported to be present in vaping products (**Table 1**; **Supplementary Table S1**). This project was a significant undertaking (development of NTA methodologies and processing of large dataset with over 14,000 chemical compounds identified), it required diverse skillsets and frequent literature reviews to better elucidate chemical information such as functional groups and possible functional roles. While some parts of this process were automated, many steps still required manual quality control and review of results to ensure accuracy and completeness. Searching for individual chemical characteristics was done using Chemical Abstracts Services (CAS number) as provided in the mass spectral libraries. Significant data clean-up was performed in order to remove duplicate CAS numbers as some compounds may have multiple CAS numbers (e.g. menthol) and different mass spectral libraries may have preferences for CAS number provided as primary one.

**FIGURE 2** | Detected chemical classes.

3.2 Chemical Space

The actual chemical space of all products tested was 1,507 unique chemical compounds. Since some chemical compounds were detected in more than one product, total number of chemicals detected in 825 samples was over 14,000. Close to 50% (734/1,507) of all chemicals were detected in just one vaping liquid, illustrating the heterogeneity of this class of consumer products and infrequency of occurrence among chemical compounds used. Only four chemical compounds were detected in over 50% of all products studied. These include nicotine, the carrier solvents propylene glycol and glycerol, as well as β -Nicotyrine, a nicotine oxidation by-product that may form during storage (Wada et al., 1959). Seven hundred and thirty-eight products were labelled as nicotine-containing, however, among these products 14 were found not to contain any detectable nicotine. The lack of detection of nicotine in these samples was not due to the sensitivity of analytical method as this scan method is able to detect nicotine down to 0.03 mg/ml. The majority of samples with this discrepancy were, in fact, labelled to contain nicotine at over 9 mg/ml. Out of 87 products labelled as nicotine free, one product was detected to contain nicotine. These discrepancies on nicotine presence are likely due to poor manufacturing practices or lack of nicotine stability, as noted elsewhere (Goniewicz et al., 2015; Kavvalakis et al., 2015). Of note is that all samples in question were collected prior to September 2018 and, when labelled, were marked as manufactured prior to this date. These products likely precede the Government of Canada's Tobacco and Vaping Products Act (Government of Canada, 2018b) which includes

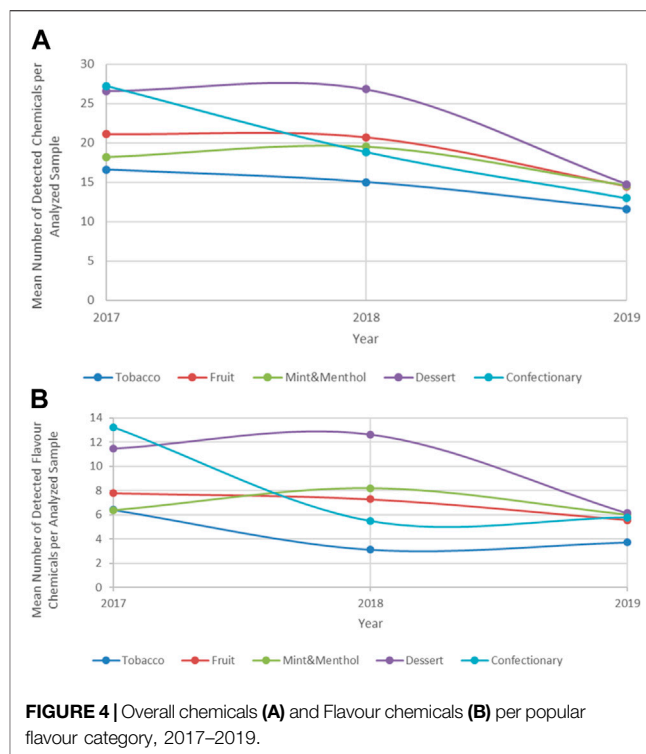


limits on nicotine concentrations and brings forward compliance and enforcement of the same.

All chemical compounds detected in the course of the study can be classified into one of 170 chemical classes. The most frequently detected chemical classes are alcohol, organooxygen, carboxylic acid and derivatives, and esters, **Figure 2**.

3.3 Chemical Roles

There were 87 (0.6%) chemical compounds for which it was not possible to assign or determine their identity using the mass spectral libraries available. In the future, samples with these compounds may be analyzed using different analytical approaches to identify them. Each chemical compound with a known identity was assigned at least one of the six functional roles using the various sources of peer-reviewed literature and supporting materials. Although identity was determined for the vast majority of detected chemicals, a functional role was not assigned to 8% of the chemicals detected as no supporting materials were found. Of note, a larger number of the chemicals with unknown roles have been previously detected in yeast (University of Washington, 2018). At this time, it is not known what the exact role or origin of yeast related chemicals in vaping liquids is. Autolyzed yeast extract is used as a flavour enhancer in foods and beverages (U.S. Food and Drug Administration, 2010; U.S. Food and Drug Administration, 2010), while microbial contamination of vaping products has been reported previously (Lee et al., 2019). Six percent of all chemicals were assigned indirect additive roles with supporting materials (Food and Drug Administration, 2017) often found among records on indirect additives on foods or food contact materials. It is likely these are found in products as a result of leaching into the vaping liquid during processing or packaging. Alkaloid roles were assigned to 10% of chemicals, which in the majority of cases included nicotine and related minor alkaloids. Thirteen percent of chemicals were found to have the natural extract role while 27% of chemicals were likely used as processing chemicals in the formulation. Examples of processing roles include emulsifiers, humectants, diluents and others. Forty-three percent of all chemicals detected were assigned a flavour or fragrance role. The number of individual chemicals per vaping liquid sample ranged between 4 and 66 compounds with a mean



of 18 chemical compounds detected per product. Although a lower number of nicotine-salt based products were analyzed ($N = 116$) when compared to free-base nicotine products ($N = 623$), nicotine-salt products were found to contain a lower number of chemicals, with a mean of 16 chemicals detected per product. The number of chemical substances present in vaping liquids (e-liquids) can be used as one of the indicators of potential toxicity of the product, as reported previously by the group of researchers from North Carolina (Sassano et al., 2018) who concluded that increasing chemical numbers were associated with increasing toxicity when compared to solvent (PG/VG) vehicle in high-throughput *in-vitro* toxicity testing. In addition to nicotine type used in the product, the number of chemicals detected varied with the liquid's flavour categories, **Figure 3**.

As expected, the unflavoured products appeared to have the least complex chemical profiles (mean number of nine chemicals), followed by the tobacco flavour category (mean number of 14 chemicals). The most complex chemical profiles were found in the categories of milk/cream (e.g., Yogurt) and spices (e.g. cinnamon), each with a mean of 22 detected chemicals. On average per product flavour category, the unflavoured category had the lowest proportion of flavour chemicals (15% of total chemicals), and energy drinks had the highest proportion of flavour chemicals (58% of total chemicals). Flavour categories such as fruit, confectionary and dessert, which may have a higher preference among youth, had higher proportions of flavour chemicals on average (48, 54 and 55% of all chemicals, respectively). This proportion of flavour compounds is somewhat lower compared to proportions (63%) reported by the Dutch study from European vaping

TABLE 2 | The top five most frequently identified chemicals in all flavour categories and the flavour/odour description from the Good Scents Company website.

CAS	Chemical name	Organoleptic properties ^a	Avg % frequency (in all liquids)	# Flavour categories detected as a top 5 chemical	Top 5 chemical in fruit (F), confectionary (C); dessert (D)
121-33-5	Vanillin	Vanilla sweet creamy spicy phenolic milky	45	14	F, C, D
4940-11-8	Ethyl maltol	Sweet burnt sugar candy jam strawberry	30	12	F, C, D
121-32-4	Ethyl Vanillin	Sweet creamy vanilla smooth caramellic	30	11	C, D
68527-74-2	Vanillin propylene glycol acetal	Sweet vanilla creamy phenolic smoky powdery	19	6	D
80-71-7	Cyclotene	Caramellic maple	14	4	None

^aThe Good Scents Company Information System (Good Scents Company, 2021).

products (Krüsemann et al., 2021). The differences could be due to the origins of the chemical datasets, as Dutch data is based on a reporting system where manufacturers provide information on ingredients added, while the non-targeted analysis based dataset results from chemical analysis which may detect impurities, indirect additives, as well as compounds that result from chemical reactions post-product formulation and product ageing (degradation, leaching and transformations). These additional compounds would increase the total number of compounds known to be present in the product, thereby decreasing the percentage of flavouring compound in the final composition.

Of note is that the mean number of chemicals detected per product has in fact changed over the years; products collected in 2017 and 2018 appear to have a significantly higher number of chemical compounds when compared to those collected in 2019. This trend is observed regardless of flavour category analyzed, **Figure 4A**. When the trend is examined for the number of flavour compounds over this time period and in the same products a similar trend emerges, suggesting a decrease in the chemical flavour complexities among this group of products, **Figure 4B**.

This trend could be in part explained by the higher frequency of nicotine-salt based products post 2018 which on average appear to contain a lower number of chemicals. Nicotine-salts are perceived to provide a less harsh and smoother sensory experience for the product users (Leventhal et al., 2021), thus it is likely they require less flavouring agents to mask the sensory experience normally associated with free-base nicotine products.

3.4 Flavour Chemicals of Concern

Vaping products on the Canadian market come in a variety of flavour categories. In the past few years, youth vaping prevalence has increased in Canada (Government of Canada, 2020a) and flavours play an important role in attracting youth to vaping products. Recent evidence suggests that youth prefer flavour categories such as fruit, confectionary and dessert (Government of Canada, 2018a; O'Connor et al., 2019). The chemicals detected in products are used to better understand flavour chemicals and their role in imparting intended or declared product flavours. Vaping product formulations are the manufacturer's interpretation of the intended or declared flavour. Our data indicates that the chemical space of each flavour category is diverse and there is a high degree of

chemical overlap between flavour categories. Similar to previously published studies (Tierney et al., 2016; Omaiye et al., 2019), our data shows that vaping liquids contain some of the same flavour chemicals despite their flavour category. Except for mint/menthol, herbal/floral and unflavoured category, across all other products, the top five most frequently detected flavour chemicals (**Table 2**) were vanillin, ethyl maltol, ethyl vanillin, vanillin propylene glycol acetal and cyclotene. Vanillin, ethyl maltol and ethyl vanillin were in the top five flavouring chemicals for more than half of the flavour categories studied. Collectively, the top five chemicals have flavour descriptors such as "sweet," "creamy" and "vanilla" (Good Scents Company, 2021). Vanillin and ethyl maltol, but not ethyl vanillin, were the most frequently detected flavour chemical in the three categories likely to be more appealing to youth. Ethyl maltol is a sweetener, with a sweet, caramellic, jammy, strawberry-like odor description and sweet, burnt cotton candy, caramel-like taste. Perception of sweet flavour in vaping products has been shown to produce greater appeal and perceived sweetness ratings among young vapers (Goldenson et al., 2016). Moreover, sweet perception and appealing flavours can enhance nicotine reward reinforcing effects in vaping and other tobacco products (Kroemer et al., 2018; Patten and De Biasi 2020).

In published studies, concentrations of ethyl maltol in vaping liquids range between undetectable to 4,200 µg/ml (Aszyk et al., 2017; Behar et al., 2018), compared to average maximum concentration ranges of 12.4–152 µg/ml in non-alcoholic beverages and baked goods, respectively, on which Flavor Extract Manufacturers Association (FEMA, The Flavor and Extract Manufacturers Association of the United States, 2021) Expert Panel based its' judgments that this substance is safe for ingestion (Oser and Ford 1977). Although generally recognized as safe for ingestion, the health effects of ethyl maltol, and more broadly the majority of flavour compounds, have not been assessed for the inhalation route (Flavor and Extract Manufacturers Association, 2021). Currently, published studies on vaping flavours focus on cytotoxic and mutagenic effects in cell models (Behar et al., 2018; Muthumalage et al., 2018); translating these study findings into a real-life setting is challenging. While inhalation toxicity data is scarce for some compounds, certain vaping flavour compounds are recognized as those of concern for human health. For example, diacetyl and 2,3

TABLE 3 | Established list of constituents identified by US FDA as harmful and potentially harmful constituents and their detection frequency in vaping liquids.

Constituent	CAS RN	Carcinogen (CA), respiratory toxicant (RT), cardiovascular toxicant (CT), reproductive or developmental toxicant (RDT), addictive (AD)	Frequency of detection (%)
Acetaldehyde	75-07-0	CA, RT, AD	1.2
Coumarin	91-64-5	Banned in food	0.6
Ethylbenzene	100-41-4	CA	4.5
Ethylene oxide	75-21-8	CA, RT, RDT	0.5
Methyl ethyl ketone	78-93-3	RT	1.0
Naphthalene	91-20-3	CA, RT	12.2
N-Nitrosodimethylamine (NDMA)	62-75-9	CA	0.1
Phenol	108-95-2	RT, CT	0.6
Quinoline	91-22-5	CA	0.5
Styrene	100-42-5	CA	0.7

pentanedione are two buttery flavours, shown to cause lung and respiratory airways damage in animal models and are associated with respiratory disease and decreased lung function in occupationally exposed employees of food flavouring and food manufacturing facilities (NIOSH, The National Institute for Occupational Safety and Health, 2016). While diacetyl was detected in two vaping liquids acquired prior to 2018, 2,3 pentanedione was not detected in any vaping liquids analyzed in the Open Characterization dataset. Another flavour, the monoterpene pulegone typically found in extracts of mint oil, has been previously detected in vaping products (Hutzler et al., 2014; Geiss et al., 2015). This chemical has been shown to induce some carcinogenic effects in mice and rats (National Toxicology Program, 2011). In the Open Characterization analysis, 11 out of 825 (1.3%) products were found to contain pulegone at unknown concentration levels, mainly mint/menthol flavoured products (9/11 products). Currently, no evidence is available that pulegone has any vaping-related health effects in humans.

3.5 Chemicals of Health Concern

Within this dataset, the quantification of all chemicals identified is untenable given the targeted study method developments may take years to complete. Chemical prioritization or screening based on known hazards was used to develop a list of chemicals for quantification. Providing exposure estimates through targeted analytical studies focused on these prioritized chemicals will provide sufficient information to better elucidate the risk. The majority of studies provide results on relative risk and comparison to tobacco cigarettes. Vaping products in fact infrequently contain tobacco specific toxicants and even in cases when they do, these are often present at much lower concentrations as observed in the exposure studies on product users (Goniewicz et al., 2018; Engineering, and Medicine National Academies of Sciences, 2018). For example, in our study there was only one product that was found to contain N-Nitrosodimethylamine (NDMA); no other nitrosamines were detected. In addition to NDMA, 9 out of 93 US FDA's

Harmful and Potentially Harmful Constituents (HPHC) (FDA, US Food and Drug Administration, 2012) were detected in Open Characterization samples (Table 3).

The reasons behind the higher frequency of detection of naphthalene compared to other HPHC chemicals are unclear at this time; this Polycyclic Aromatic Hydrocarbon is normally present in tobacco smoke, but also in the extracts of various fruits and other plants (Gómez et al., 1993; Paris et al., 2018), so it is possible that naphthalene originates from the natural extracts used to flavour the products. Of note is that other methylated and naphthalene-related structural analogs, not on the HPHC list, were also detected in vaping products studied. For example, 1-methyl naphthalene, a flavour and fragrance agent normally found in fruits (Good Scents Company, 2021), is also detected in 12% of products analyzed. Exposure of laboratory animals to 1- and 2-methylnaphthalene resulted in spleen and organ damage while mice exposed dermally for 30 weeks developed pulmonary alveolar proteinosis. Humans exposed to this compound developed skin irritation and skin photosensitization (NIH, National Institutes of Health, 2021b). In 2019, USFDA proposed the addition of 19 chemical compounds to an existing HPHC list of 93 (Food and Drug Administration, 2017), mainly to reflect potentially harmful chemicals present in vaping products. The first proposed chemical is glycidol, a probable human carcinogen (International Agency for Research on Cancer, 2000) thought to result from thermal degradation of glycerol. Glycidol has been previously detected in vaping product emissions (Sleiman et al., 2016) and was found in 3% of the liquids tested. Non-targeted studies such as this provide datasets that can inform future steps and ultimately characterize product-use specific harms. The prioritization can consider chemicals with already established health effects of concern, detection frequency or chemical presence in products with high market share. In our dataset, most chemicals of concern were not detected in the majority (>50%) of products studied, indicating that the chemicals of concern can be used to identify products for which the ingredients used may be a cause for concern. The goal is to provide information that would lead to products which

minimize the risk of vaping products for consumers looking to completely switch from combustible tobacco products.

In comparison to traditional chemical analytical methods, non-targeted analysis (NTA) methods aim to discover as many chemicals as possible in products, including those previously unknown or with limited data. To date, there has been only one published study using non-targeted screening of Canadian vaping liquids (Czoli et al., 2019). One hundred and sixty-six vaping liquids collected in 2015 were analyzed using a gas chromatography mass spectrometry instrument with limited sensitivity and resolution. Similarly, a U.S. dataset generated by the Centre for Tobacco Regulatory Science and Lung Health (Center for Tobacco Regulatory Science and Lung Health, 2021), chemically characterized approximately 300 vaping product samples; significantly fewer than the Canadian Open Characterization dataset ($N = 825$). Closed pod-system brands that make up a majority of the vaping market in Canada were not included in the U.S. dataset. In addition, limited information is available on the products tested in the U.S. including classification by flavour categories, as their product names are not self-explanatory (e.g. Carnage, Magic Dragon, etc.). Finally, it is unknown how many of these U.S. products are available for sale in Canada. These factors present challenges in comparing the two datasets. Overall, valuable information can be determined by evaluating different market datasets, however direct comparisons are challenging given the heterogeneity of vaping products within and between different regions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

REFERENCES

- Aszyk, J., Kubica, P., Kot-Wasik, A., Namieśnik, J., and Wasik, A. (2017). Comprehensive Determination of Flavouring Additives and Nicotine in E-Cigarette Refill Solutions. Part I: Liquid Chromatography-Tandem Mass Spectrometry Analysis. *J. Chromatogr. A* 1519, 45–54. doi:10.1016/j.chroma.2017.08.056
- Behar, R. Z., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2018). Analytical and Toxicological Evaluation of Flavor Chemicals in Electronic Cigarette Refill Fluids. *Sci. Rep.* 8 (1), 8288. doi:10.1038/s41598-018-25575-6
- Burdock, G. A. (2016). *Fenaroli's Handbook of Flavor Ingredients*. Boca Raton, Florida, United States: CRC Press.
- Center for Tobacco Regulatory Science and Lung Health (2021). *E-liquid Database*. University of North Carolina. Available at: <https://eliquidinfo.org/>.
- Czoli, C. D., Goniewicz, M. L., Palumbo, M., Leigh, N., Christine, M., Hammond, D., et al. (2019). Identification of Flavouring Chemicals and Potential Toxicants in E-Cigarette Products in Ontario, Canada. *Can. J. Public Health* 110 (5), 542–550. doi:10.17269/s41997-019-00208-1
- Engineering, and Medicine National Academies of Sciences (2018). *Public Health Consequences of E-Cigarettes*. The National Academies Press. Available at: <https://www.nap.edu/catalog/24952/public-health-consequences-of-e-cigarettes>.
- FDA, US Food and Drug Administration (2012). *Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke: Established List*. Silver Spring, Maryland: Food and Drug Administration (FDA).

AUTHOR CONTRIBUTIONS

IK conceived, designed and performed the chemical analysis, supervised data processing and wrote the manuscript. CK supervised the chemical analysis of open characterization study and reviewed the manuscript. XF designed and performed the chemical analysis while JM, WH, and SS performed the chemical analysis. MG, DP, and KT processed data, discussed results and revised the manuscript. TM conceived the project, supervised the project coordination, discussed results and revised the manuscript.

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- FEMA, The Flavor and Extract Manufacturers Association of the United States (2021). *The Flavor and Extract Manufacturers Association of the United States*. Washington, D.C., United States: FEMA Database.
- Flavor and Extract Manufacturers Association (2021). The Safety Assessment and Regulatory Authority to Use Flavors: Focus on E-Cigarettes. Available at: <https://www.femaflavor.org/node/24344> (Accessed June 24, 2021).
- Food and Agriculture Organization of United Nations (2021). *Combined Compendium of Food Additive Specifications*. Online Edition Available at: <http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/>.
- Food and Drug Administration (2019). *Harmful and Potentially Harmful Constituents in Tobacco Products; Established List; Proposed Additions; Request for Comments*. National Archives, Federal Register. Available at: <https://www.federalregister.gov/documents/2019/08/05/2019-16658/harmful-and-potentially-harmful-constituents-in-tobacco-products-established-list-proposed-additions>.
- Food and Drug Administration (2017). *Indirect Food Additives*. Silver Spring, Maryland: Food and Drug Administration.
- Garg, N., Sethupathy, A., Tuwani, R., Nk, R., Dokania, S., Iyer, A., et al. (2018). FlavorDB: a Database of Flavor Molecules. *Nucleic Acids Res.* 46 (D1), D1210–D1216. doi:10.1093/nar/gkx957
- Geiss, O., Bianchi, I., Barahona, F., and Barrero-Moreno, J. (2015). Characterisation of Mainstream and Passive Vapours Emitted by Selected Electronic Cigarettes. *Int. J. Hyg. Environ. Health* 218 (1), 169–180. doi:10.1016/j.ijheh.2014.10.001

- Goldenson, N. I., Kirkpatrick, M. G., Barrington-Trimis, J. L., Pang, R. D., McBeth, J. F., Pentz, M. A., et al. (2016). Effects of Sweet Flavorings and Nicotine on the Appeal and Sensory Properties of E-Cigarettes Among Young Adult Vapers: Application of a Novel Methodology. *Drug and alcohol dependence* 168, 176–180. doi:10.1016/j.drugalcdep.2016.09.014
- Gómez, E., Ledbetter, C. A., and Hartsell, P. L. (1993). Volatile Compounds in Apricot, Plum, and Their Interspecific Hybrids. *J. Agric. Food Chem.* 41 (10), 1669–1676. doi:10.1021/jf00034a029
- Goniewicz, M. L., Gupta, R., Lee, Y. H., Reinhardt, S., Kim, S., Kim, B., et al. (2015). Nicotine Levels in Electronic Cigarette Refill Solutions: A Comparative Analysis of Products from the US, Korea, and Poland. *Int. J. Drug Pol.* 26 (6), 583–588. doi:10.1016/j.drugpo.2015.01.020
- Goniewicz, M. L., Smith, D. L., Edwards, K. C., Blount, B. C., Caldwell, K. L., Feng, J., et al. (2018). Comparison of Nicotine and Toxicant Exposure in Users of Electronic Cigarettes and Combustible Cigarettes. *JAMA Netw. Open* 1 (8), e185937. doi:10.1001/jamanetworkopen.2018.5937
- Good Scents Company (2021). The Good Scents Company Information System. Available at: <http://www.thegoodscentscompany.com/index.html>.
- Government of Canada (2020a). Canada's Tobacco Strategy. Available at: <https://www.canada.ca/en/health-canada/services/publications/healthy-living/canada-tobacco-strategy.html> (Accessed 10 01, 2020).
- Government of Canada (2020b). *Canadian Tobacco and Nicotine Survey (CTNS): Summary of Results for 2019*. Canada: Health Canada.
- Government of Canada (2018a). *Canadian Tobacco, Alcohol and Drugs Survey (CTADS): Summary of Results for 2017*. Canada: Health Canada.
- Government of Canada (2018b). *Tobacco and Vaping Products Act*. Canada: Health Canada.
- Harrington, R. A., Adhikari, V., Rayner, M., and Scarborough, P. (2019). Nutrient Composition Databases in the Age of Big Data: foodDB, a Comprehensive, Real-Time Database Infrastructure. *BMJ open* 9 (6), e026652. doi:10.1136/bmjopen-2018-026652
- Hutzel, C., Paschke, M., Kruschinski, S., Henkler, F., Hahn, J., and Luch, A. (2014). Chemical Hazards Present in Liquids and Vapors of Electronic Cigarettes. *Arch. Toxicol.* 88 (7), 1295–1308. doi:10.1007/s00204-014-1294-7
- International Agency for Research on Cancer (2000). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 77. Available at: <https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Industrial-Chemicals-2000>.
- Kavvalakis, M. P., Stivaktakis, P. D., Tzatzarakis, M. N., Kouretas, D., Liesivuori, J., Alegakis, A. K., et al. (2015). Multicomponent Analysis of Replacement Liquids of Electronic Cigarettes Using Chromatographic Techniques. *J. Anal. Toxicol.* 39 (4), 262–269. doi:10.1093/jat/bkv002
- Kroemer, N. B., Veldhuizen, M. G., Dely, R., Patel, B. P., O'Malley, S. S., and Small, D. M. (2018). Sweet Taste Potentiates the Reinforcing Effects of E-Cigarettes. *Eur. Neuropsychopharmacol.* 28 (10), 1089–1102. doi:10.1016/j.euroneuro.2018.07.102
- Krusemann, E. J. Z., Boesveldt, S., de Graaf, K., and au, R. (2019). An E-Liquid Flavor Wheel: a Shared Vocabulary Based on Systematically Reviewing E-Liquid Flavor Classifications in Literature. *Nicotine Tob. Res.* 21 (10), 1310–1319. doi:10.1093/ntr/nty101
- Krusemann, E. J. Z., Havermans, A., Jeroen, L. A., de Graaf, K., Boesveldt, S., Talhout, R., et al. (2021). Comprehensive Overview of Common E-Liquid Ingredients and How They Can Be Used to Predict an E-Liquid's Flavour Category. *Tob. Control.* 30 (2), 185–191. doi:10.1136/tobaccocontrol-2019-055447
- Lee, M.-S., Allen, J. G., and Christiani, D. C. (2019). Endotoxin and (1→3)- β -D-Glucan Contamination in Electronic Cigarette Products Sold in the United States. *Environ. Health Perspect.* 127 (4), 047008. doi:10.1289/ehp3469
- Leffingwell and Associates (2011). Flavor-Base 10 - Tobacco Version. 2011. Available at: <http://www.leffingwell.com/tob2001.htm>.
- Leventhal, A. M., Madden, D. R., Peraza, N., Schiff, S. J., Lebovitz, L., Whitted, L., et al. (2021). Effect of Exposure to E-Cigarettes with Salt vs Free-Base Nicotine on the Appeal and Sensory Experience of Vaping. *JAMA Netw. Open* 4 (1), e2032757. doi:10.1001/jamanetworkopen.2020.32757
- Muthumalage, T., Prinz, M., Ansah, K. O., Gerloff, J., Sundar, I. K., and Rahman, I. (2018). Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used E-Cigarette Flavoring Chemicals and Flavored E-Liquids without Nicotine. *Front. Physiol.* 8, 1130. doi:10.3389/fphys.2017.01130
- National Toxicology Program (2011). Toxicology and Carcinogenesis Studies of Pulegone (CAS No. 89-82-7) in F344/N Rats and B6C3F1 Mice (Gavage Studies). *Natl. Toxicol. Program Tech. Rep. Ser.* 563, 1–201. Available at: <https://ntp.niehs.nih.gov/go/tr563abs>
- NIH, National Institutes of Health (2021b). *PubChem 1-Methylnaphthalene*. Available at: <https://pubchem.ncbi.nlm.nih.gov/compound/1-methylnaphthalene#section=Toxicity>.
- NIH, National Institutes of Health (2021a). *PubChem*. Available at: <https://pubchem.ncbi.nlm.nih.gov/>.
- NIOSH, The National Institute for Occupational Safety and Health (2016). *Criteria for a Recommended Standard: Occupational Exposure to Diacetyl and 2,3-Pentanedione*. Available at: https://www.cdc.gov/niosh/docs/2016-111/?s_cid=3ni7d2DiacetylPressRel102016.
- NIST, National Institute of Standards and Technology (2019). *AMDIS - Automated Mass Spectral Deconvolution and Identification System*. Available at: <https://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdms> (Accessed, 2021).
- O'Connor, R. J., Fix, B. V., McNeill, A., Goniewicz, M. L., Bansal-Travers, M., Heckman, B. W., et al. (2019). Characteristics of Nicotine Vaping Products Used by Participants in the 2016 ITC Four Country Smoking and Vaping Survey. *Addiction* 114, 15–23. doi:10.1111/add.14571
- Office of the Surgeon General (2016). *E-cigarette Use Among Youth and Young Adults*. Washington, DC, US: Centers for Disease Control and Prevention U.S. Department of Health and Human Services.
- Omaiyi, E. E., McWhirter, K. J., Luo, W., Tierney, P. A., Pankow, J. F., and Talbot, P. (2019). High Concentrations of Flavor Chemicals Are Present in Electronic Cigarette Refill Fluids. *Sci. Rep.* 9 (1), 2468–2469. doi:10.1038/s41598-019-39550-2
- Oser, B. L., and Ford, R. A. (1977). Recent Progress in the Consideration of Flavoring Ingredients under the Food Additives Amendment. 10. GRAS Substances. *Food Technol.* 31 (1), 65–74.
- Paris, A., Ledauphin, J., Poinot, P., and Gaillard, J.-L. (2018). Polycyclic Aromatic Hydrocarbons in Fruits and Vegetables: Origin, Analysis, and Occurrence. *Environ. Pollut.* 234, 96–106. doi:10.1016/j.envpol.2017.11.028
- Patten, T., and De Biasi, M. (2020). History Repeats Itself: Role of Characterizing Flavors on Nicotine Use and Abuse. *Neuropharmacology* 177, 108162. doi:10.1016/j.neuropharm.2020.108162
- Royal Society of Chemistry (2021). *ChemSpider*. Available at: <http://www.chemspider.com/>.
- Sassano, M. F., Davis, E. S., Keating, J. E., Zorn, B. T., Kochar, T. K., Wolfgang, M. C., et al. (2018). Evaluation of E-Liquid Toxicity Using an Open-Source High-Throughput Screening Assay. *Plos Biol.* 16 (3), e2003904. doi:10.1371/journal.pbio.2003904
- Sleiman, M., Logue, J. M., Montesinos, V. N., Russell, M. L., Litter, M. I., Gundel, L. A., et al. (2016). Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. *Environ. Sci. Technol.* 50 (17), 9644–9651. doi:10.1021/acs.est.6b01741
- Stein, S. (2012). Mass Spectral Reference Libraries: an Ever-Expanding Resource for Chemical Identification. *Anal. Chem.* 84 (17), 7274–7282. doi:10.1021/ac301205z
- The Flavor and Perfumer's Apprentice (2021). *The Flavor and Perfumer's Apprentice*. Available at: <https://shop.perfumersapprentice.com/>.
- Tierney, P. A., Karpinski, C. D., Brown, J. E., Luo, W., and Pankow, J. F. (2016). Flavour Chemicals in Electronic Cigarette Fluids. *Tob. Control.* 25 (e1), e10–e15. doi:10.1136/tobaccocontrol-2014-052175
- University of Washington (2018). *The Yeast Resource Center (YRC)* Available at: <http://depts.washington.edu/yeastrc/>.
- U.S. Food and Drug Administration (2010). Overview of Food Ingredients, Additives & Colors. Available at: <https://www.fda.gov/food/food-ingredients-packaging/overview-food-ingredients-additives-colors>.
- U.S. Food and Drug Administration (2021). *Substances Added to Food*. Available at: <https://www.cfsanappsexternal.fda.gov/scripts/fdc/?set=FoodSubstances>.
- U.S. Food and Drug Administration (2020). *Yeast-malt Sprout Extract* Available at: <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.590>.
- Wada, E., Kasaki, T., and Saito, K. (1959). Autoxidation of Nicotine. *Arch. Biochem. Biophys.* 79, 124–130. doi:10.1016/0003-9861(59)90385-6

Wishart, D. S., Feunang, Y. D., Marcu, A., Guo, A. C., Liang, K., Vázquez-Fresno, R., et al. (2018). HMDB 4.0: the Human Metabolome Database for 2018. *Nucleic Acids Res.* 46 (D1), D608–D617. doi:10.1093/nar/gkx1089

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EVALI Vaping Liquids Part 2: Mass Spectrometric Identification of Diluents and Additives

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The vaping liquid additive vitamin E acetate (VEA) was strongly linked to the 2019 United States nationwide outbreak of pulmonary lung illness (EVALI) associated with e-cigarettes or vaping liquids. Our laboratory received over 1,000 vaping liquid products for identification of the vaping liquid additives, including hundreds of vaping products from EVALI patients. In this work, we present results obtained for the GC-MS identification of numerous vaping liquid additives in a large subset of ca. 300 Cannabis vaping liquids, including vitamin E acetate, medium chain triglycerides oil (MCT oil), polyethylene glycols, squalane, triethyl citrate, dipropylene glycol dibenzoate (DPG dibenzoate), pine rosin acids, pine rosin methyl esters, and sucrose acetate isobutyrate (SAIB). Confirmation of DPG dibenzoate and SAIB using LC-HRMS is also presented. GC-MS analysis for additives identified as the parent compounds was conducted after separation on a commercial 5% phenyl phase. GC-MS analysis for additives identified as the trimethylsilyl derivatives was conducted after separation on a commercial 35% silphenylene phase. LC-HRMS analysis was conducted using gradient elution with either C18 or phenyl-hexyl phases and determination of exact masses for the target compounds. In addition to providing rapid methods for the identification of vaping liquid additives, this work highlights the variety of Cannabis vaping liquid additives in current use.

Keywords: EVALI, vaping liquids, e-cigarettes, vitamin E acetate (VEA), diluents, additives, GC-MS, LC-HRMS

INTRODUCTION

In Part 1 (Ciolino et al., 2021), we presented results obtained for the gas chromatography-mass spectrometry (GC-MS) analysis of a series of THC isomers in THC-vaping liquids associated with the 2019 nationwide outbreak of pulmonary lung illness (EVALI). The vaping liquids from Part 1 were a large subset (ca. 300 vapes) of over 1,000 Cannabis vaping liquids which were analyzed by our laboratory in conjunction with the EVALI health crisis and investigation.

As stated in Part 1 (Ciolino et al., 2021), the CDC concluded that the presence of the additive vitamin E acetate (VEA) in vaping liquid products was strongly linked to the EVALI outbreak (Blount et al., 2020; US Centers for Disease Control, 2020). While VEA was of particular concern, the CDC also concluded that the contribution of other chemicals in the vaping liquid products could not be ruled out in some EVALI cases (US Centers for Disease Control, 2020). Hence, in this second part, we will present results obtained for the mass spectrometric identification of numerous vaping liquid additives from this same subset of vaping liquids.

The first report of vitamin E acetate (VEA) being found as a major component in vaping liquid products used by EVALI patients was from New York (Duffy et al., 2020). Medium chain triglycerides oil (MCT oil) was also identified in many of the EVALI patient vaping fluids, alone or in combination with the VEA. The vaping liquid products were judged to originate from illicit sources (Duffy et al., 2020). There have also been reports (Duffy et al., 2020; Meehan-Atrash and Strongin, 2020) of the direct analysis of liquid additives intended for use in the formulation of Cannabis-based vaping fluids. Analysis of three “commercial diluents” (Duffy et al., 2020) identified VEA only in two of the diluents, and a combination of squalane, MCT oil, and a minor amount of triethyl citrate in the third diluent. Analysis of three “commercial thickeners” (Duffy et al., 2020) identified VEA, squalane, or the terpene α -bisabolol, respectively. Analysis of two additives (Meehan-Atrash and Strongin, 2020) described as “adulterants” obtained from a “cannabis industry source” showed one additive to be pure VEA, and the other additive to contain a pine rosin material in combination with MCT oil.

In addition to these prior reports of vaping liquid additives, a review of the patent literature shows that additives play a major role in the formulation of commercial Cannabis vaping liquids. Commercial vaping liquid additives may include solubilizers/carriers (Zumpano, 2020; Goldman et al., 2015), viscosity modifiers (Finley et al., 2016; Finley and McKee, 2019), flavoring or aroma compounds (Goldman et al., 2015; Finley and McKee, 2019; Green et al., 2020; Zumpano, 2020), terpenes (Tucker and Fulton, 2017), and other additive types (Kotra et al., 2020). A given additive may serve more than one purpose. The EVALI outbreak demonstrates the need for analytical methods which rapidly identify vaping liquid additives in order to provide the most complete information when assessing chemical exposures which may occur during vaping.

This report will provide a summary of the additives identified in the neat vaping liquids, and details of the GC-MS analytical methods used for their identification. Confirmation of selected additives using liquid chromatography-high resolution mass spectrometry (LC-HRMS) will also be presented. The additives include VEA, medium chain triglycerides oil (MCT oil), polyethylene glycols (PEGs), squalane, triethyl citrate, dipropylene glycol dibenzoate (DPG dibenzoate), pine rosin acids, pine rosin methyl esters, and sucrose acetate isobutyrate (SAIB). The peculiarities of identifying several of the more complex additives will be addressed. As analysis was conducted on the neat vaping liquids, this work is not intended to be a vaping study, nor to identify components of aerosols generated during the vaping process.

EXPERIMENTAL

Standards, Solvents, and Reagents

Vitamin E acetate (alpha-tocopherol acetate, $\geq 98\%$) was obtained from Sigma-Aldrich (St. Louis, MO) or USP (Rockville, MD). Pimaric acid ($\geq 98\%$) was obtained from Cayman Chemical Company (Ann Arbor, MI). A partially hydrogenated methyl ester rosin standard material (a complex mixture of methyl

esters of rosin acids including both methyl dihydroabietate and methyl dehydroabietate, purity not declared), trioctanoin ($\geq 93\%$), and tridecanoin ($\geq 98\%$) were obtained from TCI America. All of the remaining standards or standard materials were obtained from Sigma-Aldrich as follows: abietic acid (technical grade, 75%), dehydroabietic acid ($\geq 95\%$), isopimaric acid ($\geq 98\%$), dipropylene glycol dibenzoate (technical grade, 75%), gum rosin (acid value 165), sucrose acetate isobutyrate (food grade), squalane (96%), triethyl citrate ($\geq 98\%$), and PEG oligomers [tetraethylene glycol (99%), pentaethylene glycol (98%), hexaethylene glycol (97%), octaethylene glycol ($\geq 95\%$)].

Ethanol (200 proof, USP/ACS grade) and N,N-dimethylformamide dimethyl acetal (GC derivatization grade) were obtained from Sigma Aldrich (St. Louis, MO). Acetonitrile (HPLC grade), pyridine (certified ACS), and formic acid (99+%) were obtained from Fisher Scientific (Waltham, MA). Deionized water (18 Ω) was obtained from a Millipore filtration system fed by a service deionized water source. BSTFA reagent [99:1 N,O-bis(trimethylsilyl)trifluoroacetamide: trimethylchlorosilane] was obtained from Regis Technologies (Morton Grove, IL).

MCT Oil Reference Sample

Mixed octanoyl/decanoyl-triglycerides are common components of MCT oils, but we were unable to identify a source for these mixed triglyceride standards. An MCT oil product was purchased from a local grocery store in August 2019, and established for use as an MCT oil reference sample with respect to the mixed triglycerides. The product name was “Nature’s Way Organic MCT oil” with a label claim of “100% potency, medium chain triglycerides.” The reference MCT oil sample was analyzed by our laboratory using both GC-MS and direct analysis in real time high resolution accurate mass spectrometry (DART-HRMS) and consisted of four predominant triglycerides: trioctanoin, tridecanoin, a mixed dioctanoyl/monodecanoyl triglyceride, and a mixed monoctanoyl/didecanoyl triglyceride. Identification of the two mixed octanoyl/decanoyl triglycerides was confirmed both by the GC-MS EI spectra and the DART-HRMS accurate mass determinations and spectra. The HRMS observed vs. theoretical masses for the ammonium adducts of the mixed triglycerides were as follows: dioctanoyl/monodecanoyl triglyceride [$C_{29}H_{54}O_6 + NH_4^+$], m/z 516.4263 observed versus m/z 516.4259 theoretical, mass error +0.77 ppm, and monoctanoyl/didecanoyl triglyceride [$C_{31}H_{58}O_6 + NH_4^+$], m/z 544.4575 observed versus m/z 544.4572 theoretical, mass error +0.55 ppm. The positions of the octanoyl- and decanoyl-substituents on the triglyceride backbones for the two mixed triglycerides were not established.

Standards Preparation

Standards and MCT Oil Reference Sample for Parent Compounds Analysis

Standards for GC-MS analysis as the parent compounds were prepared from stock solutions at finished concentrations in acetonitrile in the range 50–300 $\mu\text{g/ml}$, and analyzed using GC-MS Protocol A. These include vitamin E acetate, trioctanoin, tridecanoin, dipropylene glycol dibenzoate, sucrose

acetate isobutyrate, squalane, triethyl citrate, and also included the MCT oil reference sample.

Pine Rosin Acid Methyl Esters Standards Analysis

A solution of methyl abietate was prepared at a finished concentration of 280 µg/ml in derivatizing agent by mixing 25 µl of an abietic acid stock solution, 300 µl pyridine, and 300 µl N,N-dimethylformamide dimethyl acetal in a GC vial, causing conversion of the abietic acid to methyl abietate. A standard mix (see also **Figure 5**) which contained methyl dihydroabietate, methyl dehydroabietate, and methyl abietate was prepared by mixing aliquots of the methyl abietate solution with an aliquot of a stock solution of a partially hydrogenated methyl ester rosin standard material. The partially hydrogenated methyl ester rosin standard material contained both methyl dihydroabietate and methyl dehydroabietate as primary components. The standard mix was analyzed using GC-MS Protocol A.

Standards for TMS Derivatives Analysis

For preparation of the standard TMS derivatives, an aliquot of a standard stock solution was transferred to a GC vial for derivatization in the same manner as the samples (see GC-MS Protocol B). Standards were prepared at finished concentrations in the derivatizing reagent as follows: PEG oligomers (200–350 µg/ml), pine rosin acids (10–50 µg/ml), and gum rosin standard material (230 µg/ml), and analyzed using GC-MS Protocol B.

Removal of Vaping Liquids From Vaping Devices

Used and unused vaping cartridges were received. For used cartridges, the remaining vaping liquid amounts ranged from residues to almost full cartridges. Full cartridges contained up to 1 g or 1 ml of vaping liquid. Prior to sampling for analysis, the vaping liquid contents were transferred from the cartridges or vaping devices to 2 ml autosampler glass vials (Water Corp.) for storage as follows. The receiving vial was placed in the bottom of a 15 ml conical bottom centrifuge tube (Falcon brand). A 5 ml plastic disposable pipet tip (Rainin RC-L5000) was placed into the receiving vial with the pipet tip end pointed downward. The vaping cartridge or device was disassembled, and the open end was placed into the top end of the pipet tip so as to allow flow of the vaping liquid out of the device through the pipet tip and into the receiving vial. The entire assembly was then placed in a centrifuge and spun until transfer of the vaping liquid into the receiving vial was complete (3–5 min). An IEC clinical centrifuge (dial setting 3) or Thermo Sorvall Legend RT centrifuge (2,000 rpm) was used. The amount of vaping liquid recovered from unused cartridges was in the range 0.7–1.0 g, and the amount of vaping liquid recovered from used cartridges was in the range 0.002–0.9 g. Based on visual observation of their flow behaviors, the vaping liquids we encountered typically consisted of medium to high viscosity liquids.

Preparation of Sample Concentrates for GC-MS Analysis

Due to the limited sample amounts for many of the vaping liquids, and the difficulty of sampling viscous liquids without considerable waste, an initial concentrated extract of the vaping liquid (referred to as the “sample concentrate”) was prepared in 95% ethanol. Sample concentrates were prepared in 1.0 ml or 4.0 ml glass sample vials, with vaping liquid sample weights typically in the range 10–100 mg. Solvent volumes were typically in the range 0.5–1.0 ml, resulting in finished sample concentrates generally in the range 20–100 mg vaping liquid per ml. After addition of solvent, the sample vial was capped and then briefly warmed on a hot plate as needed to speed dissolution of the vaping liquid (one or 2 min, ≤100°C). After dissolution of the vaping liquid, the sample vial was mixed on a vortexer to produce a homogeneous solution. Once prepared, aliquots of the sample concentrate were taken as described below for GC-MS qualitative analysis of the vaping liquid additives. When sufficient vaping liquid was available, duplicate preparations of sample concentrates were made, and analyzed as described.

GC-MS Sample Preparation and Analysis

Aliquots of the vaping liquid sample concentrates were taken as described below. Vitamin E acetate (VEA, alpha tocopherol acetate), medium chain triglycerides oil (MCT oil), pine rosin acid methyl esters, triethyl citrate, sucrose acetate isobutyrate (SAIB), dipropylene glycol dibenzoate isomers, and squalane were identified as the parent compounds (see GC-MS protocol A). PEG (polyethylene glycol) oligomers, and pine rosin acids were identified as the trimethylsilyl derivatives (see GC-MS protocol B). After initial identification of additive(s) in a given vaping liquid, confirmation of the additive compound(s) was achieved by concurrent analysis of the vaping liquid with the appropriate standard(s) or reference material(s).

GC-MS Protocol A

Vaping liquid sample concentrates were mixed on a vortex mixer prior to taking aliquots for subsequent analysis. A dilution of the vaping liquid sample concentrates in acetonitrile was made directly into a GC vial, with aliquot volumes generally in the range 25–100 µl and a finished volume of ca. 1.0 ml after addition of the acetonitrile. GC-MS analysis was conducted using an Agilent 7890B 70 eV EI GC-MS system with 5977B MS detector. The column was a 30 m Agilent 19091S-433HP 5 MS (5% phenyl) with 0.25 mm internal diameter and 0.25 µm film thickness. Injection volume was 1 µl splitless with an injection port temperature of 250°C. The carrier gas was helium with a flow rate of 0.8 ml/min (constant flow mode). Oven program was as follows: initial temperature 60°C with 0.5 min hold, first ramp 25°C/min to 220°C, hold for 10 min, second ramp 10°C/min to 300°C, with a final hold time of 15 min (run time 39.9 min). Transfer line temperature was 280°C. Solvent delay was 3.5 min, and MS acquisition used full scan mode with mass range 40–600 amu.

GC-MS Protocol B

A portion (generally in range 50–200 μl) of the underivatized preparation (see GC-MS protocol A) was transferred to a GC vial for derivatization. The solvent was evaporated under a stream of dry air on a Pierce Reactitherm block (nominal block temperature 70–80°C). 200 μl pyridine and 200 μl BSTFA reagent were added to the vial, the vial was capped, mixed, and incubated for 30 min (70–80°C). GC-MS analysis was conducted using an Agilent 7890B 70 eV EI GC-MS system with 5977B MS detector. The column was a 30 m Restek Rxi-35Sil MS (35% silphenylene) with 0.25 mm internal diameter and 0.25 μm film thickness. Injection volume was 1 μl splitless with an injection port temperature of 250°C. The carrier gas was helium with a flow rate of 0.8 ml/min (constant flow mode). Oven program was as follows: initial temperature 60°C with 0.5 min hold, first ramp 25°C/min to 220°C, hold for 10 min, second ramp 10°C/min to 300°C, with a final hold time of 15 min (run time 39.9 min). Transfer line temperature was 280°C. Solvent delay was 7.0 min, and MS acquisition used full scan mode with mass range 40–600 amu.

Processing of GC-MS Chromatograms for Figures

The Agilent GC-MS data files were exported as .CSV files, producing two columns of raw data corresponding to the retention times and mass spectral abundances. The .CSV files were then opened in Microsoft Excel (Excel 2016) and saved as Excel files (.xlsx). The Excel data files were used to produce Excel charts (xy scatter charts with smooth line) corresponding to the original chromatograms. For figures with more than one chromatogram, offsetting of the upper chromatogram in the display was accomplished by adding a constant arbitrary abundance value to the entire abundance data column.

DART-HRMS Identification of Mixed Octanoyl-Decanoyl Triglycerides in MCT Oil Reference Sample

Approximately 5 mg of the Nature's Way Organic MCT oil reference sample was dissolved in 1.0 ml of acetonitrile, then further diluted in acetonitrile (100 μl aliquot into final volume 1.0 ml). Acetonitrile was used as the method blank. The sample and blank preparations were sampled and analyzed using DIP-it tips (glass capillaries mounted in plastic). DART-HRMS analysis was conducted using a Thermo Scientific (Bremen, Germany) Q Exactive high resolution mass spectrometer (HRMS) equipped with a DART SVP (standardized voltage and pressure) ionization source, VAPUR interface, and linear rail, on which was mounted a module capable of holding 12 DIP-it tips, all from IonSense. Spectra were acquired with the ion source and mass analyzer operating in positive polarity. The DART SVP source was operated with helium gas at a temperature of 250°C, grid voltage of +300 V, and positioned directly in line with the VAPUR interface inlet at a distance of approximately 8.0 mm, or approximately 4.5 mm from the DIP-it tip during analysis. The mass spectrometer was operated with an inlet capillary temperature of 275°C and S-lens rf level of 80.0 (a.u.). Full scan mass spectra were acquired in profile mode with a nominal resolving power (FWHM at m/z 200) of 17,500 at a

nominal rate of 10 Hz, over the range m/z 100–1,000, with an automatic gain control (AGC) value of 10^6 and maximum injection time of 50 ms. MS/MS spectra of precursor ions (± 0.5 Da) were acquired using the same parameters and higher-energy collisional dissociation (HCD) at a collision energy of 30.0 eV. Analysis of the DIP-it tips was performed by moving the linear rail at a rate of 0.5 mm/s.

LC-HRMS Identification of DPG Dibenzoate Isomers

Approximately 5 mg of the vaping liquid was dissolved in 1.0 ml of acetonitrile, then further diluted in 50/50 acetonitrile/deionized water (10 μl aliquot into final volume 1.0 ml). The DPG dibenzoate reference standard stock solution was diluted in 50/50 acetonitrile/deionized water to yield a ca. 8 $\mu\text{g}/\text{ml}$ solution. The method blank comprised 10 μl of acetonitrile with 990 μl of 50/50 acetonitrile/deionized water. LC-HRMS analysis was conducted using a Thermo Scientific UltiMate 3000 liquid chromatograph (LC) coupled to a Q Exactive high resolution mass spectrometer (HRMS). Separation was carried out using a Zorbax Rapid Resolution HD Stablebond C18 column (1.8 μm , 2.1 mm ID $\times$ 150 mm length). Gradient elution was performed with initial conditions of 95% deionized water with 0.1% formic acid (A) and 5% acetonitrile with 0.1% formic acid (B), linearly ramped to 95% B in 25 min, then held for 15 min. Mobile phase flow rate was constant at 0.200 ml/min. Each injection was preceded by a 7.0-min equilibration at the initial conditions. The injection volume was 1.0 μl and the column was held at 40°C. The MS was equipped with a heated electrospray ionization (HESI) source operated with sheath gas flow rate of 35 units, auxiliary gas flow rate of 5 units, sweep gas flow rate of 2 units, and heater temperature of 75°C. The spray voltage was +3.25 kV and the probe was held at depth position "C" (the third farthest from the MS inlet of four marked positions). The inlet capillary temperature was 275°C. Full scan mass spectra were acquired in profile mode over the range m/z 120–1,200 with nominal resolving power of 140,000 using an automatic gain control target value of 10^6 . Data-dependent MS/MS spectra were collected in profile mode over a range determined by the precursor m/z value with nominal resolving power of 17,500 using an automatic gain control target value of 10^5 and higher-energy collisional dissociation (HCD) at a collision energy of 30.0 eV. LC-HRMS data were acquired and analyzed using Xcalibur software from Thermo Scientific.

LC-HRMS Identification of Sucrose Acetate Isobutyrate Compounds

Approximately 5 mg of the vaping liquid was dissolved in 1.0 ml of acetonitrile, then further diluted in 50/50 acetonitrile/deionized water (10 μl aliquot into final volume 1.0 ml). A stock solution of the SAIB standard material (ca. 4 mg/ml) was prepared in 95% ethanol, then further diluted in 50/50 acetonitrile/deionized water to yield a ca. 20 $\mu\text{g}/\text{ml}$ solution. The method blank comprised 10 μl of acetonitrile with 990 μl of 50/50 acetonitrile/deionized water. LC-HRMS analysis was

conducted using an Agilent 1260 Infinity liquid chromatograph (LC) coupled to a Thermo Scientific Exactive high resolution mass spectrometer (HRMS). Separation was carried out using a Phenomenex Luna phenyl-hexyl column (3.0 μ m, 2.0 mm ID $\times$ 150 mm length). Mobile phase flow rate was constant at 0.200 ml/min. Gradient elution was performed with initial conditions of 90% deionized water with 0.1% formic acid (A) and 10% acetonitrile with 0.1% formic acid (B), linearly ramped to 95% B in 20 min, then held for 10 min. Each injection was followed by a 7.0 min equilibration at the initial conditions. The injection volume was 1.0 μ l and the column was held at 40°C. The MS was equipped with a heated electrospray ionization (HESI) source operated with sheath gas flow rate of 35 units, auxiliary gas flow rate of 5 units, sweep gas flow rate of 2 units, and heater temperature of 125°C. The spray voltage was +3.25 kV and the probe was held at depth position "C" (the third farthest from the MS inlet of four marked positions). The inlet capillary temperature was 275°C. Full scan mass spectra were acquired in profile mode over the range m/z 100–2,000 with nominal resolving power of 100,000 using an automatic gain control target value of 500,000. All-ion fragmentation (AIF) spectra were collected in profile mode over the range m/z 60–1,200 with nominal resolving power of 25,000 using an automatic gain control target value of 1,000,000 and higher-energy collisional dissociation (HCD) at a collision energy of 40.0 eV. LC-HRMS data were acquired and analyzed using Xcalibur software from Thermo Scientific.

RESULTS AND DISCUSSION

It is important to emphasize that this work is not intended to be a vaping study, nor to identify components of aerosols generated during the vaping process. Rather, as analysis was conducted on the neat vaping liquids, this work is intended to provide information on the vaping liquid formulations. Obviously, for a complete understanding and assessment of the hazards associated with vaping products, it is important to determine the compositions of the neat vaping liquids, as well as conduct studies of the aerosols generated during vaping. The following paragraph provides some perspective on the volatility range which is covered by the current GC-MS methods for the identification of additives in the neat vaping liquids.

Based on visual observation of their flow characteristics, the majority of the vaping liquid additives which were identified consisted of low to medium viscosity liquids under ambient conditions. GC-MS is ideal for the analysis of these types of additives because they are sufficiently volatile for analysis as the parent compounds, or suitable for analysis as the trimethylsilyl derivatives. The oven temperature program used in GC-MS protocol A provided good retention and resolution for additives analyzed as the parent compounds (VEA, MCT oil, pine rosin acid methyl esters, triethyl citrate, sucrose acetate isobutyrate dipropylene glycol dibenzoate isomers, squalane), and also provided resolution from the parent cannabinoids. The oven temperature program used in GC-MS protocol B corresponds to the conditions used in our validated method

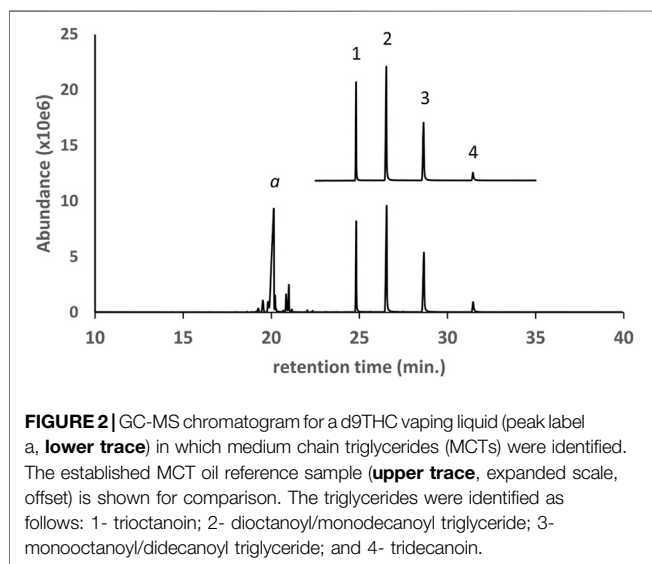
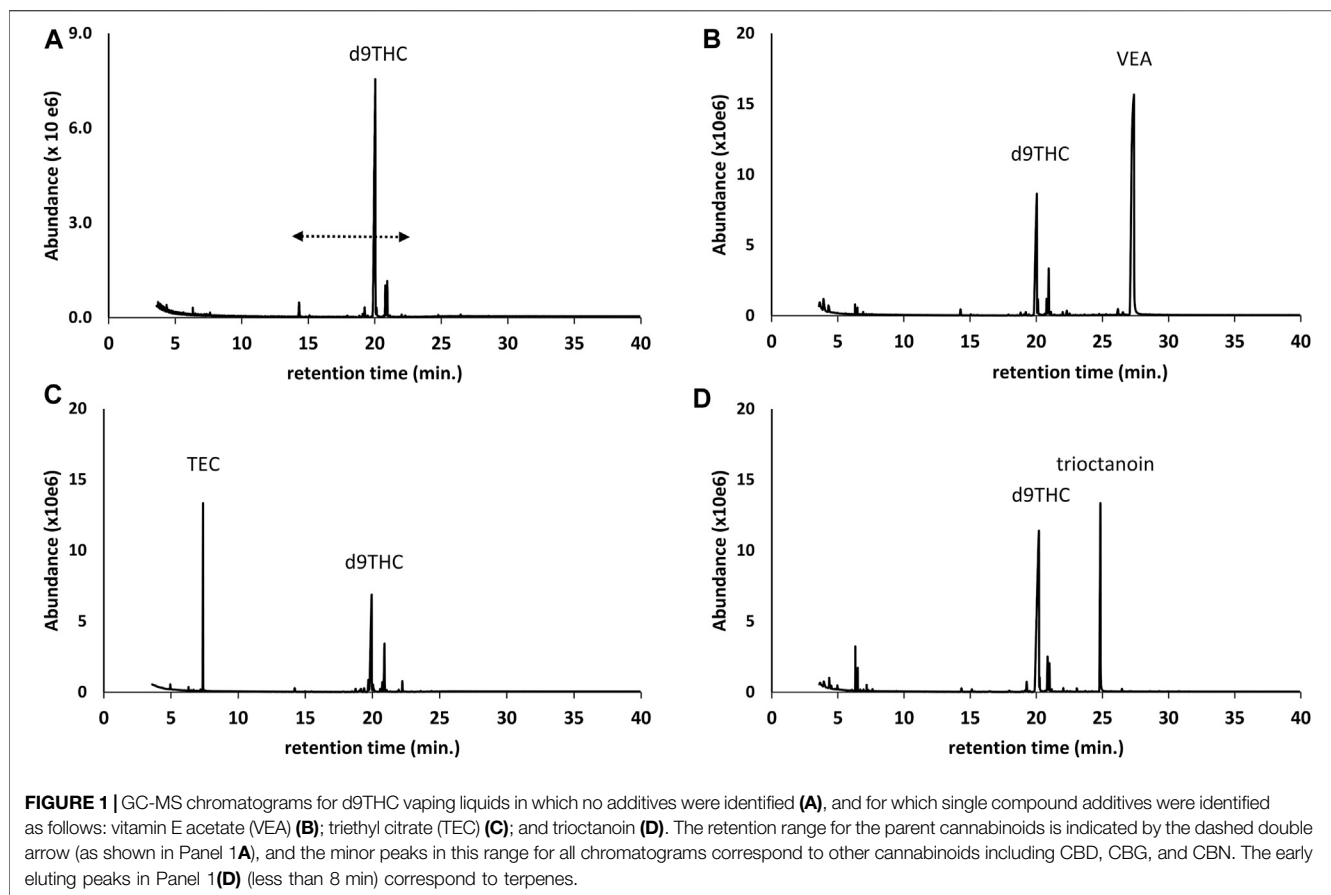
for analysis of the cannabinoids as the trimethylsilyl derivatives (Ciolino et al., 2018). GC-MS protocol B provided good retention and resolution of the additives analyzed as the trimethylsilyl derivatives (PEG oligomers and pine rosin acids), as well as resolution from the cannabinoid trimethylsilyl derivatives. In our current work with GC-MS protocol A, we established that the less volatile terpenes such as d-limonene, linalool, α -terpineol, caryophyllene, and α -bisabolol elute after the solvent delay. We also established that propylene glycol and glycerin, which are common in nicotine vaping liquids, could be detected as either the parent compounds (GC-MS protocol A) or trimethylsilyl derivatives (GC-MS protocol B, with adjustment of the solvent delay to 3.0 min). While we generally noted if any terpenes were detected in our casework, terpenes are not the focus of this work. We only encountered glycerin or propylene glycol in a few cases, where it appeared that a vaping device had been used for vaping of both nicotine and cannabis vaping liquids.

GC-MS identification of the vaping liquid additives was based on retention time and mass spectral comparison to standard materials, as well as comparison to an established reference sample for the MCT oils. Only minor shifts in GC-MS retention times were observed for the additive compounds throughout the study period (ca. 12 months). However, GC-MS confirmation of the additive compounds was achieved by concurrent analysis of the vaping liquid with the appropriate standards or reference materials, allowing for same day comparison of sample and standard retention times. All sample vs. standard or reference material retention time correspondence met our specified requirements of less than 2.0% relative difference.

The majority of the GC mass spectra are not presented here as they are well known and available in commercial libraries such as the Wiley-NIST 2010/2014 2011/2017 editions, and Designer Drug 2014 and 2017 editions. Confirmation of several more complex additives using LC-HRMS and/or DART-HRMS was also conducted, with same day comparison of sample and standard retention times for the LC-HRMS work. Both GC-MS and LC-HRMS spectra are presented for the multicomponent additive sucrose acetate isobutyrate (SAIB), as spectra for the SAIB components were not found in the commercial libraries. In the sub sections which follow, we provide additional references addressing the commercial use for each of the identified additives in Cannabis vaping liquids.

Single Component Additives (VEA, Triethyl Citrate, Trioctanoin, Squalane)

Several of the additives we encountered were single component additives, and include vitamin E acetate (alpha-tocopherol acetate), triethyl citrate, trioctanoin, or squalane. **Figure 1B** through **Figure 1D** show chromatograms for underivatized preparations of d9THC vaping liquids in which vitamin E acetate, triethyl citrate, or trioctanoin were identified, respectively. For comparison, **Figure 1A** shows a chromatogram for a vaping liquid in which no additives were identified and also shows the retention range for the cannabinoids. Identification of these single component



additives was straight forward based on retention time and mass spectral correspondence to reference standards. Use of the parent compound alpha-tocopherol (not its acetate ester) as a viscosity modifier, to inhibit oxidation, and to increase bioavailability in commercial Cannabis vaping liquids has been reported (Finley

et al., 2016; Finley and McKee, 2019). The use of triethyl citrate as a lipid solubilizer (Goldman et al., 2015), and the use of triotcanoin as a carrier liquid (Zumpano, 2019), in commercial Cannabis vaping liquids have also been reported.

Multi-Component Additives

Several complex or multi-component additives were encountered in the vaping liquids. These include MCT oils, PEG oligomers, pine rosin acids, pine rosin derived methyl esters, dipropylene glycol dibenzoate (DPG dibenzoate) isomers, and sucrose acetate isobutyrate (SAIB) compounds. Unlike the single component additives, identification of these more complex additives presented more challenges including some difficulties in obtaining standards or reference materials, and the GC-MS spectra were either not included in commercial libraries or overall less definitive for identification purposes. Additional analysis was conducted using LC-HRMS analysis for the DPG dibenzoate isomers and the SAIB compounds. In the discussion which follows, a brief description is given for each additive, including prior reports of their use in Cannabis vaping liquids.

MCT Oils

MCT oils are generally derived from natural oils which have high native contents of medium chain triglycerides, such as coconut oil. The multi-step manufacturing process involves hydrolysis of

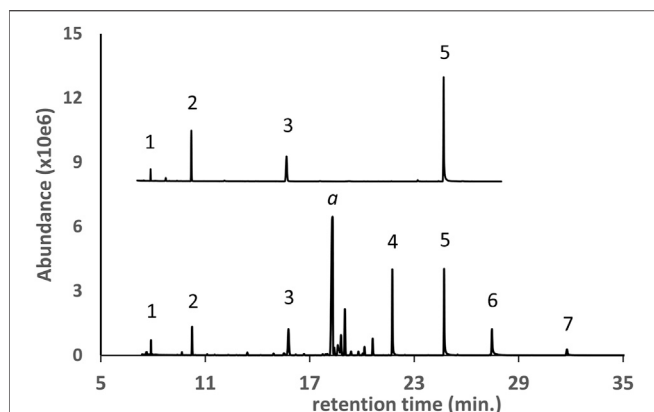


FIGURE 3 | GC-MS chromatogram for a d9THC vaping liquid (peak label a, **lower trace**) in which a series of polyethylene glycol oligomers (PEG oligomers, peak labels 1 through 7) were identified as the diTMS derivatives. PEG oligomer standard mix (**upper trace**, condensed scale, offset) as follows: 1- tetraethylene glycol; 2- pentaethylene glycol; 3- hexaethylene glycol; 5- octaethylene glycol.

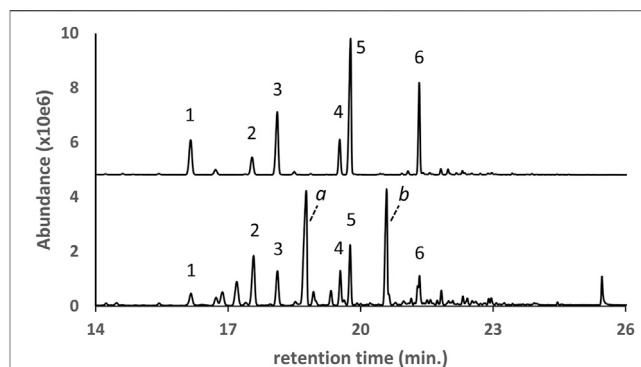


FIGURE 4 | GC-MS chromatogram for a vaping liquid (**lower trace**) in which a series of pine rosin acids (peak labels 1 through 6) were identified as the monoTMS derivatives. A gum rosin standard material (**upper trace**, condensed scale, offset) is shown for comparison. Specific pine rosin acids identified as follows: 2- isopimaric acid; 4- dehydroabietic acid; 5- abietic acid. The vaping liquid contained predominant amounts of d9THC and CBN (peak labels a and b), as well as MCT oil components (not shown).

the glycerides (mono, di, and tri) from the natural oil to produce the fatty acids, then taking the desired medium chain cut of fatty acids, typically in the C6–C12 range. The fatty acids are then reesterified with glycerin, generally to a full extent, producing the triglycerides which make up the MCT oil. The finished MCT oils are low viscosity liquids. The use of medium chain triglyceride oils (MCT oils) as carrier liquids in commercial Cannabis vaping liquids has been reported (Zumpano, 2019).

Figure 2 shows the GC-MS chromatogram for a d9THC vaping liquid (**lower trace**) in which medium chain triglycerides (MCTs) were identified. The locally purchased MCT oil reference sample (**upper trace**, expanded scale, offset) is shown for comparison. Four triglycerides (peak labels 1–4, respectively) were identified as trioctanoin, an dioctanoyl/monodecanoyl triglyceride, a monooctanoyl/didecanoyl triglyceride, and tridecanoin. The trioctanoin and tridecanoin were confirmed with reference standards, and the two mixed octanoyl/decanoyl-triglycerides were confirmed by comparison with the established MCT oil reference sample. For MCT oils in vaping liquids, this example represents the most frequent pattern of triglycerides we encountered with higher levels of the first three triglycerides and lower levels of tridecanoin. Other minor triglycerides encountered in some of the vaping liquids included hexanoin, dodecanoin, and other mixed chain length triglycerides. Some mono- and diglycerides were occasionally seen.

PEG Oligomers

Polyethylene glycols (PEGs) are synthetic oligomers/polymers of ethylene oxide which are manufactured over a wide range of chain length/molecular weights. A given PEG is typically composed of a distribution of chain lengths/molecular weights around an average value, such as PEG 400 with an average molecular weight of 400. PEGs such as PEG 200 and 400 are liquid under ambient conditions, while higher molecular weight

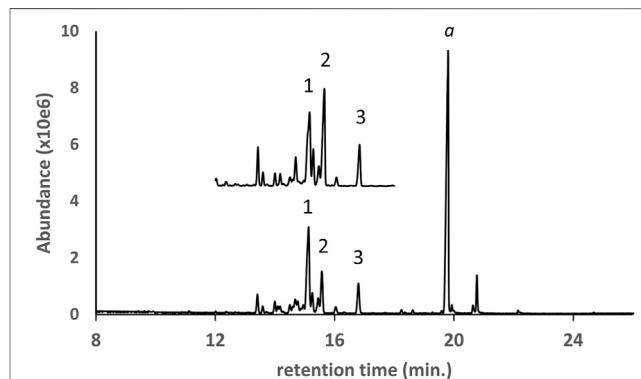


FIGURE 5 | GC-MS chromatogram for a d9THC vaping liquid (peak label a, **lower trace**) in which a series of pine rosin acid methyl esters were identified, compared to a standard mix (**upper trace**, offset, condensed scale) of a partially hydrogenated methyl ester rosinate reference material with a methyl abietate standard. The predominant esters were identified as methyl dihydroabietate, methyl dehydroabietate, and methyl abietate (peak labels 1–3, respectively).

PEGs with average molecular weights above 600 are typically solids. The use of PEG 300 and/or 400 as emulsifiers or cosolvents in commercial Cannabis eliquid or vaping formulations has been described (Llamas, 2015; Eck and Pelloni, 2020).

The GC-MS chromatogram for a vaping liquid in which polyethylene glycol oligomers (PEG oligomers, peak labels 1–7) were identified is shown in **Figure 3** (**lower trace**). While smaller PEGs may be volatile enough for analysis without derivatization, conversion to the diTMS derivatives prior to analysis provides significantly better peak shape and greatly increased signal response. A standard mix of the diTMS derivatives of tetraethylene glycol, pentaethylene glycol, hexaethylene glycol, and octaethylene glycol (**Figure 3**, **upper**

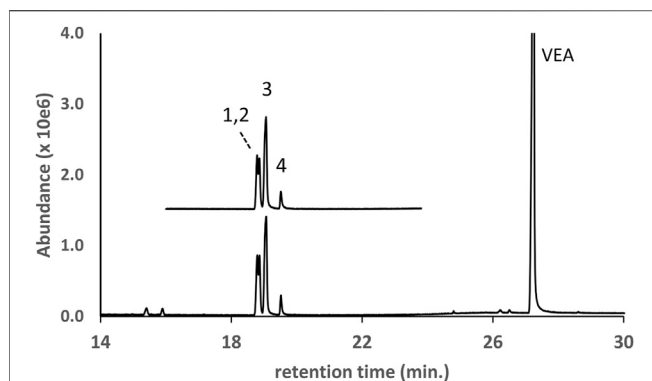


FIGURE 6 | GC-MS chromatogram for a vaping liquid (lower trace) in which multiple DPG dibenzoate isomers (peak labels 1–4) were identified in addition to VEA (VEA peak offscale). The DPG dibenzoate standard chromatogram (upper trace, offset) shows a similar distribution of the four isomers. The vaping liquid contained only a trace of d9THC (peak not visible on current scale).

trace, peak labels 1, 2, 3, and 5 respectively) is shown for comparison. The additional PEG oligomers (peak labels 4, 6, and 7) are presumed to be the hepta-, nona-, and decaethylene glycols for which standards were not available. While the GC-MS spectra of the PEG oligomer diTMS derivatives are very similar allowing assignment as PEGs, the spectra do not exhibit molecular or high mass ions which would allow an assignment of molecular weight. Our laboratory was able to confirm the PEG oligomer exact masses and identities using DART-HRMS (data not presented in this work).

Pine Gum Rosin Acids

Pine gum rosin (colophony) is derived from pine tree trunks and is a solid, resinous material which is largely nonvolatile and chiefly composed of a series of related diterpenoid acids such as abietic acid (Organic Materials Review Institute, 2014). The GC-MS chromatogram for a d9THC vaping liquid in which pine rosin acids were identified as the monoTMS derivatives is shown in **Figure 4** (lower trace). A derivatized preparation of a gum rosin standard material (**Figure 4**, upper trace), is shown for comparison. A series of six rosin acids were found in common between the vaping liquid and the gum rosin standard material including abietic acid, dehydroabietic acid and isopimaric acid (peak labels 5, 4, and 2, respectively). While standards were not available to confirm the identity of the other three rosin acids (peak labels 1, 3, and 6), the GC-MS retention times and mass spectra were consistent between the vaping liquid and the gum rosin standard material. In addition to the pine rosin material, the vaping liquid also contained MCT oil components. Note that the combination of pine rosin and MCT oil components was reported in the prior analysis of a commercial “adulterant or additive formulation” (Meehan-Atrash and Strongin, 2020).

Partially Hydrogenated Pine Rosin Acid Methyl Esters

Processing of pine rosin for industrial uses may include partial or full hydrogenation followed by esterification (Eastman Chemical Company, 2017). Natural acids found in pine rosin include

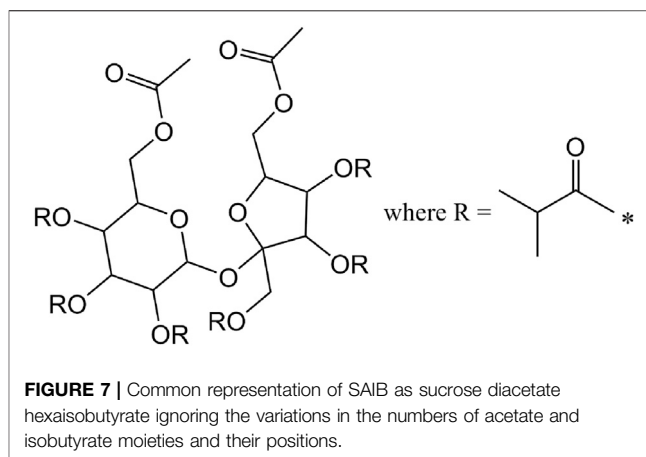


FIGURE 7 | Common representation of SAIB as sucrose diacetate hexaisobutyrate ignoring the variations in the numbers of acetate and isobutyrate moieties and their positions.

abietic acid and dehydroabietic acid, for which the chemical structures contain two or three double bonds, respectively. Abietic acid may be partially hydrogenated to form dihydroabietic acid, which contains only one double bond (Eastman Chemical Company, 2017). Esterification with methanol produces the methyl esters. The finished “methyl ester of hydrogenated rosin” materials are viscous liquids. The use of “methyl ester of partially hydrogenated rosin” as an additive in commercial Cannabis vaping compositions has been described (Cameron et al., 2019).

The GC-MS chromatogram for a d9THC vaping liquid in which both natural and partially hydrogenated pine rosin acid methyl esters were identified is shown in **Figure 5** (lower trace). The most abundant pine rosin derived methyl esters in the vaping liquid were methyl dihydroabietate, methyl dehydroabietate, and methyl abietate (peak labels 1–3, respectively). A standard mixture which contained these same three rosin esters was analyzed for comparison (upper trace). The standard mixture was prepared from a partially hydrogenated methyl ester rosin reference material and a methyl abietate standard (see *Materials and Methods* section). Several additional pine rosin methyl esters in the sample chromatogram were not confirmed with standards but the retention times and spectra were consistent with the standard mixture.

Dipropylene Glycol Dibenzoate Isomers

Dipropylene glycol dibenzoate (DPG dibenzoate) is a viscous liquid which may be derived from the esterification of dipropylene glycol with benzoic acid (Zheng et al., 2016), or the transesterification of methylbenzoate with dipropylene glycol (Hulsmann and Renckhoff, 1978). The dipropylene glycol starting material typically comprises multiple isomers (Sexton and Britton, 1953; Hulsmann and Renckhoff, 1978), which include the diprimary alcohol, the dissecondary alcohol, and the primary-secondary alcohol. As such, the finished dibenzoate ester is also a mixture of isomers. While DPG dibenzoate is used as an industrial plasticizer (Zheng et al., 2016), our literature search found no references for the use of DPG dibenzoate in vaping liquids.

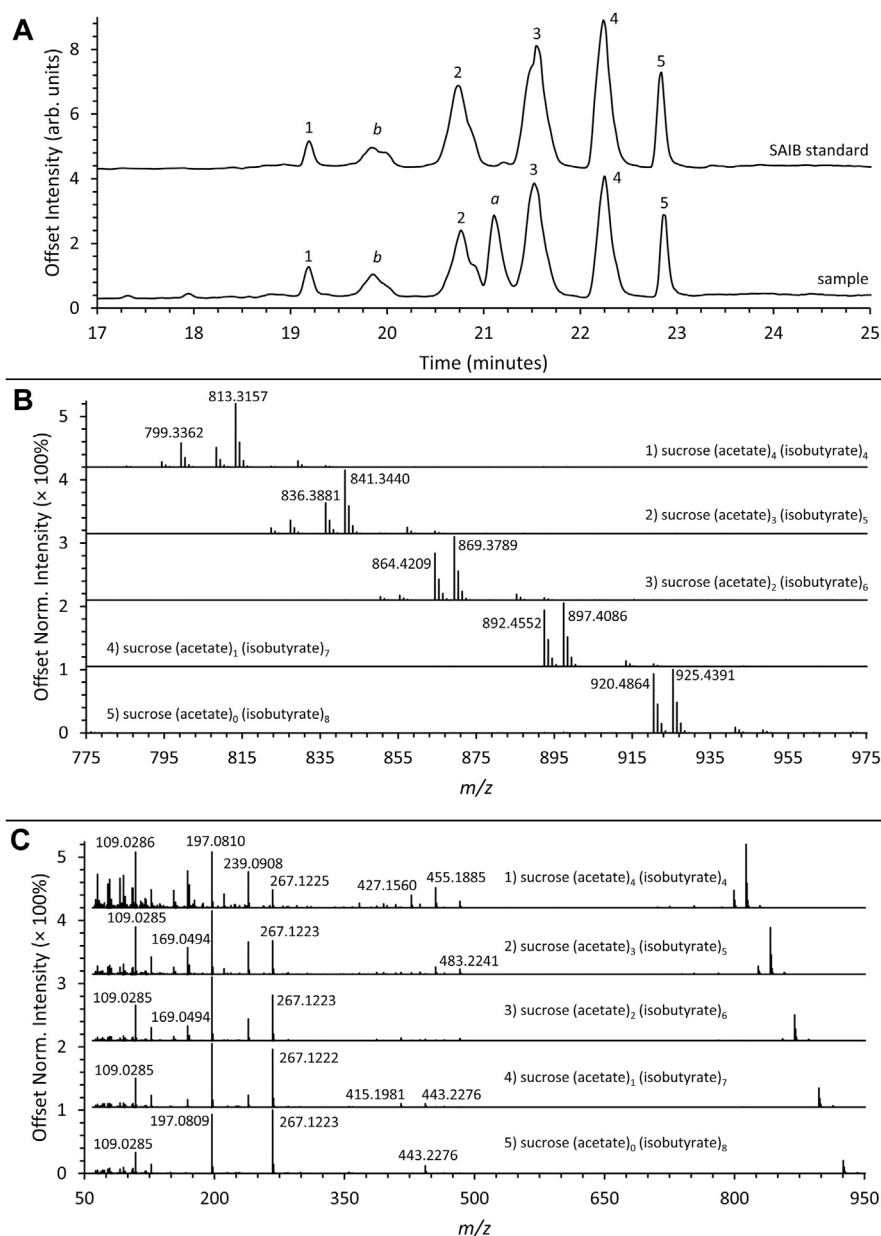


FIGURE 8 | (A) LC-HRMS total ion chromatograms of the SAIB reference standard (top) and sample (bottom) showing a series of five SAIB peaks (peak labels 1–5). Peak a corresponds to d9THC and peak b corresponds to a background impurity also present in blanks. **(B)** Mass spectra corresponding to the five SAIB chromatographic peaks. **(C)** AIF spectra corresponding to the five SAIB chromatographic peaks.

The GC-MS chromatogram for a vaping liquid in which four DPG dibenzoate isomers were identified as the parent compounds is shown in **Figure 6** (lower trace, peak labels 1–4). The DPG dibenzoate standard chromatogram (upper trace, offset) is shown for comparison, and shows a similar distribution of isomers. The vaping liquid also contained a high content of VEA with only a trace of d9THC (less than 0.1% w/w). The presence of multiple DPG dibenzoate isomers in this vaping liquid was confirmed using LC-HRMS, with exact mass confirmation of the molecular formula $C_{20}H_{22}O_5$ for the

DPG dibenzoate isomers (calculated mass error of 1.17 ppm for the H^+ adduct of the predominant isomer, m/z 343.1544 versus theoretical m/z value of 343.1540).

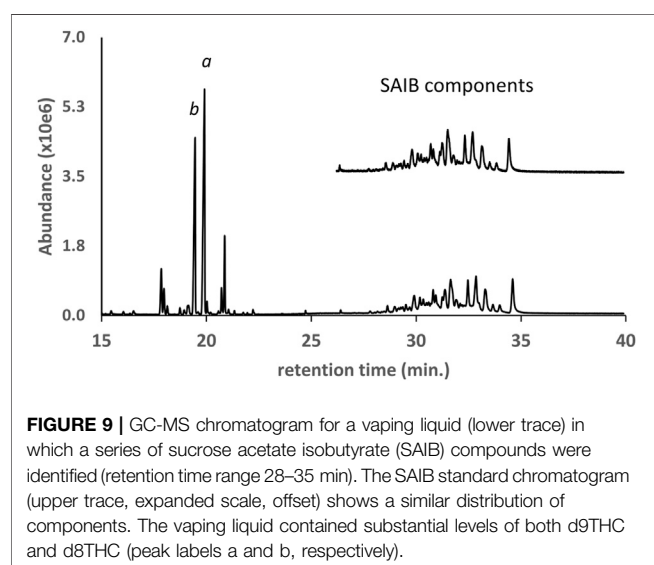
Sucrose Acetate Isobutyrate Compounds

Sucrose acetate isobutyrate (SAIB, sucrose diacetate hexaisobutyrate) is a viscous liquid which is generally derived from the esterification of sucrose with acetic and isobutyric anhydrides (Goins and Davis, 1963). Because sucrose has a total of eight reactive hydroxy sites and the synthesis is

TABLE 1 | LC-HRMS exact mass groupings of SAIB compounds and corresponding GC-MS high mass ion fragments.

SAIB compounds exact mass (molecular formula)	SAIB Na ⁺ adduct LC-HRMS exact mass theoretical/measured (Δ ppm)	# Acetates/molecule	# Isobutyrate/molecule	GC-MS SAIB compounds unit mass	# Acetates/fragment	# Isobutyrate/fragment	GC-MS high mass fragment unit mass
790.3259 (C ₃₆ H ₅₄ O ₁₉)	813.3157/813.3157 (0.0 ppm)	4	4	790	4	0	331
					3	1	359
					2	2	387
					1	3	415
					0	4	443
818.3572 (C ₃₈ H ₅₈ O ₁₉)	841.3470/841.3440 (-3.6 ppm)	3	5	818	3	1	359
					2	2	387
					1	3	415
					0	4	443
846.3885 (C ₄₀ H ₆₂ O ₁₉)	869.3783/869.3789 (0.7 ppm)	2	6	846	2	2	387
					1	3	415
					0	4	443
874.4198 (C ₄₂ H ₆₆ O ₁₉)	897.4096/897.4086 (-1.1 ppm)	1	7	874	1	3	415
					0	4	443
902.4511 (C ₄₄ H ₇₀ O ₁₉)	925.4409/925.4391 (-1.9 ppm)	0	8	902	0	4	443

Molecular ions for the SAIB compounds are not observed in EI GC-MS analysis, but the high mass ion fragments corresponding to the various tetraester distributions on the glucose and fructose fragments are observed. See text for discussion.



typically conducted with a molar ratio of 2:6 acetic:isobutyric anhydrides, SAIB is usually depicted as the diacetate hexaisobutyrate (**Figure 7**) with a corresponding unit mass of 846 g/mol. However, as our data will show, the esterification actually produces a highly complex mixture of SAIB compounds with varying numbers of acetate and isobutyrate ester substituents per sucrose molecule, as well as variations in the positions of the ester moieties. The use of SAIB as a weighting agent in commercial Cannabis vaping liquids (Goldman et al., 2015) has been reported.

A vaping liquid containing a series of SAIB compounds was analyzed using both LC-HRMS and GC-MS. The LC-HRMS total ion chromatogram for the vaping liquid is given in **Figure 8A**

(lower trace) with comparison to the SAIB standard material (upper trace). Similar profiles for a series of five SAIB peaks (retention times ~19.9, 20.7, 21.5, 22.2, and 22.8 min) were observed for both the vaping liquid and standard material. The HRMS spectra for the five peaks (**Figure 8B**) showed the presence of both Na⁺ (M + 23) and NH₄⁺ (M + 18) adducts, with higher intensities observed for the Na⁺ adducts. The Na⁺ adduct exact masses for the five peaks correspond to SAIB compounds containing varying number of acetate and isobutyrate substituents as listed in **Table 1**, first four columns. It is assumed that each of the first four SAIB peaks include coeluting SAIB compounds with the same number of acetates and butyrates per sucrose molecule, but in differing positions. The molecular weight for the fifth and last peak (902 g/mol) corresponds to the octaisobutyrate compound.

The GC-MS chromatogram for the same vaping liquid is given in **Figure 9** and shows a series of more than 15 closely spaced SAIB components (lower trace, retention range 28–35 min). Again, a very similar distribution of the SAIB components was observed in comparison with the SAIB standard material (**Figure 9**, upper trace). GC-MS mass spectra for three representative SAIB components (retention times 28.6, 31.6, and 34.4 min) are given in **Figure 10**. In the GC-MS spectra for sucrose esters including SAIB (Severson et al., 1985; Uematsu et al., 2001), molecular ions are not observed under EI conditions. Rather, the high mass ions in the EI spectra represent the esterified glucose or fructose fragments minus the bridge oxygen, which is transferred to the neutral fragments (Severson et al., 1985). Cleavage at the oxygen bridge may occur on either side such that both ionized glucose and fructose ion fragments are formed. In the case of SAIB, these high mass ions correspond to glucose and/or fructose tetraesters with varying numbers of acetate and isobutyrate moieties.

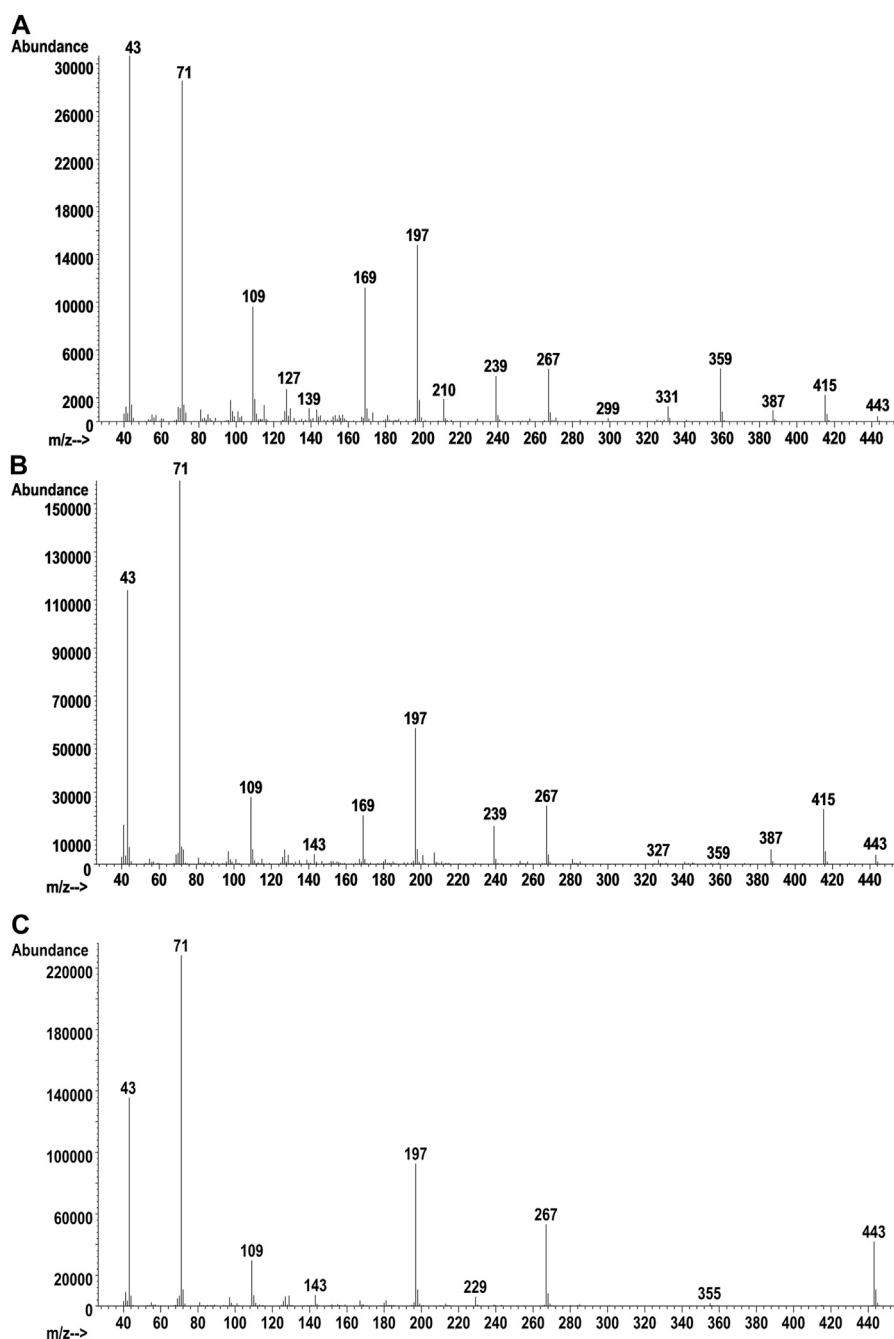
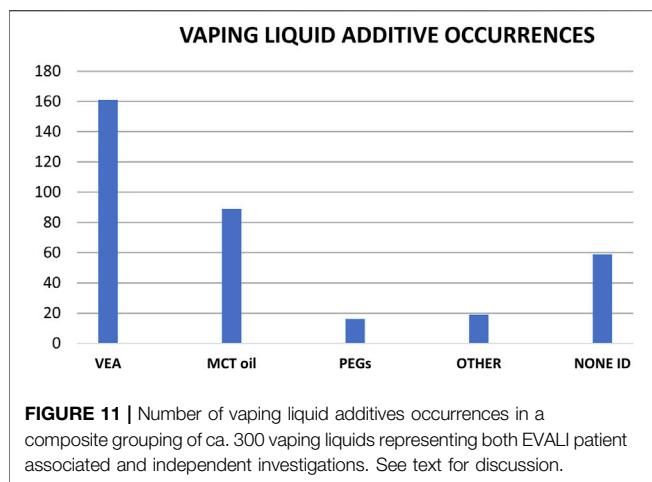


FIGURE 10 | Vaping liquid SAIB component GC-MS spectra for earliest eluting peak (A, 28.6 min), mid eluting peak (B, 31.6 min), and last eluting peak (C, 34.4 min).

Table 1 also provides the GC-MS high mass ion fragment unit masses which may be observed for various SAIB positional isomers (see last four columns) within each of the mass groupings from the LC-HRMS analysis. This listing only addresses positional isomers with respect to the numbers of acetates or isobutyrate on a glucose or fructose fragment, but does not address isomeric positions on the monosaccharides. Glucose vs. fructose fragments are also not differentiated in the

analysis. Based on molecular weight considerations, the early eluting SAIB compounds are expected to have the least number, and the late eluting compounds the most number, of isobutyrate substituents. The mass spectra are consistent with this expectation, with the earliest eluting peak showing all five of the high mass fragments listed in **Table 1** for SAIB compounds with four acetates and four isobutyrate (m/z 331, 359, 387, 415, 443), and the latest eluting peak showing



only the high mass fragment for the octaisobutyrate compound (m/z 443).

Figure 8C shows the all-ion fragmentation (AIF) spectra for the five SAIB peaks from the LC-HRMS work. The lower mass ions at m/z 267, 239, 197, 169, and 109 in both the AIF (**Figure 8C**) and the GC-MS spectra (**Figure 10**) have been described (Uematsu et al., 2001). The remaining acetate or isobutyrate moieties on the fragments are as follows: two remaining isobutyrate (m/z 267), one acetate and one isobutyrate (m/z 239), one isobutyrate (m/z 197), one acetate (m/z 169), and no remaining moieties (m/z 109).

Based on visual examination of the GC-MS chromatograms, it is apparent that the additives identified in this work were frequently major constituents in the vaping fluids. Peak heights for single component additives (**Figures 1B–D**), or cumulative peak heights for multicomponent additives (**Figures 2–6**, and **9**), may be comparable to or exceed the peak height of the vaping liquid active constituent, such as Δ^9 -tetrahydrocannabinol. In other cases, only minor amounts of additives were found. Formal quantitation of the additives in the vaping liquids was only conducted for VEA and the MCT oils, and was conducted using GC-FID methods which were developed and validated within our laboratory (Lanzarotta, 2020). VEA levels ranging from 4–88 %w/w were determined in a grouping of 127 vaping liquids, with a grand average of 50% w/w (Lanzarotta, 2020). MCT oil levels ranging from 0.2 to 66 % w/w were determined from a grouping of 55 vaping liquids, with a grand average of 15% w/w (Lanzarotta, 2020). While there is significant overlap among the ca. 300 vaping liquids reported in this study and the vaping liquids subjected to quantitative analysis for VEA and the MCT oils, the quantitative data summaries were conducted independently by the analysts conducting the quantitative work.

SUMMARY OF FINDINGS

Approximately half of the ca. 300 vaping liquids from this study were associated with EVALI patients, and the other half were obtained in the United States through independent investigations

in the time frame after the EVALI outbreak (late 2019 and early 2020). The additive findings between both groups of vaping liquids were generally consistent, and are summarized in **Figure 11** for the various additives. Note that **Figure 11** shows the composite findings for the entire group of ca. 300 vaping liquids, representing vaping liquids from both EVALI patient associated and independent investigations. No additives were identified in approximately 20% of the vaping liquids, and VEA was found in 50–60% of the vaping liquids. On average, MCT oils were found in 30% of the vaping liquids, and PEGs in about 5% of the liquids.

We found multiple additives in about 60 of the vaping liquids, with the most frequent combination being VEA and MCT oil. Other combinations included MCT with SAIB, MCT with PEG, MCT with pine rosin acids, and VEA with DPG dibenzoate. A few vaping liquids contained the three additives VEA, MCT oil, and SAIB. In addition to the additives in this report, we found terpenes in about 85% of the vaping liquids. We also saw flavoring components in some vaping liquids, and indications of various vegetable oils. Given the explosion of technological development in the commercial Cannabis industry, analytical and forensic laboratories are likely to encounter even more additive types in products such as vaping liquids. GC-MS analysis is an ideal approach for identification of a wide variety of vaping liquid additives, and LC-HRMS with exact mass determination provides definitive confirmation of identity.

This summary of findings is intended to provide information which is useful to analytical, forensic, or other testing laboratories who may conduct testing of cannabis based vaping liquids. The data sets presented here, and the summary of findings, are not intended to represent an epidemiological evaluation linked with the EVALI outbreak.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data may be considered sensitive or require a special request for public disclosure. Requests to access the datasets should be directed to laura.ciolino@fda.hhs.gov.

AUTHOR CONTRIBUTIONS

LC, TF, and TR performed conception and design of the study. LC, TR, JB, AT, and AM conducted GC-MS research and analysis of the subject vaping liquids and cutting agents. TF conducted LC-HRMS and DART-MS research and analysis of the subject vaping liquids and cutting agents. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382, 697–705. doi:10.1056/NEJMoa1916433
- Cameron, J. D., Becker, D., and Fein, F. Inventors Lunatech LLC, assignee. (2019). *Water-based Vaporizable Liquids, Methods, and Systems for Vaporizing Same*. US patent application 2019 0104762 A1. 2019 April 11.
- Ciolino, L. A., Ranieri, T. L., Brueggemeyer, J. L., Taylor, A. M., and Mohrhaus, A. S. (2021). EVALI Vaping Liquids Part 1: GC-MS Cannabinoids Profiles and Identification of Unnatural THC Isomers. *Front. Chem.* doi:10.3389/fchem.2021.746479
- Ciolino, L. A., Ranieri, T. L., and Taylor, A. M. (2018). Commercial Cannabis Consumer Products Part 1: GC-MS Qualitative Analysis of Cannabis Cannabinoids. *Forensic Sci. Int.* 289, 429–437. doi:10.1016/j.forsciint.2018.05.032
- Duffy, B., Li, L., Lu, S., Durocher, L., Dittmar, M., Delaney-Baldwin, E., et al. (2020). Analysis of Cannabinoid-Containing Fluids in Illicit Vaping Cartridges Recovered from Pulmonary Injury Patients: Identification of Vitamin E Acetate as a Major Diluent. *Toxics* 8 (1), 8–19. doi:10.3390/toxics8010008
- Eastman Chemical Company, (2017). *Eastman Rosin Products for Adhesives and Sealants*. Kingsport, TN, USA: Eastman publication WA-87.
- Eck, C. R., and Pelloni, C. L. Inventors E. P. Pharma LLC, assignee. (2020). *Cannabinoid Formulations for Inhalation*. US patent 10,588,870. 2020 March 17.
- Finley, C., and McKee, L. Inventors Finley C., McKee L., assignees. (2019). *Cannabinoid Composition and Products Including Alpha-Tocopherol*. US patent 10,238,745 B2. 2019 Mar 26.
- Finley, C., McKee, L., Poole Bestwick, H., and Roethle, P. Inventor Constance Therapeutics Inc, assignee. (2016). *Methods for Preparation of Cannabis Oil Extracts and Compositions*. WO patent application 2016 123475 A1. 2016 Aug 4.
- Goins, R. H., and Davis, H. E. Inventors Eastman Kodak Company, assignee. (1963). *Process for Manufacturing Sugar Esters*. US patent 3,096,324A (expired). 1963 July 2.
- Goldman, M. L., Marthaler, L. K., and Bjorncrantz, W. Inventors Verso PV LLC, assignee. (2015). *Personal Vaporizer Liquid for Emulsifying Oil-Soluble Compounds and Resins*. WO patent application 2015 042589 A1. 2015 March 26.
- Green, W., Alderete, S., and Freyre, J. Inventors Elevate Technologies LLC, assignee. (2020). *Terpene-based Compositions, Methods of Preparations and Uses Thereof*. US patent 10,675,264. 2020 June 9.
- Hulsmann, H. L., and Renckhoff, G. Inventors Dynamit Nobel Aktiengesellschaft, Assignee. (1978) *Process for the Manufacture of Light Colored Dipropylene glycol Dibenzate*. US patent 4,112,240. 1978 Sept 5.
- Kotra, L. P., Lewis, M. M., Wasilewski, E., and Grover, H. Inventors CannScience Innovations Inc, assignee. (2020). *Decarboxylated Cannabis Resins, Uses Thereof and Methods of Making Same*. US patent 10,537,592. 2020 Jan 21.
- Lanzarotta, A. (2020). The FDA Forensic Chemistry Center's Role during the 2019 Vaping Crisis. Invited Special Lecture at the Eastern Analytical Symposium Annual Conference (Virtual). November 18, 2020.
- Llamas, M. R. Inventor HDDC Holdings LLC, assignee. (2015). *Cannabidiol Liquid Composition for Smoking*. US patent application 2015 0181924 A1. 2015 July 2.
- Meehan-Atrash, J., and Strongin, R. M. (2020). Pine Rosin Identified as a Toxic Cannabis Extract Adulterant. *Forensic Sci. Int.* 312, 110301. doi:10.1016/j.forsciint.2020.110301
- Organic Materials Review Institute (OMRI). (2014). Wood Rosin. Technical Evaluation Report Compiled by OMRI for the USDA National Organic Program. Eugene, OR; January 22.
- Severson, R. F., Arrendale, R. F., Chortyk, O. T., Green, C. R., Thome, F. A., Stewart, J. L., et al. (1985). Isolation and Characterization of the Sucrose Esters of the Cuticular Waxes of green Tobacco Leaf. *J. Agric. Food Chem.* 33, 870–875. doi:10.1021/jf00065a026
- Sexton, A. R., and Britton, E. C. (1953). Synthesis and Identification of Dipropylene Glycol Isomers. *J. Am. Chem. Soc.* 75 (17), 4357–4358. doi:10.1021/ja01113a525
- Tucker, G., and Fulton, W. L. Inventors Metamorphic Alchemy & Distillations Inc, assignee (2017). *System and Method for Producing a Terpene-Enhanced Cannabinoid Concentrate*. US patent 9,649,349, 2017 May 16.
- Uematsu, Y., Hirata, K., Suzuki, K., Iida, K., Kan, T., and Saito, K. (2001). Determination of Sucrose Esters of Fatty Acids in Food Additive Premixes by Gas Chromatography and Confirmation of Identity by Gas Chromatography/mass Spectrometry. *JAOAC Int.* 84 (2), 498–506. doi:10.1093/jaoac/84.2.498
- US Centers for Disease Control (2020). in *Office on Smoking and Health, Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products* (Atlanta, GA: National Center for Chronic Disease Prevention and Health Promotion). February 25, 2020 Update, Available at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html (Accessed Sept 22, 2020).
- Zheng, P., Mabe, J. J., Sumner, C. E., Jr., Janka, M. E., Frye, L., Testerman, S. R., et al. Inventors Eastman Chemical Company, Assignee (2016). *Integrated Process for the Production of Benzoate Plasticizers*. US patent application 2016 0068655 A1. 2016 March 10.
- Zumpano, M. V. Inventor Entourage Bioscience LLC, assignee. (2020). *Method and Compositions for Solubilizing Non-polar Constituents*. US patent 10,561,731. 2020 Feb 18.
- Zumpano, M. V. Inventor Entourage Bioscience LLC, assignee. (2019). *Methods and Compositions for Solubilizing Non-polar Constituents*. US patent 10,376,586. 2019 Aug 13.

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Investigation of Vaping Fluids Recovered From New York State E-Cigarette or Vaping Product Use-Associated Lung Injury Patients

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E-cigarette or vaping product use-associated lung injury (EVALI) is a serious pulmonary condition that is associated with the extended use of certain vaping products. EVALI was first characterized in the summer of 2019 and has since been reported in all 50 U.S. states. From August 2019 through June 2021, the New York State Department of Health has reported more than 197 confirmed cases emanating from all regions of the state. The Wadsworth Center at the New York State Department of Health received vaping cartridges recovered from EVALI patients for chemical analysis of their contents. Untargeted analytical methods using gas chromatography-mass spectrometry and liquid chromatography-high-resolution mass spectrometry as well as targeted analyses for a variety of analytes including cannabinoids, pesticides, vitamin E acetate (VEA) and mycotoxins were used to characterize the composition of the vaping fluids and several commercial vaping fluid additives. From the analyses of the 284 e-cigarette devices recovered from patients, 82 were found to be nicotine-containing pods, and 202 devices containing cannabis oil, apparently from unauthorized or black-market dealers. The fluids from the cannabis-oil cartridges tended to have lower levels of THC_s (Δ^9 -tetrahydrocannabinol + Δ^8 -tetrahydrocannabinol) and total cannabinoids compared with those of commercially produced formulations and contained significant levels of diluents including VEA, medium-chain triglycerides, polyethylene glycol, and castor oil. VEA was the diluent most frequently detected, which was present in 132 (65.3%) of the vaping fluids that contained cannabis oil. When present, VEA ranged from 2.0 to 67.8% of the total mass of the oil with a mean content of 37.0%. In some cases, two or three diluents were detected in the same sample. The ratio of VEA to THC_s varied widely, from 0.07 to 5.34. VEA and specifically the high ratios of VEA to THC_s in black-market vaping fluids may be causative in EVALI. The safety of additional components and additives that are present in vaping fluids are likewise of concern.

Keywords: electronic cigarettes, vaping fluid, EVALI, cannabis, vitamin E acetate, diluents, terpenes, pesticides

INTRODUCTION

Coincident with the development of the e-cigarette, vaping has become a popular way to use in both nicotine and cannabis products (Gaub et al., 2019; Kumar et al., 2019; DiSilvio et al., 2021). First introduced into the US in 2006, e-cigarettes consist of a wicking material that draws the vaping fluid from a reservoir to a battery-powered metal coil that, when heated, vaporizes the fluid (Brown and Cheng 2014; Chun et al., 2017). The composition of vaping fluids varies, but they generally contain either an aqueous-based nicotine solution or a cannabis oil. E-cigarettes have been marketed as a safer alternative to traditional smoking, as the number of harmful byproducts from combustion of tobacco is greatly reduced (Giroud et al., 2015; Margham et al., 2016). However, the health effects of vaping various oils, diluents, and flavoring agents that are present in various devices are not fully understood.

Despite numerous rescheduling attempts, marijuana, or cannabis, has remained a U.S. Drug Enforcement Agency Schedule I controlled substance. As a result, cannabis vaping fluids/devices have until recently been illegal. Changing regulations now permit medical and/or adult recreational use in 47 U.S. states; however, numerous restrictions regarding cannabis use remain (NCSL 2021), and black markets for cannabis products persist, including those for illicit vaping devices. The composition of these black-market vaping fluids is a major concern, since regulators cannot provide oversight of the manufacturing practices and the additives used. Consumers may thus be exposed to significant health risks when using illicit vaping products.

The medical condition that is now known as E-cigarette or vaping product use-associated lung injury (EVALI) was first reported in June of 2019 in the U.S. states of Illinois and Wisconsin. This initial outbreak involved 98 patients, mostly young (median age 21 years) and male (79%), who presented with bilateral infiltrates upon chest imaging and had respiratory, gastrointestinal, and constitutional symptoms (Layden et al., 2020). These patients were suspected of having a malady that was not caused by an infectious agent, but rather a condition caused by a chemical component arising from the vaping fluid. As of February 18, 2020, a total of 2,807 hospitalized EVALI cases and 68 deaths had been reported to the U.S. Centers for Disease Control and Prevention (CDC) from all 50 U.S. states, the District of Columbia and the U.S. territories of Puerto Rico and the U.S. Virgin Islands (CDC 2020). The widespread occurrence of the condition spurred numerous investigations into the possible cause(s) of EVALI.

In August of 2019, the Wadsworth Center of the New York State Department of Health (NYSDOH) began receiving vaping devices associated with EVALI cases that were submitted from poison control centers and health care providers in New York State for chemical analysis. During the early investigation of EVALI, it was discovered that many of the cannabis vaping fluids that were associated with EVALI cases contained high levels of vitamin E acetate (VEA). In light of these findings, NYSDOH issued a health advisory and reported the presence of VEA in illicit vaping cartridges recovered from EVALI patients

(NYSDOH 2019; Duffy et al., 2020). VEA has since been strongly linked with the etiology of EVALI, as chemical analysis showed the presence of VEA in the vast majority of bronchoalveolar lavage (BAL) samples from the EVALI patients (Blount et al., 2019; 2020). Potential mechanisms of toxicity of VEA that may be relevant to EVALI have been identified (Wu and O'Shea, 2020; Jiang et al., 2020; Muthumalage et al., 2020).

Our previous report described the findings from the analysis of samples associated with the first 10 EVALI cases in New York State for which vaping products were available (Duffy et al., 2020). The current paper reports on a detailed analysis of the vaping fluid compositions of 284 samples from 83 EVALI patients received at the Wadsworth Center from August of 2019 through June of 2021. Both cannabinoid- and nicotine-containing products were analyzed. Our results show a variety of important analytical findings on diluents and combinations thereof in vaping fluids and the contamination of the fluids with pesticide residues. We report that VEA-containing cannabis vaping fluids associated with recent cases of EVALI in New York continue to be received and analyzed by our laboratory as of June of 2021.

MATERIALS AND METHODS

EVALI Case Identification and Sample Collection

EVALI cases reported to the NYSDOH by health care providers, and medical records provided were reviewed by medical staff of the Center for Environmental Health at the NYSDOH. EVALI diagnoses were based on criteria and case definitions established by the CDC (CDC 2019). Vaping devices provided by the patients or their guardians that were determined to be associated with EVALI cases were submitted to the Wadsworth Center for analysis after referral from poison control centers in New York State. The samples received were generally either cannabinoid-containing vaping cartridges or nicotine-containing pods. A single device or as many as 23 devices were received in association with a single EVALI case. For cannabis vaping cartridges, the units were disassembled, and a sample of the cannabis oil was recovered with the tip of a spatula. When the cartridge appeared to be empty, the reservoir section of the device was placed in a centrifuge tube and centrifuged at 2,000 $\times g$ for 2 min. This procedure often produced enough material for analysis. A portion of the viscous cannabis oil, about 10 mg or whatever was recovered, was weighed to ± 0.01 mg and dissolved in 50:50 methanol:acetonitrile to give a sample concentration of 10 mg/ml that was further diluted as appropriate for a suite of analyses. The fluids from nicotine pods were recovered using a micropipette. The recovered portion was likewise weighed to ± 0.01 mg and dissolved in 50:50 methanol:acetonitrile for further analyses.

Chemicals and Standards

The following certified cannabinoid reference standards were purchased from Cerilliant (Round Rock, TX, United States):

cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabigerol (CBG), cannabidiol (CBD), tetrahydrocannabivarin (THCV), cannabinol (CBN), Δ^9 -tetrahydrocannabinol (Δ^9 -THC), Δ^8 -tetrahydrocannabinol (Δ^8 -THC), cannabichromene (CBC), cannabidivarin (CBDV), and Δ^9 -tetrahydrocannabinolic acid-A (THCA). Primary analytical standards and ^{13}C -isotopically labeled internal standards for aflatoxins B1, B2, G1, G2, and ochratoxin A were purchased from Romer Labs (Union, MO, United States). VEA and vitamin E- d_6 (($\pm$)- α -tocopherol- d_6) were from Cerilliant (Round Rock, TX, United States). The myclobutanil analytical standard was purchased from Accustandards (New Haven, CT, United States). Polyethylene glycol (PEG), USP-grade castor oil, norgestrel, and myclobutanil-(phenyl- d_4) was purchased from MilliporeSigma (St. Louis, MO, United States). Myglol, a medium-chain triglyceride (MCT) oil, was from Warner Graham (Cockeysville, MD, United States). A Piperonyl butoxide (PBO) analytical standard was purchased from Agilent Technologies (Santa Clara, CA, United States), and the corresponding piperonyl butoxide- d_9 (PBO- d_9) internal standard was from Toronto Research Chemicals (Toronto, Canada). Mixtures containing 884 pesticide and pesticide metabolite standards for use in pesticide screening were provided by Dr. Jon Wong of the Center for Food Safety and Applied Nutrition, US Food and Drug Administration (FDA), College Park, MD, United States. Ammonium formate, formic acid, methanol, acetonitrile, and water were HPLC-grade. All other reagents used were analytical grade.

Analytical Methods

Untargeted Analysis

Untargeted analyses were performed using both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high-resolution tandem mass spectrometry (LC-HRMS/MS). For GC-MS analysis, the initial extracts were injected onto a GC-MS system comprised of a model 6890A GC interfaced with a model 5973N quadrupole mass selective detector (Agilent). Compounds were resolved on a DB5-MS column (60 m $\times$ 250 μm ID; 0.25 μm film thickness; Agilent J&W) with helium as the carrier gas at 1.5 ml/min. The MS transfer line and ion source were at 300°C and 235°C, respectively. The initial oven temperature was 90°C for 1 min, followed by a ramp of 2°C/min to 320°C and a hold at 320°C for 25 min. After a solvent delay of 7 min, full-scan mass spectra were recorded over the 50–550 m/z range in the electron ionization mode. For compound identification, data were queried against the National Institute of Science and Technology (NIST) Mass Spectral Library 11, the latest Cayman toxicology mass spectral library, and an in-house built mass spectral library.

For untargeted analysis using LC-HRMS/MS, a system comprised of a Shimadzu HPLC interfaced with a SCIEX TripleTOF 6,600 mass spectrometer was employed as described (Duffy et al., 2020). Briefly, a Poroshell EC-C18 HPLC column (Agilent, 2.1 $\times$ 100 mm; 2.7 μm particle size) was used for analyte separations. Gradient elution was performed with mobile phases A (0.1% v/v formic acid in water) and B (5 mM ammonium formate in methanol). The

mass spectrometer was operated in the positive-ion ESI mode for high-resolution MS and MS/MS acquisition. High-resolution MS and MS/MS spectra were recorded using the information-dependent acquisition technique. Data were acquired using Analyst Software (SCIEX, version 1.6.1) and data were processed using PeakView software (SCIEX, version 2.1). An accurate-mass compound library that was prepared in-house as well as other commercial and public domain databases that included data for synthetic cannabinoids, opiates, synthetic opioids, stimulants, numerous drugs of abuse and previously identified cannabis oil diluents and additives were used for compound identification.

Targeted Analysis

Quantitation of cannabinoids was performed using HPLC with photodiode array detection (NYSDOH 2018b; Li et al., 2019). This method has been certified for use in the New York State Medical Marijuana Program (NYSMMP) by the New York State Environmental Laboratory Approval Program (ELAP) according to TNI standards and has been used in the NYSMMP since 2015 for the analysis of thousands of NYSMMP samples. The method employs a Shimadzu (Kyoto, Japan) HPLC system with an SPD-M20A photodiode array detector. Cannabinoids (CBDA, CBGA, CBG, CBD, THCV, CBN, Δ^9 -THC, Δ^8 -THC, CBC, CBDV, and THCA) are resolved on an Agilent Poroshell 120 column (3.0 $\times$ 150 mm with 2.7 μm particle size) using isocratic elution at 73% v/v acetonitrile in water with 0.1% v/v formic acid and quantitation of absorbance at 227 nm relative to that of the norgestrel internal standard. Six-point calibration curves for each cannabinoid over the range of 0.19–45.0 $\mu\text{g/ml}$, plotting area ratios of the absorbance at 227 nm for the analytes to that of the internal standard against analyte concentration. The limit of detection (LOD) for each analyte was determined at the 99% confidence level from the analysis of seven blank samples that were fortified with low levels of each cannabinoid. The limit of quantitation (LOQ) for each cannabinoid was defined as five times the LOD, provided that this value was not below the lowest concentration calibrant of the calibration curve.

The analysis of mycotoxins was performed using LC-MS/MS with an ELAP-accredited method developed by NYSDOH Medical Marijuana Laboratory (NYSDOH 2018a) for the quantitation of aflatoxins B1, B2, G1, G2, and ochratoxin A in medical marijuana products, modified for use with limited amounts of sample. Quantitative analysis of the fungicide myclobutanil and the insecticide synergist PBO was conducted using a method developed and certified by the NYSDOH Medical Marijuana Laboratory using LC-MS/MS.

The quantitative analysis of VEA in vaping fluids was performed using GC-MS with electron ionization and operation in the selected-ion monitoring mode with vitamin E- d_6 as the internal standard (Duffy et al., 2020). The analytical system used was composed of a model 7890B GC with model G4513A autosampler interfaced with a 5977A MSD and Mass Hunter Version B07.01 SP/Build 7.1.524.1 software (Agilent Technologies). An Agilent HP-5MS column (30 m $\times$ 250 μm with 0.25 μm film thickness) was used with the following temperature program: an initial temperature of 90°C for 1 min

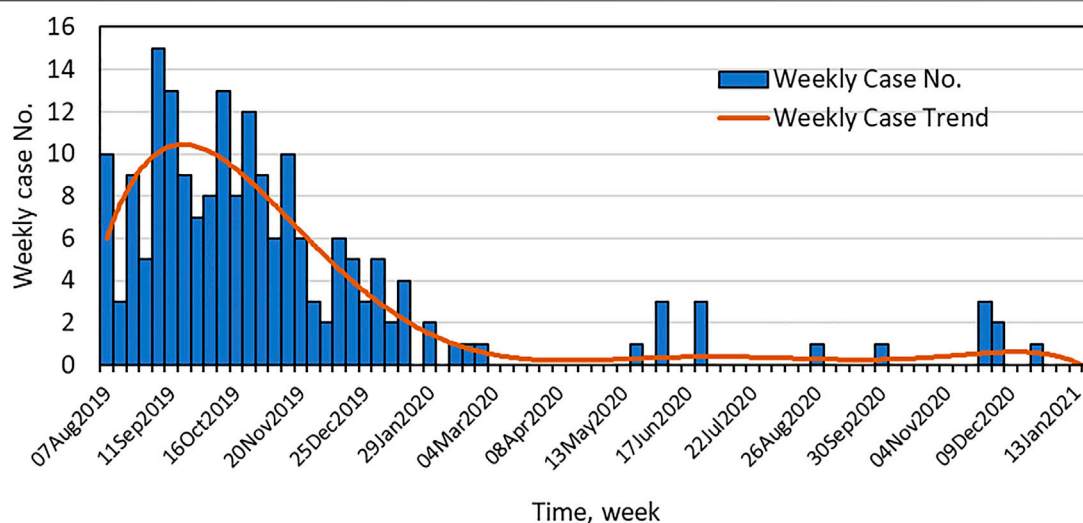


FIGURE 1 | Time course of the EVALI outbreak in New York State. Shown are the number of EVALI cases reported to the NYSDOH per week and the weekly case trend.

followed by an increase at 8°C per min to a final temperature of 290°C, a hold for 4 min, an increase at 10°C per min to 300°C, and a hold for 1 min. Ions of m/z 430 and 165 were monitored for VEA; m/z 436 and 171 were monitored for the vitamin E- d_6 internal standard. Dwell times were 50 ms. A calibration range of 0.039–2.5 µg/ml was established for VEA. The original vaping fluid solutions at 10 mg/ml typically required an additional dilution of 1,000- to 10,000-fold for analysis.

Screening for Pesticides and Pesticide Metabolites

A non-targeted data acquisition for target analysis technique using ultra high-performance liquid chromatography (UHPLC) coupled with a quadrupole-orbitrap mass spectrometer (QE-Orbitrap-MS) that is based on previous studies of screening for pesticide residues was used (Wang et al., 2019). The instrumental system used was a Vanquish UHPLC with a Hypersil GOLD column (100 × 2.1 mm with 1.9 µm particle size, Thermo Fisher Scientific, Waltham, MA, United States) interfaced with a high-resolution QE-Orbitrap-MS (Thermo Fisher Scientific) operating in the positive-ion ESI mode (Duffy et al., 2020). A pesticide database that was kindly provided by Dr. Jon Wong of the Center for Food Safety and Applied Nutrition, U.S. FDA, College Park, MD allowed identification of pesticide residues.

RESULTS AND DISCUSSION

Reported Cases of EVALI in New York State

As in many parts of the U.S., cases of EVALI first appeared in New York State in early August of 2019. The weekly number of EVALI cases reported to the NYSDOH are shown in **Figure 1**. The incidence of reported EVALI cases peaked in mid to late

TABLE 1 | Types of samples associated with EVALI cases.

Sample types submitted ^a	Number of cases (%)
Cannabinoids only	39 (47.0%)
Both cannabinoids and nicotine	24 (28.9%)
Nicotine only	20 (24.1%)
Total	83 (100.0%)

^aUntargeted screening was performed on a total of 284 vaping products from 83 patients.

September of 2019, then rapidly declined to the end of 2019, stabilizing at 1 to 3 cases or less per week by mid-January 2020. The decline in EVALI cases in New York State came soon after a press release by NYSDOH on Sept. 5th, 2019, warning against the use of black-market vaping products and announcing VEA as the focus of the investigation of lung injury associated with vaping (NYSDOH, 2019), and the preliminary report by Layden and others (Layden et al., 2020) published on-line Sept. 6th, 2019 relating pulmonary illness to e-cigarette use in Illinois and Wisconsin. A few additional EVALI cases were reported later 2020 and sporadically through mid-2021.

Characterization of EVALI Vaping Fluids—Major Components

Untargeted chemical analyses were performed on a total of 284 vaping products obtained from 83 patients. Results from the GC-MS and LC-HRMS/MS analyses confirmed the sample types as cannabinoid-containing or nicotine-containing and served to identify the major components.

When separated according to cases, the most prevalent association of EVALI was with cannabinoid-containing vaping fluids (**Table 1**). For many patients, only cannabinoid-containing vaping devices were received, while both cannabinoid-containing

TABLE 2 | Cannabinoids in vaping fluids.

Cannabinoid	N (%) ^a	Min % ^b	Max % ^c	Mean ± S.D.
CBC	11 (5.4%)	1.2	2.6	1.7 ± 0.4
CBD	25 (12.4%)	0.8	44.9	7.2 ± 9.5
CBG	102 (50.5%)	1.2	5.3	2.0 ± 0.8
CBN	86 (45.6%)	1.1	16.9	3.2 ± 2.3
Δ^8 -THC	29 (14.4%)	1.9	88.8	26.1 ± 16.6
Δ^9 -THC	194 (96.0%)	1.1	88.7	33.1 ± 18.9
THCA	17 (8.4%)	1.9	87.9	11.8 ± 22.8
THCV	1 (<1.0%)	2.3	2.3	2.3
CBDA	1 (<1.0%)	4.0	4.0	4.0
CBDV	N.D. ^d	N/A ^e	N/A	N/A
CBGA	N.D.	N/A	N/A	N/A

^aNumber of samples containing the cannabinoid and as a percentage of the total cannabinoid-containing samples.

^bMinimum concentration observed in mass%.

^cMaximum concentration observed in mass%.

^dN.D., not detected in any sample (below the reporting limit of 1.2% by mass).

^eN/A, not applicable.

and nicotine-containing devices were obtained from others. In 24% of the patients, only nicotine-containing products were received. A summary of the cannabinoid profiles found in the cannabis vaping products is presented in **Table 2**. Of cannabis vaping products obtained, 194 (96%) contained Δ^9 -THC above the LOQ of 1% by mass. The Δ^8 -THC isomeric form was also found in 28 of the samples at varying levels. The Δ^8 -THC-containing samples did not appear to represent a homogenous subgroup of samples. Varying amounts of CBG, CBN, CBD, THCA, CBC and THCV were quantified in the cannabis vaping fluids. One of the vaping fluids that contained nicotine also

contained a low level of CBD. This sample showed phase separation, which would be expected for such a sample, as cannabis oil is lipophilic in nature and is immiscible with aqueous-based solutions of nicotine and nicotine salts.

Identification of Diluents in Cannabis Vaping Fluids

All 202 cannabis vaping products appeared to be illicit or black-market products since none had packaging or markings indicative of products approved by the NYSMMP. Diluents present in vaping fluids that were not approved by the NYSMMP were identified in 185 (92%) of the vaping fluids. VEA, MCT and PEG were repeatedly identified as diluents in the EVALI-associated vaping products. The identification of PEG in a vaping fluid is shown in **Figure 2**. PEG of a polymer of the formula $H-(OCH_2CH_2)_n-OH$, in which n can vary from less than ten to several thousand. The PEG polymers identified as vaping fluid diluents were typically of average molecular mass 600. In positive-ion ESI-MS in the presence of ammonium acetate, PEG is detected as a series of peaks with the formula $[(C_{2n}H_{4n+2}O_{n+1}) + NH_4]^+$. In **Figure 2**, the peaks corresponding to PEG polymers with $n = 10$ through $n = 19$ are denoted. In each case, the m/z assignments are within 6 ppm of the theoretical values for the ammonium ion adducts of the PEG polymers.

In the initial untargeted analysis using LC-HRMS/MS, two of the vaping fluid samples showed the same prominent unknown component. Interpretation of accurate-mass data lead to the hypothesis that the unknown diluent was castor oil. The major component of castor oil is ricinolein, or ricinoleic acid

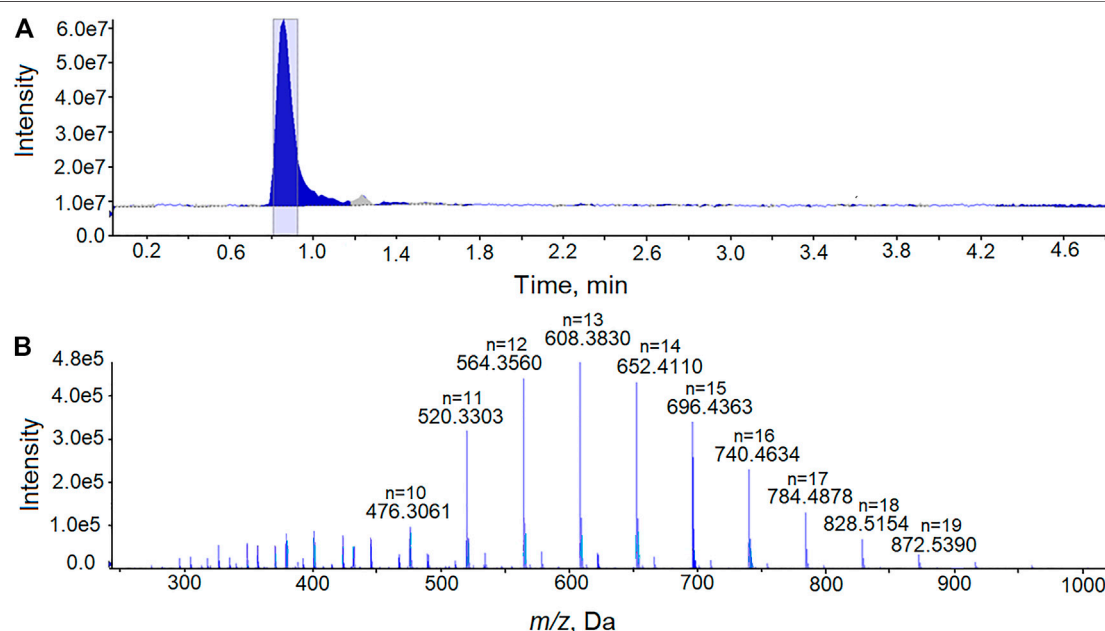


FIGURE 2 | Analysis of PEG in a cannabis vaping fluid using LC-HRMS. **(A)** The total ion current chromatogram from the analysis of an extract of a cannabis vaping fluid. **(B)** Mass spectrum recorded at retention time 0.81–0.92 min showing the $[(C_{2n}H_{4n+2}O_{n+1}) + NH_4]^+$ ions of PEG with the peaks for polymers of $n = 10$ through $n = 19$ denoted.

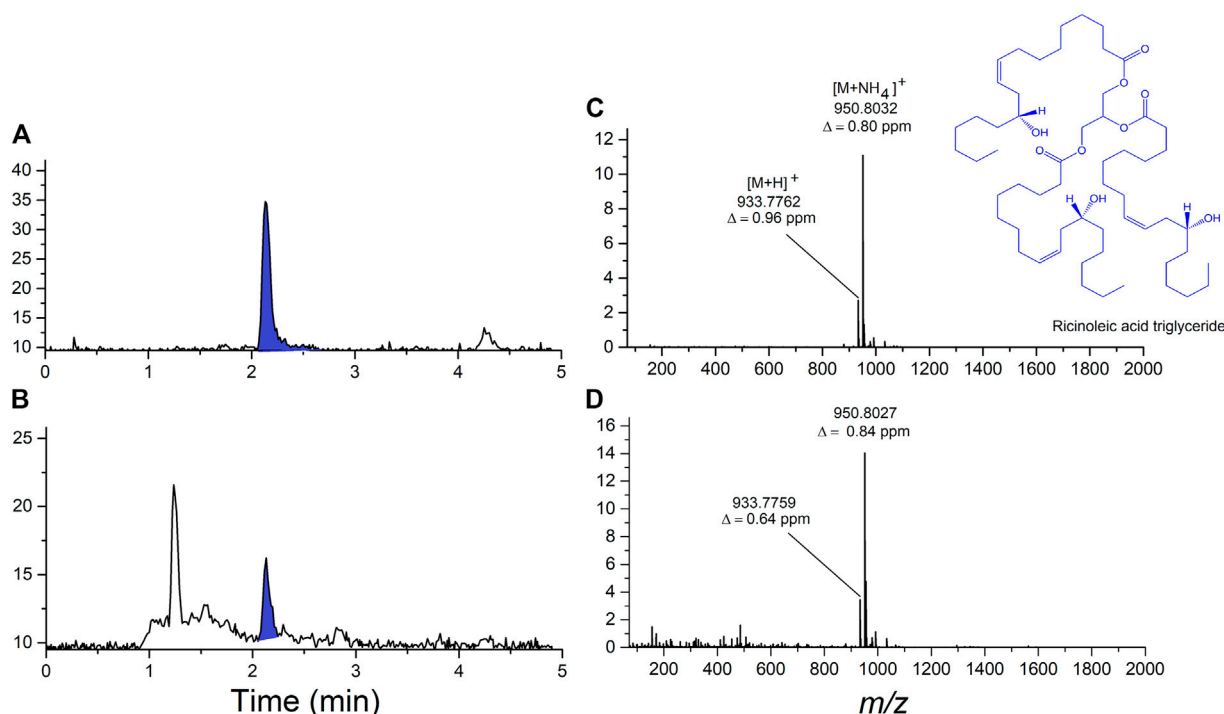


FIGURE 3 | Analysis of ricinoleic acid triglyceride, the major component of castor oil, in vaping fluid using LC-HRMS. **(A)** Total ion current chromatogram from the analysis of USP-grade castor oil standard showing the ricinoleic acid triglyceride peak shaded in blue. **(B)** Total ion current chromatogram from the analysis of an extract of a cannabinoid-containing vaping fluid. **(C)** High-resolution ESI mass spectrum of ricinoleic acid triglyceride from the castor oil standard. **(D)** High-resolution ESI mass spectrum of ricinoleic acid triglyceride present in the cannabinoid-containing vaping fluid.

TABLE 3 | Diluents found in cannabis vaping fluids submitted to NYSDOH.

Diluent	No. of samples	No. of associated cases ^a
No diluents	17	12
VEA only	107	51
MCT only	42	18
PEG only	8	4
Castor oil only	2	2
VEA + MCT	19	8
VEA + PEG	4	3
MCT + PEG	1	1
VEA + MCT + PEG	2	1

^aThe number of cases in which at least one sample has the indicated diluent profile.

triglyceride. Analysis of a vaping fluid sample together with a USP-grade castor oil standard and are shown in **Figure 3**. Ricinoleic acid triglyceride was confirmed as the major component of castor oil, eluting from the column with a retention time of 2.05 min (**Figures 3A,B**). Upon electrospray ionization in the presence of ammonium acetate, ricinoleic acid triglyceride produces a dominant $[M + NH_4]^+$ ion that was 4-fold more intense than the $[M + H]^+$ (**Figures 3C,D**). The accurate-mass measurements were consistent with the component in the vaping fluids being ricinoleic acid triglyceride, as were MS/MS spectra obtained from the $[M + H]^+$ and $[M + NH_4]^+$ ions as precursors.

VEA was the diluent most frequently detected in the cannabis vaping samples (**Table 3**). For a majority of the EVALI patients (76%), cannabis vaping fluids were associated with the condition. For EVALI cases in which cannabis vaping products were submitted to the laboratory for analysis, 84% of the time at least one VEA-containing fluid was among the samples received among the patients vaping products. MCT, PEG and castor oil were also detected as diluents in the vaping fluids, although less frequently than VEA. Some products were found to contain two or three diluents. Binary combinations of VEA, MCT and PEG were observed, as were samples in which all three of these diluents were present in combination (**Table 3**).

When diluents are used in the black-market cannabis industry, the supply of expensive cannabis oil can be extended, and profits can be maximized. VEA must have seemed to be a nearly ideal diluent for black-market cannabis vapor fluids, as it is nearly tasteless, odorless, and has very similar viscosity and color to undiluted cannabis oil, even when mixed at high ratios with cannabis extract. The vaping fluids from illicit vaping products generally had low cannabinoid content (on average, ~30% THC) and often contained as much or more VEA than total cannabinoids. The ratio of VEA to THCs varied widely, from 0.07 to 5.33 with an average of 1.35 (**Figure 4**). In the vaping fluids that contained VEA, the mean $\pm$ SD VEA concentration was 37.0 ± 15.4 mass%, and the range of values was 2.0–67.8. Since cannabis product users tend to self-titrate their dose according to the response they obtain and their tolerance

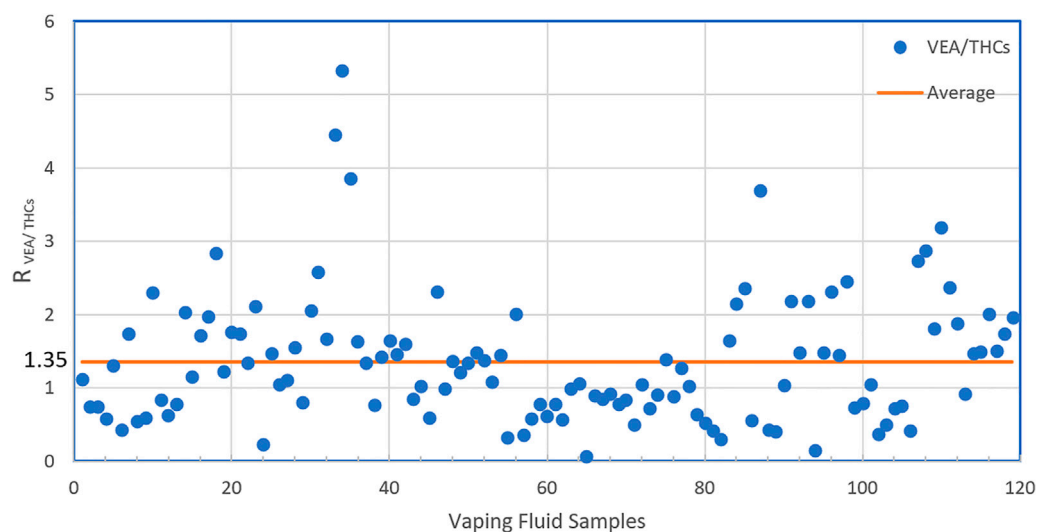


FIGURE 4 | The Ratio of VEA to THCs in illicit vaping fluids. In the vaping samples found to contain VEA, the ratios of VEA to THCs (Δ^9 -THC + Δ^8 -THC) by mass in the individual samples are shown.

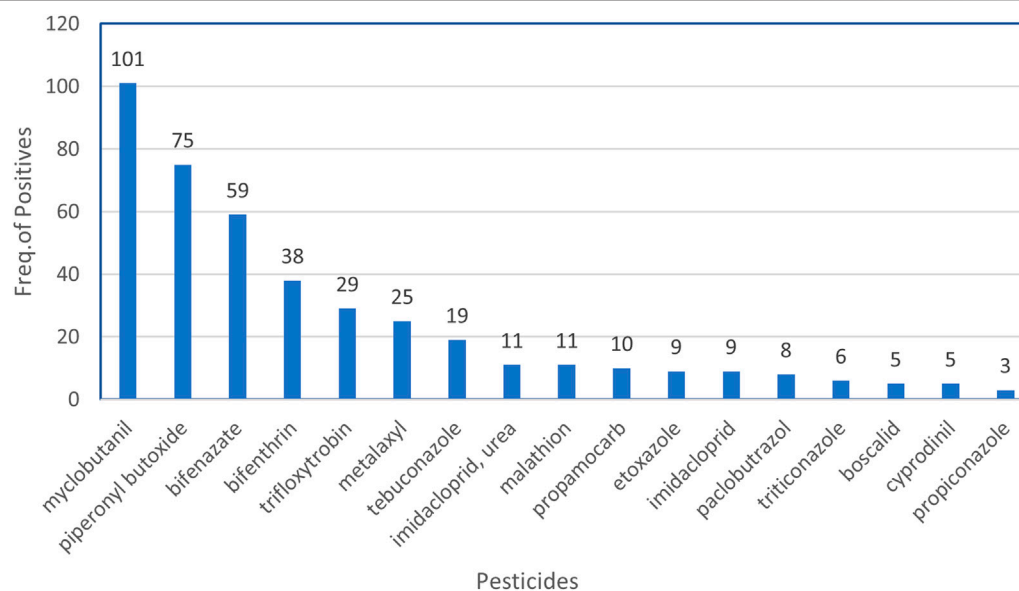


FIGURE 5 | Frequently detected pesticides in illicit cannabis vaping fluids. At a detection level of $>1 \mu\text{g/g}$, the frequencies of the most commonly detected pesticides in the 202 cannabis vaping fluids are shown.

(Barnes, 2006), as the ratio of VEA to THC in the fluid increases, cannabis users will inhale more vaping fluid, and thus more VEA, to achieve the same dose of THC. As noted, MCT and PEG were present in some of the VEA-containing fluids as additional diluents. These results are in sharp contrast to the archetypical vaping fluids analyzed for the NYSMMP, which have very high cannabinoid content, 80–90% by mass, and are excipient-free, *i.e.*, they do not contain diluents.

Contaminants in Vaping Fluids

Unlike indoor cannabis cultivation that is highly regulated under the NYSMMP, black-market cannabis products may come from indoor or outdoor growing that utilizes pesticides. Low levels of pesticides and pesticide metabolites ($>1 \mu\text{g/g}$) were found in numerous cannabis and nicotine products obtained in this study. Of 74 nicotine vaping fluids tested, 12 (16%) were positive for either propamocarb ($n = 11$) or bentranil ($n = 1$). Of the 202 cannabis vaping fluids analyzed, 159 (79%) tested

TABLE 4 | Composition of commercial vaping-fluid additives.

Additive no	Components
1	citronellyl propionate; isophytol; <i>trans</i> -phytol, <i>cis</i> -phytol
2	exo-fenchol; α -terpineol, caryophyllene; α -humulene
3	Limonene; 1,2,3-Trimethylcyclopentene; α -Terpineol; caryophyllene; α -humulene
4	β -Myrcene; α -Terpinene; Limonene; terpinolene; isoborneol; endo-borneol; α -Terpineol; caryophyllene; α -humulene; α -Bisabolol
5	β -Pinene; Limonene; 4-Carene; isoborneol; α -Terpineol; caryophyllene; <i>cis</i> - β -ocimene; D-nerolidol
6	β -Myrcene; Limonene; β -linalool; caryophyllene
7	β -Myrcene; Limonene; γ -Terpinene; neomenthol; isoborneol; α -Terpineol; caryophyllene; α -Bisabolol
8	β -Myrcene; Limonene; γ -Terpinene; <i>cis</i> -Geraniol; caryophyllene; D-nerolidol
9	β -Myrcene; Limonene; γ -Terpinene; caryophyllene; α -humulene; D-nerolidol; α -Bisabolol

TABLE 5 | Major components found in nicotine-containing vaping fluids.

Additive	Benzoic acid
Carriers	Glycerin, Propylene glycol, Benzyl alcohol
Flavors	Triacetin, Vanillin, Levomenthol, menthol, Triethyl Citrate, Ethyl maltol
Cooling agents	WS-3, WS-23

positive for various pesticides and pesticide metabolites, with individual samples containing up to 10 distinct pesticide residues. In total, 42 pesticides were detected in cannabis vaping fluids. The most detected pesticides are shown in **Figure 5**. Myclobutanil, a fungicide that is used to prevent the growth of powdery mildew on plants, was the most frequently detected, being present in 101 of 202 (50%) cannabis vaping fluids. The pesticide synergist, PBO, was detected in 75 (37%) of the samples. The pesticides bifenthrin and bifenthrin were also frequently observed in cannabis vaping fluids. Aflatoxins B1, B2, G1, G2 or ochratoxin A were not detected in any of the vaping fluids analyzed in this study. Using our LC-HRMS/MS and GC-MS screening techniques together with our database searching methods, no synthetic cannabinoids, opiates, synthetic opioids, or other controlled substances were detected in any of the vaping fluids analyzed.

Analysis of Commercial Cannabis Oil Diluents and Additives

At the onset of the NYSDOH investigation of EVALI in New York, shortly after our initial observation of VEA in EVALI case-associated vaping fluids, we questioned the source of VEA in the fluids. We noted that there were several commercial products of unknown composition marketed as “cannabis oil diluents” or “thickeners.” When these products were obtained and analyzed, several were found to be essentially pure VEA (Duffy et al., 2020). After a public health alert and subpoenas issued by the NYSDOH, these products are no longer available.

To investigate current or future chemicals that may be used in illicit vaping products, nine commercial “flavoring additives” were purchased from a commercial source. Untargeted GC-MS analysis was used to identify the components in the additives. From the results, it was apparent that most of the compounds in the flavoring additives were terpenes (**Table 4**). Caryophyllene

was the most commonly detected terpene, which was present in eight of the nine product samples. Caryophyllene is a naturally occurring terpene in cannabis extracts. It can be used to adjust the flavor of a vaping fluid to better match that of a high-grade cannabis oil (Fournier et al., 1978; Gulluni et al., 2018; Ibrahim et al., 2019; Heblinski et al., 2020). Limonene was found in seven of the nine samples. D-Limonene is an aromatic terpene found in the citrus oils and it could possibly be used to produce a citrus flavor to the vaping fluids. However, limonene can be an irritant and have a bronchoconstrictive effect (Aronson 2015). No VEA was detected in these cannabis oil “flavoring additives.”

Analysis of Nicotine Vaping Fluids

Many of the samples (82) that were submitted to our laboratory as part of our EVALI investigation were nicotine-containing pods. All of these appeared to be commercial products, and many of the samples had associated packaging and devices that supported their legitimacy as such. Nicotine vaping fluids were analyzed using untargeted GC-MS and LC-HRMS/MS. A summary of the major additives as estimated by GC-MS peak area is presented in **Table 5**. The e-liquids in nicotine-containing pods were found to contain the excipients, glycerin, propylene glycol and benzyl alcohol, along with various flavors (Margham et al., 2016). Benzoic acid, an additive to assist vaporization and improve absorption of nicotine salts, was detected in the nicotine products as has been in previous studies (Pankow et al., 2020). Benzoic acid is an acknowledged ingredient in e-liquids from Juul pods (Juul 2019). The cooling agents, WS-3 and WS-23, were also detected in nicotine-containing vaping fluids using GC-MS and confirmed using LC-HRMS/MS. These compounds provide a fast-acting cooling sensation and primarily affect the mouth and tongue (Behrendt et al., 2004; Sherkheli et al., 2010; Wang et al., 2014). When analyzed using GC-MS and LC-HRMS/MS, extracts of the nicotine pods that we received in association with EVALI cases did not reveal any chemical constituents that have not been previously reported other than trace pesticide residues.

CONCLUSION

On September 5th, 2019, based on the initial observations from our laboratory, the NYSDOH announced an update on its investigation into vaping-associated pulmonary illness and

issued the first in the nation public health advisory about VEA after high concentrations of VEA were found in the vaping devices recovered from EVALI patients (NYSDOH, 2019). In less than 2 years since that time, we have analyzed 284 samples from 83 cases of EVALI in New York State. While the overall number of cases of EVALI in New York State greatly declined over time, we observed remarkably similar rates of VEA positivity and levels of VEA content in case-associated vaping fluids as we did in the initial results leading to the health advisory. Whether the most recent samples containing VEA, *e.g.*, received June 2021, represent remaining stock of illicit products that are slowly appearing on the black market or whether there is still limited use of VEA as a diluent is unknown. In this extended study, 132 (65%) of the cannabis vaping fluids recovered from EVALI patients contained VEA, and for EVALI cases in which one or more cannabis vaping product were submitted to the laboratory for analysis, 84% of the time at least one VEA-containing fluid was among the samples received. These results continue to support the initial hypothesis that VEA is causative in EVALI.

It should be noted that only the vaping fluid samples recovered at the time of diagnosis and hospitalization were analyzed in this study. While this may provide a snapshot of the patient's chemical exposure, the fluids that were causally related to the onset of the condition may not have been submitted to the laboratory for analysis. Since the case-associated cannabis vaping products would have been illegal at the time of use, there may have been an under-submission of cannabis vaping products for analysis by the patients in favor of commercially available nicotine products (Ghinai et al., 2020). Despite these potential limitations, the association of EVALI with the use of VEA-containing cannabis vaping products is strong. The most convincing evidence for the role of VEA in EVALI came from the analyses of bronchoalveolar lavage fluids from EVALI patients. Vitamin E acetate was identified in BAL fluid obtained from 48 of 51 case patients (94%) from 16 states but not in such fluid obtained from the healthy comparator group (Blount et al., 2020).

How VEA may cause the condition of EVALI is not entirely clear; however, there are mechanistic studies that present several plausible mechanisms for VEA toxicity in vaping. At temperatures of 300 °C or higher, VEA undergoes pyrolysis and forms numerous toxic byproducts, including ketene (Wu and O'Shea, 2020) and duroquinone (Duffy et al., 2020). Ketene would be highly reactive with a variety of biomolecules. The duroquinone-durohydroquinone redox couple was observed in the vaping emissions from vitamin E acetate, which may be linked to acute oxidative stress and lung injuries (Jiang et al., 2020). While it is unclear whether inhalation of VEA causes lipid pneumonia, it is known that vaporized VEA is an irritant to the lung mucosa and bronchi and can lead to chronic hypoxia (Cannon 1940). One or more of these mechanisms may lead to the EVALI condition. To date, the strongest evidence points to VEA as causative in EVALI.

This does not rule out potential harmful effects of other vaping components, such as replacement additives and diluents. For example, given the chemical properties of ricinoleic acid

triglyceride, it could hardly be assumed that castor oil would be a safe component in the vaping scenario, as it could be expected to cause lipid pneumonia (Cannon, 1940) and/or to generate reactive intermediates at high temperature. Aromatic/volatile hydrocarbons and oils consisting of MCT, terpenes and mineral oil in cannabis vaping fluids are suspected to cause oxidative stress and inflammatory responses in the lung (Chand et al., 2020). Recent studies in rats of phytol, one of the terpenes identified in commercial additives in this study, showed significant toxicity in respiratory tissue including dose-responsive tissue degeneration and necrosis in exposed animals that were in some instances associated with mortality (Schwotzer et al., 2021). These authors recommended that phytol not be used as an excipient in vaping products, as a safe exposure range for the compound has not been established. There is also no indication that long-term vaping of even low levels of pesticide residues in vaping fluids is without impact on pulmonary health. While the evidence that VEA is causative in EVALI is very strong, a decline in the use of VEA in the illicit cannabis oil market hardly means that the black-market vaping products are now safe, as some of the replacement additives and diluents also appear to elicit pulmonary toxicity.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because; Data are associated with individual EVALI cases. Requests to access the datasets should be directed to DS, david.spink@health.ny.gov.

AUTHOR CONTRIBUTIONS

Conceptualization of the study: DS, SL, LL, and BD; methodology: LL, SL, BD, LD, MD, DP, and ED-B; method validation: LD, LL, BD, MD, and ED-B; analytical investigation: SL, LL, BD, DS, DP, LD, MD, and ED-B; resources: DS; data curation and review: LL, BD, SL, DP, LD, MD, ED-B, and DS; writing of the original draft: SL; writing, review and editing: DS, SL, LL, BD, MD, LD, and ED-B; supervision: DS and LL; and project administration: DS. All authors have read and agreed to the final version of the manuscript.

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REFERENCES

- Aronson, J. K. (2015). *Meyler's Side Effects of Drugs*. Elsevier Science, Amsterdam, Netherland. Available at: <https://www.elsevier.com/books/meylers-side-effects-of-drugs/aronson/978-0-444-53717-1>.
- Barnes, M. P. (2006). Sativex: Clinical Efficacy and Tolerability in the Treatment of Symptoms of Multiple Sclerosis and Neuropathic Pain. *Expert Opin. Pharmacother.* 7 (5), 607–615. doi:10.1517/14656566.7.5.607
- Behrendt, H.-J., Germann, T., Gillen, C., Hatt, H., and Jostock, R. (2004). Characterization of the Mouse Cold-Menthol Receptor TRPM8 and Vanilloid Receptor Type-1 VR1 Using a Fluorometric Imaging Plate Reader (FLIPR) Assay. *Br. J. Pharmacol.* 141 (4), 737–745. doi:10.1038/sj.bjp.0705652
- Blount, B. C., Karwowski, M. P., Morel-Espinoza, M., Rees, J., Sosnoff, C., Cowan, E., et al. (2019). Evaluation of Bronchoalveolar Lavage Fluid from Patients in an Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - 10 States, August-October 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68 (45), 1040–1041. doi:10.15585/mmwr.mm6845e2
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinoza, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382 (8), 697–705. doi:10.1056/NEJMoa1916433
- Brown, C. J., and Cheng, J. M. (2014). Electronic Cigarettes: Product Characterisation and Design Considerations. *Tob. Control.* 23 (Suppl. 2), ii4–ii10. doi:10.1136/tobaccocontrol-2013-051476
- Cannon, P. R. (1940). The Problem of Lipid Pneumonia. *Jama* 115 (25), 2176–2179. doi:10.1001/jama.1940.72810510009014
- CDC (2019). *2019 LUNG INJURY SURVEILLANCE PRIMARY CASE DEFINITIONS*. Atlanta, GA, USA: Centers for Disease Control and Prevention.
- CDC (2020). *Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products*. Atlanta, GA: Centers for Disease Control and Prevention.
- Chand, H. S., Muthumalage, T., Maziak, W., and Rahman, I. (2020). Pulmonary Toxicity and the Pathophysiology of Electronic Cigarette, or Vaping Product, Use Associated Lung Injury. *Front. Pharmacol.* 10, 1619. doi:10.3389/fphar.2019.01619
- Chun, L. F., Moazed, F., Calfee, C. S., Matthay, M. A., and Gotts, J. E. (2017). Pulmonary Toxicity of E-Cigarettes. *Am. J. Physiology-Lung Cell Mol. Physiol.* 313 (2), L193–L206. doi:10.1152/ajplung.00071.2017
- DiSilvio, B., Baqdunes, M., Alhajhusain, A., and Cheema, T. (2021). Smoking Addiction and Strategies for Cessation. *Crit. Care Nurs. Q.* 44 (1), 33–48. doi:10.1097/CNQ.0000000000000338
- Downs, D. (2016). *The Science behind the DEA's Long War on Marijuana*. Scientific American. United States.
- Duffy, B., Li, L., Lu, S., Durocher, L., Dittmar, M., Delaney-Baldwin, E., et al. (2020). Analysis of Cannabinoid-Containing Fluids in Illicit Vaping Cartridges Recovered from Pulmonary Injury Patients: Identification of Vitamin E Acetate as a Major Diluent. *Toxics* 8 (1), 8. doi:10.3390/toxics8010008
- Fournier, G., Lenicque, P. M., and Paris, M. R. (1978). Toxic Effects of Essential Oil of Cannabis Sativa L. And Main Constituents on Planarian (*Dugesia Tigrina*) (Author's Transl). *Toxicol. Eur. Res.* 1 (6), 385–389.
- Gaub, K. L., Hallyburton, S., Samanic, C., Paddack, D., Clark, C. R., Pence, S., et al. (2019). Patient Characteristics and Product Use Behaviors Among Persons with E-Cigarette, or Vaping, Product Use-Associated Lung Injury - Indiana, June-October 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68 (49), 1139–1141. doi:10.15585/mmwr.mm6849a3
- Ghinai, I., Navon, L., Gunn, J. K. L., Duca, L. M., Brister, S., Love, S., et al. (2020). Characteristics of Persons Who Report Using Only Nicotine-Containing Products Among Interviewed Patients with E-Cigarette, or Vaping, Product Use-Associated Lung Injury - Illinois, August-December 2019. *MMWR Morb. Mortal. Wkly. Rep.* 69 (3), 84–89. doi:10.15585/mmwr.mm6903e1
- Giroud, C., de Cesare, M., Berthet, A., Varlet, V., Concha-Lozano, N., and Favrat, B. (2015). E-cigarettes: A Review of New Trends in Cannabis Use. *Ijerp* 12 (8), 9988–10008. doi:10.3390/ijerp120809988
- Gulluni, N., Re, T., Loiacono, L., Lanzo, G., Gori, L., Macchi, C., et al. (2018). Cannabis Essential Oil: A Preliminary Study for the Evaluation of the Brain Effects. *Evidence-Based Complement. Altern. Med.* 2018, 1–11. doi:10.1155/2018/1709182
- Heblinski, M., Santiago, M., Fletcher, C., Stuart, J., Connor, M., McGregor, I. S., et al. (2020). Terpenoids Commonly Found in Cannabis Sativa Do Not Modulate the Actions of Phytocannabinoids or Endocannabinoids on TRPA1 and TRPV1 Channels. *Cannabis Cannabinoid Res.* 5 (4), 305–317. doi:10.1089/can.2019.0099
- Ibrahim, E., Wang, M., Radwan, M., Wanas, A., Majumdar, C., Avula, B., et al. (2019). Analysis of Terpenes in Cannabis Sativa L. Using GC/MS: Method Development, Validation, and Application. *Planta Med.* 85 (5), 431–438. doi:10.1055/a-0828-8387
- Jiang, H., Ahmed, C. M. S., Martin, T. J., Canchola, A., Oswald, I. W. H., Garcia, J. A., et al. (2020). Chemical and Toxicological Characterization of Vaping Emission Products from Commonly Used Vape Juice Diluents. *Chem. Res. Toxicol.* 33 (8), 2157–2163. doi:10.1021/acs.chemrestox.0c00174
- Juul (2019). Discover More about JUULpods & Flavors. Available at: <https://www.juul.com/resources/What-is-JUUL-Vape-Liquid-All-JUUL-Pod-Flavors> (Accessed July 2, 2019).
- Kumar, P. S., Clark, P., Brinkman, M. C., and Saxena, D. (2019). Novel Nicotine Delivery Systems. *Adv. Dent Res.* 30 (1), 11–15. doi:10.1177/0022034519872475
- Laughlen, G. F. (1925). Studies on Pneumonia Following Naso-Pharyngeal Injections of Oil. *Am. J. Pathol.* 1 (4), 401407–401411.
- Layden, J. E., Ghinai, I., Pray, I., Kimball, A., Leyer, M., Tenforde, M. W., et al. (2020). Pulmonary Illness Related to E-Cigarette Use in Illinois and Wisconsin - Final Report. *N. Engl. J. Med.* 382 (10), 903–916. doi:10.1056/NEJMoa1911614
- Li, L., Duffy, B. C., Durocher, L. A., Dittmar, M. A., Acosta, R. A., Delaney, E. R., et al. (2019). Potency Analysis of Medical Marijuana Products from New York State. *Cannabis Cannabinoid Res.* 4 (3), 195–203. doi:10.1089/can.2018.0037
- Margham, J., McAdam, K., Forster, M., Liu, C., Wright, C., Mariner, D., et al. (2016). Chemical Composition of Aerosol from an E-Cigarette: A Quantitative Comparison with Cigarette Smoke. *Chem. Res. Toxicol.* 29 (10), 1662–1678. doi:10.1021/acs.chemrestox.6b00188
- Muthumalage, T., Friedman, M. R., McGraw, M. D., Ginsberg, G., Friedman, A. E., and Rahman, I. (2020). Chemical Constituents Involved in E-Cigarette, or Vaping Product Use-Associated Lung Injury (EVALI). *Toxics* 8 (2), 25. doi:10.3390/toxics8020025
- NCSL (2021). State Medical Marijuana Laws. Washington, DC: National Conference of State Legislatures. Available at: <http://www.ncsl.org/research/health/state-medical-marijuana-laws.aspx>.
- NYSDOH (2019). *New York State Department of Health Announces Update on Investigation into Vaping-Associated Pulmonary Illnesses*. Albany, NY: New York State Department of Health. Available at: https://www.health.ny.gov/press/releases/2019/2019-09-05_vaping.htm.
- NYSDOH (2018c). *Measurement of Phytocannabinoids in Medical Marijuana Using HPLC-PDA*. NYS DOH MML-300; Revision 3. Albany, NY, USA: New York State Department of Health—Wadsworth Center. Available at: <https://www.wadsworth.org/sites/default/files/WebDoc/NYS%20DOH%20MML-300-03.pdf>.
- NYSDOH (2018b). *New York State Department of Health—Wadsworth Center. Determination of the Pesticide Myclobutanil and the Synergist Piperonyl Butoxide in Medical Marijuana Using LC-MS/MS with Positive Ion Detection*. NYS DOH MML-307-02. Revision 4. Albany, NY, USA: New York State Department of Health—Wadsworth Center. Available at: <https://www.wadsworth.org/sites/default/files/WebDoc/NYS%20DOH%20MML-307-02.pdf>.
- NYSDOH (2018a). *New York State Department of Health—Wadsworth Center. Measurement of Mycotoxins in Medical Marijuana by LC-MS/MS*. Albany, NY, USA: New York State Department of Health—Wadsworth Center. Available at: <https://www.wadsworth.org/sites/default/files/WebDoc/NYS%20DOH%20MML-303-02.pdf>.
- Pankow, J. F., Duell, A. K., and Peyton, D. H. (2020). Free-Base Nicotine Fraction afb in Non-aqueous versus Aqueous Solutions: Electronic Cigarette Fluids without versus with Dilution with Water. *Chem. Res. Toxicol.* 33 (7), 1729–1735. doi:10.1021/acs.chemrestox.0c00008
- Schwotzer, D., Gigliotti, A., Irshad, H., Wendy Dye, W., Dye, W., and McDonald, J. (2021). Phytol, Not Propylene Glycol, Causes Severe Pulmonary Injury after Inhalation Dosing in Sprague-Dawley Rats. *Inhalation Toxicol.* 33 (1), 33–40. doi:10.1080/08958378.2020.1867260
- Sherkheili, M. A., Vogt-Eisele, A. K., Bura, D., Beltrán Márques, L. R., Gisselmann, G., and Hatt, H. (2010). Characterization of Selective TRPM8 Ligands and Their Structure Activity Response (S.A.R) Relationship. *J. Pharm. Pharm. Sci.* 13 (2), 242–253. doi:10.18433/j3n88n

- Wang, J., Chow, W., Wong, J. W., Leung, D., Chang, J., and Li, M. (2019). Non-target Data Acquisition for Target Analysis (nDATA) of 845 Pesticide Residues in Fruits and Vegetables Using UHPLC/ESI Q-Orbitrap. *Anal. Bioanal. Chem.* 411, 1421–1431. doi:10.1007/s00216-019-01581-z
- Wang, T., Wang, B., and Chen, H. (2014). Menthol Facilitates the Intravenous Self-Administration of Nicotine in Rats. *Front. Behav. Neurosci.* 8, 437. doi:10.3389/fnbeh.2014.00437
- Wu, D., and O'Shea, D. F. (2020). Potential for Release of Pulmonary Toxic Ketene from Vaping Pyrolysis of Vitamin E Acetate. *Proc. Natl. Acad. Sci. USA* 117 (12), 6349–6355. doi:10.1073/pnas.1920925117

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New Analytical Method for Quantifying Flavoring Chemicals of Potential Respiratory Health Risk Concerns in e-Cigarette Liquids

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Numerous flavoring chemicals are added to e-cigarette liquids to create various flavors. Flavorings provide sensory experience to users and increase product appeal; however, concerns have been raised about their potential inhalation toxicity. Estimating potential health risk of inhaling these chemicals has been challenging since little is known about their actual concentrations in e-cigarette products. To date, a limited number of analytical methods exist to measure the concentrations of flavoring chemicals in e-cigarette products. We have developed an analytical method that accurately and precisely measures the concentrations of 20 flavoring chemicals of potential inhalation risk concerns: 2,3,5-trimethylpyrazine, acetoin, benzaldehyde, benzyl alcohol, butanoic acid, dl-limonene, ethyl maltol, ethyl salicylate, ethyl vanillin, eucalyptol, eugenol, furaneol, isovanillin, l-menthol, maltol, methyl salicylate, pulegone, *trans*-cinnamaldehyde, triacetin, and vanillin. Calibration and QC solutions were prepared in 50:50 propylene glycol (PG):vegetable glycerin (VG) and 5% H₂O and flavoring concentrations ranging from 0.02 to 10.00 mg/ml. Samples of commercial e-cigarette liquids, calibration and QC solutions were combined with 30 μ L of an internal standard mix (benzene-d₆, pyridine-d₅, chlorobenzene-d₅, naphthalene-d₈ and acenaphthene-d₁₀; 1 mg/ml each) and were diluted 100-fold into methanol. Analysis was performed on an Agilent 7890B/7250 GC/Q-TOF using a DB-624UI column (30 m x 0.25 mmID x 1.4 μ m film thickness), with a total runtime of 13.5 min. Calibration curves were fit using a weighted quadratic model and correlations of determination (r^2) values exceeded 0.990 for all chemicals. Bias and precision tests yielded values less than 20% and lower limits of quantitation (LLOQ) ranged from 0.02 to 0.63 mg/ml. Over 200 commercially available products, purchased or collected from adult e-cigarette users and spanning a range of flavor categories, were evaluated with this method. Concentrations of pulegone, a suspected carcinogen, varied from below limit of quantitation (BLOQ) to 0.32 mg/ml, while acetoin and vanillin, known precursors to more cytotoxic byproducts, ranged from BLOQ to 1.52 mg/ml and from BLOQ to 16.22 mg/ml, respectively. This method features a wide dynamic working range and allows for a rapid routine analysis of flavoring additives in commercial e-cigarette liquids.

Keywords: flavors, flavorings, flavoring chemicals, electronic cigarettes, e-cigarettes, E-liquids, E-cigarette refill solutions, vaping (Min 5–Max 8)

INTRODUCTION

Flavoring chemicals are a main constituent of e-cigarette liquids and help impart either a characteristic flavor or contribute to the overall sensory experience of e-cigarette users (vapers). Over 7,000 e-cigarette liquid flavors are available to consumers (Zhu et al., 2014), with unlimited variations of added flavoring chemicals and their concentrations. The accessibility of flavors has led to higher likability of e-cigarette products (Kim et al., 2016) and higher initiation rates of vaping (Leventhal et al., 2019). As a result, an increase in preference and usage of flavored e-cigarettes has been shown among multiple population groups (Harrell et al., 2017), observed most strikingly among the youth population (Ambrose et al., 2015; Harrell et al., 2017). Users more frequently cite flavors as their reason for initiation and usage (Pepper et al., 2016; Harrell et al., 2017; Russell et al., 2018) and the exposure from increased consumption of flavored liquids and their chemical flavorings, is worrisome from a public health perspective.

Previous qualitative methods have established the identities of the flavoring chemicals commonly used in tobacco products (Krüsemann et al., 2018), including e-cigarette liquids (Hutzler et al., 2014; Czoli et al., 2019). Among the most frequently reported flavorings in e-cigarette liquids, aldehydes (ethyl vanillin, vanillin, benzaldehyde and cinnamaldehyde), alcohols (l-menthol, benzyl alcohol and furaneol), esters (triacetin), ketones (ethyl maltol, maltol, acetoin) and terpenes (limonene, pulegone) are the most common (Hutzler et al., 2014; Tierney et al., 2016; Behar et al., 2018; Omaiye et al., 2019b; Czoli et al., 2019; Hua et al., 2019; Krüsemann et al., 2021). While considered “generally recognized as safe” (GRAS) for consumption, concerns have been raised about the potential inhalation toxicity associated with these chemicals. Initial findings suggest a link between inhalation toxicity of e-cigarettes and flavorings (Bahl et al., 2012; Leigh et al., 2016), while more recent *in vitro* studies have demonstrated specific chemicals such as cinnamaldehyde, benzaldehyde, ethyl vanillin, ethyl maltol and vanillin to be highly cytotoxic to respiratory cells (Behar et al., 2018; Hua et al., 2019; Rickard et al., 2021), as well as disruptive to normal cellular and immune function (Gerloff et al., 2017; Hickman et al., 2019). Selected flavoring chemicals have been also shown to form free radicals when heated in e-cigarette devices (Muthumalage et al., 2017; Bitzer et al., 2018), as well as known carcinogens such as benzene (Pankow et al., 2017), while others react with solvents used in e-cigarette liquids to form more highly cytotoxic acetal byproducts (Erythropel et al., 2019; Jabba et al., 2020). Further, ethyl vanillin and vanillin have been revealed to contain potentially addictive properties (Truman et al., 2019).

While exposure to flavoring chemical classes such as aldehydes and alcohols are more widely studied, the use of additional chemicals in e-cigarette liquids are also concerning. For example, pulegone is a suspected carcinogen at high concentrations and has subsequently been banned from food products in 2018 (Kidwell, 2018). Its presence in e-cigarette liquids; however, remains unregulated as the margin of exposure in some marketed e-cigarette liquids have been shown to far exceed that found in food (Jabba and Jordt,

2019). Further, acetoin is a known precursor to diacetyl formation in e-cigarette liquids (Vas et al., 2019), where diacetyl has been identified in a large number of sweet flavored liquids (Farsalinos et al., 2015). Importantly, occupational inhalation of diacetyl has previously demonstrated to cause serious human respiratory outcomes (bronchiolitis obliterans) (Kreiss et al., 2002). Similarly, the addition of triacetin to e-cigarette liquids has been correlated with increases in harmful smoke constituents, such as formaldehyde hemiacetals, acrolein and acetaldehyde (Vreeke et al., 2018), owing to the degradation of triacetin at high temperatures (Laino et al., 2012).

As research continues to focus on the health effects of flavoring chemicals used in flavored e-cigarette liquids, accurately estimating potential toxicity has been challenging since concentrations of these chemicals are generally not known. E-cigarette liquid manufacturers are not required to report chemical constituents or concentrations. Several studies have published concentrations of common flavoring chemical additives; however, validation of the methods used to determine accuracy of the reported results are limited. This study aimed to develop an accurate and highly efficient method, spanning a wide concentration range for the following 20 chemicals commonly found in e-cigarette liquids that are of potential inhalation concern: 2,3,5-trimethylpyrazine, acetoin, benzaldehyde, benzyl alcohol, butanoic acid, dl-limonene, ethyl maltol, ethyl salicylate, ethyl vanillin, eucalyptol, eugenol, furaneol, isovanillin, l-menthol, maltol, methyl salicylate, pulegone, *trans*-cinnamaldehyde, triacetin and vanillin. Using this validated method, over 200 commercial e-cigarette liquids were assessed for concentrations of these 20 chemicals.

MATERIALS AND METHODS

Chemicals

Neat standards for the 20 flavoring chemicals and internal standards were purchased from Acros Organics (Fair Lawn, NJ), Alfa Aesar (Haverhill, MA), Cambridge Isotopes (Tewksbury, MA), Santa Cruz Biotechnology (Dallas, TX), Sigma Aldrich (St. Louis, MO), and TCI (Portland, OR) (**Supplementary Table S1**). The solvents methanol (LCMS-grade) and water (HPLC-grade) were obtained from Fisher Chemical (Waltham, MA), propylene glycol (PG) from Acros Organics and vegetable glycerin (VG) from Alfa Aesar.

Preparation of Working Solutions

A solvent solution of 50:50 (%:%) propylene glycol (PG) and vegetable glycerin (VG) was first prepared by combining 475 ml of each along with 50 ml of HPLC-grade water and mixing for 15 min using a magnetic stir plate (Fisher Scientific Isotemp™, Waltham, MA) at 350 rpm and ambient temperature. The solution settled for 0.5 h to allow removal of trapped air bubbles from the mixing process. This solution was utilized for subsequent preparation of calibration, quality control and fortified matrix samples used in the validation of this method.

Storage of the solution was in ambient dark conditions and prepared as needed.

A 20 mg/ml working solution containing the follow chemicals was prepared by weighing 2.0000 ± 0.0005 g of each solid neat standard using a precision balance (0.008–220 g, Mettler-Toledo, Columbus, OH) and dissolving into methanol: acetoin, ethyl maltol, ethyl vanillin, furaneol, isovanillin, maltol, l-menthol and vanillin. L-menthol was first crushed to a fine powder using a ceramic mortar and pestle. The mixture was hand-vortexed at 3,200 rpm using a vortex mixer (Fisher Scientific) for a minimum of 5 min or until all visible granules were dissolved. The working solution was stored in dark, 4 °C conditions and prepared monthly.

A 1 mg/ml working internal standard (IS) solution was prepared into methanol, where 100 μ L of benzene-d₆, chlorobenzene-d₅ and pyridine-d₅ and 100.0 ± 0.05 mg of naphthalene-d₈ and acenaphthene-d₁₀ were measured and the solution hand-vortexed for a minimum of 5 min. Internal standard solution was kept in ambient dark conditions and prepared yearly.

Preparation of Working Calibration and Quality Control Standards

Ten calibration and nine quality control (QC) concentrations were prepared ranging from 0.02 to 10.00 mg/ml and 0.03–8.00 mg/ml, respectively by serial dilutions (2-fold) starting with the most concentrated level (**Supplementary Table S2**). Here, 100 and 80 μ L of each liquid neat standard (**Supplementary Table S1**) and 4 ml of the 20 mg/ml working solution were gently mixed with 3.7 and 4.96 ml of 50:50 PG:VG solution, respectively for 0.5 h using a vertical multi-function rotator (Grant Instruments, Shepreth, United Kingdom). Given the high concentration of the flavoring chemicals in the calibration standards as well as high concentrations expected in e-cigarette liquids, detector saturation with direct injection was of concern. To reduce this effect, each standard was diluted 100-fold prior to injection using similar methodology to dilute-and-shoot LCMS (Greer et al., 2021), by adding 30 μ L of each to 30 μ L of internal standard solution and 3 ml of methanol. Calibration and QC standards were stored in 4 °C dark conditions and prepared monthly.

Preparation of Fortified Matrix Samples for Method Validation

Fortified matrix samples to validate the bias and precision of the method were prepared in triplicate at the following three concentrations within the instrument linear range: 1. approximately 3 times the lowest level, 2. middle of the range 3. within at least 70% of the highest level. Given the complexity of the ranges, this required the preparation of six fortified samples (at concentrations of 0.04, 0.10, 0.88, 1.75, 3.50 and 7.00 mg/ml). The most concentrated fortified sample was prepared first by adding each neat standard (70 μ L) and 20 mg/ml working standard (3.5 ml) into the PG:VG solution (5.59 ml) and gently mixing for 0.5 h, followed by subsequent serial dilutions. To assess the lower limit of quantitation (LLOQ) for

each chemical, additional fortified matrix samples were prepared at concentrations 0.5–2 times the lowest level in the instrument linear range. This required the preparation of five samples (at concentrations of 0.01, 0.04, 0.07, 0.10 and 0.27 mg/ml) and were prepared from individual dilutions of a working intermediate (1 mg/ml). Dilution capability of the method was evaluated by preparing independent fortified matrix samples at concentrations of 5 and 10 mg/ml, using previously described procedures, and 18.6 mg/ml, where solid and liquid neat standards were dissolved directly into 50:50 PG:VG. Similar to calibration and QC standards, all fortified matrix samples were diluted 100-fold with methanol and internal standard solution prior to analysis.

Selection of Commercial E-Cigarette Liquids

To test the capacity of the method, previously obtained e-cigarette liquids were selected for analysis based on the availability of popular flavors. Roughly half of the liquids were either purchased online or in vape shops (53%), while the remainder (47%) were collected from participants from observational studies of adult e-cigarette users. Most liquids were from the US (90%), with several from Australia (7%), the United Kingdom (3%) and one liquid from Canada. This included 215 in total and incorporated 13 of 16 flavor categories from a recently published e-cigarette liquid flavor wheel (Krüsemann et al., 2019), increasing the probability of detecting the targeted flavorings chemicals of this method. Such flavor categories from the flavor wheel included Fruit (further delineated as tropical, berry, citrus and other), Dessert, Candy, and Menthol/Mint, in addition to Tobacco. E-cigarette liquids were stored in 4 °C dark conditions prior to analysis and were brought to room temperature and mixed for 1 hour using a vertical multi-function rotator. As with the calibration standards and fortified matrix samples, e-cigarette liquids were diluted 100-fold with methanol and internal standard solution prior to analysis.

Instrumental Analysis

Sample analysis was performed on a 7890B/7250 GC/Q-TOF (Agilent Technologies, Santa Clara, CA), equipped with a PAL RSI 120 autosampler (CTC Analytics, Zwingen, Switzerland). Separation of chemicals was achieved using an Agilent DB-624UI (30 m x 0.25 mmID x 1.4 μ m film thickness) column. To ensure adequate settling of the stationary phase between injections and more reproducible retention times of early eluting chemicals, the column equilibrated for 2 min at initial conditions between injections. After pre-rinsing the needle with methanol, 1 μ L of the prepared aliquot was injected into 320 °C with a split of 20:1. Initial oven conditions started at 60 °C and were held for 1 min. The temperature was then increased at a rate of 30 °C/min until reaching 225 °C and held for an additional 4 min. Post-acquisition, the oven was ramped to 280 °C and held for 1 min to help clean residual carryover. Total analysis time was 13.5 min. Elution from the column into the mass spectrometer occurred at 250 °C and source and quadrupole temperatures were held at 230 °C and 150 °C, respectively. Positive ionization was performed

using low-EI (15 eV) with emission of 0.2 μ A. Q/TOF scan range was between 50 and 250 amu, with acquisition rate and time of five spectra/sec and 200 ms/spectrum, respectively. Carrier flow (helium) was held constant at 2.0 ml/min, while quench (helium) and collision (nitrogen) gases were held at 2.0 ml/min and 1.5 ml/min, respectively. Needle rinses post-injection were first in acetone and followed by methanol.

Each acquisition batch consisted of up to 75 e-cigarette liquids, as well as one complete set of calibration (0.02–10.00 mg/ml) and QC (0.03–8.00 mg/ml) standards injected prior to the e-cigarette liquids and one complete set injected after. Using the responses from both sets of calibration standards, a calibration curve was plotted to measure the concentrations of chemicals identified in the e-cigarette liquids in the batch. Data review was performed using Agilent MassHunter software (Quantitation, v10.2) and automated method processing. Computer generated peak assignments and integrations were reviewed and corrected when applicable. Calibration curves were plotted for each chemical, using peak area and the internal standard method. Commercial liquids with chemicals exceeding the upper quantitation limit of the calibration curve range were diluted to a concentration near the middle of the calibration range (1 mg/ml). Dilutions of 2, 5 and 10X were performed with reduction of initial e-cigarette liquid volume, while dilutions of 20 and 50X also required adjusted final volumes of the solvent. To account for differing final volumes, the volume of the internal standard solution added was adjusted likewise to allow recovery within $\pm 20\%$ compared to the calibration. Diluted liquids were re-injected with corresponding calibrators and QC.

METHOD VALIDATION

Validation of this method followed recommendations from the Scientific Working Group for Forensic Toxicology (SWGTOX) (Scientific Working Group for Forensic, 2013). To assess the suitability of internal standard concentration and assignments, relative response factors (RRF) across calibration standards for each chemical were calculated using the following equation:

$$RRF = \frac{A_s \times C_{is}}{A_{is} \times C_s}$$

Where:

A_s = area response of the chemical.

A_{is} = area response of the internal standard.

C_s = concentration of the chemical.

C_{is} = concentration of the internal standard.

Relative standard error (RSE) was calculated to determine the acceptability of the curve models, using the following equation:

$$\% RSE = 100 \times \sqrt{\frac{\sum_{i=1}^n \left[\frac{x'_i - x_i}{x_i} \right]^2}{(n - p)}}$$

Where:

n = number of calibration points

x_i = expected concentration of chemical in calibration level i

x'_i = measured concentration of chemical in calibration level i
 p = number of term in the fitting equation (average = 1, linear = 2, quadratic = 3).

For all 20 chemicals, $p = 3$

Fortified matrix samples were analyzed in triplicate per batch, where seven batches in total were injected on separate days. Percent recovery of each calibrator, QC and fortified matrix sample were compared within batch (inter-day) and between batches (intra-day) and coefficient of variation (CV) was used to determine precision and accuracy, where a range of $\pm 20\%$ was considered acceptable (Scientific Working Group for Forensic, 2013). Precision was calculated using the following equation:

$$\% CV = \frac{\text{Average Concentration}}{\text{Standard Deviation}} \times 100$$

Bias was determined using the following equation:

$$\% Bias = \frac{\text{Average Concentration} - \text{Expected Concentration}}{\text{Expected Concentration}} \times 100$$

LLOQ values for the working calibration range were primarily determined from the lowest calibration level meeting $\pm 20\%$ recovery and CV, and secondly from the results of the LLOQ and carryover analyses. Carryover was assessed with three 50:50 PG:VG and three methanol blank matrix samples following injection of the highest working standards (8 and 10 mg/ml). Dilution capability was determined by targeting concentrations within the working calibration range with two- and 5-fold dilutions of each the 5, 10 and 18.6 mg/ml fortified samples, while 10- and 50-fold dilutions were performed on the 10 mg/ml and 18.6 ml samples only. Stability was assessed by percent recovery of several calibrators injected after 1 month against a newly prepared calibration.

RESULTS

Chemical Identification

Spectral identification and retention times (RTs) were established from independent analysis of each chemical (Table 1). The largest ion was selected for quantitation when possible and secondary ions were chosen at a minimum relative abundance of 10% of the quantitation ion, with exception of benzene-d6 (6.2%). Five chemicals were further assigned tertiary ions meeting the minimum threshold. A m/z window of ± 10 ppm was applied to allow slight instrument measurement variations. The earliest eluting peak after internal standard benzene-d6 (2.722 min) was acetoin (3.382 min) while the concluding peak isovanillin eluted at 8.747 min (Figure 1). PG and VG, while not included in the calibration, were observed at RTs of 3.759 and 5.599 min, respectively. Several unknown peaks were observed at RTs of 5.892, 6.431, 6.929, 7.603 and 9.130 min. Tentative identification by comparing spectral breakdown (with the most abundant ions of 121.0654, 123.0810, 121.0655, 147.0810 and 167.0709 m/z , respectively) to the National Institute of Standards and Technology (NIST) library suggest these may be benzaldehyde

TABLE 1 | Retention time, Resolution, Quantitation and Qualitative Ions and Internal Standard Assignments for Twenty Flavoring Chemicals.

	CAS	Molecular Formula	Molecular Weight (g/mol)	RT (min)	Peak resolution ^a	m/z (± 10 ppm)					IS Assignment
						1 ^o (Quant)	2 ^o (Qual)	Relative abundance (%)	3 ^o (Qual)	Relative abundance (%)	
Acetoin	513-86-0	C4H8O2	88.11	3.382	4.08	88.0530	73.0295	15.0	---	---	Benzene-d6
Butanoic Acid	107-92-6	C4H8O2	88.11	3.928	10.96	60.0211	73.0293	30.0	---	---	Pyridine-d5
Benzaldehyde	100-52-7	C7H6O	106.12	5.141	2.35	106.0409	77.0395	84.0	105.0335	11.9	Chlorobenzene-d5
2,3,5-Trimethylpyrazine	14667-55-1	C7H10N2	122.17	5.203	3.96	122.0834	81.0583	11.1	---	---	Chlorobenzene-d5
D-Limonene	5989-27-5	C10H16	136.23	5.287	3.91	93.0705	121.1022	28.5	136.1257	33.5	Chlorobenzene-d5
L-Limonene	5989-54-8	C10H16	136.23	5.287	3.91	93.0705	121.1022	28.5	136.1257	33.5	Chlorobenzene-d5
Eucalyptol	470-82-6	C10H18O	154.25	5.381	5.73	139.1129	154.1363	90.2	---	---	Chlorobenzene-d5
Benzyl Alcohol	100-51-6	C7H8O	108.14	5.657	3.58	108.0566	79.0546	33.1	107.0498	62.0	Chlorobenzene-d5
Furaneol	3658-77-3	C6H8O3	128.13	5.738	17.42	128.0478	85.0294	38.9	---	---	Chlorobenzene-d5
Maltol	118-71-8	C6H6O3	126.11	6.132	8.74	126.0310	71.0136	15.2	---	---	Chlorobenzene-d5
L-Menthol	2216-51-5	C10H20O	156.26	6.334	6.31	123.1180	109.1024	37.7	138.1415	64.8	Chlorobenzene-d5
Methyl Salicylate	119-36-8	C8H8O3	152.15	6.489	4.52	120.0206	152.0469	79.1	---	---	Naphthalene-d8
Ethyl Maltol	4940-11-8	C7H8O3	140.14	6.597	6.75	140.0471	139.0399	32.1	---	---	Naphthalene-d8
(+)-Pulegone	89-82-7	C10H16O	152.23	6.752	5.51	81.0706	152.1203	87.2	---	---	Naphthalene-d8
Ethyl Salicylate	118-61-6	C9H10O3	166.17	6.887	2.02	120.0204	166.0624	64.4	---	---	Naphthalene-d8
trans-Cinnamaldehyde	104-55-2	C9H8O	132.16	7.072	6.56	131.0499	132.0576	56.5	---	---	Naphthalene-d8
Triacetin	102-76-1	C9H14O6	218.20	7.230	8.86	103.0398	145.0506	62.8	---	---	Naphthalene-d8
Eugenol	97-53-0	C10H12O2	164.20	7.459	6.25	164.0830	149.0607	19.4	---	---	Naphthalene-d8
Vanillin	121-33-5	C8H8O3	152.15	8.130	14.91	152.0474	151.0399	55.4	---	---	Naphthalene-d8
Ethyl Vanillin	121-32-4	C9H10O3	166.17	8.571	0.33	137.0244	138.0321	66.6	---	---	Naphthalene-d8
Isovanillin	621-59-0	C8H8O3	152.50	8.747	11.91	152.0475	151.0400	59.4	---	---	Acenaphthene-d10
Internal Standards (IS)											
Benzene-d6	1076-43-3	C6H6	84.15	2.722	27.66	84.0851	85.0886	6.2	---	---	N/A
Pyridine-d5	7291-22-7	C5H5N	84.13	3.477	7.53	84.0739	56.0568	13.0	---	---	N/A
Chlorobenzene-d5	3114-55-4	C6H5Cl	117.59	4.184	42.81	117.0396	119.0369	29.5	---	---	N/A
Naphthalene-d8	1146-65-2	C10H8	136.22	6.478	0.58	136.1131	137.1168	10.1	---	---	N/A
Acenaphthene-d10	15067-26-2	C12H10	164.27	8.585	5.40	164.1416	165.1449	11.8	---	---	N/A

^aCalculated using peak width (W) (determined from full width at half maximum height (FWHM)) and retention time (RT) from the peak immediately following, using the equation $R_s = (RT_2 - RT_1) / 0.5(W_1 + W_2)$.

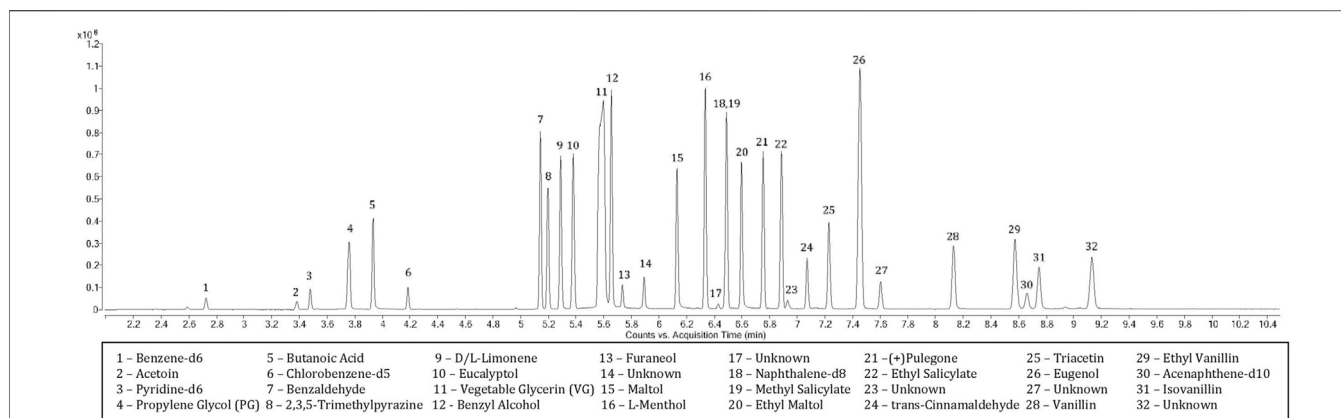


FIGURE 1 | Total Ion Chromatogram of Targeted Flavoring Chemicals and Internal Standards.

dimethyl acetal (CAS# 1125-88-8), isopulegone (CAS# 29606-79-9), 2'-hydroxybutyrophenone (CAS# 2887-61-8), cinnamaldehyde dimethyl acetal (CAS# 4364-06-1) and 4-(2-hydroxyethyl)-2,6-dimethoxyphenol (CAS# 20824-4-7), respectively. Identities of these peaks were determined from the analysis of the total ion chromatogram (TIC) rather than through deconvolution processes, which could have revealed additional peaks at the specified retention times. Further, because the NIST was developed using nominal mass and an ionization energy of 70eV, the spectral match to the data produced with high resolution (accurate) mass and low eV may not adequately identify these peaks. Peak resolution, calculated from the difference in retention times of the later and earlier eluting chemicals, divided by the average of the peak widths (Carle, 1972), was greater than 1.5 between each extracted ion current profile (EICP) with exception of ethyl vanillin and naphthalene-d8 (Table 1). Internal standard assignments are listed in Table 1.

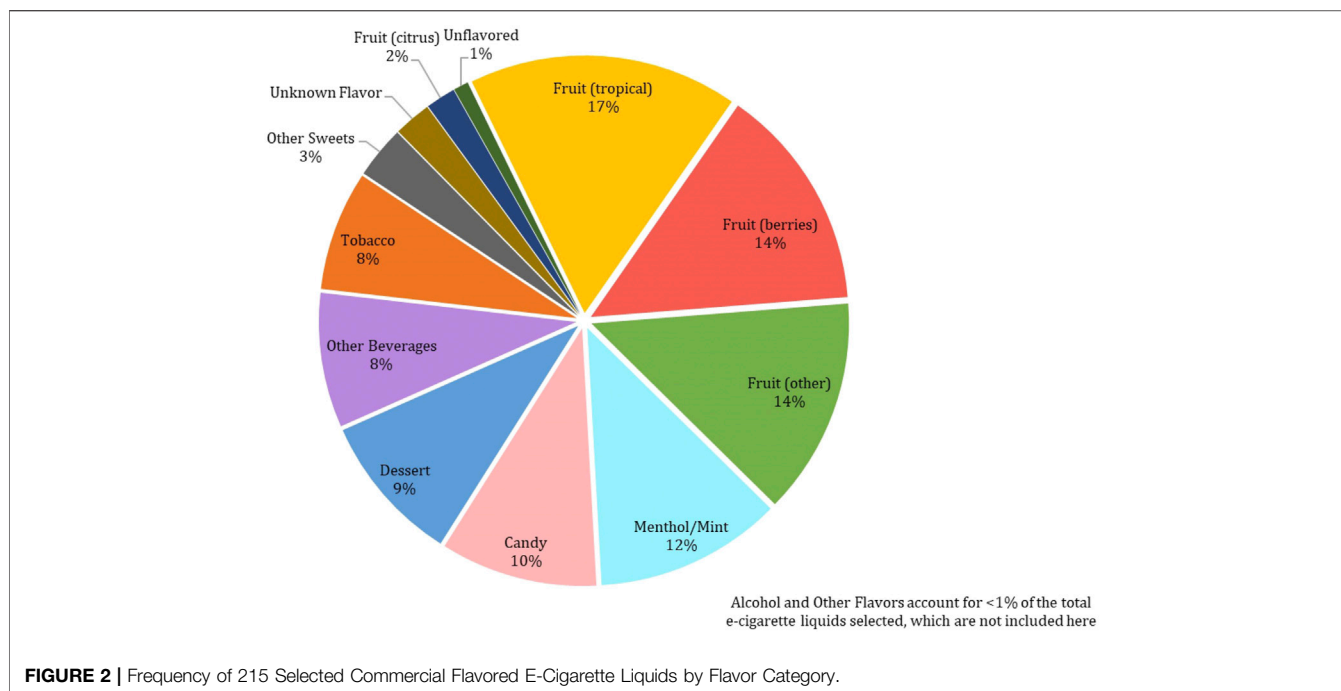
Calibration

Coefficient of determination (r^2) values measured among the seven batches consistently exceeded 0.990 for all chemicals when fit using a weighted quadratic ($1/x^2$) model (Supplementary Table S3, Supplementary Figure S1). Average RRF ranged from 0.03 (acetoin) to 0.70 (2,3,5-trimethylpyrazine) and RSE averaged below 10% for each chemical. Instrument linearity was established over the entire final concentration range (after 100-fold dilution) of 0.02 and 10 mg/ml for 20% of the chemicals. Benzyl alcohol, furaneol and *trans*-cinnamaldehyde reached detector saturation at a final concentration of 5 mg/ml, while the remaining chemicals met linearity up to 10 mg/ml but had varying lower limits (Supplementary Table S3). The lowest values in the instrument linear range were determined from average percent recovery and bias (CV) across seven calibrations (Supplementary Table S4). Quality control levels within the established instrumental range recovered within $\pm 20\%$ for each chemical. For 60% of the chemicals, the working calibration range met the same range as the instrument linear range. Butanoic acid, ethyl maltol, ethyl vanillin, isovanillin,

maltol, *trans*-cinnamaldehyde, triacetin and vanillin each had tighter working calibration ranges (Supplementary Table S3), where additional lower levels were excluded based on results from the LLOQ and carryover method validation analyses.

Method Validation

Fortified matrix samples (0.04, 0.10, 0.88, 1.75, 3.5 and 7.0 mg/ml) showed high precision and low bias (within $\pm 20\%$) when compared within and between batches for 95% (19/20) of chemicals (Supplementary Table S5). Eucalyptol was within $\pm 30\%$ among three batches, while intra-day precision and bias remained within 20%. Greater variability was observed with recoveries of LLOQ fortified matrix samples (0.01, 0.04, 0.07, 0.10 and 0.27 mg/ml) where nine chemicals exceeded $\pm 20\%$ inter- and/or intra-batch precision (Supplementary Table S5). However, for seven of these chemicals, variability ($\pm 30\%$) was observed only in samples with concentrations below the instrumental linear range, where recoveries were considered estimated. Triacetin exceeded 30% inter- and intra-batch precision in 5/7 batches, resulting in a reduced working calibration range for this chemical. Bias across batches was within $\pm 20\%$ for all chemicals except eugenol, where two LLOQ fortified matrix samples (0.10 and 0.27 mg/ml) recovered within $\pm 30\%$ of the true concentration. Carryover was observed for butanoic acid, ethyl maltol, ethyl vanillin, isovanillin, maltol and vanillin when PG:VG blanks were analyzed proceeding concentrated samples (Supplementary Table S6). Likewise, ethyl maltol, isovanillin, maltol, *trans*-cinnamaldehyde and vanillin demonstrated carryover in methanol blanks. Accordingly, LLOQ values in the working calibration range for these chemicals were raised so that carryover accounted for $< 5\%$ of the measured concentration. Dilutions of two- and 5-fold of each concentrated standard yielded intra-batch precision within $\pm 20\%$ for each chemical (Supplementary Table S7), except for benzyl alcohol and furaneol. Likewise, 10-fold dilutions of the 10 and 18.6 mg/ml samples were highly precise, with benzyl alcohol the sole chemical with higher variability between batches. Among the 50-fold dilutions, high precision was observed for 16/20 chemicals.



Bias among all dilutions was within $\pm 20\%$ for 7/20 chemicals, while 14/20 were within $\pm 30\%$. Stability over 1 month was observed in 18/20 chemicals where recovery was within 80–120%. Acetoin and furaneol presumably degraded, recovering below 80% of the expected concentration.

To assess the effect of the quadratic model on recovery, one acquisition batch was re-calculated after universally applying the linear ($1/x^2$) model. Subsequent recovery of each calibration standard was then compared to the previously reported result. Sixteen chemicals had r^2 values exceeding 0.990 when fit using calibration standards within the working calibration range. Further, percent recoveries were within $\pm 20\%$ of the expected concentration for each calibration and QC standard. Compared to the average recoveries reported in **Supplementary Table S4**, CV was within $\pm 20\%$ for each standard of these 16 chemicals, with exception of the lowest calibration standard (0.04 mg/ml) for triacetin (25% CV). DL-limonene, eucalyptol, furaneol and *trans*-cinnamaldehyde had calculated r^2 values of 0.853, 0.985, 0.981 and 0.971 respectively, when calculated using a linear model. Concentrations of multiple standards when calculated against the linear fit were more variable, exceeding $\pm 30\%$ recovery and CV compared to quadratic recoveries.

To determine the variation between calibration curves analyzed in the same acquisition batch, percent drift was calculated using the opening calibration as the reference. Here, concentration of each calibration standard in the second calibration was subtracted from the corresponding standard from the first calibration and divided by the concentration of the first standard. The resulting drift for each chemical across concentrations within their respective working calibration ranges were within $\pm 20\%$ for all chemicals except dl-limonene (41% drift with 0.08 mg/ml standard), eucalyptol (69, 29 and 27% drift for

0.04, 0.16 and 0.31 mg/ml standards, respectively), benzyl alcohol (22% drift for 0.02 mg/ml standard), l-menthol (22% drift for 0.08 mg/ml standard) and pulegone (28% drift for 0.02 mg/ml standard).

Flavoring Chemicals in Commercial E-Cigarette Liquids

Among the 215 e-cigarette liquids selected, fruit flavors were most predominately analyzed, with tropical, berry and other-flavors (such as watermelon) comprising of 17, 14 and 13%, respectively of the total liquids (**Figure 2**). Between the remaining liquids, similar distributions across flavor categories were observed, where Menthol/Mint, Candy, Dessert, Other Beverages and Tobacco flavors encompassed 12, 10, 9, 8 and 8% of the liquids, respectively. All chemicals except for ethyl salicylate, isovanillin and *trans*-cinnamaldehyde were detected above the LLOQ in at least one e-cigarette liquid. Benzyl alcohol was the most abundant chemical found (in 41% of the products), followed by ethyl maltol (32%) and triacetin (29%) (**Figure 3**). L-menthol (detected in 21% of products) had the highest average concentration (4.83 mg/ml), followed by ethyl maltol (3.84 mg/ml), vanillin (3.81 mg/ml), triacetin (3.56 mg/ml) and ethyl vanillin (3.24 mg/ml) (**Figure 4**). Eight chemicals contained average concentrations below 1 mg/ml where 2,3,5-trimethylpyrazine averaged the lowest among liquids with 0.10 mg/ml. The highest individual concentrations were found with ethyl maltol (32.49 mg/ml, Candy-flavored), triacetin (23.15 mg/ml, Citrus fruit-flavored), ethyl vanillin (19.07 mg/ml, Other Sweets-flavored) and l-menthol (19.01 mg/ml, Menthol/Mint-flavored) (**Supplementary Table S8**). The lowest concentrations identified were 2,3,5-trimethylpyrazine

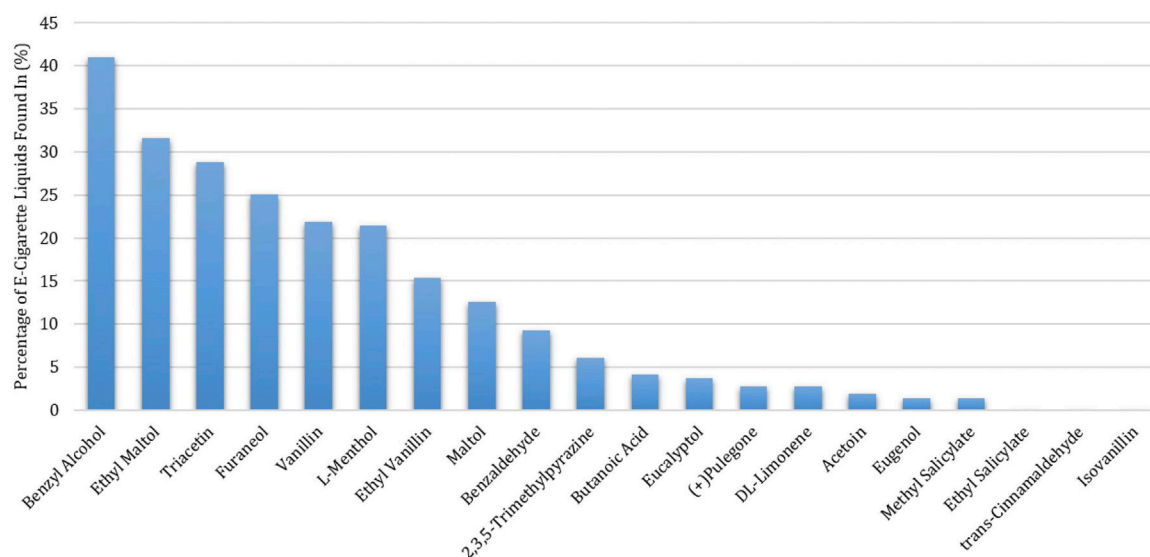


FIGURE 3 | Percentage of Detections of Twenty Flavoring Chemicals Found in 215 Commercial Flavored E-Cigarette Liquids.

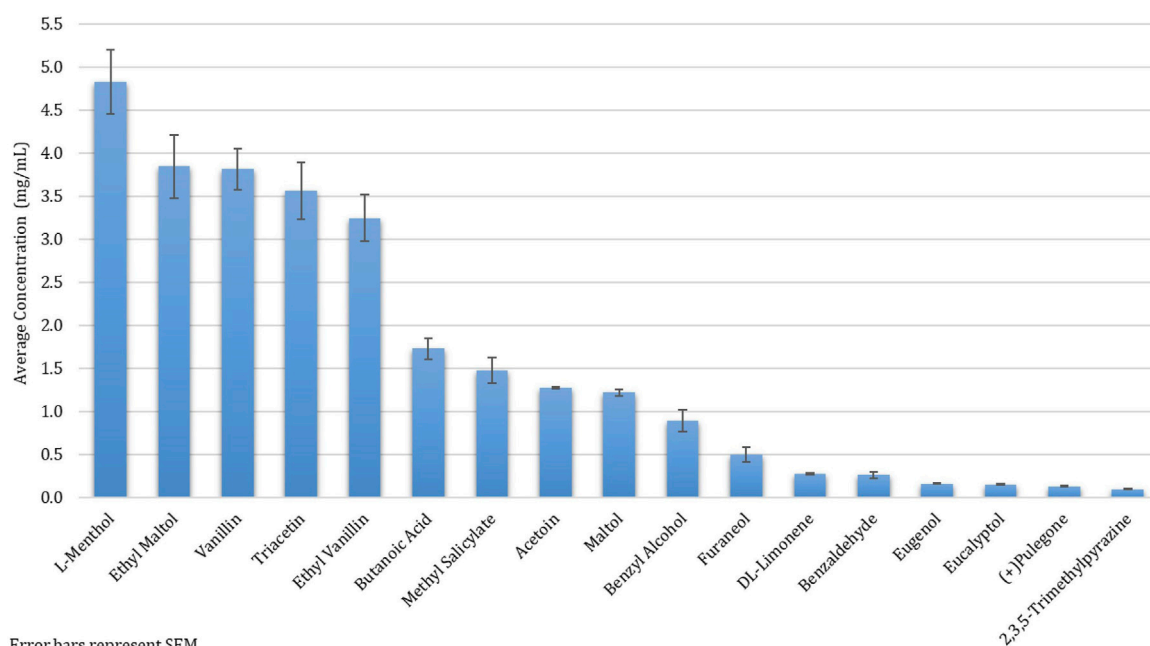


FIGURE 4 | Average Concentration of Twenty Flavoring Chemicals found in 215 Selected Commercial Flavored E-Cigarette Liquids.

and pulegone, where each were detected at 0.02 mg/ml. Several chemicals were identified in at least half of the liquids assigned to a single flavor category and included eugenol (100% in Fruit (tropical)), eucalyptol (100% in Menthol/Mint), pulegone (83% in Menthol/Mint), 2,3,5-trimethylpyrazine (62% in Tobacco), acetoin (50% in Dessert), and l-menthol (48% in Menthol/Mint).

DISCUSSION

The validated method demonstrates repeatable and accurate measurement of 20 commonly added flavoring chemicals of potential inhalation concern, where precision and accuracy of flavoring chemicals across batches were consistently within 20%. The large dynamic concentration range provides sensitivity for

multiple chemicals in a single injection, while improving efficiency by reducing the frequency of re-analyses owing to overrange concentrations. As identified with this study, concentrations can vary by at least 1000-fold. Nearly 500 detections were calculated and less than 5% of the measured e-cigarette liquids required subsequent dilution. Further, our results suggest benzyl alcohol, ethyl maltol, ethyl vanillin, l-menthol, triacetin and vanillin are more likely to be added to e-cigarette liquids in concentrations greater than 10 mg/ml. This method has demonstrated precision and accuracy within $\pm 20\%$ across multiple dilutions for these six chemicals, with exception of benzyl alcohol. Here, values greater than 20% are found predominately with dilutions of the 10 mg/ml sample, suggesting a preparation issue with this chemical.

Quadratic calibration models were selected for several chemicals, rather than using a curve splitting technique for wide calibration concentration ranges (Basu et al., 2012) resulting in expediting data review. This model was universally applied throughout the method validation process to all chemicals for consistency. Calibration curves for many chemicals, however, were observed to follow linear trajectories (**Supplemental Figure S1**). To understand the difference in curve models, re-calculation of data in a single acquisition batch was performed using linear calibration curves for each chemical. After comparison to previous quadratic-fit data, concentrations of most chemicals (16/20) did not vary substantially, indicating minimal bias with the use of the quadratic model. Future assessment of the method should include the use of linear models for these chemicals. Recoveries of dl-limonene, eucalyptol, furaneol and *trans*-cinnamaldehyde varied more substantially after applying a linear model (without the use of curve splitting techniques), indicating a clear difference in instrumental response and suggestive of potential bias in the reported quadratic-based concentrations. However, recoveries from the fortified matrix samples at concentrations spanning the working calibration range were within $\pm 20\%$ of expected concentrations. Based on this validation, minimal bias is assumed with the quadratic model.

Benzyl alcohol, which had a reduced upper limit of quantitation, was relatively linear across validation batches. Two out of the seven acquisition batches used in the method validation experienced detector saturation beyond a final concentration of 5 mg/ml, for unknown reasons. Recoveries of benzyl alcohol from these batches were not excluded, leading to higher variability when assessing precision and bias. Based on these results, the upper level of quantitation (ULOQ) was lowered to 5 mg/ml. Subsequent analyses using this method has continued to show linearity beyond 5 mg/ml and method validation should be repeated for this chemical to establish high precision and low bias at a final concentration of 10 mg/ml.

Minimal carryover in methanol-only and PG/VG-only blanks injected immediately following the highest calibration standard was observed for six chemicals. Butanoic acid and ethyl vanillin appeared in PG/VG blanks only, suggesting that methanol alone, if used to assess carryover, is not sufficient to remove these chemicals from the system. Since e-cigarette liquids contain PG/VG, high concentrations of these chemicals may cause

biased results in the subsequent injection. This has not been reported in previously published studies. To reduce this effect, LLOQ values were elevated such that reported concentrations were greater than 5% of possible carryover. Increased LLOQ values reduces the sensitivity of the method; however, several of these chemicals (ethyl maltol, ethyl vanillin and vanillin) were frequently found in higher concentrations, or not detected (isovanillin and *trans*-cinnamaldehyde) and are therefore not likely affected.

Multiple internal standards were included so that elution would span the chromatographic run and allow better representation of similarly eluting target chemicals. Relative retention times (RRT) of flavoring chemicals compared to their assigned internal standard were within the EPA suggested range of 0.80–1.20 (EPA, 2014) for 50% of the chemicals, while the furthest chemical was 1.51 (l-menthol) relative to its internal standard. Relative response factors (RRF) were calculated to assess the suitability of these assignments and selected concentration. Internal standard response should fall below 100 times the response of the target chemical, corresponding to a minimum value of 0.01 and an ideal value of 1 (EPA, 2014). Our method exceeds the minimum value for all chemicals, the lowest reaching 0.03 (acetoin). Validation batches exhibited consistent RRF values (data not shown), which further demonstrates the repeatability and stability of the instrument response over time.

The analytical column used in this method (DB-624 UI) was selected for the stationary phase, which is considered of intermediate polarity (Sigma-Aldrich, 2013) and designed for the rapid separation of volatile chemicals (Agilent_Products, 2021). Ultra Inert (UI) provides improved bonding and crosslinking of the stationary phase, leading to less column bleed and a lower baseline signal. Dimensions were selected to improve the efficiency of separations, which included a column length of 30 m and internal diameter of 0.25 mm (Rahman et al., 2015). Given the high concentrations of flavoring chemicals expected in e-cigarette liquids, a relatively large film thickness (1.4 μm) was selected to allow maximal loading capacity. Increased film thickness tends to increase peak width as chemical species are retained longer on the column, leading to reduced resolution, however; the chromatography observed in our method did not suffer.

This method takes advantage of innovative low-EI technology available in GC coupled MS instrumentation. Having a reduced applied ionization voltage (15 eV versus the traditional 70 eV) lowers the energy delivered to subsequent collisions of electrons with incoming chemical species and reduces the efficiency of ionization (Lau et al., 2019). This provides a two-fold advantage. First, given the high expected abundance of most flavoring chemicals in e-cigarette liquids, fewer chemical species are ionized, reducing potential detector saturation. Secondly, given the relatively small mass of the targeted flavoring chemicals, lower voltages may lead to softer fragmentation and increased abundance of the molecular ion. A comparison between ionization energies to understand fragmentation patterns of the flavoring chemicals was not performed in this study.

Detector sensitivity to matrix interference was not a concern in this method. The 7250 GC/Q-TOF, the newest of Q-TOF

instrumentation from Agilent (currently), improves on the detector sensitivity of previous generations and has been utilized in low level detections of environmental contaminants. Here, the instrument has been shown to detect concentrations less than 10 ppb, while demonstrating sufficient separation from background noise (Agilent_Promotions, 2021). In contrast, the lowest detectable concentration required by our method is 2000-fold higher (20,000 ppb).

Few quantitative methods are available to measure concentrations of popular flavor additives in e-cigarette liquids, mainly using GC-MS (Bansal et al., 2019). Several studies followed a developed method containing 90 chemicals (Tierney et al., 2016) which was later expanded to 178 (Behar et al., 2018; Omaiye et al., 2019a; Omaiye et al., 2019b; Hua et al., 2019). Authentication standards were used to establish identifications; however, each study references the same published method for calibration procedures (Brown et al., 2014), where neither calibration or method validation information was provided. Further, this method was developed to measure chemicals in tobacco products and did not evaluate the e-cigarette liquid matrix. Likewise, an early published method used authentication standards to verify detections; however, only a three-point calibration was prepared (Schober et al., 2014) where concentrations were not reported. The wide range of concentrations found in their e-cigarette liquids combined with the lack of validation results yields uncertainty in the accuracy of the method. Conversely, the method published by Aszyk et al. includes a comprehensive method validation, offering bias and precision information for 46 chemicals, but excludes key flavoring chemicals with inhalation concern, such as ethyl maltol, ethyl vanillin, l-menthol, and vanillin (Aszyk et al., 2017). Important method parameters such as a reduced calibration range and use of acetonitrile limits time and cost effectiveness, which our method improves upon. Krüsemann et al. published a study which evaluated the validity of their method, but similarly had a limited calibration range (10-fold) and reduced target list (10 flavoring chemicals) (Krüsemann et al., 2020). In each of these published methods, the calibration curves have been prepared in the same organic solvent used to dilute the liquid. Our method is the first to prepare calibration levels using a similar matrix as the e-cigarette liquids themselves. Aszyk et al. evaluated matrix effects that impact the reported concentrations as part of their method validation. Our method accounts for this, therefore providing more accurate values for several chemicals identified with high matrix effects, such as benzyl alcohol (34%) and eugenol (133%) (Aszyk et al., 2017).

Our results confirm previous findings that high concentrations of several concerning flavoring chemicals are found in e-cigarette liquids (ethyl maltol, ethyl vanillin, triacetin, and vanillin). Further, pulegone was found almost exclusively in menthol/mint-flavored liquids at concentrations ranging from 0.02 to 0.32 mg/ml. Cinnamaldehyde, demonstrated to be highly cytotoxic (Behar et al., 2016) and disruptive to the immune response (Clapp et al., 2017), was not detected in any liquid tested in our study. This is not surprising since e-cigarette liquids with this characterizing cinnamon flavor were not included here. Our lower quantitation limits, however, allows for surveillance of

such chemicals that may be added without the purpose of characterizing taste. For example, eugenol, with a flavor descriptor of spicy clove (The_Good_Scents_Company, 1980) is a common flavoring chemical found in clove cigarettes (Stanfill et al., 2006). While clove-flavored e-cigarette liquids were not included, this chemical was measured in low concentrations (<1 mg/ml) among several liquids exclusively characterized as tropical fruit-flavored. This is concerning as eugenol in clove cigarettes has been associated with pulmonary edema (LaVoie et al., 1986; McDonald and Heffner, 1991) and further acts as an anesthetic (Guidotti et al., 1989), allowing for deeper inhalation and more severe lung effects (infection and respiratory damage) (Hendee, 1988). Presence of this chemical in e-cigarette liquids, particularly in highly popular fruit flavors (Nguyen et al., 2019), may create a similar anesthetic effect, to which we have not identified published research relevant to e-cigarette users.

Limitations

The stability of stock standards was not verified with newly purchased standards. Although storage followed vendor recommendations between use, the shelf-life of opened standards is generally unknown. This is particularly true of furaneol, where reactions with oxygen are visually observed with physical changes over time, despite storage under inert gas. Stability tests of calibration standards further demonstrates this loss, with less than 80% recovery after 1 month. Known degradation of acetoin was also observed after 1 month in calibration standards; however, the conversion to diacetyl was not assessed. Additionally, conversion of several aldehydes to their acetal forms was not determined with this method; however, acetals were observed qualitatively throughout the method validation process. Given the stability of the aldehydes in calibration solutions over 1 month, the conversion to acetals may be relatively quick as previously demonstrated (Erythropel et al., 2019). Reduced initial concentrations could lead to high biased measurements in e-cigarette liquids. Similarly, the e-cigarette liquids included here were previously obtained and degradation of flavoring chemicals are possible. Stability assessments of these 20 chemicals in commercial e-cigarette liquids is an ongoing project. Finally, the impact of PG/VG ratio in each calibration level is unknown. Since the initial level was prepared with nearly 50:50 methanol:PG/VG and increasing PG/VG volume for subsequent levels, the density differs between calibrators. However, linearity was established for all chemicals and multiple validation samples prepared with differing methanol to PG/VG ratios did not indicate substantial differences in recovery.

CONCLUSION

Our newly developed method allows for the precise and accurate measurement of a wide range of concentrations for twenty flavoring chemicals of inhalation concern in commercial flavored e-cigarette liquids. With greater accuracy in the measurement among the liquid, the percentage of conversion to the aerosol and subsequent inhalation by the user can further

assessed. This method can be applied to an assessment of inhalation exposure to flavoring chemicals in e-cigarette users.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

The methodology and design of this project was developed by MG, who also oversaw its completion. The work was performed, and manuscript drafted by MP. Review and edits of the manuscript were done by both authors.

REFERENCES

- Agilent_Products (2021). Agilent_Products DB-624 Ultra Inert Columns. Available: <https://www.agilent.com/en/product/gc-columns/ultra-inert-gc-columns/db-624-ultra-inert-columns> (Accessed September 22, 2021).
- Agilent_Promotions (2021). Agilent_Promotions Agilent 7250 GC/Q-TOF System. See the Whole Picture. Available: <https://www.agilent.com/en/promotions/7250-gc-q-tof> (Accessed September 22, 2021).
- Ambrose, B. K., Day, H. R., Rostron, B., Conway, K. P., Borek, N., Hyland, A., et al. (2015). Flavored Tobacco Product Use Among US Youth Aged 12-17 Years, 2013-2014. *JAMA* 314 (17), 1871-1873. doi:10.1001/jama.2015.13802
- Aszyk, J., Woźniak, M. K., Kubica, P., Kot-Wasik, A., Namieśnik, J., and Wasik, A. (2017). Comprehensive Determination of Flavouring Additives and Nicotine in E-Cigarette Refill Solutions. Part II: Gas-Chromatography-Mass Spectrometry Analysis. *J. Chromatogr. A* 1517, 156-164. doi:10.1016/j.chroma.2017.08.057
- Bahl, V., Lin, S., Xu, N., Davis, B., Wang, Y.-h., and Talbot, P. (2012). Comparison of Electronic Cigarette Refill Fluid Cytotoxicity Using Embryonic and Adult Models. *Reprod. Toxicol.* 34 (4), 529-537. doi:10.1016/j.reprotox.2012.08.001
- Bansal, V., Hashemi, B., Raza, N., Kim, K.-H., Raza, W., Kumar, P., et al. (2019). Review of the Analytical Methods for and Clinical Impact of Additives and Flavors Used in Electronic Cigarettes. *Expo. Health* 12 (4), 593-615. doi:10.1007/s12403-019-00331-x
- Basu, S., Basit, A., Ravindran, S., Patel, V. B., Vangala, S., and Patel, H. (2012). Split Calibration Curve: an Approach to Avoid Repeat Analysis of the Samples Exceeding ULOQ. *Bioanalysis* 4 (19), 2375-2389. doi:10.4155/bio.12.219
- Behar, R. Z., Luo, W., Lin, S. C., Wang, Y., Valle, J., Pankow, J. F., et al. (2016). Distribution, Quantification and Toxicity of Cinnamaldehyde in Electronic Cigarette Refill Fluids and Aerosols. *Tob. Control* 25 (Suppl. 2), ii94-ii102. doi:10.1136/tobaccocontrol-2016-053224
- Behar, R. Z., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2018). Analytical and Toxicological Evaluation of Flavor Chemicals in Electronic Cigarette Refill Fluids. *Sci. Rep.* 8 (1), 8288. doi:10.1038/s41598-018-25575-6
- Bitzer, Z. T., Goel, R., Reilly, S. M., Elias, R. J., Silakov, A., Foulds, J., et al. (2018). Effect of Flavoring Chemicals on Free Radical Formation in Electronic Cigarette Aerosols. *Free Radic. Biol. Med.* 120, 72-79. doi:10.1016/j.freeradbiomed.2018.03.020
- Brown, J. E., Luo, W., Isabelle, L. M., and Pankow, J. F. (2014). Candy Flavorings in Tobacco. *N. Engl. J. Med.* 370 (23), 2250-2252. doi:10.1056/NEJMc1403015
- Carle, G. C. (1972). Determination of Chromatographic Resolution for Peaks of Vast Concentration Differences. *Anal. Chem.* 44 (11), 1905-1906. doi:10.1021/ac60319a030
- Clapp, P. W., Pawlak, E. A., Lackey, J. T., Keating, J. E., Reeber, S. L., Glish, G. L., et al. (2017). Flavored E-Cigarette Liquids and Cinnamaldehyde Impair Respiratory Innate Immune Cell Function. *Am. J. Physiology-Lung Cell Mol. Physiol.* 313 (2), L278-L292. doi:10.1152/ajplung.00452.2016

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- Czoli, C. D., Goniewicz, M. L., Palumbo, M., Leigh, N., White, C. M., and Hammond, D. (2019). Identification of Flavouring Chemicals and Potential Toxicants in E-Cigarette Products in Ontario, Canada. *Can. J. Public Health* 110 (5), 542-550. doi:10.17269/s41997-019-00208-1
- EPA (2014). *SW-846 Test Method 8000D: Determinative Chromatographic Separations*. Washington, DC: US EPA.
- Erythropel, H. C., Jabba, S. V., DeWinter, T. M., Mendizabal, M., Anastas, P. T., Jordt, S. E., et al. (2019). Formation of Flavorant-Propylene Glycol Adducts with Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids. *Nicotine Tob. Res.* 21 (9), 1248-1258. doi:10.1093/ntr/nty192
- Farsalinos, K. E., Kistler, K. A., Gillman, G., and Voudris, V. (2015). Evaluation of Electronic Cigarette Liquids and Aerosol for the Presence of Selected Inhalation Toxins. *Nicotine Tob. Res.* 17 (2), 168-174. doi:10.1093/ntr/ntu176
- Gerloff, J., Sundar, I. K., Freter, R., Sekera, E. R., Friedman, A. E., Robinson, R., et al. (2017). Inflammatory Response and Barrier Dysfunction by Different E-Cigarette Flavoring Chemicals Identified by Gas Chromatography-Mass Spectrometry in E-Liquids and E-Vapors on Human Lung Epithelial Cells and Fibroblasts. *Appl. Vitro Toxicol.* 3 (1), 28-40. doi:10.1089/aivt.2016.0030
- Greer, B., Chevallier, O., Quinn, B., Botana, L. M., and Elliott, C. T. (2021). Redefining Dilute and Shoot: The Evolution of the Technique and its Application in the Analysis of Foods and Biological Matrices by Liquid Chromatography Mass Spectrometry. *Trac Trends Anal. Chem.* 141, 116284. doi:10.1016/j.trac.2021.116284
- Guidotti, T. L., Laing, L., and Prakash, U. B. (1989). Clove Cigarettes. The Basis for Concern Regarding Health Effects. *West. J. Med.* 151 (2), 220-228.
- Harrell, M. B., Weaver, S. R., Loukas, A., Creamer, M., Marti, C. N., Jackson, C. D., et al. (2017). Flavored E-Cigarette Use: Characterizing Youth, Young Adult, and Adult Users. *Prev. Med. Rep.* 5, 33-40. doi:10.1016/j.pmedr.2016.11.001
- Hendee, W. (1988). Evaluation of the Health hazard of Clove Cigarettes. *JAMA* 260, 3641. doi:10.1001/jama.1988.03410240111042
- Hickman, E., Herrera, C. A., and Jaspers, I. (2019). Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chem. Res. Toxicol.* 32 (6), 982-985. doi:10.1021/acs.chemrestox.9b00171
- Hua, M., Omaiye, E. E., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2019). Identification of Cytotoxic Flavor Chemicals in Top-Selling Electronic Cigarette Refill Fluids. *Sci. Rep.* 9 (1), 2782. doi:10.1038/s41598-019-38978-w
- Hutzler, C., Paschke, M., Kruschinski, S., Henkler, F., Hahn, J., and Luch, A. (2014). Chemical Hazards Present in Liquids and Vapors of Electronic Cigarettes. *Arch. Toxicol.* 88 (7), 1295-1308. doi:10.1007/s00204-014-1294-7
- Jabba, S. V., Diaz, A. N., Erythropel, H. C., Zimmerman, J. B., and Jordt, S. E. (2020). Chemical Adducts of Reactive Flavor Aldehydes Formed in E-Cigarette Liquids Are Cytotoxic and Inhibit Mitochondrial Function in Respiratory Epithelial Cells. *Nicotine Tob. Res.* 22 (Suppl. 1), S25-S34. doi:10.1093/ntr/ntaa185
- Jabba, S. V., and Jordt, S. E. (2019). Risk Analysis for the Carcinogen Pulegone in Mint- and Menthol-Flavored E-Cigarettes and Smokeless Tobacco Products.

- JAMA Intern. Med.* 179 (12), 1721–1723. doi:10.1001/jamainternmed.2019.3649
- Kidwell, J. (2018). Synthetic Flavoring Agents and Adjuvants. *FDA. Food Additive Regul. ed* 83, 50490–50503.
- Kim, H., Lim, J., Buehler, S. S., Brinkman, M. C., Johnson, N. M., Wilson, L., et al. (2016). Role of Sweet and Other Flavours in Liking and Disliking of Electronic Cigarettes. *Tob. Control.* 25 (Suppl. 2), ii55–ii61. doi:10.1136/tobaccocontrol-2016-053221
- Kreiss, K., Gomaa, A., Kullman, G., Fedan, K., Simoes, E. J., and Enright, P. L. (2002). Clinical Bronchiolitis Obliterans in Workers at a Microwave-Popcorn Plant. *N. Engl. J. Med.* 347 (5), 330–338. doi:10.1056/nejmoa020300
- Krüsemann, E. J., Visser, W. F., Cremers, J. W., Pennings, J. L., and Talhout, R. (2018). Identification of Flavour Additives in Tobacco Products to Develop a Flavour Library. *Tob. Control.* 27 (1), 105–111. doi:10.1136/tobaccocontrol-2016-052961
- Krüsemann, E. J. Z., Boesveldt, S., de Graaf, K., and Talhout, R. (2019). An E-Liquid Flavor Wheel: A Shared Vocabulary Based on Systematically Reviewing E-Liquid Flavor Classifications in Literature. *Nicotine Tob. Res.* 21 (10), 1310–1319. doi:10.1093/ntr/nty101
- Krüsemann, E. J. Z., Havermans, A., Pennings, J. L. A., de Graaf, K., Boesveldt, S., and Talhout, R. (2021). Comprehensive Overview of Common E-Liquid Ingredients and How They Can Be Used to Predict an E-Liquid's Flavour Category. *Tob. Control.* 30 (2), 185–191. doi:10.1136/tobaccocontrol-2019-055447
- Krüsemann, E. J. Z., Pennings, J. L. A., Cremers, J. W. J. M., Bakker, F., Boesveldt, S., and Talhout, R. (2020). GC-MS Analysis of E-Cigarette Refill Solutions: A Comparison of Flavoring Composition between Flavor Categories. *J. Pharm. Biomed. Anal.* 188, 113364. doi:10.1016/j.jpba.2020.113364
- Laino, T., Tuma, C., Moor, P., Martin, E., Stolz, S., and Curioni, A. (2012). Mechanisms of Propylene Glycol and Triacetin Pyrolysis. *J. Phys. Chem. A.* 116 (18), 4602–4609. doi:10.1021/jp300997d
- Lau, H., Liu, S. Q., Tan, L. P., Lassabliere, B., Sun, J., and Yu, B. (2019). A Systematic Study of Molecular Ion Intensity and Mass Accuracy in Low Energy Electron Ionisation Using Gas Chromatography-Quadrupole Time-Of-Flight Mass Spectrometry. *Talanta* 199, 431–441. doi:10.1016/j.talanta.2019.02.089
- LaVoie, E. J., Adams, J. D., Reinhardt, J., Rivenston, A., and Hoffmann, D. (1986). Toxicity Studies on Clove Cigarette Smoke and Constituents of Clove: Determination of the LD50 of Eugenol by Intratracheal Instillation in Rats and Hamsters. *Arch. Toxicol.* 59 (2), 78–81. doi:10.1007/bf00286727
- Leigh, N. J., Lawton, R. I., Hershberger, P. A., and Goniewicz, M. L. (2016). Flavourings Significantly Affect Inhalation Toxicity of Aerosol Generated from Electronic Nicotine Delivery Systems (ENDS). *Tob. Control.* 25 (Suppl. 2), ii81–ii87. doi:10.1136/tobaccocontrol-2016-053205
- Leventhal, A. M., Goldenson, N. I., Barrington-Trimis, J. L., Pang, R. D., and Kirkpatrick, M. G. (2019). Effects of Non-tobacco Flavors and Nicotine on E-Cigarette Product Appeal Among Young Adult Never, Former, and Current Smokers. *Drug and Alcohol Dependence* 203, 99–106. doi:10.1016/j.drugalcdep.2019.05.020
- Mcdonald, J. W., and Heffner, J. E. (1991). Eugenol Causes Oxidant-Mediated Edema in Isolated Perfused Rabbit Lungs. *Am. Rev. Respir. Dis.* 143, 806–809. doi:10.1164/ajrccm/143.4_pt_1.806
- Muthumalage, T., Prinz, M., Ansah, K. O., Gerloff, J., Sundar, I. K., and Rahman, I. (2017). Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used E-Cigarette Flavoring Chemicals and Flavored E-Liquids without Nicotine. *Front. Physiol.* 8, 1130. doi:10.3389/fphys.2017.01130
- Nguyen, N., McKelvey, K., and Halpern-Felsher, B. (2019). Popular Flavors Used in Alternative Tobacco Products Among Young Adults. *J. Adolesc. Health* 65 (2), 306–308. doi:10.1016/j.jadohealth.2019.05.004
- Omaie, E. E., McWhirter, K. J., Luo, W., Pankow, J. F., and Talbot, P. (2019a). High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations. *Chem. Res. Toxicol.* 32 (6), 1058–1069. doi:10.1021/acs.chemrestox.8b00381
- Omaie, E. E., McWhirter, K. J., Luo, W., Tierney, P. A., Pankow, J. F., and Talbot, P. (2019b). High Concentrations of Flavor Chemicals Are Present in Electronic Cigarette Refill Fluids. *Sci. Rep.* 9 (1), 2468. doi:10.1038/s41598-019-39550-2
- Pankow, J. F., Kim, K., McWhirter, K. J., Luo, W., Escobedo, J. O., Strongin, R. M., et al. (2017). Benzene Formation in Electronic Cigarettes. *PLoS One* 12 (3), e0173055. doi:10.1371/journal.pone.0173055
- Pepper, J. K., Ribisl, K. M., and Brewer, N. T. (2016). Adolescents' Interest in Trying Flavoured E-Cigarettes. *Tob. Control.* 25 (Suppl. 2), ii62–ii66. doi:10.1136/tobaccocontrol-2016-053174
- Rahman, M. M., Abd El-Aty, A. M., Choi, J.-H., Shin, H.-C., Shin, S. C., and Shim, J.-H. (2015). Basic Overview on Gas Chromatography Columns. *Analytical Separation Science*, 3, 823–834.
- Rickard, B. P., Ho, H., Tiley, J. B., Jaspers, I., and Brouwer, K. L. R. (2021). E-cigarette Flavoring Chemicals Induce Cytotoxicity in HepG2 Cells. *ACS Omega* 6 (10), 6708–6713. doi:10.1021/acsomega.0c05639
- Russell, C., McKeganey, N., Dickson, T., and Nides, M. (2018). Changing Patterns of First E-Cigarette Flavor Used and Current Flavors Used by 20,836 Adult Frequent E-Cigarette Users in the USA. *Harm Reduct J.* 15 (1), 33. doi:10.1186/s12954-018-0238-6
- Schober, W., Szendrei, K., Matzen, W., Osiander-Fuchs, H., Heitmann, D., Schettgen, T., et al. (2014). Use of Electronic Cigarettes (E-cigarettes) Impairs Indoor Air Quality and Increases FeNO Levels of E-Cigarette Consumers. *Int. J. Hyg. Environ. Health* 217 (6), 628–637. doi:10.1016/j.ijheh.2013.11.003
- Scientific Working Group for Forensic (2013). Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. *J. Anal. Toxicol.* 37 (7), 452–474. doi:10.1093/jat/bkt054
- Sigma-Aldrich (2013). *GC Column Selection Guide*. Available: <https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/marketing/global/documents/891/300/t407133.pdf> (Accessed September 22, 2021).
- Stanfill, S. B., Brown, C. R., Yan, X., Watson, C. H., and Ashley, D. L. (2006). Quantification of Flavor-Related Compounds in the Unburned Contents of Bidi and Clove Cigarettes. *J. Agric. Food Chem.* 54 (22), 8580–8588. doi:10.1021/jf060733o
- The_Good_Scents_Company (1980). *The_Good_Scents_Company*. Available: <http://www.thegoodscentscompany.com/data/rw1004991.html> (Accessed August 23, 2021).
- Tierney, P. A., Karpinski, C. D., Brown, J. E., Luo, W., and Pankow, J. F. (2016). Flavour Chemicals in Electronic Cigarette Fluids. *Tob. Control.* 25 (e1), e10–e15. doi:10.1136/tobaccocontrol-2014-052175
- Truman, P., Stanfill, S., Heydari, A., Silver, E., and Fowles, J. (2019). Monoamine Oxidase Inhibitory Activity of Flavoured E-Cigarette Liquids. *Neurotoxicology* 75, 123–128. doi:10.1016/j.neuro.2019.09.010
- Vas, C. A., Porter, A., and McAdam, K. (2019). Acetoin Is a Precursor to Diacetyl in E-Cigarette Liquids. *Food Chem. Toxicol.* 133, 110727. doi:10.1016/j.fct.2019.110727
- Vreeke, S., Peyton, D. H., and Strongin, R. M. (2018). Triacetin Enhances Levels of Acrolein, Formaldehyde Hemiacetals, and Acetaldehyde in Electronic Cigarette Aerosols. *ACS Omega* 3 (7), 7165–7170. doi:10.1021/acsomega.8b00842
- Zhu, S.-H., Sun, J. Y., Bonnevill, E., Cummins, S. E., Gamst, A., Yin, L., et al. (2014). Four Hundred and Sixty Brands of E-Cigarettes and Counting: Implications for Product Regulation. *Tob. Control.* 23 (Suppl. 3), iii3–iii9. doi:10.1136/tobaccocontrol-2014-051670

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A Retrospective Analysis of Chemical Constituents in Regulated and Unregulated E-Cigarette Liquids

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E-cigarette or vaping use-associated lung injury (EVALI) was identified with the incidents of a multi-state outbreak of acute lung injuries associated with the use of electronic cigarettes (e-cigs) and attributed to vitamin E acetate in off-market cannabis-based e-liquids. Aside from EVALI, hypersecretion of mucus, irritated nasal passages, and watery, red eyes have been defined as complaints associated with vaping standard nicotine-based e-liquids. The chemical composition of e-liquids varies between manufacturers and robust oversight of ingredients is lacking. Manufacturers use chemicals deemed “generally recognized as safe” (GRAS) by the FDA, a designation for chemicals used in foodstuffs to be ingested. Most “GRAS” chemicals are associated with at least one Global Harmonization System (GHS) warning class, ranging from irritant to toxic. Untargeted chemical analysis is critical to evaluate e-liquid products to determine chemical composition; equally important is the quantitation of components to help elucidate the potential harms from exceeding recommended exposure limits. Untargeted screening of e-liquids was accomplished using gas chromatography-mass spectrometry (GC-MS) and Direct Analysis in Real Time-AccuTOF™ mass spectrometry (DART-ToF-MS) and has identified 350 chemical constituents from 241 products analyzed. Nicotine, caffeine, menthol, and vitamin E were confirmed and quantitated by GC-MS, ethanol was confirmed and quantitated by headspace-gas chromatography-dual flame ionization detection (HS-GC-FID), and olivetol and cannabinoids were confirmed and quantitated by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Maximum identified concentrations of nicotine, caffeine, menthol, vitamin E, ethanol, olivetol, Δ^9 -tetrahydrocannabinol, and cannabidiol were 56.4, 26.9, 4.28, 307.9, 217.2, 399.6, 497.7, and 332.6 mg/ml, respectively. Evaluation of untargeted analysis and quantitation of unlabeled chemical components of e-liquids is essential to improving etiology of acute lung injury and less severe impacts of vaping, both short-term and long-term. The historical documentation of unlabeled ingredients can provide some insight for a retrospective analysis of health consequences and inform policy discussions.

Keywords: e-cig, olivetol, vitamin E, menthol, caffeine, ethanol, cannabinoids, vaping

INTRODUCTION

The modern electronic cigarette evolved rapidly after its introduction in 2003 in the United States. Four device types are recognized by the Centers for Disease Control and Prevention (CDC). The first-generation device, the “cig-alike”, was low voltage and disposable and the second generation was re-fillable while the third generation enabled a user to select power, wicking material, and coil type. The fourth generation, called the “pod mod” allowed the user discreet vaping with small concealable devices and lack of associated aerosol cloud (Centers for Disease Control and Prevention, 2020a). Each type of e-cigarette allowed the consumer to make choices in line with their preferences. A fifth class of e-cigarette devices that facilitate the consumption of drugs other than nicotine (DOTN) and drug formulations that include waxes, dabs, crystals, and plant materials emerged from the highly customizable third generation device (Poklis et al., 2017a; Harrell and Eissenberg, 2018).

The liquid formulations used in the products, often referred to as e-liquids, also evolved as user preferences and public health sentiment changed, the industry evolved, and looming regulations became enforced. In addition to nicotine, typical e-liquid compositions contain humectants such as propylene glycol (PG) and vegetable glycerin (VG), an array of flavoring compounds, and other chemicals that are solvents for the flavorants or serve unknown purposes (Allen et al., 2016; Farsalinos et al., 2016; Peace et al., 2016; Poklis et al., 2017b; Peace et al., 2018; Fagan et al., 2018; Omaiye et al., 2019a; Holt et al., 2021). The cannabis industry drove an evolution of e-liquid formulations. Cannabinoids like Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and cannabidiol (CBD) do not easily dissolve in PG and VG, which are hygroscopic. Medium chain triglycerides (MCT) and polyethylene glycol (PEG) have been used to dissolve the cannabinoids more easily in PG and VG or as stand-alone carriers (Troutt and DiDonato, 2017; Erickson, 2019; Muthumalage et al., 2020a).

Adverse effects from vaping nicotine-based e-liquids have been reported to include cough, airway irritation, mucus hypersecretion, red eyes, sinus irritation, cardiovascular damage, and pulmonary granulomas (Lestari et al., 2018; Thirion-Romero et al., 2019; Lin et al., 2020; Münzel et al., 2020; Overbeek et al., 2020).

In 2019 in the United States, e-cigarette or vaping use-associated lung injury (EVALI), a new type of lung injury directly related to e-cig use, emerged. As of February 18, 2020, 2,807 cases of EVALI hospitalizations, including 68 deaths, were reported by the CDC (Centers for Disease Control and Prevention, 2020b). Illicit cannabis products were most commonly reported by EVALI patients (82%), though some reported only using nicotine-based products (14%) (Ellington et al., 2020; Krishnasamy et al., 2020). In late-2019, the Minnesota Department of Health evaluated 46 THC-containing products submitted by 12 EVALI patients, identifying vitamin E acetate (VEA) in 52% of products, MCTs in 43%, CBD in 43%, and alpha-tocopherol (vitamin E, VE) in 37% (Taylor et al., 2019). Similar studies have also identified VEA in a high percentage of THC-containing products (Muthumalage et al., 2020a; Duffy et al., 2020). In 2020, the CDC concluded that VEA was a

likely cause, as it was identified in most EVALI patients' bronchoalveolar-lavage fluid along with a high percentage of products, but recognized that there may be more than one cause and that continued research is necessary (Blount et al., 2020; Ellington et al., 2020).

In May 2016, the FDA promulgated regulations to govern e-liquids, yet product approval deadlines were slated for May 2020, marking a significant 4 years delay in required compliance (United States Food and Drug Administration, 2020b). The flavoring ban instituted in January 2020 was an attempt to thwart adolescent usage, but only governed pod-based products (Yingst et al., 2021). While chemicals used to achieve particular flavor profiles were banned in pod-based formulations, chemicals used in e-liquid formulations were not dictated or restricted by the FDA's regulatory language. The majority of flavorants used in e-liquids are substances which are Generally Recognized As Safe (GRAS) by the FDA. This designation is only applicable to food and food additives to be consumed orally (Flavor and Extracts Manufacturers Association of the United States, 2021). The presence of ethanol in e-liquids is documented, along with a number of other pharmacologically active chemicals (Peace et al., 2017; Poklis et al., 2017b; Poklis et al., 2017a; Poklis et al., 2019). Many e-liquids are marketed as containing vitamins and other health supplements, such as caffeine or melatonin. The addition of DOTNs less soluble in PG or VG required manufacturers to begin using other carriers, thus expanding the potential ingredient list consumer's may be exposed to.

In addition to flavoring chemicals, e-liquids contain chemicals to achieve a desired consistency and pharmacological profile. Nicotine content in e-liquids increased with the introduction of nicotine salts, which are reported to make higher concentrations of nicotine more palatable to users (Harvanko et al., 2020). Other countries, like Canada and England, have regulations regarding allowable nicotine content in e-liquid formulation (Institute for Global Tobacco Control, 2018). To date, the FDA has not defined a maximum nicotine concentration. As vaping devices have evolved, so too have the options for using these devices to administer drugs other than nicotine (DOTN).

The following report highlights the findings from the untargeted evaluation of e-liquid products submitted or purchased for a comprehensive chemical analysis since 2014. Chemical profiles were generated using Direct Analysis in Real Time-AccuTOF™ mass spectrometry (DART-ToF-MS), gas chromatography mass spectrometry (GC-MS), head space gas chromatography with a flame ionization detector (HS GC-FID). Quantitation of targeted analytes was performed by GC-MS or liquid chromatograph tandem mass spectrometry (LC-MS/MS). The implications to health and safety of specific chemicals or classes of chemicals are also discussed.

MATERIALS

Since 2014, 241 e-liquids were submitted for analysis by individuals, purchased directly from manufacturers, or purchased from local retailers for product characterization.

All glassware, tubing, and fritted glass dispersion tubes were purchased from Colonial Scientific (Richmond, VA, United States). United States Pharmacopeia (USP) grade propylene glycol (PG) and vegetable glycerin (VG) were purchased from Wizard Labs (Altamonte Springs, FL, United States). HPLC grade acetone and Optima grade formic acid, isopropanol and methanol were purchased from Fisher Scientific (Hanover Park, IL, United States). 200-proof ethanol was purchased from Decon Labs (King of Prussia, PA, United States). T-butanol was purchased from Honeywell Riedel-de Haën (Seelze, Germany). Air, helium, hydrogen, and nitrogen gases were purchased from Praxair (Richmond, VA, United States) or AirGas (Richmond, VA, United States). Type 1 water was generated in-house using a Millipore Direct-Q3 system. (-)-Nicotine [$\geq 99\%$ (GC)], quinoline (reagent grade, 98%), caffeine, caffeine-(trimethyl-d₉), menthol, and *trans*-anethole were purchased from Sigma (St. Louis, MO, United States). Certified reference materials for a quality assurance test mix containing amitriptyline, diazepam, fluoxetine, methadone, nicotine, nordiazepam, norfluoxetine, nortriptyline, paroxetine, and trazodone were acquired from Cerilliant (Round Rock, TX, United States), as were CBD, CBD-d₃, Δ^9 -THC, Δ^9 -THC-d₃, cannabigerol (CBG), cannabidivarin (CBDV), cannabichromene (CBC), cannabinol (CBN), CBN-d₃, VE, VE-d₆, and VEA. Tetrahydrocannabinolic acid (THCA), Δ^8 -tetrahydrocannabinol (Δ^8 -THC), cannabidiolic acid (CBDA), and cannabigerolic acid (CBGA) were purchased from Cayman Chemical (Ann Arbor, MI, United States). Olivetol was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States) and olivetol-d₉ was purchased from Toronto Research Chemicals (Toronto, ON, Canada).

METHODS

Lab protocol is to screen new products to elucidate chemical constituents and prioritize further analyses. Additional methods include confirmation and quantitation of nicotine and cannabinoids in all samples which had positive screen results. Volatiles and other chemicals of interest were also confirmed and quantitated. New quantitative methods were developed and validated as chemicals of interest were identified.

Screening by DART-MS

Initial screening of e-liquids was performed on a JEOL JMS T100LC Accu-ToF DART-MS using a previously published method (Poklis et al., 2015). In brief, a capillary tube was dipped directly into the e-liquid and then introduced into the helium stream for analysis to identify components' exact mass. The data was analyzed by creating an averaged, background subtracted, centroided mass spectra that was calibrated using PEG 600. Data was evaluated using National Institute of Standards and Technology (NIST) and Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) libraries. Full method details can be found in **Supplementary Material A**.

Screening by GC-MS

E-liquids were also screened using a simple dilute-and-shoot preparation with an untargeted analysis method employed on a Shimadzu QP-2020 GC-MS (Kyoto, Japan). Samples were processed using NIST 17-1, 17-2, and 17s libraries and SWGDRUG 3.5-3.9 libraries for identification. Full method details can be found in **Supplementary Material A**.

Quantitation of Nicotine by GC-MS

Quantitation of nicotine was accomplished by GC-MS using a Shimadzu QP-2020 GC-MS following previously published parameters (Pagano et al., 2016). Full method details can be found in **Supplementary Material A**.

Quantitation of Caffeine and Menthol by GC-MS

Quantitation of caffeine and menthol was accomplished by developing a single-ion-monitoring method for GC-MS using a Shimadzu QP-2020 GC-MS. Full method details can be found in **Supplementary Material A**.

Quantitation of Vitamin E and Vitamin E Acetate by GC-MS

Quantitation of VE and VEA was accomplished by developing a single-ion-monitoring method for GC-MS using a Shimadzu QP-2020 GC-MS. Full method details can be found in **Supplementary Material A**.

Quantitation of Volatiles by HS-GC-FID

Quantitation of acetone, ethanol, isopropanol, and methanol was accomplished using a modified version of a previously published method for headspace gas chromatography-flame ionization detector (HS-GC-FID) (Poklis et al., 2017b) and employed a Shimadzu HS-20 headspace sampler attached to a Nexis 2030 GC-dual FID (Shimadzu Corp., Kyoto, Japan). Full method details can be found in **Supplementary Material A**.

Quantitation of Olivetol by LC-MS/MS

Quantitation of olivetol was accomplished by developing a multiple-reaction-monitoring method for LC-MS/MS using a Shimadzu LC-MS 8050. Full method details can be found in **Supplementary Material A**.

Quantitation of Cannabinoids by LC-MS/MS

Quantitation of cannabinoids was accomplished using a modified version of a previously published method with a Shimadzu LC-MS 8050 (Poklis et al., 2010). Full method details can be found in **Supplementary Material A**.

RESULTS

In all, 350 chemicals were identified among the 241 products evaluated (**Supplementary Material C**). Some products contained novel psychoactive substances (NPS), pharmaceuticals, or

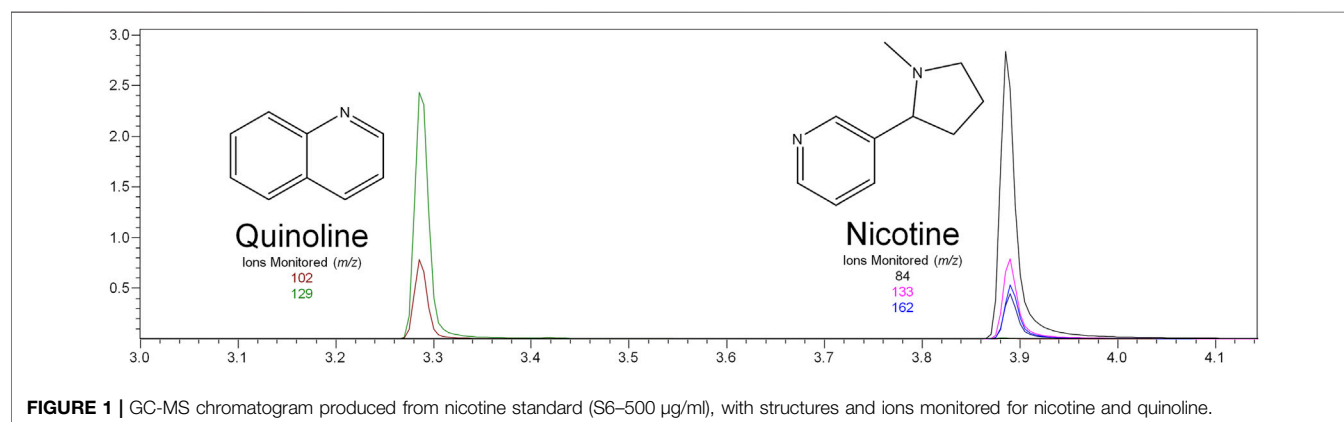
TABLE 1 | Pharmacologically active chemicals identified in products through screening, with product type and frequency of detection.

Compound	Hazard class	Uses/Description	Product type								Additive
			Nic (refill)	Nic (pod)	Non-nic (refill)	Nic/CBD (pod)	Cannab. (pod/cart)	Cannab. (refill)	Nic/DOTN (refill)	DOTN/non-nic (refill)	
4-Fluoroisocathinone		Structurally mimics substituted cathinones and phenethylamines but avoids current legal issues by being neither							1	1	
4F-MDMB-BINACA		aka-4F-MDMB-BUTICANA or 4F-ADB; synthetic cannabinoid									1
5F-ADB	Irritant	aka-5F-MDMB-PINACA or 5F-ADB-PINACA; synthetic cannabinoid					1	2			1
5F-EDMB-PINACA		Synthetic cannabinoid									1
Apomorphine	Acute Toxic, Irritant, Health Hazard	Used as an alpha-adrenergic drug, a serotonergic drug, a dopamine agonist, and an emetic								1	
Caffeine	Irritant	CNS stimulant; anti-inflammatory and legal psychoactive that alters fatigue, mood, alertness, motor reaction time, vascular hemodynamics, and pain sensation		6							
Cannabidiol (CBD)	Acute Toxic, Irritant, Health Hazard	Active phytocannabinoid in Cannabis, but not psychoactive				1	11	11			2
Dextromethorphan (DXM)	Acute Toxic	Active ingredient in cough medicine; structural similarity to codeine and morphine									1
EMB-FUBINACA		aka - AEB-FUBINACA, FUB-AEB; synthetic cannabinoid									1
Ethanol	Flammable	Solvent/preservative; bactericidal activity/topical disinfectant; CNS depressant	32	2	7	1				5	
FUB-AMB	Irritant	aka - AMB-FUBINACA, MMB-FUBINACA; synthetic cannabinoid									1
γ-Butyrolactone (GBL)	Corrosive, Acute Toxic, Irritant	Flavorant; prodrug of Schedule 1 GHB; numerous legitimate industrial uses		3							
MDMB-FUBINACA		Synthetic cannabinoid								3	
MFUBINAC		Synthetic cannabinoid									1
Mitragynine	Irritant	A major component of kratom that acts via opioid receptors; stimulatory, antinociceptive, and opiate-like effects							2	2	
MMB-FUBICA		aka - AMB-FUBICA; synthetic cannabinoid									1
Nicotine	Acute Toxic, Environmental Hazard	Highly addictive CNS stimulant with many side effects; major component of cigarettes and often added to e-liquids	59	28		1					

(Continued on following page)

TABLE 1 | (Continued) Pharmacologically active chemicals identified in products through screening, with product type and frequency of detection.

Compound	Hazard class	Uses/Description	Product type								
			Nic (refill)	Nic (pod)	Non-nic (refill)	Nic/ CBD (pod)	Cannab. (pod/ cart)	Cannab. (refill)	Nic/ DOTN (refill)	DOTN/ non-nic (refill)	Additive
Nuciferine	Acute Toxic Irritant	alkaloid of Blue Lotus									1
Olivetol		Precursor in various syntheses of tetrahydrocannabinol; people are claiming it can be used like Narcan for cannabis - helping to reduce a "raging high"				1	6				
Paynantheine		Major alkaloid found in kratom; thought to have cardiotoxic effects							1	1	
Tetrahydrocannabinol (d9-THC)	Irritant, Health Hazard	Schedule 1 drug; principal psychoactive compound in cannabis				1	8	3			
Tetrahydrocannabinolic acid (THCA)	Irritant, Health Hazard	Precursor of THC that converts through decarboxylation via heating					5	1			
Theobromine	Irritant, Health Hazard	Purine alkaloid derived from cacao and other plants. Is a vasodilator, diuretic, heart stimulator, bronchodilator, muscle stimulant. Similar to caffeine		6							

**FIGURE 1 |** GC-MS chromatogram produced from nicotine standard (S6–500 µg/ml), with structures and ions monitored for nicotine and quinoline.

pharmacologically active herbal compounds. **Table 1** highlights the major pharmacologically active ingredients identified through the screening process.

Nicotine has been quantitated in 90 products, with concentrations as high as 56.4 mg/ml. **Figure 1** displays chromatography produced by the nicotine quantitation method, and individual product concentrations can be seen in **Table 2**. Nineteen products were evaluated for caffeine and menthol content, with quantitated concentrations as high as 26.9 µg/ml and 4.28 mg/ml, respectively. **Figure 2** displays chromatography produced by the caffeine and menthol quantitation method, and individual product results can be

seen in **Table 3**. Five products were evaluated for VE content, with quantitated concentrations as high as 307.9 µg/ml. VEA was not detected in any samples. **Figure 3** displays chromatography produced by the VE and VEA quantitation method, and individual product concentrations can be seen in **Table 4**. 66 samples were evaluated for volatile content, in which ethanol has been quantitated in concentrations up to 217.2 mg/ml. Methanol and isopropanol were also identified but were below the limit of quantitation. **Figure 4** displays chromatography produced by the volatiles quantitation method, and individual product results can be seen in **Table 5**. Olivetol has been quantitated for five samples, in

TABLE 2 | Actual nicotine concentrations in e-liquids vs. labeled concentrations.

Brand	Product name	Labeled nicotine ^a (mg/ml)	Actual nicotine ^b (mg/ml)	% Difference	Product type
Adirondak	Delta	12.0	7.7	-43.7%	Nicotine e-liquid (refill)
AVAIL Vapor	Blueberry Cupcake	12.0 (1.2%)	12.0	0.0%	Nicotine e-liquid (refill)
AVAIL Vapor	Bombshell	6.0 (0.6%)	5.4	-10.5%	Nicotine e-liquid (refill)
AVAIL Vapor	Captain's Cut	24.0 (2.4%)	24.0	0.0%	Nicotine e-liquid (refill)
AVAIL Vapor	Continental Breakfast	24.0 (2.4%)	24.0	0.0%	Nicotine e-liquid (refill)
AVAIL Vapor	Midnight Splash	24.0 (2.4%)	24.0	0.0%	Nicotine e-liquid (refill)
AVAIL Vapor	Persian Winter	24.0 (2.4%)	24.0	0.0%	Nicotine e-liquid (refill)
AVAIL Vapor	Sapphire Morning	18.0 (1.8%)	18.0	0.0%	Nicotine e-liquid (refill)
AVAIL Vapor	Wave Runner	3.0 (0.3%)	3.2	6.5%	Nicotine e-liquid (refill)
Bluumpod	Tobacco	50.0 (5%)	19.4	-88.2%	Nicotine/CBD e-liquid (pod)
Bombies	White Gummy Bear	6.0	5.0	-18.2%	Nicotine e-liquid (refill)
Bryce's	Vanilla Cream Custard	6.0	5.9	-1.7%	Nicotine e-liquid (refill)
Coval Vapes	Mayflower	6.0	4.3	-33.0%	Nicotine e-liquid (refill)
Craft Sorbet	Skull Juice Watermelon Ice Cream	6.0	5.3	-12.4%	Nicotine e-liquid (refill)
Crafty E-Liquids	Watermelon	6.0	4.7	-24.3%	Nicotine e-liquid (refill)
Criss Cross	Original Tobacco	0.0	ND	—	Nicotine e-liquid (refill)
Elate Vapes	Helcats	6.0	6.3	4.9%	Nicotine e-liquid (refill)
Fennet	High Janty	12.0	12.4	3.3%	Nicotine e-liquid (refill)
Five Pawns	Grandmaster	6.0	7.8	26.1%	Nicotine e-liquid (refill)
Glas	Menthol	50.0 (5%)	49.1	-1.8%	Nicotine e-liquid (pod)
Glas	Royal	50.0 (5%)	47.1	-6.0%	Nicotine e-liquid (pod)
Glas	Signature Tobacco	50.0 (5%)	40.7	-20.5%	Nicotine e-liquid (pod)
Good Life Vapor	El Kamino	12.0	8.7	-31.9%	Nicotine e-liquid (refill)
Gremlin Juice	Birthday Cake	12.0	11.6	-3.4%	Nicotine e-liquid (refill)
Gremlin Juice	Kentucky Mint Julep	6.0	6.3	4.9%	Nicotine e-liquid (refill)
Gremlin Juice	Vanilla Custard	12.0	9.1	-27.5%	Nicotine e-liquid (refill)
Hel Vapes	Nic Salt 50 mg	50.0	30.5	-48.4%	Nicotine e-liquid (refill)
Hel Vapes	Nic Salt 15 mg	15.0	8.8	-52.1%	Nicotine e-liquid (refill)
Hurricane	M B	18.0	4.4	-121.4%	Nicotine e-liquid (refill)
Hurricane	Watermelon	12.0	1.5	-155.6%	Nicotine e-liquid (refill)
Hurricane	Whiskey	12.0	3.3	-113.7%	Nicotine e-liquid (refill)
Indigo Vapor	Birthday Cake	12.0	10.8	-10.5%	Nicotine e-liquid (refill)
Indigo Vapor	Captain Ron	12.0	11.0	-8.7%	Nicotine e-liquid (refill)
Indigo Vapor	Sunset	6.0	6.0	0.0%	Nicotine e-liquid (refill)
Juice Head	Strawberry Kiwi	25	24.5	-2.0%	Nicotine e-liquid (refill)
Juice Mafia	Peach Tobacco	12.0	8.9	-29.7%	Nicotine e-liquid (refill)
Juice Mafia	Turkish Tobacco	12.0	11.2	-6.9%	Nicotine e-liquid (refill)
JUUL	Classic Tobacco 5% Nicotine	59.0	54.7	-7.6%	Nicotine e-liquid (pod)
JUUL	Classic Tobacco 3% Nicotine	35.0	32.3 [30.4–34.2]	-8.0%	Nicotine e-liquid (pod)
JUUL	Menthol 5% Nicotine	59.0	50.6	-15.3%	Nicotine e-liquid (pod)
JUUL	Menthol 3% Nicotine	35.0	34.3 [33.1–35.5]	-2.0%	Nicotine e-liquid (pod)
JUUL	Virginia Tobacco 5% Nicotine	59.0	56.4 [50.4–51.1]	-4.5%	Nicotine e-liquid (pod)
JUUL	Virginia Tobacco 3% Nicotine	35.0	31.6	-10.2%	Nicotine e-liquid (pod)
Mighty Vapors Salts	Hulk Tears	50	47.0	-6.2%	Nicotine e-liquid (refill)
Mt. Baker	GWAR Spew	12.0	13.3	10.3%	Nicotine e-liquid (refill)
My Ohms	Pink Melon	12.0	8.4	-35.3%	Nicotine e-liquid (refill)
NJOY	Blueberry	50.0 (5%)	46.1 [39.9–49.8]	-8.1%	Nicotine e-liquid (pod)
NJOY	Classic Tobacco	50.0 (5%)	44.7	-11.2%	Nicotine e-liquid (pod)
NJOY	Menthol	50.0 (5%)	46.2	-7.9%	Nicotine e-liquid (pod)
NJOY	Watermelon Twist	50.0 (5%)	47.6 [47.1–48.4]	-4.9%	Nicotine e-liquid (pod)
OG CBD Oil	Cookies 'N Cream	0.0	ND	—	CBD e-liquid (pod)
OG CBD Oil	Mango	0.0	ND	—	CBD e-liquid (pod)
OG CBD Oil	Pineapple Express	0.0	ND	—	CBD e-liquid (pod)
OG CBD Oil	Pink Lemonade	0.0	ND	—	CBD e-liquid (pod)
Ritchy Group	Liqua Vanilla	0.0	<LOQ	—	Nicotine e-liquid (refill)
S&S Mods	Grumpy's Hooch	12.0	9.0	-28.6%	Nicotine e-liquid (refill)
Seduce Juice	Jango	12.0	12.6	4.9%	Nicotine e-liquid (refill)
Seduce Juice	Pharaoh	12.0	10.7	-11.5%	Nicotine e-liquid (refill)
Seduce Juice	Snake Eyes	12.0	10.1	-17.2%	Nicotine e-liquid (refill)
Seduce Juice	Snake Oil	12.0	10.5	-13.3%	Nicotine e-liquid (refill)
Shosha	Mango	12.0	12.0	0.0%	Nicotine e-liquid (refill)
Shosha	Seedless	18.0	16.3	-9.9%	Nicotine e-liquid (refill)
Shosha	USA Mix	12.0	13.4	11.0%	Nicotine e-liquid (refill)

(Continued on following page)

TABLE 2 | (Continued) Actual nicotine concentrations in e-liquids vs. labeled concentrations.

Brand	Product name	Labeled nicotine ^a (mg/ml)	Actual nicotine ^b (mg/ml)	% Difference	Product type
Shosha	VG UAS	16.0	15.2	-5.1%	Nicotine e-liquid (refill)
Shosha	VG Virginia	16.0	15.2	-5.1%	Nicotine e-liquid (refill)
Sky Pod	Blue Lemonade	60 (6%)	33.2	-57.5%	Nicotine e-liquid (pod)
StL Vapor	Spearmint	22.0	10.0	-75.0%	Nicotine e-liquid (refill)
Supreme Nicotine	258 Rally Squirrel	16.0	10.3	-43.3%	Nicotine e-liquid (refill)
Top Vapor	Unflavored PG	6.0	8.3	32.2%	Nicotine e-liquid (refill)
Triumph	Juicy Peach	11.0	8.4	-26.8%	Nicotine e-liquid (refill)
VapeWell	Cheery	18.0	14.7	-20.2%	Nicotine e-liquid (refill)
Vapourium NZ	Caffiend	12.0	10.8	-10.5%	Nicotine e-liquid (refill)
Vapourium NZ	Deez Melons	12.0	11.1	-7.8%	Nicotine e-liquid (refill)
Vapourium NZ	Jamaican Rum	6.0	5.2	-14.3%	Nicotine e-liquid (refill)
Vapourium NZ	So Fresh So Clean	12.0	12.4	3.3%	Nicotine e-liquid (refill)
Vapourium NZ	Stoned Fruits	12.0	11.4	-5.1%	Nicotine e-liquid (refill)
Vapourium NZ	Vanilla Beanie	12.0	10.4	-14.3%	Nicotine e-liquid (refill)
Velvet Cloud Vapor	Vanilla Tobacco	6.0	6.5	8.0%	Nicotine e-liquid (refill)
Wizard Labs	VG	12.0	10.3	-15.2%	Nicotine e-liquid (refill)

^aNicotine concentrations advertised as a (percentage) have been converted to mg/ml for comparison.

^bAverage of multiple samples/lots, with [range].

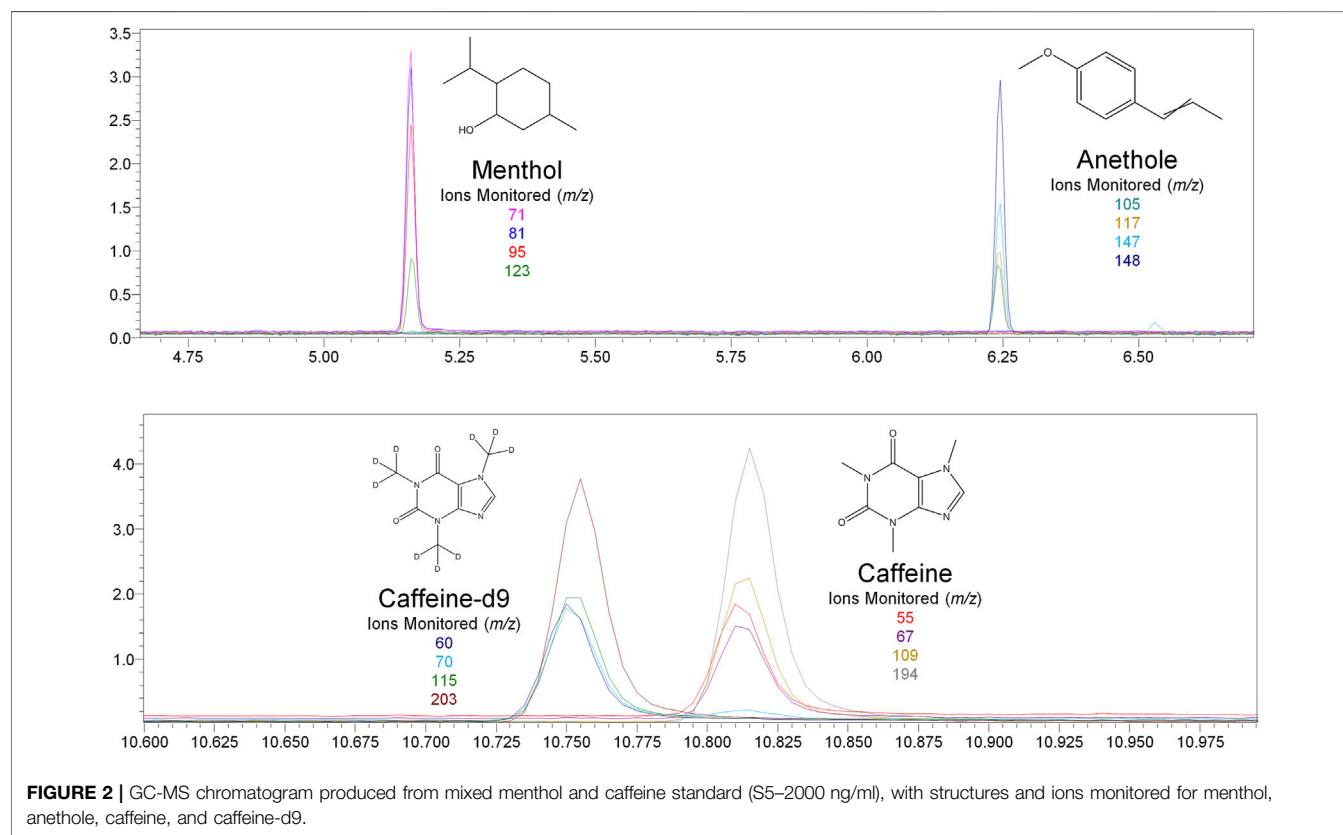


FIGURE 2 | GC-MS chromatogram produced from mixed menthol and caffeine standard (S5–2000 ng/ml), with structures and ions monitored for menthol, anethole, caffeine, and caffeine-d9.

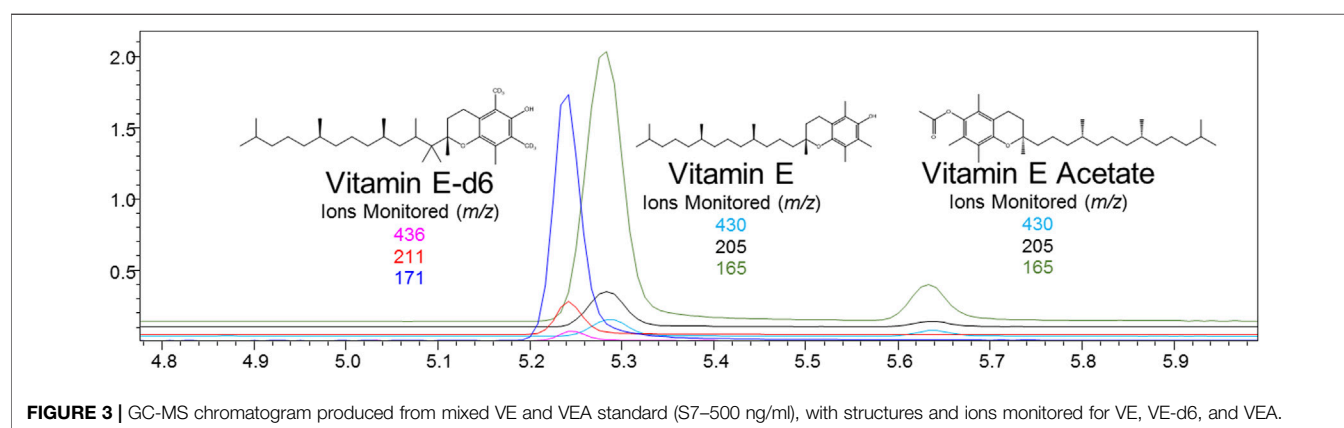
concentrations as high as 399.6 µg/ml. **Figure 5** displays chromatography produced by the olivetol quantitation method, and individual product results can be seen in **Table 6**. Cannabinoid content in the eleven products evaluated was found to vary greatly in both concentrations and compositions. CBD and Δ⁹-THC were the most identified cannabinoids and have been found in

concentrations as high as 332.6 and 497.7 mg/ml, respectively. Cannabinoid concentrations identified through quantitation were often different from labeled values, as can be seen in **Supplementary Material D**. **Figure 6** displays a chromatogram produced by the cannabinoid quantitation method. Individual cannabinoid transition ions monitored can be found in **Supplementary Material B**.

TABLE 3 | Caffeine and menthol concentrations in e-liquids.

Product ^a	Caffeine (µg/ml)	Menthol (µg/ml)	Product type
Appalachian Sunshine	ND	139.4 ± 6.1	CBD vape cart
Bluumpod "Tobacco"	ND	ND	Nicotine/CBD e-liquid
Glas "Fresh Menthol"	ND	4,278.6 ± 109.9	Nicotine e-liquid (pod)
Juul "Classic Tobacco" 3%	26.9 ± 1.3	49.9 ± 0.9	Nicotine e-liquid (pod)
Juul "Classic Tobacco" 3%	24.7 ± 0.6	96.2 ± 8.3	Nicotine e-liquid (pod)
Juul "Classic Tobacco" 5%	25.3 ± 0.1	118.4 ± 2.9	Nicotine e-liquid (pod)
Juul "Menthol" 3%	10.7 ± 0.5	1,361.8 ± 52.1	Nicotine e-liquid (pod)
Juul "Menthol" 3%	9.7 ± 0.2	1995.4 ± 18.6	Nicotine e-liquid (pod)
Juul "Menthol" 5%	8.8 ± 0.3	1,391.7 ± 31.4	Nicotine e-liquid (pod)
Liberty	ND	3.8 ± 0.4	CBD vape cart
Mighty Vapors Salts "Hulk Tears"	ND	ND	Nicotine e-liquid
Myle Mini-unknown flavor	ND	2,208.3 ± 126.3	Nicotine disposable
NJOY "Blueberry"	ND	182.1 ± 4.8	Nicotine e-liquid (pod)
NJOY "Blueberry"	ND	117.5 ± 3.73	Nicotine e-liquid (pod)
NJOY "Menthol"	ND	1750.2 ± 32.9	Nicotine e-liquid (pod)
NJOY "Watermelon Twist"	ND	3.2 ± 0.3	Nicotine e-liquid (pod)
NJOY "Watermelon Twist"	ND	10.8 ± 0.1	Nicotine e-liquid (pod)
Twisted CBD "Watermelon"	ND	24.4 ± 1.0	CBD vape cart
Vuse "Mixed Berry" 5%	ND	118.0 ± 1.3	Nicotine e-liquid (pod)

^aDuplicate products from separate submissions or product lots.

**FIGURE 3** | GC-MS chromatogram produced from mixed VE and VEA standard (S7–500 ng/ml), with structures and ions monitored for VE, VE-d6, and VEA.**TABLE 4** | Vitamin E concentrations in e-liquids.

Product	Concentration (µg/ml)	Product type
Appalachian Sunshine	236.7 ± 7.5	CBD vape cart
Diamond CBD	2.9 ± 0.1	Dietary supplement/vape additive
Liberty	226.6 ± 23.1	CBD vape cart
MMS Elemental "Blue Dream"	307.9 ± 11.0	CBD vape cart
Unidentified THC product	101.6 ± 0.5	THC vape cart

DISCUSSION

All e-liquids were analyzed by DART-MS and GC-MS with an untargeted method to characterize the chemical profile. An example of screening results can be seen in **Figure 7**. Once the chemicals were identified, quantitative analysis was performed by HS GC-FID, GC-MS, or LC-MS/MS. The

chemicals identified in this study can be classified as carriers or humectants, flavorants/organoleptics, preservatives, additives/enhancers, or as pharmacologically active. Several chemicals, such as menthol, have multiple properties, making attribution of their use in the e-liquid difficult. **Supplementary Material C** lists compounds identified, associated GHS hazard classes, and reported uses of compounds. Untargeted analytical methods are

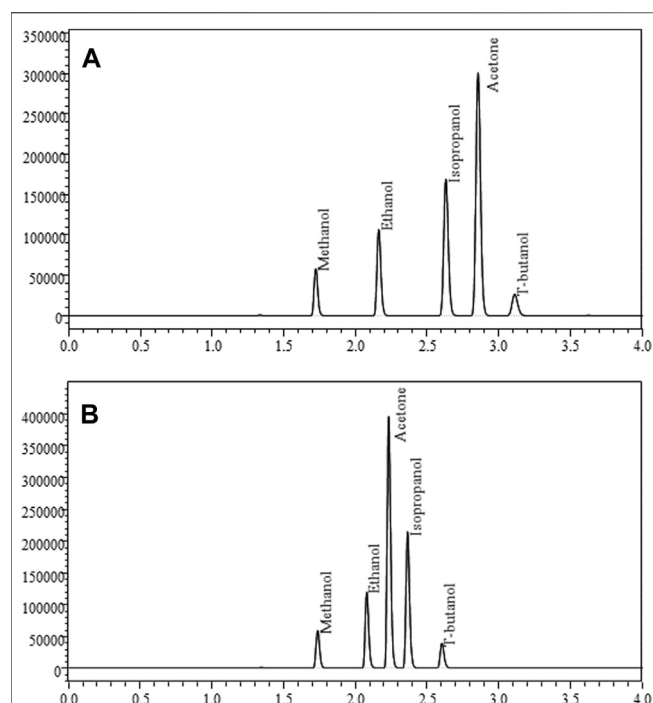


FIGURE 4 | GC-FID chromatograms produced from quantitation of methanol, ethanol, isopropanol, and acetone using t-butanol as an internal standard. Figure A is a standard chromatogram produced by FID 1, and Figure B is a standard chromatogram produced by FID 2. Differences in analyte retention times are produced by the different separation techniques of the two chromatographic columns utilized.

necessary to evaluate products for compliance with regulations and to assess unregulated products. With widely varied and constantly changing products like e-liquids, a critical evaluation of chemical composition is crucial for public health and safety. Although not reported here, analysis of the aerosols can identify if compounds in the e-liquid will be inhaled by the user. The aerosol analysis can also determine if new, unique compounds are formed from the vaping process, such as pyrolytic, degradant, or adduct compounds. Chemicals identified in e-liquids can indicate user exposure, though in the absence of aerosol studies, results should be interpreted with caution.

Of products evaluated in this study, the more specifically regulated nicotine e-liquids found in pod style products have trended toward more simple compositions. Nicotine based refill formulations, potentially associated with small-batch craft products, have continued to have complex chemical profiles, often containing more flavoring chemicals. For example, 10 chemicals were identified in a JUUL Menthol pod, whereas 26 chemicals were identified in Mighty Vapors “Hulk Tears”, a refill formulation. Cannabinoid-containing products also tend to have more chemical constituents, as many of the terpenes from cannabis are extracted along with the desired cannabinoids. For example, MMS Elemental “Blue Dream” contained 35 identified chemicals, 23 of which were terpenes, and 9 of which were cannabinoids. These terpenes can naturally add to the flavor and aroma profiles, but may also be added to

formulations to simulate desired profiles. As with other compounds, these terpenes can have multiple properties, including GHS health hazards. The lack of regulations governing product ingredients provides manufacturers opportunity to create complicated products with potentially harmful chemicals.

Most carriers and flavorants identified in products are “generally recognized as safe” by the FDA, meaning “the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excepted from the definition of a food additive” (United States Food and Drug Administration, 2019; Flavor and Extracts Manufacturers Association of the United States, 2021). GRAS status only applies to foodstuffs to be ingested orally and does not translate to any other route of administration. Extrapolation of oral ingestion safety to inhalation safety is fraught with assumptions and false equivalencies.

A variety of chemical compounds with pharmacologically active properties were found in addition to nicotine, as seen in **Table 1**. This study identified cannabinoids (CBD, Δ^9 -THC, Δ^8 -THC, CBG, CBDA, CBN, CBC, THCV, and THCA), caffeine, dextromethorphan, kratom alkaloids (mitragynine and 7-hydroxy-mitragynine), the blue lotus alkaloids apomorphine and nuciferine, gamma-Butyrolactone (GBL), a variety of synthetic cannabinoids (5F-ADB, MDMB-FUBINACA, FUB-AMB, EMB-FUBINACA, MFUBINAC, MMB-FUBICA, 4F-MDMB-BINACA, 5F-EDMB-PINACA) and the synthetic cathinone 4-fluoroisocathinone in products. Nicotine, Δ^9 -THC, and CBD were the only active ingredients identified on the product labels. All other pharmacologically active ingredients were unlisted on the products, meaning consumers did not know what they were consuming. Some of the pharmacologically active ingredients are illicit substances, some are legal herbal compounds, and others are co-opted therapeutic drugs used for recreational purposes.

Carriers, Diluents, and Thickeners

Propylene glycol and vegetable glycerin were the most commonly identified carriers in e-liquids. Both are considered GRAS and used in food products, pharmaceuticals, and health and beauty products. Though considered harmless based on years of use in food and medicine, use of these chemicals in e-cigs and vaporizers is not inherently safe. Studies have demonstrated that when heated to high temperatures, like those of a heated e-cig coil, PG and VG can produce carbonyls such as formaldehyde and acetaldehyde (Kosmider et al., 2014; Geiss et al., 2016; Troutt and DiDonato, 2017; Qu et al., 2019; Jiang et al., 2020). Device settings have been found to directly affect the production of these harmful chemicals, and therefore may lead to the risks of increased exposures to these carcinogens, as carbonyl formation has been shown to increase directly with increasing battery output voltages (Kosmider et al., 2014; Qu et al., 2019).

PG and VG were the only known carriers identified in nicotine and flavorant-only formulations. In addition to PG and VG, MCTs, PEG, and squalene were identified in cannabinoid and DOTN formulations, as well as formulations that contained both

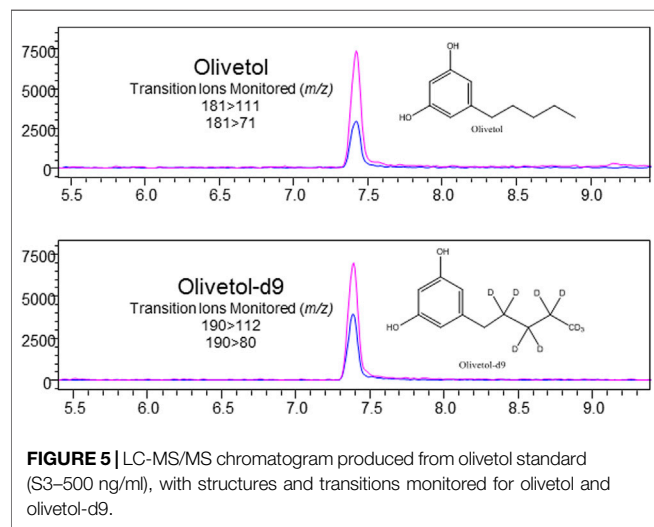
TABLE 5 | Ethanol concentrations in e-liquids.

Product	Average concentration (mg/ml)				Product type
	Methanol	Ethanol	Isopropanol	Acetone	
Aqua "Flow"	ND	1.7 ± 0.4	ND	ND	Nicotine e-liquid (refill)
Blue CBD "Crystals Isolate" 1,000 mg	ND	ND	ND	ND	CBD Additive
Blue CBD "Crystals Isolate" 250 mg	ND	ND	ND	ND	CBD Additive
Blue Monkey Vapes "Dr. Freeze-Ice Menthol"	ND	2.7 ± 0.1	ND	ND	Nicotine e-liquid (refill)
Bluumpod "Tobacco"	ND	<LOQ	ND	ND	Nicotine/CBD e-liquid
Cereal Killa "Duchess"	ND	9.6 ± 0.7	ND	ND	Nicotine e-liquid (refill)
Clown "Pennywise Circus Salts"	ND	13.1 ± 0.1	ND	ND	Nicotine e-liquid (refill)
Craft Sorbet Skull Juice "Watermelon Ice Cream"	<LOQ	<LOQ	ND	ND	Nicotine e-liquid (refill)
Crafty "Watermelon"	ND	5.5 ± 0.3	ND	ND	Nicotine e-liquid (refill)
Diamond CBD "Hemp Infused Liquid" 1,000 mg	ND	ND	ND	ND	CBD Additive
Diamond CBD "Hemp Infused Liquid" 50 mg	ND	ND	ND	ND	CBD Additive
Diamond CBD Vape Additive	ND	ND	ND	ND	CBD Additive
Directors Cut "The Devil"	ND	4.7 ± 0.1	ND	ND	Nicotine e-liquid (refill)
Elate Vape "Hell-Cats"	ND	ND	ND	ND	Nicotine e-liquid (refill)
Fresh Pressed "Fruit Finale"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
Galaxy	ND	ND	ND	ND	Vape additive
Geeked Out "Dork Breath"	ND	3.7 ± 0.2	ND	ND	Nicotine e-liquid (refill)
HEL Vape "Breaking Bad" 15 mg Nic salt	ND	ND	ND	ND	Nicotine e-liquid (refill)
HEL Vape "Breaking Bad" 50 mg Nic salt	ND	ND	ND	ND	Nicotine e-liquid (refill)
Hurricane "M B"	ND	ND	ND	ND	Nicotine e-liquid (refill)
Hurricane "Watermelon"	ND	ND	ND	ND	Nicotine e-liquid (refill)
Hurricane "Whiskey"	ND	ND	ND	ND	Nicotine e-liquid (refill)
Jango	ND	ND	ND	ND	Nicotine e-liquid (refill)
JUUL Menthol	ND	2.0 ± 0.04	ND	ND	Nicotine e-liquid (pod)
JUUL Virginia Tobacco	ND	32.8 ± 0.7	ND	ND	Nicotine e-liquid (pod)
Kai's Virgin Vapor "Blue Vango" 0 mg Nic	ND	2.6 ± 0.03	ND	ND	Non-nicotine e-liquid (refill)
Kai's Virgin Vapor "Blue Vango" 12 mg Nic	ND	2.3 ± 0.1	ND	ND	Nicotine e-liquid (refill)
Kai's Virgin Vapor "Caramel Kona Milkshake" 0 mg Nic	ND	89.5 ± 3.2	ND	ND	Non-nicotine e-liquid (refill)
Kai's Virgin Vapor "Caramel Kona Milkshake" 12 mg Nic	ND	37.0 ± 1.4	ND	ND	Nicotine e-liquid (refill)
Kai's Virgin Vapor "Celestial Honeydew" 0 mg Nic	ND	16.1 ± 0.7	ND	ND	Non-nicotine e-liquid (refill)
Kai's Virgin Vapor "Celestial Honeydew" 12 mg Nic	ND	16.5 ± 0.6	ND	ND	Nicotine e-liquid (refill)
Kai's Virgin Vapor "Chocolate Grasshopper" 0 mg Nic	ND	46.5 ± 1.4	ND	ND	Non-nicotine e-liquid (refill)
Kai's Virgin Vapor "Chocolate Grasshopper" 12 mg Nic	ND	19.6 ± 1.9	ND	ND	Nicotine e-liquid (refill)
Kai's Virgin Vapor "French Vanilla Kiss" 0 mg Nic	ND	214.3 ± 13.6	ND	ND	Non-nicotine e-liquid (refill)
Kai's Virgin Vapor "French Vanilla Kiss" 12 mg Nic	ND	65.7 ± 3.4	ND	ND	Nicotine e-liquid (refill)
Kai's Virgin Vapor "Plum Crazy" 0 mg Nic	ND	2.5 ± 0.04	ND	ND	Non-nicotine e-liquid (refill)
Kai's Virgin Vapor "Plum Crazy" 12 mg Nic	ND	3.4 ± 0.2	ND	ND	Nicotine e-liquid (refill)
Kai's Virgin Vapor "Raspberry Mocha Whip" 0 mg Nic	ND	39.2 ± 4.3	ND	ND	Non-nicotine e-liquid (refill)
Kentucky Route "Strawberry Fields"	ND	ND	ND	ND	Vape additive
Koi "Blue Raspberry Dragon Fruit"	ND	<LOQ	ND	ND	Vape additive
Lotus Extracts "Areca Nut"	ND	ND	ND	ND	DOTN e-liquid
Lotus Extracts "Blue Lotus"	ND	1.9 ± 0.05	ND	ND	DOTN e-liquid
Lotus Extracts "Damiana"	ND	ND	ND	ND	DOTN e-liquid
Lotus Extracts "Klip Dagga"	ND	<LOQ	ND	ND	DOTN e-liquid
Lotus Extracts "Kra Thum Kok"	ND	<LOQ	ND	ND	DOTN e-liquid
Lotus Extracts "Kra Thum Na"	ND	2.3 ± 0.1	<LOQ	ND	DOTN e-liquid
Lotus Extracts "Wild Lettuce"	ND	ND	ND	ND	DOTN e-liquid
My Ohm "Pink Melon"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
R.A. Royal CBD "Classic"	ND	ND	ND	ND	Vape additive
Ritchy "Liqua Vanilla"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
Shosha "Mango"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
Shosha "Seedless"	ND	8.8 ± 0.5	ND	ND	Nicotine e-liquid (refill)
Shosha "UAS Mix"	ND	1.5 ± 0.02	ND	ND	Nicotine e-liquid (refill)
Shosha "USA Mix"	ND	1.6 ± 0.07	ND	ND	Nicotine e-liquid (refill)
Shosha "Virginia"	<LOQ	1.8 ± 0.07	ND	ND	Nicotine e-liquid (refill)
Triumph "Juicy Peach"	ND	ND	ND	ND	Nicotine e-liquid (refill)
Vapourium "Caffiend"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
Vapourium "Deez Melons"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
Vapourium "Jamaican Rum"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
Vapourium "So Fresh So Clean"	ND	28.7 ± 0.7	ND	ND	Nicotine e-liquid (refill)
Vapourium "Stoned Fruits"	ND	17.0 ± 0.9	ND	ND	Nicotine e-liquid (refill)
Vapourium "Vanilla Beanle"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)
Whispers "Razzel Dazzel"	ND	<LOQ	ND	ND	Nicotine e-liquid (refill)

(Continued on following page)

TABLE 5 | (Continued) Ethanol concentrations in e-liquids.

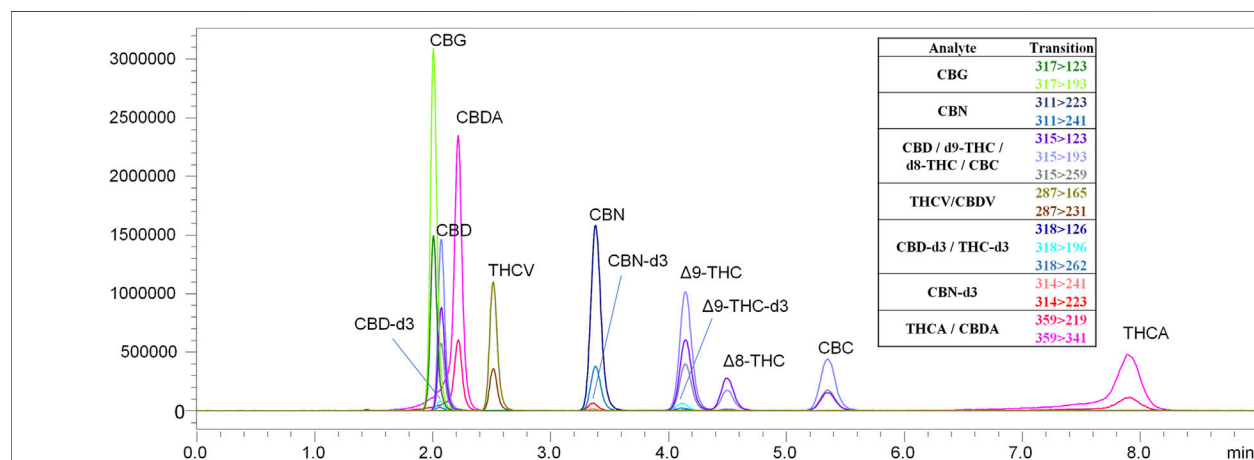
Product	Average concentration (mg/ml)				Product type
	Methanol	Ethanol	Isopropanol	Acetone	
Yami Vapor "Joy Trio" 35 mg Nic	ND	2.3 ± 0.1	ND	ND	Nicotine e-liquid (refill)
Yami Vapor "Joy Trio" 50 mg Nic	ND	2.5 ± 0.1	ND	ND	Nicotine e-liquid (refill)
Yami Vapor "Taruto"	ND	16.5 ± 1.4	ND	ND	Nicotine e-liquid (refill)



nicotine and a DOTN. These carriers may be used to dissolve more lipophilic compounds, including cannabinoids, before adding them to PG and VG. MCTs may also be used because they are attributed with health benefits. MCTs have been touted online as a safer, healthier alternative to PG and VG (Zachar, 2018). MCTs produce harmful volatile organic compounds and increase interleukin-8 (IL-8) and interleukin-6 (IL-6) levels which are biomarkers for lung inflammation and injury. MCTs also decrease transepithelial electrical resistance and increase lipid-laden macrophage formation which can lead to lipoid pneumonia (Muthumalage et al., 2020b). MCT aerosols were found to contain alkyl alcohols, which are surfactant-like and can produce cytotoxic effects. MCTs and PEG both produce harmful carbonyls when aerosolized (Troutt and DiDonato, 2017; Jiang et al., 2020). PEG was found to produce levels of formaldehyde that neared those seen by traditional combustion cigarettes, with one puff exposing the user to 1.12% of the daily exposure limit set forth by the United States Occupational and

TABLE 6 | Olivitol concentrations in e-liquids.

Product	Concentration (µg/ml)	Product type
Appalachian Sunshine	3,880.9 ± 185.7	CBD vape cart
Bluempod CBD "Tobacco"	<LOQ	Nicotine/CBD e-liquid
Liberty	8.6 ± 0.4	CBD vape cart
Twisted CBD "Watermelon"	21.4 ± 3.0	CBD vape cart
Western Cultured "Seatown Lemon Haze"	<LOQ	CBD vape cart



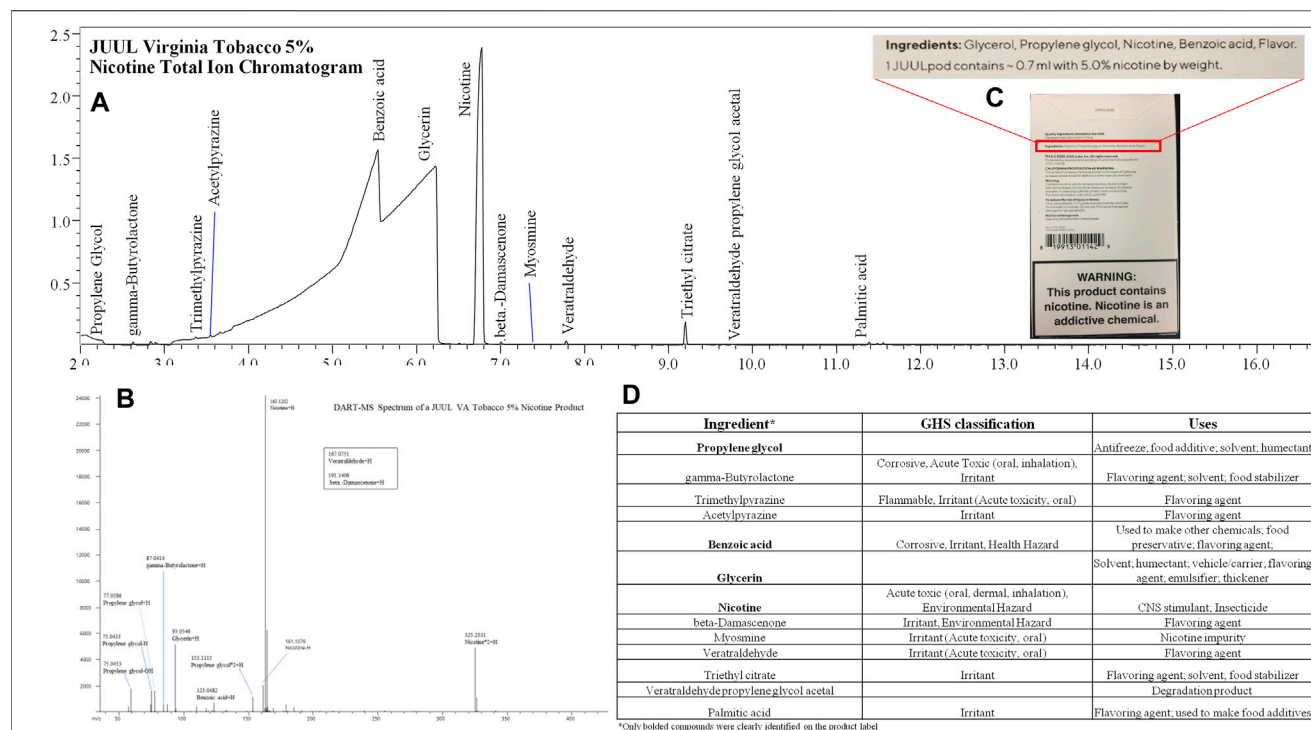


FIGURE 7 | (A) JUUL Virginia Tobacco 5% Nicotine Total Ion Chromatogram, **(B)** DART-MS Spectrum, **(C)** Packaging, and **(D)** GHS classification and Uses of Identified Ingredients. This exemplifies the typical results of product screening.

Safety Health Administration (OSHA) (Troutt and DiDonato, 2017). Squalene, a clear, slightly yellow oily substance, has been implicated in causing exogenous lipid pneumonia (Cha et al., 2019).

Recent studies have documented the presence of both VE and VEA in THC products (Taylor et al., 2019; Muthumalage et al., 2020a; Duffy et al., 2020). VEA is thought to be used as a diluent that mimics the consistency of a high purity THC oil, thus deceiving the consumer into believing they are purchasing a high-grade THC product and allowing a larger profit for the manufacturer by extending the supply (Duffy et al., 2020). VEA is also implicated as one of the primary chemicals thought to be responsible for the EVALI epidemic (Blount et al., 2019; Blount et al., 2020; Muthumalage et al., 2020b). Though VEA was not identified in any samples, VE was identified in multiple cannabinoid-containing products. Both VE and VEA produce quinone-like compounds, which can produce reactive oxygen species that increase cytotoxicity (Jiang et al., 2020).

Flavorants

Trends in the use of chemicals used as flavorants are difficult to discern due to limited sample size (241 products) compared to products available commercially, the variety of chemicals available, the constantly changing formulations, and evolving product regulations. Though most flavorants have been designated as GRAS, the majority of the flavorants identified through screening are associated with at least one GHS

classification, such as irritant, corrosive, or acutely toxic, which contraindicates their safety.

Several studies have demonstrated that flavorants produce varying degrees of toxicity to cells through different mechanisms (Behar et al., 2016; Leigh et al., 2016; Sherwood and Boitano, 2016; Behar et al., 2018). Omaiye et al. reported total concentrations of flavor chemicals in refill fluids were found as high as 362.3 mg/ml, while pod and cartomizer formulations evaluated (JUUL and Vuse, respectively) only contained total flavor chemical concentrations of 0.2–15.7 mg/ml (Omaiye et al., 2019a). They also reported that all 8 evaluated JUUL liquid formulations and the corresponding aerosols were cytotoxic. Cinnamaldehyde is already widely reported as cytotoxic, and a study evaluating cinnamaldehyde in e-liquids found that increasing battery output voltage further increased cytotoxicity (Behar et al., 2016). Costigan et al. pointed out that use of the same e-cigarette liquid in different devices will alter the aerosol formation (Costigan and Meredith, 2015). These studies' findings suggest that a e-liquid toxicity may vary based on the device and operational settings.

Another flavorant-related health concern identified in this study is the formation of flavorant-carrier adduct products. Compounds such as vanillin propylene glycol acetal are formed post-production, sometimes within hours of initial product mixing, through an acetalization reaction between an aldehyde and PG. The formation of these acetal products was reported to be dependent on the ratio of PG present in the formulation (Erythropel et al., 2019). Adducts were shown to

aerosolize with similar efficiency as the parent aldehyde and proved more effective at activating respiratory irritant receptors than the respective parent aldehyde, suggesting e-cig liquids may become more harmful to the user as time passes and flavorant-PG adducts form. The Flavor Extract Manufacturers Association (FEMA) lists some flavorant-carrier adducts as their own unique entity, which can be purchased for use as a flavorant (Flavor and Extracts Manufacturers Association of the United States, 2021). This can further complicate the determination of product ingredients in terms of what is added by the manufacturer or what was formed post-production. Additionally, if these chemicals are intentionally added, their concentration within the products may rise above the manufacturers intended level due to the post-production formation.

The combinations of flavorants may potentiate the harmful or toxic effects of one another. Manufacturers are currently not compelled to release e-liquid formulation recipes nor list all ingredients used within products on labels. Relevant toxicity studies are impossible to conduct as the proprietary formulations rapidly evolve.

Nicotine

Nicotine, while a common constituent, is not always present. Some e-cig liquids are manufactured as nicotine-free, flavor-only options, and sometimes other pharmacologically active ingredients are used, as exemplified by the numerous cannabinoid liquid options available today. In this study, one e-liquid contained nicotine even though the product label claimed it did not contain any amount of nicotine. The presence of nicotine in this product could be due to contamination during the manufacturing process, incomplete/insufficient labeling, or the intention of including a known pharmacologically active ingredient to elucidate some effect. Other products were found to have higher or lower concentrations of nicotine than were labeled on the product, again demonstrating poor quality assurance and quality control standards in the industry (Peace et al., 2016; Peace et al., 2018).

Nicotine concentrations increased as manufacturers switched to nicotine salt formulations, which use nicotine with an organic acid, such as benzoic or lactic acid. These formulations allow manufacturers to significantly increase the nicotine concentration while reportedly reducing the harshness of such high nicotine amounts (Harvanko et al., 2020). The highest levels observed in this study were from JUUL liquid formulations, which use nicotine salts. The concentrations observed in these products correlate with concentrations identified in other studies (Omaiye et al., 2019a).

While nicotine use and dependence are well documented in the scientific literature, toxicity and poisonings, especially as it pertains to the e-cigarette industry, are worth discussing. Higher nicotine concentrations increase the chance of accidental nicotine poisoning, both through inhalation or ingestion of the liquid. The American Association of Poison Control Centers has reported thousands of poisoning cases about e-liquids since 2011, when e-cig use became more prevalent in the United States. As of May 31st of this year, 2063 cases have been reported (National Poison

Data System, American Association of Poison Control Centers, 2021). Many poisoning cases involve young children ingesting the products accidentally, while some cases involve someone intentionally ingesting or injecting the liquid for a means of self-harm. Two cases of poisoning are reported following inhalation of a nicotine product by active duty military personnel, leading to clinical nicotine toxicity requiring emergency medical services (Bendel et al., 2021). Dermal contact from spilled or leaky pods is a concern that should not be overlooked, as nicotine readily absorbs through the skin, leading to both localized and systemic health concerns. Attempted homicide by nicotine liquid being poured directly onto the skin of the victim has been reported, with the victim describing the liquid as sticky with a spicy flavor (FOX 9, 2021).

Caffeine

Caffeine was identified and quantitated in JUUL Menthol and Classic Tobacco liquid formulations. JUUL Classic Tobacco products contained an average of 23.5 µg/ml caffeine, while JUUL Menthol products contained an average of 9.3 µg/ml. In another study, caffeine in JUUL Menthol and Classic Tobacco aerosols were found in concentrations of 0.037 and 0.090 mM, respectively, showing it is able to both aerosolize and be inhaled by users (Omaiye et al., 2019a). Caffeine was not labeled on the e-liquid products.

Caffeine affects the cardiovascular, renal, nervous, and respiratory systems (Ueno et al., 2020), and is widely consumed throughout the world as a legal stimulant. In addition to the caffeine consumed in coffees, teas, sodas, and energy drinks, it can be found as a dietary supplement and is used in narcolepsy and asthma therapies.

Issues regarding caffeine in e-cig liquids should be considered. Inhalation of caffeine increases its bioavailability. An *in vivo* study using mice demonstrated caffeine inhalation, via a nebulizer, was an effective way of administering caffeine and produced greater spontaneous activity compared to the same dose administered intraperitoneally (Ueno et al., 2020). Additionally, caffeine was identified as an unlisted ingredient. Individuals with caffeine sensitivity or underlying medical conditions that require a caffeine-free lifestyle could be endangered by inhaling caffeine.

The addition of caffeine to e-cig liquids could act as an initiation primer, leading to increased caffeine seeking and consumption and chances of caffeine addiction. Caffeine consumption has been reported to increase the odds of smoking, the urge to smoke, and the subjective reinforcement from smoking (Treloar et al., 2014). A correlation between combined inhalation of caffeine and smoking with promotion of coronary heart disease and severe vascular lesions has also been reported (Pan et al., 2021).

Menthol

Menthol was identified in a variety of products evaluated, sometimes as a listed ingredient, sometimes unlisted. Menthol was quantitated in concentrations as high as 4.48 mg/ml. Another study reported finding menthol in concentrations up to 68 mg/ml (Omaiye et al., 2019b). In that study, the cytotoxic properties of several flavorants were evaluated. Menthol was identified as toxic

in cells studied in concentrations 30 times lower than their highest identified concentration, meaning all products evaluated with menthol in concentrations greater than approximately 2.5 mg/ml would be considered cytotoxic.

Menthol has been identified as having other important pharmacological properties related to smoking and vaping. Therefore, its identification as an additive in non-menthol flavored products is not surprising. Menthol is reported as imparting a cooling sensation with analgesic or counterirritant effects, reducing the perceived harshness of the nicotine and smoke or aerosol (Ton et al., 2015; Taylor et al., 2021). Menthol's effects are thought to allow users to inhale deeper, hold the smoke or aerosol in the lungs longer, and use products with higher nicotine content, all which may allow toxic or carcinogenic chemicals to have a longer duration of exposure (Ton et al., 2015). Menthol, while itself is an irritant, can also work as a counterirritant for other chemicals commonly encountered in e-cig liquids.

Menthol has been reported to reduce nicotine metabolism both *in-vitro* and *in-vivo*, leading to increased systemic nicotine exposure, reduced clearance, and longer durations of pharmacological effects (Benowitz et al., 2004; Alsharari et al., 2015). Menthol has been associated with reduced Cytochrome-P450 2A6 isoform activity (MacDougall et al., 2003; Benowitz et al., 2004). This same enzyme is responsible for nicotine metabolism, thus co-ingestion of menthol with nicotine will alter nicotine metabolism and elimination and therefore prolong the pharmacological effect of nicotine felt by the consumer.

Ethanol

Ethanol has been identified in many of the products evaluated in this study, including nicotine refill formulations, nicotine pods, and DOTN formulations, with concentrations ranging from not-present up to 217.2 mg/ml. Previous studies have also reported on ethanol concentrations in e-cig liquids, and have also identified concentrations of ethanol greater than 20% (Valentine et al., 2016; Poklis et al., 2017b).

Some flavoring chemicals use ethanol as part of the manufacturing process, such as vanilla, which is required by FDA Code of Federal Regulations to contain “not less than 35% by volume” (United States Food and Drug Administration, 2020a). While ethanol may be present in formulations due to their use in flavorings, it can also be added to thin the liquid, to help dissolve other substances that are not miscible with typical carriers, or for intentional consumption of ethanol. Do-it-yourself (DIY) e-liquids may contain higher concentrations of ethanol than those found in the manufactured products reported in the literature.

There is a dearth of knowledge regarding ethanol pharmacokinetics and intoxication from inhalation. Inhalation bypasses first-pass metabolism and increases bioavailability of ethanol. It has also been suggested that co-administration of ethanol with nicotine could lead to increased dependence and addiction liability for both substances due to their synergistic nature (McKee et al., 2006; Oliver et al., 2013), especially as ethanol has been

found to potentiate several of the rewarding effects of nicotine (Rose et al., 2004).

A clinical study from 2017 compared the effects of inhaling a nicotine e-liquid with high or trace amounts of alcohol (Valentine et al., 2016). They found no difference in subjective effects between high and trace alcohol groups. Their findings suggest that users of high alcohol concentration e-cig liquids may experience some alteration in psychomotor function without recognizing any subjective effects to alert them to the impairment. Additionally, they found the metabolites of ethanol in the urine of three participants out of the eight exposed to the high alcohol e-liquid.

Olivetol

Olivetol was identified and quantitated in five cannabinoid-based e-liquid products. Concentrations ranged from below the limit of quantitation to 3.9 mg/ml. Olivetol is a naturally occurring organic compound that can be used as a precursor for synthesizing various cannabinoids (Tadayon and Ramazani, 2021). Commonly found in lichen, it also exists for a short time in cannabis plants before conversion to CBGA, the precursor to THCA and CBDA. Its presence in e-liquid products may be due to an incomplete chemical reaction used by manufacturers when synthesizing THC, and thus could be considered an impurity. Olivetol may also be an intentional ingredient in e-liquid products. It is touted as an “antidote” to purportedly reduce unwanted effects from the consumption of high concentration THC products, such as anxiety, paranoia, or feeling overly “high” from a THC overload (Carberry, 2018; Royal Queen Seeds, 2019). Olivetol is thought to act on cannabinoid CB1 and/or CB2 receptors (Carberry, 2018). Evidence of olivetol's ability to reduce some effects of THC was reported in the UNDOO, LLC product patent application (Carberry, 2018). No clinical trials evaluating olivetol's effects have been conducted to support these claims. UNDOO reports volunteer testimonies from real-life, non-clinical trials in which participants either smoked or ingested THC, then took known amounts of olivetol, and reported on their subjective experiences.

Olivetol is listed in the National Institute of Health's PubChem database as a GHS irritant, causing skin, eye, mucous membrane, and respiratory irritation that could lead to severe tissue destruction and specific target organ toxicity with a single exposure (National Institute of Health, 2021). It emits carbon monoxide and carbon dioxide when heated to decomposition, and it is recommended to use a NIOSH-approved respirator equipped with an organic vapor/acid gas cartridge when handling neat olivetol (National Oceanic and Atmospheric Administration, 2021).

Cannabinoids

Cannabinoids have been identified and quantitated in several products evaluated in this study. CBD and THC were the most abundant in both prevalence and concentration and were quantitated in concentrations as high as 332.6 and 497.7 mg/ml, respectively. Cannabinoid-based e-liquid formulations have existed since the advent of the modern e-cigarette. JUUL is a spin-off company and product of PAX, a discreet cannabis

vaporizer launched in 2012. Gresco Science also officially launched the THC vaporizer in 2012, after years of product development and testing (Freedman, 2014; Farah, 2017; Bobrow, 2019; Hartman, 2021). The launch of these cannabis-based e-cigs coincided with adult-use legalization in Colorado and Washington. Even though almost every state in the United States has legalized some form of *C. sativa*, whether medical or adult use, the regulation of cannabis and cannabis products vary by the state.

The National Institute on Drug Abuse (NIDA) Monitoring the Future survey data indicates that while “any vaping”, “vaping nicotine”, and “vaping flavors” trends appear to be steady, or maybe even slightly decreasing, between 2019 and 2020, “vaping marijuana” is still on the rise among 8th, 10th, and 12th graders (National Institute of Health, 2020).

Like other chemicals, cannabinoid labeling accuracy has significant deficiencies. In this study, products were analyzed that were labeled with the wrong concentration, or listed as THC-free, though THC was identified. **Supplementary Material D** lists bottle/package claims and results of screening and cannabinoid quantitation. Eight out of nine products analyzed that indicated total volume of product or cannabinoid content were over-labeled. By comparison, one study reported nearly half of the CBD products analyzed were under-labeled and about 25% were over-labeled, reporting “vaporizing liquids” to be the most frequently mislabeled (85%) and oil the most frequently labeled accurately (Bonn-Miller et al., 2017). Additionally, they reported THC was present in 18 of 84 products tested, though they were listed as THC free. The current study found THC in five samples out of nine that indicated they were “THC free” or contained “0% THC”. Inaccurate product labeling demonstrates the lack of quality assurance and quality control required in the industry and poses a significant danger to consumers who may consume a higher dose than intended.

Chronic use of products containing trace concentrations of THC can result in failed urine drug tests (Spindle et al., 2020; Sholler et al., 2021). Additionally, higher peak blood concentrations have been reported from vaping THC compared to the same dose of smoked cannabis (Spindle et al., 2019). This increase in delivery efficiency is thought to be a product of minimized sidestream smoke and lack of drug pyrolysis, both of which reduce the possible dose to be inhaled in traditional combustion delivery methods (Pomahacova et al., 2009). Compared to combustion smoking conditions, vaping cannabis has been found to increase the frequency of testing above immunoassay cutoff levels in a clinical setting (Spindle et al., 2020).

Some marketed “cannabis” products contain synthetic novel psychoactive substances (NPS) as the active drug. Many NPSs are not scheduled, and therefore legal, at the time of product manufacturing. By the time these chemicals are identified and federally scheduled, manufacturers have adopted another NPS which is not scheduled, allowing manufacturers to skirt federal DEA regulations. These NPS can be more potent and can lead to severe and life-threatening situations. Some consumers have information that certain

products contain NPSs and knowingly choose to use those products. Some consumers are unaware that products purchased for relief contain NPSs but experience untoward effects (Poklis et al., 2019).

Cannabinoid-based products evaluated in this study have generally become more complicated over the years. Early formulations were mainly comprised of PG, VG, cannabinoids, and terpenes. More recent formulations contain a variety of carriers, sometimes mixing multiple carriers in one product, as well as extra active ingredients and flavorants. Terpenes found in older products are thought to be carried over from extraction methods (Peace et al., 2016). Recent formulations market terpene-specific profiles to appeal to flavor preferences or purported health benefits. Until these products are federally regulated, formulations can only be limited to individual States’ regulations.

Lung Injury

The carriers, diluents, thickeners, flavorants, and solvents identified have been generally considered as safe because of their accepted safety for oral ingestion, yet there is minimal to no evidence for long-term consequences from inhaling these substances. These chemicals can create injury to the lung tissue. They can prevent proper oxygen flow, disrupt cell membranes, cause irritation and inflammation to the lung tissue, mucosa, and bronchi, and induce lipoid pneumonia (Erythropel et al., 2019; Thirion-Romero et al., 2019; Muthumalage et al., 2020b). Two mechanisms for lung pathogenesis have been proposed. The first hypothesis describes acute exposure that creates a direct chemical injury that results in negative health effects (Alexander et al., 2020). The second mechanism describes a change to the immune cells in the alveoli due to chronic exposure to a chemical, which may or may not result in symptomology recognized by the vapor. With the addition of a new chemical, the body reaches some threshold that triggers a pathologic inflammatory response, precipitating neutrophil recruitment, edema, and necrosis (Johnson and Matthey, 2010; Alexander et al., 2020).

CONCLUSION

Increasing concentrations of pharmacologically active ingredients, the risk for complex drug-drug interactions both from individual products and co-administration with other drugs, and the general unknown implications of vaping GRAS chemicals underscore the need for transparent reporting of chemical constituents. The absence of regulatory oversight of specific ingredients and labeling requirements make the demonstration of general safety of such products difficult. Unsuspecting consumers can and are experiencing untoward and unexpected effects. Physicians may not understand and attribute the etiology of reported symptoms, leading to misdiagnoses and/or incomplete treatment regimens. Continued studies evaluating chronic and acute exposure of both singular ingredients and chemical mixtures are critical.

With these considerations, constant product surveillance incorporating untargeted chemical analyses of products intended for public consumption is critical for understanding what chemical ingredients are being used in these products and the potential health and safety impacts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AH was responsible for developing and validating several methods, compiling research results, and drafting the manuscript. MP and JP oversaw all research and edited the manuscript for clarity and content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.752342/full#supplementary-material>

REFERENCES

- Alexander, L. E. C., Bellinghausen, A. L., and Eakin, M. N. (2020). What Are the Mechanisms Underlying Vaping-Induced Lung Injury?. *J. Clin. Invest.* 130, 2754–2756. doi:10.1172/JCI138644
- Allen, J. G., Flanagan, S. S., LeBlanc, M., Vallarino, J., MacNaughton, P., Stewart, J. H., et al. (2016). Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-Pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes. *Environ. Health Perspect.* 124, 733–739. doi:10.1289/ehp.1510185
- Alsharari, S. D., King, J. R., Nordman, J. C., Muldoon, P. P., Jackson, A., Zhu, A. Z. X., et al. (2015). Effects of Menthol on Nicotine Pharmacokinetic, Pharmacology and Dependence in Mice. *PLoS ONE* 10, e0137070. doi:10.1371/journal.pone.0137070
- Behar, R. Z., Luo, W., Lin, S. C., Wang, Y., Valle, J., Pankow, J. F., et al. (2016). Distribution, Quantification and Toxicity of Cinnamaldehyde in Electronic Cigarette Refill Fluids and Aerosols. *Tob. Control.* 25, ii94–ii102. doi:10.1136/tobaccocontrol-2016-053224
- Behar, R. Z., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2018). Analytical and Toxicological Evaluation of Flavor Chemicals in Electronic Cigarette Refill Fluids. *Sci. Rep.* 8, 8288. doi:10.1038/s41598-018-25575-6
- Bendel, G. S., Hiller, H. M., and Ralston, A. (2021). Nicotine Toxicity Secondary to Aftermarket Modifications to a Vaping Device. *Mil. Med.*, 1–4. doi:10.1093/milmed/usab223
- Benowitz, N. L., Herrera, B., and Jacob, P. (2004). Mentholated Cigarette Smoking Inhibits Nicotine Metabolism. *J. Pharmacol. Exp. Ther.* 310, 1208–1215. doi:10.1124/jpet.104.066902
- Blount, B. C., Karwowski, M. P., Morel-Espinosa, M., Rees, J., Sosnoff, C., Cowan, E., et al. (2019). Evaluation of Bronchoalveolar Lavage Fluid from Patients in an Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - 10 States, August–October 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 1040–1041. doi:10.15585/mmwr.mm6845e2
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382, 697–705. doi:10.1056/NEJMoa1916433
- Bobrow, W. (2019). Chris Folkerts, CEO of Grenco Science Digs Deeply into Five Questions. *Forbes*. Available at: <https://www.forbes.com/sites/warrenbobrow/2019/08/13/chris-folkerts-ceo-of-grenco-science-digs-deeply-into-five-questions/> (Accessed June 8, 2021).
- Bonn-Miller, M. O., Loflin, M. J. E., Thomas, B. F., Marcu, J. P., Hyke, T., and Vandrey, R. (2017). Labeling Accuracy of Cannabidiol Extracts Sold Online. *JAMA* 318, 1708–1709. doi:10.1001/jama.2017.11909
- Carberry, J. J. (2018). Composition of Olivetol and Method of Use to Reduce or Inhibit the Effects of Tetrahydrocannabinol in the Human Body. U.S. Patent No 9918947. Washington, DC: U.S. Patent and Trademark Office. Available at: <https://patents.justia.com/patent/9918947> (Accessed May 26, 2020).
- Centers for Disease Control and Prevention (2020a). E-cigarette, or Vaping, Products Visual Dictionary. Available at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/pdfs/ecigarette-or-vaping-products-visual-dictionary-508.pdf (Accessed May 19, 2020).
- Centers for Disease Control and Prevention (2020b). Smoking and Tobacco Use; Electronic Cigarettes. Office on Smoking and Health. Available at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html (Accessed July 28, 2021).
- Cha, S. I., Choi, S. H., Kim, H. J., Kim, Y. J., Lim, J. K., Yoo, S. S., et al. (2019). Clinical and Radiological Manifestations of Lipoid Pneumonia According to Etiology: Squalene, omega-3-acid Ethyl Esters, and Idiopathic. *Clin. Respir. J.* 13, 328–337. doi:10.1111/crj.13015

- Costigan, S., and Meredith, C. (2015). An Approach to Ingredient Screening and Toxicological Risk Assessment of Flavours in E-Liquids. *Regul. Toxicol. Pharmacol.* 72, 361–369. doi:10.1016/j.yrtph.2015.05.018
- Duffy, B., Li, L., Lu, S., Durocher, L., Dittmar, M., Delaney-Baldwin, E., et al. (2020). Analysis of Cannabinoid-Containing Fluids in Illicit Vaping Cartridges Recovered from Pulmonary Injury Patients: Identification of Vitamin E Acetate as a Major Diluent. *Toxics* 8, 8. doi:10.3390/toxics8010008
- Ellington, S., Salvatore, P. P., Ko, J., Danielson, M., Kim, L., Cyrus, A., et al. (2020). Update: Product, Substance-Use, and Demographic Characteristics of Hospitalized Patients in a Nationwide Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - United States, August 2019-January 2020. *MMWR Morb. Mortal. Wkly. Rep.* 69, 44–49. doi:10.15585/mmwr.mm6902e2
- Erickson, B. E. (2019). Cannabis Industry Gets Crafty with Terpenes. *Chem. Eng. News*. Available at: <https://cen.acs.org/biological-chemistry/natural-products/Cannabis-industry-crafty-terpenes/97/i29> (Accessed August 28, 2021).
- Erythropel, H. C., Jabba, S. V., DeWinter, T. M., Mendizabal, M., Anastas, P. T., Jordt, S. E., et al. (2019). Formation of Flavorant-Propylene Glycol Adducts with Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids. *Nicotine Tob. Res.* 21, 1248–1258. doi:10.1093/ntr/nty192
- Fagan, P., Pokhrel, P., Herzog, T. A., Moolchan, E. T., Cassel, K. D., Franke, A. A., et al. (2018). Sugar and Aldehyde Content in Flavored Electronic Cigarette Liquids. *Nicotine Tob. Res.* 20, 985–992. doi:10.1093/ntr/ntx234
- Farah, T. (2017). A Brief History of Weed Vapes. Available at: <https://www.vice.com/en/article/43d4dm/a-brief-history-of-weed-vapes> (Accessed June 8, 2021).
- Farsalinos, K. E., Gillman, I. G., Hecht, S. S., Polosa, R., and Thornburg, J. (2016). *Analytical Assessment of E-Cigarettes: From Contents to Chemical and Particle Exposure Profiles*. Saint Louis, UNITED STATES: Elsevier. Available at: <http://ebookcentral.proquest.com/lib/vcu/detail.action?docID=4745405> (Accessed March 10, 2021).
- Flavor and Extract Manufacturers Association of the United States (2021). About FEMA GRAS Program. Available at: <https://www.femaflavor.org/gras#concept> (Accessed May 28, 2020).
- FOX 9 (2021). Officials: Poison Used in Attempted Murder Was Liquid Nicotine. Available at: <https://www.fox9.com/news/officials-substance-used-in-attempted-poisoning-was-liquid-nicotine> (Accessed July 28, 2021).
- Freedman, D. H. (2014). Ploom's Vaporizers Tackle a Marketing Challenge. Available at: <https://www.inc.com/magazine/201405/david-freedman/james-monsees-ploom-ecigarette-company-marketing-dilemma.html> (Accessed June 8, 2021).
- Geiss, O., Bianchi, I., and Barrero-Moreno, J. (2016). Correlation of Volatile Carbonyl Yields Emitted by E-Cigarettes with the Temperature of the Heating Coil and the Perceived Sensorial Quality of the Generated Vapours. *Int. J. Hyg. Environ. Health.* 219, 268–277. doi:10.1016/j.ijheh.2016.01.004
- Harrell, P. T., and Eissenberg, T. (2018). Automated Dripping Devices for Vapers: RDTAs, Bottomfeeders, Squonk Mods and Dripboxes. *Tob. Control.* 27, 480–482. doi:10.1136/tobaccocontrol-2017-053817
- Hartman, M. (2021). Cannabis Overview. Available at: <https://www.ncsl.org/research/civil-and-criminal-justice/marijuana-overview.aspx> (Accessed June 8, 2021).
- Harvanko, A. M., Havel, C. M., Jacob, P., and Benowitz, N. L. (2020). Characterization of Nicotine Salts in 23 Electronic Cigarette Refill Liquids. *Nicotine Tob. Res.* 22, 1239–1243. doi:10.1093/ntr/ntz232
- Holt, A. K., Poklis, J. L., Cobb, C. O., and Peace, M. R. (2021). Identification of Gamma-Butyrolactone in JUUL Liquids. *J. Anal. Toxicol.* 45, 892–900. doi:10.1093/jat/bkab067
- Institute for Global Tobacco Control (2018). Product Regulation (Nicotine Volume/concentration, safety/hygiene, Ingredients/flavors). Available at: <https://www.globaltobaccocontrol.org/e-cigarette/product-regulation-nicotine-volumeconcentration-safetyhygiene-ingredientsflavors> (Accessed July 28, 2021).
- Jiang, H., Ahmed, C. M. S., Martin, T. J., Canchola, A., Oswald, I. W. H., Garcia, J. A., et al. (2020). Chemical and Toxicological Characterization of Vaping Emission Products from Commonly Used Vape Juice Diluents. *Chem. Res. Toxicol.* 33, 2157–2163. doi:10.1021/acs.chemrestox.0c00174
- Johnson, E. R., and Matthay, M. A. (2010). Acute Lung Injury: Epidemiology, Pathogenesis, and Treatment. *J. Aerosol Med. Pulm. Drug Deliv.* 23, 243–252. doi:10.1089/jamp.2009.0775
- Kosmider, L., Sobczak, A., Fik, M., Knysak, J., Zaciera, M., Kurek, J., et al. (2014). Carbonyl Compounds in Electronic Cigarette Vapors: Effects of Nicotine Solvent and Battery Output Voltage. *Nicotine Tob. Res.* 16, 1319–1326. doi:10.1093/ntr/ntu078
- Krishnasamy, V. P., Hallowell, B. D., Ko, J. Y., Board, A., Hartnett, K. P., Salvatore, P. P., et al. (2020). Update: Characteristics of a Nationwide Outbreak of E-Cigarette, or Vaping, Product Use-Associated Lung Injury - United States, August 2019-January 2020. *MMWR Morb. Mortal. Wkly. Rep.* 69, 90–94. doi:10.15585/mmwr.mm6903e2
- Leigh, N. J., Lawton, R. I., Hershberger, P. A., and Goniewicz, M. L. (2016). Flavours Significantly Affect Inhalation Toxicity of Aerosol Generated from Electronic Nicotine Delivery Systems (ENDS). *Tob. Control.* 25, ii81–ii87. doi:10.1136/tobaccocontrol-2016-053205
- Lestari, K. S., Humairo, M. V., and Agustina, U. (2018). Formaldehyde Vapor Concentration in Electronic Cigarettes and Health Complaints of Electronic Cigarettes Smokers in Indonesia. *J. Environ. Public Health* 2018, 1–6. doi:10.1155/2018/9013430
- Lin, C., Arrossi, V., Yadav, R., and Choi, H. (2020). Vaping-related Pulmonary Granulomatous Disease. *Respir. Med. Case Rep.* 31, 101179. doi:10.1016/j.rmcr.2020.101179
- MacDougall, J. M., Fandrick, K., Zhang, X., Serafin, S. V., and Cashman, J. R. (2003). Inhibition of Human Liver Microsomal (S)-Nicotine Oxidation by (–)-Menthyl and Analogues. *Chem. Res. Toxicol.* 16, 988–993. doi:10.1021/tx0340551
- McKee, S. A., Krishnan-Sarin, S., Shi, J., Mase, T., and O'Malley, S. S. (2006). Modeling the Effect of Alcohol on Smoking Lapse Behavior. *Psychopharmacology* 189, 201–210. doi:10.1007/s00213-006-0551-8
- Münzel, T., Hahad, O., Kuntic, M., Keaney, J. F., Deanfield, J. E., and Daiber, A. (2020). Effects of Tobacco Cigarettes, E-Cigarettes, and Waterpipe Smoking on Endothelial Function and Clinical Outcomes. *Eur. Heart J.* 41, 4057–4070. doi:10.1093/eurheartj/ehaa460
- Muthumalage, T., Friedman, M. R., McGraw, M. D., Ginsberg, G., Friedman, A. E., and Rahman, I. (2020a). Chemical Constituents Involved in E-Cigarette, or Vaping Product Use-Associated Lung Injury (EVALI). *Toxics* 8, 25. doi:10.3390/toxics8020025
- Muthumalage, T., Lucas, J. H., Wang, Q., Lamb, T., McGraw, M. D., and Rahman, I. (2020b). Pulmonary Toxicity and Inflammatory Response of Vape Cartridges Containing Medium-Chain Triglycerides Oil and Vitamin E Acetate: Implications in the Pathogenesis of EVALI. *Toxics* 8, 46. doi:10.3390/toxics8030046
- National Institute of Health (2020). National Institute on Drug AbuseMonitoring the Future Study: Trends in Prevalence of Various Drugs. Available at: <https://www.drugabuse.gov/trends-statistics/monitoring-future/monitoring-future-study-trends-in-prevalence-various-drugs> (Accessed May 13, 2020).
- National Institute of Health (2021). National Library of Medicine. Available at: <https://pubchem.ncbi.nlm.nih.gov/> (Accessed March 11, 2021).
- National Oceanic and Atmospheric Administration (2021). OLIVETOL. Available at: <https://cameochemicals.noaa.gov/chemical/20821> (Accessed June 4, 2021).
- National Poison Data System, American Association of Poison Control Centers (2021). E-cigarettes and Liquid Nicotine. Available at: <https://aapcc.org/track/ecigarettes-liquid-nicotine> (Accessed June 16, 2021).
- Oliver, J. A., Blank, M. D., Van Rensburg, K. J., MacQueen, D. A., Brandon, T. H., and Drobos, D. J. (2013). Nicotine Interactions with Low-Dose Alcohol: Pharmacological Influences on Smoking and Drinking Motivation. *J. Abnormal Psychol.* 122, 1154–1165. doi:10.1037/a0034538
- Omaiye, E. E., McWhirter, K. J., Luo, W., Pankow, J. F., and Talbot, P. (2019a). High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations. *Chem. Res. Toxicol.* 32, 1058–1069. doi:10.1021/acs.chemrestox.8b00381
- Omaiye, E. E., McWhirter, K. J., Luo, W., Tierney, P. A., Pankow, J. F., and Talbot, P. (2019b). High Concentrations of Flavor Chemicals Are Present in Electronic Cigarette Refill Fluids. *Sci. Rep.* 9, 2468. doi:10.1038/s41598-019-39550-2
- Overbeek, D. L., Kass, A. P., Chiel, L. E., Boyer, E. W., and Casey, A. M. H. (2020). A Review of Toxic Effects of Electronic Cigarettes/vaping in Adolescents and Young Adults. *Crit. Rev. Toxicol.* 50, 531–538. doi:10.1080/10408444.2020.1794443
- Pagano, T., DiFrancesco, A. G., Smith, S. B., George, J., Wink, G., Rahman, I., et al. (2016). Determination of Nicotine Content and Delivery in Disposable Electronic

- Cigarettes Available in the United States by Gas Chromatography-Mass Spectrometry. *Nictob* 18, 700–707. doi:10.1093/ntr/ntv120
- Pan, S.-c., Cui, H.-h., Si, G.-g., Qiu, C.-g., and Qin, Z.-s. (2021). Association of Long-Term Inhalation of Caffeine-Sodium Benzoate with Coronary Heart Disease in Men. *Cp* 59, 247–253. doi:10.5414/CP203805
- Peace, M. R., Baird, T. R., Smith, N., Wolf, C. E., Poklis, J. L., and Poklis, A. (2016). Concentration of Nicotine and Glycols in 27 Electronic Cigarette Formulations. *J. Anal. Toxicol.* 40, 403–407. doi:10.1093/jat/bkw037
- Peace, M. R., Krakowiak, R. I., Wolf, C. E., Poklis, A., and Poklis, J. L. (2017). Identification of MDMB-FUBINACA in Commercially Available E-Liquid Formulations Sold for Use in Electronic Cigarettes. *Forensic Sci. Int.* 271, 92–97. doi:10.1016/j.forsciint.2016.12.031
- Peace, M. R., Mulder, H. A., Baird, T. R., Butler, K. E., Friedrich, A. K., Stone, J. W., et al. (2018). Evaluation of Nicotine and the Components of E-Liquids Generated from E-Cigarette Aerosols. *J. Anal. Toxicol.* 42, 537–543. doi:10.1093/jat/bky056
- Poklis, J. L., Mulder, H. A., Halquist, M. S., Wolf, C. E., Poklis, A., and Peace, M. R. (2017a). The Blue Lotus Flower (*Nymphaea Caerulea*) Resin Used in a New Type of Electronic Cigarette, the Re-buildable Dripping Atomizer. *J. Psychoactive Drugs*. 49, 175–181. doi:10.1080/02791072.2017.1290304
- Poklis, J. L., Mulder, H. A., and Peace, M. R. (2019). The Unexpected Identification of the Cannabimimetic, 5F-ADB, and Dextromethorphan in Commercially Available Cannabidiol E-Liquids. *Forensic Sci. Int.* 294, e25–e27. doi:10.1016/j.forsciint.2018.10.019
- Poklis, J. L., Raso, S. A., Alford, K. N., Poklis, A., and Peace, M. R. (2015). Analysis of 25I-NBOMe, 25B-NBOMe, 25C-NBOMe and Other Dimethoxyphenyl-N-[(2-Methoxyphenyl) Methyl]Ethanamine Derivatives on Blotter Paper. *J. Anal. Toxicol.* 39, 617–623. doi:10.1093/jat/bkv073
- Poklis, J. L., Thompson, C. C., Long, K. A., Lichtman, A. H., and Poklis, A. (2010). Disposition of Cannabichromene, Cannabidiol, and 9-Tetrahydrocannabinol and its Metabolites in Mouse Brain Following Marijuana Inhalation Determined by High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *J. Anal. Toxicol.* 34, 516–520. doi:10.1093/jat/34.8.516
- Poklis, J. L., Wolf, C. E., and Peace, M. R. (2017b). Ethanol Concentration in 56 Refillable Electronic Cigarettes Liquid Formulations Determined by Headspace Gas Chromatography with Flame Ionization Detector (HS-GC-FID). *Drug Test. Anal.* 9, 1637–1640. doi:10.1002/dta.2193
- Pomahacova, B., Van der Kooy, F., and Verpoorte, R. (2009). Cannabis Smoke Condensate III: The Cannabinoid Content of vaporised Cannabis Sativa. *Inhalation Toxicol.* 21, 1108–1112. doi:10.3109/08958370902748559
- Qu, Y., Szulejko, J. E., Kim, K.-H., and Jo, S.-H. (2019). The Effect of Varying Battery Voltage Output on the Emission Rate of Carbonyls Released from E-Cigarette Smoke. *Microchemical J.* 145, 47–54. doi:10.1016/j.microc.2018.10.019
- Rose, J., Brauer, L., Behm, F., Cramblett, M., Calkins, K., and Lawhon, D. (2004). Psychopharmacological Interactions between Nicotine and Ethanol. *Nicotine Tob. Res.* 6, 133–144. doi:10.1080/14622200310001656957
- Royal Queen Seeds (2019). Uncomfortably High? Olivetol May Be Able to Bring You Down. Available at: <https://www.royalqueenseeds.com/blog-uncomfortably-high-olivetol-may-be-able-to-bring-you-down-n1154> (Accessed May 26, 2020).
- Sherwood, C. L., and Boitano, S. (2016). Airway Epithelial Cell Exposure to Distinct E-Cigarette Liquid Flavorings Reveals Toxicity Thresholds and Activation of CFTR by the Chocolate Flavoring 2,5-dimethylpyrazine. *Respir. Res.* 17, 57. doi:10.1186/s12931-016-0369-9
- Sholler, D. J., Spindle, T. R., Cone, E. J., Goffi, E., Kuntz, D., Mitchell, J. M., et al. (2021). Urinary Pharmacokinetic Profile of Cannabidiol (CBD), Δ^9 -tetrahydrocannabinol (THC) and Their Metabolites Following Oral and Vaporized CBD and Vaporized CBD-Dominant Cannabis Administration. *J. Anal. Toxicol.* [Preprint]. doi:10.1093/jat/bkab059
- Spindle, T. R., Cone, E. J., Schlienz, N. J., Mitchell, J. M., Bigelow, G. E., Flegel, R., et al. (2019). Acute Pharmacokinetic Profile of Smoked and Vaporized Cannabis in Human Blood and Oral Fluid. *J. Anal. Toxicol.* 43, 233–258. doi:10.1093/jat/bky104
- Spindle, T. R., Cone, E. J., Schlienz, N. J., Mitchell, J. M., Bigelow, G. E., Flegel, R., et al. (2020). Urinary Excretion Profile of 11-Nor-9-Carboxy- Δ^9 -Tetrahydrocannabinol (THCCOOH) Following Smoked and Vaporized Cannabis Administration in Infrequent Cannabis Users. *J. Anal. Toxicol.* 44, 1–14. doi:10.1093/jat/bkz038
- Tadayon, N., and Ramazani, A. (2021). A Review on the Syntheses of Dronabinol and Epidiolex as Classical Cannabinoids with Various Biological Activities Including Those against SARS-COV-2. *J. Iran. Chem. Soc.* 18, 2517–2534. doi:10.1007/s13738-021-02212-0
- Taylor, A., Dunn, K., and Turfus, S. (2021). A Review of Nicotine-containing Electronic Cigarettes-Trends in Use, Effects, Contents, Labelling Accuracy and Detection Methods. *Drug Test. Anal.* 13, 242–260. doi:10.1002/dta.2998
- Taylor, J., Wiens, T., Peterson, J., Saravia, S., Lunda, M., Hanson, K., et al. (2019). Characteristics of E-Cigarette, or Vaping, Products Used by Patients with Associated Lung Injury and Products Seized by Law Enforcement - Minnesota, 2018 and 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 1096–1100. doi:10.15585/mmwr.mm6847e1
- Thirion-Romero, I., Pérez-Padilla, R., Zabert, G., and Barrientos-Gutiérrez, I. (2019). Respiratory Impact of Electronic Cigarettes and Low-Risk Tobacco. *Ric* 71, 17–27. doi:10.24875/RIC.18002616
- Ton, H. T., Smart, A. E., Aguilar, B. L., Olson, T. T., Kellar, K. J., and Ahern, G. P. (2015). Menthol Enhances the Desensitization of Human $\alpha 3 \beta 4$ Nicotinic Acetylcholine Receptors. *Mol. Pharmacol.* 88, 256–264. doi:10.1124/mol.115.098285
- Treloar, H. R., Piasecki, T. M., McCarthy, D. E., and Baker, T. B. (2014). Relations Among Caffeine Consumption, Smoking, Smoking Urge, and Subjective Smoking Reinforcement in Daily Life. *J. Caffeine Res.* 4, 93–99. doi:10.1089/jcr.2014.0007
- Troutt, W. D., and DiDonato, M. D. (2017). Carbonyl Compounds Produced by Vaporizing Cannabis Oil Thinning Agents. *J. Altern. Complement. Med.* 23, 879–884. doi:10.1089/acm.2016.0337
- Ueno, H., Takahashi, Y., Suemitsu, S., Murakami, S., Kitamura, N., Wani, K., et al. (2020). Caffeine Inhalation Effects on Locomotor Activity in Mice. *Drug Dev. Ind. Pharm.* 46, 788–794. doi:10.1080/03639045.2020.1753064
- United States Food and Drug Administration (2020a). CFR - Code of Federal Regulations Title 21. Available at: <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=169.175> (Accessed May 25, 2021).
- United States Food and Drug Administration (2020b). Enforcement Priorities for Electronic Nicotine Delivery Systems (ENDS) and Other Deemed Products on the Market without Premarket Authorization (Revised): Guidance for Industry 52. Available at: <https://www.fda.gov/tobacco-products/products-guidance-regulations/rules-regulations-and-guidance> (Accessed July 28, 2021).
- United States Food and Drug Administration (2019). Generally Recognized as Safe (GRAS). Available at: <https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras> (Accessed May 28, 2020).
- Valentine, G. W., Jatlow, P. I., Coffman, M., Nadim, H., Gueorgieva, R., and Sofuoglu, M. (2016). The Effects of Alcohol-Containing E-Cigarettes on Young Adult Smokers. *Drug. Alcohol Dependence* 159, 272–276. doi:10.1016/j.drugalcdep.2015.12.011
- Yingst, J. M., Bordner, C. R., Hobkirk, A. L., Hoglen, B., Allen, S. I., Krebs, N. M., et al. (2021). Response to Flavored Cartridge/Pod-Based Product Ban Among Adult JUUL Users: "You Get Nicotine However You Can Get it". *Ijerph* 18, 207. doi:10.3390/ijerph18010207
- Zachar, L. (2018). Why Choose MCT over PG VG as Your Vape Juice Carrier(2018). Available at: <https://cbdtesters.co/2018/08/30/why-choose-mct-over-pg-vg/> (Accessed May 14, 2020).

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An Automated Aerosol Collection and Extraction System to Characterize Electronic Cigarette Aerosols

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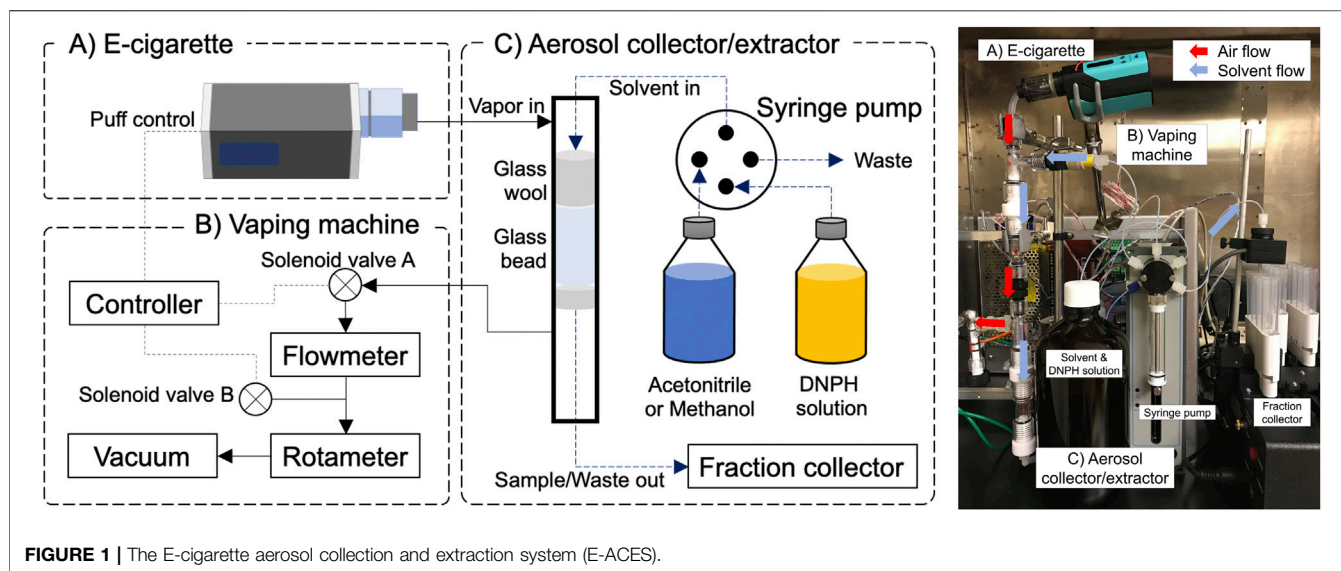
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Electronic cigarette (e-cigarette) market increased by 122% during 2014–2020 and is expected to continue growing rapidly. Despite their popularity, e-cigarettes are known to emit dangerous levels of toxic compounds (e.g., carbonyls), but a lack of accurate and efficient testing methods is hindering the characterization of e-cigarette aerosols emitted by a wide variety of e-cigarette devices, e-liquids, and use patterns. The aim of this study is to fill this gap by developing an automated E-cigarette Aerosol Collection and Extraction System (E-ACES) consisting of a vaping machine and a collection/extraction system. The puffing system was designed to mimic e-cigarette use patterns (i.e., power output and puff topography) by means of a variable power-supply and a flow control system. The sampling system collects e-cigarette aerosols using a combination of glass wool and a continuously wetted denuder. After the collection stage, the system is automatically washed with absorbing and extracting liquids (e.g., methanol, an acetaldehyde-DNPH solution). The entire system is controlled by a computer. E-ACES performance was evaluated against conventional methods during measurements of nicotine and carbonyl emissions from a tank type e-cigarette. Nicotine levels measured using glass fiber filters and E-ACES were not significantly different: 201.2 ± 6.2 and 212.5 ± 17 $\mu\text{g}/\text{puff}$ ($p = 0.377$), respectively. Differences in formaldehyde and acetaldehyde levels between filter-DNPH cartridges and the E-ACES were 14% ($p = 0.057$) and 13% ($p = 0.380$), respectively. The E-ACES showed reproducible nicotine and carbonyl testing results for the selected e-cigarette vaping conditions.

Keywords: electronic cigarette, carbonyl, aldehyde, nicotine, testing, denuder, DNPH, public health

INTRODUCTION

Popularity of electronic cigarettes (e-cigarettes) has been rapidly increasing, with sales per 4 weeks interval increasing from 7.7 million in 2014 to 17.1 million units in 2020 (Ali et al., 2020). Despite the popularity, e-cigarettes are known to emit potentially harmful compounds including heavy metals (Olmedo et al., 2018; Zhao et al., 2019), carbonyls (Geiss et al., 2016; Khlystov and Samburova 2016; Sleiman et al., 2016; Son et al., 2020), vaporized flavoring chemicals (Allen et al., 2015; Klager et al., 2017), and reactive oxygen species (ROS) (Lerner et al., 2015; Anderson et al., 2016; Son et al., 2019) in concentrations that could cause numerous adverse health impacts on respiratory, cardiovascular, neurological and immune system (Hua and Talbot, 2016; NASEM, 2018). In order to protect public health, the U.S. Food



and Drug Administration (FDA) introduced a deeming rule regulating tobacco products including e-cigarette products requiring e-cigarette product testing and reporting potentially harmful substances (FDA, 2016).

In line with the regulatory efforts, scientists have been reporting a number of potentially harmful compounds in e-cigarette emissions. For instance, carbonyls are the most commonly reported and abundant harmful or potentially harmful compounds found in e-cigarette emissions (NASEM, 2018). However, there is a lack of standardized e-cigarette testing methods that can efficiently address a wide range of e-cigarette device settings (e.g., power output, coil type, and coil surface area, etc.), e-liquid compositions (e.g., base material, nicotine content, and flavoring, etc.), and vaping topography (i.e., puff duration, volume, and interval). For carbonyl measurements, most studies used either 2,4-dinitrophenyl-hydrazone (DNPH) cartridges (Goniewicz et al., 2014; Geiss et al., 2016; Khlystov and Samburova, 2016; Sleiman et al., 2016; Beauval et al., 2019) or impingers containing DNPH solution (Gillman et al., 2016; Flora et al., 2017; Farsalinos et al., 2018) to measure carbonyls in e-cigarette aerosol. While these methods are widely accepted, they are labor and cost intensive to allow quick and efficient testing of the rapidly evolving e-cigarette products under the wide range of use conditions. It is also worth mentioning that current commercially available smoking machines have been originally designed to target conventional cigarettes that do not produce large amounts of liquid particulates and large amounts of condensable gases that could hinder their performance.

The aim of this study was to develop a fully automated E-cigarette Aerosol Collection and Extraction System (E-ACES) which combines an e-cigarette vaping machine with an aerosol collection/extraction system. We developed and evaluated a prototype of E-ACES using a fourth generation “mod” type device filled with a tobacco flavored e-liquid. The

performance of E-ACES for nicotine and carbonyl measurements was compared with conventional testing methods.

MATERIALS AND METHODS

The E-Cigarette Aerosol Collection and Extraction System

The E-ACES consists of an e-cigarette vaping machine and an aerosol collector/extractor (Figure 1). The vaping machine has two 24 V DC solenoid valves connected to a vacuum source. Air flow rates were monitored and controlled using a flowmeter (TSI, Shoreview, MN) and a rotameter. The solenoid valves, as well as an e-cigarette, were controlled using a U6 multifunction DAQ device with a PS12DC power switching board (LabJack Corporation, Lakewood, CO) connected to a laptop. To initiate a puff, the controller opened the solenoid valve A and closed the solenoid valve B (Figure 1), while simultaneously activating the e-cigarette using a relay channel for a “mod”-device or a power supply channel for a 510-thread type device. To terminate the puff, the controller de-activates the e-cigarette, closes the valve A and opens valve B. A Python script was used to operate the controller according to a pre-defined vaping topography.

Sample Collection Using E-Cigarette Aerosol Collection and Extraction System

E-cigarette aerosol samples for nicotine and aldehyde analysis were collected using the E-ACES. A “mod” type e-cigarette device [ReuLeaux RX200 (WISMEC Electronics, Guangdong, China) and an Aspire Cleito atomizer (Shenzhen Eigate Technology, Shenzhen, China)] with a tobacco flavored e-liquid [3:7 = propylene glycol (PG): vegetable glycerin (VG), 6 mg/ml nicotine] at 50 W power output was used to test the E-ACES. We used 4 s puff duration,

100 ml puff volume, and 30 s puff interval based on the reported e-cigarette vaping topography (Dautzenberg and Bricard, 2015; Son et al., 2020). For the nicotine analysis, the aerosol collection part was rinsed with 5 ml methanol (LC/MS grade, Fisher Chemical, Waltham, MA) before aerosol generation. 5-puffs of e-cigarette aerosol were collected on the continuously wetted collector (methanol, 0.5 ml/min rate), then extracted three times with 2 ml methanol and the extract collected in the fraction collector. After that, the system was flushed with 5 ml methanol to assure no carry-over to the next analysis. Carbonyls in e-cigarette aerosols were collected as follows: 1) the collector was rinsed with 5 ml acetonitrile, 2) the glass wool and beads were soaked with 1 ml DNPH solution [22 mM DNPH (Spectrum, New Brunswick, NJ) in acetonitrile with 25 mM hydrochloric acid (Sigma-Aldrich, MO, United States), pH3-4], then 3) 5-puffs of e-cigarette aerosol were generated while the collector was continuously wetted with the DNPH solution (0.5 ml/min rate). After the collection step, the collector was extracted with 2 ml acetonitrile three times, then flushed with 5 ml acetonitrile. Extracted samples were stored in centrifuge tubes using the fraction collector. Nicotine and carbonyl sample collection and extraction were continuously done in triplicate (6 sampling cycles in total, 5 min sampling time per one condition) without any interruption (e.g., changing collection media, etc.). Collected sample volumes were consistent (less than 5% variation) across the samples.

Sample Collection Using Conventional Testing Methods

For verification purposes, we also analyzed e-cigarette nicotine and carbonyl emissions using conventional methods. E-cigarette aerosols were generated using the vaping machine under the same vaping conditions (i.e., a “mod” device with tobacco flavored e-liquid, 50 W power output, 4 s puff duration, 100 ml volume, and 30 s interval). Nicotine samples were collected using glass fiber filter (GFF) pads (47 mm, MilliporeSigma, Burlington, MA). The sample filters were spiked with 40 µg of quinoline (98%, Sigma-Aldrich, MO, United States) as an internal standard. For carbonyl analysis, DNPH-coated glass fiber filters (ORBO 827, SUPELCO, CA, United States) followed by DNPH cartridges (Sep-Pak XPoSure Plus Short Cartridge, Waters, Milford, MA, United States) were used to assure collection of both particle- and gas-phase carbonyls (Son et al., 2020). All measurements were done in triplicate.

Nicotine Analysis

The GFF were extracted with 4 ml methanol. 1 µl of the E-ACES extracts or the filter extracts were injected into an HPLC system (Waters 2,690 Alliance System with a model 996 photodiode array detector) equipped with an Agilent Polaris 3 column (C18-A, 3 µm, 100 × 2.0 mm). The mobile phase A was pH 7.9 phosphate buffer [8.5 mM Na₂HPO₄ (Electron Microscopy Sciences, PA, United States), 1.5 mM KH₂PO₄ (Beantown Chemical Corporation, NH, United States)] and the mobile

phase B was methanol. The solvent gradient (0.1 ml/min flow rate) was 70% mobile phase A at 0-min and hold for 2.5 min, increase to 95% in 3.5 min, decrease to 30% in 2 min and hold for 4 min, increase to 95% in 3 min and hold for 5 min, and decrease to 70% in 5 min and hold for 5 min. The total run time was 30 min. External standards of nicotine (99%, Sigma-Aldrich, MO, United States) and quinoline were prepared and quantified at 260 and 220 nm wavelengths, respectively. Limits of detection (LOD) and limits of quantification (LOQ) were estimated by adding three- and ten-times the standard deviation of seven measurements of the lowest calibration standard, respectively, to the mean blank sample value. LOD and LOQ for nicotine were 0.44 µg/ml and 1.47 µg/ml, respectively.

Carbonyl Analysis

DNPH-filters and DNPH-cartridges were extracted with 2 ml of acetonitrile. The HPLC system described above were used to quantify carbonyl compounds. Sample injection and mobile phase flow rates were 2 µl and 0.25 ml/min, respectively. Acetonitrile (mobile phase A) and ultrapure water (mobile phase B) was used to separate carbonyl compounds. Mobile phase gradients were 42% phase A at 0 min and hold for 9 min, increase to 55% in 7 min and hold for 2 min, increase to 90% in 1 min and hold for 6 min, decrease to 42% in 1 min and hold for 4 min. Carbonyls (i.e., formaldehyde, acetaldehyde, acrolein, propionaldehyde, 2-butanone, benzaldehyde, glyoxal, and hexaldehyde) were detected at 360 nm wavelength and full spectrum readings (210–400 nm) were used to confirm individual compounds. A certified carbonyl calibration mixture (AccuStandard, CT, United States) was used to generate calibration curves. LOD and LOQ for the eight carbonyl compounds were estimated using the same method described above and ranged from 0.011 to 0.022 µg/ml and from 0.037 to 0.074 µg/ml, respectively.

Statistical Analysis

Two-tailed Student's t-tests were conducted to compare nicotine and carbonyl emission levels measured with the conventional methods and the E-ACES using the R software package version 3.4.3 (R Development Core Team, Vienna, Austria). Significances were determined at $p = 0.05$.

RESULTS

Figure 2 shows nicotine and carbonyl levels emitted from a “mod” type e-cigarette device. Measured nicotine levels were not significantly different, with values of 201.2 ± 6.2 and 212.5 ± 17 µg/puff ($p = 0.377$) for the conventional method (i.e., GFF method) and the E-ACES, respectively. Carbonyl emission levels measured using the DNPH-filter/cartridge method and the E-ACES were not significantly different except benzaldehyde. Formaldehyde and acetaldehyde, which are known carcinogens, were 0.854 ± 0.034 and 0.305 ± 0.031 µg/puff for DNPH-filter/cartridge method and 0.995 ± 0.069 and 0.350 ± 0.064 µg/puff for the E-ACES method, respectively (p -values > 0.057). Acrolein, propionaldehyde, and 2-butanone levels measured using the

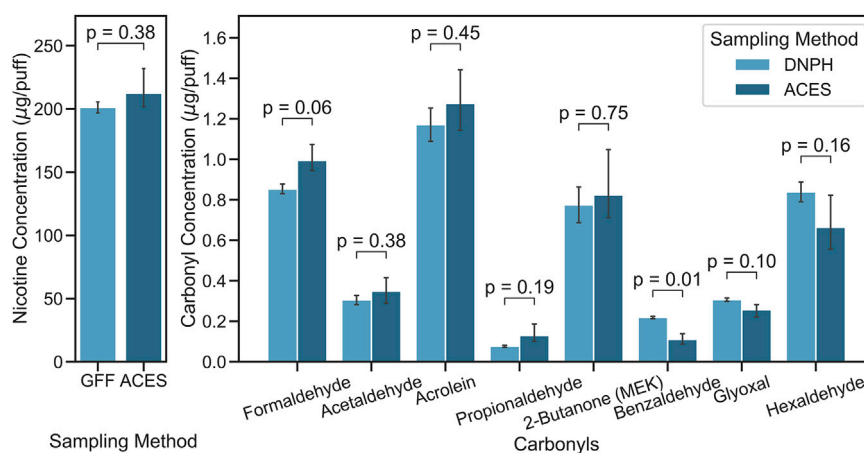


FIGURE 2 | E-cigarette nicotine and carbonyl emission levels measured using the conventional methods [glass fiber filter (GFF) or DNPH-filer/cartridge (DNPH)] and the E-cigarette Aerosol Collection and Extraction System (E-ACES).

E-ACES method were slightly higher than the conventional method without significance (p -values > 0.193). Benzaldehyde levels determined using the conventional method were significantly higher than the E-ACES method ($0.219 \pm 0.008 \mu\text{g/puff}$ vs. $0.111 \pm 0.026 \mu\text{g/puff}$, $p = 0.011$). Conventional method could capture higher levels of glyoxal and hexaldehyde from e-cigarette aerosol than the E-ACES method (p -values > 0.102). Variabilities between the two methods were 5.6, 14.2, and 12.7% for nicotine, formaldehyde, and acetaldehyde, respectively.

DISCUSSION

We developed the fully automated E-cigarette Aerosol Collection and Extraction System (E-ACES) to characterize two types of e-cigarette emissions (i.e., nicotine and carbonyls). The results of the study show that the E-ACES method provides measurements that are in a good agreement with the conventional methods for nicotine and most of the measured carbonyl compounds. A low variability ($< 15\%$) between the E-ACES and the conventional methods for nicotine and the two main toxic aldehyde (formaldehyde and acetaldehyde) levels provides confidence in the reliability of the developed system. The E-ACES system detection limit (LOD) for nicotine was $0.088 \mu\text{g/puff}$ and for aldehydes it was 0.016 – $0.244 \mu\text{g/puff}$ (formaldehyde and acetaldehyde were 0.016 and $0.032 \mu\text{g/puff}$, respectively) using a 5-puff aerosol collection (4 s puff duration and 30 s interval) and 6 ml extraction. The system LOD is sufficiently low to detect nicotine and aldehydes emitted from most e-cigarette devices (Khlystov and Samburova, 2016; El-Hellani et al., 2018; Beauval et al., 2019; Gillman et al., 2020) except for acetaldehyde and glyoxal from a “pod” device (i.e., JUUL) (Son et al., 2020). The high sensitivity of the E-ACES system could allow cost and labor efficient sample collection (e.g., 5 min sampling/condition) for most e-cigarette products. The system sensitivity could be further improved by increasing the number of collected puffs. For instance, the system LOD for acetaldehyde will be $0.009 \mu\text{g/puff}$ if 35 puffs are collected (15 min sampling/condition),

which will be sufficient for detection of acetaldehyde in JUUL aerosols containing $0.01 \pm 0.01 \mu\text{g}$ acetaldehyde/puff (Son et al., 2020). A modular construction (vaping machine and aerosol collector/extractor) of the E-ACES is one of its strengths. The vaping machine could be applied to other sampling devices and/or methods. The aerosol collector/extractor could be applied to other tobacco products or even air pollution research to collect and extract samples automatically.

To the best of our knowledge, there is no such automated system for e-cigarette emission testing in a high-throughput format. Most studies reported so far have been using labor intensive sampling methods employing filters, cartridges, or impingers (Goniewicz et al., 2012; Uchiyama et al., 2013; Geiss et al., 2016; Khlystov and Samburova, 2016; Flora et al., 2017; Farsalinos et al., 2018; Qu et al., 2018; Son et al., 2018; Gillman et al., 2020). Havel et al. (2017) developed an e-cigarette-specific vaping machine that consists of a solenoid relay and a valve to generate e-cigarette aerosols. The vaping machine could operate e-cigarettes under a wide variety of vaping conditions. However, their sample collection and extraction were still manual and fairly labor intensive, involving three impingers containing 40 ml HCl solution, to test nicotine, propylene glycol (PG), and vegetable glycerin (VG). In another study, a direct e-cigarette aerosol collection method was evaluated (Olmedo et al., 2016). Strength of that collection method is that the system could collect undiluted e-cigarette aerosol samples, but the method needs long sampling times (15–20 min) and post sample processing steps for chemical analysis. Unlike these methods, the E-ACES automatically activates and puffs e-cigarettes, as well as collects and extracts e-cigarette aerosol samples. It also provides an opportunity to be interfaced with an online analytical instrument, thus providing a fully automated sample collection, extraction and analysis system.

The developed E-ACES prototype still has room for improvement. First, the E-ACES vaping machine needs a more sophisticated flow rate controller, such as a programmable mass flow controller. The current prototype system employed a flow meter and a rotameter to control air flows through the system. The manual flow rate controller

cannot address flow rate variations due to the pressure drop across the sample collection system. Even though we didn't observe a significant pressure drop during testing, accurate and consistent flow rate would be desired because flow rate could change e-cigarette chemical emissions (Zhao et al., 2016; Son et al., 2020).

Second, particle collection efficiency of the aerosol collector needs to be improved. The glass wool plug proved to be sufficiently efficient to collect particles emitted from the "mod" type e-cigarette. However, the glass wool plug showed particle breakthrough for a "pod" type device (e.g., JUUL). This is because the "pod" type device generates smaller particles than the "mod" type device due to the lower power output (Floyd et al., 2018), which the glass wool could not capture. The glass wool filter could be replaced with a finer pore material such as a fritted glass in-line column filter.

Third, the collection efficiency could be further improved by optimization of derivatization and/or sampling methods. Our results showed that the E-ACES could collect similar or slightly higher levels of low-molecular carbonyls (e.g., formaldehyde and acetaldehyde), but lower amounts of large-molecular compounds (e.g., benzaldehyde and hexaldehyde). This could be due to differences in chemical properties of the target analytes. For instance, formaldehyde and acetaldehyde have high solubility (i.e., 13.2 M and 22.7 M, respectively) that could increase absorption rate, and thus their collection efficiency, while benzaldehyde has a low solubility (i.e., 0.06 M) and could be less efficiently absorbed during the contact time with DNPH (i.e., 20–40 min) (de Andrade and Tanner, 1992). Materials improving chemical sorption (e.g., silica gel beads) could help to capture chemicals emitted from e-cigarettes (Uchiyama et al., 2010). Collection efficiency of the DNPH solution-wetted filter/denuder type sampler could also be affected by pH, humidity, and characteristics of target carbonyl compounds (Kallinger and Niessner, 1997; John et al., 2020). Reaction between DNPH and carbonyl compounds are more efficient at acidic conditions than at neutral pH (Bicking et al., 1988). Our continuously wetting system supplying fresh DNPH solution to the filter/denuder minimizes pH changes over time, but the impacts of nicotine ($pK_a = 8.0$) and other e-liquid constituents on pH need to be studied. Humid conditions (60% relative humidity [RH]) could accelerate DNPH-carbonyl derivatization reactions relative to dry conditions (0% RH) (John et al., 2020). E-liquids are known to contain different levels of water (Crenshaw et al., 2016). The impact of e-liquid water content should be evaluated to optimize the carbonyl collection in our system.

Lastly, there is an emerging need of testing metallic nanoparticles in e-cigarette aerosols due to their health risks (Mikheev et al., 2016; Olmedo et al., 2018; Wilson et al., 2019). The E-ACES system was originally designed to test e-cigarette chemical compound emissions (e.g., nicotine and carbonyls), but the developed system can be potentially adapted to measuring the aerosol metal content by using nitric acid as an extraction solution. Such an adaptation will require

additional testing to characterize metal particle collection and extraction efficiency.

A limitation of this work is the limited number of e-cigarette devices and vaping conditions tested. A testing method and/or instrument should be evaluated systematically to prove their reliability. We have tested a "mod" type e-cigarette device with a tobacco flavored e-liquid under a single vaping topography (4 s puff duration, 100 ml volume, and 30 s interval). The tested e-cigarette is one of the most popular devices (i.e., "mod" and "pod" type device) and we used a vaping topography mimicking the common use patterns (Dautzenberg and Bricard, 2015; Robinson et al., 2015). This study aimed to demonstrate the capabilities of the new E-ACES method. We plan to further improve the E-ACES and evaluate it using different devices, e-liquids, and vaping conditions.

In conclusion, the E-ACES was developed to improve our ability to test for potentially harmful chemicals in e-cigarette aerosols that is critical for understanding the potential risks of e-cigarette use. Despite the limitations stated above, the E-ACES showed a good agreement with the conventional methods in measuring nicotine and carbonyls in e-cigarette aerosols. The developed instrument could benefit public health and tobacco regulatory science by accurately and rapidly testing a large variety of e-cigarette devices and e-liquids under different conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YS and AK contributed to conception and design of the study. YS performed the experiment and data analysis. YS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

- Ali, F. R. M., Diaz, M. C., Vallone, D., Tynan, M. A., Cordova, J., Seaman, E. L., et al. (2020). E-cigarette Unit Sales, by Product and Flavor Type - United States, 2014-2020. *MMWR Morb. Mortal. Wkly. Rep.* 69, 1313-1318. doi:10.15585/mmwr.mm6937e2
- Allen, J. G., Flanagan, S. S., LeBlanc, M., Vallarino, J., MacNaughton, P., Stewart, J. H., et al. (2015). Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-Pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes. *Environ. Health Perspect.* 124, 733-739. doi:10.1289/ehp.1510185
- Anderson, C., Majeste, A., Hanus, J., and Wang, S. (2016). E-cigarette Aerosol Exposure Induces Reactive Oxygen Species, DNA Damage, and Cell Death in Vascular Endothelial Cells. *Toxicol. Sci.* 154 (2), 332-340. doi:10.1093/toxsci/kfw166
- Beauval, N., Verrière, M., Garat, A., Fronval, I., Dusautoir, R., Anthérieu, S., et al. (2019). Influence of Puffing Conditions on the Carbonyl Composition of E-Cigarette Aerosols. *Int. J. Hyg. Environ. Health* 222, 136-146. doi:10.1016/j.ijheh.2018.08.015
- Bicking, M. K. L., Marcus Cooke, W., Kawahara, F. K., and Longbottom, J. E. (1988). Effect of pH on the Reaction of 2,4-Dinitrophenylhydrazine with Formaldehyde and Acetaldehyde. *J. Chromatogr. A* 455, 310-315. doi:10.1016/s0021-9673(01)82130-0
- Crenshaw, M. D., Tefft, M. E., Buehler, S. S., Brinkman, M. C., Clark, P. I., and Gordon, S. M. (2016). Determination of Nicotine, Glycerol, Propylene Glycol and Water in Electronic Cigarette Fluids Using Quantitative ^1H NMR. *Magn. Reson. Chem.* 54, 901-904. doi:10.1002/mrc.4498
- Dautzenberg, B., and Bricard, D. (2015). Real-Time Characterization of E-Cigarettes Use: The 1 Million Puffs Study. *J. Addict. Res. Ther.* 06, 229. doi:10.4172/2155-6105.1000229
- de Andrade, J. B., and Tanner, R. L. (1992). Determination of Formaldehyde by HPLC as the DNPH Derivative Following High-Volume Air Sampling onto Bisulfite-Coated Cellulose Filters. *Atmos. Environ. A. Gen. Top.* 26, 819-825. doi:10.1016/0960-1686(92)90242-d
- El-Hellani, A., Salman, R., El-Hage, R., Talih, S., Malek, N., Baalbaki, R., et al. (2018). Nicotine and Carbonyl Emissions from Popular Electronic Cigarette Products: Correlation to Liquid Composition and Design Characteristics. *Nicotine Tob. Res.* 20, 215-223. doi:10.1093/ntr/ntw280
- Farsalinos, K. E., Kistler, K. A., Pennington, A., Spyrou, A., Kouretas, D., and Gillman, G. (2018). Aldehyde Levels in E-Cigarette Aerosol: Findings from a Replication Study and from Use of a New-Generation Device. *Food Chem. Toxicol.* 111, 64-70. doi:10.1016/j.fct.2017.11.002
- FDA (2016). "Deeming Tobacco Products to Be Subject to the Federal Food, Drug, and Cosmetic Act, as Amended by the Family Smoking Prevention and Tobacco Control Act; Restrictions on the Sale and Distribution of Tobacco Products and Required Warning Statements for Tobacco Products," in *Food and Drug Administration*. Final rule. Federal register 81 (90), 28973-29106.
- Flora, J. W., Wilkinson, C. T., Wilkinson, J. W., Lipowicz, P. J., Skapars, J. A., Anderson, A., et al. (2017). Method for the Determination of Carbonyl Compounds in E-Cigarette Aerosols. *J. Chromatogr. Sci.* 55, 142-148. doi:10.1093/chromsci/bmw157
- Floyd, E. L., Queimado, L., Wang, J., Regens, J. L., and Johnson, D. L. (2018). Electronic Cigarette Power Affects Count Concentration and Particle Size Distribution of Vaping Aerosol. *PLoS one* 13, e0210147. doi:10.1371/journal.pone.0210147
- Geiss, O., Bianchi, I., and Barrero-Moreno, J. (2016). Correlation of Volatile Carbonyl Yields Emitted by E-Cigarettes with the Temperature of the Heating Coil and the Perceived Sensorial Quality of the Generated Vapours. *Int. J. Hyg. Environ. Health* 219, 268-277. doi:10.1016/j.ijheh.2016.01.004
- Gillman, I. G., Kistler, K. A., Stewart, E. W., and Paolantonio, A. R. (2016). Effect of Variable Power Levels on the Yield of Total Aerosol Mass and Formation of Aldehydes in E-Cigarette Aerosols. *Regul. Toxicol. Pharmacol.* 75, 58-65. doi:10.1016/j.yrtph.2015.12.019
- Gillman, I. G., Pennington, A. S. C., Humphries, K. E., and Oldham, M. J. (2020). Determining the Impact of Flavored E-Liquids on Aldehyde Production during Vaping. *Regul. Toxicol. Pharmacol.* 112, 104588. doi:10.1016/j.yrtph.2020.104588
- Goniewicz, M. L., Kuma, T., Gawron, M., Knysak, J., and Kosmider, L. (2012). Nicotine Levels in Electronic Cigarettes. *Nicotine Tob. Res.* 15, 158-166. doi:10.1093/ntr/nts103
- Goniewicz, M. L., Knysak, J., Gawron, M., Kosmider, L., Sobczak, A., Kurek, J., et al. (2014). Levels of Selected Carcinogens and Toxicants in Vapour from Electronic Cigarettes. *Tob. Control.* 23, 133-139. doi:10.1136/tobaccocontrol-2012-050859
- Havel, C. M., Benowitz, N. L., Jacob, P., III, and St Helen, G. G. (2017). An Electronic Cigarette Vaping Machine for the Characterization of Aerosol Delivery and Composition. *Nicotine Tob. Res.* 19, 1224-1231. doi:10.1093/ntr/ntw147
- Hua, M., and Talbot, P. (2016). Potential Health Effects of Electronic Cigarettes: A Systematic Review of Case Reports. *Prev. Med. Rep.* 4, 169-178. doi:10.1016/j.pmedr.2016.06.002
- John, E., Coburn, S., Liu, C., McAughey, J., Mariner, D., McAdam, K. G., et al. (2020). Gas-Particle Partitioning of Formaldehyde in Mainstream Cigarette Smoke. *Beiträge zur Tabakforschung International/Contributions Tob. Res.* 29, 2-20. doi:10.2478/cttr-2020-0002
- Kallinger, G., and Niessner, R. (1997). Development and Laboratory Investigation of a Denuder Sampling System for the Determination of Carbonyl Compounds in Stack Gas. *Fresenius' J. Anal. Chem.* 358, 687-693. doi:10.1007/s002160050492
- Khlystov, A., and Samburova, V. (2016). Flavoring Compounds Dominate Toxic Aldehyde Production during E-Cigarette Vaping. *Environ. Sci. Technol.* 50, 13080-13085. doi:10.1021/acs.est.6b05145
- Klager, S., Vallarino, J., MacNaughton, P., Christiani, D. C., Lu, Q., and Allen, J. G. (2017). Flavoring Chemicals and Aldehydes in E-Cigarette Emissions. *Environ. Sci. Technol.* 51, 10806-10813. doi:10.1021/acs.est.7b02205
- Lerner, C. A., Sundar, I. K., Yao, H., Gerloff, J., Ossip, D. J., McIntosh, S., et al. (2015). Vapors Produced by Electronic Cigarettes and E-Juices with Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung Epithelial Cells and in Mouse Lung. *PLoS one* 10, e0116732. doi:10.1371/journal.pone.0116732
- Mikhchev, V. B., Brinkman, M. C., Granville, C. A., Gordon, S. M., and Clark, P. I. (2016). Real-Time Measurement of Electronic Cigarette Aerosol Size Distribution and Metals Content Analysis. *Nicotine Tob. Res.* 18, 1895-1902. doi:10.1093/ntr/ntw128
- NASEM (2018). Public Health Consequences of E-Cigarettes. *National Academies of Sciences, Engineering, and Medicine* (Washington, DC: National Academies Press).
- Olmedo, P., Goessler, W., Tanda, S., Grau-Perez, M., Jarmul, S., Aherrera, A., et al. (2018). Metal Concentrations in E-Cigarette Liquid and Aerosol Samples: the Contribution of Metallic Coils. *Environ. Health Perspect.* 126 (2), 027010. doi:10.1289/ehp2175
- Olmedo, P., Navas-Acien, A., Hess, C., Jarmul, S., and Rule, A. (2016). A Direct Method for E-Cigarette Aerosol Sample Collection. *Environ. Res.* 149, 151-156. doi:10.1016/j.envres.2016.05.008
- Qu, Y., Kim, K.-H., and Szulejko, J. E. (2018). The Effect of Flavor Content in E-Liquids on E-Cigarette Emissions of Carbonyl Compounds. *Environ. Res.* 166, 324-333. doi:10.1016/j.envres.2018.06.013
- Robinson, R. J., Hensel, E. C., Morabito, P. N., and Roundtree, K. A. (2015). Electronic Cigarette Topography in the Natural Environment. *PLoS one* 10, e0129296. doi:10.1371/journal.pone.0129296
- Sleiman, M., Logue, J. M., Montesinos, V. N., Russell, M. L., Litter, M. I., Gundel, L. A., et al. (2016). Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. *Environ. Sci. Technol.* 50, 9644-9651. doi:10.1021/acs.est.6b01741
- Son, Y., Mishin, V., Laskin, J. D., Mainelis, G., Wackowski, O. A., Delnevo, C., et al. (2019). Hydroxyl Radicals in E-Cigarette Vapor and E-Vapor Oxidative Potentials under Different Vaping Patterns. *Chem. Res. Toxicol.* 32 (6), 1087-1095. doi:10.1021/acs.chemrestox.8b00400
- Son, Y., Weisel, C., Wackowski, O., Mainelis, G., Schwander, S., Delnevo, C., et al. (2018). Evaluation of E-Vapor Nicotine and Nicotyrine Concentrations Emitted under Various E-Liquid Compositions, Device Settings and Vaping Topographies. *Chem. Res. Toxicol.* 31 (9), 861-868. doi:10.1021/acs.chemrestox.8b00063

- Son, Y., Bhattarai, C., Samburova, V., and Khlystov, A. (2020). Carbonyls and Carbon Monoxide Emissions from Electronic Cigarettes Affected by Device Type and Use Patterns. *Int. J. Environ. Res. Public Health* 17, 2767. doi:10.3390/ijerph17082767
- Uchiyama, S., Inaba, Y., and Kunugita, N. (2010). Determination of Acrolein and Other Carbonyls in Cigarette Smoke Using Coupled Silica Cartridges Impregnated with Hydroquinone and 2,4-dinitrophenylhydrazine. *J. Chromatogr. A* 1217, 4383–4388. doi:10.1016/j.chroma.2010.04.056
- Uchiyama, S., Ohta, K., Inaba, Y., and Kunugita, N. (2013). Determination of Carbonyl Compounds Generated from the E-Cigarette Using Coupled Silica Cartridges Impregnated with Hydroquinone and 2,4-Dinitrophenylhydrazine, Followed by High-Performance Liquid Chromatography. *Anal. Sci.* 29, 1219–1222. doi:10.2116/analsci.29.1219
- Wilson, M. D., Prasad, K. A., Kim, J. S., and Park, J. H. (2019). Characteristics of Metallic Nanoparticles Emitted from Heated Kanthal E-Cigarette Coils. *J. Nanoparticle Res.* 21, 1–11. doi:10.1007/s11051-019-4598-y
- Zhao, D., Navas-Acien, A., Ilievski, V., Slakovich, V., Olmedo, P., Adria-Mora, B., et al. (2019). Metal Concentrations in Electronic Cigarette Aerosol: Effect of Open-System and Closed-System Devices and Power Settings. *Environ. Res.* 174, 125–134. doi:10.1016/j.envres.2019.04.003
- Zhao, T., Shu, S., Guo, Q., and Zhu, Y. (2016). Effects of Design Parameters and Puff Topography on Heating Coil Temperature and Mainstream Aerosols in Electronic Cigarettes. *Atmos. Environ.* 134, 61–69. doi:10.1016/j.atmosenv.2016.03.027
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Modeled Respiratory Tract Deposition of Aerosolized Oil Diluents Used in Δ^9 -THC-Based Electronic Cigarette Liquid Products

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Electronic cigarette, or vaping, products (EVP) heat liquids (“e-liquids”) that contain substances (licit or illicit) and deliver aerosolized particles into the lungs. Commercially available oils such as Vitamin-E-acetate (VEA), Vitamin E oil, coconut, and medium chain triglycerides (MCT) were often the constituents of e-liquids associated with an e-cigarette, or vaping, product use-associated lung injury (EVALI). The objective of this study was to evaluate the mass-based physical characteristics of the aerosolized e-liquids prepared using these oil diluents. These characteristics were particle size distributions for modeling regional respiratory deposition and puff-based total aerosol mass for estimating the number of particles delivered to the respiratory tract. Four types of e-liquids were prepared by adding terpenes to oil diluents individually: VEA, Vitamin E oil, coconut oil, and MCT. A smoking machine was used to aerosolize each e-liquid at a predetermined puff topography (volume of 55 ml for 3 s with 30-s intervals between puffs). A cascade impactor was used to collect the size-segregated aerosol for calculating the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD). The respiratory deposition of EVP aerosols on inhalation was estimated using the Multiple-Path Particle Dosimetry model. From these results, the exhaled fraction of EVP aerosols was calculated as a surrogate of secondhand exposure potential. The MMAD of VEA (0.61 μm) was statistically different compared to MCT (0.38 μm) and coconut oil (0.47 μm) but not to Vitamin E oil (0.58 μm); $p < 0.05$. Wider aerosol size distribution was observed for VEA (GSD 2.35) and MCT (GSD 2.08) compared with coconut oil (GSD 1.53) and Vitamin E oil (GSD 1.55). Irrespective of the statistical differences between MMADs, dosimetry modeling resulted in the similar regional and lobular deposition of particles for all e-liquids in the respiratory tract. The highest (~ 0.08 or more) fractional deposition was predicted in the pulmonary region, which is consistent as the site of injury among EVALI cases. Secondhand exposure calculations indicated that a substantial amount of EVP aerosols could be exhaled, which has potential implications for bystanders. The number of EVALI cases has declined with the removal of VEA; however, further research is required to investigate the commonly available commercial ingredients used in e-liquid preparations.

Keywords: e-cigarette, EVALI, vitamin E acetate, particle size distributions, lung deposition, secondhand exposure estimates

INTRODUCTION

Electronic cigarette, or vaping, products (EVP) work by aerosolizing a liquid that is inhaled into the lungs by the user. The liquid used in an EVP, also known as e-liquid, can contain humectants, nicotine, flavorings, and other types of chemicals. EVP can be modified to aerosolize e-liquids that contain various forms of cannabis plant extracts, oil diluents, and other substances and additives. One of these extracts, delta-9-tetrahydrocannabinol (Δ^9 -THC), used in some e-liquids, contains mind-altering psychoactive properties that give a “high” (1). Along with reports that most EVALI patients (85%) were 18 years or older, ever use of Δ^9 -THC among youth (8.9%), and use of Δ^9 -THC among EVP users (30.6%), are indicative of potential health risks in the United States (2–5). Perrine et al. (6) stated that among college students, 75% of EVP users consumed various products of cannabis extracts in e-liquids. He et al. first reported a case of acute respiratory failure in a person who inhaled aerosolized Δ^9 -THC in 2017 (7). Subsequently, in 2019, the United States experienced an epidemic of acute lung injury termed as “e-cigarette or vaping use-associated lung injury” (EVALI) among persons who reportedly inhaled aerosolized Δ^9 -THC or nicotine e-liquids (8). As of January 14, 2020, among 2,668 hospitalized EVALI cases or deaths, about half were younger than 24 years, and 82% reported using an EVP to inhale Δ^9 -THC (9–11).

Δ^9 -THC extracts are hydrophobic, highly viscous, and semi-solid, and require thinning by dilution to be used in e-liquids aerosolized by EVP. Oils such as Vitamin-E acetate (VEA), Vitamin E oil, medium chain triglycerides (MCT), and coconut oil are used to dilute Δ^9 -THC extracts to create e-liquids. Heating these diluents to aerosolize Δ^9 -THC oils produces harmful chemicals, such as acetone, duroquinone, durohydroquinone, short chain esters, short chain alkanes, and ethenone (12–16).

Blount et al. (13, 14) measured several possible toxic substances as exposure markers in bronchoalveolar lavage fluid (BALF) samples from EVALI patients and an unaffected comparison group. These included Δ^9 -THC and e-liquid constituents such as VEA, MCT, coconut oil (identified as a common MCT), and terpenes such as limonene. VEA, coconut oil, and limonene were quantified in 94, 2, and 3% of EVALI-patient BALF samples, respectively, but were not detected in BALF samples from a comparison group. Among these oil diluents, tasteless, and odorless VEA is likely preferred by manufacturers because its viscosity profile makes it difficult to differentiate between pure Δ^9 -THC extracts and diluted products (14, 17).

A joint investigation conducted by the Centers for Disease Control and Prevention (9), the U.S. Food and Drug Administration, and the state health authorities identified VEA as strongly linked to the clinical presentation among EVALI cases and it also led to pulmonary damage *in vivo* using a mouse model (18). Although EVALI appeared to resolve by stopping the use of VEA, other diluents could potentially play a role or have other toxic effects (9, 10, 19). While studies have shown the formation of potentially toxic gases when these diluents were heated, information is lacking on the physical and chemical

properties of inhaled aerosol particles after aerosolizing diluent oils (20).

EVP aerosol is a two-phase mixture of gases and particles (21–23). Sosnowski and Kramek-Romanowska (24) highlighted the need to understand the size distribution of inhaled EVP aerosols as an influential factor for estimating their regional deposition in the respiratory tract. Other studies have mentioned sites of regional lung depositions for inhaled, and fractions for exhaled, micron-sized particles (25, 26). Measurement of the size distribution of the EVP aerosol can be challenging because aerosolized liquid droplets change their native size, depending on various conditions such as evaporation and hygroscopic growth. Evaporation of liquid droplets in the EVP aerosol during sampling results in an under-estimation of particle size, while hygroscopic growth results in an over-estimation of particle size (23). These deviations in size distribution, in turn, result in errant predictions of regional deposition in the respiratory tract. The native physical and chemical properties of particles should be maintained as intact as possible during measurement to determine the accurate size distribution of the emitted EVP aerosol (27, 28). Oldham et al. (27) predicted gas and particle phases as a function of the mass of collected aerosols without dilution, and therefore unadulterated mass-based aerosol size distribution is considered as an important parameter to determine their lung deposition (29, 30).

Recently, one study has assessed particle size distribution of aerosolized VEA from a commercially available EVP using a combination of a differential mobility spectrometer and an electrical low-pressure impactor. The authors noticed a substantial decrease in particle size for three out of four tested vape-pens, a type of commercially available EVP, because of air dilution caused by high puffing flow rates (31). In the current study, mass-based particle size distribution was directly measured with as little dilution as possible for several common oil diluents used as a constituent of EVP e-liquids for inhalation of Δ^9 -THC. We then estimated the location and mass concentration of deposited inhaled EVP aerosols in the respiratory tract. In addition, we estimated the exhaled fraction as secondhand exposure fraction, which can potentially affect the health of the bystanders and workers at certain occupational settings, such as vape shops and smoking centers.

MATERIALS AND METHODS

Diluents and Simulated e-Liquid Preparation

VEA was purchased from Sigma-Aldrich (St. Louis, MO, United States), and the Vitamin E oil (42,900 IU, 100% pure & natural, Chandler, AZ, United States), MCT (100% organic unflavored, Garden of Life LLC, FL, United States), and coconut oil (Organic unflavored, Carrington Farms, Closter, NJ, United States) were purchased from Amazon (Seattle, WA, United States). To more closely mimic herein prepared simulated e-liquids with commonly used e-liquids, these oils were thinned with ethanol (200 proof, ACS/USP grade, CAS# 64-17-5, Pharmaco-Aaper, Brookfield, CT, United States) at 0.6% w/w and with terpenes:

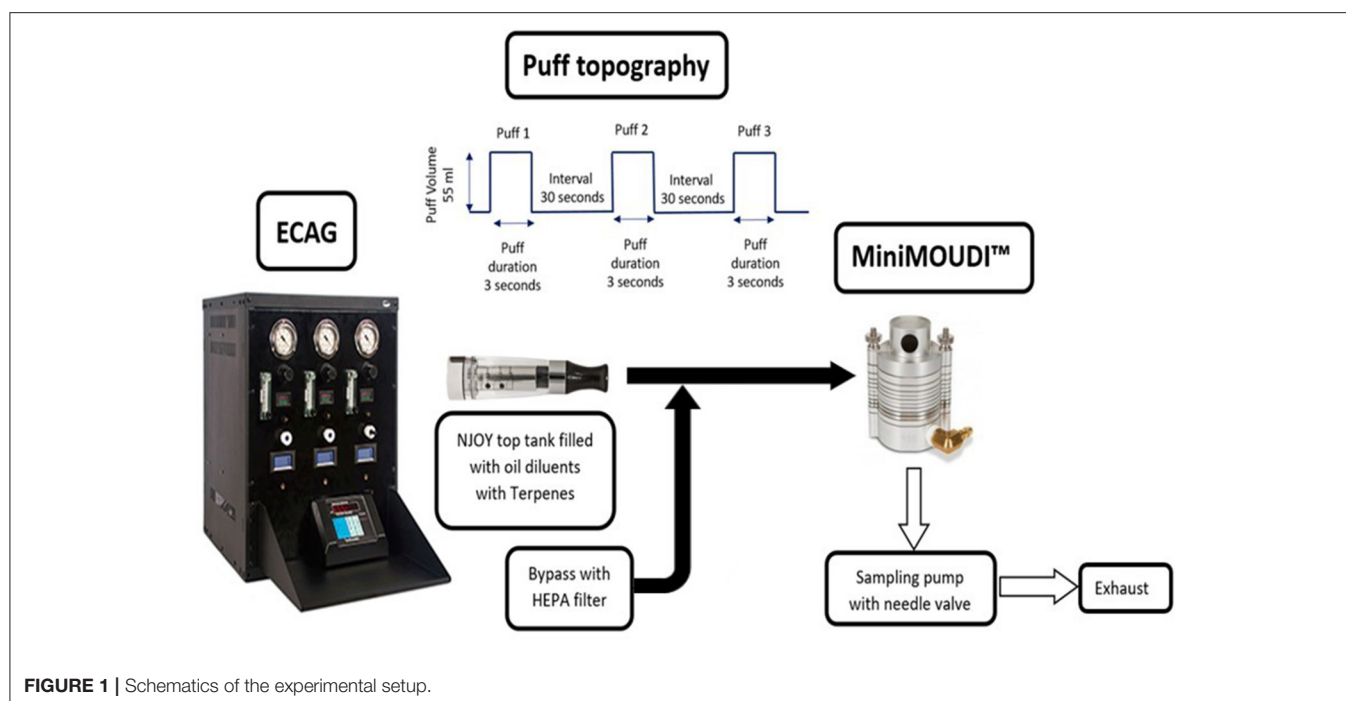


FIGURE 1 | Schematics of the experimental setup.

d-limonene (ACS grade, CAS# 5969-27-5, Sigma-Aldrich) at 0.2% w/w and α -pinene (ACS grade, CAS# 80-56-8, Sigma-Aldrich) at 0.2% w/w. We chose to simulate e-liquid formulation because ethanol is a solvent commonly used to extract and solubilize Δ^9 -THC oils, and terpenes are used to make Δ^9 -THC miscible in e-liquids (14, 17). Simulated e-liquids were prepared gravimetrically using a Mettler Toledo XS 205 dual-range microbalance capable of measuring to 0.01 mg (Mettler-Toledo LLC, Columbus, OH, United States) and homogenized for 1 h using a ThermoScientific rotator, Model 4152110 (Dubuque, IA, United States). The density of the diluent oils was measured in grams per cubic centimeter (g/cm^3): Vitamin E oil ($1.21 \text{ g}/\text{cm}^3$), VEA ($0.96 \text{ g}/\text{cm}^3$), coconut oil ($0.94 \text{ g}/\text{cm}^3$), and MCT ($0.91 \text{ g}/\text{cm}^3$).

Experimental Setup

The U.S. National Institute of Drug Abuse has developed a Standardized Research E-Cigarette (SREC) and considered NJOY (NJOY Inc., Scottsdale, AZ, United States) as a reference EVP (32). Studies reported the prevalent use of “Dank Vapes” among EVALI cases, which are Δ^9 -THC-containing pre-filled cartridges that operate below one ohm of resistance (33, 34). We used NJOY top tanks in our study because they are refillable and compatible with the sub-ohm resistance of “Dank Vapes” devices although they are a different EVP brand. NJOY top tanks, Model # UVTB02, can be filled with 1.6 ml of e-liquid. An automated e-cigarette aerosol generator (ECAG; e~Aerosols LLC, Central Valley, NY, United States) was programmed to aerosolize each simulated e-liquid. The ECAG works on positive pressure to aerosolize the simulated e-liquids by heating the coil at 3.7 volts (set) of electric current at a determined puff topography. Puff topography was calibrated daily to 55 ml puff volume within

3 s (1 puff) with a 30-s puff delay (35), using a soap-bubble flow meter (Borgwalt KC GmbH, Hamburg, Germany) as a primary volumetric flow calibration device. Three puffs were directly sampled without dilution into a MiniMOUDI (MSP Corporation, Shoreview, MN, United States), a type of low-flow cascade impactor, to preserve the native physical and chemical properties of the aerosol intact (27, 28). The MiniMOUDI was used to size fractionate e-cigarette aerosol (size range: $0.056\text{--}10 \mu\text{m}$) at a sampling flow rate of 2 liters per minute (LPM). The mass of aerosols deposited on each impactor stage at cut off particle diameter (D_p : $0.056\text{--}10 \mu\text{m}$) was measured on a 37-mm aluminum filter using a Mettler Toledo XS 205 dual range microbalance with a mass resolution of 0.01 mg (Mettler-Toledo LLC, Columbus, OH, United States).

A new NJOY tank was used to fill $1.3 \pm 0.5 \text{ ml}$ for each laboratory prepared e-liquid, which was puffed for 3 min before conducting the trials. Five trials were conducted for each of the e-liquids. The second set of five trials was conducted with a single VEA e-liquid preparation to assess reproducibility across each day of testing. There was no significant difference (p -value = 0.19) between the average mass median aerodynamic diameter (MMAD) of aerosolized VEA across days ($0.71 \mu\text{m}$ on day 1 and $0.61 \mu\text{m}$ on day 2), which indicated that the size distribution based on mass for VEA was reproducible.

Figure 1 shows a schematic of the experimental setup. The ECAG provided power to the device and forced air with an established puff topography through the tank into the MiniMOUDI. When the ECAG was operating, 1.1 LPM of EVP aerosol was sent directly to the MiniMOUDI along with 0.9 LPM of high-efficiency particulate air (HEPA: Whatman Schleicher & Schuell; Stockbridge, GA, United States)-filtered bypass air. During the puff delay, the impactor sampled 2.0 LPM

from the bypass air that did not result in any mass loading on the aluminum filters for any size of aerosols. To avoid aerosol losses, the mouthpiece of the tank was connected to the inlet of the MiniMOUDI using a small piece of flexible, black conductive silicone tubing with an inside diameter of 0.5 cm. A bypass HEPA-air filter was attached to allow uninterrupted flow to the impactor and to alleviate pressure drops and volume flow differences between the aerosol supply and the sampler requirements.

Statistical Analyses

Data were log transformed and analyzed using JMP 13.0 (SAS Institute Inc., Cary, NC, United States). To determine particle size distributions, the MMAD and GSD were calculated by including each cutoff size of the MiniMOUDI using a probit model for five trials on each e-liquid. To consider the significant differences ($p < 0.05$) between the particle size distribution of e-liquids, the average MMAD ($n = 5$) between e-liquids was compared using a one-way ANOVA and Tukey's HSD multiple comparisons. Deposited mass (in mg) of EVP aerosols for each size bin (D_p) was calculated by measuring aluminum substrate before (pre) and after (post) sampling. The total deposited mass of e-liquid aerosol was calculated by adding the mass collected at each particle cut-off diameter (μm) from $D_{p_i} = 0.056$ –10 for every trial ($n = 5$). Puff mass (mg/puff) was calculated for e-liquids according to Equation 1. Consideration of total mass collection per puff-based particle size distribution was intended to allow for the comparison between studies that researched mass-based particle size distribution.

$$\text{Puff mass} \left(\frac{\text{mg}}{\text{puff}} \right) \text{ for every trial } (n = 5) = \left[\frac{\sum_{D_{p_i}=0.056}^{10} (\text{Post mass (mg)} - \text{Pre mass (mg)})}{\# \text{ of puffs}} \right] \quad (1)$$

Lung Deposition Modeling

Based on the MMAD and GSD, the fraction of inhaled particles that could deposit in different sites of the human respiratory tract was predicted using the Multiple-Path Particle Dosimetry (MPPD), version 3.04 (ARA, Albuquerque, NM).

Estimates for regional deposition as the fraction of inhaled EVP aerosols were considered according to the Yeh–Schum model. The Yeh–Schum single-path model considers the whole human lung as a symmetric tree; therefore, respective regional depositions are average values for three regions: the head, trachea—bronchial (TB), and pulmonary regions (36). Regional deposition in the head includes mouth, nose, larynx, and pharynx to the trachea (generation 0). The TB region is from the trachea (generation 0) to the bronchioles (generation 16). The pulmonary region is from the terminal bronchioles onward to the alveoli. Deposition estimates are average values for each generation. Total respiratory tract deposition was calculated by summing three regional depositions. Based on the predicted deposition fractions, we modeled the conceptual estimation of EVP aerosol mass concentration (mg/puff) deposited in the respiratory tract as a product of deposition fraction and puff

mass yield (Equation 1). For example, the mass of EVP aerosol deposited in the head region was modeled by multiplying the regional deposition fraction in the head with puff mass (mg/puff) yield for each e-liquid. Based on the total respiratory tract deposition upon inhalation by the EVP user, we could also estimate the fraction of EVP aerosol that is potentially exhaled out. The exhaled particles fraction was estimated using Equation 2. Both the estimated exhaled EVP aerosol fraction and the modeled mass concentrations could serve as indicators of potential secondhand exposure.

$$\text{Secondhand exposure fraction} = 1 - \text{Total respiratory deposition fraction} \quad (2)$$

Unlike regional deposition, Yeh–Schum lobular deposition pattern characterized the segmental bronchi within each lobe as a single symmetric path to report the mass deposited in each of the five lobes of the human lungs: right upper (RU), right middle (RM), right lower (RL), left upper (LU), and left lower (LL) (36). The total lobar deposition includes deposition in the TB and pulmonary regions of each lung lobe but not the initial airways as they do not belong to any lobe. Default parameters for Yeh–Schum model were as follows: forced residual capacity = 3,300 ml, upper respiratory tract volume = 50 ml, breaths per minute (bpm) = 12, and tidal volume = 625 ml.

RESULTS

Figure 2 shows a representative particle size distribution (in the x -axis) and size-segregated mass (mg) deposition (in the y -axis) for all the e-liquids evaluated in the study. The average MMAD and standard deviation for five trials evaluating e-liquids were as follows: Vitamin E containing e-liquids (VEA: $0.61 \pm 0.16 \mu\text{m}$) and Vitamin E oil ($0.58 \pm 0.05 \mu\text{m}$) and without Vitamin E containing e-liquids (coconut oil: $0.47 \pm 0.00 \mu\text{m}$) and MCT ($0.38 \pm 0.03 \mu\text{m}$). One-way ANOVA ($p = 0.0012$) and Tukey's test resulted in a statistically significant difference (at $p < 0.05$) between the MMADs for VEA and MCT, VEA, and coconut oil, and Vitamin E oil and MCT (**Supplementary Table S2**). However, we detected no significant statistical difference between the MMADs of VEA and Vitamin E oil ($p = 0.24$). Additionally, there was a wider aerosol size distribution emitted by e-liquids for VEA (GSD 2.35) and MCT (GSD 2.08) compared with coconut oil (GSD 1.53) and Vitamin E oil (GSD 1.55). Results of MMAD and GSD values for individual trials for all the e-liquids are presented in **Supplementary Table S1**.

Data (average, standard deviation for $n = 5$) in **Figure 2** shows the total mass and puff mass yield (mg/puff) of EVP aerosol emitted by all the e-liquids. We found that both these attributes correlated with the MMADs of the e-liquids. MMAD of VEA was the largest ($0.61 \mu\text{m}$) and resulted in the highest averaged total mass collected and puff mass yield for VEA (5.60 mg) compared with other e-liquids. Additionally, VEA and Vitamin E oil (VEA at 5.60 mg and Vitamin E oil at 4.58 mg) resulted in the comparable total mass collection and puff mass yield (VEA: 1.87 mg/puff and Vitamin E oil: 1.53 mg/puff). Because of the potential hygroscopic nature of VEA particles, larger-sized EVP

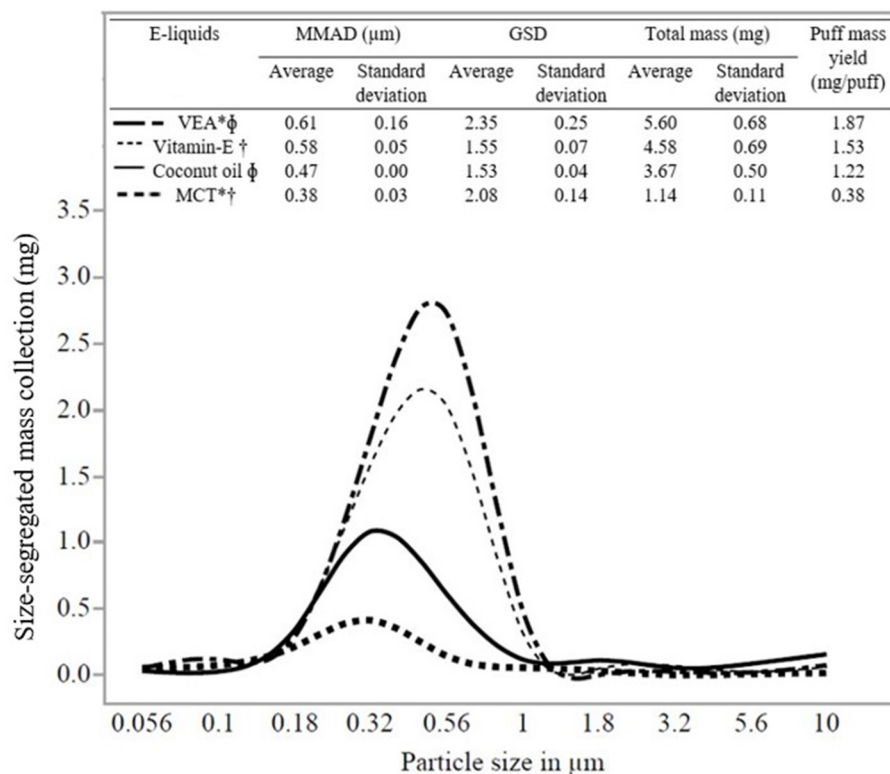


FIGURE 2 | Size distribution (MMAD and GSD), puff mass yield, and statistical comparisons of e-liquids. Note that the total (non-size-segregated) mass presented at the top is different from the size-segregated mass collection presented in the y-axis. E-liquids connected by the same symbols are significantly different. *VEA and MCT are significantly different. φVEA and coconut oil are significantly different. †Vitamin E oil and MCT are significantly different.

TABLE 1 | Predicted* deposition fraction and modeled dose deposition for e-liquids.

E-liquids	Predicted* fraction					Modeled mass (mg/puff)				
	Regional deposition			Total deposition	Secondhand exposure	Regional deposition			Total deposition	Secondhand exposure
	Head	TB	Pulmonary			Head	TB	Pulmonary		
VEA	0.09	0.06	0.11	0.26	0.74	0.17	0.11	0.21	0.49	1.38
Vitamin E oil	0.05	0.06	0.09	0.20	0.80	0.08	0.09	0.14	0.31	1.22
Coconut oil	0.04	0.06	0.08	0.18	0.82	0.05	0.07	0.10	0.22	1.00
MCT	0.05	0.06	0.10	0.21	0.79	0.02	0.02	0.04	0.08	0.30

*As per MPPD, version 3.04 (ARA, Albuquerque, NM).

Modeled mass (mg/puff) = predicted fraction * puff-mass yield (mg/puff).

aerosols have more condensable material available for particle mass-growth, which corresponds well with the finding of the higher mass collection with larger MMAD (23, 24, 37, 38). MCT and coconut oil aerosolized into smaller particles and because they have lower liquid densities (relative to VEA and vitamin E oil), they resulted in comparably less mass collection. The results of mass collection for the individual trials for all the e-liquids are presented in **Supplementary Table S1**.

Table 1 presents the predicted regional and total respiratory deposition (inhaled), as well as secondhand exposure (exhaled) fraction of EVP aerosols for the various e-liquids. Dosimetry

modeling predicted that, out of the total respiratory deposition, higher fractions of particles were estimated to deposit in deeper lung regions (pulmonary and TB) compared with the head region. Among these e-liquids, VEA had the highest total respiratory tract deposition (0.26). Out of the total respiratory tract depositions, the majority (~0.14–0.17) of the aerosolized e-liquids were estimated to deposit in the TB (~0.06) and pulmonary (~0.08–0.11) regions.

Based on the predicted deposition fractions (**Table 1**) and calculated puff mass yield (**Figure 2**), modeled EVP mass concentrations (~mg/puff) for regional, total, and secondhand

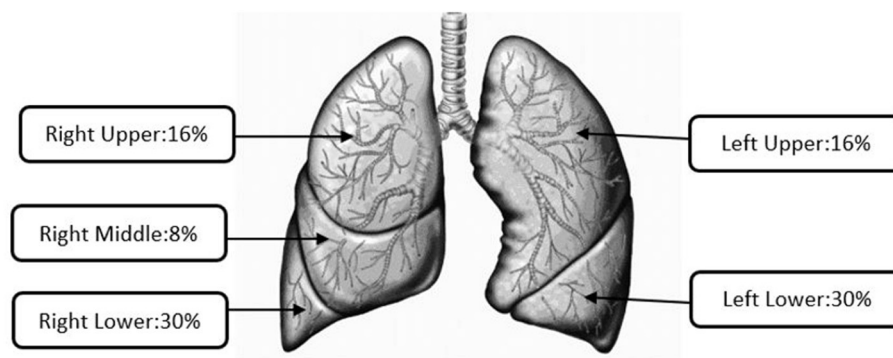


FIGURE 3 | Average lobular deposition for all e-liquids.

exposure are presented in **Table 1**. For e-liquids, puff mass yields (mg/puff) were as follows: VEA (1.87), Vitamin E oil (1.53), coconut oil (1.22), and MCT (0.38), and therefore were predicted to deposit more mass per puff in the respiratory tract (total and regional) of the EVP user as well as exhalation for secondhand exposure conditions. Out of the total inhaled EVP mass concentrations (~ 0.08 – 0.49 mg/puff), considerable amounts were deposited in the deeper (pulmonary and TB) lung regions, ~ 0.08 – 0.33 mg/puff, compared with the head region (~ 0.02 – 0.16 mg/puff) for all the e-liquids. For e-liquids studied in this work, the total mass inhaled per puff estimated to deposit in pulmonary regions (mg/puff) were: VEA (0.21/0.49), Vitamin E oil (0.14/0.31), coconut oil (0.10/0.22), and MCT (0.04/0.08) compared with the head and TB regions.

Indication of considerable physical deposition of herein studied EVP aerosols into the TB and pulmonary regions (predicted fraction: ~ 0.14 – 0.17 and modeled mass concentration: ~ 0.08 – 0.33 mg/puff) could explain respiratory illnesses, including BALF investigations associated with EVALI (13, 14, 17). Dosimetry analysis calculations indicated that MCT (MMAD: $0.38 \mu\text{m}$) had a high proportion of pulmonary region deposition (~ 0.10) out of the total respiratory tract deposition (~ 0.21) because of its smaller size. However, compared to other e-liquids, estimates for MCT (puff mass yield: 0.38 mg/puff) translated to less mass concentration inhaled (~ 0.08 mg/puff) and deposited in pulmonary regions (~ 0.04 mg/puff). One of the other possibilities for these smaller-sized particles was reported to be exhaled with greater chances than larger-sized particles (25, 26). Consideration of exhalation of the smaller-sized particles is addressed in this study by presenting estimates of secondhand exposure fractions. Like MCT, similar observations were noticed for coconut and vitamin E oils regarding higher predicted pulmonary deposition fraction (~ 0.08 or more) and modeled mass concentrations (~ 0.10 – 0.14 mg/puff) and considerable secondhand exposure conditions (~ 0.80 or more).

Note that the predicted total respiratory tract deposition estimates and the secondhand exposure condition are inversely related, that is, the lower the total respiratory tract deposition fraction, the higher the secondhand exposure fraction. For example, with coconut oil only a small fraction of the particles

estimated to account for total respiratory tract deposition (fraction: ~ 0.18 and mass concentration: ~ 0.22 mg/puff) so more particles were estimated to be exhaled out (fraction: ~ 0.82 and mass concentration: ~ 1.00 mg/puff). For all the e-liquids, the predicted total respiratory tract deposition fraction was 0.21 ± 0.04 and the estimated secondhand exposure fraction was ≥ 0.74 . MCT resulted in the smallest MMAD and the least total mass collection, but modeling projected a considerable amount ($\sim 0.10/0.21$) of emitted MCT particles that would deposit in the pulmonary region. Because of its smaller size, deposition of particles emitted by aerosolizing MCT is predicted deep in the pulmonary region and exhaled out as well, which explains estimates for the regional deposition, lower total respiratory tract deposition (~ 0.21), and total mass concentration (~ 0.08 mg/puff). It is noteworthy that we did not detect a significant difference between MMAD of vitamin E oil ($0.58 \mu\text{m}$) and VEA ($0.61 \mu\text{m}$), and like VEA (~ 0.11), a considerable amount of total inhaled vitamin E oil aerosols (~ 0.20) was also predicted to deposit in the pulmonary region (~ 0.09). Additionally, a large fraction (~ 0.80) of aerosolized vitamin E oil particles could potentially account for secondhand exposure conditions. Exhaled EVP aerosols fraction and mass per puff can be a potential indicator of secondhand exposure conditions for bystanders including those in occupational settings.

Figure 3 presents the average lobular aerosol mass depositions of all the studied e-liquids. However, higher percentages of the aerosol mass were found to be deposited in right-sided lung lobes (RU, RM, and RL = 54%) compared with the left-sided lung lobes (LU and LL = 46%). Manigrasso et al. have presented right lung lobes as the sites where effects of the EVP aerosol occur more likely than left lung lobes (39). The highest percentages of lobular deposition of emitted aerosols from all the oils were predicted in the lower lobes (right: $30 \pm 0.2\%$ and left: $30 \pm 0.2\%$) compared to other lobes of the lungs (RU $16 \pm 0.2\%$; RM $8 \pm 0.1\%$; and LU $16 \pm 0.2\%$). For particle sizes in the range from 0.2 to $1 \mu\text{m}$, higher deposition fractions in lower lobes compared to the upper lobes were also documented, as observed in our results and prior studies (36, 40–43).

DISCUSSION

In the CDC update on February 2020, 68 confirmed deaths were reported out of a total of 2,807 hospitalized EVALI cases (9). Studies have quantified some toxic chemicals present in BALF at the time of clinical examination from EVALI cases and confirmed that VEA was strongly linked to EVALI (9, 14, 17). However, the physiological mechanism by which aerosolized diluent oils found in BALF of EVALI patients actually injured lungs remains unclear. Not only does the pathophysiology of these chemically-induced damages remain unclear, but adequate research on the physical characteristics of the EVP aerosols, necessary to understand regional lung depositions, has, until now, been lacking.

Mikheev et al. (31) evaluated the particle size distribution and chemical composition of aerosolized VEA using four commercially available vape pens. This group measured particle size distribution by sampling eight puffs of aerosolized VEA with a 60-s puff interval, which required high flow rates (20–40 LPM). The two puff flow rates considered in the study of Mikheev et al. were 20 ml/s and 40 ml/s for 5 s with a 60-s puff delay. Although particle size distribution was also dependent on the types of commercial EVP devices and their heating capability, the authors noticed a strong influence of puffing flow rate on the size of aerosolized VEA particles that resulted in particle sizes smaller than 50 nm at the higher puffing flow rate (40 ml/s) (31). In contrast to the study of Mikheev et al., we sampled three puffs of EVP aerosols at a puff flow rate of 18.33 ml/s with a 30-s puff delay with no dilution flow. Our study focused on directly measuring mass-based particle size, to the extent feasible without deviation, from their native size aerosolized from simulated e-liquids. The intention was to mimic the composition of oil diluent constituents consistent with products associated with EVALI cases but without the presence of Δ^9 -THC for safety reasons. Rather than using commercially available e-liquids, we prepared e-liquids in the laboratory that contained each diluent oil, to evaluate the specific influence of that diluent oil on MMAD and lung deposition. This study showed no statistical difference ($p = 0.24$) between the MMAD values of VEA (0.61 μm) and Vitamin E oil (0.58 μm), which translated into similar estimates regarding respiratory deposition behavior. Compared to MCT and coconut oil, the aerosolization of Vitamin E-containing e-liquids (VEA and Vitamin E oil) was observed to report larger MMADs and a greater total mass of EVP aerosol.

Variations in EVP aerosol generation and characterization methods have led to a lack of reproducibility. Therefore, the ability to compare various studies or to integrate information is difficult (44–46). The puff-based mass collection presented in this study was an attempt to compare the results between existing studies within the given experimental parameters. Using the same type of cascade impactor, (37) found size distributions of two commercial e-cigs comparable to the size distributions measured by Ingebrethsen et al. (38) using spectral extinction with a slightly variable puff topography. As presented in **Figure 2**, we observed a similar trend between MMAD and mass collected for all the studied e-liquids. For VEA with MMAD of 0.61 μm , puff mass yield resulted in 1.87 mg/puff, which is comparable with the study

of Alderman et al. results for one commercially available e-cig with MMAD of 0.63 μm that yielded puff mass of 2.16 mg/puff. Apart from different experimental parameters and sampling methods, differences were noticed in puff-mass measurements between these two studies for the same EVP devices. It was also hypothesized that the possibility of growth for larger-sized particles resulted in more mass collection. Similar to the study of Alderman et al., we observed more mass collection [Puff mass yield: VEA (1.87 mg/puff), vitamin E oil (1.53 mg/puff), coconut oil (1.22 mg/puff), and MCT (0.38 mg/puff)] for larger MMAD [VEA (0.61 μm), vitamin E oil (0.58 μm), coconut oil (0.47 μm), and MCT (0.38 μm)] of diluent oils, respectively. Our laboratory-prepared e-liquids included common diluent oils but not polypropylene glycol (PG), vegetable glycerin (VG), or nicotine. Particle size distribution of EVP aerosol depends on e-liquid compositions. This being one of the first studies to address the mass-based direct measurement of the particle size distribution for diluent oils, it is important to present the information in a way that could be compared with further research considering different experimental parameters. Moreover, by considering the accurate measurement of native particle size as close as possible, we provided conceptual estimates for deposition fraction and modeled per puff mass–dose deposition.

Out of the total respiratory tract deposition for all the diluent oils, the highest fraction (~ 0.08 – 0.11) and mass per puff (mg/puff: 0.04–0.21) were predicted in the pulmonary region where gas exchange occurs. Lewis et al. (29) noted that a maximum probability of particle deposition in the pulmonary region was for particles with MMAD $< 3 \mu\text{m}$ and GSD < 3 . For smaller-sized particles with MMAD $< 2 \mu\text{m}$, Raabe et al. (47) concluded that the highest fraction of total deposited particles is reported in the pulmonary region. Dosimetry results presented in this study for VEA were consistent with these inhalation toxicological evaluations. We observed that VEA resulted in the highest total mass collected (5.60 mg) *via* cascade impactors and the highest total respiratory tract deposition (~ 0.26) *via* dosimetry analysis, compared with all the e-liquids included in the study. Literature showed the higher lobular depositions of submicron size particles in lower lobes than upper lobes of the lungs (36, 40–43), which is the same as our study results. Using number concentrations, Manigrasso et al. estimated size-segregated aerosols emitted from commercially available EVP as a function of the airway generation number in the lung lobes. They concluded that, for both the TB and pulmonary regions, twice as many particles were deposited in RU compared to the LU and ~ 0.20 more particles deposited in RL compared to LL. Cumulatively, right lung and lobar bronchi were documented as sites where PG–VG-based e-liquid aerosols may likely affect more than the left lung, which is consistent with our study results.

It is possible that pulmonary deposition of constituents of aerosolized e-liquids alters airway homeostasis, changes surfactant integrity, and provokes oxidative or inflammatory damages or contributes to the formation of lipid-laden macrophages (14, 48–50). These histological findings might be consistent with BALF investigations and clinical presentations, such as chemical pneumonitis, among EVALI cases (14, 51). Two research studies conducted in mice presented similar

lung pathologies to EVALI patients following the inhalation of aerosolized VEA (18, 52).

Jiang et al. (15) reported that the aerosolization of MCT, Vitamin E oil, and VEA yielded the cytotoxic products short chain esters, duroquinone, and durohydroquinone, respectively, one or more of which might explain cellular damages among the reported EVALI cases (15). In the current study, a greater fraction of aerosolized MCT (~ 0.10), VEA (~ 0.11), and Vitamin E oil (~ 0.09) were predicted to deposit in the pulmonary region of the lung. If one or more of the cytotoxic products reported by Jiang et al. contribute to EVALI, our data support this exposure pathway based on these similar respiratory particle deposition patterns. Regarding MCT, a fraction (~ 0.21) of aerosolized particle sizes $< 1 \mu\text{m}$ were estimated to be deposited in the respiratory tract, which could be a reason why coconut oil was observed in BALF samples for one of the EVALI cases (reference).

Herein discussed assessments aligning with the available literature indicate that particles smaller than $1 \mu\text{m}$ can deposit in the pulmonary region of the lung and are also exhaled from the lung with greater probability than particles larger than $1 \mu\text{m}$ (25, 26). These exhaled particles could serve as a source of potential secondhand exposure to nearby people (53, 54). Secondhand exposures are not only important in maintaining indoor air quality for bystanders at domestic settings where vulnerable populations are in proximity to EVP users but also at certain occupational settings such as vape shops (20, 53, 54). In that regard, the greater fraction (~ 0.74 or more) of the aerosols emitted from all the e-liquids studied were estimated to be a source of potential secondhand exposure. This high predicted fraction of exhaled particles could also explain why analyses found coconut oil (compared to VEA) in the BALF sample of only one EVALI case (the lowest total respiratory tract deposition at ~ 0.18 and highest second-hand exposure fraction at ~ 0.82) (13, 14, 17). Direct measurement of exhaled aerosols should be conducted to determine a true particle size distribution that could contribute to secondhand exposure because the primary aerosol inhaled by an EVP user likely differs from the secondhand aerosol exhaled by a user.

Study Limitations

The results of this laboratory study are limited to one fixed puff topography from the CORESTA method: 3 s puff, 30 s interval, and 55 ml puff volume. However, without standardized experimental protocols, parameters included in any study could be a source of limitation, which also causes a lack of reproducibility. At times, contrasting observations were noticed between studies that addressed the effects of the EVP settings, such as voltage, power, and coil resistance, that influence heating of e-liquids, on the particle size of the EVP aerosol that is generated and lung depositions (55–59). Unlike Floyd et al. and Lechasseur et al., Mulder et al. purported that the compositions of e-liquids, such as the various proportions of PG–VG, significantly impact particle size distributions, and not the voltage and coil resistance. Studies also showed that commercially available e-liquids do not have their ingredients and their proportion of ingredients fully documented, which limits their influence on conclusions drawn (60–65). Therefore,

in this study, we prepared e-liquids in the laboratory that contained each diluent oil with terpenes to validate their influence on particle emission after heating at 3.6 V with a particular puff topography. Li et al. (66) evaluated the effects of heating PG–VG-based e-liquids at different puff volumes, puff duration, and interval on the particle size of aerosols and mass deposition in the respiratory tract. One of their puff profiles was exactly what we used in this study, but further investigations are necessary to evaluate the effects of puff profile on the particle size distribution and lung deposition using e-liquids containing diluent oils. We addressed the particle size distribution of diluent oils in e-liquids intended to mimic products used by EVALI cases and revealed that more experiments regarding different puff topographies are needed in the future.

For any study of EVP aerosols, the determination of the particle size distribution in its native state is complex because of the dynamics involved in generating and measuring a mist from a variety of EVP devices (23). Protano et al. (45) demonstrated that there were significant variations in puff-to-puff EVP aerosol generation within a single device with all other parameters held constant. Considering impaction and spectral transmissions, various studies using different EVP models and brands, reported particle sizes that ranged from 0.1 to $0.9 \mu\text{m}$ and utilizing sampling methods without the dilution of the emitted aerosol (24, 37, 38, 67). Using a low-flow impactor, MMADs observed in our studies for all e-liquids ranged from 0.38 to $0.61 \mu\text{m}$, which was comparable with the study results of Alderman et al. (MMAD range: 0.43– $0.63 \mu\text{m}$) for similar puff topography (with 3 s puff, 30 s interval, and 50 ml of puff volume). MMAD results (0.38– $0.61 \mu\text{m}$) presented in this study using NJOY top tank sub-ohm EVP devices were comparable with previous reports (37, 38).

The EVP construction material such as ceramic vs. non-ceramic coil used to heat e-liquid could be a source of variability that influences the size distribution of aerosol e-liquids. VEA and the other oils tested are more typically aerosolized using a ceramic cell EVP device that usually functions at a higher temperature than a sub-ohm resistance device. One limitation of our study is that we evaluated a sub-ohm resistance NJOY top tank EVP device, not a ceramic cell device, which could modify the size distribution generated for the prearranged puff topography.

Finally, the estimated respiratory deposition fractions using MPPD were not modified for hygroscopic growth and evaporation according to the human lung environment. Dosimetry analysis did not consider various factors such as aerosol temperature, hygroscopicity, relative humidity, and gas–vapor interchange, all of which can impact modeled lung deposition from the oral cavity throughout the respiratory tract (68–70). Our approach was to accurately measure the native size distribution of particles emitted after aerosolizing diluent oils and to predict respiratory deposition fraction and mass–dose per puff. Future studies should incorporate, to the extent feasible, these factors that influence deposition into models (where available) to provide more accurate dose estimates. Within the presented experimental parameters, our results bolster ongoing EVALI investigations as well as provide valuable data on the physical deposition of particles in the deep regions of

the respiratory tract, which, when coupled with toxicological investigations associated with diluent oils in e-liquids, provided insights into the disease.

CONCLUSIONS

Diluent oils such as VEA, Vitamin E oil, coconut oil, and MCT are mixed with Δ^9 -THC extracts, so the thinned down products can be used as a constituent of e-liquids. Although various toxicological investigations and histopathological studies have reported evidence of lung damage from inhalation of these diluent oils in aerosolized e-liquids, particle size distribution, which is necessary to understand regional depositions in the respiratory tract, has not been addressed. This study focused on determining and comparing particle size distributions of aerosol emitted from simulated e-liquids that contained VEA, Vitamin E oil, coconut oil, or MCT with terpenes. Based on MMADs, particle size distribution for VEA (0.61 μm) was significantly different than coconut oil (0.47 μm) and MCT (0.38 μm) but not Vitamin E oil (0.58 μm).

Dosimetry analysis predicted that $\sim 60\%$ of total respiratory depositions of particles were in the pulmonary ($\sim 42\%$) and TB ($\sim 20\%$) regions for VEA. Irrespective of statistical difference in their size distribution, aerosolized particles were predominantly ($\sim 69\%$ or more) deposited in lower lobes (Right: $\sim 30\%$ and Left: $\sim 30\%$) of the lungs. These observed particle deposition patterns were consistent with previous inhalation toxicological studies and with characterization of BALF of EVALI cases, which support the pulmonary region of the lung as the site of injury. The study results presented herein help to explain existing clinical presentations and pathological findings by providing particle size distribution of diluent oils and their respiratory depositions. Additionally, EVP aerosol sizes less than 1 μm , which have high probability of being inhaled then exhaled,

could pose secondhand exposure risk to persons in proximity to EVP users in occupational and non-occupational settings. While elimination of VEA in e-liquid products seemed to mitigate the EVALI outbreak, further research is required to investigate the usage of other commonly available oil diluents in Δ^9 -THC-based e-liquid preparations, which could also be potentially harmful for users and bystanders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpubh.2021.744166/full#supplementary-material>

REFERENCES

- Breitbarth AK, Morgan J, Jones AL. E-cigarettes—an unintended illicit drug delivery system. *Drug Alcohol Depend.* (2018) 192:98–111. doi: 10.1016/j.drugalcdep.2018.07.031
- Cullen KA, Gentzke AS, Sawdey MD, Chang JT, Anic GM, Wang TW, et al. E-cigarette use among youth in the United States. *JAMA.* (2019) 322:2095–103. doi: 10.1001/jama.2019.18387
- Singh T, Arrazola RA, Corey CG, Husten CG, Neff LJ, Homa DM, et al. Tobacco use among middle and high school students—United States, 2011–2015. *MMWR Morb Mortal Wkly Rep.* (2016) 65:361–7. doi: 10.15585/mmwr.mm6514a1
- Trivers KF, Phillips E, Gentzke AS, Tynan MA, Neff LJ. Prevalence of cannabis use in electronic cigarettes among US youth. *JAMA Pediatr.* (2018) 172:11:1097–9. doi: 10.1001/jamapediatrics.2018.1920
- Trivers KF, Gentzke AS, Phillips E, Tynan M, Marynak KL, Schauer GL. Substances used in electronic vapor products among adults in the United States, 2017. *Addict Behav Rep.* (2019) 10:100222. doi: 10.1016/j.abrep.2019.100222
- Perrine C, Pickens C, Boehmer T, King B, Jones C, DeSisto C, et al. Characteristics of a multistate outbreak of lung injury associated with e-cigarette use, or vaping—United States. *Morb Mortal Wkly Rep.* (2019) 68:860–4. doi: 10.15585/mmwr.mm6839e1
- He T, Oks M, Esposito M, Steinberg H, Makaryus M. “Tree-in-Bloom”: severe acute lung injury induced by vaping cannabis oil. *Ann Am Thorac Soc.* (2017) 14:468–70. doi: 10.1513/AnnalsATS.201612-974LE
- Schier JG, Meiman JG, Layden J, Mikosz CA, VanFrank B, King BA, et al. Severe pulmonary disease associated with electronic-cigarette-product use—interim guidance. *MMWR Morb Mortal Wkly Rep.* (2019) 68:787–90. doi: 10.15585/mmwr.mm6836e2
- Centers for Disease Control, and Prevention. *Outbreak of Lung Injury Associated With the Use of e-Cigarette, or Vaping, Products.* (2020). Cincinnati, OH: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available online at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html (accessed March 2020).
- Krishnasamy VP, Hallowell BD, Ko JY, Board A, Hartnett KP, Salvatore PP, et al. Update: characteristics of a nationwide outbreak of e-cigarette, or vaping, product use-associated lung injury—United States. *Morb Mortal Wkly Rep.* (2020) 69:90–4. doi: 10.15585/mmwr.mm6903e2

11. Moritz ED, Zapata LB, Lekachvili A, Glidden E, Annor FB, Werner AK, et al. Update: Characteristics of patients in a national outbreak of e-cigarette, or vaping, product use-associated lung injuries—United States, October 2019. *Morb Mortal Wkly Rep.* (2019) 68:985–9. doi: 10.15585/mmwr.mm6843e1
12. Attfield KR, Chen W, Cummings KJ, Jacob P, O'Shea DE, Wagner J, et al. Potential of ethenone (ketene) to contribute to electronic cigarette, or vaping, product use–associated lung injury. *Am J Respir Crit Care Med.* (2020) 202:1187–9. doi: 10.1164/rccm.202003-0654LE
13. Blount BC, Karwowski MP, Morel-Espinosa M, Rees J, Sosnoff C, Cowan E, et al. Evaluation of bronchoalveolar lavage fluid from patients in an outbreak of e-cigarette, or vaping, product use-associated lung injury—10 states, August–October 2019. *MMWR Morb Mortal Wkly Rep.* (2019) 68:1040–1. doi: 10.15585/mmwr.mm6845e2
14. Blount BC, Karwowski MP, Shields PG, Morel-Espinosa M, Valentin-Blasini L, Gardner M, et al. Vitamin E acetate in bronchoalveolar-lavage fluid associated with EVALI. *N Engl J Med.* (2020) 382:697–705. doi: 10.1056/NEJMoa1916433
15. Jiang H, Ahmed CMS, Martin TJ, Canchola A, Oswald IWH, Garcia JA, et al. Chemical and toxicological characterization of vaping emission products from commonly used vape juice diluents. *Chem Res Toxicol.* (2020) 33:2157–63. doi: 10.1021/acs.chemrestox.0c00174
16. Troutt WD, DiDonato MD. Carbonyl compounds produced by vaporizing cannabis oil thinning agents. *J Altern Complement Med.* (2017) 23:879–84. doi: 10.1089/acm.2016.0337
17. Duffy B, Li L, Lu S, Durocher L, Dittmar M, Delaney-Baldwin E, et al. Analysis of cannabinoid-containing fluids in illicit vaping cartridges recovered from pulmonary injury patients: identification of vitamin E acetate as a major diluent. *Toxics.* (2020) 8:8. doi: 10.3390/toxics8010008
18. Bhat TA, Kalathil SG, Bogner PN, Blount BC, Goniewicz ML, Thanavala YM. An animal model of inhaled Vitamin E acetate and EVALI-like lung injury. *Engl J Med.* (2020) 382:1175–7. doi: 10.1056/NEJMc2000231
19. Taylor J, Wiens T, Peterson J, Saravia S, Lunda M, Hanson K, et al. Characteristics of e-cigarette, or vaping, products used by patients with associated lung injury and products seized by law enforcement—Minnesota, 2018 and 2019. *Morb Mortal Wkly Rep.* (2019) 68:1096–100. doi: 10.15585/mmwr.mm6847e1
20. Stefaniak AB, LeBouf RE, Ranpara A, Leonard SS. Toxicology of flavoring- and cannabis-containing e-liquids used in electronic delivery systems. *Pharmacol Ther.* (2021) 224:107838. doi: 10.1016/j.pharmthera.2021.107838
21. Pankow JF. Calculating compound dependent gas-droplet distributions in aerosols of propylene glycol and glycerol from electronic cigarettes. *J Aerosol Sci.* (2017) 107:9–13. doi: 10.1016/j.jaerosci.2017.02.003
22. Pankow JF, Kim K, Luo W, McWhirter KJ. Gas/particle partitioning constants of nicotine, selected toxicants, and flavor chemicals in solutions of 50/50 propylene glycol/glycerol as used in electronic cigarettes. *Chem Res Toxicol.* (2018) 31:985–90. doi: 10.1021/acs.chemrestox.8b00178
23. Sosnowski TR, Jablczynska K, Odziomek M, Schlage WK, Kuczaj AK. Physicochemical studies of direct interactions between lung surfactant and components of electronic cigarettes liquid mixtures. *Inhal Toxicol.* (2018) 30:159–68. doi: 10.1080/08958378.2018.1478916
24. Sosnowski TR, Kramek-Romanowska K. Predicted deposition of e-cigarette aerosol in the human lungs. *J Aerosol Med Pulm Drug Deliv.* (2016) 29:299–309. doi: 10.1089/jamp.2015.1268
25. Chow AHL, Tong HHY, Chattopadhyay P, Shekunov BY. Particle engineering for pulmonary drug delivery. *Pharm Res.* (2007) 24:411–37. doi: 10.1007/s11095-006-9174-3
26. Haughney J, Price D, Barnes NC, Virchow JC, Roche N, Chrystyn H. Choosing inhaler devices for people with asthma: Current knowledge and outstanding research needs. *Respir Med.* (2010) 104:1237–45. doi: 10.1016/j.rmed.2010.04.012
27. Oldham M, Zhang J, Rusyniak MJ, Kane DB, Gardner WP. Particle size distribution of selected electronic nicotine delivery system products. *Food Chem Toxicol.* (2018) 113:236–40. doi: 10.1016/j.fct.2018.01.045
28. Zhao J, Zhang Y, Sisler JD, Shaffer J, Leonard SS, Morris AM, et al. Assessment of reactive oxygen species generated by electronic cigarettes using acellular and cellular approaches. *J Hazard Mater.* (2018) 344:549–57. doi: 10.1016/j.jhazmat.2017.10.057
29. Lewis TR, Morrow PE, McClellan RO, Raabe OG, Kennedy GL, Schwetz BA, et al. Establishing aerosol exposure concentrations for inhalation toxicity studies. *Toxicol Appl Pharmacol.* (1989) 99:377–83. doi: 10.1016/0041-008X(89)90147-6
30. OECD. *Environment, Health, and Safety Publications. Series on Testing and Assessment.* (2002). No. 39 B. Draft guidance document on acute inhalation toxicity testing. Organization for Economic Co-operation and Development. Available online at: <http://www.oecd.org/chemicalsafety/testing/2765785.pdf>. (accessed March 20, 2021).
31. Mikheev VB, Klupinski TP, Ivanov A, Lucas EA, Strozier ED, Fix C. Particle size distribution and chemical composition of aerosolized vitamin E acetate. *Aerosol Sci Technol.* (2020) 54:993–8. doi: 10.1080/02786826.2020.1783431
32. NIDA. *NIDA Drug Supply Program.* (2017). Available online at: <https://www.drugabuse.gov/research/research-data-measures-resources/nida-drug-supply-program/> (accessed March 20, 2021).
33. Balmes JR. Vaping-induced acute lung injury: an epidemic that could have been prevented. *Am J Respir Crit Care Med.* (2019) 200:1342–4. doi: 10.1164/rccm.201910-1903ED
34. Ghinai I, Pray IW, Navon L, O'Laughlin K, Saathoff-Huber L, Hoots B, et al. E-cigarette product use, or vaping, among persons with associated lung injury—Illinois and Wisconsin. *Morb Mortal Wkly Rep.* (2019) 68:865–9. doi: 10.15585/mmwr.mm6839e2
35. Cooperation Centre for Scientific Research Relative to Tobacco. CORESTA recommended method N° 81. In: *CORESTA E-Cigarette Task Force Technical Report.* (2015). Available online at: https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf (accessed March 2020).
36. Yeh HC, Schum GM. Models of human lung airways and their application to inhaled particle deposition. *Bull Math Biol.* (1980) 42:461–80. doi: 10.1016/S0092-8240(80)80060-7
37. Alderman SL, Song C, Moldoveanu SC, Cole SK. *Particle size distribution of E-Cigarette aerosols and the relationship to Cambridge filter pad collection efficiency.* (2015) 26. doi: 10.1515/cttr-2015-0006
38. Ingebrethsen BJ, Cole SK, Alderman SL. Electronic cigarette aerosol particle size distribution measurements. *Inhal Toxicol.* (2012) 24:976–84. doi: 10.3109/08958378.2012.744781
39. Manigrasso M, Buonanno G, Stabile L, Morawska L, Avino P. Particle doses in the pulmonary lobes of electronic and conventional cigarette users. *Environ.* (2015) 202:24–31. doi: 10.1016/j.envpol.2015.03.008
40. Bennett WD, Messina MS, Smaldone GC. Effect of exercise on deposition and subsequent retention of inhaled particles. *J Appl Physiol.* (1985) 59:1046–54. doi: 10.1152/jappl.1985.59.4.1046
41. Chang YH, Yu CP. A model of ventilation distribution in the human lung. *Aerosol Sci Technol.* (1999) 30:309–19. doi: 10.1080/027868299304660
42. Gerrity TR, Garrard CS, Yeates DB. Theoretic analysis of sites of aerosol deposition in the human lung. *Chest.* (1981) 80:898–901. doi: 10.1378/chest.80.6.898
43. Subramaniam RP, Asgharian B, Freijer JI, Miller FJ, Anjilvel S. Analysis of lobar differences in particle deposition in the human lung. *Inhal Toxicol.* (2008) 15:1–21. doi: 10.1080/089583703004451
44. Peace MR, Haley A, Mulder HA, Baird TR, Butler KE, Friedrich AK, et al. Evaluation of nicotine and the components of e-liquids generated from e-cigarette aerosols. *J Anal Tox.* (2018) 42:537–43. doi: 10.1093/jat/bky056
45. Protano C, Avino P, Manigrasso M, Vivaldi V, Perna F, Valeriani F, et al. Environmental electronic vape exposure from four different generations of electronic cigarettes: airborne particulate matter levels. *Int J Environ Res Public Health.* (2018) 15:2172. doi: 10.3390/ijerph15102172
46. Zervas E, Litsiou E, Konstantopoulos K, Pouloupoulos S, Katsaounou P. Physical characterization of the aerosol of an electronic cigarette: impact of refill liquids. *Inhal Toxicol.* (2018) 30:218–23. doi: 10.1080/08958378.2018.1500662
47. Raabe OG, Yeh HC, Newton GJ, Phalen RF, Velasquez DF. Deposition of inhaled monodisperse aerosols in small rodents. (1975) 4:3–21.
48. Chand HS, Muthumalage T, Maziak W, Rahman I. Pulmonary toxicity and the pathophysiology of electronic cigarette, or vaping product, use associated lung injury. *Front Pharmacol.* (2020) 10:1619. doi: 10.3389/fphar.2019.01619

49. Hickman E, Herrera CA, Jaspers I. Common E-cigarette flavoring chemicals impair neutrophil phagocytosis and oxidative burst. *Chem Res Toxicol.* (2019) 32:982–5. doi: 10.1021/acs.chemrestox.9b00171
50. Butt YM, Smith ML, Tazelaar HD, Vaszar LT, Swanson KL, Cecchini MJ, et al. Pathology of Vaping-Associated Lung Injury. *N Engl J Med.* (2019) 381:1780–1. doi: 10.1056/NEJMc1913069
51. Mukhopadhyay S, Mehrad M, Dammert P, Arrossi AV, Sarda R, Brenner DS, et al. Lung biopsy findings in severe pulmonary illness associated with e-cigarette use (vaping): a report of eight cases. *Am J Clin Pathol.* (2019) 153:30–9. doi: 10.1093/ajcp/aqz182
52. Matsumoto S, Fang X, Traber MG, Jones KD, Langelier C, Serpa PH, et al. Dose-dependent pulmonary toxicity of aerosolized Vitamin E acetate. *Am J Respir Cell Mol Biol.* (2020) 63:748–57. doi: 10.1165/rcmb.2020-0209OC
53. Flouris AD, Chorti MS, Poulanioti KP, Jamurtas AZ, Kostikas K, Tzatzarakis MN, et al. Acute impact of active and passive electronic cigarette smoking on serum cotinine and lung function. *Inhal Toxicol.* (2013) 25:91–101. doi: 10.3109/08958378.2012.758197
54. Melstrom P, Koszowski B, Thanner MH, Hoh E, King B, Bunnell R, et al. Measuring PM_{2.5} ultrafine particles, nicotine air and wipe samples following the use of electronic cigarettes. *Nicotine Tob Res.* (2017) 19:1055–61. doi: 10.1093/ntr/ntx058
55. Floyd EL, Queimado L, Wang J, Regens JL, Johnson DL. Electronic cigarette power affects count concentration and particle size distribution of vaping aerosol. *PLoS ONE.* (2018) 13:e0210147. doi: 10.1371/journal.pone.0210147
56. Lechasseur A, Altmeld S, Turgeon N, Buonanno G, Morawska L, Brunet D, et al. Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by an electronic cigarette. *Physiol Rep.* (2019) 7:14093. doi: 10.14814/phy2.14093
57. Mulder HA, Patterson JL, Halquist MS, Kosmider L, Turner JB, Poklis JL, et al. The effect of electronic cigarette user modifications and e-liquid adulteration on the particle size profile of an aerosolized product. *Sci Rep.* (2019) 9:10221. doi: 10.1038/s41598-019-46387-2
58. Hua M, Talbot P. Potential health effects of electronic cigarettes: a systematic review of case reports. *Prev Med Rep.* (2016) 4:169–78. doi: 10.1016/j.pmedr.2016.06.002
59. Carvalho, T. C., Peters, J. I., and Williams, R. O. (2011). Review: Influence of particle size on regional lung deposition—What evidence is there? *Int J Pharm.* 406, 1–10. doi: 10.1016/j.ijpharm.2010.12.040
60. Cheng T. Chemical evaluation of electronic cigarettes. *Tob Control.* (2014) 23:iii1–7. doi: 10.1136/tobaccocontrol-2013-051482
61. Crenshaw M, Tefft M, Buehler S, Brinkman M, Clark P, Gordon S. Determination of nicotine, glycerol, propylene glycol and water in electronic cigarette fluids using quantitative ¹H NMR. *Magn Reson Chem.* (2016) 54:901–4. doi: 10.1002/mrc.4498
62. Hutzler C, Paschke M, Kruschinski S, Henkler F, Hahn J, Luch A. Chemical hazards present in liquids and vapors of electronic cigarettes. *Arch Toxicol.* (2014) 88:1295–308. doi: 10.1007/s00204-014-1294-7
63. LeBouf RF, Burns DA, Ranpara A, Attfield K, Zwack L, Stefaniak AB. Headspace analysis for screening of volatile organic compound profiles of electronic juice bulk material. *Anal.* (2018) 410:5951–60. doi: 10.1007/s00216-018-1215-3
64. Peace M, Baird T, Smith N, Wolf C, Poklis J, Poklis A. Concentration of nicotine and glycols in 27 electronic cigarette formulations. *J Anal Toxicol.* (2016) 40:403–7. doi: 10.1093/jat/bkw037
65. Raymond B, Collette-Merrill K, Harrison R, Jarvis S, Rasmussen R. The nicotine content of a sample of e-cigarette liquid manufactured in the United States. *J of Addict Med.* (2018) 12:127–31. doi: 10.1097/ADM.0000000000000376
66. Li Y, Cui H, Wang X, Si X, Fan M, Chen L, et al. Effects of puffing parameters on physical properties and deposition in human respiratory tract of e-cigarette aerosols. *Tob Sci & Technol.* (2020) 53:10.
67. Sundahl M, Berg E, Svensson M. Aerodynamic particle size distribution and dynamic properties in aerosols from electronic cigarettes. *J Aerosol Sci.* (2017) 103:141–50. doi: 10.1016/j.jaerosci.2016.10.009
68. Asgharian B, Price O, Rostami A, Pithawalla Y. Deposition of inhaled electronic cigarette aerosol in the human oral cavity. *J Aerosol Sci.* (2018) 116:34–47. doi: 10.1016/j.jaerosci.2017.11.014
69. Asgharian B, Price O, Rostami A, Pithawalla Y. Regional deposition of inhaled aerosol constituents from Electronic Nicotine Delivery Systems (ENDS) in the respiratory tract. *J Aerosol Sci.* (2018) 126:7–20. doi: 10.1016/j.jaerosci.2018.08.006
70. Asgharian B, Hofmann W, Bergmann R. Particle deposition in a multiple-path model of the human lung. *Aerosol Sci Technol.* (2001) 34:332–9. doi: 10.1080/02786820119122

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Effect of Puffing Behavior on Particle Size Distributions and Respiratory Depositions From Pod-Style Electronic Cigarette, or Vaping, Products

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The current fourth generation (“pod-style”) electronic cigarette, or vaping, products (EVPs) heat a liquid (“e-liquid”) contained in a reservoir (“pod”) using a battery-powered coil to deliver aerosol into the lungs. A portion of inhaled EVP aerosol is estimated as exhaled, which can present a potential secondhand exposure risk to bystanders. The effects of modifiable factors using either a prefilled disposable or refillable pod-style EVPs on aerosol particle size distribution (PSD) and its respiratory deposition are poorly understood. In this study, the influence of up to six puff profiles (55-, 65-, and 75-ml puff volumes per 6.5 and 7.5 W EVP power settings) on PSD was evaluated using a popular pod-style EVP (JUUL® brand) and a cascade impactor. JUUL® brand EVPs were used to aerosolize the manufacturers’ e-liquids in their disposable pods and laboratory prepared “reference e-liquid” (without flavorings or nicotine) in refillable pods. The modeled dosimetry and calculated aerosol mass median aerodynamic diameters (MMADs) were used to estimate regional respiratory deposition. From these results, exhaled fraction of EVP aerosols was calculated as a surrogate of the secondhand exposure potential. Overall, MMADs did not differ among puff profiles, except for 55- and 75-ml volumes at 7.5 W ($p < 0.05$). For the reference e-liquid, MMADs ranged from 1.02 to 1.23 μm and dosimetry calculations predicted that particles would deposit in the head region (36–41%), in the trachea-bronchial (TB) region (19–21%), and in the pulmonary region (40–43%). For commercial JUUL® e-liquids, MMADs ranged from 0.92 to 1.67 μm and modeling predicted that more particles would deposit in the head region (35–52%) and in the pulmonary region (30–42%). Overall, 30–40% of the particles aerosolized by a pod-style EVP were estimated to deposit in the pulmonary region and 50–70% of the inhaled EVP aerosols could be exhaled; the latter could present an inhalational hazard to bystanders in indoor occupational settings. More research is needed to understand the influence of other modifiable factors on PSD and exposure potential.

Keywords: e-cigarette, pod-style e-cigarette, JUUL®, particle size distributions, respiratory deposition, secondhand exposure estimates

INTRODUCTION

Electronic cigarette, or vaping, products (EVPs) heat liquids (“e-liquids”) using a battery-operated coil and deliver the aerosolized particles to the lungs. By 2014, EVPs were the most popular tobacco product among youth in the USA. The evolution of internal design and external appearance of EVPs have occurred with each consequent modification, referred to as “generations” (1–3). The current, fourth generation, EVPs or “pod-mod” or “pod-style” device type includes two parts: a heating coil/e-liquid reservoir assembly and a flow-activated, rechargeable battery. The coil/e-liquid reservoir assembly is referred to as a “pod” and is either a prefilled disposable pod or refillable pod. For consistency in our study, the fourth generation EVP design type is described as pod-style. A pod-style design such as JUUL[®] brand has been popular for its sleek design, user-friendly function, desirable flavors, and ability to be used for “stealth vaping” (4–14).

Studies have characterized the constituents of JUUL[®] e-liquids and documented the different proportions of propylene glycol (PG) and vegetable glycerin (VG); other constituents include flavorings, nicotine, and benzoic acid (BA) (15–20). In the presence of BA, nicotine forms a salt, which forms protonated nicotine, rather than free-base nicotine, thereby allowing high levels of nicotine to be inhaled with less irritation or harsh “throat hit” as compared with traditional tobacco cigarettes and earlier generation (first, second, and third) EVP designs (2, 17, 20–23). The literature has conveyed that the presence of nicotinic salts in JUUL[®] e-liquids maximized nicotine uptake to the blood (8, 17, 21–24). The literature contains reports of the chemical toxicity associated with particles emitted by aerosolizing JUUL[®] e-liquids (16, 25–28).

Studies have emphasized mass-based particle size distribution (PSD) of EVP aerosols as an influential factor for estimating their regional respiratory deposition during inhalation and exhalation (29–33). Determining the PSD of EVP aerosols has been complex because these liquid droplets can deviate from their innate size depending on various conditions, such as evaporation and hygroscopic growth (34–36). The evaporation of liquid droplets in the EVP aerosol during sampling results in an underestimation of particle size, while hygroscopic growth results in an overestimation of particle size (36). These deviations in measuring PSD, in turn, result in errant predictions of regional depositions in the respiratory tract. Hence, the innate properties of particles should be maintained as intact as possible while measuring to determine an accurate PSD of the emitted EVP aerosols (37, 38). Oldham et al. (37) and Zhao et al. (38) predicted gas and particle phases as a function of the mass of collected aerosols without dilution to keep the physical and chemical components of the aerosol intact.

Previous studies evaluated the effects of multiple interlinked factors such as e-liquid compositions, puffing behavior (puff topography), and EVP electric settings (voltage, power, coil temperature, etc.) on PSD by aerosolizing e-liquid using first, second, and third generation EVPs (39–43). Fuoco et al. (39) found that flavorings from the products studied have a negligible influence on EVP particle emission. Lechasseur et al. (40)

discovered that together nicotine and flavorings in 30:70 PG–VG e-liquid did not affect PSD in any of the studied EVP power settings. Robinson et al. (42) presented an empirical correlation model of the dependence of whole aerosol mass emissions as a function of parameters, such as puff flow conditions, device operating power, and e-liquid composition, from the five different types across different EVP generations, including pod-style (JUUL[®]). This group considered the lack of information regarding puff topography for JUUL[®] as a limitation that needed to be addressed for a better functionality of their model. Vargas-Rivera et al. (43) provided puff topographical data from 21 college-aged (18–24 years) JUUL[®] users and reported that JUUL[®] flavored e-liquid usage did not seem to significantly affect puffing behavior, i.e., puff volumes, puff duration, interval, and number of puffs. Hence, EVP users’ puffing behavior and PSD may not be affected by the presence of both flavorings and nicotine in PG–VG-based e-liquids. Lechasseur et al. (40) and Son et al. (44) have evaluated multifactor effects on PSD and lung deposition, but these studies did not include pod-style EVPs. Therefore, it is critical to assess the impact of multiple factor variations on measuring the PSD of pod-style EVPs to better understand their role in aerosol deposition in the respiratory tract.

Considering the extensive use of JUUL[®] pods and the availability of other refillable pods (identical or not with JUUL[®] brand), the impact of influential factors affecting PSD and ultimately respiratory deposition as a significant knowledge gap must be addressed. Through this study, we offer insights into inhaled respiratory depositions and exhaled potential exposure fraction of aerosols impacted by puff volumes and power settings using both types of pod-style products: refillable or prefilled. We also assess lung deposition and, thereby potential secondhand exposure fraction, as a function of mass-based PSD of a JUUL[®]-like laboratory prepared reference e-liquid using refillable pods compared with commercially available prefilled JUUL[®] pods impacted by various puff behavior patterns. Stefaniak et al. (16) conveyed the mismatch between laboratory prepared study materials and widely used market EVPs, limiting the generalizability of research applications in real-world scenarios. By including both, we captured the standardized approach of controlling the preparation of e-liquids for testing and the generalizability of testing commercial e-liquids contained in the sealed pod-style devices, which more closely matches real-world conditions.

MATERIALS AND METHODS

Reference e-Liquid in Refillable Pods

As noted, the constituents of JUUL[®] e-liquids include PG–VG, acids, ethanol, flavorings, nicotine, and water (15, 16, 19, 20). Talih et al. (17) reported that the ratio of PG–VG was 30:70–27:73 in both liquid and aerosol for the JUUL[®] products sold in the USA (17, 18). Mallock et al. (7) reported that the PG–VG ratio of JUUL[®] e-liquid was 25:60 and the concentration of BA was ~9.4 mg/ml. Reilly et al. (26) evaluated toxicant emission considering 30:70 PG–VG to mimic JUUL[®] e-liquids. As flavor and nicotine together do not affect puff topography

TABLE 1 | Details of 12 JUUL[®] pod types used in this study.

JUUL [®] pod type	Nicotine %	Market availability
Menthol	3 and 5	Available since 2019
Virginia Tobacco	3 and 5	Available since 2019
Classic Tobacco	3 and 5	Discontinued May 8, 2020
Mint	3 and 5	Discontinued November 7, 2019
Crème Brûlée	5	Discontinued October 17, 2019
Fruit Medley	5	Discontinued October 17, 2019
Mango	5	Discontinued October 17, 2019
Classic Menthol	5	Limited Edition available in 2019

and PSD, they were not included in the preparation of the reference e-liquid (40, 43). Hence, we prepared an e-liquid, known as the “reference e-liquid,” which mimics the makeup of JUUL[®] using a composition of 25:73 PG (ACS grade, CAS# 57-55-6, Fisher, Hampton, NH, USA)/VG (certified ACS grade, CAS# 56-81-5, Fisher, Hampton, NH, USA) or PG-VG, and 1 part each of 200 proof ethanol (ACS/USP grade, CAS# 64-17-5, Pharmaco-Aaper, Brookfield, CT, USA) and 18 mΩ water with BA concentration at 9.4 mg/ml. Preparations were made using a Mettler Toledo XS 250 dual range microbalance (Mettler-Toledo LLC, Columbus, OH, USA). Although JUUL[®] does not explicitly list its constituent concentrations, the reference e-liquid constituent concentrations were selected to mimic the suspected composition of commercially available JUUL[®] pods. Approximately 0.7 ± 0.03 ml of the reference e-liquid was placed into refillable pods (Blankz! brand pods), which simulate the volume, appearance, and style of JUUL[®] brand pods. The density (1.13 ± 0.02 g/ml) of the reference e-liquid was determined by measuring the gravimetric mass of the e-liquid at a volume of 100 μl in triplicate. The volume of the reference e-liquid in the pod was calculated by dividing the pre- and post-mass difference by the density.

JUUL[®] e-Liquid in Disposable Pods (JUUL Labs, Inc., San Francisco, CA, USA)

We studied 12 commercially available JUUL[®] pod types of varying flavors and nicotine content, which were currently or previously commercially available. In November 2019, JUUL[®] voluntarily stopped the sale of all but Classic Menthol-, Classic Tobacco-, and Virginia Tobacco- flavored e-liquids. The US Food and Drug Administration (FDA) announced on January 2, 2020, its final enforcement policy for removing prefilled, flavored e-liquid cartridge-based products from the US market (except Menthol and Tobacco flavors) (45). Moreover, consumers can still purchase concentrated humectants or flavorings to dilute and mix their own desired flavored e-liquid, and then fill their pods at home (46). Three independent measurements were conducted to calculate the density (g/ml) of JUUL[®] e-liquids by measuring the gravimetric mass of the JUUL e-liquid at a fixed deliverable volume. Details of the commercial availability of JUUL[®] pod e-liquids used in this study are described in **Table 1**.

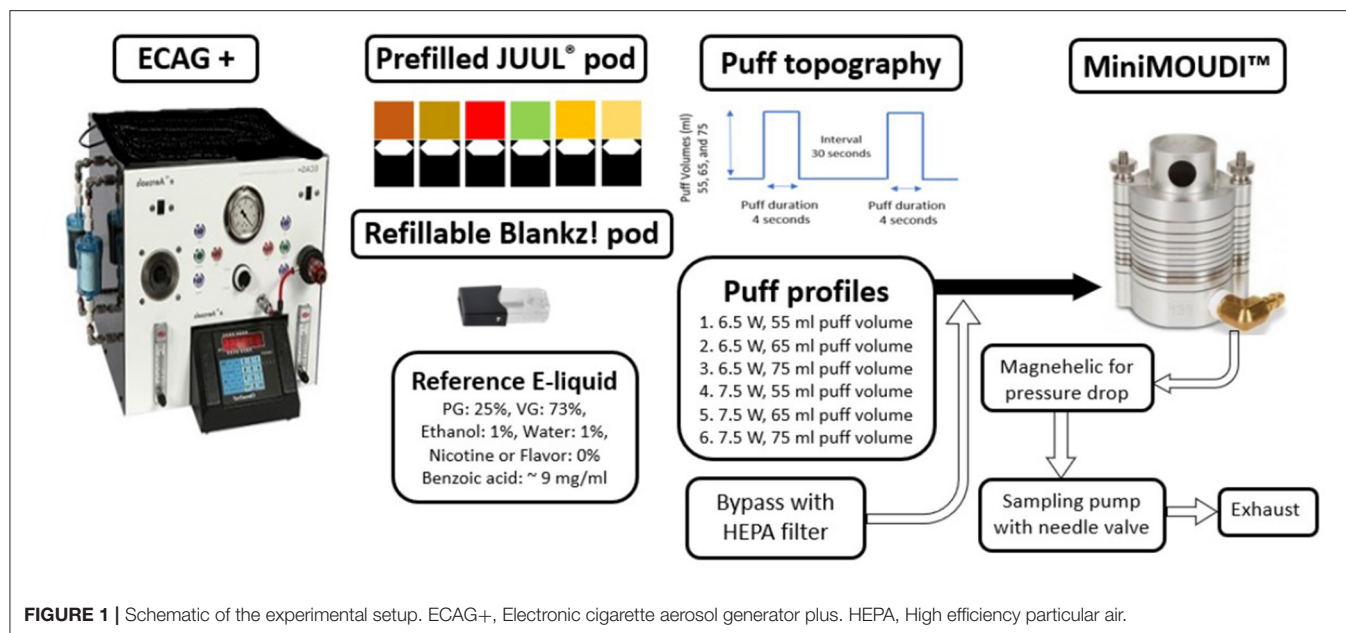
Experimental Design

An automated e-cigarette aerosol generator (ECAG+, e~Aerosols LLC, Central Valley, NY, USA) was programmed to aerosolize the reference e-liquid and JUUL[®] e-liquids. The ECAG+ works using positive pressure to aerosolize e-liquids. Prior to conducting trials, both JUUL[®] pods and the reference e-liquid pod were puffed 10 times to ensure that the coil was heated at the calibrated puff topography. Puff volumes were calibrated using a soap-bubble flow meter (Borgwaldt KC GmbH, Hamburg, Germany) as a primary volumetric flow calibration device.

JUUL[®], as a puff flow-activated, low-powered EVP, has been described by the manufacturer as operating at 6–8 W of power settings. However, the user of a JUUL device has no control over the power setting at which e-liquid gets aerosolized between 6 and 8 W. Talih et al. (17) reported the peak operating temperature to $\sim 215^{\circ}\text{C}$ while conducting their study with JUUL[®] EVPs at the maximum power (i.e., 8.1 W). In this study, reference e-liquid was aerosolized by heating it at 6.5 and 7.5 W [presumably to a temperature $<215^{\circ}\text{C}$ based on the report by Talih et al. (17)]; at three puff volumes, 55, 65, and 75 ml; and with 4-s puff durations that were 30 s apart, which is comparable to previous studies (42, 43, 47–49) and in agreement with the modified CORESTA method (50). Puff flow rates at the three tested puff volumes: 55, 65, and 75 ml with a 4-s puff duration were 0.8, 1.0, and 1.1 LPM, respectively. For commercially available JUUL[®] pods, one puff topography (65 ml at 7.5 W) was used to aerosolize the e-liquid. This profile was chosen after analyzing the results of the puff profiles for the reference e-liquid.

In our temperature- and humidity-controlled laboratory study, EVP aerosols were directly sampled with minimal dilution into a low-flow cascade impactor to keep the native physical properties of the aerosol intact as detailed in previous studies (37, 38, 51). MiniMOUDI[™] (MSP Corporation, Shoreview, MN, USA) was used to size fractionate EVP aerosols (size range: 0.056–10 μm) at a sampling flow rate of 2.0 LPM. The mass of aerosols deposited on each impactor stage was measured on a 37-mm aluminum filter using a Mettler Toledo XS 205 dual range microbalance with a mass resolution of 0.01 mg (Mettler-Toledo LLC, Columbus, OH, USA). Five trials with five puffs per trial were conducted for each JUUL[®] pod type, and three trials with three puffs per trial were conducted using the reference e-liquid in a refillable pod. Considering intra-device variability with the aerosol generation with JUUL[®] pods reported by Mallock et al. (7), we conducted five trials with five puffs per trial using JUUL[®] pods compared to three trials with three puffs per trial using the reference e-liquid in a refillable pod.

Figure 1 shows a schematic of the experimental setup. The ECAG+ powered the pod and forced the emitted EVP aerosols with the established puff topography into the MiniMOUDI[™]. While the ECAG+ was operating, there was a puff flow rate of 0.8, 1.0, and 1.1 LPM at the puff volumes of 55, 65, and 75 ml, respectively. The rest of the sampling flow rate of 2.0 LPM entered from a bypass air flow that passes through a high efficiency particulate air filter (HEPA; Whatman[®] Schleicher & Schuell; Stockbridge, GA, USA) to allow uninterrupted flow to



the MiniMOUDI™ and to alleviate pressure drops because of differences between the EVP aerosol puff flow rate and sampling flow rate. During the puff delay, the impactor sampled 2.0 LPM from bypass air, which did not result in any mass loading on the aluminum filters. To minimize aerosol loss, the pods were connected to an inlet of the MiniMOUDI™ using flexible, black conductive silicone tubing with an inside diameter of 0.5 cm and a length of 70 cm.

Evaporative Aerosol Mass Loss During Sampling for Reference e-Liquid

Depending on sampling and experimental parameters, the evaporation and hygroscopic growth of liquid droplets in the EVP aerosol result in a biased estimation of PSD (36), and eventually, in a biased estimation of lung deposition. For similar types of EVPs, Oldham et al. (37) reported a larger particle size while sampling at low dilution, whereas Mikheev et al. (41) noted a reduced size of particles because of evaporation at elevated flow rates of more than 25 ml/s. Flow rates used in this study correspond to 14–19 ml/s. We measured mass loss in the collected EVP aerosol at each puff volume of 7.5 W to determine whether it affected mass median aerodynamic diameters (MMADs) and associated respiratory deposition using the reference e-liquid. *Post-hoc*, we only compared evaporative mass loss for 55- and 75-ml puff profiles at 7.5 W based on a significant influence of these parameters on MMADs (see Section Results). Mass loss trials were conducted by collecting reference e-liquid aerosols using an impactor in the same manner as described previously. After collection, the filter stages were returned to an impactor and clean air was sampled at a flow rate of 2.0 LPM, at increasing times (1, 5, and 15 min). Deposited mass on each filter stage was measured after loading the EVP aerosol followed by clean air at increasing time intervals. Linear regression models were used to calculate the mass deposited for

each size bin at time zero, which was the mass used to calculate an adjusted MMAD. Non-adjusted MMADs were compared to the adjusted MMADs for each puff profile.

Data Analysis

Data were log transformed and managed using JMP 15.1.0 (SAS Institute, Inc., Cary, NC, USA). Deposited mass (in mg) of EVP aerosols for each impactor stage of the MiniMOUDI™ was calculated by measuring aluminum substrate before (pre-) and after (post-) sampling. To demonstrate mass-based PSD, MMAD and geometric SD (GSD) were calculated by including each cutoff size for the impactor stages of the MiniMOUDI™ using a probit model (52). Data acquisition of trials ($n = 3$ or $n = 5$) was done at least in triplicate, and significant differences ($p < 0.05$) between MMADs were determined using an one-way ANOVA and Tukey's HSD multiple comparisons.

Respiratory Deposition

The fraction of particles estimated to deposit in the respiratory tract was calculated using the Multiple Path Particle Dosimetry (MPPD), version 3.04 (ARA, Albuquerque, NM, USA). Regional depositions were reported as mass deposited in the head, trachea-bronchial (TB), and pulmonary regions using a default adult human symmetric model as an oronasal mouth breather (the Yeh–Schum model) in an upright position. The Yeh–Schum model uses a symmetric tree for the whole lung, as given by Yeh and Schum (53), and the estimated results correspond with the results from realistic lung structures (54). Regions from the mouth, nose, larynx, pharynx to trachea (generation 0) are considered as head. Regions from the trachea (generation 0) to the bronchioles (generation 16) are considered as TB. Regions beyond terminal bronchioles (from generations 16–23) are considered as pulmonary. Total respiratory deposition fractions were calculated by summing the regional deposition

fractions. Regional deposition (%) was calculated by the fraction of aerosols deposited in each region (head, TB, and pulmonary) divided by the total respiratory deposition. Statistical differences between the respective regional depositions of the reference e-liquid and JUUL® e-liquids at each studied puff profile were tested using an one-way ANOVA at $p < 0.05$. Based on the total respiratory deposition, the exhaled aerosol fraction was estimated using Equation 1.

$$\begin{aligned} &\text{Exhaled aerosol fraction} \\ &= 1 - \text{Total respiratory deposition fraction} \quad (1) \end{aligned}$$

The Yeh–Schum single path model considered the whole lung as a symmetric tree; therefore, respective regional depositions are the average values for each generation in the head, TB, and pulmonary regions (53). Unlike regional deposition, the Yeh–Schum lobular deposition pattern characterized the segmental bronchi within each lobe as a single symmetric path to report mass deposited in each of the five lobes of the lungs: right upper (RU), right middle (RM), right lower (RL), left upper (LU), and left lower (LL) (53). The total lobular deposition includes depositions in the TB and pulmonary regions of each lung lobe but not the initial airways as they do not belong to any lobe. Default parameters for the Yeh–Schum model were as follows: forced residual capacity = 3,300 ml, upper respiratory tract volume = 50 ml, breaths per minute (bpm) = 12, and tidal volume = 625 ml.

RESULTS

PSD and Respiratory Depositions for Reference e-Liquid

Of the six puff profiles studied, larger MMADs and wider GSDs were reported as puff volumes increased from 55 to 65 ml when heating the reference e-liquid at 7.5 W compared with 6.5 W (Table 2). We measured a statistical difference between MMADs for 55- (MMAD $1.23 \mu\text{m}$) and 75-ml (MMAD $1.02 \mu\text{m}$) puff volumes when heated at 7.5 W. The results for evaporative mass loss adjusted trials for these two puff profiles at 7.5 W are also reported in Table 2. This observation suggested a significant impact of puff volume (55 and 75 ml) on the PSD of the reference e-liquid aerosolized at 7.5 W. Irrespective of power settings, a 75-ml puff volume resulted in a narrower PSD (GSD 1.45–1.56) compared with other puff volumes (GSD 1.60–1.70). The largest average MMAD $\pm$ SD ($1.23 \pm 0.02 \mu\text{m}$) and GSD (1.70) resulted when heating the reference e-liquid at 7.5 W at a 55-ml puff volume across all the tested puff profiles. The smallest average MMAD $\pm$ SD ($1.02 \pm 0.08 \mu\text{m}$) and GSD (1.45) for all the tested puff profiles were reported on heating the reference e-liquid at 7.5 W at a 75-ml puff volume. We did not notice a statistical significance among MMADs and mass loss adjusted MMADs at these two puff volumes: 55 (MMAD 1.23 vs. $1.23 \mu\text{m}$) and 75 ml (MMAD 1.02 vs. $1.00 \mu\text{m}$) at 7.5 W. Because evaporative mass loss did not affect MMADs at the puff flow rates of 14 and 19 ml/s (55 and 75 ml volumes over 4 s), no loss correction was made to the rest of the trial mass data.

Particle size distributions did not differ among the puff volumes 55, 65, and 75 ml when the reference e-liquid was

aerosolized at 6.5 W (Table 2). Furthermore, we did not observe statistically significant differences between the MMADs of the reference e-liquid aerosolized at a 65-ml puff volume when compared with the 55- and 75-ml puff volumes, irrespective of power settings. MMAD and GSD results for individual trials, including mass loss trials for all e-liquids, are presented in Supplementary Table 1.

Based on MMADs and GSDs, the dosimetry analysis estimated more mass fractional deposition in the pulmonary region (40–43%) than in the head (36–41%) and TB regions (19–21%). Generally, across all the studied puff profiles (except the 55-ml puff volume at 7.5 W), the highest regional deposition was estimated to be at the pulmonary region. Deposition in the pulmonary region accounted for ~40% or more of the total deposited aerosol. Dosimetry analysis revealed the highest pulmonary (43%) and TB (21%) deposition at a 75-ml puff volume at 7.5 W. At this puff profile, smaller particle sizes with an average MMAD $\pm$ SD ($1.02 \pm 0.08 \mu\text{m}$) and a tighter aerosol size distribution with lesser GSD (1.45) resulted in higher mass deposition in pulmonary and TB regions compared with the head. A wider PSD with a greater GSD (1.70) and a higher MMAD ($1.23 \mu\text{m}$) was noticed with a higher deposition in the head compared with the other regions of 55-ml puff volume at 7.5 W. In the same puff profile, the only exception resulted in the highest deposition in the head region (41%), based on the average MMAD $\pm$ SD ($1.23 \pm 0.02 \mu\text{m}$) and GSD (1.70) at the 65- and 75-ml puff volumes. Although regional respiratory deposition was comparable between pulmonary (40%) and head regions (41%) for the puff profile of a 55-ml puff volume at 7.5 W. As expected, a larger MMAD $\pm$ SD ($1.23 \pm 0.02 \mu\text{m}$) and GSD (1.70) from a 55-ml puff volume at 7.5 W resulted in a higher deposition in the head.

Comparing 55- and 75-ml puff volumes between the power settings, a wider range in the estimated total respiratory deposition was noticed at 7.5 W (28–36%) compared to 6.5 W (31–32%). Generally, all the tested puff profiles resulted in 19–21% deposition in the TB region. Puff profiles with smaller MMADs resulted in a higher pulmonary deposition, lower total respiratory deposition, and higher percentage of exhaled aerosol.

Inhaled total respiratory deposition and the percentage of exhaled EVP aerosols were inversely related. Hence, the lower the total deposition, the higher the percentage of exhaled EVP aerosols. Depending on MMAD and GSD, the highest total respiratory deposition (36%) and thereby the lowest percentage of exhaled EVP aerosols (64%) were from a puff profile of 55-ml puff volume at 7.5 W. Inversely, the lowest total respiratory deposition (28%) and thereby the highest percentage of exhaled EVP aerosols (72%) resulted from a puff profile of 75-ml puff volume at 7.5 W. Like MMAD and GSD values, the remaining puff profiles, other than those with 55 and 75 ml of puff volumes at 7.5 W, have similar regional depositions (head: 38–39%, TB: 20%, and pulmonary: 41–42%), total respiratory depositions (31–32%), and the percentage of exhaled EVP aerosols (68–69%). Operating at a higher power setting of 7.5 W was an influential factor affecting PSD; and 65 ml of puff volume, at which the PSD did not result into a statistical difference across other puff profiles, was considered to aerosolize the prefilled JUUL® pods

TABLE 2 | Particle size distribution (PSD), regional respiratory deposition, and exhaled aerosol for the reference e-liquid at different puff profiles with three puffs ($n = 3$ per profile).

Puff profiles	Power (W)	Puff volume (ml)	Average MMAD (μm) $\pm$ SD	Average GSD	Head (%)	TB (%)	Pulmonary (%)	Total respiratory (%)	Exhaled aerosol (%)
1	6.5	55	$1.10 \pm 0.01^{A,B}$	1.60	38	20	42	32	68
2		65	$1.11 \pm 0.03^{A,B}$	1.60	38	20	42	31	69
3		75	$1.10 \pm 0.07^{A,B}$	1.56	38	20	42	31	69
4	7.5	55	1.23 ± 0.02^A	1.70	41	19	40	36	64
4*		55	1.23 ± 0.03^A	1.70	41	19	40	36	64
5		65	$1.13 \pm 0.06^{A,B}$	1.63	39	20	41	32	68
5*		65	$1.11 \pm 0.08^{A,B}$	1.58	39	20	41	32	68
6		75	1.02 ± 0.08^B	1.45	36	21	43	28	72
6*		75	1.00 ± 0.07^B	1.45	36	21	43	28	72

*Indicates puff profiles evaluated for evaporative mass loss.

MMAD, mass median aerodynamic diameter; SD, standard deviation; GSD, geometric SD; TB, trachea-bronchial region.

MMAD, if not connected by the same letter (A or B), are significantly different at $p < 0.05$. Resistance (Ohm: Ω) of the reference e-liquid containing Blankz! pod: Average $\pm$ SD ($1.87\Omega \pm 0.02\Omega$) with relative SD 1.30%.

TABLE 3 | PSD, regional respiratory deposition, the percentage of exhaled electronic cigarette, or vaping, products (EVPs) aerosols, and statistical comparisons of JUUL[®] pod types ($n = 5$ puffs per pod type) and the reference e-liquid at the same puff profile (65-ml puff volume at 7.5 W).

JUUL [®] pod type	Nicotine %	Density of e-liquid (g/ml)	Average MMAD (μm) $\pm$ SD	GSD	Head (%)	TB (%)	Pulmonary (%)	Total respiratory (%)	Exhaled aerosol (%)
Virginia Tobacco	3	1.10	$1.49 \pm 0.30^{A,B}$	2.50	49	18	33	45	55
Virginia Tobacco	5	1.01	$1.20 \pm 0.45^{A,B}$	2.27	43	21	37	36	64
Menthol	3	1.42	$1.11 \pm 0.11^{A,B}$	2.45	43	20	37	39	61
Menthol	5	1.27	0.92 ± 0.19^B	1.69	35	23	42	28	72
Classic Tobacco	3	1.17	$1.47 \pm 0.34^{A,B}$	2.54	49	18	33	44	56
Classic Tobacco	5	1.26	$1.40 \pm 0.14^{A,B}$	2.57	48	18	34	45	55
Mint	3	1.07	$1.21 \pm 0.20^{A,B}$	2.46	45	19	36	40	60
Mint	5	1.06	$1.41 \pm 0.25^{A,B}$	2.64	49	18	33	45	55
Crème Brûlée	5	1.26	$1.31 \pm 0.29^{A,B}$	2.71	48	18	33	42	58
Fruit Medley	5	1.02	1.67 ± 0.53^A	2.75	52	18	30	47	53
Mango	5	1.03	1.55 ± 0.11^A	2.65	50	18	32	48	52
Classic Menthol	5	1.11	1.59 ± 0.20^A	2.57	50	18	32	47	53
Reference	0	1.13	$1.13 \pm 0.06^{A,B}$	1.63	39	20	41	32	68

MMAD, mass median aerodynamic diameter; SD, standard deviation; GSD, geometric SD; TB, trachea-bronchial region.

JUUL[®] pod types and the reference e-liquid not connected by the same letter (A or B) had significantly different MMADs at $p < 0.05$. Resistance (Ω) for JUUL[®] pod types: Average $\pm$ SD (Ω : 1.81 ± 0.03) with relative SD 1.87%.

for evaluating PSD, and thus, their *vis-à-vis* comparisons for respiratory depositions.

PSD and Respiratory Depositions for JUUL[®] e-Liquids

Table 3 shows comparisons of e-liquid densities, MMAD, and GSD (for $n = 5$) of the prefilled commercial JUUL[®] pods with 3 and 5% nicotine strengths aerosolized with a 65-ml puff volume emitted at 7.5 W. Overall, for all the flavors, higher e-liquid density (g/ml) resulted for the 3% nicotine strength compared with the 5%, except for the flavor Classic Tobacco (3%: 1.17 vs. 5%: 1.26). Of 12 JUUL[®] pod types, the largest averages of MMAD $\pm$ SD ($1.67 \pm 0.53\mu\text{m}$) and GSD (2.75) were reported with the Fruit Medley flavor with a 5% nicotine

strength (Fruit Medley 5%). The smallest averages of MMAD and SD ($0.92 \pm 0.19\mu\text{m}$) and GSD (1.69) were for Menthol with a 5% nicotine strength across all the studied JUUL[®] pod types. At 5% nicotine, significant differences (at $p < 0.05$) were observed among MMADs for Menthol ($0.92\mu\text{m}$) and the three flavor types: Fruit Medley ($1.67\mu\text{m}$; $p = 0.01$), Classic Menthol ($1.59\mu\text{m}$; $p = 0.02$), and Mango ($1.55\mu\text{m}$; $p = 0.03$). We detected no significant differences among MMADs for the rest of the JUUL[®] pod flavors at each nicotine strength. When compared with Menthol at a 5% nicotine strength (Menthol 5%: GSD 1.69), the rest of the JUUL[®] pod flavors had wider PSDs based on the GSD values (2.27–2.75).

Table 3 also shows comparisons between MMADs of 3% and 5% nicotine for the same JUUL[®] flavor types; there was no

significant difference between the average MMAD of 3% and 5% nicotine for JUUL[®] flavor types: Classic Tobacco ($p = 0.65$), Virginia Tobacco ($p = 0.27$), Mint ($p = 0.20$), and Menthol ($p = 0.08$). Other than Mint, all JUUL[®] e-liquid flavors with 5% nicotine strength had smaller MMADs compared with that of the 3% nicotine strength; for Menthol flavor, the average MMAD and GSD at 5% nicotine (0.92 and 1.69 μm) were less than those at 3% nicotine (1.11 and 2.45 μm). A similar observation resulted for the Virginia Tobacco flavor when comparing average MMAD and GSD between 5% nicotine (1.20 and 2.27 μm) and 3% nicotine (1.49 and 2.50 μm). Likewise, Classic Tobacco resulted in a smaller average MMAD and GSD (1.40 and 2.57 μm) at 5% nicotine when compared to 3% nicotine (1.47 and 2.54 μm). However, with Mint flavor type, this trend reversed, and 3% nicotine resulted in a smaller average MMAD and GSD (1.21 and 2.46 μm) than the average MMAD and GSD at 5% nicotine (1.41 and 2.64 μm).

Based on MMAD and GSD, the highest regional respiratory deposition was generally in the head (35–52%), compared with pulmonary (30–42%) and TB regions (18–23%). With the smallest MMAD and GSD, Menthol 5% (0.92 and 1.69 μm) had the highest pulmonary (42%) and TB (23%) depositions and the lowest head (35%) depositions. A smaller particle size with a smaller average MMAD and a tighter PSD with a smaller GSD resulted in higher regional depositions in the pulmonary and TB region when compared with the head. A wider PSD with a larger GSD and a higher MMAD were observed with a higher deposition in the head region compared with the other regions. The dosimetry analysis of the Fruit Medley pod type resulted in the lowest pulmonary (30%) deposition and the highest head (52%) deposition because of a wider PSD and the highest MMAD (1.67 and 2.75 μm). According to the dosimetry analysis, the range of total respiratory deposition for the studied JUUL[®] pods was estimated to be between 28% (Menthol 5%) and 48% (Mango 5%) with an average of $42 \pm 6\%$. This assessment suggests that ~52–72% of exhaled aerosol with an average of 68% could serve as an aerosol available for secondhand exposure to bystanders.

The average measured density of the reference e-liquid at 1.13 ± 0.02 g/ml was comparable to all the studied JUUL[®] brand e-liquids at 1.15 ± 0.13 g/ml. None of the studied JUUL[®] pods were found to have a statistically significant difference from the reference e-liquid and filled in blank pods, when aerosolized at the same puff profile (65-ml puff volume at 7.5 W). Depending on PSD, most of the JUUL[®] pods with the exception of Menthol with 5% nicotine resulted in a higher regional deposition in the head (43–52%) and a higher total respiratory deposition (36–48%), but a lower percentage of exhaled aerosol (52–64%) when compared with the reference e-liquid, respectively, 39% (head), 32% (total), and 68% (exhaled). These higher regional depositions in the head were attributed to higher MMADs and higher GSD values for JUUL[®] pods compared to the reference e-liquid (GSD 1.63) with the exception of Menthol at 5% nicotine (GSD 1.69 and the head deposition percentage of 35%). The pulmonary deposition of the reference e-liquid (41%) and Menthol 5% (42%) was similar at the same puff profile (65-ml puff volume at 7.5 W). However, Menthol 5% resulted in lesser total respiratory deposition (28 vs. 32%) and thereby a higher

percentage of exhaled EVP aerosols (72 vs. 68%) compared with the reference e-liquid.

The average measured density of the reference e-liquid at 1.13 ± 0.02 g/ml was comparable to all the studied JUUL[®] brand e-liquids at 1.15 ± 0.13 g/ml. None of the studied JUUL[®] pods were found to have a statistically significant difference from the reference e-liquid and filled in blank pods, when aerosolized at the same puff profile (65-ml puff volume at 7.5 W). Depending on PSD, most of the JUUL[®] pods with the exception of Menthol with 5% nicotine resulted in a higher regional deposition in the head (43–52%) and a higher total respiratory deposition (36–48%), but a lower percentage of exhaled aerosol (52–64%) when compared with the reference e-liquid, respectively, 39% (head), 32% (total), and 68% (exhaled). These higher regional depositions in the head were attributed to higher MMADs and higher GSD values for JUUL[®] pods compared to the reference e-liquid (GSD 1.63) with the exception of Menthol 5% (GSD 1.69 and the head deposition percentage of 35%). The pulmonary deposition of the reference e-liquid (41%) and Menthol 5% (42%) was similar at the same puff profile (65-ml puff volume at 7.5 W). However, for inversely related dosimetry attributes, Menthol 5% resulted in lesser total respiratory deposition (28 vs. 32%) and thereby a higher percentage of exhaled EVP aerosols (72 vs. 68%) compared with the reference e-liquid.

Lobular Depositions

Figure 2 represents lobular aerosol deposition of all the studied puff profiles for the reference e-liquid as well as commercially available prefilled JUUL[®] pods, which were not significantly different from each other. A slightly higher percentage (8%) of the mass deposited in right-sided lung lobes (sum of RU, RM, and RL = 54%) than left-sided lung lobes (sum of LU and LL = 46%). Manigrasso et al. (55) presented right lung lobes as the sites where the effects of EVP aerosols occur more frequently. For all the studied puff profiles for the reference e-liquid, the MMAD ranged from 1.02 to 1.23 μm and for all commercially available prefilled JUUL[®] pods, the MMAD ranged from 0.92 to 1.67 μm . The highest percentages of lobular deposition of the emitted aerosols were predicted in the lower lobes (right $30\% \pm 0.2\%$ and left $30\% \pm 0.2\%$) compared with other lobes of the lungs (RU $16\% \pm 0.2\%$, RM $8\% \pm 0.1\%$, and LU $16\% \pm 0.2\%$).

DISCUSSION

Electronic cigarette, or vaping, products are currently the most popular tobacco product among youth in the USA (56–58). Implications of EVP use also extend to workplaces. Romberg et al. (59) concluded that vaping and vaping exposures are common in US workplaces. Employees, especially non-users, reported reduced productivity because of workplace vaping. Previous studies also purported potential harmful health effects to bystanders because of an exposure to toxic chemical and aerosol emissions from using the fourth generation EVPs, which also negatively affect indoor air quality (59, 60). As such, understanding the emission characteristics of EVPs, specifically PSD, is critical to evaluate aerosol dosimetry for users and bystanders.

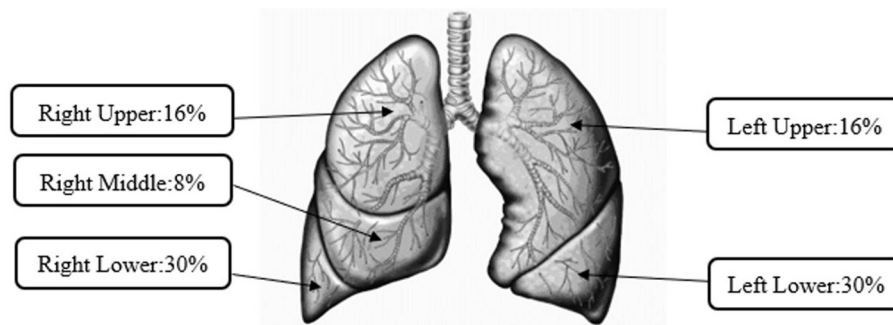


FIGURE 2 | Average lobular deposition of aerosols from pod-style electronic cigarette, or vaping, products (EVPs).

The choice of EVP and e-liquid to study aerosol PSD is complex. The current study focused on pod-mod or pod-style EVPs as these fourth generation devices are the most current designs on the market. The choice of e-liquid includes reference or standardized e-liquids or commercially available products and each has its own unique advantages and disadvantages. Several investigators have created reference or standardized e-liquids for testing earlier generation EVPs (15, 61). Reilly et al. (26) found no differences in free radicals and carbonyl yields when a commercial JUUL[®] e-liquid was replaced with a laboratory prepared e-liquid with a 30:70 PG–VG mixture that mimicked the PG:VG ratio in the JUUL[®] e-liquid. This observation was important because commercial brands such as JUUL[®] do not reveal the formulation of their e-liquids, and therefore it can be difficult to interpret study results when conclusions are made in circumstances where e-liquid composition was not controlled. Building on the results of Reilly et al. (26), we incorporated both attributes of reference and commercial e-liquids using pod-style EVPs: (1) laboratory-prepared reference e-liquid that mimics commercially available JUUL[®] e-liquid and (2) commercially available JUUL[®] e-liquids. Our approach had helped to better understand the factors that influence the PSD of these e-liquids measured under the conditions that maintain their native state and provided comparisons of respiratory deposition estimates for users and bystanders.

Reference e-Liquids

Our study observed a significant influence of puff volumes (55 and 75 ml) on MMADs when heated at a higher power setting (7.5 vs. 6.5 W) ($p < 0.05$). Modifiable factors of the puffing behavior considered in this study such as puff volumes (55, 65, and 75 ml), puff duration (4 s), and puff interval (30 s) were recommended by methods such as CORESTA, previous empirical studies, and/or documented in cytotoxic investigations for EVPs-associated research applications (26, 42, 43, 47, 48, 50, 62–65). Though the pod-style EVP user has no control over power settings, one of the goals of this study was to assess the influence of puff volumes (55, 65, and 75 ml) by heating the reference e-liquid at power settings (6.5 and 7.5 W) on PSD and respiratory deposition using the pod-style EVP. MMADs for all the puff profiles with the reference e-liquid were $\sim 1 \mu\text{m}$,

which is consistent with the finding presented by Lechasseur et al. (40). In their study, the authors noted e-liquids with a higher VG component with a PG:VG ratio of 30:70 resulted in the emission of larger particles than a 70:30 PG:VG ratio. Our observation is that with 55- and 65-ml puff volumes, increasing the power delivery could generate larger particles, possibly because of higher particle density by aerosolizing more e-liquid material, is consistent with the earlier studies that evaluated an EVP design prior to a pod-style design (40, 66). Despite not statistically significant, MMAD with a 75-ml puff volume was $1.02 \mu\text{m}$ at 7.5 W compared to $1.10 \mu\text{m}$ at 6.5 W, possibly because of the emission of smaller-sized particles at a higher puff volume, which lowered the size distribution at a higher power. Mikheev et al. (41) documented a reduction in particle size at a higher puff flow rate beyond 25 ml/s because of evaporation, presumably a result of higher dilution while sampling aerosols. Compared to Mikheev's study, we sampled the three puffs of reference e-liquid EVP aerosols with puff volumes of 55, 65, and 75 ml within 4 s, each 30 s apart. This led us to sample a total of 165, 195, or 225 ml for every trial at a constant sampling flow rate of two LPMs to evaluate MMAD using MiniMOUDI[™]. Additionally, at given experimental parameters, we did not measure significant differences in MMADs because of evaporation with different puff profiles aerosolizing the reference e-liquid at 7.5 W. At higher power settings (7.5 W), our measurement at a higher puff volume ($75 > 65 > 55 \text{ ml}$) caused the particle size to decrease (MMAD (μm) $1.02 < 1.13 < 1.23$), which was consistent with the findings from Li et al. (48). Higher puff volumes, possibly providing less time for particle collision or coagulation, coupled with a higher power increased the heating of the e-liquid and decreased the aerosol size emitted.

Complexity in determining the respiratory deposition of EVP aerosols based on their size distribution has been addressed in previous studies (29–34, 36). Raabe et al. (67) concluded that the highest pulmonary deposition fraction was for particles with MMAD smaller than $2 \mu\text{m}$, which would deposit in the lower respiratory tract. At particle sizes ranging from 0.2 to $1 \mu\text{m}$, higher deposition fractions in the lower lobes compared to the upper lobes were also documented, as observed in our results (53, 68–71). Lechasseur et al. (40) presented the conditions

that an increase in the particle size aerosolized by heating e-liquids with a PG–VG ratio of 30:70 at higher power settings led to a reduction in pulmonary deposition. Dosimetry results presented in this study for various puff profiles are consistent with these authors' observations. Other than the highest MMAD (1.23 μm), 55-ml puff volume at 7.5 W, all puff profiles resulted in the highest pulmonary deposition. Compared with other puff profiles, this puff profile resulted in 41% of mass deposition in the head, which is comparable with 40% in pulmonary regions. The literature has evidentially presented that particles smaller than 1 μm are not only known to result in deep lung deposition but also are able to be exhaled with a greater chance (29, 30). Smaller-sized particles could serve as a potential secondhand exposure on exhalation to nearby people, especially in occupational settings such as vape shops. More than 64% of the aerosols emitted from all the puff profiles studied were estimated to be exhaled. The direct measurement of exhaled aerosols should be conducted to determine the correct PSD of secondhand exposure conditions as primary aerosols inhaled differ from the aerosol exhaled by an EVP user. The results of the percentage of exhaled aerosol presented here indicated that pod-style EVP aerosols can potentially serve as a secondhand exposure for employees in occupational settings (e.g., vape shops, bars, and restaurants) that allow to use in indoors.

Commercially Available JUUL[®] e-Liquids

After FDA restrictions on the sale of flavored prefilled pods in 2020, other fourth generation EVP devices became available and popular in the market that can aerosolize various flavored e-liquids, other than the prefilled JUUL[®] brand pods flavored with Menthol and Tobacco (45, 72, 73). Furthermore, nicotine salt mixed with a custom-made e-liquid bulk material to be used in refillable pod-style devices could provide all the flavors reported in a previous study (74). Additionally, this ban only applied to the flavored cartridges for use in pod-style EVP devices and does not apply to manufacturers of any other flavored e-liquid that is attached to the mouthpiece of JUUL[®] and other brand pod-style EVP devices. It is noteworthy that other manufacturers have developed the flavored e-liquid pods that are attached to the mouthpiece of JUUL[®] and other brand pod-style EVPs (75). However, adequate research on the physical characteristics of the particles, such as PSD emitted from the pod-style EVPs, has until now been lacking and this information is necessary to understand regional lung depositions. We studied commercial JUUL[®] pods, either currently available or not, to have an idea about PSD and lung deposition by aerosolizing the prefilled flavored JUUL[®] e-liquids.

Menthol-containing JUUL[®] flavors resulted in lower MMADs [5%: 0.92 μm (the lowest); 3%: 1.11 μm] compared with the other studied JUUL[®] pods. This result was consistent with a previous observation by Lechasseur et al. (40) evaluating the effect of menthol on e-liquid with nicotine in 30:70 PG–VG. Lamb et al. (76) studied the cytotoxicity of currently available JUUL[®] flavor aerosols: Menthol and Virginia Tobacco. The authors indicated that an exposure to Menthol-flavored JUUL[®] pods causes considerable mitochondrial dysfunction in lung epithelial cells compared with Virginia Tobacco. Depending

on MMAD and GSD, of all the studied JUUL[®] pods, Menthol 5% resulted in the highest pulmonary (42%) and TB (23%) depositions and the lowest head (35%) and total respiratory (28%) depositions. An inversely related percentage of exhaled EVP aerosols (72%), a secondhand exposure estimate, was the highest for Menthol 5%. A consideration of the exhalation of smaller-sized particles was addressed in this study by presenting the estimates of exhaled fraction as a surrogate for potential secondhand exposure conditions. Inversely related to total respiratory deposition, an average of 58%, EVP aerosols ranged between 52% (the lowest with Mango 5% nicotine) and 72% (the highest with Menthol 5% nicotine). Other than Menthol 5%, the rest of the studied JUUL pods resulted in larger sized particles with MMAD > 1 μm and wider size distributions with GSD > 2. Lechasseur et al. (40) indicated larger sized particles deposited at regions other than the pulmonary region. In our dosimetry evaluations, all the JUUL pods other than Menthol 5% resulted in a higher deposition in the head (43–52%) rather than in the pulmonary region (30–37%). Some studies have observed an association between an EVP user and respiratory symptoms among adolescents (77, 78), a reduced pulmonary immune function (65, 79), and an increased risk of mood and attention symptoms (80) as well as potential long-term effects on brain development for cognitive behavior (81, 82). Pearce et al. (83) characterized the aerosolized JUUL[®] Fruit Medley flavored e-liquid and documented a reduced cellular metabolic activity in a dose-dependent manner. Stefaniak et al. (16) reviewed the toxicology of flavorings used in e-liquids. Apart from the existing cytotoxicity studies, our findings of pulmonary deposition could help explain the development or exacerbation of respiratory and systemic toxicity from the use of aerosolized JUUL[®] e-liquids. Though some JUUL[®] flavors are restricted from sale in the USA, the currently available Menthol and Virginia Tobacco JUUL[®] flavors can potentially lead to considerable health damages (76). Additionally, the aerosolization of flavored e-liquids using refillable blank pod-style EVPs can be as harmful as JUUL[®] flavors (75).

CHALLENGES AND LIMITATIONS

For the fourth generation EVPs, a reference model is not available like those of the second and third generation EVPs (84). Study results may deviate using the different types of blank pod-style devices than those used in this study. The refillable pods from other brands may not have identical characteristics, such as coil material and surface area, compared to the JUUL[®] brand. However, we used blank pods comparable to JUUL[®] brand pods in reservoir size and electric capability to aerosolize the reference e-liquid.

Without standardized experimental protocols, parameters included in any study could be a source of limitations that can cause a lack of reproducibility and comparability among different studies. The ECAG+ used in our study was based on the principles of positive pressure to generate aerosols rather than the negative pressure used by humans. We are unaware of any study that has compared PSDs generated by positive and

negative pressure devices for the same e-liquid so the influence of this experimental parameter on results is unknown at this time. Depending on these experimental and sampling parameters, the size distribution of EVP aerosols deviates because of hygroscopic growth and evaporation in the human lung environment. These deviations could be impacted by various experimental parameters such as the composition of e-liquids, puff volume, and power settings (32, 36, 40, 85, 86). These factors play a significant role in the determination of PSD and therefore the regional deposition of the aerosols in the respiratory tract. Influences of variables such as the puff volume and power setting are more relatable to JUUL[®] and other pod-style devices, which are flow-activated, low-powered EVPs.

Even with all variables held constant, Protano et al. (87) demonstrated significant variations in puff-to-puff aerosol generation within a single EVP device. Mass measurement with JUUL[®] EVPs was challenging because of a variability in aerosol generation, as documented in a previous study (7). To better understand PSD, two different power settings were considered in this study, however, to avoid damages to the physical integrity of a pod-style EVP, we could not report the temperatures of e-liquid while puffing at 6.5 and 7.5 W. As JUUL[®] devices are flow-activated EVP design types, it was difficult to determine estimates for power settings and thereby the temperature of e-liquid while puffing at the given puff profiles among EVP users in the real-world scenarios. Therefore, for JUUL[®] pods, the results of this study are limited to one fixed puff topography, which was a 4-s puff, 30-s interval, and 65-ml puff volume by aerosolizing e-liquid at 7.5 W.

As the focus of our study was to evaluate the influential puffing parameters affecting PSD using pod-style EVPs, we considered using a puff profile that included those influential parameters related to determining PSD and ultimately respiratory deposition. The influential parameter conditions (e.g., puff volumes, puff flow rates, electrical settings, and devices) considered herein might be different from those found in real-world scenarios or used by other research studies. Hence, PSD depending on these parameters might be affected and this would impact aerosol respiratory deposition estimates. Additionally, the estimated respiratory deposition fractions using the MPPD software were not modified for hygroscopic growth and evaporation according to the human lung environment. The dosimetry analysis did not consider the clearance mechanisms that may impact total respiratory deposition and exhaled aerosol estimates. This report, being unique in addressing the PSD of pod-style EVPs, should stimulate additional experiments regarding different puff topographies for JUUL[®] pods in the future and focus on characterizing the chemical content of the vapor exhaled by an individual using a pod-style EVP.

SUMMARY

After FDA restrictions on prefilled EVP flavor pods other than Menthol and Tobacco, refillable pods have been more popular

to aerosolize various flavors of homemade or commercially available e-liquids. Although toxicological studies have reported evidence of deleterious health effects on heating e-liquids in pod-style EVP design—either prefilled in JUUL[®] or simulated JUUL[®]-like e-liquids, the PSD of emitted EVPs aerosol as a significant determinant for their regional respiratory depositions has not been addressed adequately (28, 65). This study evaluated respiratory tract depositions as a function of the MMAD and GSD of particles emitted by heating the simulated reference e-liquid that mimicked JUUL[®] e-liquid at 6.5 and 7.5 W with the three puff volumes (55, 65, and 75 ml). The higher 7.5 W power setting was an influential factor that significantly impacted MMADs at 55- (1.23 μm) and 75-ml (1.02 μm) puff volumes. In general, for all puff profiles with the reference e-liquid, the dosimetry analysis predicted that 40–43% of total respiratory depositions of particles were in the pulmonary regions where toxicological implications have been reported. With wider size distributions for JUUL[®] e-liquids (GSD: 1.69–2.75), dosimetry modeling predicted comparable particle depositions in the head (35–52%) and in the pulmonary regions (30–42%). Irrespective of statistical differences in their size distribution, the emitted aerosols from heating the reference e-liquid or JUUL[®] e-liquids are predominantly (60%) deposited in the lower lobes (Right 30% and Left 30%) of the lungs. Inversely related to the estimated total respiratory deposition, more than 52% of the aerosols were exhaled, which could potentially serve as secondhand exposure conditions at workplace and hence needs to be assessed in indoor environments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpubh.2021.750402/full#supplementary-material>

Supplementary Table 1 | Mass median aerodynamic diameter (MMAD) and geometric SD (GSD) for individual trials for puff profiles of the reference e-liquid.

REFERENCES

- Bhatnagar A, Whitsel LP, Ribisl KM, Bullen C, Chaloupka F, Piano MR, et al. Electronic cigarettes: a policy statement from the American Heart Association. *Circulation*. (2014) 130:1418–36. doi: 10.1161/CIR.000000000000107
- Schmidt S. Vaper, beware: the unique toxicological profile of electronic cigarettes. *Environ Health Perspect*. (2020) 128. doi: 10.1289/EHP6628
- Schraufnagel DE, Blasi F, Drummond MB, Lam DC, Latif E, Rosen MJ, et al. Electronic cigarettes. A position statement of the forum of international respiratory societies. *Am J Respir Crit Care Med*. (2014) 190:611–8. doi: 10.1164/rccm.201407-1198PP
- Fadus MC, Smith TT, Squeglia LM. The rise of e-cigarettes, pod mod devices, and JUUL among youth: factors influencing use, health implications, and downstream effects. *Drug Alcohol Depend*. (2019) 201:85–93. doi: 10.1016/j.drugalcdep.2019.04.011
- Huang J, Duan Z, Kwok J, Binns S, Vera LE, Kim Y, et al. Vaping versus JUULing: how the extraordinary growth and marketing of JUUL transformed the US retail e-cigarette market. *Tob Control*. (2019) 28:146–51. doi: 10.1136/tobaccocontrol-2018-054382
- Leavens ELS, Stevens EM, Brett EI, Hebert ET, Villanti AC, Pearson JL, et al. JUUL electronic cigarette use patterns, other tobacco product use, and reasons for use among ever users: results from a convenience sample. *Addict Behav*. (2019) 95:178–83. doi: 10.1016/j.addbeh.2019.02.011
- Mallock N, Trieu HL, Macziol M, Malke S, Katz A, Laux P, et al. Trendy e-cigarettes enter Europe: chemical characterization of JUUL pods and its aerosols. *Arch Toxicol*. (2020) 94:1985–94. doi: 10.1007/s00204-020-02716-3
- Ramamurthi D, Chau C, Jackler RK. JUUL and other stealth vaporisers: hiding the habit from parents and teachers. *Tob Control*. (2019) 28:610–6. doi: 10.1136/tobaccocontrol-2018-054455
- Cullen KA, Gentzke AS, Sawdey MD, Chang JT, Anic GM, Wang TW, et al. E-cigarette use among youth in the United States, 2019. *J Am Med Assoc*. (2019) 322:2095–103. doi: 10.1001/jama.2019.18387
- Roberts ME, Keller-Hamilton B, Ferketich AK, Berman ML. JUUL and the upsurge of e-cigarette use among college undergraduates. *J Am Coll Health*. (2020) 2020:1726355. doi: 10.1080/07448481.2020.1726355
- Vallone DM, Cuccia AF, Briggs J, Xiao H, Schillo BA, Hair EC. Electronic cigarette and JUUL use among adolescents and young adults. *J Am Med Assoc Pediatr*. (2020) 174:277–86. doi: 10.1001/jamapediatrics.2019.5436
- Ali FRM, Diaz MC, Vallone D, Tynan MA, Cordova J, Seaman EL, et al. E-cigarette unit sales, by product and flavor type—United States, 2014–2020. *Morb Mortality Weekly Rep*. (2020) 69:1313–8. doi: 10.15585/mmwr.mm6937e2
- Barrington-Trimis JL, Leventhal AM. Adolescents' use of "pod mod" e-cigarettes - urgent concerns. *N Engl J Med*. (2018) 379:1099–102. doi: 10.1056/NEJMp1805758
- Ickes M, Hester JW, Wiggins AT, Rayens MK, Hahn EJ, Kavuluru R. Prevalence and reasons for Juul use among college students. *J Am Coll Health*. (2020) 68:455–9. doi: 10.1080/07448481.2019.1577867
- Soulet S, Duquesne M, Toutain J, Paireud C, Lalo H. Experimental method of emission generation calibration based on reference liquids characterization. *Int J Environ Res Public Health*. (2019) 16:2262. doi: 10.3390/ijerph16132262
- Stefaniak AB, LeBouf RE, Ranpara A, Leonard SS. Toxicology of flavoring- and cannabis-containing e-liquids used in electronic delivery systems. *Pharmacol Ther*. (2021) 2021:107838. doi: 10.1016/j.pharmthera.2021.107838
- Talih S, Salman R, El-Hage R, Karam E, Karaoghlanian N, El-Hellani A, et al. Characteristics and toxicant emissions of JUUL electronic cigarettes. *Tob Control*. (2019) 28:678–80. doi: 10.1136/tobaccocontrol-2018-054616
- Talih S, Salman R, El-Hage R, Karam E, Salam S, Karaoghlanian N, et al. A comparison of the electrical characteristics, liquid composition, and toxicant emissions of JUUL USA and JUUL UK e-cigarettes. *Sci Rep*. (2020) 10:7322. doi: 10.1038/s41598-020-64414-5
- Varlet V, Farsalinos K, Augsburger A, Thomas A, Etter JF. Toxicity assessment of refill liquids for electronic cigarettes. *Int J Environ Res Public Health*. (2015) 12:4796–815. doi: 10.3390/ijerph120504796
- El-Hellani A, El-Hage R, Baalbaki R, Salman R, Talih S, Shihadeh A, et al. Free-base and protonated nicotine in electronic cigarette liquids and aerosols. *Chem Res Toxicol*. (2015) 28:1532–7. doi: 10.1021/acs.chemrestox.5b00107
- Gotts JE, Jordt SE, McConnell R, Tarran R. What are the respiratory effects of e-cigarettes? *BMJ*. (2019) 366:l5275. doi: 10.1136/bmj.l5275
- Harvanko AM, Havel CM, Jacob P, Benowitz NL. Characterization of nicotine salts in 23 electronic cigarette refill liquids. *Nicotine Tob Res*. (2020) 22:1239–43. doi: 10.1093/ntr/ntz232
- Jackler RK, Ramamurthi D. Nicotine arms race: JUUL and the high-nicotine product market. *Tob Control*. (2019) 28:623–8. doi: 10.1136/tobaccocontrol-2018-054796
- Duell AK, Pankow JF, Peyton DH. Free-base nicotine determination in electronic cigarette liquids by ¹H NMR spectroscopy. *Chem Res Toxicol*. (2018) 31:431–4. doi: 10.1021/acs.chemrestox.8b00097
- Omaiye EE, McWhirter KJ, Luo W, Pankow JF, Talbot P. Toxicity of JUUL fluids and aerosols correlates strongly with nicotine and some flavor chemical concentrations. *bioRxiv*. (2018) 2018:490607. doi: 10.1101/490607
- Reilly SM, Bitzer ZT, Goel R, Trushin N, Richie JP Jr. Free radical, carbonyl, and nicotine levels produced by JUUL electronic cigarettes. *Nicotine Tob Res*. (2019) 21:1274–8. doi: 10.1093/ntr/nty221
- Omaiye EE, McWhirter KJ, Luo W, Pankow JF, Talbot P. High-nicotine electronic cigarette products: toxicity of JUUL fluids and aerosols correlates strongly with nicotine and some flavor chemical concentrations. *Chem Res Toxicol*. (2019) 32:1058–69. doi: 10.1021/acs.chemrestox.8b00381
- Rao P, Liu J, Springer ML. JUUL and combusted cigarettes comparably impair endothelial function. *Tob Regul Sci*. (2020) 6:30–7. doi: 10.18001/TRS.6.1.4
- Chow AHL, Tong HHY, Chattopadhyay P, Shekunov BY. Particle engineering for pulmonary drug delivery. *Pharm Res*. (2007) 24:411–37. doi: 10.1007/s11095-006-9174-3
- Haughney J, Price D, Barnes NC, Virchow JC, Roche N, Chrystyn H. Choosing inhaler devices for people with asthma: current knowledge and outstanding research needs. *Respir Med*. (2010) 104:1237–45. doi: 10.1016/j.rmed.2010.04.012
- Lewis TR, Morrow PE, McClellan RO, Raabe OG, Kennedy GL, Schwetz BA, et al. Establishing aerosol exposure concentrations for inhalation toxicity studies. *Toxicol Appl Pharmacol*. (1989) 99:377–83. doi: 10.1016/0041-008X(89)90147-6
- Sosnowski TR, Kramek-Romanowska K. Predicted deposition of e-cigarette aerosol in the human lungs. *J Aerosol Med Pulm Drug Deliv*. (2016) 29:299–309. doi: 10.1089/jamp.2015.1268
- Organisation for Economic Co-operation and Development. *Environment, Health, and Safety Publications. Series on testing and assessment. No. 39 B. Draft guidance document on acute inhalation toxicity testing. Organisation for Economic Co-operation and Development*. Paris, France: OECD, Environment, Health and Safety Division. (2002). Available online at: <http://www.oecd.org/chemicalsafety/testing/2765785.pdf> (accessed March 2021).
- Pankow JF. Calculating compound dependent gas-droplet distributions in aerosols of propylene glycol and glycerol from electronic cigarettes. *J Aerosol Sci*. (2017) 107:9–13. doi: 10.1016/j.jaerosci.2017.02.003
- Pankow JF, Kim K, Luo W, McWhirter KJ. Gas/particle partitioning constants of nicotine, selected toxicants, and flavor chemicals in solutions of 50/50 propylene glycol/glycerol as used in electronic cigarettes. *Chem Res Toxicol*. (2018) 31:985–90. doi: 10.1021/acs.chemrestox.8b00178
- Sosnowski TR, Jablczynska K, Odziomek M, Schlage WK, Kuczaj AK. Physicochemical studies of direct interactions between lung surfactant and components of electronic cigarettes liquid mixtures. *Inhal Toxicol*. (2018) 30:159–68. doi: 10.1080/08958378.2018.1478916
- Oldham M, Zhang J, Rusyniak MJ, Kane DB, Gardner WP. Particle size distribution of selected electronic nicotine delivery system products. *Food Chem Toxicol*. (2018) 113:236–40. doi: 10.1016/j.fct.2018.01.045
- Zhao J, Zhang Y, Sisler JD, Shaffer J, Leonard SS, Morris AM, et al. Assessment of reactive oxygen species generated by electronic cigarettes using acellular and cellular approaches. *J Hazard Mater*. (2018) 344:549–57. doi: 10.1016/j.jhazmat.2017.10.057
- Fuoco FC, Buonanno G, Stabile L, Vigo P. Influential parameters on particle concentration and size distribution in the mainstream of e-cigarettes. *Environ Pollut*. (2014) 184:523–9. doi: 10.1016/j.envpol.2013.10.010
- Lechasseur A, Altmeld J, Surgeon N, Buonanno G, Morawska L, Brunet D, et al. Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by an electronic cigarette. *Physiol Rep*. (2019) 7:e14093. doi: 10.14814/phy2.14093

41. Mikheev VB, Ivanov A, Lucas EA, South PL, Colijn HO, Clark PI. Aerosol size distribution measurement of electronic cigarette emissions using combined differential mobility and inertial impaction methods: smoking machine and puff topography influence. *Aerosol Sci Technol.* (2018) 52:1233–48. doi: 10.1080/02786826.2018.1513636
42. Robinson RJ, Eddingsaas NC, DiFrancesco AG, Jayasekera S, Hensel EC Jr. A framework to investigate the impact of topography and product characteristics on electronic cigarette emissions. *PLoS ONE.* (2018) 13:e0206341. doi: 10.1371/journal.pone.0206341
43. Vargas-Rivera M, Ebrahimi Kalan M, Ward-Peterson M, Osibogun O, Li W, Brown D, et al. Effect of flavour manipulation on ENDS (JUUL) users' experiences, puffing behaviour and nicotine exposure among US college students. *Tob Control.* (2020) 30:55551. doi: 10.1136/tobaccocontrol-2019-055551
44. Son Y, Mainelis G, Delnevo C, Wackowski OA, Schwander S, Meng Q. Investigating E-cigarette particle emissions and human airway depositions under various E-cigarette-use conditions. *Chem Res Toxicol.* (2020) 33:343–52. doi: 10.1021/acs.chemrestox.9b00243
45. US Food and Drug Administration. *FDA Finalizes Enforcement Policy on Unauthorized Flavored Cartridge-Based E-Cigarettes That Appeal to Children, Including Fruit and Mint.* Washington, DC: U.S. Food and Drug Administration. (2020). Available online at: <https://www.fda.gov/news-events/press-announcements/fda-finalizes-enforcement-policy-unauthorized-flavored-cartridge-based-e-cigarettes-appeal-children/> (accessed September, 2020).
46. Noël JC, Rainer D, Gstir R, Rainer M, Bonn G. Quantification of selected aroma compounds in e-cigarette products and toxicity evaluation in HUVEC/Tert2 cells. *Biomed Chromatogr.* (2020) 34:e4761. doi: 10.1002/bmc.4761
47. Talih S, Balhas ZM, Eissenberg T, Salman R, Karaoghlanian N, El Hellani A, et al. Effects of user puff topography, device voltage, and liquid nicotine concentration on electronic cigarette nicotine yield: measurements and Model Predictions. *Nicotine Tob Res.* (2014) 17:150–7. doi: 10.1093/ntr/ntu174
48. Li Y, Cui H, Wang X, Si X, Fan M, Chen L, et al. Effects of puffing parameters on physical properties and deposition in human respiratory tract of e-cigarette aerosols. *Tob Sci Technol.* (2020) 53:32–38. doi: 10.16135/j.issn1002-0861.2019.0529
49. Farsalinos KE, Gillman G, Poulas K, Voudris V. Tobacco-specific nitrosamines in electronic cigarettes: comparison between liquid and aerosol levels. *Int J Environ Res Public Health.* (2015) 12:9046–53. doi: 10.3390/ijerph120809046
50. Cooperation Centre for Scientific Research Relative to Tobacco. *CORESTA recommended method N° 81. CORESTA E-Cigarette Task Force Technical Report.* (2015). Available online at: https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf (accessed August, 2020).
51. Ranpara A, Stefaniak AB, Williams K, Fernandez E, LeBouf RF. Modeled respiratory tract deposition of aerosolized oil diluents used in 19-THC-based electronic cigarette liquid products. *Front Public Health.* (2021) 9:744166. doi: 10.3389/fpubh.2021.744166
52. O'Shaughnessy PT, Raabe OG. A comparison of cascade impactor data reduction methods. *Aerosol Sci Technol.* (2003) 37:187–200. doi: 10.1080/02786820300956
53. Yeh HC, Schum GM. Models of human lung airways and their application to inhaled particle deposition. *Bull Math Biol.* (1980) 42:461–80. doi: 10.1016/S0092-8240(80)80060-7
54. Asgharian B, Hofmann W, Bergmann R. Particle deposition in a multiple-path model of the human lung. *Aerosol Sci Technol.* (2001) 34:332–9. doi: 10.1080/02786820119122
55. Manigrasso M, Buonanno G, Stabile L, Morawska L, Avino P. Particle doses in the pulmonary lobes of electronic and conventional cigarette users. *Environ Pollut.* (2015) 202:24–31. doi: 10.1016/j.envpol.2015.03.008
56. Centers for Disease Control and Prevention. *E-cigarettes Shaped like USB Flash Drives: Information for Parents, Educators and Health Care Providers.* (2020). Available online at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/factsheet/pdfs/e-cigarettes-usb-flash-p.pdf (accessed September, 2021).
57. Lanza HI, Leventhal AM, Cho J, Braymiller JL, Krueger EA, McConnell R, et al. Young adult e-cigarette use: a latent class analysis of device and flavor use, 2018–2019. *Drug Alcohol Depend.* (2020) 216:108258. doi: 10.1016/j.drugalcdep.2020.108258
58. Miech R, Leventhal A, Johnston L, O'Malley PM, Patrick ME, Barrington-Trimis J. Trends in use and perceptions of nicotine vaping among US youth from 2017 to 2020. *J Am Med Assoc Pediatr.* (2021) 175:185–90. doi: 10.1001/jamapediatrics.2020.5667
59. Romberg AR, Diaz MC, Briggs J, Stephens DK, Rahman B, Graham AL, et al. Vaping in the workplace prevalence and attitudes among employed US adults. *JOEM.* (2021) 63:2061. doi: 10.1097/JOM.0000000000002061
60. Chen R, Aherrera A, Isichei C, Olmedo P, Jarmul S, Cohen JE, et al. Assessment of indoor air quality at an electronic cigarette (Vaping) convention. *J Expo Sci Environ Epidemiol.* (2018) 28:522–9. doi: 10.1038/s41370-017-0005-x
61. Kim JJ, Sabatelli N, Tutak W, Giuseppetti A, Frukhtbeyn S, Shaffer I, et al. Universal electronic-cigarette test: physiochemical characterization of reference e-liquid. *Tob Induc Dis.* (2017) 15:119. doi: 10.1186/s12971-017-0119-x
62. Farsalinos KE, Romagna G, Allifranchini E, Ripamonti E, Bocchietto E, Todeschi S, et al. Comparison of the cytotoxic potential of cigarette smoke and electronic cigarette vapour extract on cultured myocardial cells. *Int J Environ Res Public Health.* (2013) 10:5146–62. doi: 10.3390/ijerph10105146
63. Farsalinos KE, Romagna G, Tsiapras D, Kyrzopoulos S, Voudris V. Evaluation of electronic cigarette use (vaping) topography and estimation of liquid consumption: implications for research protocol standards definition and for public health authorities' regulation. *Int J Environ Res Public Health.* (2013) 10:2500–14. doi: 10.3390/ijerph10062500
64. Larcombe AN, Janka MA, Mullins BJ, Berry LJ, Bredin A, Franklin PJ. The effects of electronic cigarette aerosol exposure on inflammation and lung function in mice. *Am J Physiol Lung Cell Mol Physiol.* (2017) 313:L67–79. doi: 10.1152/ajplung.00203.2016
65. Laube BL, Afshar-Mohajer N, Koehler K, Chen G, Lazarus P, Collaco JM, et al. Acute and chronic *in vivo* effects of exposure to nicotine and propylene glycol from an E-cigarette on mucociliary clearance in a murine model. *Inhal Toxicol.* (2017) 29:197–205. doi: 10.1080/08958378.2017.1336585
66. Floyd EL, Queimado L, Wang J, Regens JL, Johnson DL. Electronic cigarette power affects count concentration and particle size distribution of vaping aerosol. *PLOS ONE.* (2018) 2018:210147. doi: 10.1371/journal.pone.0210147
67. Raabe OG, Yeh HC, Newton GJ, Phalen RF, Velasquez DF. Deposition of inhaled monodisperse aerosols in small rodents. In: Walton WH, editor, *Inhaled Particles, Vol. IV. Part I:1-2 I.* Oxford: Pergamon (1975). Available online at: <https://pubmed.ncbi.nlm.nih.gov/1236165/> (accessed August, 2017).
68. Bennett WD, Messina MS, Smaldone GC. Effect of exercise on deposition and subsequent retention of inhaled particles. *J Appl Physiol.* (1985) 59:1046–54. doi: 10.1152/jappl.1985.59.4.1046
69. Chang YH, Yu CP. A model of ventilation distribution in the human lung. *Aerosol Sci Technol.* (1999) 30:309–19. doi: 10.1080/027868299304660
70. Gerrity TR, Garrard CS, Yeates DB. Theoretic analysis of sites of aerosol deposition in the human lung. *Chest.* (1981) 80:898–901. doi: 10.1378/chest.80.6.898
71. Subramaniam RP, Asgharian B, Freijer JJ, Miller FJ, Anjilvel S. Analysis of lobar differences in particle deposition in the human lung. *Inhal Toxicol.* (2008) 15:1–21. doi: 10.1080/089583703004451
72. Dai H, Hao J. Online popularity of JUUL and Puff Bars in the USA: 2019–2020. *Tob Control.* (2020) 2020:55727. doi: 10.1136/tobaccocontrol-2020-055727
73. Delnevo C, Giovenco DP, Hrywna M. Rapid proliferation of illegal pod-mod disposable e-cigarettes. *Tob Control.* (2020) 29:e150–1. doi: 10.1136/tobaccocontrol-2019-055485
74. LeBouf RF, Burns DA, Ranpara A, Attfield K, Zwack L, Stefaniak AB, et al. Headspace analysis for screening of volatile organic compound profiles of electronic juice bulk material. *Anal Bioanal Chem.* (2018) 410:5951–60. doi: 10.1007/s00216-018-1215-3
75. Cwalina SN, Leventhal AM, Barrington-Trimis JL. E-cigarette flavour enhancers: flavoured pod attachments compatible with JUUL and other pod-based devices. *Tob Control.* (2020) 29:e127–8. doi: 10.1136/tobaccocontrol-2020-055618

76. Lamb T, Muthumalage T, Rahman I. Pod-based menthol and tobacco flavored e-cigarettes cause mitochondrial dysfunction in lung epithelial cells. *Toxicol Lett.* (2020) 333:303–11. doi: 10.1016/j.toxlet.2020.08.003
77. Wang M, Ho S, Leung L, Lam T. Electronic cigarette use and respiratory symptoms in chinese adolescents in hong kong. *J Am Med Assoc Pediatr.* (2016) 170:89–91. doi: 10.1001/jamapediatrics.2015.3024
78. Cho J, Paik S. Association between electronic cigarette use and asthma among high school students in south korea. *PLoS ONE.* (2016) 11:0151022. doi: 10.1371/journal.pone.0151022
79. Hwang JH, Lyes M, Sladewski K, Enany S, McEachern E, Mathew DP, et al. Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria. *J Mol Med.* (2016) 94:667–79. doi: 10.1007/s00109-016-1378-3
80. National Academies of Sciences, Engineering, and Medicine. *Public Health Consequences of e-cigarettes.* Washington, DC: National Academies Press (2018).
81. Yuan M, Cross S, Loughlin S, Leslie F. Nicotine and the adolescent brain. *J Physiol.* (2015) 593:3397–412. doi: 10.1113/JP270492
82. Squeglia LM, Gray KM. Alcohol and drug use and the developing brain. *Curr Psychiatry Rep.* (2016) 18:46. doi: 10.1007/s11920-016-0689-y
83. Pearce K, Gray N, Gaur P, Jeon J, Suarez A, Shannahan J, et al. Toxicological analysis of aerosols derived from three electronic nicotine delivery systems using normal human bronchial epithelial cells. *Toxicol in vitro.* (2020) 69:104997. doi: 10.1016/j.tiv.2020.104997
84. National Institute on Drug Abuse. *NIDA Drug Supply Program.* (2017). Available online at: <https://www.drugabuse.gov/research/research-data-measures-resources/nida-drug-supply-program/> (accessed July, 2020).
85. Mulder HA, Patterson JL, Halquist MS, Kosmider L, Turner JB, Poklis JL, et al. The effect of electronic cigarette user modifications and e-liquid adulteration on the particle size profile of an aerosolized product. *Sci Rep.* (2019) 9:10221. doi: 10.1038/s41598-019-46387-2
86. Carvalho TC, Peters JI, Williams RO. Influence of particle size on regional lung deposition – what evidence is there? *Int J Pharm.* (2011) 406:1–10. doi: 10.1016/j.ijpharm.2010.12.040
87. Protano C, Avino P, Manigrasso M, Vivaldi V, Perna F, Valeriani F, et al. Environmental electronic vape exposure from four different generations of electronic cigarettes: airborne particulate matter levels. *Int J Environ Res Public Health.* (2018) 15:2172. doi: 10.3390/ijerph15102172

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Vitamin E Acetate Determination in Vaping Liquids and Non-targeted Analysis of Vaping Emissions of Diluents of Concern, Vitamin E Acetate and Medium-Chain Triglycerides Oil

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During the summer of 2019, cases of lung injury associated with vaping emerged in North America, including among individuals who reported exclusive use of nicotine vaping liquids. Once vitamin E acetate was identified as a potential causative agent a quantitative method based on a simple sample dilution, separation by gas chromatography and analysis by triple quadrupole mass spectrometry (GC MSMS) was developed. Method detection limit (MDL) and limit of quantification (LOQ) were determined at 0.159 µg/mL and 0.505 µg/mL, respectively. The analysis was performed on a subset of 203 commercially sourced nicotine containing vaping liquids of various flavour profile and nicotine range (nicotine free-59 mg/mL) from an internal inventory. The target analyte, Vitamin E Acetate, was not detected in any samples analyzed, as expected, given the reported detection in literature and high association of the chemical with cannabis and not nicotine containing vaping products.

Keywords: vaping, vitamin E acetate, medium chain triglycerides, aerosol, nicotine, gc ms, emission, cannabis

Emissions profiles of vitamin E acetate and Medium Chain Triglycerides (MCT) oil were also characterized via non-targeted analysis, using GC-MS/MS, to identify potential chemical analytes generated when liquids were heated. The emissions were generated under standardized conditions using a vaping machine and a vaporizer device designed for use with cannabis concentrates. Vitamin E acetate emissions mainly contained target analyte itself, while MCT oil emissions were more chemically complex, as expected due to the presence of various triglycerides in the un-vaped oil. Of note even following rigorous cleaning procedures of the device and contact parts with a number of chemical solvents and replacement of consumable parts between MCT oil and vitamin E acetate vaping sessions, cross-contamination was observed. This cross contamination and residue persistence, is indicative of a real-life scenario where users may share or use the same device with a number of different products or formulations and not be aware of potential exposures to diluents of concern.

INTRODUCTION

E-cigarettes or vaping products are battery-powered, alternative nicotine delivery systems that vaporize a formulation of vaping liquid or e-liquid. The liquids most commonly contain carrier

solvents, propylene glycol (PG) and/or vegetable glycerine (VG), nicotine and flavours. Once heated, the formulation generates additional chemical compounds in the emissions, resulting in a complex exposure pattern to users of these products. Nevertheless, in spite of potential health risks, vaping provides nicotine delivery with lower exposures to chemicals of concern for the individuals who smoke (Goniewicz et al., 2018). One of the main appealing characteristics of vaping products is the fact that they come in thousands of flavours and in various nicotine concentrations. Although some vaping products are available in a more closed, pod format, many products and flavour choices are available in open format. Users of open systems can refill or customize vaping liquids to be used, the most frequently, with a modifiable, custom power setting, vaping device. The vaping or heating principle is also used for delivery of other substances such as those containing cannabidiol (CBD) and tetrahydrocannabinol (THC) cannabis vaping products.

Vitamin E acetate or alpha tocopherol acetate is the stabilized form of vitamin E often used as food additive or nutrient supplement (National Library of Medicine 2021c). Medium chain triglycerides and vitamin E acetate are, reportedly, added to the vaping products in order to dilute the illicit THC-containing liquids and lower their costs. Moreover, diluents are also used in order to improve products' appearance and flavour (Blount et al., 2020). Although ingestion of vitamin E acetate is thought to have nutritional and health benefits, the inhalation exposure studies in animals suggest a severe lung damage and impaired function (Bhat et al., 2020). Moreover, the recent studies on various diluents (coconut oil, medium-chain triglycerides, Vitamin E Acetate, etc) in *in-vitro* models have observed an increased cytotoxicity in human airway epithelial cells (Jiang et al., 2020) however, their effects in humans are still being investigated.

During the summer of 2019, cases of lung injury associated with vaping (vaping-associated lung illness (VALI (Canada)) or e-cigarette associated lung injury [EVALI (United States)] emerged in North America. In the course of investigation, illegal cannabis vaping products containing vitamin E acetate were found to be strongly associated and thought to be the most likely cause of the outbreak in the majority of cases in the United States (CDC 2020), as observed through laboratory analysis of patients' biological and illicit THC product samples (Blount et al., 2020). MCT oil, to date, has not been reported as a cause of related adverse occurrence.

In 2019, when vitamin E acetate from the illicit products was identified as a potential causative agent of EVALI in the United States, new analytical method to quantify this target analyte in the Canadian vaping samples was developed. At the time, cannabis vaping products were illegal in Canada, so the analysis was carried out on the nicotine-containing products. Moreover, since the data on chemical transformations and constituents of emissions generated from heating of the pure vitamin E acetate were lacking, we characterized the same and identified chemicals relevant for the inhalation exposures. In addition to Vitamin E Acetate emission, MCT oil emission

was characterized as well, and the main constituents are reported here.

MATERIALS AND METHODS

Chemicals and Reagents

99.7% pure propylene glycol and 99.2% pure glycerol were purchased from Sigma-Aldrich (Oakville, Canada). HPLC grade methanol, acetonitrile and hexane were purchased from Fisher Scientific (Ottawa, Canada). Vitamin E acetate (D, L- α -Tocopherol acetate) ($\geq 99\%$) analytical standard was purchased from Sigma-Aldrich (Oakville, Canada).

Samples

Two hundred and three vaping liquids from over 70 manufacturers were collected between 2017 and 2019 from online and brick and mortar stores in Canada. These products were chosen at random from a larger pool of samples collected for a project on characterization of nicotine vaping liquids. The on-product labelled nicotine concentration varied between nicotine-free and 59 mg/mL (**Supplementary Figure S1**) and 20% of products were labelled to contain nicotine salts.

A majority of samples were packaged in glass or plastic 30 mL, refillable bottles and each one was classified into one of 18 flavour categories as per manufacturer's declared flavour or based on product descriptions (**Supplementary Figure S2**). Flavour classification wheel was adapted for vaping products on Canadian market and based on previously reported approach (Krüsemann et al., 2019).

Medium Chain Triglyceride (MCT) oil from coconut oil (60% caprylic acid (C8)/40% capric acid (C10)-St. Francis, Herb Farm) was obtained from a local health food store in Ottawa, Canada. Vaping device used to generate emissions was Pax 3 (PAX, Labs) dual-use vaporizer device with concentrate insert consisting of aluminum oven purchased in vaping store in Ottawa, Canada.

Chemical Analysis

Two analytical methods were developed one for targeted analysis and determination of concentration of vitamin E acetate in vaping liquids, and another one for characterization of emissions generated from MCT oil and vitamin E acetate.

Targeted Analysis of Vaping Liquids

The sample preparation was a simple dilution in methanol. Following a thorough sample mixing, 40 mg of sample measured to the nearest 0.1 mg was diluted in a 20 mL volumetric flask and made up to volume with methanol. Upon dilution and vortex mixing, 1 μ L of diluted sample was injected and analyzed using Thermo Ultra Trace GC (gas chromatograph) coupled to Quantum TSQ tandem mass spectrometer (MS/MS) (Thermo Elec. Mississauga, Canada). The chromatographic separation was achieved on a Zebtron ZB-1HT GC capillary column (15 $\times$ 0.25 mm $\times$ 0.1 μ m) from Phenomenex (CA, United States). The injector temperature was set at 280°C and injection mode used was splitless. The GC oven temperature program was as follows: 120–200°C at 20°C/min, followed by

10°C/min to 290°C and holding for 1 min, and reaching 320°C at 20°C/min and finally holding for 3 min. Helium was the carrier gas at a flow rate of 1 mL/min in constant flow mode. The source and GC interface temperatures were set at 180 and 250°C, respectively. The mass spectrometer was operated in electron ionization at 70 eV in multiple-reaction monitoring (MRM) mode using argon as a collision gas. Xcalibur data system was used for data acquisition and processing. The following MRM transitions were optimized for vitamin E acetate quantifier ion: 430.3 → 165.05 with collision energy of 20 eV and qualifier ion: 472.1 → 430.75 with collision energy of 10 eV. The calibration was achieved using the standard addition method. Each individual calibration standard level was prepared using the aliquots of diluted sample and fortifying with target analyte, Vitamin E acetate. The calibration curve was linear in the concentration range 0.025–12.5 µg/mL.

Method Performance and Validation

Method performance was assessed according to the EPA Regulation 40 CFR part 136 (Appendix B) method (USEPA 2011). Eight replicates of vaping sample with no detectable levels of vitamin E acetate were fortified with vitamin E acetate at 0.50 µg/mL, diluted with methanol and analyzed. The standard deviation associated with eight replicate analyses of fortified vaping liquid, was multiplied by the Student's *t* value of 2.998 (appropriate for a 99% confidence level with 7 degrees of freedom). The method detection limit (MDL) for vitamin E acetate was calculated to be 0.159 µg/mL or 0.18 µg/g. The limit of quantitation (LOQ) was calculated according to the US EPA method, where the standard deviation associated with the eight replicate analyses of fortified vaping liquid was multiplied by a factor of 10. The LOQ was calculated to be 0.505 µg/mL.

Laboratory blanks consisting of methanol and vaping liquid containing pG/VG (50/50 v/v) prepared in laboratory were used to assess carry-over between injections and any possible contamination. Since the analytical methodology did not employ the use of internal standard nor extraction sample clean up, the measures of analyte recovery were irrelevant. However, using the standard addition calibration and quality control sample fortified at 0.50 µg/mL, processed as eight repeats on a single day and as a repeated QC sample in 4 analysed sample batches, accuracy and precision (%RSD) were determined as 6.9 and 18.1%, respectively.

Characterization of Vitamin E Acetate and Medium-Chain Triglycerides Oil, and Vaping Emissions

Emission Generation

Approximately 150 mg of vitamin E acetate or MCT oil were placed in the aluminum, concentrate, oven insert of a Pax 3 device. The mouthpiece was connected to a CETI 8 vaping machine (Cerulean, United States) and the Pax 3 was set to 215°C, (maximum device temperature setting). Generation of vaped emissions was achieved using the ISO 20768 puffing regimen of 55 ml square wave puff, over 3 s, once every 30 s (ISO 2018). Ten puffs of each vitamin E acetate or MCT oil were

collected on solvent pre-cleaned collection pads. Device weight was recorded prior and after vaping. Collection pads were extracted twice with 5 mL of methanol. Extracts were combined and 1 µL of extract was injected and analyzed using GC MS.

Analysis of Diluents of Concern and Their Emissions

The Quantum TSQ MS/MS instrument coupled to a Trace GC Ultra gas chromatograph (Thermo Electron Corp.) was operated in a full-scan acquisition mode. The oven ramp programming was identical to the one used for targeted analysis of Vitamin E Acetate. The MS was operated in Electron Ionization, full-scan mode with scan range 35–600 *m/z* and emission current set at 100 µA. GC separation was performed using Zebron ZB-1HT GC capillary column (15 × 0.25 mm × 0.1 µm) from Phenomenex (CA, United States). The injector temperature was set at 280°C with splitless injection mode. GC carrier gas was helium operated in constant flow mode at 1 mL/min rate. The spectrum of detected compounds was matched against spectra from the National Institute of Standards and Technology (NIST 17) library reference peaks.

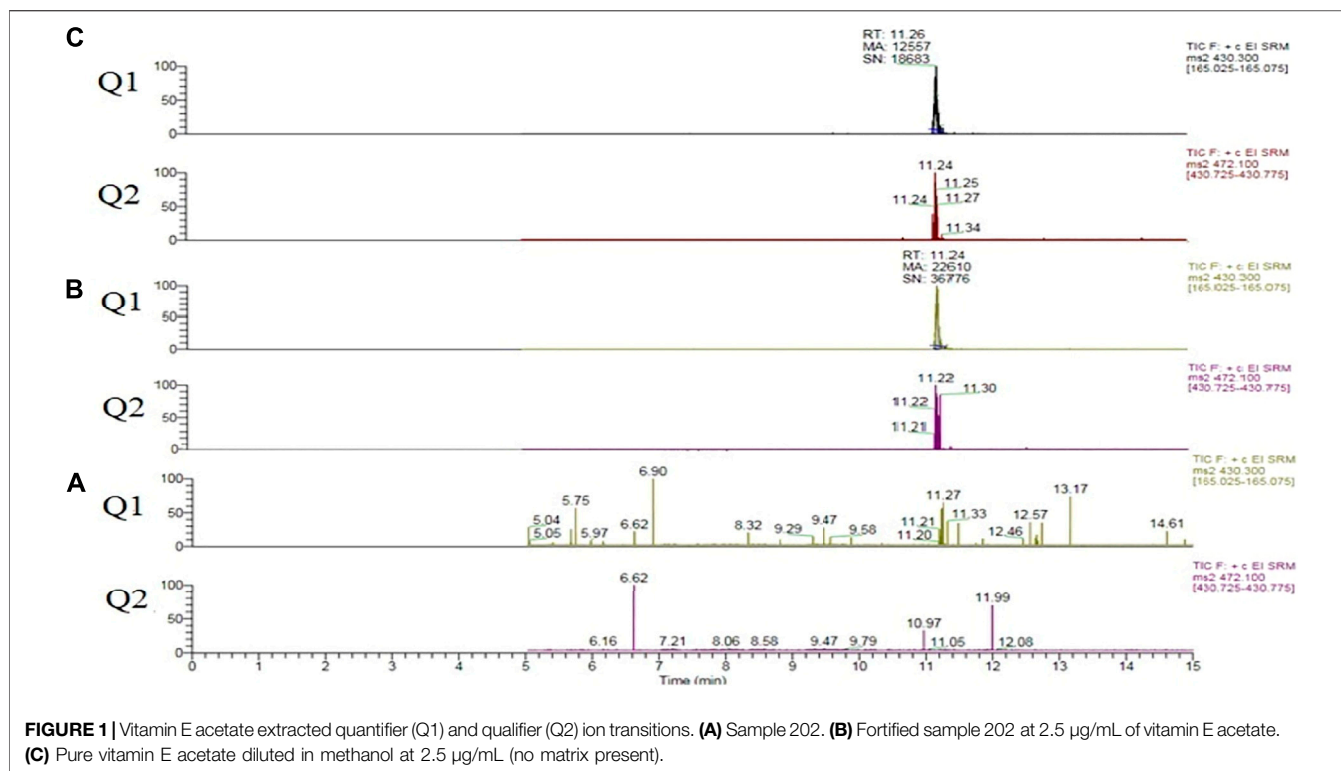
RESULTS AND DISCUSSION

Vitamin E Acetate in Nicotine-Containing Vaping Liquids

During the development of the targeted method matrix effects were observed due to the presence of carrier solvents PG and VG, **Figure 1**.

In order to account for matrix effects the six level calibration was performed using the standard addition method, where diluted samples were fortified with known concentrations of Vitamin E Acetate. The analytical blank samples revealed no background contamination or in-between sample carry-over.

During the early reporting of EVALI, it was not clear which vaping product's (nicotine or cannabis) constituents were related to the cases of the outbreak. Since the cannabis vaping products only became legal for sale in Canada in late 2019 (Government of Canada 2019) and would only have entered the marketplace towards mid-to end of December 2019 or early 2020, we screened the legal nicotine-containing products. The analytical method was applied to 203 nicotine-containing vaping liquids in order to quantify Vitamin E Acetate. The target analyte was not detected above limit of detection (0.159 µg/mL) in any products analyzed. To date and to our knowledge no nicotine-vaping liquids have been reported to contain Vitamin E Acetate. In fact nicotine-containing vaping products' ingredient reports from cartridges and refill containers (41,809), were assessed for the presence of vitamin E acetate and THC by United Kingdom Centre for Tobacco and Alcohol Studies (Nyakutsikwa et al., 2020). Although no actual chemical analysis was conducted, the assessment of reported ingredients had found no Vitamin E Acetate and THC present. The chemical matrix of cannabis vaping liquids differs from that of nicotine containing vaping products due to distinct chemical properties of active ingredients added to the products. Cannabinoids are hydrophobic and



typically diluted with oils or other, more hydrophobic diluents, while nicotine is diluted in more polar, alcohol-based diluents. Nicotine formulations in fact, often contain water (Crenshaw et al., 2016). While vitamin E acetate is a suspected cause of majority of EVALI cases in United States, more than half of cases reported in Canada appear to be associated with nicotine-containing vaping liquids (Government Of Canada 2020). Up to the August of 2020, 20 cases of VALI were reported in Canada, with 11 cases associated with nicotine containing vaping liquids only, however this is based on self-reporting and is not laboratory verified through testing of patients' biological samples for presence of active substances (THC, nicotine, etc) or substances of concern (Vitamin E Acetate). The causes of these cases are an area of continued monitoring.

Reported analytical methods for the targeted analysis or quantification of vitamin E Acetate in vaping liquids and generated aerosols are limited, particularly for nicotine-containing products. In the VALI cases, mainly in the illicit cannabis vaping products, Vitamin E Acetate was quantified in relatively high concentrations since it was used as a diluent (Duffy et al., 2020). In analyzing such samples, analytical method's detection sensitivity may not be of a concern; however, to date, toxicologically relevant concentrations of Vitamin E Acetate for inhalation exposures remain unknown; therefore, analytical methods that can detect the low levels of Vitamin E Acetate may be of interest as well. In a study by German federal institute for risk assessment (BfR), LC-MS/MS based method was developed to detect Vitamin E and Vitamin E Acetate in e-liquids with limit of detection for latter of 0.3 ng per Gram of e-liquid (The German Federal Institute for Risk Assessment (BfR) 2020).

Although the method is more sensitive than method presented here (MDL = 18 ng per Gram of liquid), BfR method requires sample mass of 500 mg for analysis compared to 40 mg required in our methodology. Developing the methods that use small sample volume or weight is advantageous as often times, in the real cases of EVALI, forensic labs will only have a residue of vaping liquid, i.e. what remains in the device to be used for testing and not necessarily significant amounts of the product left. In other studies from the United States, LC-MS/MS methods were developed for analysis of Vitamin E Acetate in vaping products that contain tetrahydrocannabinol (THC) acquired from informal sources from Minnesota (Taylor et al., 2019) and in trapped aerosol emissions from e-cigarette, or vaping products associated with EVALI cases in United States (Puetz et al., 2021). Vitamin E acetate was also detected in THC cartridges and in bronchoalveolar lavage fluid from EVALI patients in Wisconsin United States (Pray et al., 2020). THC/VEA mixtures were vaped at elevated power levels in vaping device to study the VEA decomposition by GC/MS in Forensic Chemistry Center, United States (Lynch et al., 2021). Most of the reported studies lack detailed information on the method detection limits, validation data, sample preparation, and sample size.

Medium Chain Triglyceride Oil

The saturated fatty acids of mixed triacylglycerols with 6–10 carbons chains are collectively referred to as medium chain triglyceride oil. MCT oil is included in a generally recognized as safe (GRAS) substances for human consumption (Traul et al., 2000). They are sourced from plant oil sources such as coconut

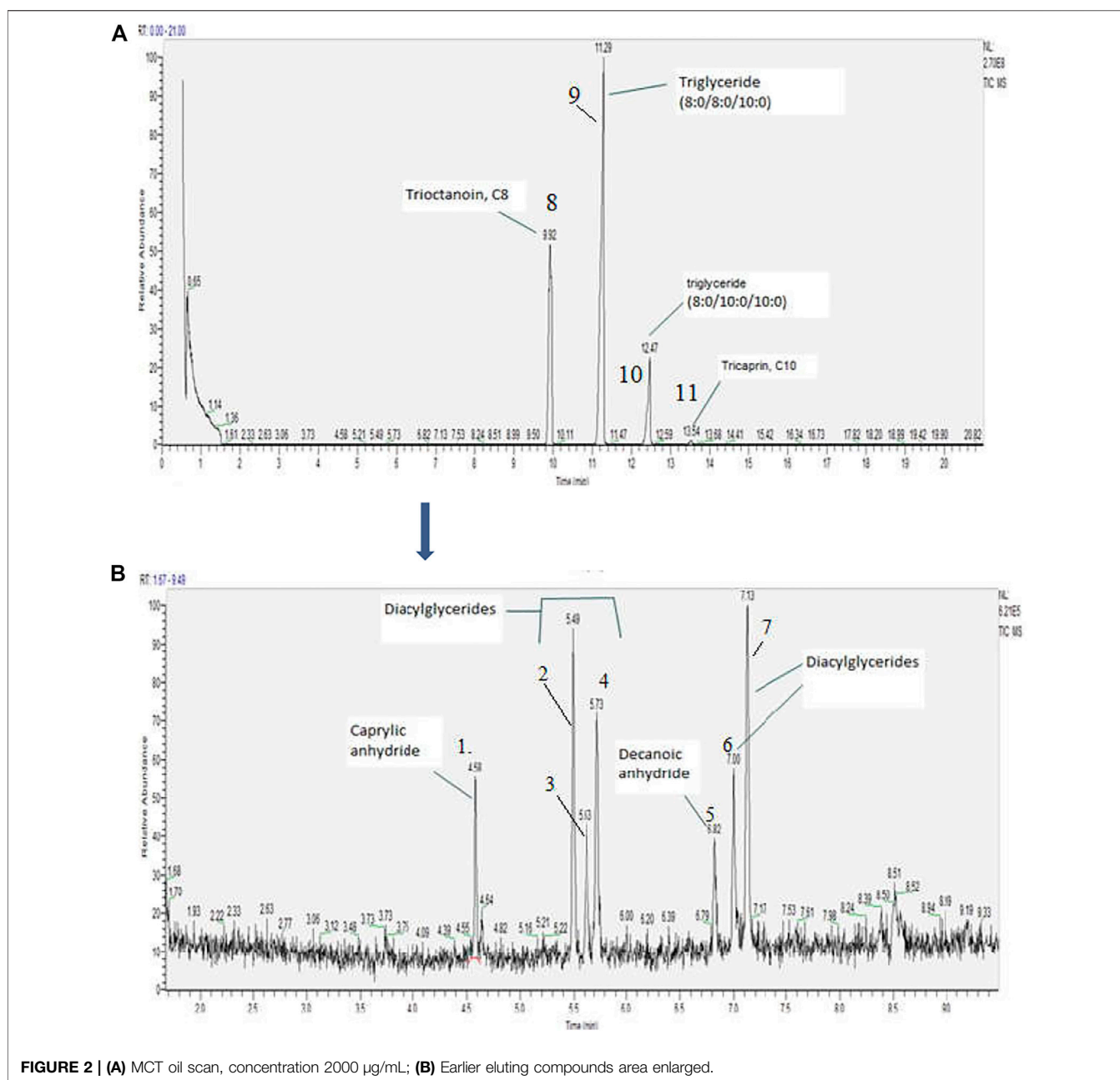


FIGURE 2 | (A) MCT oil scan, concentration 2000 µg/mL; **(B)** Earlier eluting compounds area enlarged.

and palm oils. This study used MCT oil from coconut which according to product label, was comprised of 60% caprylic acid (C8) and 40% capric acid (C10) triglycerides. Prior to heating and emission generation, chemical characterization of this oil was conducted to better understand the starting composition. Four major mixed triacylglyceride constituents were detected (Trioctanoin (8:0/8:0/8:0), glyceryl 2-caprate dicaprylate (8:0/8:0/10:0); glyceryl 2-caprylate dicaprate (10:0/10:0/8:0) and tricaprin (10:0/10:0/10:0), **Figure 2A**). In addition to major constituents, earlier eluting chemical compounds included two anhydrides caprylic and decanoic, as well as octanoic acid, 2-propenyl ester (imitation fatty, fruity,

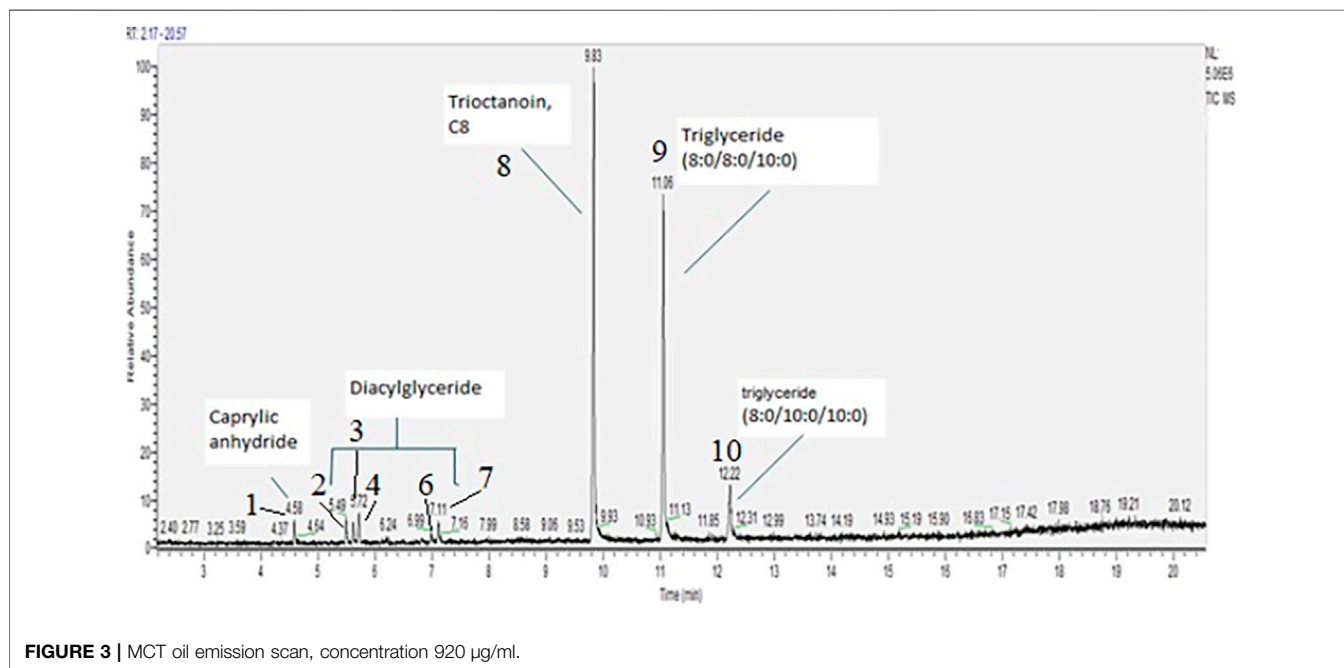
pineapple flavour agent) (The Good Scents Company 2021b) and four diacylglycerides, **Figure 2B**).

Table 1 contains detailed information on detected chemical compounds. Open source databases, Pubchem (National Library of Medicine 2021b), NIST Chemistry WebBook ((NIST) 2021) and The Good Scents Company Information System (The Good Scents Company 2021a) were used to extract more information about and typical use for detected chemicals.

Generated emissions of heated MCT oil in Pax 3 device were captured, extracted in methanol and analyzed (**Figure 3**). The analysis of ten puffs revealed presence of nine of eleven chemical compounds detected in the unvaped MCT oil.

TABLE 1 | Chemical compounds detected in MCT oil (Refer to **Figures 2, 3**).

Peak	IUPAC Name	Common Name	CASRN	More information
1	Octanoyl octanoate	n-Caprylic Anhydride	623-66-5	Listed in cosmetic products patents
2	Prop-2-enyl octanoate	Octanoic acid, 2-propenyl ester	4230-97-1	Flavour and fragrance agent; Fatty, fruity, pineapple tropical-like flavour
3	[(2S)-3-hydroxy-2-octanoyloxypropyl] octanoate	Octanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester, (S)-	60514-48-9	Common food additives used to blend together certain ingredients, such as oil and water, which would not otherwise blend well. Diacylglycerols are often found in bakery products, beverages, ice cream, chewing gum, shortening, whipped toppings, margarine, and confections
4	(2-hydroxy-3-octanoyloxypropyl) octanoate	1,3-Dioctanoin	1429-66-9	Metabolite of rapeseed
5	Decanoyl decanoate	Decanoic anhydride	2082-76-0	—
6	(1-hydroxy-3-octanoyloxypropan-2-yl) decanoate	Decanoic acid, 1-(hydroxymethyl)-2-[(1-oxooctyl)oxy]ethyl ester	177717-46-3	Patents list personal care product and emulsion formulations use
7	(2-hydroxy-3-octanoyloxypropyl) decanoate	Glyceryl 1-caprate-3-caprylate	93980-84-8	Patents list use in surfactants
8	2,3-di(octanoyloxy)propyl octanoate	Trioctanoin	538-23-8	Has a role as a plant metabolite. Used as diluent, as carrier for flavours and essential oils
9	1,3-di(octanoyloxy)propan-2-yl decanoate	Glyceryl 2-caprate dicaprylate	33368-87-5	Triglyceride 8:0/8:0/10:0, patents list use in printing inks and varnishes
10	(3-decanoyloxy-2-octanoyloxypropyl) decanoate	Glyceryl 2-caprylate dicaprate	33368-86-4	Triglyceride 8:0/10:0/10:0, patents list use in printing inks and varnishes
11	2,3-di(decanoyloxy)propyl decanoate	Tricaprin	621-71-6	Precursor of decanoic acid (DA), a 10-carbon fatty acid (Triglyceride 10:0/10:0/10:0) and major component of medium chain triglyceride oils. Used in dietary and cosmetic products as emollient and solvent



Two chemicals that were not detected in the emissions were tricaprins (10:0/10:0/10:0 triglyceride), and decanoic anhydride. Comparing the chemical profile of unvaped MCT oil and compounds generated in the emissions it is evident that the absolute ratio of triglycerides changes, presumably due to the transformation during heating process or perhaps due to the

varying degrees of transfer from oil into emissions given the differences in boiling points among the triglycerides. The exact concentrations of each generated chemical were not quantified as calibration and genuine analytical standards were not used. Instead, the overall concentration of MCT oil mixture was estimated using the mass loss before and after vaping and by weighing the collection pad.

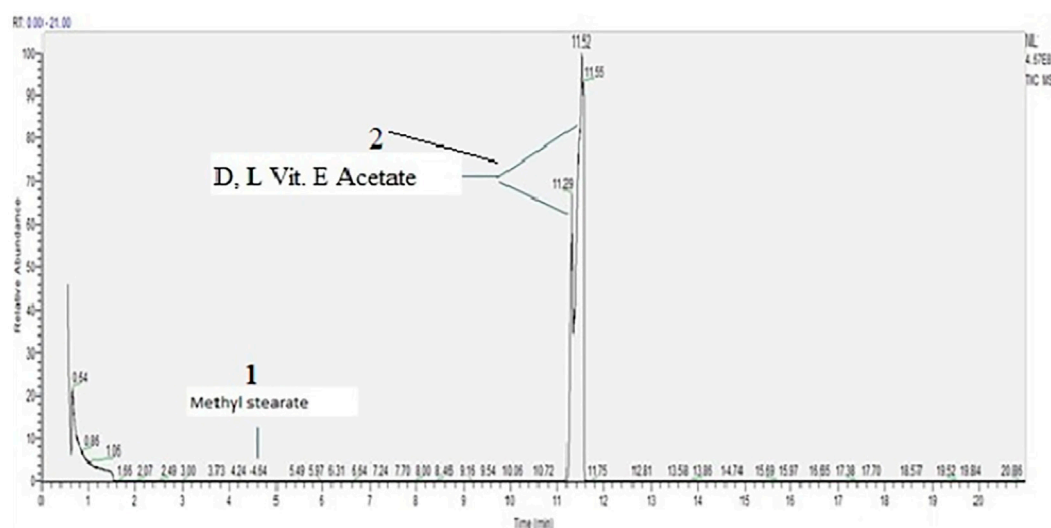


FIGURE 4 | D, L Vitamin E acetate scan, concentration 2000 µg/ml.

TABLE 2 | Chemical compounds detected in Vitamin E Acetate (Figure 4).

Peak	IUPAC Name	Common Name	CASRN	More information
1	Methyl octadecanoate	Methyl stearate	112-61-8	Found in cloves. Used as cosmetics and fragrance agent. Plant metabolite
2	[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydrochromen-6-yl] acetate	D,L Vitamin E acetate or (±)-α-Tocopherol acetate	7695-91-2	Synthetic D,L form. Antioxidant, nutritional supplement and preservative

Thinning agents such as vegetable glycerine, propylene glycol or poly ethylene glycol are used as diluents of cannabis vaping oils (Erickson 2019; Jiang et al., 2020). MCT oils have also been reported to be used in some cannabis products, however, to date and in comparison with Vitamin E Acetate, MCT oil has been less frequently detected in the samples associated with cases of EVALI in the United States (Blount et al., 2020).

Vitamin E Acetate

The same procedure was followed with vitamin E acetate as with MCT oil. Vitamin E acetate was diluted in methanol and analyzed using the non-targeted or scanning protocol. This substance was of a high grade and purity as confirmed by the analysis where two isomers, dexter (D) and laevus (L) were detected as major compounds. Only one other compound was detected in the tested material and that is methyl stearate, flavouring and cosmetics agent as well as plant metabolite (National Library of Medicine 2021a), **Figure 4** and **Table 2**.

Aluminium oven from Pax 3 device was filled with Vitamin E Acetate, weight recorded and used to generate ten puffs on CETI 8 vaping machine. The emissions were collected on the chemically pre-cleaned collection pad which in turn was extracted with methanol and analyzed using the scanning protocol. It is important to note that the same Pax3 device was used to vape MCT oil first, and then D, L Vitamin E

Acetate. Five chemical compounds were detected in D, L vitamin E acetate emission, **Figure 5** and **Table 3**.

The major constituent of the emission was one racemic structure (D or L) of vitamin E acetate and a minor constituent was Methyl isostearate. Additional three chemicals detected in the emissions were most likely due to the cross-contamination with MCT oil emissions as they have been previously detected in the oil itself and its' emission. Although in between the samples, the mouthpiece was replaced, an aluminium concentrate insert heating oven was thoroughly cleaned as per manufacturer's instructions, and through the use of various laboratory chemical solvents (hexane, acetonitrile, methanol), the analysis of vitamin E acetate generated emissions, unfortunately, revealed the presence of MCT oil compounds as well. The cross-contamination did not result from the GC MS instrument carry over or instrument contamination as the analytical blanks injected between samples showed no detected MCT oil compounds. Although it was not confirmed through laboratory analysis, it is likely that the contamination originated from the heating element on the outside of the oven or from the other parts of the device that could have gotten into contact through emission path. Some interior device parts were not rinsed thoroughly with solvents as they could be damaged, and therefore, were a likely source of MCT oil triglycerides.

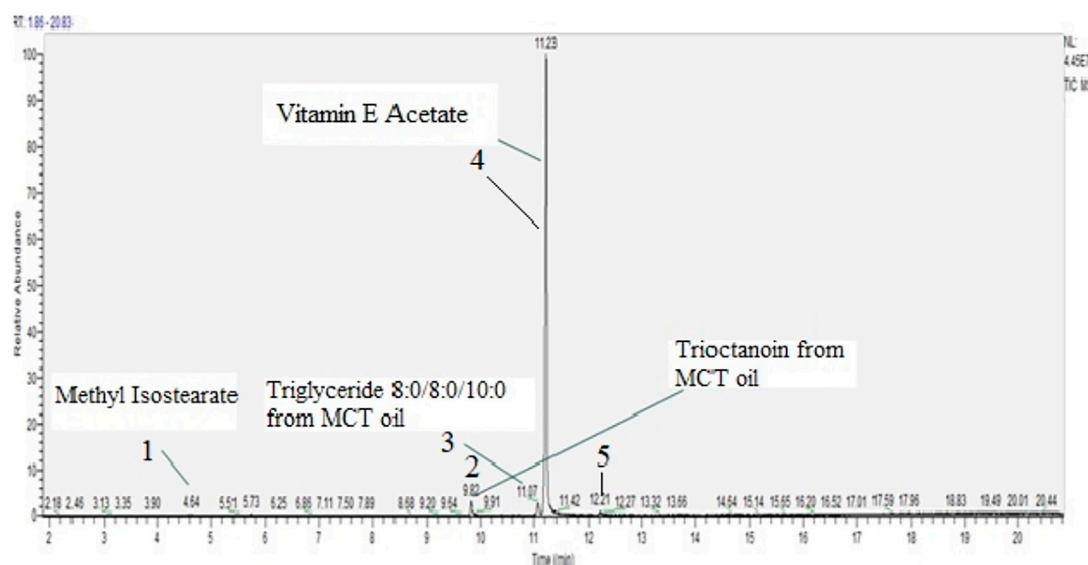


FIGURE 5 | D, L vitamin E acetate emission scan, concentration 870 µg/mL, including two MCT oil cross-contaminants.

TABLE 3 | Chemical compounds detected in vitamin E acetate emission (Figure 5).

Peak	IUPAC Name	Common Name	CASRN	More information
1	Methyl 16-methylheptadecanoate	Methyl isostearate	5129-61-3	Natural substance and extractive, used in cosmetic products
2	2,3-di(octanoyloxy)propyl octanoate	Trioctanoin	538-23-8	Has a role as a plant metabolite. Used as diluent, as carrier for flavours and essential oils
3	1,3-di(octanoyloxy)propan-2-yl decanoate	Glyceryl 2-caprate dicaprylate	33368-87-5	Triglyceride 8:0/8:0/10:0, patents list use in printing inks and varnishes
4	[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydrochromen-6-yl] acetate	D,L Vitamin E Acetate or (±)-α-Tocopherol acetate	7695-91-2	Synthetic D,L form. Antioxidant, nutritional supplement and preservative
5	(3-decanoyloxy-2-octanoyloxypropyl) decanoate	Glyceryl 2-caprylate dicaprate	33368-86-4	Triglyceride 8:0/10:0/10:0, patents list use in printing inks and varnishes

The cross contamination, although unfortunate, can be indicative of a real-life occurrence where users may use the same device to vaporize or vape a number of different products or formulations, and unintentionally get exposed to chemical transformation products that may not necessarily match vaping liquid they are consuming in spite of a thorough device cleaning. Of a particular concern may also be the cases where a device is shared or borrowed among users - a social behaviour previously documented among youth who vape (Pepper et al., 2019). In such cases, cross-contamination or persistence of diluent of concern in the vaping device may lead to lack of awareness and unintentional exposure of additional users.

Limitations

The vitamin E acetate targeted method presented here was not applied to any cannabis-containing vaping products, therefore, no conclusions can be made on presence of this diluent in cannabis products in Canada. Vitamin E acetate (D, L) emissions in our study were generated using 96% pure compound. The chemical grade of vitamin E acetate used as diluent for illicit cannabis vaping products

may contain more impurities and be of a lower grade. In the absence of real cannabis sample or type of diluent used, it is difficult to conclude whether the impurities from the technical grade product would be present in the emissions.

While this study provides an improved understanding of chemical profiles of emissions of vaping diluents, caution should be taken when interpreting these results in real-world conditions. In order to study formation of chemical transformations, the liquid diluents were studied individually and not in vaping liquid mixtures which most frequently will include other diluents (PG or VG), flavours, processing agents and active ingredients (nicotine, THC, etc).

Recent studies on pyrolysis of vitamin E acetate have hypothesized and detected a presence of a simple ketene-ethenone, an acute lung irritant and highly reactive compound (Wu and O'Shea 2020). We did not detect this chemical compound in the emissions as it is likely that our pad capturing method was not sufficient to collect this highly volatile compound. Another reason could also be that the device used in our study was heating only to 215°C that may not have been sufficient temperature to generate this compound (Attfield et al.,

2020). Further studies are required to capture and characterize the highly volatile chemical compounds generated in the vaped emissions.

CONCLUSION

In our study, Vitamin E Acetate was not detected above detection limit (0.159 µg/mL) in any nicotine containing vaping liquids analyzed. Two diluents of concern-Vitamin E Acetate and medium chain triglycerides, were analyzed as pure substances in order to better characterize their emissions. Both diluents when heated in vaping device in fact, generate high concentrations of diluents as well as other chemical compounds typically present in unvaped precursor material. Of a note is that there was an observed cross-contamination of vaping device with diluents used which may have implications for device sharing. Although Vitamin E Acetate has been identified as a potential causative agent in the occurrence of EVALI, MCT oil, to date has not been directly implicated in EVALI. Moreover, to date the cause of cases related to the exclusive use of nicotine-containing vaping products has not been determined. Future studies should characterize vaped emissions from the products of interest in order to better elucidate chemicals of concern.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

REFERENCES

- Attfield, K. R., Chen, W., Cummings, K. J., Jacob, P., 3rd, O'Shea, D. F., Wagner, J., et al. (2020). Potential of Ethenone (Ketene) to Contribute to Electronic Cigarette, or Vaping, Product Use-Associated Lung Injury. *Am. J. Respir. Crit. Care Med.* 202 (8), 1187–1189. doi:10.1164/rccm.202003-0654le
- Bhat, T. A., Kalathil, S. G., Bogner, P. N., Blount, B. C., Goniewicz, M. L., and Thanavala, Y. M. (2020). An Animal Model of Inhaled Vitamin E Acetate and EVALI-like Lung Injury. *N. Engl. J. Med.* 382 (12), 1175–1177. doi:10.1056/nejmc2000231
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382 (8), 697–705. doi:10.1056/nejmoa1916433
- CDC, Centers for Disease Control and Prevention (2020). Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products. Available at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html.
- Duffy, B., Li, L., Lu, S., Durocher, L., Dittmar, M., Delaney-Baldwin, E., et al. (2020). Analysis of Cannabinoid-Containing Fluids in Illicit Vaping Cartridges Recovered from Pulmonary Injury Patients: Identification of Vitamin E Acetate as a Major Diluent. *Toxics* 8 (1), 8. doi:10.3390/toxics8010008
- Erickson, Britt. E. (2019). Cannabis Industry Gets Crafty with Terpenes. *C&EN News* 97 (29), 18–21. doi:10.1021/cen-09729-feature1
- Goniewicz, M. L., Smith, D. M., Edwards, K. C., Blount, B. C., Caldwell, K. L., Feng, J., et al. (2018). Comparison of Nicotine and Toxicant Exposure in Users of Electronic Cigarettes and Combustible Cigarettes. *JAMA Netw. Open* 1 (8), e185937. doi:10.1001/jamanetworkopen.2018.5937

AUTHOR CONTRIBUTIONS

IK conceived, designed and performed the chemical analysis, supervised data processing and wrote the manuscript. CK supervised the targeted analysis of vitamin E acetate and reviewed the manuscript. GK performed the chemical analysis and revised the manuscript. DP generated the emissions, performed the analysis and revised the manuscript. TM conceived the project, supervised the project coordination, discussed results and revised the manuscript.

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- Government Of Canada (2019). Final Regulations: Edible Cannabis, Cannabis Extracts, Cannabis Topicals. Available at: <https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/resources/regulations-edible-cannabis-extracts-topicals.html>.
- Government Of Canada (2020). Vaping-associated Lung Illness. Available at: <https://www.canada.ca/en/public-health/services/diseases/vaping-pulmonary-illness.html>.
- (ISO), The International Organization for Standardization (2018). *ISO 20768:2018 Vapour Products - Routine Analytical Vaping Machine - Definitions and Standard Conditions*.
- Jiang, H., Ahmed, C. M. S., Martin, T. J., Canchola, A., Oswald, I. W. H., Garcia, J. A., et al. (2020). Chemical and Toxicological Characterization of Vaping Emission Products from Commonly Used Vape Juice Diluents. *Chem. Res. Toxicol.* 33 (8), 2157–2163. doi:10.1021/acs.chemrestox.0c00174
- Krusemann, E. J. Z., Boesveldt, S., De Graaf, K., and Talhout, R. (2019). An E-Liquid Flavor Wheel: a Shared Vocabulary Based on Systematically Reviewing E-Liquid Flavor Classifications in Literature. *Nicotine Tob. Res.* 21 (10), 1310–1319.
- Lynch, J., Lorenz, L., Brueggemeyer, J. L., Lanzarotta, A., Falconer, T. M., and Wilson, R. A. (2021). Simultaneous Temperature Measurements and Aerosol Collection during Vaping for the Analysis of Δ^9 -Tetrahydrocannabinol and Vitamin E Acetate Mixtures in Ceramic Coil Style Cartridges. *Front. Chem.* 9, 734793. doi:10.3389/fchem.2021.734793
- National Library of Medicine (2021c). Alpha-Tocopherol Acetate. Available at: <https://pubchem.ncbi.nlm.nih.gov/compound/alpha-Tocopherol-acetate#section=Food-Additives-and-Ingredients>.
- National Library of Medicine (2021a). Methyl Stearate. Available at: <https://pubchem.ncbi.nlm.nih.gov/compound/Methyl-stearate#section=Use-Classification>.
- National Library of Medicine (2021b). PubChem. Available at: <https://pubchem.ncbi.nlm.nih.gov/>.

- (NIST), National Institute of Science and Technology (2021). *NIST Chemistry WebBook Standard Reference Database* 69. Available at: <https://webbook.nist.gov/chemistry/>.
- Nyakutsikwa, B., Britton, J., Bogdanovica, I., and Langley, T. (2020). Vitamin E Acetate Is Not Present in Licit E-cigarette Products Available on the UK Market. *Addiction* 115 (4), 782–783. doi:10.1111/add.14920
- Pepper, J. K., Coats, E. M., Nonnemaker, J. M., and Loomis, B. R. (2019). How Do Adolescents Get Their E-Cigarettes and Other Electronic Vaping Devices? *Am. J. Health Promot.* 33 (3), 420–429. doi:10.1177/0890117118790366
- Pray, I. W., Atti, S. K., Tomasallo, C., and Meiman, J. G. (2020/2019). E-cigarette, or Vaping, Product Use-Associated Lung Injury Among Clusters of Patients Reporting Shared Product Use - Wisconsin, 2019. *MMWR Morb. Mortal. Wkly. Rep.* 69 (9), 236–240. doi:10.15585/mmwr.mm6909a4
- Puetz, Andrew., Espinosa, Maria. Morel, Watson, Clifford., Benjamin, C. Blount., and Valentin-Blasini, Liza. (2021). Development, Validation, and Application of a Novel Method for the Analysis of Vitamin E Acetate and Other Tocopherols in Aerosol Emissions of E-Cigarettes, or Vaping Products Associated with Lung Injury. *Front. Chem.* 636. doi:10.3389/fchem.2021.730954
- Taylor, J., Wiens, T., Peterson, J., Saravia, S., Lunda, M., Hanson, K., et al. (2019). Characteristics of E-Cigarette, or Vaping, Products Used by Patients with Associated Lung Injury and Products Seized by Law Enforcement - Minnesota, 2018 and 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68 (47), 1096–1100. doi:10.15585/mmwr.mm6847e1
- The German Federal Institute for Risk Assessment (BfR) (2020). Determination of Vitamin E and Vitamin E Acetate in E-Liquids Using LC-MS/MS. Available at: <https://www.bfr.bund.de/cm/349/Determination-of-Vitamin-E-and-Vitamin-E-Acetate%20.pdf>.
- The Good Scents Company (2021b). Allyl Octanoate. Available at: <http://www.thegoodscentscompany.com/data/rw1002871.html>.
- The Good Scents Company (2021a). TGSC Information System. Available at: <http://www.thegoodscentscompany.com/index.html>.
- Traul, K. A., Driedger, A., Ingle, D. L., and Nakhasi, D. (2000). Review of the Toxicologic Properties of Medium-Chain Triglycerides. *Food Chem. Toxicol.* 38, 79–98. doi:10.1016/s0278-6915(99)00106-4
- USEPA, United States Environmental Protection Agency (2011). 40 CFR Appendix B to Part 136 - Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11. Available at: <https://www.govinfo.gov/app/details/CFR-2011-title40-vol23/CFR-2011-title40-vol23-part136-appB>.
- Wu, D., and O'Shea, D. F. (2020). Potential for Release of Pulmonary Toxic Ketene from Vaping Pyrolysis of Vitamin E Acetate. *Proc. Natl. Acad. Sci. USA* 117 (12), 6349–6355. doi:10.1073/pnas.1920925117
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Chemical Emissions From Heated Vitamin E Acetate—Insights to Respiratory Risks From Electronic Cigarette Liquid Oil Diluents Used in the Aerosolization of Δ^9 -THC-Containing Products

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As of February 18, 2020, the e-cigarette, or vaping, product use associated lung injury (EVALI) outbreak caused the hospitalization of a total of 2,807 patients and claimed 68 lives in the United States. Though investigations have reported a strong association with vitamin E acetate (VEA), evidence from reported EVALI cases is not sufficient to rule out the contribution of other chemicals of concern, including chemicals in either THC or non-THC products. This study characterized chemicals evolved when diluent oils were heated to temperatures that mimic e-cigarette, or vaping, products (EVPs) to investigate production of potentially toxic chemicals that might have caused lung injury. VEA, vitamin E, coconut, and medium chain triglyceride (MCT) oil were each diluted with ethanol and then tested for constituents and impurities using a gas chromatograph mass spectrometer (GC/MS). Undiluted oils were heated at 25°C (control), 150°C, and 250°C in an inert chamber to mimic a range of temperatures indicative of aerosolization from EVPs. Volatilized chemicals were collected using thermal desorption tubes, analyzed using a GC/MS, and identified. Presence of identified chemicals was confirmed using retention time and ion spectra matching with analytic standards. Direct analysis of oils, as received, revealed that VEA and vitamin E were the main constituents of their oils, and coconut and MCT oils were nearly identical having two main constituents: glycerol tricaprylate and 2-(decanoyloxy) propane-1,3-diyl dioctanoate. More chemicals were measured and with greater intensities when diluent oils were heated at 250°C compared to 150°C and 25°C. Vitamin E and coconut/MCT oils produced different chemical emissions. The presence of some identified chemicals is of potential health consequence because many are known respiratory irritants and acute respiratory toxins. Exposure to a mixture of hazardous chemicals may be relevant to the development or exacerbation of EVALI, especially when in concert with physical damage caused by lung deposition of aerosols produced by aerosolizing diluent oils.

Keywords: e-cigarettes, e-liquids, vitamin E acetate, EVALI, chemical emissions

INTRODUCTION

The e-cigarette, or vaping, product use associated lung injury (EVALI) outbreak was a public health crisis that caused the hospitalization of 2,807 people and was responsible for 68 deaths in the United States as of February 18, 2020 (1). Inhalation of vitamin E acetate (VEA) is strongly associated with the EVALI cases described during the outbreak (2) and EVALI-like syndromes have been produced in mouse models by VEA (3). Evidence is not sufficient to rule out the contribution of other chemicals of concern, including chemicals in either THC or non-THC products, in some of the reported EVALI cases and mechanisms of lung injury in EVALI are incompletely understood (4). Blount et al. (2) hypothesize one possible mechanism of injury could be that the aliphatic tail of VEA could penetrate a layer of lung surfactant to align the molecule in parallel with phospholipids, thereby interfering with surfactant function. However, all users of VEA-containing e-liquids presumably did not develop EVALI, which may indicate unrecognized risk factors, or a difference in individual susceptibilities to the stressors. Public health messaging, the removal of VEA from electronic cigarette liquid (e-liquid) formulations, and law enforcement practices might have led to the decline in emergency department visits related to e-cigarette, or vaping, products (EVPs) (1). As of January 14, 2020, 82% (1,658/2,022) of hospitalized EVALI patients reported using products containing Δ^9 -tetrahydrocannabinol (THC), 33% reported exclusive use of THC-containing products, 57% reported using any nicotine-containing product, and 14% reported using only nicotine-containing products (1). According to the (5), the cannabis plant is referred to as “marijuana” when the plant contains more than 0.3% by dry weight of Δ^9 -THC, the main psychoactive cannabinoid, and referred to as “hemp” when the plant contains <0.3% of Δ^9 -THC (2018). Marijuana is the most popular illicit/recreational drug in the United States with ~123 million people (age 12 + years) who have tried it at least once in their lifetime and ~41 million people who have used it in the past year (6). In 2014, among adults who reported using marijuana, 9.9% reported using “vaporizers” (devices used for drug delivery via inhalation) or other electronic devices. Of current adult EVP users, 17% reported using marijuana and 70% reported using nicotine (7). Among college students, 75% of those who reported using a substance other than nicotine in EVPs responded that they used marijuana or Δ^9 -THC-containing products (8).

EVPs for nicotine delivery heat an e-liquid composed of propylene glycol and vegetable glycerin. Cannabis extracts are hydrophobic, semi-solid materials. During the time of the EVALI outbreak, illicit Δ^9 -THC products were shown to commonly be diluted with terpenes, VEA, medium chain triglycerides, and polyethylene glycol (9, 10). VEA is strongly associated with EVALI and has been identified in bronchoalveolar lavage specimens from 48 of 51 EVALI cases in 16 states, giving direct evidence at the site of injury (2). Bhat et al. provided additional evidence of lung injury consistent with EVALI by exposing mice to VEA aerosols but recognized the need to characterize chemical emissions from heated VEA (3).

THC oil is aerosolized in EVPs that contain a reservoir with a wick, a heating element, and a battery. For propylene glycol and vegetable glycerin based e-liquids commonly used with nicotine, top-coil or clearomizer-coil temperatures range from 322 to 1,008°C for dry conditions, 145–334°C for wet-through-wick conditions, and 110–185°C for full-wet conditions (11). Lynch et al. observed that temperatures ranged from 375 to 569°C when THC/VEA were aerosolized with ceramic coil cartridges (12). The inhaled aerosol contains liquid droplets that will deposit throughout the respiratory tract based, in part, on their aerodynamic diameter, and gas-phase substances, including volatile organic compounds (VOCs). Hence, it is likely that both the physical (particle size) and chemical constituents of inhaled aerosol have deleterious individual and combined respiratory and systemic effects. For example, VEA, when aerosolized using third-generation EVPs, produces ethenone (C_2H_2O) gas, a type of ketene gas and respiratory irritant, which is hypothesized to be a contributing factor to EVALI (13). Attfield et al. also hypothesized that thermal degradation of VEA may be important in EVALI and suggested that acetate moieties are precursors for high temperature formation of ethenone gas. The researchers also note that the reaction could be amplified at high temperatures in the presence of catalytic metals, including chromium and nickel, and/or ceramic surfaces present in heating coils of EVPs. According to Wu & O'Shea (13), additional thermal transformation products produced from aerosolized VEA included benzene, butadiene, and formaldehyde. All of these chemicals are respiratory irritants and potential occupational carcinogens (14). Chemical emissions from heating oil diluents during aerosolization are composed of the unadulterated chemical, thermal breakdown and rearrangement products from the original chemical, and new products formed in the presence of oxygen. In this study, we elucidated several chemical emissions of VEA and other oil diluents as well as assigned relative hazard designations to identified chemicals. This information provides novel insights for investigators to study chemical toxicity in concert with aerosol deposition modeling to better understand the mechanism(s) of lung injury observed among EVALI patients.

METHODS

Materials

VEA (α -tocopherol acetate), $\geq 99\%$, CAS# 7695-91-2, was purchased from Sigma-Aldrich (St. Louis, MO). Vitamin E oil (42900IU, 100% pure & natural, Chandler, AZ), medium chain triglycerides (MCT, 100% organic unflavored, Garden of Life LLC, FL), and coconut oil (Organic unflavored, Carrington Farms, Closter, NJ) were purchased from Amazon.com, Inc. (Seattle, WA).

Chemical standards and reagents were purchased from various suppliers:

Sigma-Aldrich (St. Louis, MO): O-2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride (PFBHA), $\geq 99\%$, CAS# 57981-02-9; N,N-dimethylbenzamide, CAS# 611-74-5; glyceryl trioctanoate, CAS# 538-23-8; hexamethylcyclotrisiloxane, CAS#

541-05-9; nonanal, CAS# 124-19-6; acetic anhydride, CAS# 108-24-7; isoamyl ether, CAS# 544-01-4; 2,6,10-trimethyldodecane, CAS# 3891-98-3; methylpropenal (methacrolein), CAS# 78-85-3; isovaleraldehyde, CAS# 590-86-3; 1-ethyl-2-pyrrolidinone, CAS# 2687-91-4; undecanoic acid, CAS# 112-37-8; 2-butyl-1-octanol, CAS# 3913-02-8, diacetyl, CAS# 431-03-8, methylglyoxal, CAS# 78-98-8, 2-butanone, CAS# 78-93-3.

Accela ChemBio (San Diego, CA): hexahydrofarnesyl acetone, CAS# 502-69-2; 2-hexyl-1-octene, CAS# 19780-80-4; and 2-ethylcrotonaldehyde, CAS# 19780-25-7.

Alfa-Chemistry (Stony Brook, NY): 2-methylpentyl formate, CAS# 381670-34-4.

Cayman Chemical (Ann Arbor, MI): hexadecanal, CAS# 629-80-1.

Crescent Chemical (Islandia, NY): pristane, CAS# 1921-70-6.

Fisher Scientific (Pittsburgh, PA): acetic acid, CAS# 64-19-7; 1,2-epoxyhexane (Acros Organics), CAS# 1436-34-6; 2-methylheptane (Acros Organics), CAS# 592-27-8; 2-nonanone (Acros Organics), CAS# 821-55-6.

Oakwood Chemical (Estill, SC): 1,2-epoxyoctadecane, CAS# 7390-81-0; 2-methoxyacetic acid, CAS# 625-45-6.

Tokyo Chemical Industry Co. (Montgomeryville, PA): 1-eicosene, CAS# 3452-07-1.

Bulk Oil Analysis

We performed analysis of oils to evaluate constituents and impurities by diluting with ethanol to 10^{-3} , 10^{-4} , and 10^{-5} . We injected the diluted oil and analyzed using a 7890B/5977B gas chromatograph/mass spectrometer (GC/MS, Agilent Technologies, Inc., Santa Clara, CA). The column was a Restek (Bellefonte, PA) Rxi[®]-1ms (0.32 mm I.D., 60 m long, 1.0 μ m film thickness) with ultra-high purity helium flowing at 2 mL min⁻¹. The oven program was 58.25 min long and started at 30°C for 5 min, 5°C min⁻¹ to 170°C, 20°C min⁻¹ to 250°C, hold for 10 min, 40°C min⁻¹ to 300°C, hold for 10 min. Electron impact (EI) ionization spectra were collected from m/z 30–500 with an MS source temperature of 300°C and quadrupole temperature of 150°C.

Experimental Setup and Design

Aluminum weigh boats (Fischer Scientific, Waltham, MA) were conditioned for 1 h in an oven at 250°C, allowed to cool to room temperature, and used immediately for chamber testing. A small amount (100–300 mg) of each oil was placed in an aluminum boat and heated in a micro-chamber/thermal extractor (M-CTE250, Markes International Inc., Gold River, CA) to 25°C (control), 150°C, or 250°C (**Figure 1**) with sample collection beginning immediately with the first of four tubes as described below. Because of fouling from the oils at high temperatures, the microchamber was cleaned with methylene chloride and baked at 250°C for one h between trials. VEA, vitamin E oil, coconut oil, and MCT were tested. Some of the trials were repeated to check reproducibility of chemical emission profiles: VEA at 250°C, vitamin E at 250°C, coconut oil at 150°C, and MCT at 150°C.

General VOC Testing

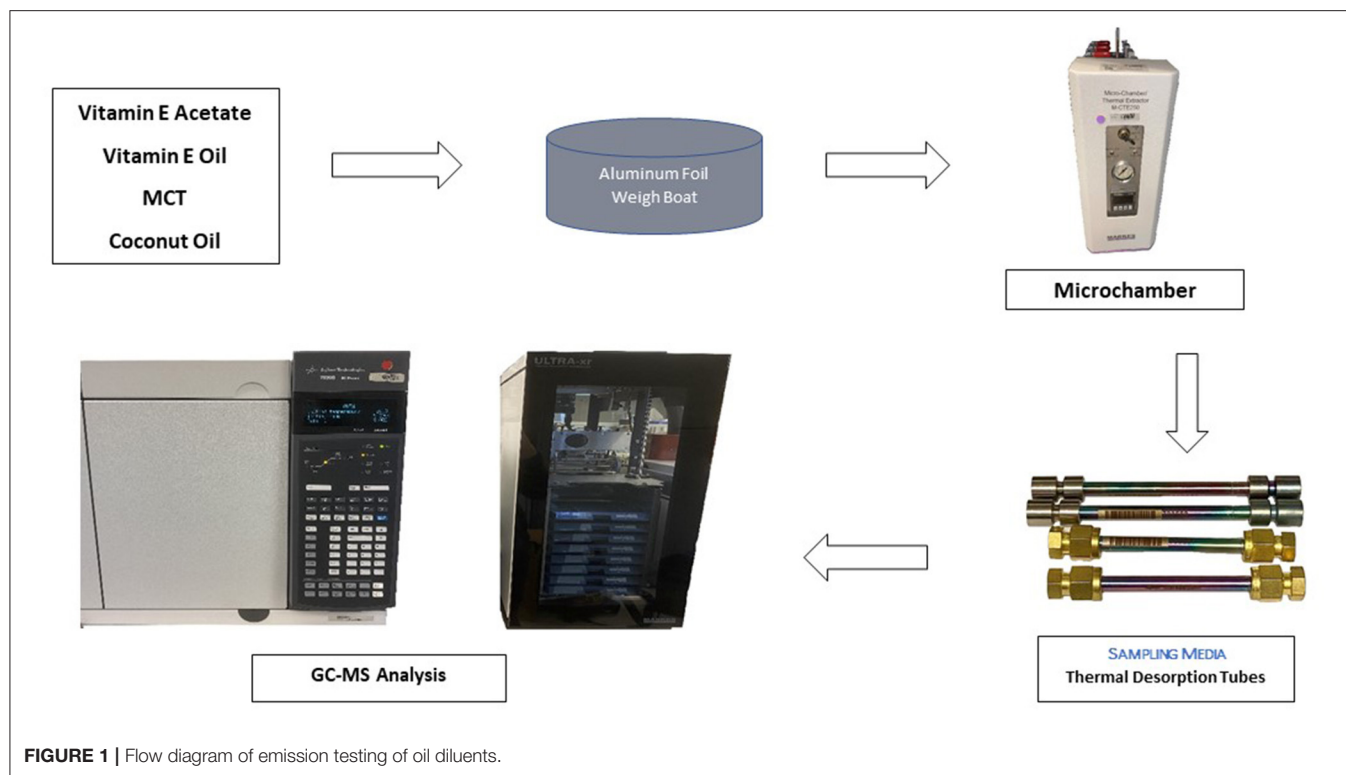
Prior to testing, two background air samples were collected on separate Markes Universal thermal desorption (TD) tubes (Markes International, Inc., Sacramento, CA): one for 20 min and one for 30 s. Prior to placing the oil sample in the microchamber, a background emission sample of a conditioned aluminum foil weigh boat was collected for 30 s. Chemical emissions were collected on four TD tubes every 30 s after placing the room temperature (22°C) oil into the heated chamber. Sampling rate was measured to be 36.0 ± 2.7 mL min⁻¹ (mean $\pm$ standard deviation) equivalent to ~ 18 mL of sample collected on each tube.

TD tubes were analyzed using an Ultra-xr autosampler and Unity-xr thermal desorber (Markes International, Inc.) with an Air Toxics cold trap (U-T15ATA-2S, Markes International, Inc.) at a 20:1 split ratio. The general TD parameters included standby split flow of 10 mL min⁻¹, flow path temperature at 150°C, and a minimum carrier pressure of 5 psi. The pre-desorption TD parameters included a pre-purge time of 2 min at a split flow of 50 mL min⁻¹. The TD tube desorption parameters consisted of a desorb time of 7 min at 280°C with a trap flow of 50 mL min⁻¹. The TD trap parameters consisted of a trap purge for 2 min at 50 mL min⁻¹ with trap low temperature set at 25°C, followed by a MAX trap heating rate to 290°C, and desorbed for 3 min with a desorb split flow rate of 38 mL min⁻¹. The TD system was attached to a GC/MS operated at the same conditions described above for the bulk oil analysis.

Carbonyl Testing

For carbonyl analysis, samples were collected for VEA heated to 250°C from the microchamber into a 60-mL Teflon[®] impinger (Saville, Eden Prairie, MN) containing 5 mL of deionized water at 40 mL min⁻¹ for 3 min. Vitamin E oil, MCT, and coconut oil diluents were not tested for carbonyls because VEA was more strongly associated with EVALI than the other oils. Two independent trials were conducted one week apart to check reproducibility of chemical emission testing. After collection, samples were decanted into 16 mL vials, then derivatized with 200 microliters (μ L) aqueous 250 mM PFBHA. Vials were left overnight to complete derivatization. The next day, 3 mL of methyl tert-butyl ether (MTBE) was added to each vial. The vial was then shaken for 30 s and allowed to separate into a MTBE layer and aqueous layer. The MTBE was removed and put into a 7-mL vial, blown to dryness, then reconstituted with 100 μ L of toluene. The toluene layer was then removed with a pipette and placed in a 2-mL autosampler vial with a 250- μ L glass insert (Restek, Bellefonte, PA).

All PFBHA-derivatized samples were analyzed using a Varian (Palo Alto, CA) 3800/Saturn 2000 GC/MS system operated in the EI mode. Compound separation was achieved by an Agilent (Santa Clara, CA) HP-5MS (0.25 mm I.D., 30 m long, 0.25 μ m film thickness) column and the following GC oven parameters: 40°C for 2 min., then 5°C min⁻¹ to 200°C, then 25°C min⁻¹ to 280°C and held for 5 min. One μ L of each sample was injected in the splitless mode, and the GC injector was returned to split mode 1 min after sample injection, with the following injector temperature parameters: 130°C for 2 min then 200°C min⁻¹ to



300°C and held for 10 min. The Saturn 2,000 ion trap mass spectrometer was tuned using perfluorotributylamine (FC-43). Full-scan EI ionization spectra were collected from m/z 40–650.

Data Analysis

We performed confirmation of identified compounds emitted from heated oils using retention time matching for 34 compounds of interest (**Supplementary Table 1**) by comparing sample mass spectra to the NIST11 mass spectral library using Masshunter Unknowns Analysis software, manually interrogating the chromatograms using Masshunter Qualitative Analysis Navigator (Agilent Technologies, Inc.), and interrogating the mass spectra for PFBHA loss from derivatized compounds for carbonyls. Carbonyl masses are reported as underivatized mass to charge ratios (m/z). Identified compounds are reported for the fourth TD tube (collected beginning at 90 s and ending at 120 s after oil was added to chamber) to allow for temperature equilibration and are reported when the match factor was $\geq 75\%$ or the compound retention time was matched using an analytical standard. Acetic acid in both VEA 250°C trials was not correctly identified by the automated Masshunter Unknowns Analysis software because of triangular peak shape, overall intensity, and mass spectral noise. It was correctly identified in lower temperature samples (e.g., VEA 150°C). For acetic acid in both VEA 250°C trials, an extracted ion chromatogram for m/z 61, 60, 45, 43, and 42 in Masshunter Qualitative Analysis Navigator was used for peak integration between 7.9 and 10.0 min and qualitative match factors. For VOC retention time matching, analytical standards were diluted

in methanol or ethanol to ~ 500 ng/sample and fortified on the front of a TD tube with a 1 μL injection. TD tube spiked samples had nitrogen passed over them for 5 min at 100 mL min^{-1} to push the analytes onto the sorbent beds.

We extracted Globally Harmonized System (GHS) hazard classes (H codes) from PubChem for each identified chemical emission and assigned a hazard group (“–” < “+” < “++”) with the highest hazard class being used for designation (i.e., a higher hazard group can also include a lower hazard class): “–” = physical hazard only (H220, H225, H226), or environmental hazard only (H400, H410, H411, H412, H413), or no hazards noted in PubChem; “+” = oral acute toxicity (H301, H302), skin corrosion/irritation (H314, H315, H316), skin sensitization (H317), serious eye damage/eye irritation (H319), respiratory tract irritation or narcotic effects from a single exposure with specific target organ toxicity (H335, H336), germ cell mutagenicity (H340, H341), carcinogenicity (H350, H351), reproductive toxicity (H360D, H360FD, H361, H361d, H361f), and/or repeated exposure with specific target organ toxicity (H372, H373); and “++” = aspiration hazard (H304) and/or acute inhalation toxicity (H330, H331, H332, or H333).

RESULTS

Bulk Oil Analysis

For VEA, we confirmed ($\pm$)- α -tocopherol acetate as the main constituent of the oil tested, at a retention time of 54.7 min with a small amount of vitamin E. NIST mass spectral matching revealed only a 40% match for VEA but the characteristic m/z

pattern of 472 (molecular ion), 430, 207, and 165 (base peak) was evident. For vitamin E oil, we confirmed vitamin E as the sole constituent at a retention time of 52.0 min with a NIST mass spectral matching of 32% but the characteristic m/z pattern of 430 (molecular ion), 205, and 165 (base peak) were present. For coconut oil and MCT, we identified two main constituents: glycerol tricaprylate (CAS# 538-23-8) at 43.8 min with a 94% match and 2-(decanoyloxy) propane-1,3-diyl dioctanoate (CAS# 33368-87-5) at 51.2 min with an 88% match.

General VOC Testing

As expected, the chromatogram for the TD tube instrument blank (**Figure 2A**) did not show the presence of any VOCs or carbonyls. The background air samples collected for 30 s (**Figure 2B**) had a few chromatogram peaks at retention times 36–43 min and 48–52 min; however, peaks were minimal in magnitude and number compared to the instrument blank. A second, longer duration (higher volume) background air sample was collected for 20 min (**Figure 2C**) and the chromatogram contained multiple peaks. Though peaks were present in this 20-min duration blank, their magnitude was much lower compared to the emissions in the chromatogram for VEA heated at 250°C (**Figure 2D**). These results demonstrated that the chemicals identified in the (30-s and 20-min) background samples did not appreciably influence the emissions identified from the heated VEA samples. For example, acetic acid in the 30-s background accounted for only 0.2% of the integrated area of the total ion chromatogram of acetic acid in the sample collected when VEA was heated at

250°C. Though not shown for brevity, background air samples were similarly low for the tested vitamin E, coconut, and MCT oils.

Chemical emissions confirmed with retention time matching to neat chemical standards as well as hazards associated with those chemicals for heated oil diluents are presented in **Table 1**. The chemicals elucidated included alcohols, aldehydes, ketones, and saturated and unsaturated hydrocarbons. When VEA was heated in an inert chamber at 250°C in the presence of oxygen (i.e., air flowing over the material), chemical emissions included thermal degradation products such as acetic acid and oxidation products such as 6,10,14-trimethyl-2-pentadecanone (**Table 1**). Seven hazardous chemical emissions were identified and confirmed from VEA: acetic acid, acetone, formic acid, methylpropenal, isovaleraldehyde, farnesane, and 2-methylheptane. Methylpropenal, farnesane, and 2-methylheptane were not measured during 25°C or 150°C trials. These three chemicals were also categorized into the most hazardous group [i.e., “++,” which is an aspiration hazard (H304) and/or acute inhalation toxicity (H330, H331, H332, or H333)]. One of these hazardous chemicals, 2-Methylheptane, was observed in vitamin E oil emissions at area responses 3–4 times lower than VEA emissions ($5.42\text{E}+07$ compared to $1.60\text{E}+08$ or $2.22\text{E}+08$). Acetic acid had the greatest peak area for VEA ($2.64\text{E}+09$ in the first trial and $2.25\text{E}+09$ in the second trial). Vitamin E oil, coconut, and MCT had some of the same chemical emissions (e.g., acetic acid, formic acid) as VEA but at much lower areas (**Table 1**). Nonanal and 2-nonanone emissions

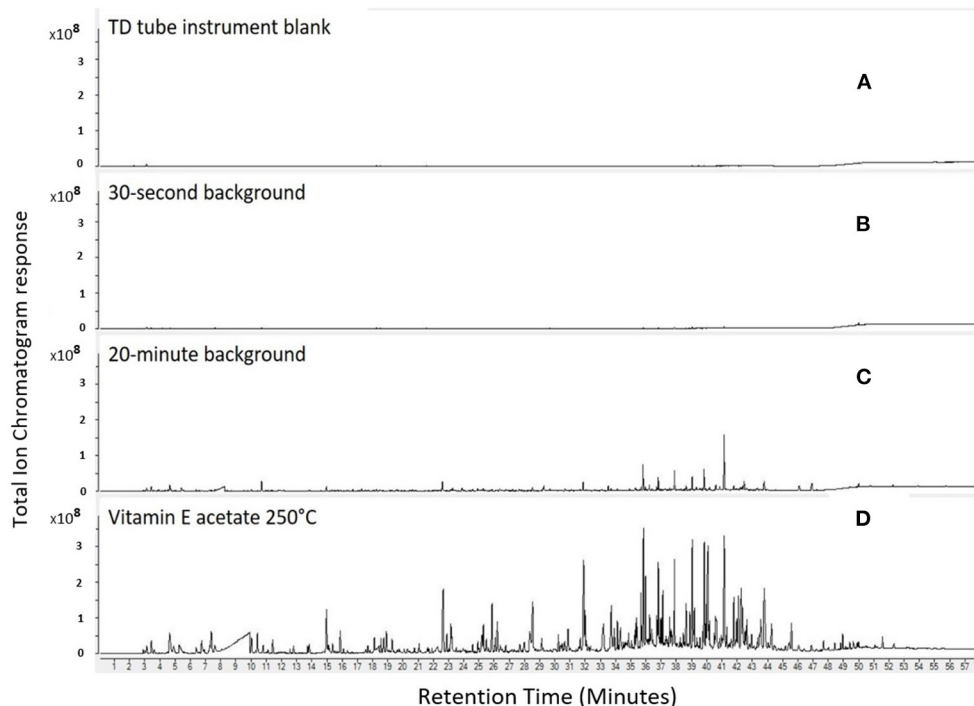


FIGURE 2 | Comparison of TD tube instrument blank (**A**), 30-s background (**B**), 20-min background (**C**), and VEA emissions at 250°C sampled between 90 and 120 s after oil was added to chamber (**D**).

TABLE 1 | Hazards associated with compounds identified and confirmed by retention time matching in heated emissions of oil diluents at 250°C.

Oil	Trial	Common name	Formula	CAS#	Hazard*	Match Factor	RT	Area
VEA	First	Acetic acid	C2H4O2	64-19-7	+	75	7.7	2.64E+09
VEA	Second	Acetic acid	C2H4O2	64-19-7	+	81	7.7	2.25E+09
VEA	First	Acetone	C3H6O	67-64-1	+	41 ^a	4.6	3.97E+08
VEA	Second	Acetone	C3H6O	67-64-1	+	78	4.7	1.60E+08
VEA	First	Formic acid	CH2O2	64-18-6	+	99	5.3	2.11E+08
VEA	Second	Formic acid	CH2O2	64-18-6	+	99	5.3	1.30E+08
VEA	First	Methylpropenal (methacrolein)	C4H6O	78-85-3	++	63	6.7	1.61E+08
VEA	Second	Methylpropenal (methacrolein)	C4H6O	78-85-3	++	97	6.8	1.40E+08
VEA	First	Isovaleraldehyde	C5H10O	590-86-3	+	96	10.1	1.52E+08
VEA	Second	Isovaleraldehyde	C5H10O	590-86-3	+	94	10.1	1.10E+08
VEA	First	2-Methylheptane	C8H18	592-27-8	++	98	15.9	2.22E+08
VEA	Second	2-Methylheptane	C8H18	592-27-8	++	98	15.9	1.60E+08
VEA	First	Farnesane	C15H32	3891-98-3	++	19 ^a	35.7	4.24E+08
VEA	Second	Farnesane	C15H32	3891-98-3	++	80	35.7	2.80E+08
VEA	First	Hexahydrofarnesyl acetone ^b	C18H36O	502-69-2	–	81	42.6	2.64E+08
Vitamin E	First	Acetone	C3H6O	67-64-1	+	97	4.9	1.50E+07
Vitamin E	First	Acetic acid	C2H4O2	64-19-7	+	98	8.6	3.24E+07
Vitamin E	Second	Acetic acid	C2H4O2	64-19-7	+	98	8.1	1.02E+08
Vitamin E	Second	2-Methylheptane	C8H18	592-27-8	++	93	15.9	5.42E+07
Vitamin E	Second	Pristane	C19H40	1921-70-6	+	94	39.6	3.13E+08
Vitamin E	First	Hexahydrofarnesyl acetone	C18H36O	502-69-2	–	98	41.5	2.37E+08
Vitamin E	Second	Hexahydrofarnesyl acetone	C18H36O	502-69-2	–	97	41.1	5.05E+08
Vitamin E	Second	Phytol	C20H40O	150-86-7	+	85	42.3	1.57E+08
Coconut	First	Formic acid	CH2O2	64-18-6	+	99	5.4	8.94E+07
Coconut	First	Acetic acid	C2H4O2	64-19-7	+	97	8.4	2.00E+08
Coconut	First	2-Nonanone	C9H18O	821-55-6	+	98	27.4	1.46E+08
Coconut	First	Nonanal	C9H18O	124-19-6	+	99	27.9	1.56E+08
MCT	First	Ethanol	C2H6O	64-17-5	–	99	4.2	4.51E+06
MCT	First	Formic acid	CH2O2	64-18-6	+	98	5.5	1.09E+07
MCT	First	Acetic acid	C2H4O2	64-19-7	+	98	7.9	3.09E+07
MCT	First	2-Nonanone	C9H18O	821-55-6	+	98	27.4	2.12E+07
MCT	First	Nonanal	C9H18O	124-19-6	+	98	27.9	1.22E+07

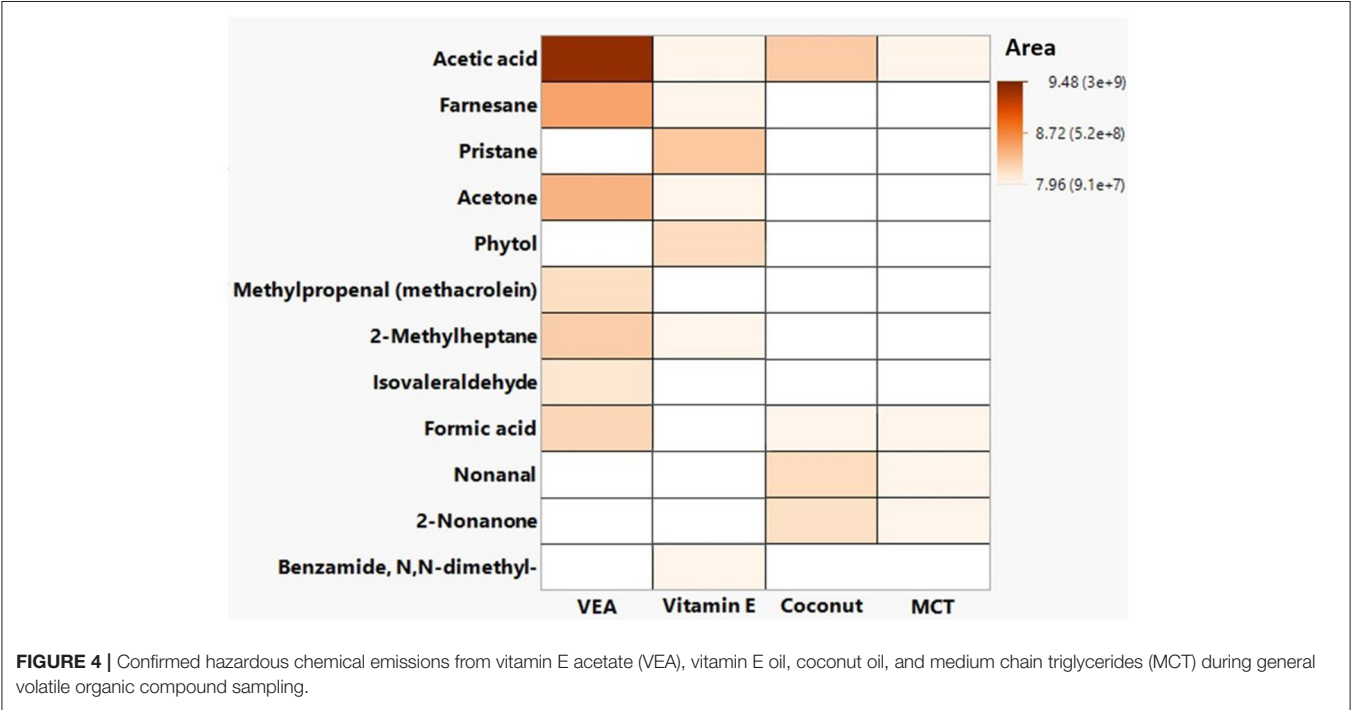
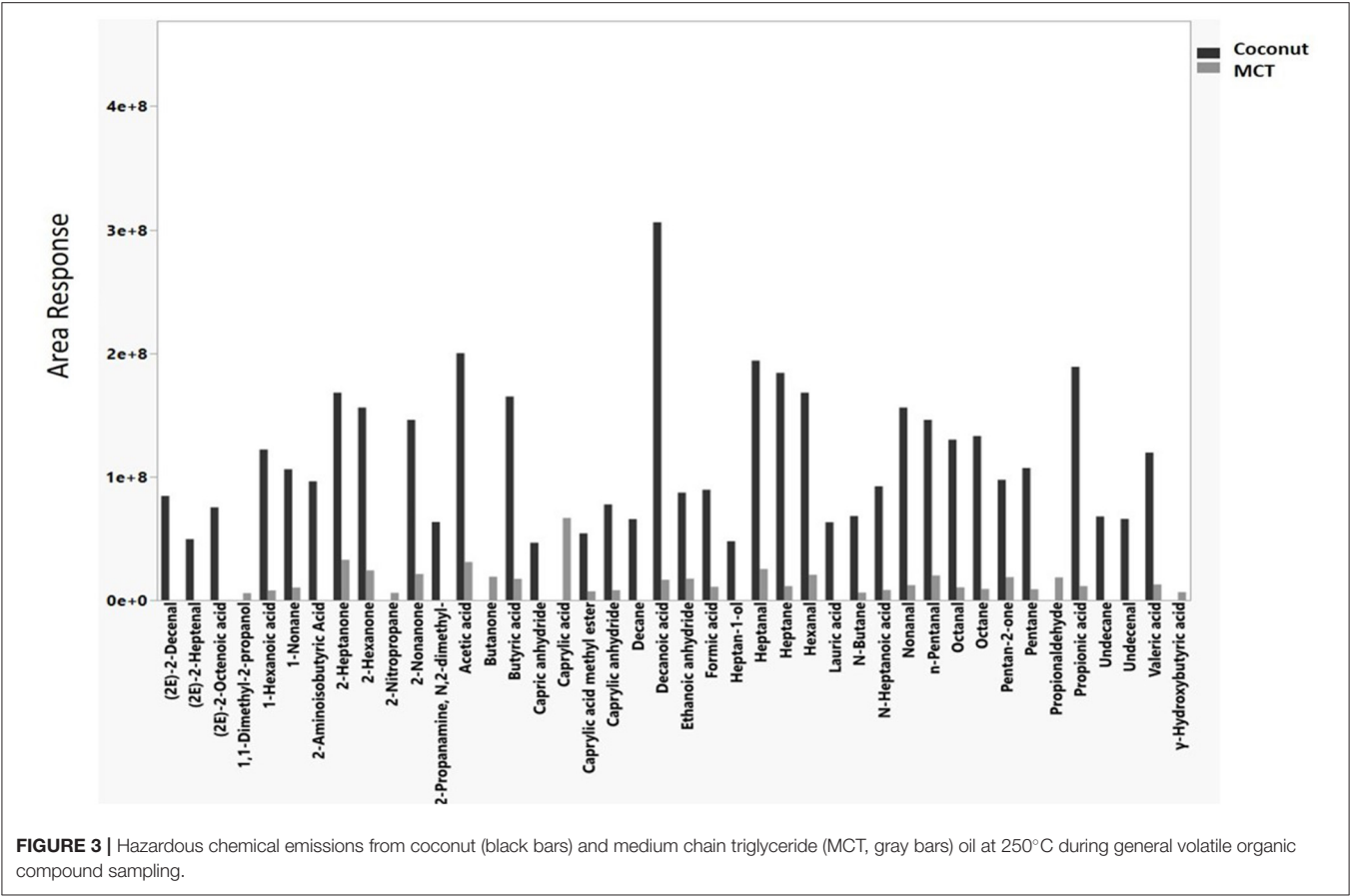
*Hazard assigned to groups using PubChem and Globally Harmonized System (GHS) classification hazard class with the highest hazard class being used for designation (i.e., higher hazard group can include lower hazard class): “–” = Physical hazard only (H220, H225, H226), or environmental hazard only (H400, H410, H411, H412, H413), or no hazards noted in PubChem; “+” = Oral acute toxicity (H301, H302), skin corrosion/irritation (H314, H315, H316), skin sensitization (H317), serious eye damage/eye irritation (H319), respiratory tract irritation or narcotic effects from a single exposure with specific target organ toxicity (H335, H336), germ cell mutagenicity (H340, H341), carcinogenicity (H350, H351), reproductive toxicity (H360D, H360FD, H361, H361d, H361f), and/or repeated exposure with specific target organ toxicity (H372, H373); and “++” = Aspiration hazard (H304) and/or acute inhalation toxicity (H330, H331, H332, or H333). ^aThese low match factors were a result of noisy mass spectrum compared to NIST11 library. ^bIdentified as 1,2-epoxynonadecane, CAS#67860-04-2, in second trial but not confirmed with retention time matching (see **Supplementary Table 3**). Common name taken from Chemspider (<http://www.chemspider.com/>). Area is the component area after mass spectral deconvolution. Match factor is the automated NIST mass spectral quality factor ranging from 0 to 100 with higher numbers indicating a better match with standard spectra. RT is the retention time of the deconvoluted peak in minutes.

were unique to coconut oil and MCT (**Table 1**). Even though coconut and MCT direct analysis of diluted oil revealed the same main constituents, the chemical emissions at 250°C for coconut oil had more hazardous compounds and greater areas (more intense) than MCT (**Figure 3**). A list of hazards associated with compounds identified in emissions for each oil trial at 250°C can be found in **Supplementary Tables 2–8**.

VEA emissions had many chemicals that were unique from the other three oils as can be seen in **Figure 4** and **Table 1**. The unique emissions from heated VEA compared to the other oils included: (1) three chemicals that were identified and confirmed

(methylpropenal, isovaleraldehyde, and 6,10,14-Trimethyl-2-pentadecanone); and (2) 30 chemicals that were identified but not confirmed, of which eight have known hazards associated with exposure (2-hexyl-1-octene, 2-ethylcrotonaldehyde, 3-methylbutanoic acid, 6-methyl-3-heptanol, 3,4-dimethylhex-3-en-2-one, disparture, dodecan-1-ol, and cis-13-octadecanal).

VEA emissions at 250°C were complex in terms of the number and magnitude of chemicals emitted when compared to 150°C and 25°C (**Figure 5**). Increasing the temperature from 25°C to 250°C substantially increased emissions from VEA (as evidenced by increased chromatographic peak area response),



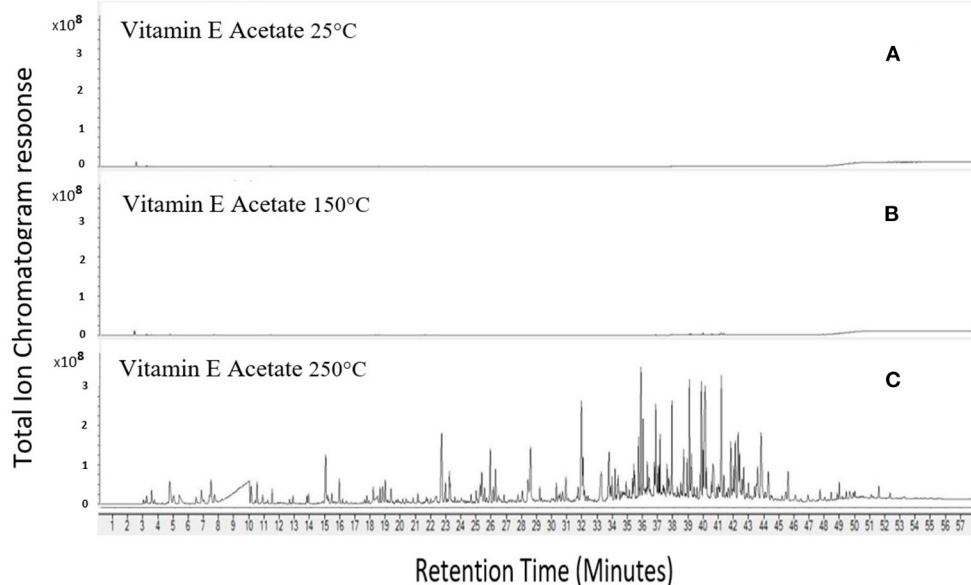


FIGURE 5 | Increasing emissions from VEA with increasing temperature. 25°C (A), 150°C (B), 250°C (C).

Acetic acid peak area response was five times more abundant at 150°C compared to 25°C and 418 times more abundant at 250°C compared to 150°C. Acetone peak area increased by ~580 times from 25°C to 250°C. The other six chemicals positively confirmed by retention time matching (6,10,14-trimethyl-2-pentadecanone; formic acid; methylpropenal; isovaleraldehyde; 2-methylheptane; farnesane) were produced at 250°C but not at 25°C or 150°C. VEA emissions at 250°C were reproducible as evidenced by a similar pattern of chemical peaks between replicate tests (**Figure 6**). On heating VEA at 250°C, we elucidated and confirmed ten hazardous chemicals: acetone, acetic acid, formic acid, methylpropenal, isovaleraldehyde, 2-methylheptane, farnesane, methylglyoxal, diacetyl, and 2-butanone. The latter three chemicals were a direct result of carbonyl testing.

Carbonyl Testing

As with VOC testing results, VEA emissions at 250°C were complex and several oxygenated chemicals were observed when compared to blank air samples (**Figure 7**). Although majority of peaks were not identified in PFBHA-derivatized samples, four carbonyl compounds were elucidated using neat standards: acetone (13.9 min), 2-butanone (16.1 and 16.3 min), methylglyoxal (32.3 and 32.8 min), and diacetyl (33.6 min). Other major carbonyl peaks observed were at 20.6 and 20.7 min ($m/z = 100$), 23.5 and 23.8 min ($m/z = 128$), 24.5 and 24.6 min ($m/z = 98$), and 37.3 min ($m/z = 222$). Using PFBHA as a derivatization agent and subsequently extracting the 181 ion from mass spectra (**Figure 7B**), aids in identifying that peaks observed were attributable to carbonyl species.

Acetone, 2-butanone, methylglyoxal, and diacetyl were not observed in blank air samples collected from micro-chamber,

which indicated that they were generated from oxidation of VEA at 250°C. Additionally, the other major carbonyl species were only observed when VEA was present and heated.

DISCUSSION

In this study, diluent oils were heated to temperatures consistent with wet-through-wick (145–334°C) and full-wet conditions (110–185°C) (11). The temperature at the center of a coil can be up to 100°C hotter than the sides (15). THC-extract oil should be heated between 220 and 230°C to maximize aerosolization and minimize combustion (16). Diluent oils heated to temperatures representative of use in EVPs emitted hazardous chemicals that were a result of volatilization, thermal degradation, or reactions (oxidation or rearrangements). The elucidated chemicals included alcohols, aldehydes, ketones, and saturated and unsaturated hydrocarbons, that when inhaled simultaneously together might have deleterious effects locally on the respiratory system or systemically. We positively identified seven hazardous chemicals emitted from VEA when heated to 250°C that were not present at 150°C, and the emissions were reproducible, although the exposure time to that temperature was longer than typical puff durations ($\sim 3.5 \pm 1.4$ s) observed for nicotine-containing e-cigarettes (17).

Extended temperature exposure times used in this study (i.e., 30 s) could change the heated chemical emissions compared to shorter puff durations typically employed by users. However, several other researchers have observed heated VEA emissions similar to our results. Riordan-Short et al. identified formic acid, acetone, isovaleraldehyde, acetic acid, and methylpropenal (i.e., methacrolein) in the headspace of heated VEA at 300°C (18). Jiang et al. observed thermal decomposition and oxidation

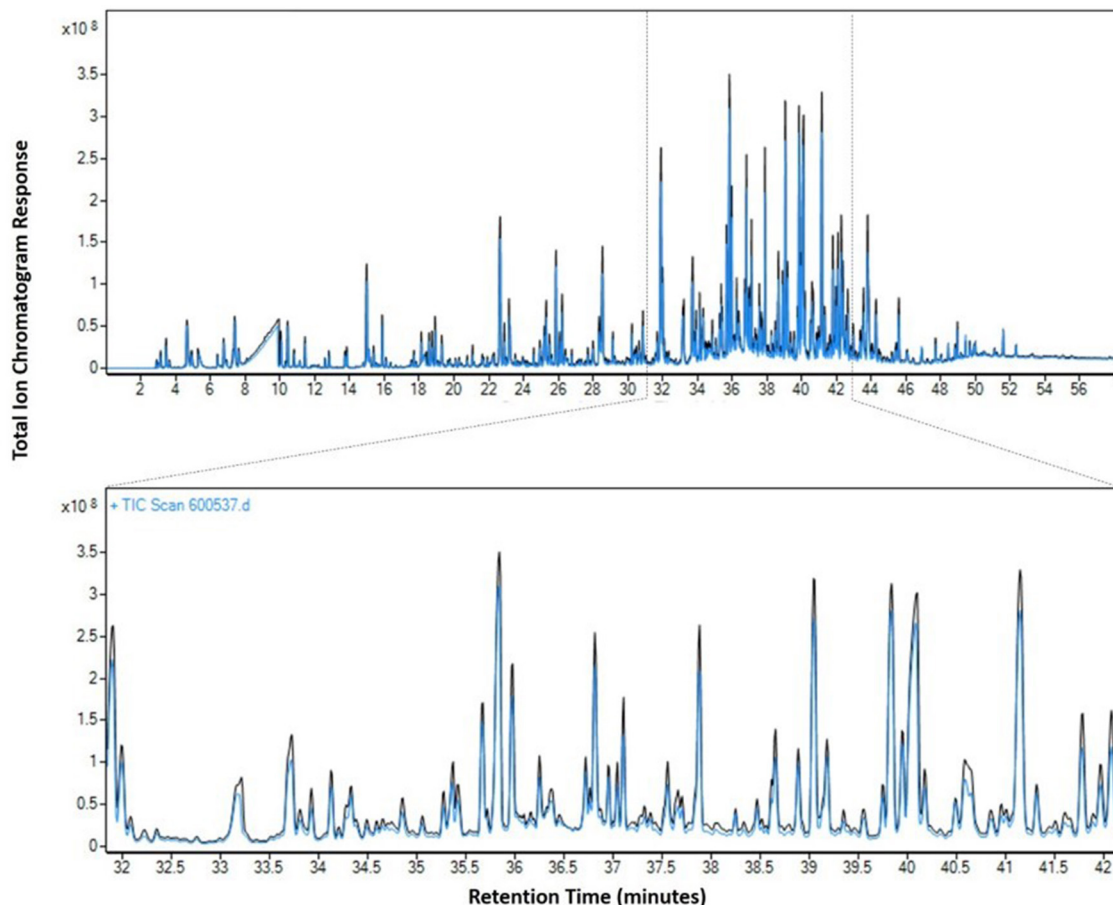


FIGURE 6 | Reproducibility of VEA emissions at 250°C. Blue line = emission test on 11/5/2019 and black line = emission test on 11/14/2019 with a zoomed region from 32 to 42 min.

product formation including carbonyls from aerosolization of oil diluents using a commercially available vape pen (19). Similar to this study, they observed acetone emissions from vitamin E oil and VEA as well as butanone, n-pentanal, 2-pentanone, and 2-heptanone from MCT oil. Mikheev et al. observed duroquinone and 1-pristene, similar to Wu and Shea (13) but also durohydroquinone monoacetate, from direct extraction of vaped VEA condensate using methylene chloride with no derivatization (20). Lynch et al. observed duroquinone, 1-pristene, and durohydroquinone monoacetate in vaped THC/VEA aerosol condensate from commercially available ceramic coil vaping cartridges (12). They used an elevated power setting of 16 W corresponding to 415–476°C at 5 V with temperatures exceeding those used in this study and measured derivatized condensate instead of measuring gas-phase native species of heated emissions as in this study. Direct comparisons to studies of vaped emission condensate that are native or derivatized and collected at different temperatures should be made with caution but data from our study complements information obtained elsewhere. Although we did not observe duroquinone or durohydroquinone in this study, we did observe quinone-containing compounds from vitamin E oil (**Supplementary Table 5**) but not from VEA.

Of note, hexahydrofarnesyl acetone, C₁₈H₃₆O, CAS# 502-69-2 observed in this study is an oxidized form of, and structurally similar to, 1-pristene, a degradation product of VEA, observed elsewhere (12, 20).

Hazards associated with these emitted chemicals included aspiration hazards and/or acute inhalation toxicity. While aspiration hazards can cause acute effects in the lungs, in the context of EVALI, early reports suggested exogenous lipoid pneumonia from aspiration of e-liquids, though subsequently it was shown that this pathology was not consistent with EVALI (21, 22). Rather, lung biopsies of EVALI cases indicated acute lung injury, including organizing pneumonia and/or diffuse alveolar damage, which suggested that inhalation of aerosols is the more likely cause of lung damage (21, 22). The importance of the inhalation pathway in EVALI highlights the importance of understanding the chemistry of emissions from oil diluents. VEA is associated with EVALI (2, 3), though whether the effects are from aerosolized VEA, its gas-phase thermal degradation products, or a combined effect is unclear. In this study, heating VEA to a temperature consistent with user device settings emitted a myriad of gas-phase chemicals (alcohols, aldehydes, ketones, and saturated and unsaturated hydrocarbons) associated

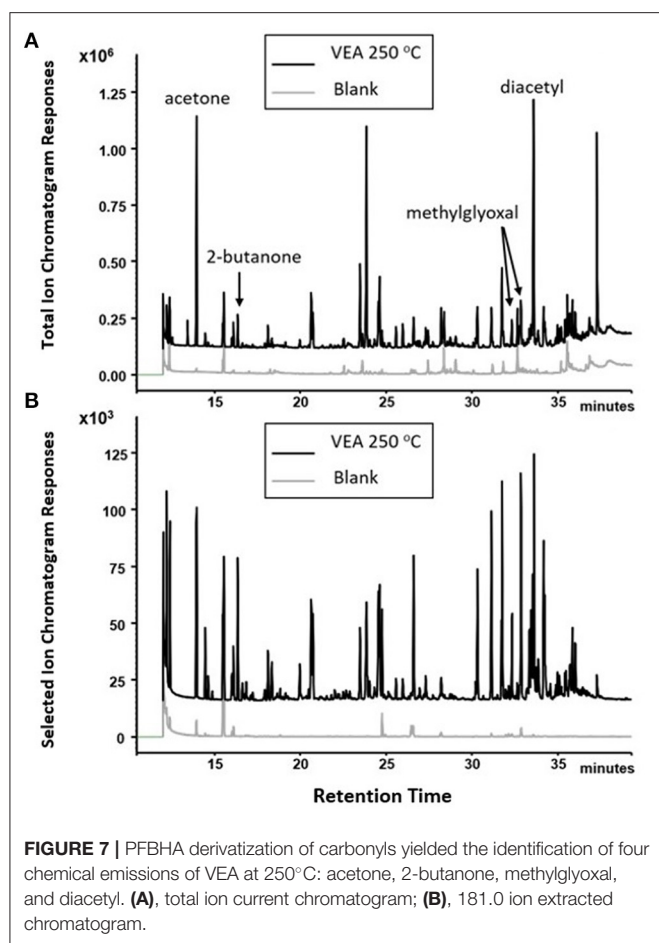


FIGURE 7 | PFBHA derivatization of carbonyls yielded the identification of four chemical emissions of VEA at 250°C: acetone, 2-butanone, methylglyoxal, and diacetyl. (A), total ion current chromatogram; (B), 181.0 ion extracted chromatogram.

with adverse respiratory health effects. None of these compounds individually yield the same pathology observed with EVALI, though whether the combined effects on the respiratory system could contribute to EVALI remains unclear and warrants further investigation. The findings of relatively high emissions of acetone and acetic acid are significant given both are known precursors for ethenone (23). These gas-phase emissions can deposit along the respiratory tract and reach the deep lung (i.e., small airways) depending on partitioning behavior of the chemicals. A portion of these gas-phase compounds will remain partitioned in aerosolized liquid droplets (24) produced by heating VEA. These liquid droplets will deposit in the respiratory tract, and as described in our companion paper, have sub-micron scale, and will therefore predominantly reach the lung alveoli, which is the site of injury of EVALI (25).

Dicarbonyls such as methylglyoxal and diacetyl have been shown to cause adverse respiratory health effects (26). Exposure to methylglyoxal and diacetyl can induce necrosis in lung epithelial cells (26). Furthermore, exposure to diacetyl can induce development of bronchiolitis obliterans, a severe respiratory illness characterized by damage to the small airways proximal to the alveoli (26, 27). In the current study, diacetyl was generated as an emission product when VEA was heated to 250°C, but we did not measure concentrations making it difficult to say whether the concentrations were high enough to invoke damage.

The pathology of bronchiolitis obliterans appears to differ from the pathology of EVALI, the latter which is thought to be chemical pneumonitis, that for most patients, manifested as damage in the alveoli (21, 22, 28). Some EVALI cases with diffuse alveolar damage and organizing pneumonia also had evidence of bronchiolitis (21, 28), but it is unclear if this pathology indicates a possible role for diacetyl.

Given the increase in marijuana use and legalization across the United States, adverse respiratory health effects associated with inhalation of aerosolized oils could continue to be an issue. VEA has been used to dilute THC oil and has been identified in a high proportion (94%) of bronchoalveolar lavage fluid samples from EVALI cases (29). Separate from the recent outbreak in EVALI, a severe case of bronchiolitis has been observed in a Canadian youth who aerosolized THC oil and flavorings, which highlights the need to understand the influence of flavoring components of these complex mixtures (30).

This study had several limitations. First, VEA was heated in an aluminum dish within a heated chamber instead of an aerosolization device designed for inhalation of THC-containing products. Attfield et al. dissected cartridges used for aerosolizing THC-containing products and analyzed the materials chemistry (23). The authors noted that the cartridges contained nickel and chromium coils that were encased in charred oil-soaked, silica ceramic, which is favorable to ethenone formation (but not present in aluminum dishes used in the current study). Consequently, any effects of aerosolized products derived from the device and components used in the devices to aerosolize THC-containing products were not mimicked by our protocol. Second, Lynch et al. observed that unknowns analysis software can incorrectly identify chemicals (12). We attempted to manually investigate the chromatograms to identify compounds but were sometimes unsuccessful because of noisy mass spectra or coeluting peaks. These identities have been assigned based on a mass spectral deconvolution and matching software that might have been influenced by noisy mass spectra from column bleed or by detector saturation. In addition, the TD tube sampling technique did not capture reactive species such as the proposed formation of ketene from pyrolysis of VEA (13). Finally, our work focused only on individual oils used for dilution of THC-containing products and did not examine other THC-based mixtures believed to be involved in EVALI. Muthumalage et al. (10) evaluated a counterfit vape cartridge and reported a significant increase in toxicity from the aerosolized mixture compared with MCT or VEA alone.

CONCLUSION

VEA and other oil diluents emit a wide variety of hazardous chemicals when heated to a temperature mimicking electronic delivery devices for THC-aerosolization. Numerous evolved compounds are known respiratory irritants, acute toxins, and known precursors to highly reactive ketenes. This information provides novel insights for investigators to study chemical toxicity in concert with aerosol deposition modeling to better understand the mechanism(s) of EVALI. This data further supports current Centers for Disease Control and Prevention

(CDC) guidance to not add VEA or any other substances not intended by the manufacturer to the vaping products (1).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

RL and AS contributed to the conception of the study. RL, JH, and AS wrote the first draft of the manuscript. MA, KW, EF, and

JH collected and analyzed the samples. DB and AR consulted on data collection and analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpubh.2021.765168/full#supplementary-material>

REFERENCES

- Centers for Disease Control and Prevention. *Outbreak of Lung Injury Associated With the Use of E-Cigarette, or Vaping, Products*. (2021). Available online at: https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html (accessed June 2021).
- Blount BC, Karwowski MP, Shields PG, Morel-Espinosa M, Valentin-Blasini L, Gardner M, et al. Vitamin E acetate in bronchoalveolar-lavage fluid associated with EVALI. *N Engl J Med*. (2020) 382:697–705.
- Bhat TA, Kalathil SG, Bogner PN, Blount BC, Goniewicz ML, Thanavala YM. An animal model of inhaled vitamin E acetate and EVALI-like lung injury. *N Engl J Med*. (2020) 382:1175–7. doi: 10.1056/NEJMc2000231
- Stefaniak AB, LeBouf RF, Ranpara A, Leonard SS. Toxicology of flavoring- and cannabis-containing e-liquids used in electronic delivery systems. *Pharmacol Ther*. (2021) 2021:107838. doi: 10.1016/j.pharmthera.2021.107838
- Agricultural Improvement Act of. Public Law 115-334 (2018). <https://uscode.house.gov/statutes/pl/115/334.pdf> (accessed June 2021).
- National Survey of Drug Use Health. *Results from the 2018 national survey on drug use health: detailed tables*. Substance Abuse and Mental Health Services Administration Center for Behavioral Health Statistics and Quality, Rockville, Maryland, United States. Available Online at: <https://www.samhsa.gov/data/sites/default/files/cbhsq-reports/NSDUHDetailedTabs2018R2/NSDUHDetailedTabs2018.pdf> (accessed June 15, 2020).
- Trivers KE, Gentzke AS, Phillips E, Tynan M, Marynak KL, Schauer GL. Substances used in electronic vapor products among adults in the United States, 2017. *Addict Behav Rep*. (2019) 10:100222. doi: 10.1016/j.abrep.2019.100222
- Perrine C, Pickens C, Boehmer T, King B, Jones C, Desisto C, et al. Characteristics of a multistate outbreak of lung injury associated with e-cigarette use, or vaping - United States, 2019. *Morb Mortal Wkly Rep*. (2019) 68:860–4. doi: 10.15585/mmwr.mm6839e1
- Guo W, Vrdoljak G, Liao V-C, Moezzi B. Major constituents of cannabis vape oil liquid, vapor and aerosol in California vape oil cartridge samples. *Front Chem*. (2021) 9:694905. doi: 10.3389/fchem.2021.694905
- Muthumalage T, Friedman MR, McGraw MD, Ginsberg G, Friedman AE, Rahman I. Chemical constituents involved in e-cigarette, or vaping product use-associated lung injury (EVALI). *Toxics*. (2020) 8:25. doi: 10.3390/toxics8020025
- Chen W, Wang P, Ito K, Fowles J, Shusterman D, Jaques PA, et al. Measurement of heating coil temperature for e-cigarettes with a “top-coil” clearomizer. *PLoS ONE*. (2018) 13:e0195925. doi: 10.1371/journal.pone.0195925
- Lynch J, Lorenz L, Brueggemeyer JL, Lanzarotta A, Falconer TM, Wilson RA. Simultaneous temperature measurements and aerosol collection during vaping for the analysis of δ 9-tetrahydrocannabinol and vitamin E acetate mixtures in ceramic coil style cartridges. *Front Chem*. (2021) 9:643. doi: 10.3389/fchem.2021.734793
- Wu D, O’Shea DF. Potential for release of pulmonary toxic ketene from vaping pyrolysis of vitamin E acetate. *Proc Natl Acad Sci USA*. (2020) 117:6349–55. doi: 10.1073/pnas.1920925117
- National Institute for Occupational Safety and Health. Pocket Guide to Chemical Hazards (2021). Available online at: <http://www.cdc.gov/niosh/npg/default.html> (accessed June 2021).
- Dibaji SAR, Guha S, Arab A, Murray BT, Myers MR. Accuracy of commercial electronic nicotine delivery systems (ENDS) temperature control technology. *PLoS ONE*. (2018) 13:e0206937. doi: 10.1371/journal.pone.0206937
- Troutt WD, Didonato MD. Carbonyl compounds produced by vaporizing cannabis oil thinning agents. *JACM*. (2017) 23:879–84. doi: 10.1089/acm.2016.0337
- St. Helen G, Havel C, Dempsey D, Jacob P, Benowitz N. Nicotine delivery, retention and pharmacokinetics from various electronic cigarettes. *Addiction*. (2016) 111:535–44. doi: 10.1111/add.13183
- Riordan-Short S, Nguyen TD, Sagar N, Noestheden M, O’Brien R. *Thermal Decomposition of Vitamin E Acetate in a Surrogate Vaping Environment. Thermo-Scientific Customer Application Note 73327*. (2020). Available online at: <https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/can-73327-thermal-decomposition-vitamin-e-acetate-can73327-en.pdf> (accessed June 2021).
- Jiang H, Ahmed CMS, Martin TJ, Canchola A, Oswald IWH, Garcia JA, et al. Chemical and toxicological characterization of vaping emission products from commonly used vape juice diluents. *Chem Res Toxicol*. (2020) 33:2157–63. doi: 10.1021/acs.chemrestox.0c00174
- Mikheev VB, Klupinski TP, Ivanov A, Lucas EA, Strozier ED, Fix C. Particle size distribution and chemical composition of aerosolized vitamin E acetate. *Aerosol Sci Technol*. (2020) 54:993–8. doi: 10.1080/02786826.2020.1783431
- Reagan-Steiner S, Gary J, Matkovic E, Ritter JM, Shieh WJ, Martinez RB, et al. Pathological findings in suspected cases of e-cigarette, or vaping, product use-associated lung injury (EVALI): a case series. *Lancet Res*. (2020) 8:1219–32. doi: 10.1016/S2213-2600(20)30321-0
- Mukhopadhyay S, Mehrad M, Dammert P, Arrossi AV, Sarda R, Brenner DS, et al. Lung biopsy findings in severe pulmonary illness associated with e-cigarette use (vaping). *Am J Clin Pathol*. (2019) 153:30–9. doi: 10.1093/ajcp/aqz182
- Attfield KR, Chen W, Cummings KJ, Jacob P, O’Shea DF, Wagner J, et al. Potential of ethenone (Ketene) to contribute to electronic cigarette, or vaping, product use-associated lung injury. *Am J Respir Crit Care Med*. (2020) 202:1187–9. doi: 10.1164/rccm.202003-0654LE
- Pankow JF. Calculating compound dependent gas-droplet distributions in aerosols of propylene glycol and glycerol from electronic cigarettes. *J Aerosol Sci*. (2017) 107:9–13. doi: 10.1016/j.jaerosci.2017.02.003
- Ranpara A, Stefaniak AB, Williams K, Fernandez E, LeBouf RF. Modeled respiratory tract deposition of aerosolized oil diluents used in 19-THC-based electronic cigarette liquid products. *Front. Public Health*. (2021) 9:744166. doi: 10.3389/fpubh.2021.744166
- Hubbs AF, Kreiss K, Cummings KJ, Fluharty KL, O’Connell R, Cole A, et al. Flavorings-related lung disease: a brief review and new mechanistic data. *Toxicol Pathol*. (2019) 47:1012–6. doi: 10.1177/0192623319879906
- Cichocki JA, Morris JB. Inhalation dosimetry modeling provides insights into regional respiratory tract toxicity of inhaled diacetyl. *Toxicology*. (2017) 388:30–39. doi: 10.1016/j.tox.2016.11.007

28. Butt YM, Smith ML, Tazelaar HD, Vaszar LT, Swanson KL, Cecchini MJ, et al. Pathology of vaping-associated lung injury. *N Engl J Med.* (2019) 381:1780–81. doi: 10.1056/NEJMc1913069
29. Blount BC, Karwowski MP, Morel-Espinosa M, Al. E. Evaluation of bronchoalveolar lavage fluid from patients in an outbreak of e-cigarette, or vaping, product use – associated lung injury – 10 States, August–October 2019. *Morb Mortal Wkly Rep.* (2019) 68:1040–1. doi: 10.15585/mmwr.mm6845e2
30. Landman ST, Dhaliwal I, Mackenzie CA, Martinu T, Steele A, Bosma KJ. Life-threatening bronchiolitis related to electronic cigarette use in a Canadian youth. *Can Med Assoc J.* (2019) 191:E1321–31. doi: 10.1503/cmaj.191402

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Influence of E-Liquid Humectants, Nicotine, and Flavorings on Aerosol Particle Size Distribution and Implications for Modeling Respiratory Deposition

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Electronic cigarette, or vaping, products are used to heat an e-liquid to form an aerosol (liquid droplets suspended in gas) that the user inhales; a portion of this aerosol deposits in their respiratory tract and the remainder is exhaled, thereby potentially creating opportunity for secondhand exposure to bystanders (e.g., in homes, automobiles, and workplaces). Particle size, a critical factor in respiratory deposition (and therefore potential for secondhand exposure), could be influenced by e-liquid composition. Hence, the purposes of this study were to (1) test the influence of laboratory-prepared e-liquid composition [ratio of propylene glycol (PG) to vegetable glycerin (VG) humectants, nicotine, and flavorings] on particle size distribution and (2) model respiratory dosimetry. All e-liquids were aerosolized using a second-generation reference e-cigarette. We measured particle size distribution based on mass using a low-flow cascade impactor (LFCI) and size distribution based on number using real-time mobility sizers. Mass median aerodynamic diameters (MMADs) of aerosol from e-liquids that contained only humectants were significantly larger compared with e-liquids that contained flavorings or nicotine ($p = 0.005$). Humectant ratio significantly influenced MMADs; all aerosols from e-liquids prepared with 70:30 PG:VG were significantly larger compared with e-liquids prepared with 30:70 PG:VG ($p = 0.017$). In contrast to the LFCI approach, the high dilution and sampling flow rate of a fast mobility particle sizer strongly influenced particle size measurements (i.e., all calculated MMAD values were < 75 nm). Dosimetry modeling using LFCI data indicated that a portion of inhaled particles will deposit throughout the respiratory tract, though statistical differences in aerosol MMADs among e-liquid formulations did not translate into large differences in deposition estimates. A portion of inhaled aerosol will be exhaled and could be a source for secondhand exposure. Use of laboratory-prepared e-liquids and a reference e-cigarette to standardize aerosol generation and a LFCI to measure particle size distribution without dilution represents an improved method to characterize physical properties of volatile aerosol particles and permitted determination of MMAD values more representative of e-cigarette aerosol *in situ*, which in turn, can help to improve dose modeling for users and bystanders.

Keywords: electronic cigarette, vaping, secondhand or passive exposure, occupational exposure, cascade impactor

INTRODUCTION

Electronic cigarette, or vaping, products (e-cigarettes) heat a liquid (e-liquid) to form an aerosol that the user inhales. Since their introduction, the internal design and external appearance of e-cigarettes has evolved continuously (1–3). Currently, there are four “generations” of e-cigarettes that differ in external appearance and internal design (4, 5); however, all generations have in common: (1) a battery, (2) a chamber that contains a heating coil, (3) a cartridge to store the e-liquid, and (4) a mouthpiece through which the user inhales (6). When the user inhales through the device, the battery heats the coil that is wrapped in a wick, which is wetted with e-liquid from the cartridge, and vaporizes the e-liquid; as it travels to the mouthpiece, the vapor cools and condenses to form an aerosol (liquid droplets suspended in gas) that is delivered to the respiratory tract (7, 8).

E-liquids are composed of humectants, and sometimes water, ethanol, flavorings, and/or nicotine (9). Humectants are hygroscopic substances that retain moisture and constitute the main ingredients in e-liquids (10, 11). Propylene glycol (PG) and/or vegetable glycerin (VG) are the most common humectants, and their relative proportions in an e-liquid can be tailored to the user's personal experiences and preferences (2, 7, 10). Water and ethanol are added to the humectants as diluents for flavorings (11). Flavorings are added to the e-liquid to impart taste and aromas to the inhaled aerosol (11–14). Nicotine, when present, is in either the free-base (basic pH ~8–10) or salt (acidic pH) form; e-liquids used in third and prior generation e-cigarettes contained up to 95% of their total nicotine in free-base form (15), whereas e-liquids for fourth generation e-cigarettes contain nicotine in the acidic salt form (16–18).

Upon inhalation of aerosol generated by an e-cigarette, a portion of the particles (liquid droplets) and gases deposit throughout the respiratory tract of the user and the remainder is exhaled (19, 20). This exhaled portion creates potential for secondhand exposures among persons in proximity to e-cigarette users. Secondhand exposures can occur in home environments (5, 21–23) and in occupational environments that span a range of industries such as hospitality venues (e.g., convention centers), bars, restaurants, and nightclubs as well as businesses adjacent to e-cigarette retail stores that permit their use indoors (24–29). E-cigarette aerosol that settles onto surfaces in homes, vehicles, or workplaces can serve as a source of dermal exposure from skin contact with residues (30, 31).

Particle size, in part, influences where e-cigarette aerosol liquid droplets will deposit in the respiratory tract (8, 19, 32–35). Hence, understanding factors that influence particle size distribution (PSD) of aerosol generated by e-cigarettes are needed to model dosimetry. These factors can be broadly categorized as consumable-related (e.g., e-liquid chemical composition), device-related (e.g., e-cigarette power setting), and user-related (e.g., puff topography). The focus of the current research is on the influence of consumable-related factors on aerosol PSD, specifically humectants, nicotine, and flavorings. Literature on the influence of these e-liquid constituents on PSD are conflicting. Some studies reported that particle size decreased

as the percentage of PG in the e-liquid increased (4, 7, 33, 36–38), whereas other studies indicated that PSD increased as the percentage of PG in the e-liquid increased (39), or was not influenced by the ratio of PG and VG in the e-liquid (35, 40). The presence of nicotine in e-liquids is reported to increase particle size (33, 41, 42), decrease particle size (41), or have no effect (8, 43–47). Several studies reported that aerosol characteristics were not influenced by the presence of flavorings in e-liquids (8, 33, 35, 45, 47, 48), though one study reported that the presence of vanillin drastically increased particle size. Note that this inter-comparison of reviewed literature results is difficult because observed inconsistencies could reflect that the commercially available e-liquids contained undisclosed ingredients or ingredients with unknown purity that influenced PSD and/or that other critical factors that influenced PSD were not consistent among prior studies.

The primary purpose of this study was to evaluate the hypothesis that e-liquid composition (humectant ratio, flavorings, and nicotine) influenced aerosol particle size. To test this hypothesis, laboratory-prepared e-liquids and a reference second-generation e-cigarette were used to generate aerosols. A cascade impactor was used to measure the mass-based aerosol PSD and real-time instruments were used to monitor number-based PSD during puffing. The secondary purpose of this work was to use the PSD data to model the regional deposition of aerosol particles in the respiratory tract of e-cigarette users. An estimate of the exhaled fraction of aerosol was derived from these modeling results as an indicator of potential for secondhand exposure.

MATERIALS AND METHODS

Existing literature indicated that the PG:VG humectant ratio of e-liquids can range from 100:0 to 0:100 (9, 49). PG:VG ratios of 70:30 and 30:70 are commonly used to evaluate size distribution (33) and toxicity (50) of e-cigarette aerosols. An analysis of 800 commercially available e-liquids indicated that the total volume fraction of water and ethanol never exceeded 10% in the products, i.e., either 10% water, 10% ethanol, or 5% water and 5% ethanol (11). E-liquids for third and prior generation e-cigarettes contained ~1–4% (10–40 mg/mL) flavorings and 0.6–3% nicotine (6–30 mg/mL), though current fourth generation e-cigarettes can contain 5–7% (50–70 mg/mL) nicotine (51, 52). This wide variability in composition (and ambiguity of ingredient purity and presence of undisclosed ingredients) of commercially available e-liquids can present challenges for experimental studies to elucidate the influence of specific constituents on aerosol particle size. As such, for experimental investigations, “model” or “reference” e-liquids with proportions that mimic commercially available products that are prepared in the laboratory under controlled conditions are useful surrogates (recognizing that an ideal laboratory reference e-liquid has less “real-world” generalizability than commercially available products) (11, 53, 54). Hence, for the current study, we chose to use laboratory-prepared e-liquids composed of varying ratios of humectants with water and

ethanol and/or flavorings and nicotine in accordance with the Association Française de Normalization (AFNOR) standard D90-300-2 (55). The sources and purity of reagents were as follows: PG (ACS grade, CAS# 57-55-6, Fisher Scientific, Pittsburgh, PA, USA), VG (Certified ACS grade, CAS# 56-81-5, Fisher Scientific), 200 proof ethanol (ACS/USP grade, CAS# 64-17-5, Pharmaco-Aaper, Brookfield, CT), nicotine (>99% GC grade liquid, CAS# 54-11-5), vanillin (99%, CAS# 121-33-5), 3-methyl-1-butanol (99%, CAS# 123-51-3), 2-methylbutyric acid (98%, CAS# 1730-91-2), 2,3-butanedione (99%, CAS# 431-03-8), 2,3-pentanedione (97%, CAS# 600-14-6), and 2,3-hexanedione (99%, CAS# 3848-24-6) flavorings (all from ACROS Organics™, Geel, Belgium). Two proportions of PG and VG humectants (30:70, 70:30) with 18 milli-Ohm (MΩ) water (1% w/w) and 200 proof ethanol (1% w/w) but no nicotine or flavorings were prepared gravimetrically using a microbalance with mass resolution of 0.1 mg (XS 250, Mettler-Toledo LLC, Columbus, OH, USA). Each e-liquid was vortexed for 1 min to mix. To evaluate the influence of nicotine on PSD, the humectants e-liquids were also prepared with nicotine (2.4% w/w). To evaluate the influence of flavorings on PSD, 1% (w/w) of each flavoring (vanillin, 3-methyl-1-butanol, 2-methylbutyric acid, 2,3-butanedione, 2,3-pentanedione, and 2,3-hexanedione) was dissolved in PG then diluted with VG to achieve PG:VG ratios of 30:70 and 70:30; the final concentration of each flavoring in the e-liquids prepared in this manner was 0.3% w/w. E-liquids prepared with nicotine or flavorings were homogenized for 1 h using a rotator (Model 4152110, Thermo Scientific, Dubuque, IA) to mix.

Aerosol Generation

To achieve comparable and repeatable aerosol generation, an automated E-cigarette Aerosol Generator (ECAG; e ~ Aerosols, LLC, Central Valley, NY, USA) was used to control power delivered to the e-cigarette heating coil and maintain a consistent coil temperature. The ECAG works on positive pressure to aerosolize an e-liquid at a user-defined puff topography by heating the coil at 3.7 V (set) and 1.6 A (measured) of electric current. Six measurement trials were performed for each e-liquid. For each trial, 1.2 mL of e-liquid was added to the chamber (tank) of an NJOY top tanks (NJOY, Inc., Scottsdale, AZ) second-generation reference e-cigarette from the NIDA Drug Supply Program (56). A separate new NJOY chamber was used for each e-liquid formulation (i.e., one new NJOY chamber was used for all six trials with 30:70 PG:VG, a separate new chamber was used for all trials with 70:30 PG:VG, and so on). The puff topography was set to 55 ml puff volume for 3 s (1 puff) with a 30 s puff delay. For each e-liquid, 2 puffs were generated per trial to measure the mass-based PSD using a low flow cascade impactor (LFCI) and ~30 puffs were generated per trial to measure the number-based PSD using mobility analyzers (57). The mass of e-liquid in the e-cigarette chamber was weighed on the microbalance prior to and after each trial.

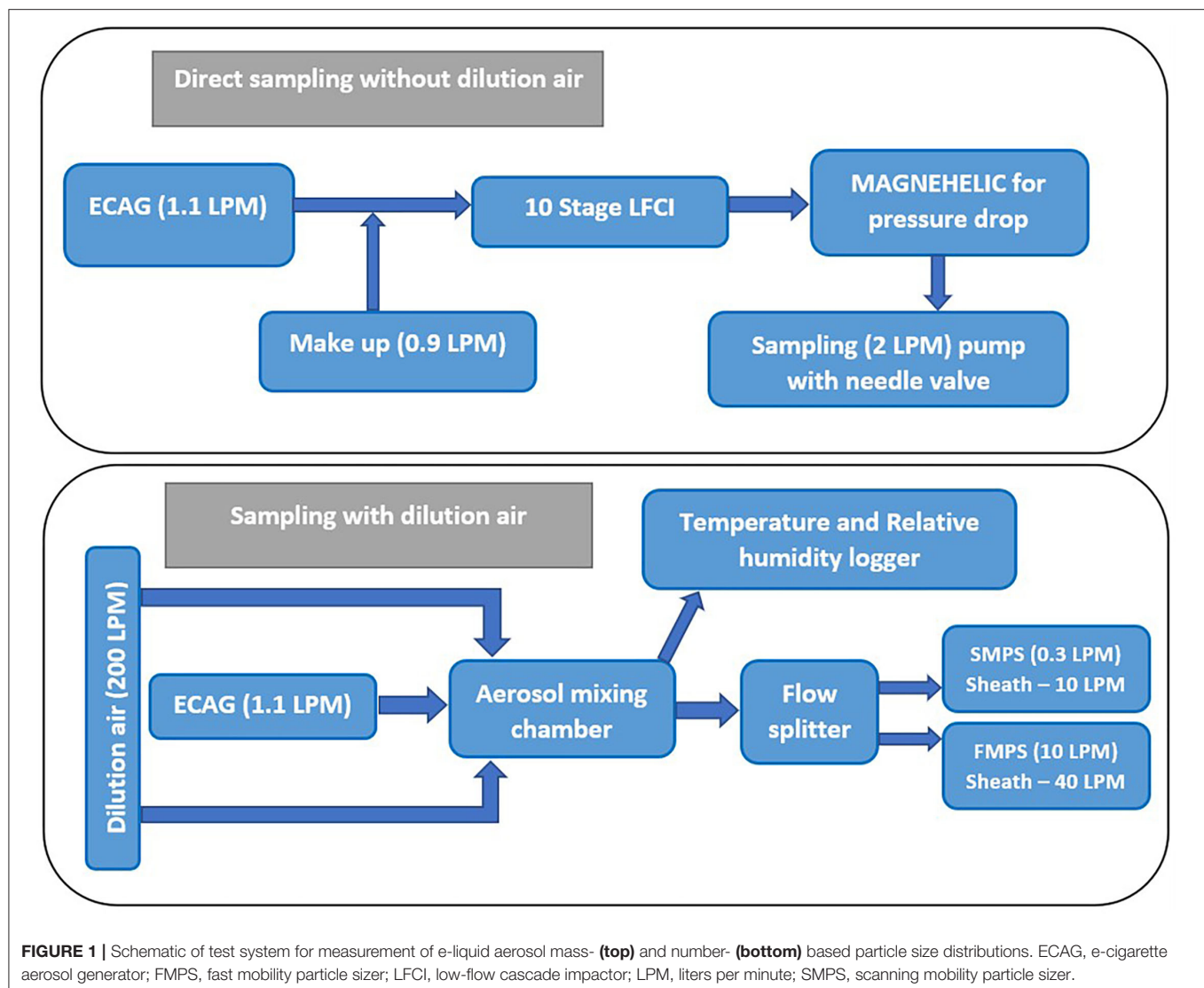
Aerosol Characterization

Accurate measurement of e-cigarette aerosol characteristics (mass, number, size) is challenging because (1) a high droplet

number concentration is generated during each puff, (2) some droplets contain constituents that are highly volatile, and (3) humectants are hygroscopic (8, 19, 36, 43, 58–60). Given the presence of volatile constituents, e-cigarette aerosol properties can change because of evaporation within aerosol sampling instruments, which in turn, can significantly distort PSD measurements (61). Hence, for e-cigarettes, the choice of measurement approach is an important consideration in the experimental design. To date, various approaches have been utilized and included real-time instruments and cascade impactors (4, 7, 8, 36, 58, 62, 63). Real-time instruments such as mobility sizers operate at high sampling flow rates and often require dilution of the aerosol to bring the number concentration within the measurement range of the instrument; both high sampling flow rate and high dilution can promote evaporation of aerosol droplets, thereby introducing bias into PSD measurements (7, 8, 35, 36, 63). In contrast, cascade impactors are generally not affected by high particle number concentrations, and if a low-flow impactor is used for aerosol collection and its sampling flow rate is closely matched to the e-cigarette puff flow rate, evaporative losses from dilution can be minimized, and PSD determined more accurately. Additionally, since the impactor stages must be analyzed gravimetrically, further losses of very volatile constituents can be minimized by quickly measuring mass or by applying an experimentally derived correction factor (4, 7, 8, 35, 36, 58, 63). For additional details on the relative advantages and disadvantages of sampling e-cigarette aerosols, the reader is referred to prior literature (8, 35, 64). For the purposes of this study, a LFCI was used to characterize aerosol mass-based size distribution to understand the influence of e-liquid constituents on PSD and to model aerosol deposition in the respiratory tract. Real-time mobility sizers were used to measure aerosol number concentration and the data used to calculate PSD values for comparison to the impactor results.

Determination of Mass-Based Aerosol Particle Size Distribution

A LFCI (MiniMOUDI™, TSI Incorporated, Shoreview, MN, USA) with 37-mm diameter aluminum collection substrates (Fisher Scientific) was used to size-separate the aerosol generated by the e-cigarette into 10 size fractions (d_{50} cut-points = 0.056, 0.1, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2, 5.6, and 10 μ m) at the default sampling flow rate of 2 liters/min (LPM). Prior to sampling, the NJOY e-cigarette was filled with 1.2 mL of e-liquid and puffed for 10 min to condition the e-cigarette and ECAG system. Aerosol from each e-liquid was sampled directly into the LFCI without further dilution by connecting the inlet of the impactor to the e-cigarette mouthpiece using flexible, black conductive silicone tubing that was 70 cm long with 0.5 cm (inner diameter) to minimize aerosol wall losses. To maintain a constant sampling flow rate of 2 LPM for the cascade impactor, a high efficiency particulate air (HEPA)-filtered air bypass was used to provide 0.9 LPM of laboratory air during puffing with 1.1 LPM air per puff provided by the ECAG. During the inter-puff interval, the same calibrated sampling pump provided 2 LPM air flow from the HEPA-filtered bypass to the impactor (Figure 1). After



the last puff of a trial, the mass of aerosol collected on each pre-weighed aluminum substrate was quickly determined using a microbalance (XS 250, Mettler-Toledo) within minutes of sampling to minimize evaporation. For each trial, the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated from the log-transformed gravimetric measurements of sample mass collected on each stage of the LFCI using a probit model as described previously (65).

To verify that the LFCI impactor flow rate and gravimetric weighing procedure did not substantially bias PSD determinations, a mass loss experiment was performed ($n = 3$ trials) in which three puffs per e-liquid were collected with the impactor and masses were measured 1, 5, and 15-min post-collection all while 2 LPM of air flowed through the impactor. For purposes of this experiment, only the masses of e-liquid aerosol collected on the impactor stages with d_{50} cut-points of 0.32, 0.56, and 1 μm were measured as these stages accounted for more than 89% of the mass deposited in the impactor for

all e-liquids and trials. This observation that most mass was limited to a few impactor stages was consistent with Baassiri et al. who reported that 76% of the aerosol mass collected in their study was on LFCI stages with d_{50} cutoffs of 0.5–2.5 μm (7). For each e-liquid, the total mass loss among the three impactor stages combined did not exceed 10% (**Supplementary Figure 1**). Therefore, no correction factor was applied to the MMAD estimates for each e-liquid formulation.

Determination of Number-Based Aerosol Particle Size Distribution

A scanning mobility particle sizer spectrometer (SMPS; Model 3080, TSI Inc., Shoreview, MN, USA) and fast mobility particle sizer spectrometer (FMPS; Model 3091, TSI Inc.) were used to continuously measure mobility size during e-liquid aerosolization. The SMPS is capable of measuring particles in the size range 2–1,000 nm in 128 channels with scan time of 195 s at a sampling flow rate of 0.3 LPM and sheath flow rate

of 10 LPM. The FMPS is capable of measuring particles with size 5.6–560 nm in 32 channels on a second-by-second basis at a sampling flow rate of 10 LPM and sheath flow rate of 40 LPM. To permit inter-comparison of data, the measurement size range of the SMPS was truncated to 6–560 nm to match the FMPS and the integrating time for the FMPS was adjusted to 195 s to match the SMPS. The aerosol count median diameter (CMD) for each trial was obtained from the instrument software. Prior to measuring e-cigarette aerosol, the inlet of each instrument was connected to a high-efficiency particulate air filter (HEPA-CAP™ 36, GE Healthcare Whatman™, Fisher Scientific) to verify operability. To bring the aerosol number concentration within the measurement range of these instruments, the aerosol generated by the ECAG was diluted with ~200 LPM of HEPA-filtered air. Using a flow splitter, the SMPS and FMPS were connected to the diluter and sampled air for at least 5 min to measure background particle concentrations, next the ECAG was turned on and the e-cigarette with an e-liquid was puffed (same topography as for mass measurements) for 1 h, followed by post-puffing background air monitoring for 5 min. Six trials were conducted for each e-liquid per mobility sizer instrument. The mass median diameter (MMD) was calculated from the average CMD value for each e-liquid trial using the Hatch-Choate equation (Equation 1), moment average, $b = 3$, and geometric standard deviation, σ_g :

$$\text{MMD} = \text{CMD} \cdot \exp\left(b \cdot \ln^2 \sigma_g\right). \quad (1)$$

Values of the MMAD were calculated by multiplying the MMD by the square root of the weighted average density of humectants in the e-liquids (70:30 PG:VG = 1.11 g/cm³, 30:70 PG:VG = 1.19 g/cm³).

Using Equation 2 below, the mass of aerosol collected per LFC stage (M_{stage}), in units of $\mu\text{g}/\text{cm}^3$ was calculated as the difference in mass of the aluminum substrate after sampling (M_{post}) compared with its mass before sampling (M_{pre}) divided by average ECAG sampling volume (V_{ECAG}). The total aerosol mass concentration for each e-liquid formulation was calculated by summing the calculated concentration values for all impactor stages for a trial.

$$M_{\text{stage}} = \frac{M_{\text{post}} (\text{mg}) - M_{\text{pre}} (\text{mg})}{V_{\text{ECAG}} (\text{cm}^3)} \cdot \frac{1000 \mu\text{g}}{\text{mg}} \quad (2)$$

Dose Modeling

Knowledge of particle size, coupled with physiological data on respiratory tract characteristics (anatomy, ventilation parameters), can be used to model dosimetry for e-cigarette users. When known, the PSD of exhaled aerosol from e-cigarette users can be used to model dosimetry for bystanders. Several models exist for estimating particle deposition throughout the respiratory tract, including computational fluid dynamics (CFD) approaches, the International Commission for Radiological Protection (ICRP) human respiratory tract model, and the multiple path particle dosimetry model (MPPD) (34). Among these models, the MPPD model is based on realistic

lung geometry, physiology and deposition mechanisms, and it provides estimates of both the whole-lung and the regional particle deposition fractions that were validated with experimental data (34). Hence, for our purposes, the freely available MPPD model (version 3.04, ARA) was used to conceptually estimate regional and total particle deposition throughout the respiratory tract of e-cigarette users and derive an estimate of the exhaled particle fraction.

The mass fraction of particles that could deposit in the head (H), tracheobronchial (TB), and pulmonary (P) regions were estimated using in MPPD using the Yeh/Schum symmetric lung model for an oronasal-mouth breather. This model was chosen rather than oral-only inhalation because available evidence indicates histological changes in the nasal cavity epithelial lining and oral mucosal damage among e-cigarette users (6, 66, 67). **Supplementary Table 1** summarizes the details of the physiological parameters used for particle deposition modeling. The total amount of particles that could deposit in the respiratory tract was calculated by summing the H, TB, and P regional deposition fractions. The mass fraction of particles that could be exhaled by the e-cigarette user was conceptually estimated as 1—total deposited fraction, to provide a rough indicator of secondhand exposure potential (68).

It is important to note that the CFD, ICRP, and MPPD models are intended for reasonably diluted non-volatile particles; however, aerosol generated by e-cigarettes do not meet this condition because the high number concentration produced by a puff behaves as a “cloud” or bolus and droplets may change in size *via* coagulation during mouth hold and/or absorption of water, conductive heat and diffusive/convective vapor transport, and dilution/mixing as they travel throughout the respiratory tract, which in turn will affect estimates of the amount deposited in a given region (19, 20, 59). Hence, estimates of particle deposition in the respiratory tract and subsequent exhalation fraction provided herein are intended only to illustrate these concepts and the numerical values reported should be interpreted with caution.

Statistical Analyses

Data acquired from LFCI trials ($n = 6$ per e-liquid type) were compared using least squares linear regression models and Tukey's HSD to account for multiple comparisons. Statistics were computed using JMP 13.0 and SAS 9.4 (SAS Institute Inc., Cary, NC) at $\alpha = 0.05$ as the level of significance.

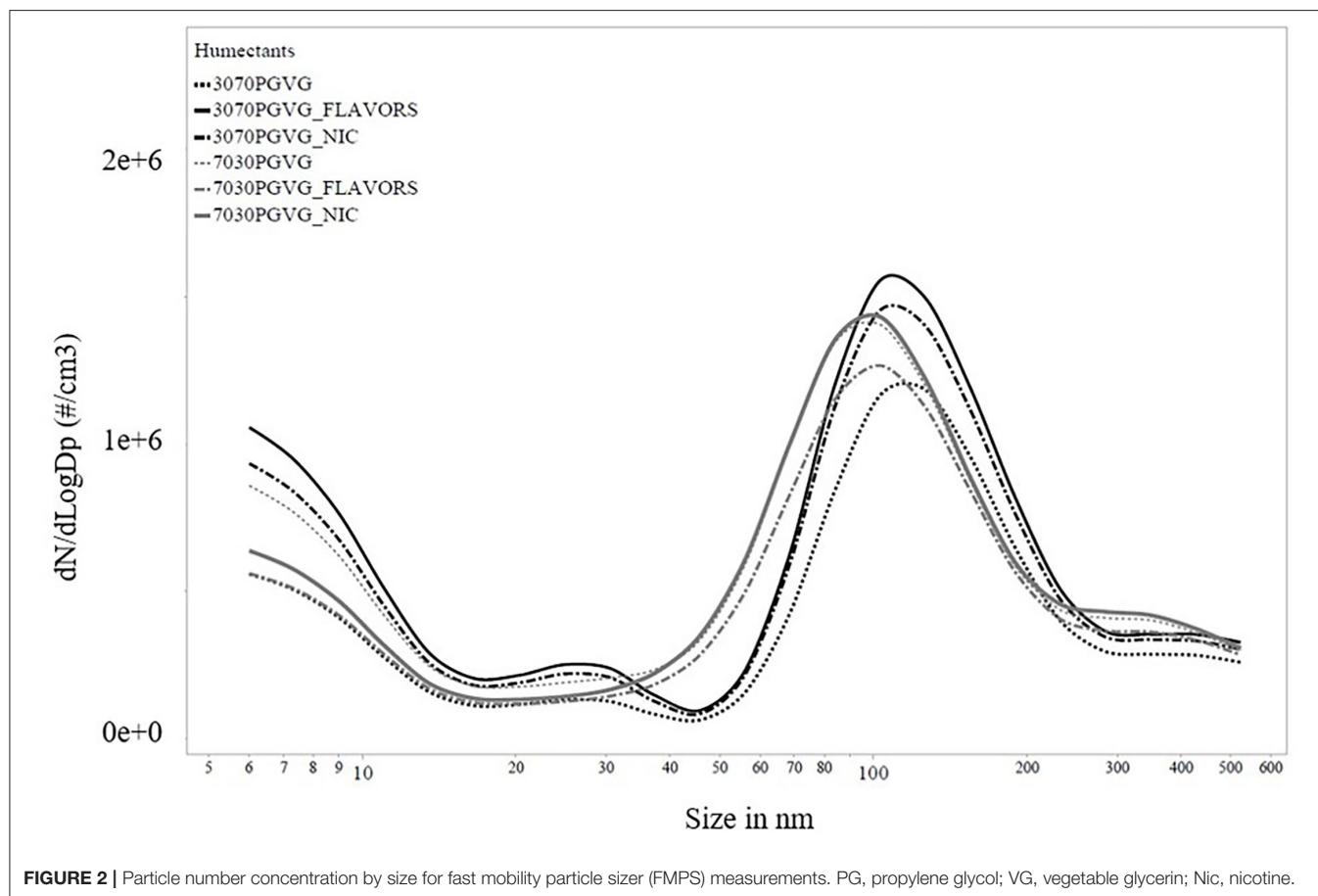
RESULTS

Table 1 summarizes the mass-based aerosol characteristics measured using a LFCI for each e-liquid. The mass of e-liquid consumed ranged from 2,578 $\mu\text{g}/\text{puff}$ (30:70 PG:VG) to 3,971 $\mu\text{g}/\text{puff}$ (70:30 PG:VG); amounts did not differ by e-liquid type. The average aerosol mass concentration per puff ranged from ~50 to over 90 $\mu\text{g}/\text{cm}^3$. For the 70:30 PG:VG e-liquids, the presence of flavorings and nicotine resulted in significantly higher mass concentrations per puff compared with the 70:30 PG:VG e-liquid. In general, variability (coefficient of variation) in mass concentrations per puff were higher

TABLE 1 | Influence of e-liquid formulation on mass-based aerosol characteristics measured using a low-flow cascade impactor ($n = 6$ trials/e-liquid).

E-liquid	E-liquid consumed ($\mu\text{g}/\text{puff}$)		Concentration per puff ($\mu\text{g}/\text{cm}^3$)		MMAD (μm)	GSD
	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)		
30:70 PG:VG	3,906 $\pm$ 1,233 ^A	32	67.4 $\pm$ 21.6 ^{A,B,C}	32	0.93 ^{A,B}	1.43
30:70 PG:VG w/flavorings	2,578 $\pm$ 451.3 ^A	18	51.0 $\pm$ 7.8 ^C	15	0.88 ^B	1.38
30:70 PG:VG w/nicotine	3,272 $\pm$ 220.6 ^A	6.7	84.5 $\pm$ 22.7 ^A	27	0.86 ^B	1.36
70:30 PG:VG	3,931 $\pm$ 1,648 ^A	42	51.7 $\pm$ 3.0 ^{B,C}	6	1.00 ^A	1.43
70:30 PG:VG w/flavorings	3,328 $\pm$ 284.7 ^A	8.5	78.8 $\pm$ 5.3 ^{A,B}	7	0.93 ^{A,B}	1.36
70:30 PG:VG w/nicotine	3,597 $\pm$ 560.0 ^A	16	90.3 $\pm$ 19.5 ^A	22	0.91 ^{A,B}	1.38

SD, standard deviation; CV, coefficient of variation; MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation; PG, propylene glycol; VG, vegetable glycerin. Levels not connected by same letter (A, B, C) are significantly different. For main effects on comparisons of MMADs, the absence of flavoring or nicotine in the humectant significantly increased MMADs of aerosol from e-liquids ($p = 0.005$). Also, significantly increased MMADs were observed for 70:30 PG:VG compared with aerosol from e-liquids prepared with 30:70 PG:VG ($p = 0.017$).

**FIGURE 2** | Particle number concentration by size for fast mobility particle sizer (FMPS) measurements. PG, propylene glycol; VG, vegetable glycerin; Nic, nicotine.

for 30:70 PG:VG e-liquids compared to the 70:30 PG:VG e-liquids. The presence of flavorings or nicotine influenced particle size, i.e., MMADs of aerosol from e-liquids that contained humectants only were significantly larger compared with aerosol from e-liquids that contained flavorings or nicotine ($p = 0.005$). MMADs were also influenced by the relative proportion of humectants. Specifically, aerosols from e-liquids prepared with 70:30 PG:VG had significantly larger MMADs compared

with aerosol from e-liquids prepared with 30:70 PG:VG ($p = 0.017$).

Figures 2, 3 show the particle number concentration from FMPS and SMPS measurements, respectively. Major peaks were on the order of a few hundred nanometers for both instruments. As summarized in **Table 2**, CMD values from all mobility sizer measurements were below $0.15 \mu\text{m}$. The MMAD values calculated from the FMPS data were all below 75 nm, and MMAD

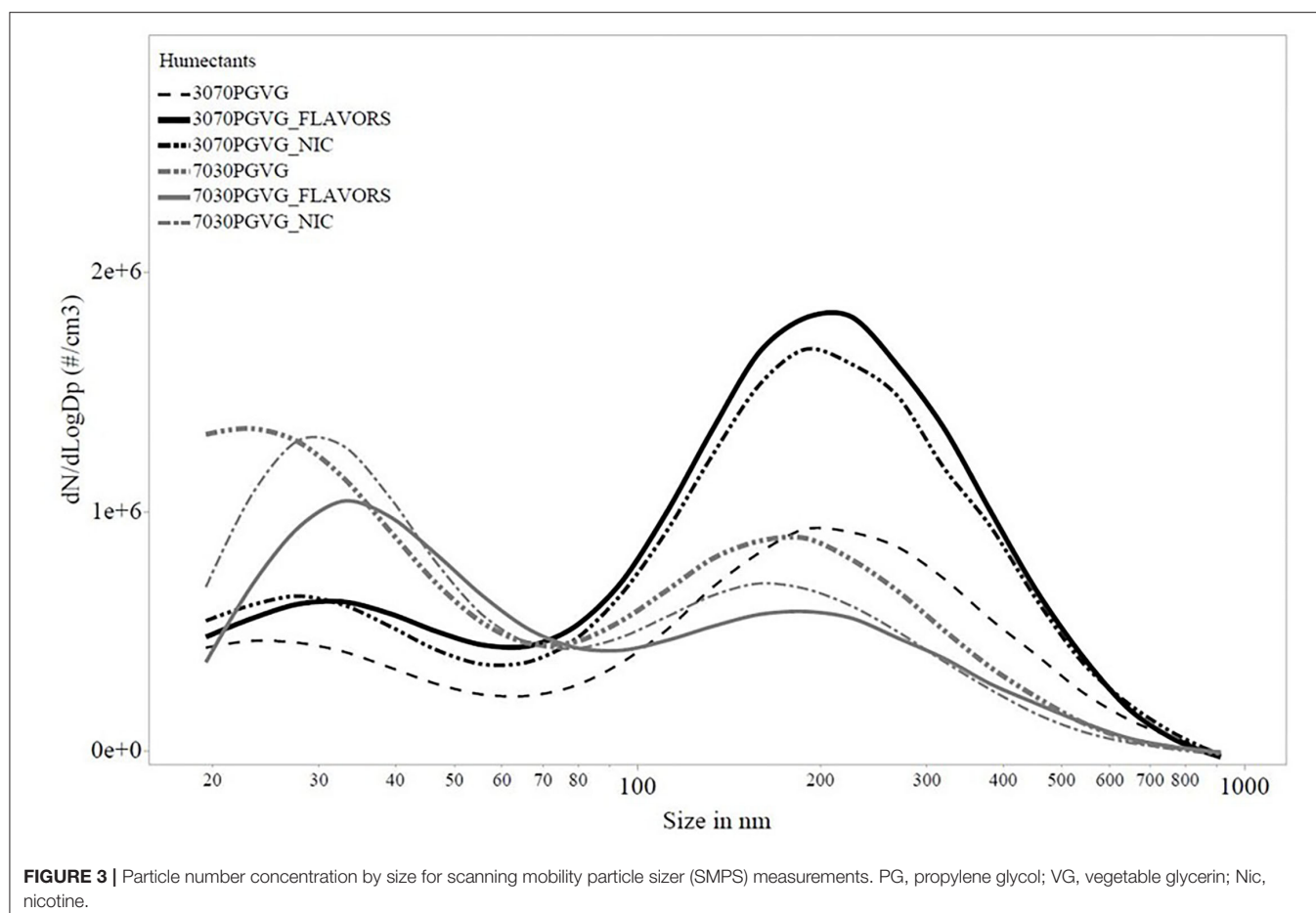


TABLE 2 | Number and calculated mass-based aerosol characteristics from fast mobility particle sizer (FMPS) and scanning mobility particle sizer (SMPS) measurements ($n = 6$ trials/e-liquid).

E-liquid	FMPS			SMPS		
	CMD (μm)	GSD	MMAD (μm)	CMD (μm)	GSD	MMAD (μm)
30:70 PG:VG	0.075	1.004	0.082	0.132	2.6	2.23
30:70 PG:VG w/ flavorings	0.060	1.005	0.066	0.141	2.4	1.54
30:70 PG:VG w/ nicotine	0.062	1.005	0.068	0.140	2.4	1.52
70:30 PG:VG	0.064	1.004	0.067	0.076	2.6	1.24
70:30 PG:VG w/ flavorings	0.073	1.004	0.077	0.078	2.5	1.02
70:30 PG:VG w/ nicotine	0.072	1.004	0.076	0.071	2.5	0.93

CMD, count median diameter; GSD, geometric standard deviation; MMAD, mass median aerodynamic diameter; PG, propylene glycol; VG, vegetable glycerin.

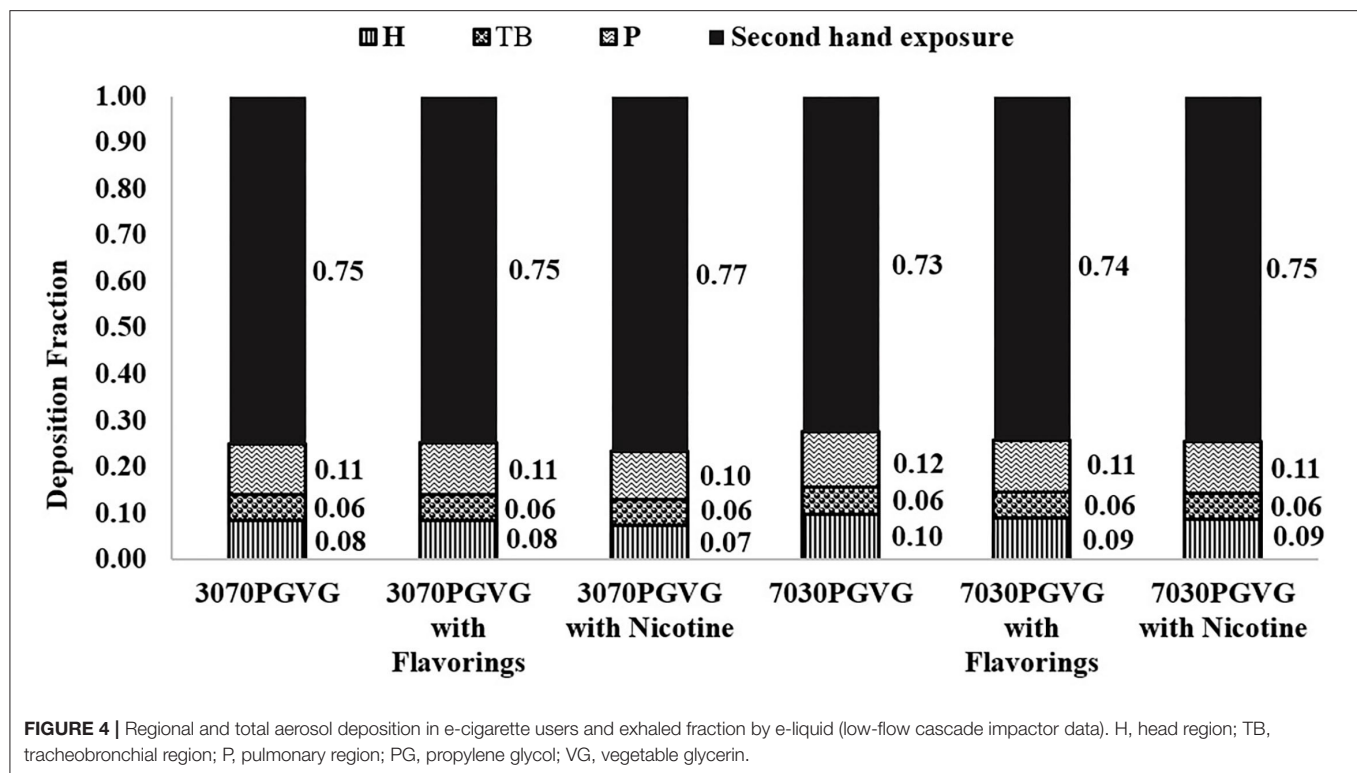
values calculated from the SMPS data ranged from 0.93 to 2.23 μm .

Figure 4 summarizes the regional and total particle deposition estimates in the respiratory tract for each e-liquid that was determined from the LFCI data and assuming a symmetrical lung model. Though significant differences were observed in MMAD values among some e-liquid formulations, regional and total deposition estimates were similar, i.e., ~8–10% of particles will deposit in the H region, 6% will deposit in the TB region, and 10–12% will deposit in the P region, with total deposition of 23–27%.

Assuming that any undeposited aerosol is exhaled, the remainder of particles could contribute to potential secondhand exposure.

DISCUSSION

The primary purpose of this study was to evaluate the hypothesis that e-liquid composition (humectant ratio, flavorings, and nicotine) influenced aerosol particle size. To test this hypothesis, laboratory-prepared e-liquids and a reference second-generation e-cigarette were used to generate aerosols that were sampled



using a LFCI to measure the mass-based aerosol PSD and real-time instruments to monitor number-based PSD during puffing. LFCI measurements indicated that humectant ratio influenced PSD of laboratory-prepared e-liquids aerosolized using a second-generation e-cigarette. In the current study, MMADs determined using a LFCI were larger for e-liquids that contained a higher proportion of PG (Table 1). This observed influence of PG on mass-based PSD is consistent with a prior report (39), but contrasts observations from other investigators that mass-based particle size measured using impactors decreased as the percentage of PG in the e-liquid increased (4, 7, 36, 58). The exact reason for this divergence in experimental results is unknown at this time but could be related to the hygroscopic and/or volatility properties of the humectant constituents (33). The presence of nicotine or flavorings yielded significantly smaller MMADs (LFCI data) compared with e-liquids composed of only humectants (Table 1). Larcombe et al. observed that for VG-based e-liquids, particle size measured *in situ* with an optical particle spectrometer was smaller in the presence of nicotine (41). Other reports indicated that particle size for e-liquids measured using an optical particle sizer or mobility particle sizers increased in the presence of nicotine (33, 41, 42). Some investigators have reported that the presence of nicotine had no effect on particle size measured using mobility and aerodynamic particle sizers or low-pressure impactors (8, 43–47). Fuoco et al. observed that flavorings did not influence PSD of aerosolized e-liquids (45), whereas Lechasseur et al. reported that the presence of vanillin dramatically increased

number-based particle size (33). The reason for the observed inconsistent influence of vanillin on PSD between our study and Lechasseur et al. is unknown but could be related to the measurement methods and/or the concentration of vanillin in the tested e-liquids. In our study, the concentration of vanillin in e-liquids was 0.3%, whereas in the Lechasseur et al. study, the concentration of vanillin in e-liquids was 1%. It is difficult to interpret the meaning of similarities and differences in results from the current study with published literature. In the current study, e-liquids were prepared in the laboratory to maintain control over composition and a reference e-cigarette was used to facilitate future replication of our work. However, even with this standardization, inter-comparison of measurement among studies is complicated because of differences in e-cigarette device power settings, differences in experimental setups (e.g., amount of dilution air or puff topography), and differences in aerosol monitoring approaches (e.g., the same sampler or monitor operated under different conditions or use of different samplers and monitors that measure PSD based on some other principle).

MMADs measured using a LFCI ranged from 0.86 to 1.00 μm (Table 1) and importantly, were obtained with minimal evaporative loss during sampling. These data are generally consistent with MMADs reported in other studies that measured PSD using a LFCI, e.g., Alderman et al. (0.5–0.6 μm), Oldham et al. (0.9–1.2 μm), Kane and Li (0.5–0.9 μm) for various e-cigarette devices, and Pourchez et al. (0.7–1.2 μm) for various e-liquids that were aerosolized at different power settings (4, 35, 36, 58).

In contrast, Mikheev et al. reported MMADs of $\sim 0.3 \mu\text{m}$ for several different flavored e-liquids and Baassiri et al. reported MMADs that ranged from 2.3 to $3.6 \mu\text{m}$ (7, 8). It is important to note that it is difficult to inter-compare measurement results among studies because other factors that could influence PSD such as e-liquid composition (including purities and impurities in commercial products) and e-cigarette generation and device power settings were not standardized among studies in the literature.

Despite significant differences in PSD determined among e-liquid formulations (Table 1), the modeled regional and total aerosol particle deposition fractions in the respiratory tract were similar (Figure 4). Baassari et al. also noted that despite influences of certain factors on aerosol PSD, these variations might not translate to clinically important differences in lung deposition patterns (7). Hence, when modeling particle lung deposition, future efforts should be placed on improving understanding of those consumable-, device-, and user-related factors that have large impacts on PSD (i.e., sufficient to alter estimates of respiratory tract deposition).

MMADs calculated from the SMPS data (Table 2) tended to be smaller for e-liquids prepared using 70:30 PG:VG compared with e-liquids prepared using 30:70 PG:VG. PG has a lower boiling point and higher vapor pressure compared with VG, so more of the 70:30 PG:VG e-liquids evaporated, yielding relatively smaller MMADs. For the e-liquids with prepared with 30:70 PG:VG, the higher boiling point and vapor pressure of VG would translate into less evaporation, yielding relatively larger MMAD values. In general, MMADs calculated from the SMPS data (Table 2) tended to be similar or larger to MMADs measured without dilution using a LFCI (Table 1). This observation was somewhat surprising given the need for dilution air to sample aerosol using the SMPS and likely reflects the larger GSD of the SMPS data (~ 2.5 for all e-liquids) compared with the GSD of the LFCI data (~ 1.4 for all e-liquids). From the Hatch-Choate equation (Equation 1), conversion of CMD values from the SMPS to MMD values depends on $\text{Ln}(\text{GSD})^2$, so a larger GSD will yield a larger calculated MMD value. All MMADs calculated from the FMPS data were below 75 nm and were monodisperse, i.e., GSDs < 1.005 (Table 2). Both the FMPS and SMPS sampled diluted aerosol with sheath flow of, respectively, 40 and 10 LPM, though the inlet sampling flowrate of the FMPS was 10 LPM compared with the SMPS at 0.3 LPM, which resulted in more dilution ($400\times$ compared with $3\times$) of the sampled aerosol (Figure 1). These data illustrate that the high dilution of the e-cigarette aerosol necessary for measurement using an FMPS and the high sampling flow rate of this instrument resulted in extensive evaporation independent of humectant composition to the point where only the non-volatile components of droplets remained during measurement. Previously, Ingebrethsen et al. reported that average particle diameters determined for e-cigarette aerosols by an electrical mobility sizer were $\sim 50 \text{ nm}$, which was believed to result from almost complete particle evaporation at the dilution levels and conditions of their measurements (60). Mikheev et al. (63) also observed that e-cigarette aerosol particles contained both volatile and less volatile compounds and

when the volatile compounds evaporated at high sampling flow rates and dilution using a mobility sizer, the remaining particles were nanoparticles.

Based on our dosimetry modeling using the LFCI data, it was estimated that $\sim 75\%$ of aerosol particles breathed in by a user could be exhaled and serve as a source of secondhand exposure (Figure 4). Similarly, Sundahl et al. (68) modeled nicotine deposition in the respiratory tract of e-cigarette users and reported that 75–90% of nicotine droplets would be exhaled into the surrounding atmosphere. Dosimetry studies on secondhand exposure to e-cigarette aerosols are scarce (6). One study used a smoking machine to generate aerosol from an e-cigarette and measured PSD *in situ* using laser diffraction. The authors used the PSD data to model passive exposure and predicted total respiratory deposition of 15–30% (of which, 7–10% was in the P region) to a bystander (69). Note that in this study design, changes in aerosol PSD during inhalation and exhalation were not captured by using a smoking machine. Another study had e-cigarette users exhale aerosol in a room and measured PSD at a distance of 2 meters away using a FMPS to simulate a bystander; based on size distribution measurements, the authors predicted 20% of particles would deposit in the H region, 32% in the TB region, and 48% in the P region of a passively exposed person (70). As demonstrated in the current study, e-cigarette aerosol size values determined using a FMPS were likely smaller than *in situ* because of extensive evaporation of volatile constituents during sampling, which in turn, could yield higher estimates of regional particle deposition throughout the respiratory tract. Regardless, the data presented herein, and these cited studies conceptually support the potential for secondhand exposure from e-cigarettes to bystanders in homes and employees in workplace settings; however, estimates of magnitude should be interpreted with caution because aerosol PSD input to these dosimetry models were strongly influenced by the choice of sampling method and modeling required the assumption that e-cigarette aerosol behaved as non-volatile particles.

Study Limitations

The current study used MPPD to conceptually demonstrate particle deposition in the respiratory tract of an e-cigarette user and estimate the exhaled fraction, which could serve as a source of secondhand exposure to bystanders. It is important to understand that models for non-volatile aerosols such as MPPD are not suitable for making accurate predictions of e-cigarette deposition in the respiratory tract (69). Recently, investigators have developed dynamic models for e-cigarette aerosols that contain volatile constituents (19, 20, 59). These dynamic models account for the high number concentration produced by an e-cigarette puff, the cloud behavior of aerosol that can change PSD *via* coagulation during mouth hold (i.e., increase in particle size and decrease in particle number), hygroscopic growth of droplets from absorption of water in the humid environment of the lung, conductive heat and diffusive/convective vapor transport of volatile constituents, and dilution/mixing in residual air in the lung as particles travel throughout the respiratory tract. Additionally, droplet chemistry is an important factor because

the thermodynamics of vapor-liquid partitioning of constituents has a major influence on the deposition characteristics of vapor constituents in the respiratory tract. Several studies have demonstrated that droplet chemistry varies with PG:VG ratio (54, 71, 72). Hence, for e-cigarette aerosol, dosimetry modeling based on PSD alone is insufficient to accurately predict the fate of inhaled particles (59).

By accounting for both physical and chemical factors, dynamic models can be applied to realistic scenarios for e-cigarette use, i.e., puff withdrawal into the oral cavity, mouth hold, dilution of the e-cigarette puff in the mouth with the subsequent dilution from inhaled air, inhalation of the diluted puff into the lower respiratory tract, lung-hold, and exhalation of aerosol into the surrounding air (19, 59). Results of dynamic modeling indicated that particle size gets progressively larger as aerosol travels from puff withdrawal from the e-cigarette into the oral cavity (smallest particle size) to after puff to mouth hold to reaching the alveolar region to exhaled fraction (largest particle size); the smallest particles will coagulate during the puffing and mouth hold phases and grow in size (19, 20, 59). As these particles travel through the lungs, their size will continue to grow from absorption of water in the humid lung, and the net effect is that exhaled particles will be larger than inhaled particles (19, 20, 59). This growth in particle size has implications for dosimetry modeling for e-cigarette users and for secondhand exposure potential. For example, modeling by Asgharian et al. indicated that for a 1 μm e-cigarette aerosol particle (similar to the size reported in **Table 1**), total deposition calculated using a dynamic model was $\sim 85\%$; however, the total deposition predicted using an insoluble particle model was $\sim 35\%$. For the P region, the dynamic model predicted 65% particle deposition, whereas the insoluble model predicted 25% particle deposition (19). Assuming that all the non-deposited particles are exhaled, the dynamic model indicated $\sim 15\%$ of particles would be available for secondhand exposure, whereas the insoluble model indicated 65% of particles could be a source of secondhand exposure (similar to our results presented in **Figure 4**). Pichelstorfer et al. (20) also compared dynamic and insoluble particle models and reported that dynamic models yielded median number-based and mass-based particle sizes that were 242 and 466% larger than situations where dynamics were ignored, which again means that the actual amount deposited would be higher than predicted using a non-volatile particle model. The primary reason for this difference in deposition predictions between dynamic and insoluble models is the volatility of e-cigarette aerosol constituents and the corresponding contribution to deposition from vapor uptake, which were not accounted for in the insoluble particle models (19). Currently, these dynamic models are not yet widely available for use by the research community though it is expected that in the future, if available, their use will be adopted to improve dosimetry estimates.

Finally, it is worth noting that in the current work, we only focused on the influence of consumable-related factors on aerosol PSD, though it is recognized that device- and user-related factors can also influence aerosol particle size. For example, PSD is reported to be influenced by device coil power setting and temperature (4, 33, 39, 62) as well as puff flow rate (8, 35, 73).

SUMMARY

Laboratory-prepared e-liquids and a second-generation reference e-cigarette were used to test the hypothesis that e-liquid composition (humectant ratio, flavorings, and nicotine) influenced aerosol PSD. Results from LFCI measurements demonstrated that the proportion of humectants and the presence of nicotine or flavorings significantly influenced MMAD values; however, these differences did not translate into meaningful differences in estimates of regional particle deposition throughout the respiratory tract of e-cigarette users. Notably, use of a LFCI permitted determination of mass-based MMADs with minimal bias from evaporation during sampling. In contrast, monitoring using a FMPS demonstrated significant evaporation of volatile aerosol particle constituents, which yielded PSDs that were an order of magnitude smaller than the native state of droplets produced during puffing. This improved method to characterize physical properties of volatile aerosol particles yielded MMAD values more representative of e-cigarette aerosol *in situ*, which in turn, can help to improve validity of size distribution values input to dosimetry models to estimate exposures to users and bystanders. Particle deposition modeling assuming non-volatile particles conceptually supported the premise that e-cigarettes can be a source of secondhand exposure to persons in proximity to users, whether at home, in a vehicle, or in a workplace. Use of a model developed for non-volatile aerosol particles precluded accurate estimation of the magnitude of aerosol deposition in the respiratory tract of users and the exhaled fraction that could serve a source of secondhand exposure. As dynamic models become more readily available to researchers, understanding of the fate of aerosol generated by e-cigarettes will improve.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AR, AS, and RL contributed to conception and design of the study. AR performed the statistical analysis under guidance from MV. AR and AS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpubh.2022.782068/full#supplementary-material>

REFERENCES

- Bhatnagar A, Whitsell LP, Ribisl KM, Bullen C, Chaloupka F, Piano MR, et al. Electronic cigarettes: a policy statement from the American Heart Association. *Circulation*. (2014) 130:1418–36. doi: 10.1161/CIR.000000000000107
- Schmidt S. Vaper, beware: the unique toxicological profile of electronic cigarettes. *Environ Health Perspect*. (2020) 128:052001. doi: 10.1289/EHP6628
- Schraufnagel DE, Blasi F, Drummond MB, Lam DC, Latif E, Rosen MJ, et al. Electronic cigarettes. A position statement of the forum of international respiratory societies. *Am J Respir Crit Care Med*. (2014) 190:611–8. doi: 10.1164/rccm.201407-1198PP
- Pourchez J, Parisse S, Sarry G, Perinel-Ragey S, Vergnon JM, Clotagatide A, et al. Impact of power level and refill liquid composition on the aerosol output and particle size distribution generated by a new-generation e-cigarette device. *Aerosol Sci Technol*. (2018) 52:359–69. doi: 10.1080/02786826.2017.1422857
- Protano C, Avino P, Manigrasso M, Vivaldi V, Perna F, Valeriani F, et al. Environmental electronic vape exposure from four different generations of electronic cigarettes: airborne particulate matter levels. *Int J Environ Res Public Health*. (2018) 15:2172. doi: 10.3390/ijerph15102172
- Stefaniak AB, LeBouf RF, Ranpara AC, Leonard SS. Toxicology of flavoring- and cannabis-containing e-liquids used in electronic delivery systems. *Pharmacol Ther*. (2021) 224:107838. doi: 10.1016/j.pharmthera.2021.107838
- Baassiri M, Talih S, Salman R, Karaoghlanian N, Saleh R, El Hage R, et al. Clouds and “throat hit”: Effects of liquid composition on nicotine emissions and physical characteristics of electronic cigarette aerosols. *Aerosol Sci Technol*. (2017) 51:1231–9. doi: 10.1080/02786826.2017.1341040
- Mikheev VB, Ivanov A, Lucas EA, South PL, Colijn HO, Clark PI. Aerosol size distribution measurement of electronic cigarette emissions using combined differential mobility and inertial impaction methods: smoking machine and puff topography influence. *Aerosol Sci Technol*. (2018) 52:1233–48. doi: 10.1080/02786826.2018.1513636
- El-Hellani A, Salman R, El-Hage R, Talih S, Malek N, Baalbaki R, et al. Nicotine and carbonyl emissions from popular electronic cigarette products: correlation to liquid composition and design characteristics. *Nicotine Tob Res*. (2018) 20:215–23. doi: 10.1093/ntr/ntw280
- Bonner E, Chang Y, Christie E, Colvin V, Cunningham B, Elson D, et al. The chemistry and toxicology of vaping. *Pharmacol Therap*. (2021) 225:e107837. doi: 10.1016/j.pharmthera.2021.107837
- Soulet S, Duquesne M, Toutain J, Paireaud C, Lalo H. Experimental method of emission generation calibration based on reference liquids characterization. *Int J Environ Res Public Health*. (2019) 16:2262. doi: 10.3390/ijerph16132262
- Girvalaki C, Tzatzarakis M, Kyriakos CN, Vardavas AI, Stivaktakis PD, Kavvalakis M, et al. Composition and chemical health hazards of the most common electronic cigarette liquids in nine European countries. *Inhal Toxicol*. (2018) 30:361–9. doi: 10.1080/08958378.2018.1527879
- Havermans A, Krusemann EJZ, Pennings J, de Graaf K, Boesveldt S, Talhout R. Nearly 20 000 e-liquids and 250 unique flavour descriptions: an overview of the Dutch market based on information from manufacturers. *Tob Control*. (2021) 30:57–62. doi: 10.1136/tobaccocontrol-2019-055303
- Krusemann EJZ, Boesveldt S, de Graaf K, Talhout R. An E-liquid flavor wheel: a shared vocabulary based on systematically reviewing E-liquid flavor classifications in literature. *Nicotine Tob Res*. (2019) 21:1310–9. doi: 10.1093/ntr/nty101
- El-Hellani A, El-Hage R, Baalbaki R, Salman R, Talih S, Shihadeh A, et al. Free-base and protonated nicotine in electronic cigarette liquids and aerosols. *Chem Res Toxicol*. (2015) 28:1532–7. doi: 10.1021/acs.chemrestox.5b00107
- Harvanko AM, Havel CM, Jacob P, Benowitz NL. Characterization of nicotine salts in 23 electronic cigarette refill liquids. *Nicotine Tob Res*. (2020) 22:1239–43. doi: 10.1093/ntr/ntz232
- Pankow JF, Duell AK, Peyton DH. Free-base nicotine fraction α (fb) in non-aqueous versus aqueous solutions: electronic cigarette fluids without versus with dilution with water. *Chem Res Toxicol*. (2020) 33:1729–35. doi: 10.1021/acs.chemrestox.0c00008
- Romberg AR, Miller Lo EJ, Cuccia AF, Willett JG, Xiao H, Hair EC, et al. Patterns of nicotine concentrations in electronic cigarettes sold in the United States, 2013–2018. *Drug Alcohol Depend*. (2019) 203:1–7. doi: 10.1016/j.drugalcdep.2019.05.029
- Asgharian B, Rostami AA, Price OT, Pithawalla YB. Regional deposition of inhaled aerosol constituents from Electronic Nicotine Delivery Systems (ENDS) in the respiratory tract. *J Aerosol Sci*. (2018) 126:7–20. doi: 10.1016/j.jaerosci.2018.08.006
- Pichelstorfer L, Winkler-Heil R, Boy M, Hofmann W. Aerosol dynamics simulations of the anatomical variability of e-cigarette particle and vapor deposition in a stochastic lung. *J Aerosol Sci*. (2021) 158:105706. doi: 10.1016/j.jaerosci.2020.105706
- Bhatt JM, Ramphul M, Bush A. An update on controversies in e-cigarettes. *Paediatr Respir Rev*. (2020) 36:75–86. doi: 10.1016/j.prrv.2020.09.003
- Protano C, Manigrasso M, Avino P, Vitali M. Second-hand smoke generated by combustion and electronic smoking devices used in real scenarios: ultrafine particle pollution and age-related dose assessment. *Environ Int*. (2017) 107:190–5. doi: 10.1016/j.envint.2017.07.014
- Tzortzi A, Teloniatis S, Matiampa G, Bakelas G, Tzavara C, Vyzikidou VK, et al. Passive exposure of non-smokers to E-Cigarette aerosols: sensory irritation, timing and association with volatile organic compounds. *Environ Res*. (2020) 182:108963. doi: 10.1016/j.envres.2019.108963
- Chen R, Aherrera A, Isichei C, Olmedo P, Jarmul S, Cohen JE, et al. Assessment of indoor air quality at an electronic cigarette (Vaping) convention. *J Expo Sci Environ Epidemiol*. (2018) 28:522–9. doi: 10.1038/s41370-017-0005-x
- Johnson JM, Naeher LP, Yu X, Sosnoff C, Wang L, Rathbun SL, et al. A biomonitoring assessment of secondhand exposures to electronic cigarette emissions. *Int J Hyg Environ Health*. (2019) 222:816–23. doi: 10.1016/j.ijheh.2019.04.013
- Li L, Nguyen C, Lin Y, Guo Y, Fadel NA, Zhu Y. Impacts of electronic cigarettes usage on air quality of vape shops and their nearby areas. *Sci Total Environ*. (2021) 760:143423. doi: 10.1016/j.scitotenv.2020.143423
- Logue JM, Sleiman M, Montesinos VN, Russell ML, Litter MI, Benowitz NL, et al. Emissions from electronic cigarettes: assessing vapers' intake of toxic compounds, secondhand exposures, and the associated health impacts. *Environ Sci Technol*. (2017) 51:9271–9. doi: 10.1021/acs.est.7b00710
- Soule EK, Maloney SF, Spindle TR, Rudy AK, Hiler MM, Cobb CO. Electronic cigarette use and indoor air quality in a natural setting. *Tob Control*. (2017) 26:109–12. doi: 10.1136/tobaccocontrol-2015-052772
- Tigova O, Amalia B, Castellano Y, Fu M, Nogueira SO, Kyriakos CN, et al. Secondhand exposure to e-cigarette aerosols among smokers: a cross-sectional study in six European countries of the EUREST-PLUS ITC Europe Surveys. *Tob Induc Dis*. (2018) 16:A11. doi: 10.18332/tid/99117
- Khachatoorian C, Jacob P, III, Sen A, Zhu Y, Benowitz NL, Talbot P. Identification and quantification of electronic cigarette exhaled aerosol residue chemicals in field sites. *Environ Res*. (2019) 170:351–8. doi: 10.1016/j.envres.2018.12.027
- Melstrom P, Koszowski B, Thanner MH, Hoh E, King B, Bunnell R, et al. Measuring PM_{2.5} ultrafine particles, nicotine air and wipe samples following the use of electronic cigarettes. *Nicotine Tobacco Res*. (2017) 19:1055–61. doi: 10.1093/ntr/ntx058
- Kleinstreuer C, Feng Y. Lung deposition analyses of inhaled toxic aerosols in conventional and less harmful cigarette smoke: a review. *Int J Environ Res Public Health*. (2013) 10:4454–85. doi: 10.3390/ijerph10094454

33. Lechasseur A, Altmeld S, Turgeon N, Buonanno G, Morawska L, Brunet D, et al. Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by an electronic cigarette. *Physiol Rep.* (2019) 7:e14093. doi: 10.14814/phy2.14093
34. Li Y, Cui H, Chen L, Fan M, Cai J, Guo J, et al. Modeled respiratory tract deposition of smoke aerosol from conventional cigarettes, electronic cigarettes and heat-not-burn products. *Aerosol Air Qual Res.* (2021) 21:241. doi: 10.4209/aaqr.200241
35. Oldham MJ, Zhang J, Rusyniak MJ, Kane DB, Gardner WP. Particle size distribution of selected electronic nicotine delivery system products. *Food Chem Toxicol.* (2018) 113:236–40. doi: 10.1016/j.fct.2018.01.045
36. Alderman SL, Song C, Moldoveanu SC, Cole SK. Particle size distribution of e-cigarette aerosols and the relationship to cambridge filter pad collection efficiency. *Beitrage zur Tabakforschung Int/Contrib Tobacco Res.* (2014) 26:183–90. doi: 10.1515/cttr-2015-0006
37. Zervas E, Litsiou E, Konstantopoulos K, Pouloupoulos S, Katsaounou P. Physical characterization of the aerosol of an electronic cigarette: impact of refill liquids. *Inhal Toxicol.* (2018) 30:218–23. doi: 10.1080/08958378.2018.1500662
38. Zhang Y, Sumner W, Chen DR. *In vitro* particle size distributions in electronic and conventional cigarette aerosols suggest comparable deposition patterns. *Nicotine Tob Res.* (2013) 15:501–8. doi: 10.1093/ntr/nts165
39. Mulder HA, Patterson JL, Halquist MS, Kosmider L, Turner JBMG, Poklis JL, et al. The effect of electronic cigarette user modifications and E-liquid adulteration on the particle size profile of an aerosolized product. *Sci Rep.* (2019) 9:10221. doi: 10.1038/s41598-019-46387-2
40. Prévôt N, de Oliveira F, Perinel-Ragey S, Basset T, Vergnon JM, Pourchez J. Nicotine delivery from the refill liquid to the aerosol via high-power e-cigarette device. *Sci Rep.* (2017) 7:2592. doi: 10.1038/s41598-017-03008-0
41. Larcombe AN, Janka MA, Mullins BJ, Berry LJ, Bredin A, Franklin PJ. The effects of electronic cigarette aerosol exposure on inflammation and lung function in mice. *Am J Physiol Lung Cell Mol Physiol.* (2017) 313:L67–79. doi: 10.1152/ajplung.00203.2016
42. Manigrasso M, Buonanno G, Fuoco FC, Stabile L, Avino P. Electronic cigarettes: age-specific generation-resolved pulmonary doses. *Environ Sci Pollut Res.* (2017) 24:13068–79. doi: 10.1007/s11356-017-8914-8
43. Belka M, Lizal F, Jedelsky J, Jicha M, Pospisil J. Measurement of an electronic cigarette aerosol size distribution during a puff. In: *Paper Presented at the EPJ Web of Conferences.* Marienbad (2017).
44. Bertholon JF, Becquemin MH, Roy M, Roy F, Ledur D, Annesi Maesano I, et al. Comparison of the aerosol produced by electronic cigarettes with conventional cigarettes and the shisha. *Rev Mal Respir.* (2013) 30:752–7. doi: 10.1016/j.rmr.2013.03.003
45. Fuoco FC, Buonanno G, Stabile L, Vigo P. Influential parameters on particle concentration and size distribution in the mainstream of e-cigarettes. *Environ Pollut.* (2014) 184:523–9. doi: 10.1016/j.envpol.2013.10.010
46. Laube BL, Afshar-Mohajer N, Koehler K, Chen G, Lazarus P, Collaco JM, et al. Acute and chronic *in vivo* effects of exposure to nicotine and propylene glycol from an E-cigarette on mucociliary clearance in a murine model. *Inhal Toxicol.* (2017) 29:197–205. doi: 10.1080/08958378.2017.1336585
47. Manigrasso M, Buonanno G, Fuoco FC, Stabile L, Avino P. Aerosol deposition doses in the human respiratory tree of electronic cigarette smokers. *Environ Pollut.* (2015) 196:257–67. doi: 10.1016/j.envpol.2014.10.013
48. Lee MS, LeBouf RF, Son YS, Koutrakis P, Christiani DC. Nicotine, aerosol particles, carbonyls and volatile organic compounds in tobacco- and menthol-flavored e-cigarettes. *Environ Health.* (2017) 16:42. doi: 10.1186/s12940-017-0249-x
49. Cheah NP, Chong NW, Tan J, Morsed FA, Yee SK. Electronic nicotine delivery systems: regulatory and safety challenges: Singapore perspective. *Tob Control.* (2014) 23:119–25. doi: 10.1136/tobaccocontrol-2012-050483
50. Szafran BN, Pinkston R, Perveen Z, Ross MK, Morgan T, Paulsen DB, et al. Electronic-cigarette vehicles and flavoring affect lung function and immune responses in a murine model. *Int J Mol Sci.* (2020) 21:6022. doi: 10.3390/ijms21176022
51. Jackler RK, Ramamurthi D. Nicotine arms race: JUUL and the high-nicotine product market. *Tob Control.* (2019) 28:623–8. doi: 10.1136/tobaccocontrol-2018-054796
52. Tierney PA, Karpinski CD, Brown JE, Luo W, Pankow JF. Flavour chemicals in electronic cigarette fluids. *Tob Control.* (2016) 25:e10–15. doi: 10.1136/tobaccocontrol-2014-052175
53. Kim JJ, Sabatelli N, Tutak W, Giuseppetti A, Frukhtbeyn S, Shaffer I, et al. Universal electronic-cigarette test: physicochemical characterization of reference e-liquid. *Tob Induc Dis.* (2017) 15:14. doi: 10.1186/s12971-017-0119-x
54. Ooi BG, Dutta D, Kazipeta K, Chong NS. Influence of the E-cigarette emission profile by the ratio of glycerol to propylene glycol in E-liquid composition. *ACS Omega.* (2019) 4:13338–48. doi: 10.1021/acsomega.9b01504
55. (AFNOR), AFdN. *Cigarettes Electroniques et E-Liquides. Partie 3: Exigences et Méthodes D'essais Relatives aux Emissions.* Paris: AFNOR (2016). p. 46.
56. NIDA. *NIDA Standard Drug Program.* (2021). Available online at: <https://www.drugabuse.gov/research/research-data-measures-resources/nida-drug-supply-program/> (accessed July 15, 2021).
57. CORESTA. *Recommended Method No. 81.* CORESTA, Paris, France (2015).
58. Kane DB, Li W. Particle size measurement of electronic cigarette aerosol with a cascade impactor. *Aerosol Sci Technol.* (2021) 55:205–14. doi: 10.1080/02786826.2020.1849536
59. Asgharian B, Price OT, Rostami AA, Pithawalla YB. Deposition of inhaled electronic cigarette aerosol in the human oral cavity. *J Aerosol Sci.* (2018) 116:34–47. doi: 10.1016/j.jaerosci.2017.11.014
60. Ingebrethsen BJ, Cole SK, Alderman SL. Electronic cigarette aerosol particle size distribution measurements. *Inhal Toxicol.* (2012) 24:976–84. doi: 10.3109/08958378.2012.744781
61. Biswas P, Jones CL, Flagan RC. Distortion of size distributions by condensation and evaporation in aerosol instruments. *Aerosol Sci Technol.* (1987) 7:231–46. doi: 10.1080/02786828708959161
62. Floyd EL, Queimado L, Wang J, Regens JL, Johnson DL. Electronic cigarette power affects count concentration and particle size distribution of vaping aerosol. *PLoS ONE.* (2018) 13:e0210147. doi: 10.1371/journal.pone.0210147
63. Mikheev VB, Brinkman MC, Granville CA, Gordon SM, Clark PI. Real-time measurement of electronic cigarette aerosol size distribution and metals content analysis. *Nicotine Tob Res.* (2016) 18:1895–902. doi: 10.1093/ntr/ntw128
64. Sosnowski TR, Odziomek M. Particle size dynamics: toward a better understanding of electronic cigarette aerosol interactions with the respiratory system. *Front Physiol.* (2018) 9:853. doi: 10.3389/fphys.2018.00853
65. O'Shaughnessy PT, Raabe OG. A comparison of cascade impactor data reduction methods. *Aerosol Sci Technol.* (2003) 37:187–200. doi: 10.1080/02786820300956
66. Ebersole J, Samburova V, Son Y, Cappelli D, Demopoulos C, Capurro A, et al. Harmful chemicals emitted from electronic cigarettes and potential deleterious effects in the oral cavity. *Tob Induc Dis.* (2020) 18:41. doi: 10.18332/tid/116988
67. Rouabhia M, Piché M, Corriveau MN, Chakir J. Effect of e-cigarettes on nasal epithelial cell growth, Ki67 expression, and pro-inflammatory cytokine secretion. *Am J Otolaryngol.* (2020) 41:102686. doi: 10.1016/j.amjoto.2020.102686
68. Sundahl M, Berg E, Svensson M. Aerodynamic particle size distribution and dynamic properties in aerosols from electronic cigarettes. *J Aerosol Sci.* (2017) 103:141–50. doi: 10.1016/j.jaerosci.2016.10.009
69. Sosnowski TR, Kramek-Romanowska K. Predicted deposition of E-cigarette aerosol in the human lungs. *J Aerosol Med Pulm Drug Deliv.* (2016) 29:299–309. doi: 10.1089/jamp.2015.1268
70. Protano C, Manigrasso M, Avino P, Sernia S, Vitali M. Second-hand smoke exposure generated by new electronic devices (IQOS® and e-cigs) and traditional cigarettes: submicron particle behaviour in human respiratory system. *Ann Ig.* (2016) 28:109–12. doi: 10.7416/ai.2016.2089
71. Conklin DJ, Ogunwale MA, Chen Y, Theis WS, Nantz MH, Fu XA, et al. Electronic cigarette-generated aldehydes: the contribution of e-liquid components to their formation and the use of urinary aldehyde metabolites as biomarkers of exposure. *Aerosol Sci Technol.* (2018) 52:1219–32. doi: 10.1080/02786826.2018.1500013
72. Jensen RP, Strongin RM, Peyton DH. Solvent chemistry in the electronic cigarette reaction vessel. *Sci Rep.* (2017) 7:2549. doi: 10.1038/srep42549

73. Fisenko SP, Rostami AA, Kane DB, Pithawalla YB, Maximoff SN, Li W, et al. Model of aerosol evolution in high supersaturated glycerol-air vapor mixtures. *Aerosol Sci Technol.* (2021) 55:871–85. doi: 10.1080/02786826.2021.1904130

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The Quantitation of Squalene and Squalane in Bronchoalveolar Lavage Fluid Using Gas Chromatography Mass Spectrometry

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Chemicals of unknown inhalational toxicity are present in electronic cigarette and vaping products. E-cigarettes typically contain nicotine and other relatively hydrophilic chemicals while vaping products typically contain cannabinoids and other hydrophobic chemicals. For example, vaping products can include hydrophobic terpenes such as squalane (SQA) and squalene (SQE). However, little is known about the SQA and SQE transmission from liquid to aerosol. SQA and SQE are used in commercial products that are applied dermally and ingested orally, but limited information is available on their inhalational exposure and toxicity. We developed and validated a quantitative method to measure SQE and SQA in bronchoalveolar lavage fluid to assess if these chemicals accumulate in lung epithelial lining fluid after inhalation. Calibration curves spanned a range of 0.50–30.0 µg analyte per mL bronchoalveolar lavage fluid. Recoveries were found to be 97–105% for SQE and 81–106% for SQA. Limits of detection were 0.50 µg/mL for both SQE and SQA. The method was applied to bronchoalveolar lavage fluid samples of patients from the 2019 outbreak of e-cigarette, or vaping, product use-associated lung injury (EVALI) and a comparison group. Neither SQA nor SQE was detected above the method LOD for any samples analyzed; conversely, SQA or SQE were reproducibly measured in spiked quality control BAL fluids (relative standard deviations <15% for both analytes). Further applications of this method may help to evaluate the potential toxicity of SQA and SQE chronically inhaled from EVPs.

Keywords: electronic cigarettes, electronic vaping products, bronchoalveolar lavage fluid, gas-chromatography mass spectrometry, squalene, squalane

INTRODUCTION

Use of electronic cigarettes (e-cigarettes), or vaping, products (EVPs) in the United States has increased since e-cigarettes were introduced into the U.S. market (Cullen et al., 2019; Knapp et al., 2019; Meier et al., 2019; Schulenberg et al., 2019; Spindle et al., 2019; Braak et al., 2020). These devices deliver inhalable aerosols that contain active ingredients such as nicotine and cannabinoids. E-cigarettes typically contain nicotine and other relatively hydrophilic chemicals while vaping products typically contain cannabinoids and other hydrophobic chemicals. In a 2018 study of adults using e-cigarettes, 7.1% of participants also report vaping some form of cannabis (Uddin et al., 2020).

A more recent study of U.S. youth finds that 19.6% of high school and 4.7% of middle school students reported vaping in the past 30 days (Wang et al., 2020) and 4.2% of 8th graders, 23.5% of 10th graders, 12.2% of 12th graders reported vaping cannabis in the past 30 days (Miech et al., 2021). Furthermore, both adults and adolescents who use cannabis preferred vaping e-liquids (e.g., concentrates from cannabis flower or plant extract) over smoking cannabis (Lee et al., 2016; Morean et al., 2017; Knapp et al., 2019). Multiple reasons could explain the increase in use of both nicotine and cannabinoid delivery devices, including perception of reduced risk compared with smoking and the ease of concealing these potentially-illicit devices. The non-medical cannabis legalization by multiple states and the ease of product customization have likely contributed to the increase in vaping of cannabis (Morean et al., 2017; Cullen et al., 2018; King et al., 2018; Romeh et al., 2018).

Increased demand for EVPs has contributed to a proliferation of sources of EVP liquids, including legitimate vendors, off-market sources, and “DIY” liquids (Kong et al., 2017; King et al., 2018; Romeh et al., 2018). EVP liquids and their components are widely available online and over-the-counter, thus enabling use of unregulated off-market and DIY liquids. Active ingredients are often diluted or “cut” with miscible diluents (hydrophobic or hydrophilic depending on product type) such as polyethylene glycol (PG), vegetable glycerin (VG), medium-chain triglycerides (MCT), and terpene mixtures to increase vaping efficiency, provide aroma and flavors, enhance the appearance of products, and/or to lower product cost (Kosmider et al., 2014; Erythropel et al., 2019).

Squalene (SQE) is a naturally-occurring polyunsaturated triterpene formed by many plant and animal cells as a biochemical precursor of sterols. Topical and oral products have included SQE as an ingredient for decades without reports of adverse health effects (Reddy and Couvreur, 2009; Wu et al., 2016). Squalane (SQA) is the completely saturated form of SQE and is naturally formed by plants and animals (albeit in lesser amounts than SQE). SQA can also be chemically synthesized through hydrogenation of SQE. SQA is more widely used in dermatological products than is SQE, perhaps because of better skin absorption, ability to enhance flexibility of and to moisturize human skin, to act as a carrier to increase absorption of other substances, and its long shelf-life (Reddy and Couvreur, 2009; Wu et al., 2016). Conversely, physical properties such as viscosity, miscibility with other oily substances, and higher boiling point make SQE more likely than SQA to be used as a diluent for hydrophobic EVP liquids.

The U.S. recently experienced an outbreak of e-cigarette, or vaping, products use-associated lung injury (EVALI), with over 2,800 hospitalized cases reported (King et al., 2020; Reagan-Steiner et al., 2020; Werner et al., 2020). Some patients diagnosed with EVALI exhibited respiratory symptoms resembling those previously reported by patients who had aspirated mineral oil (Aberegg et al., 2020; Gay et al., 2020; Shah et al., 2020). In an urgent attempt to identify the cause of the outbreak-associated lung injury, bronchoalveolar lavage (BAL) fluid samples from case patients were analyzed by the Centers for Disease Control and Prevention (CDC) for multiple

EVP-associated chemicals (DeJesús et al., 2020a; DeJesús et al., 2020b; Holder et al., 2021; Morel-Espinosa et al., 2021; Xia et al., 2021; Brosius et al., 2022). Limited clinical observations indicate that SQE can cause lipoid pneumonia following accidental inhalation, and thus SQE and SQA were considered potential substances of interest (Lee et al., 1999; Kanaji et al., 2008; Cha et al., 2018).

SQE has been quantified in various matrices including olive oil, human hair, human serum, and incense smoke by means of gas chromatography flame ionization detection and gas chromatography-mass spectrometry (GC-MS) (Wu et al., 2016; Santivanez-Veliz et al., 2017; Budge and Barry, 2019; Pacetti et al., 2019; Aresta et al., 2020). Qualitative confirmation of SQE in human BAL fluid (Kanaji et al., 2008) has been reported using GC-MS. However, quantitative analytical measurements of SQA and SQE have not been previously reported in BAL fluid. Therefore, we rapidly developed and validated a novel, accurate, and precise method for quantifying squalane and squalene in BAL fluid using GC-MS. The intended purpose of this method is to measure SQA and SQE in BAL fluid at concentrations capable of causing lung injury and/or lipoid pneumonia. Therefore, the method was applied to BAL fluids collected from people with EVP-related lung injury and from otherwise healthy comparators.

MATERIALS AND METHODS

Chemicals and Reagents

Reference standards of squalene ($\geq 98\%$ purity) and squalane ($\geq 96\%$ purity) were purchased from Sigma (St. Louis, MO). Cannabinol- D^3 (CBN- D^3) 100 $\mu\text{g/ml}$ solution in methanol was purchased from Cerilliant Corp. (Round Rock, Texas). Simulated lung fluid—Gamble’s formulation (not stabilized) was purchased from Pickering Laboratories Inc. (Mountain View, CA). Solvents including hexanes and methanol (Optima for GC) were purchased from Fisher Scientific (Pittsburgh, PA). Research-grade helium (He) and ultra-high purity grade nitrogen (N_2) gases were obtained from Airgas, Inc. (Hapeville, GA).

Instrumentation

The BAL fluids were analyzed using an Agilent 7890B GC System with an Agilent Sampler 80 and interfaced Agilent 7000C triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). Chromatographic separation was achieved using a HP-5MS UI (30 mm $\times$ 0.25 μm $\times$ 0.25 mm) capillary column with a 1.2 ml/min flow of helium carrier gas. GC inlet was operated at 250°C in split mode with a 20:1 split ratio. The initial oven temperature was 150°C and was ramped at 40°C/min to 250°C with a 2 min hold, followed by a second ramp of 5°C/min until reaching a maximum temperature of 300°C. Analytes eluted at 7.693 min (CBN- D^3), 8.05 min (SQA), and 9.89 min (SQE) with a total run time of 14.5 min. The mass spectrometer source and transfer line were maintained at 280°C and quadrupoles temperatures were 150°C. The MS was operated in positive electron ionization (EI) mode and the resulting ions analyzed by single-ion monitoring (SIM) scan mode. Electron multiplier

voltage gain factor was 10 for all ions monitored (SQA: 113 *m/z* (quantitative), 183, 127, 422 *m/z* (confirmation); SQE: 137 (quantitative), 410, 411 *m/z* (confirmation); CBN-D³ 298 *m/z* (quantitative), 313, 312 *m/z* (confirmation). Data acquisition and analysis were conducted using Agilent MassHunter Workstation Software (Agilent Technologies, Santa Clara, CA).

Standard Solutions Preparation

Neat materials were weighed gravimetrically, and all solutions were made volumetrically in hexane and stored at -70°C . Individual stock solutions of SQA and SQE were prepared and used to prepare a mixture of the two analytes (1.00 ng/ml) and two additional stock dilutions (0.100 and 0.005 ng/ml). Gamble's solution is a synthetic fluid that represents the interstitial fluid deep within the lungs that is commonly used in particle inhalation effect studies. Eight calibration standards were prepared daily by spiking 0.500 ml of Gamble's Solution with the appropriate dilution and 25 μl aliquot of internal standard (ISTD) CBN-D³ (100 $\mu\text{g/ml}$ methanol, as purchased) (Boisa et al., 2014). Final calibrator concentrations covered a range of 0.5–30 $\mu\text{g/ml}$.

Quality Control Material Preparation

Separate sets of reference materials, stock solutions, mixtures, and dilutions were used in all validation experiments and in the preparation of two quality control (QC) spiking pools. These pools were used to spike commercial synthetic BAL fluid (ml) at a low (QCL) and a high (QCH) analyte concentration. Mean concentrations (SQE: 1.27 and 22 $\mu\text{g/ml}$; SQA: 1.26 and 20 $\mu\text{g/ml}$) and the 95th (1.96 σ) and 99th (2.96 σ) percentile control limits for each pool were determined from five runs over five different days. Each QC pool was analyzed in duplicate with each analytical run and the resulting QC data compared to the established control limits to evaluate the validity of analyses using modified Westgard rules (Westgard et al., 1981; Caudill et al., 2008). All solutions were stored at -70°C .

Bronchoalveolar Lavage Fluid Collection and Storage

BAL fluid was obtained from 55 EVALI patients as part of their clinical treatment. The lavage procedures were not standardized across the multiple institutions that collected samples because the samples were being collected as part of emergency care or diagnosis of ill patients. Residual BAL fluids were sent to CDC as coded specimens with no personally identifiable information. Samples were refrigerated or frozen after collection and shipped on dry ice to CDC. A CDC human-subjects research review panel judged this collection of samples to be a non-research public health response activity.

Additional BAL fluids had previously been collected from 99 non-EVALI comparators ages ranging from 21 to 45 years. These comparators either did not use tobacco products (52), currently exclusively used nicotine-containing e-cigarette products (18), or currently exclusively smoked cigarettes (29) (Tsai et al., 1999; Song et al., 2020). BAL fluid was transported to the CDC laboratory on ice and processed within 30 min upon

arrival by centrifugation, to remove l; cellular debris pellets and supernatant were immediately placed in storage at -80°C (Blount et al., 2020). All non-EVALI participants provided written informed consent before participation. The study of healthy comparators was approved by the institutional review board at the Ohio State University, NCT02596685 (Song et al., 2020).

Bronchoalveolar Lavage Fluid Sample Preparation

For SQA/SQE analysis, 0.500 ml BAL fluid was thawed to room temperature, vortexed, transferred to a 16 $\times$ 125 mm disposable culture tube, and spiked with 25 μl CBN-D³ ISTD. Two milliliters of hexane were added, and samples were extracted by 360-degree rotation for 30 min. Samples were held at room temperature until the solvent phases separated; subsequently, the hexane layer was transferred to clean 16 $\times$ 125 mm centrifugation tubes using an automated liquid handling and solid phase extraction system (Gilson 274). The hexane layer was dried to completion under a stream of nitrogen in a turbo evaporator equipped with a heated water bath. Samples were reconstituted in 200 μl hexane, vortexed, transferred to amber sample vials with glass inserts and added to the GC-MS autosampler tray for analysis. Solvent blanks were injected after every EVALI case patient BAL fluid to confirm no analyte carryover and to help flush residual lipids from the column.

Quantitation

All SQA, SQE, and CBN-D³ data was evaluated for accuracy integration and manually reintegrated if necessary. Quantitation was based on a set of eight calibration standards (0.5–30 $\mu\text{g/ml}$) prepared in commercial synthetic BAL fluid same as unknown samples with the exception that standards were spiked with known concentrations of SQA and SQE prior to sample preparation. Calibration curves were constructed using quadratic regression of the analyte-to-ISTD response ratio versus known standard concentrations with 1/*X* weighting. Results were reported in concentration units (ng analyte per ml BAL fluid).

METHOD VALIDATION

Method validation parameters: analytical specificity, sensitivity, recovery, precision and repeatability, and storage stability were studied to confirm that the performance characteristics of the method were accurate and fit-for-purpose as described below.

Method Specificity

Specificity was verified by comparing chromatograms of 10 random human BAL fluids, pooled BAL fluid, and commercial synthetic BAL fluid to those spiked with SQA and SQE (1.5 $\mu\text{g/ml}$).

Accuracy

Accuracy was evaluated using spiked recoveries in hexane, matrix (commercial synthetic BAL fluid), and pooled human BAL at two levels (hexane 2.0 and 4.0 $\mu\text{g/ml}$; matrix 1.0 and 2.0 $\mu\text{g/ml}$; pooled human BAL 6.0 and 15.0 $\mu\text{l/mg}$ $n \geq 3$). Recovery was calculated as the ratio of experimental concentration and the nominal spiked

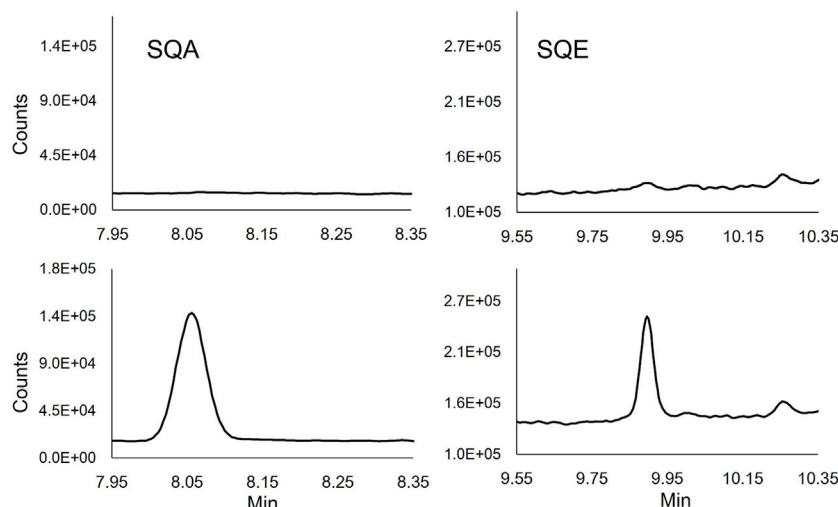


FIGURE 1 | Extracted ion chromatograms of SQE (137 m/z) and SQA (113 m/z) quantifying ions in unmodified (Top chromatograms) and spiked (1.5 µg/ml) human BALF (Lower chromatograms).

concentration and was considered acceptable between 85–115% (80–120% at 3 times LOD).

Precision

Method precision was evaluated as repeatability and intermediate precision of 10 QC samples at two levels over a period of 5 days. Repeatability was measured and reported as the percent relative standard deviation (%RSD) of within-run duplicate samples and intermediate precision was calculated as the %RSD of the mean of duplicate samples over 5 separate runs.

Stability

Analyte stability was assessed by two levels of prepared QC samples at room temperature (GC autosampler tray) for 48 h and at -20°C for 13 days. These conditions were chosen to evaluate analyte stability in prepared samples as the samples were queued for GCMS analysis or stored at -20°C . All samples were sequenced for instrument analysis immediately after preparation in this study.

METHOD APPLICATION

The validated method was used to analyze a total of 182 BAL fluid samples during the CDC response to the 2019 EVALI outbreak. Fifty-five of the analyzed samples were collected from probable or confirmed EVALI patients from 17 different states. Comparator BAL fluids were also collected from 99 volunteers with no major clinical illness.

RESULTS AND DISCUSSION

The specificity of the method was evaluated in synthetic commercial BAL fluid, pooled human BAL fluids, and 10

individual human BAL fluid samples. The resulting chromatographic data (e.g., baseline interferences, matrix interferences, and peak shape) was compared between samples prepared with only the addition of ISTD and those spiked with relatively low levels of each standard (1.5 µg/ml). No interfering peaks were identified in either the quantitation or confirmation ions for SQE, SQA or ISTD in the pooled and individual BAL fluid samples (**Figure 1** and **Supplementary Figure S1**). No chromatographic peak shape distortions or other visible matrix interferences were observed.

The method was developed to respond to a public health emergency and thus BAL fluid was not readily available to use as a solvent for matrix equivalency experiments. Despite this challenge, validation experiments document that the method performed without significant bias in BAL fluid matrix. **Table 1** shows acceptable accuracy following spiking analytes into hexane and synthetic BAL fluid (SQA: 80.6–106%; SQE: 93.2–106%). The lowest calculated recoveries were consistently from SQA spiked into hexane. Additional recovery experiments spiked in pooled human BAL fluid yielded similar results, and all were within the acceptable range (SQA: 91.9–103%; SQE: 83.4–102%). Within-run repeatability was determined to be acceptable with all % RSD being less than 4.5% for all both analytes. Intermediate precision over 5 days was calculated to be below 13% (SQA) and 9% (SQE). Thus, the internal standard was able to produce accurate and precise quantitation in both synthetic and actual BAL fluids despite potential differences in matrix compositions.

The minimum reporting level (MRL) for both SQA and SQE was set at the lowest calibrator concentration, 0.50 µg/ml BAL fluid. This value was verified by evaluating seven separately prepared calibration curves for the lowest calibrator that resulted in a chromatographic peak with a signal-to-noise (S/N) ratio greater than three and a calculated concentration accuracy of $\pm 40\%$ of the expected concentration. The absence

TABLE 1 | Analyte recovery for three fortified matrices ($n \geq 3$).

Analyte	Matrix	Spike value ($\mu\text{g/ml}$)	Recovered value Mean ($\mu\text{g/ml}$)	Recovery (%)	RSD (%)
SQA	Hexane	2	1.94	97.2	1.40
		4	3.22	80.6	1.30
	Synthetic BAL	1	0.860	86.3	11.4
		2	2.12	106	11.2
SQE	Hexane	2	2.16	106	3.80
		4	3.72	93.2	3.30
	Synthetic BAL	1	0.980	97.0	11.3
		2	2.10	105	14.5

of published methods quantitating these analytes in lung fluid makes it difficult to compare the MRLs reported here to similar methods; the 0.50 $\mu\text{g/ml}$ MRLs are, however, within the range of other analytical methods reporting the detection of SQE in biological samples, such as human hair (0.1 $\mu\text{g/ml}$), plasma (0.026 $\mu\text{g/ml}$), and bile (0.104 $\mu\text{g/ml}$) (Liu et al., 1976; Pacetti et al., 2019).

To ensure analyte stability in prepared samples during the study, a stability study was conducted at room-temperature (21°C in GC autosampler tray) and at -20°C. Both analytes were found to be stable in prepared BAL fluids stored at -20°C for 14 days with low percent changes in analyte concentrations from day 1 to day 14 (<6% decrease for SQA (1.93–5.66%) and less than 11% for SQE (11.30–6.65%). For prepared samples stored at room temperature for 48 h, we observed no differences in measured concentrations. Based on these results, SQA and SQE were considered stable in prepared BAL fluids under the conditions tested and were suitable for the intended application of the method; however, a longer stability study is needed to evaluate the stability of the analytes for longer periods of time. Due to the urgency of the results, all case-related BAL fluids were prepared within 24 h of receipt and were analyzed immediately after preparation.

APPLICATION RESULTS

The validated method was applied to analyze SQA and SQE in BAL fluids collected from 55 EVALI case patients and 99 comparators with no EVALI symptoms. Neither the case-related nor the comparator BAL fluids contained SQA or SQE above the method MRL; however, these findings do not indicate that the method was inadequately sensitive. Quality control samples were analyzed in the same analytical batch as unknown BAL fluid samples and yielded expected analyte levels within the stated imprecision of the method (Supplementary Figures S3, S4). Furthermore, the analytical sensitivity of each analysis was confirmed by an acceptable internal standard signal (Supplementary Figure S2). Thus, the effectiveness of the method was confirmed for each batch of analyses (QC quantitation) and for each individual analysis (internal standard signal). The intended purpose of this method was to look for evidence of inhaled SQA and SQE accumulating in BAL fluid to concentrations capable of causing lung injury and/or

lipoid pneumonia. Therefore, the focus was on a rapid method that could be developed, validated, and applied to clinical specimens in a matter of weeks. The resulting MRL was 0.5 $\mu\text{g/ml}$ of BAL fluid for both SQA and SQE; Thus, the method is adequately sensitive to detect the level of SQE identified in BAL fluid collected from a patient with lipoid pneumonia caused by SQE aspiration (Kanaji et al., 2008). Another way to evaluate the need for methodological sensitivity is to consider the likely daily inhalation of SQE based on the emissions of EVPs associated with EVALI case patients. EVALI-associated EVPs that contained SQE produced an aerosol with mean SQE concentration of 33 ng/ml puffed air (Cowan et al., 2022). Therefore, an EVALI case patient using one of these SQE-containing EVPs would inhale approximately 27,500 ng SQE for every 15 puffs. By comparison, the method that can detect as little as 0.125 ng of SQE or SQA on column. The finding of no detectable SQE or SQA likely indicates that these chemicals are being cleared from the lungs. Lastly, the method is able to detect SQE and SQA at much lower concentrations than the levels of vitamin E acetate that are attributed to be the primary cause of EVALI (Bhat et al., 2020; Blount et al., 2020; Morel-Espinosa et al., 2021).

Our findings clearly show no measurable accumulation of SQA or SQE in BAL fluids collected from EVALI case patients. These results do not preclude harm caused by vaping EVPs that contain SQA and SQE. Aspirated SQE has been shown to cause lipoid pneumonia (Kanaji et al., 2008; Kim et al., 2009; Cha et al., 2018) and has been detected in EVP aerosol emissions (Cowan et al., 2022). Thus, more research is needed to understand the health consequences of chronically inhaling these chemicals as constituents of EVP-produced aerosol. Additional work is also needed to better understand analyte stability over longer periods of time and to further optimize the gas chromatography liner and injection method to increase sensitivity of the method.

CONCLUSION

SQE and SQA are readily transmitted from vape products to aerosols that users inhale. Furthermore, accidental inhalation of large amounts of SQE are associated with lipoid pneumonia and many EVALI case patients presented with lipoid pneumonia-like symptoms. Therefore, we developed and validated an analytical method for measuring SQA and SQE in human BAL fluid to see if SQE or SQA were accumulating in EVALI case patient lungs. This rapidly developed

method was adequately sensitive, accurate, and precise for the intended purpose of investigating EVP-associated lung injury. The method was applied to BAL fluid samples from both relatively healthy comparators and patients presenting with EVALI. The concentration of SQA and SQE was found to be below the MRL for all BAL fluids. The results from this method helped to focus our public health response away from SQA and SQE and toward the likely primary causal agent, vitamin E acetate. Future application of this method will help to characterize the exposure and health consequences of chronically inhaling SQA and SQE as constituents of EVP-produced aerosol.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board at the Ohio State University, NCT02596685.

REFERENCES

- Aberegg, S. K., Cirulis, M. M., Maddock, S. D., Freeman, A., Keenan, L. M., Pirozzi, C. S., et al. (2020). Clinical, Bronchoscopic, and Imaging Findings of E-Cigarette, or Vaping, Product Use-Associated Lung Injury Among Patients Treated at an Academic Medical Center. *JAMA Netw. Open* 3 (11), e2019176–13. doi:10.1001/jamanetworkopen.2020.19176
- Aresta, A., Damascelli, A., De Vietro, N., and Zamboni, C. (2020). Measurement of Squalene in Olive Oil by Fractional Crystallization or Headspace Solid Phase Microextraction Coupled with Gas Chromatography. *Int. J. Food Properties* 23 (1), 1845–1853. doi:10.1080/10942912.2020.1833033
- Bhat, T. A., Kalathil, S. G., Bogner, P. N., Blount, B. C., Goniewicz, M. L., and Thanavala, Y. M. (2020). An Animal Model of Inhaled Vitamin E Acetate and EVALI-like Lung Injury. *N. Engl. J. Med.* 382 (12), 1175–1177. doi:10.1056/NEJMc2000231
- Blount, B. C., Karwowski, M. P., Shields, P. G., Morel-Espinosa, M., Valentin-Blasini, L., Gardner, M., et al. (2020). Vitamin E Acetate in Bronchoalveolar Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* 382 (8), 697–705. doi:10.1056/NEJMoa1916433
- Boisa, N., Elom, N., Dean, J. R., Deary, M. E., Bird, G., and Entwistle, J. a. (2014). Development and Application of an Inhalation Bioaccessibility Method (IBM) for lead in the PM10 Size Fraction of Soil. *Environ. Int.* 70, 132–142. doi:10.1016/j.envint.2014.05.021
- Braak, D., Michael Cummings, K., Nahhas, G. J., Reid, J. L., and Hammond, D. (2020). How Are Adolescents Getting Their Vaping Products? Findings from the International Tobacco Control (ITC) Youth Tobacco and Vaping Survey. *Addict. Behaviors* 105, 106345–106347. doi:10.1016/j.addbeh.2020.106345
- Brosius, C. R., Caron, K. T., Sosnoff, C. S., Blount, B. C., and Wang, L. (2022). Rapid Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method to Measure Cannabinoids in Bronchoalveolar-Lavage Fluid of Patients with E-Cigarette, or Vaping, Product Use-Associated Lung Injury. *ACS Omega* 7, 443–452. doi:10.1021/acsomega.1c04815
- Budge, S. M., and Barry, C. (2019). Determination of Squalene in Edible Oils by Transmethylation and GC Analysis. *MethodsX* 6, 15–21. doi:10.1016/j.mex.2018.12.001

AUTHOR CONTRIBUTIONS

EC: methodology, validation, data analysis, investigation, writing—original draft. HT: validation, investigation, sample analysis. CW: conceptualization, writing—review and editing, oversight. BB: conceptualization, visualization, funding acquisition, supervision, writing—review and editing. LB: visualization, supervision, project administration, funding acquisition, formal analysis, writing—review and editing.

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- Caudill, S. P., Schleicher, R. L., and Pirkle, J. L. (2008). Multi-rule Quality Control for the Age-Related Eye Disease Study. *Statist. Med.* 27, 4094–4106. doi:10.1002/sim.3222
- Cha, S. I., Choi, S. H., Kim, H. J., Kim, Y. J., Lim, J. K., Yoo, S. S., et al. (2019). Clinical and Radiological Manifestations of Lipoid Pneumonia According to Etiology: Squalene, omega-3-acid Ethyl Esters, and Idiopathic. *Clin. Respir. J.* 13, 328–337. doi:10.1111/crj.13015
- Cowan, E. A., Tran, H., Gray, N., Perez, J. J., Watson, C., Blount, B. C., et al. (2022). A Gas Chromatography-Mass Spectrometry Method for Quantifying Squalene and Squalene in Aerosol Emissions of Electronic Cigarette, or Vaping, Products. *Talanta* 238 (1), 122985. doi:10.1016/j.talanta.2021.122985
- Cullen, K. A., Ambrose, B. K., Gentzke, A. S., Appelberg, B. J., Jamal, A., and King, B. A. (2018). *Notes From the Field: Use of Electronic Cigarettes and Any Tobacco Product Among Middle and High School Students - United States, 2011–2018*. *MMWR Morb. Mortal. Wkly. Rep.* 67, 1276–1277. doi:10.15585/mmwr.mm6745a5
- Cullen, K. A., Gentzke, A. S., Sawdey, M. D., Chang, J. T., Anic, G. M., Wang, T. W., et al. (2019). E-Cigarette Use Among Youth in the United States, 2019. *Jama* 322 (21), 2095–2103. doi:10.1001/jama.2019.18387
- De Jesús, V. R., Chambers, D. M., Reese, C., Braselton, M., Espinosa, P., Corstvet, J., et al. (2020a). Novel Methods for the Analysis of Toxicants in Bronchoalveolar Lavage Fluid Samples from E-Cigarette, or Vaping, Product Use-Associated Lung Injury Cases: Selected Petroleum Distillates. *Rapid Commun. Mass. Spectrom.* 34 (19), e8898. doi:10.1002/rcm.8898
- De Jesús, V. R., Silva, L. K., Newman, C. A., and Blount, B. C. (2020b). Novel Methods for the Analysis of Toxicants in Bronchoalveolar Lavage Fluid Samples from E-cigarette, or Vaping, Product Use Associated Lung Injury (EVALI) Cases: Terpenes. *Rapid Commun. Mass. Spectrom.* 34 (19). doi:10.1002/rcm.8879
- Erythropel, H. C., Jabba, S. V., DeWinter, T. M., Mendizabal, M., Anastas, P. T., Jordt, S. E., et al. (2019). Formation of Flavorant-Propylene Glycol Adducts with Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids. *Nicotine Tob. Res.* 21 (9), 1248–1258. doi:10.1093/ntr/nty192
- Gay, B., Field, Z., Patel, S., Alvarez, R. M., Nasser, W., Madruga, M., et al. (2020). Vaping-Induced Lung Injury: A Case of Lipoid Pneumonia Associated with

- E-Cigarettes Containing Cannabis. *Case Rep. Pulmonology* 2020, 1–4. doi:10.1155/2020/7151834
- Holder, C., Adams, A., Allison, C., Cote, O., Lippens, R., Blount, B. C., et al. (2021). A Novel UHPLC-MS/MS Method for Measuring 8-Iso-Prostaglandin F2 α in Bronchoalveolar Lavage Fluid. *Front. Chem.* 9, 695940. doi:10.3389/fchem.2021.695940
- Kanaji, N., Bandoh, S., Takano, K., Ishida, T., Kadota, K., Haba, R., et al. (2008). Positron Emission Tomography-Positive Squalene-Induced Lipoid Pneumonia Confirmed by Gas Chromatography-Mass Spectrometry of Bronchoalveolar Lavage Fluid. *Am. J. Med. Sci.* 335 (4), 310–314. doi:10.1097/maj.0b013e31811ec1a0
- Kim, E. S., Kim, K. W., Song, T. W., Cho, S. H., Kim, M.-J., Kim, K.-E., et al. (2009). Squalene-induced Exogenous Lipoid Pneumonia in an Infant. *Pediatr. Int.* 51 (5), 751–753. doi:10.1111/j.1442-200X.2009.02902.x
- King, B. A., Gammon, D. G., Marynak, K. L., and Rogers, T. (2018). Electronic Cigarette Sales in the United States, 2013–2017. *Jama* 320 (13), 1379–1380. doi:10.1001/jama.2018.10488
- King, B. A., Jones, C. M., Baldwin, G. T., and Briss, P. A. (2020). The EVALI and Youth Vaping Epidemics - Implications for Public Health. *N. Engl. J. Med.* 382 (8), 689–691. doi:10.1056/NEJMp1916171
- Knapp, A. A., Lee, D. C., Borodovsky, J. T., Auty, S. G., Gabrielli, J., and Budney, A. J. (2019). Emerging Trends in Cannabis Administration Among Adolescent Cannabis Users. *J. Adolesc. Health* 64, 487–493. doi:10.1016/j.jadohealth.2018.07.012
- Kong, G., Morean, M. E., Cavallo, D. A., Camenga, D. R., and Krishnan-Sarin, S. (2017). Sources of Electronic Cigarette Acquisition Among Adolescents in Connecticut. *Tobacco Reg. Sci.* 3 (1), 10–16. doi:10.18001/TRS.3.1.2
- Kosmider, L., Sobczak, A., Fik, M., Knysak, J., Zaciara, M., Kurek, J., et al. (2014). Carbonyl Compounds in Electronic Cigarette Vapors: Effects of Nicotine Solvent and Battery Output Voltage. *Nicotine Tob. Res.* 16 (10), 1319–1326. doi:10.1093/ntr/ntu078
- Lee, D. C., Crosier, B. S., Borodovsky, J. T., Sargent, J. D., and Budney, A. J. (2016). Online Survey Characterizing Vaporizer Use Among Cannabis Users. *Drug and Alcohol Dependence* 159, 227–233. doi:10.1016/j.drugalcdep.2015.12.020
- Lee, J. S., Im, J.-G., Song, K. S., Seo, J. B., and Lim, T.-H. (1999). Exogenous Lipoid Pneumonia: High-Resolution CT Findings. *Eur. Radiol.* 9, 287–291. doi:10.1007/s003300050669
- Liu, G. C., Ahrens, E. H., Crouse, J. R., and Crouse, J. R. (1976). Measurement of Squalene in Human Tissues and Plasma: Validation and Application. *J. Lipid Res.* 17, 38–45. doi:10.1016/s0022-2275(20)37014-0
- Meier, M. H., Docherty, M., Leischow, S. J., Grimm, K. J., and Pardini, D. (2019). Cannabis Concentrate Use in Adolescents. *Pediatrics* 144 (3), 338. doi:10.1542/peds.2019-0338
- Miech, R., Leventhal, A., Johnston, L., O'Malley, P. M., Patrick, M. E., and Barrington-Trimis, J. (2021). Trends in Use and Perceptions of Nicotine Vaping Among US Youth from 2017 to 2020. *JAMA Pediatr.* 175 (2), 185–190. doi:10.1001/jamapediatrics.2020.5667
- Morean, M. E., Lipshie, N., Josephson, M., and Foster, D. (2017). Predictors of Adult E-Cigarette Users Vaporizing Cannabis Using E-Cigarettes and Vape-Pens. *Substance Use & Misuse* 52 (8), 974–981. doi:10.1080/10826084.2016.1268162
- Morel Espinosa, M., Blount, B. C., and Valentin-Blasini, L. (2021). Liquid Chromatography-Tandem Mass Spectrometry Method for Measuring Vitamin E Acetate in Bronchoalveolar Lavage Fluid. *J. Chromatogr. B* 1171, 1–5. doi:10.1016/j.jchromb.2021.122607
- Pacetti, D., Scortichini, S., Boarelli, M. C., and Fiorini, D. (2019). Simple and Rapid Method to Analyse Squalene in Olive Oils and Extra virgin Olive Oils. *Food Control* 102, 240–244. doi:10.1016/j.foodcont.2019.03.005
- Reagan-Steiner, S., Gary, J., Matkovic, E., Ritter, J. M., Shieh, W.-J., Martinez, R. B., et al. (2020). Pathological Findings in Suspected Cases of E-Cigarette, or Vaping, Product Use-Associated Lung Injury (EVALI): a Case Series. *Lancet Respir. Med.* 8 (12), 1219–1232. doi:10.1016/S2213-2600(20)30321-0
- Reddy, L. H., and Couvreur, P. (2009). Squalene: A Natural Triterpene for Use in Disease Management and Therapy. *Adv. Drug Deliv. Rev.* 61, 1412–1426. doi:10.1016/j.addr.2009.09.005
- Romeh, F., Diaz, M. C., Vallone, D., Tynan, M. A., Cordova, J., Seaman, E. L., et al. (2018). “E-cigarette Unit Sales, by Product and Flavor Type- United States, 2014–2020,” in *Morbidity and Mortality Weekly Report, MMWR*.
- Santivañez-Veliz, M., Moreno-Viguri, E., Pérez-Silanes, S., Varela, J., Cerecetto, H., González, M., et al. (2017). Development, Validation and Application of a GC-MS Method for the Simultaneous Detection and Quantification of Neutral Lipid Species in *Trypanosoma Cruzi*. *J. Chromatogr. B* 1061–1062, 225–232. doi:10.1016/j.jchromb.2017.07.031
- Schulenberg, J. E., Johnston, L. D., O'Malley, P. M., Bachman, J. G., Miech, R. A., and Patrick, M. E. (2019). “College Students & Adults Ages 19–60,” in *Monitoring the Future National Survey Results on Drug Use 1975–2019: Volume II* (Ann Arbor: Institute for Social Research: The University of Michigan).
- Shah, J., Mullen, M., and Lu, M. (2020). E-cigarette or Vaping Product Use-Associated Lung Injury: A Case Study and Review of the Literature. *J. Radiol. Nurs.* 39 (4), 305–308. doi:10.1016/j.jradnu.2020.09.005
- Song, M.-A., Freudenheim, J. L., Brasky, T. M., Mathe, E. A., McElroy, J. P., Nickerson, Q. A., et al. (2020). Biomarkers of Exposure and Effect in the Lungs of Smokers, Nonsmokers, and Electronic Cigarette Users. *Cancer Epidemiol. Biomarkers Prev.* 29 (2), 443–451. doi:10.1158/1055-9965.EPI-19-1245
- Spindle, T. R., Bonn-Miller, M. O., and Vandrey, R. (2019). Changing Landscape of Cannabis: Novel Products, Formulations, and Methods of Administration. *Curr. Opin. Psychol.* 30, 98–102. doi:10.1016/j.copsyc.2019.04.002
- Tsai, M., Song, M.-A., McAndrew, C., Brasky, T. M., Freudenheim, J. L., Mathé, E., et al. (2019). Electronic versus Combustible Cigarette Effects on Inflammasome Component Release into Human Lung. *Am. J. Respir. Crit. Care Med.* 199 (7), 922–925. doi:10.1164/rccm.201808-1467LE
- Uddin, S. M. I., Osei, A. D., Obisesan, O. H., El-Shahawy, O., Dzaye, O., Cainzos-Achirica, M., et al. (2020). Prevalence, Trends, and Distribution of Nicotine and Marijuana Use in E-Cigarettes Among US Adults: The Behavioral Risk Factor Surveillance System 2016–2018. *Prev. Med.* 139, 106175–106177. doi:10.1016/j.ypmed.2020.106175
- Wang, T. E., Neff, L. J., Park-Lee, E., Ren, C., Cullen, K. A., and King, B. A. (2020). “E-cigarette Use Among Middle and High School Students - United States, 2020,” in *Morbidity and Mortality Weekly Report, MMWR*. doi:10.15585/mmwr.mm6937e1
- Werner, A. K., Koumans, E. H., Chatham-Stephens, K., Salvatore, P. P., Armatas, C., Byers, P., et al. (2020). Hospitalizations and Deaths Associated with EVALI. *N. Engl. J. Med.* 382 (17), 1589–1598. doi:10.1056/NEJMoa1915314
- Westgard, J. O., Barry, P. L., Hunt, M. R., and Groth, T. (1981). A Multi-Rule Shewhart Chart for Quality Control in Clinical Chemistry. *Clin. Chem.* 27 (3), 493–501. doi:10.1093/clinchem/27.3.493
- Wu, Y., Chen, G., Ji, C., Hoptroff, M., Jones, A., Collins, L. Z., et al. (2016). Gas Chromatography-Mass Spectrometry and Raman Imaging Measurement of Squalene Content and Distribution in Human Hair. *Anal. Bioanal. Chem.* 408, 2357–2362. doi:10.1007/s00216-016-9335-0
- Xia, B., Blount, B. C., and Wang, L. (2021). Sensitive Quantification of Nicotine in Bronchoalveolar Lavage Fluid by Acetone Precipitation Combined with Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry. *ACS Omega* 6 (22), 13962–13969. doi:10.1021/acsomega.0c05696

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Minor Tobacco Alkaloids as Biomarkers to Distinguish Combusted Tobacco Use From Electronic Nicotine Delivery Systems Use. Two New Analytical Methods

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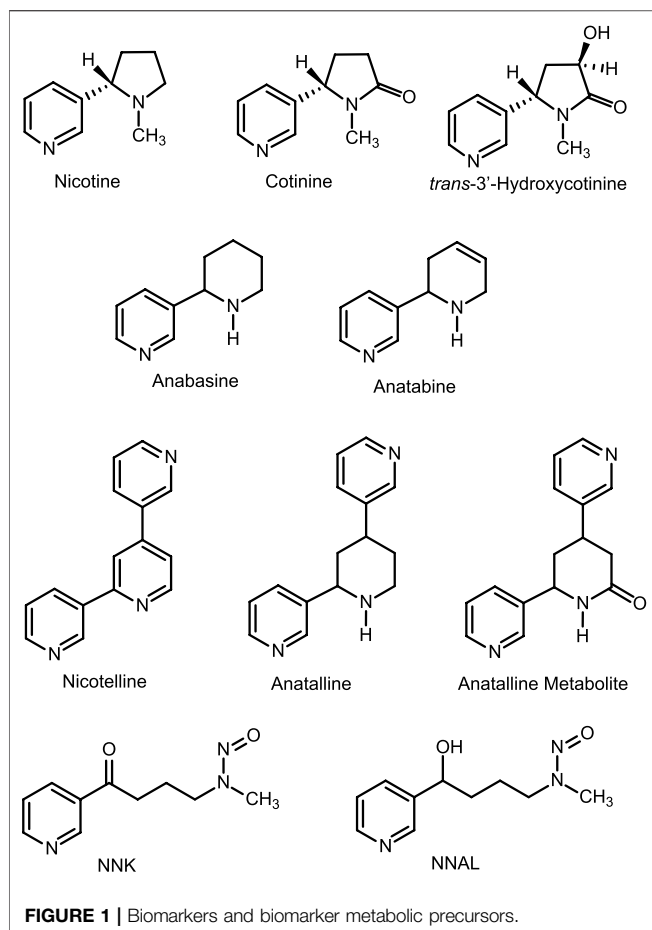
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Biomarkers for the use of electronic nicotine delivery systems (ENDS) are desirable for studies of the health effects of electronic cigarettes and related devices. However, the aerosols inhaled from these devices do not contain substances that are unique to this class of products, *i.e.*, substances that are not present in cigarette smoke or those that do not have common environmental or dietary sources. Consequently, identifying selective biomarkers for ENDS use remains a challenge. If co-use of conventional tobacco products can be definitively ruled out, then nicotine and its metabolites are suitable for assessing exposure. Self-reports from questionnaires are often used to obtain information on product use. But self-reports may not always be accurate, and are not amenable to obtaining quantitative information on exposure. An alternative approach is to use selective biomarkers for conventional tobacco products to definitively rule out their use. In this article, we describe two new LC-MS/MS methods for the minor tobacco alkaloids anabasine, anatabine, nicotelline, anatabine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a tobacco-specific nitrosamine metabolite, all biomarkers that are selective for the use of conventional tobacco products. Applications of these biomarkers in studies of ENDS use and dual use of ENDS and conventional tobacco products are also discussed.

Keywords: tobacco, e-cigarettes, biomarkers of exposure, tobacco alkaloids, liquid chromatography -tandem mass spectrometry

INTRODUCTION

During the past several years, a variety of new tobacco products and nicotine delivery devices have been introduced. These include ENDS, heated tobacco products such as Philip Morris' IQOS and British American Tobacco's Glo, and oral nicotine delivery products such as Zyn, On! and Velo. Of these, ENDS, in particular electronic cigarettes are the most widely used. Most but not all public health researchers are of the opinion that these new products are generally less harmful than conventional tobacco products. The extent to which they could reduce harm is unknown, largely because some adverse health effects, in particular cancer and chronic lung disease, take many years to

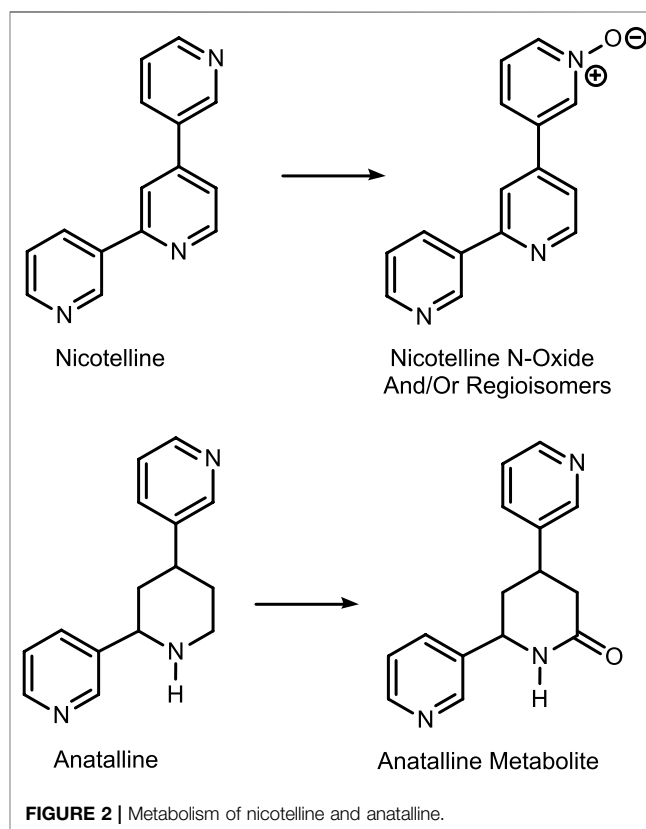


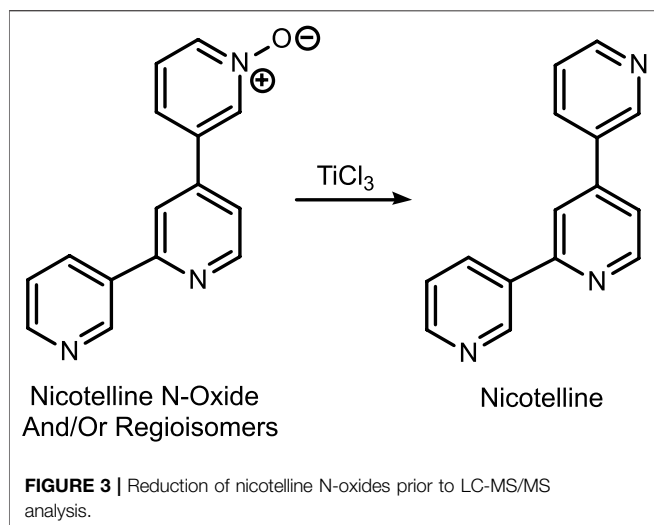
develop. Therefore, thorough epidemiological studies have not been possible. Furthermore, dual use of ENDS with combusted cigarettes is commonplace, and the extent of reduced exposure and potential harm in ENDS users who continue to smoke cigarettes is difficult to assess. (Goniewicz et al., 2018; Borland et al., 2019; Piper et al., 2019; Smith et al., 2021) In short term studies of acute effects of novel products such as ENDS, (Hajek et al., 2017; St Helen et al., 2020a) it is important to determine recent use of tobacco products as well as the extent of dual use of both products. For exposure assessment, self-reported use of particular products can be useful, but they are of limited utility for obtaining quantitative data. In this regard, biomarkers of exposure are useful. Biomarkers would be especially important to assess recent dual use in clinical trials of novel products and for epidemiological studies of health effects. Specific biomarkers have not been identified for e-cigarettes and other ENDS, because the substances in these products are nicotine, solvents used to generate the aerosols (propylene glycol and glycerol), and flavoring compounds which are found in conventional tobacco products and have dietary sources as well. (Schick et al., 2017) Pyrolysis reactions transform components of the e-liquids into various products during aerosol formation, but as yet no pyrolysis products unique to ENDS have been identified that could serve as selective biomarkers. Consequently, other than self-reports, which are

of limited value, the only viable approach is to use biomarkers specific to tobacco products to identify and estimate the extent of their use in people using ENDS.

Tobacco contains a number of pyridine alkaloids other than the major alkaloid nicotine. (Schmeltz and Hoffmann, 1977; Rodgman and Perfetti, 2013) These minor alkaloids, which include anabasine, anatabine, anatabine, and nicotelline (Figure 1) are present in cigarette tobacco in concentrations ranging from about 1 to 1000 µg/g, compared to concentrations of about 15 mg/g for nicotine. (Jacob et al., 2013; Lisko et al., 2013) Although nicotine in nearly all e-liquids used in ENDS is derived from tobacco, the nicotine in most (but not all) products has been purified sufficiently that minor alkaloid concentrations are low compared to the amounts present in tobacco. (Palazzolo et al., 2019; Jacob et al., 2020) Consequently, minor alkaloids may be used as biomarkers for the use of conventional tobacco products in people using ENDS. (Berlin et al., 2019; Jacob et al., 2020) In this article we describe two new methods for quantitation of nicotine-related minor tobacco alkaloids in urine that can be used in this approach. One of these methods also measures concentrations of the tobacco-specific carcinogen metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). The goal of our studies was to develop methods to simultaneously quantify multiple biomarkers useful in studies of dual use of ENDS and combusted cigarettes.

The first method is based on our published LC-MS/MS method for nicotelline, (Jacob et al., 2013) an alkaloid that we have proposed as a biomarker to distinguish ENDS use from use





of combusted cigarettes. (Jacob et al., 2020) Since little if any nicotelline is excreted unchanged, and the metabolites identified so far are N-oxides (Figure 2), the method involves treating urine with titanium trichloride to convert the N-oxides back to nicotelline (Figure 3) that can be readily extracted and measured. Chromatography and mass spectrometry parameters were modified to include other minor tobacco alkaloid analytes. The second LC-MS/MS method utilizes a derivatization with hexanoic anhydride, developed for the carcinogen biomarker NNAL to enhance sensitivity. (Jacob et al., 2008) This derivatization also converts the secondary amine alkaloids anabasine, anatabine, and anattaline into amides, which results in improved chromatography, and allows simultaneous determination of these alkaloids with NNAL. (Figure 4) An advantage of these new methods is simultaneous determination of multiple biomarkers that have a wide range of biological half-lives, ranging from 2–3 h for nicotelline, to more than 10 days for NNAL. This can be important if measures of long-term exposure and recent exposure are desired. Another advantage is higher sensitivity (lower limits of quantitation) than previously reported methods, thus facilitating low-level exposure assessment. These advantages of the two new methods should make them especially useful in studies of dual use of ENDS and conventional tobacco products. We also introduce the alkaloid anattaline as a new, highly selective biomarker for tobacco exposure.

MATERIALS AND METHODS

Reagents and Standards

Analytical standards and internal standards are available commercially (Toronto Research Chemicals, North York, ON, Canada, and other sources) or can be synthesized by published methods. (Surya Prakash Rao et al., 1997; Jacob et al., 2013) The analyte standards were anabasine (internal standard anabasine- d_4 , pyridine ring labeled), anatabine (internal standard anatabine- d_4 , pyridine ring labeled),

nicotelline (internal standard nicotelline- d_8 , pyridine rings labeled), anattaline (internal standard anattaline- d_4 , pyridine ring labeled), anattaline metabolite: 4,6-di-3-pyridinyl-2-piperidinone (internal standard anattaline metabolite- d_4 , pyridine ring labeled), NNAL: 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanol (internal standard NNAL- d_3 , N-methyl labeled). Reagents and solvents used for sample extractions and for preparing LC mobile phases were of analytical reagent grade or HPLC grade.

Instrumentation

LC-MS/MS analyses were carried out with a Thermo Accela UPLC pump and Pal Autosampler interfaced to a Thermo Vantage triple-stage quadrupole mass spectrometer, or with a Thermo/Dionex UltiMate 3000 RS Pump UPLC+ Focused UPLC and CTC/Dionex UltiMate 3000 XRS Open Autosampler interfaced to a Thermo Quantiva triple-stage quadrupole mass spectrometer. Evaporations were carried out using a centrifugal vacuum evaporator, Thermo-Fisher Speedvac concentrator SPD 2010.

Extraction Procedure, Method 1

The internal standards, in 0.01 N aqueous HCl, 100 μ L of a mixture of anabasine- d_4 , (100 ng/ml) anatabine- d_4 (100 ng/ml), nicotelline- d_8 (10 ng/ml), anattaline- d_4 (4 ng/ml) and anattaline metabolite- d_4 (150 ng/ml), were added to 0.5 ml sample of urine. 100 μ L titanium (III) chloride, 20% w/v solution in 2 N hydrochloric acid (ACROS Organics) were added to fortified

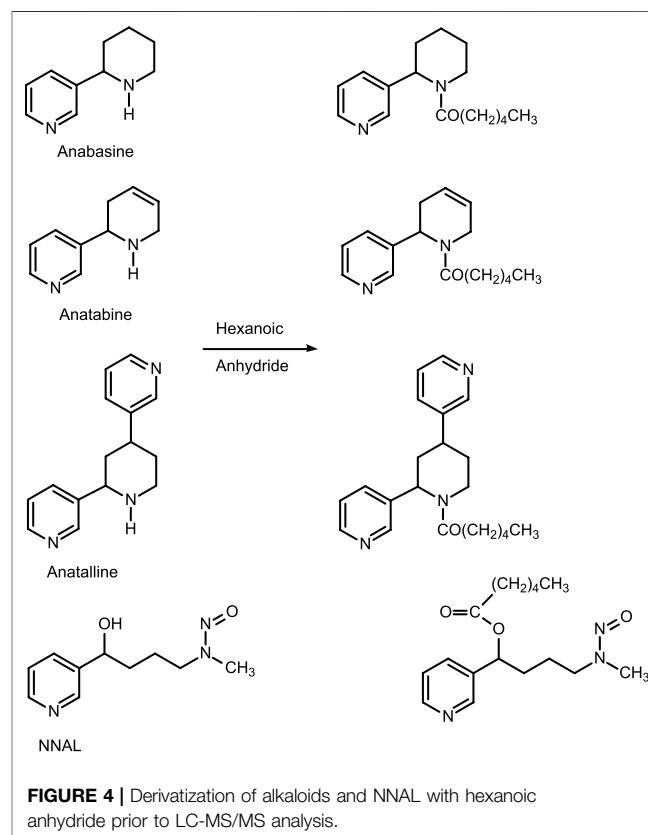


TABLE 1 | SRM transitions and collision energies (CE) for analytes and internal standards.

Analyte	Method 1			Method 2		
	Parent	Product	CE	Parent	Product	CE
Anabasine	163	146	14	261	120	30
Anabasine-d ₄	167	150	14	265	124	30
Anatabine	161	144	14	259	144	30
Anatabine-d ₄	165	148	14	263	148	30
Anatalline	240	197	18	338	197	30
Anatalline-d ₄	244	201	18	342	201	30
Anatalline Metabolite	254	195	25	NA	NA	NA
Anatalline Metabolite-d ₄	258	199	25	NA	NA	NA
Nicotelline	234	207	30	NA	NA	NA
Nicotelline-d ₈	242	214	30	NA	NA	NA
NNAL	NA	NA	NA	308	162	11
NNAL-d ₃	NA	NA	NA	311	165	11

urine sample, were mixed and incubated 30 min at room temperature. Saturated aqueous tetrasodium EDTA/concentrated ammonium hydroxide (4:1, 500 μ L) was added next. Toluene/ethyl acetate (2:1, 4.5 ml) was added, the tubes were vortexed 5 min, centrifuged at 4,000 g for 10 min, and the aqueous phase frozen by immersion in a dry ice/acetone bath. The organic phase was poured to a new tube containing 0.5 ml 1 M sulfuric acid. The mixture was vortexed, centrifuged, and the upper layer was poured out and discarded after freezing the aqueous layer in dry ice/acetone. The acid phase containing the analytes was made basic with 0.5 ml 50% potassium carbonate, and 4 ml pentane/dichloromethane (1:1) was added. The mixture was vortexed, centrifuged, and placed in a dry ice/acetone bath to freeze the lower aqueous layer. The organic phase poured into a tube containing 100 μ L 10% HCl in methanol (to prevent evaporation of the analytes by converting them to non-volatile salts) before evaporating to dryness. The residues were reconstituted in 200 μ L 200 mM ammonium formate in 10% MeOH that had been adjusted to pH 9 with concentrated aqueous ammonia. Standards and QC samples were prepared by spiking pooled non-smokers' urine with the analytes, spanning the expected concentration ranges. QC sample concentrations were for anabasine, anatabine, and anatalline, in ng/mL: 30, 5, 0.25, 0.1, and 0 = blank urine matrix. For nicotelline they were, in ng/mL: 3, 0.5, 0.025, 0.01, 0 = blank urine matrix. For the anatalline metabolite they were, in ng/mL: 3, 0.5, 0 = blank urine matrix. Duplicate standards and QCs were extracted and analyzed with each sample run.

Chromatography and Mass Spectrometry, Method 1

A 20 μ L aliquot of the extract was injected *via* the autosampler into the LC-MS/MS system, Vantage or Quantiva system. Chromatography was performed on an X-Bridge BEH C18 column (2.5 μ m particle size, 3 mm $\times$ 150 mm, Waters, United States) at 50°C with a flow rate of 0.6 ml/min, applying a gradient consisting of 20 mM ammonium formate in 10% methanol with pH 9 (A) and methanol (B). Preparation of 1 L of mobile phase A involves mixing 1.25 g of ammonium formate,

0.5 ml concentrated aqueous ammonia, 100 ml methanol and HPLC grade water to volume. Gradient conditions were as follows: 0 min: 100% A, 0–10 min: 100–40% A, 10–11 min: 40–0% A, 11–13 min: 0% A, 13–13.5 min: 0–100% A, 13.5–17 min: 100% A. Positive electrospray ionization (ESI) was used. The spray voltage was 3000, the vaporizer temperature was 450°C, the capillary temperature was 350°C, the sheath gas pressure was 45 psi, the auxiliary gas pressure was 5 psi, and the ion sweep gas pressure was 2 psi. The resolution of the first quadrupole, FWHM, was set at 0.4 amu, the resolution of the third quadrupole was set at 0.7 amu FWHM. The MS/MS system was run in the selected reaction monitoring (SRM) mode. Mass transitions for the analytes and internal standards are in **Table 1**.

Data Analysis Method 1

The Thermo XCalibur/LC Quan software was used to generate calibration curves and calculate concentrations using peak area ratios of analyte/internal standard. Linear regression with 1/X weighting, “ignore origin” was used. Blanks (pooled non-smokers' urine) were included in the standard curves and “ignore origin” was used to correct for the small amounts of analytes that might be present in non-smokers' urine used to prepare standards, due to secondhand smoke exposure. Eight concentrations spanning the calibration range for each analyte were used, and standards were run in duplicate. Typically, one set of standards was injected at the beginning of the run, and one set following injection of the clinical study samples. Concentrations of the standards, equations and correlation coefficients for representative calibration curves are in the Supplementary Material document.

Extraction Procedure, Method 2

The internal standards, in 0.01 N HCl, 100 μ L of a mixture of anabasine-d₄ (100 ng/ml), anatabine-d₄ (100 ng/ml), anatalline-d₄ (4 ng/ml) and NNAL d₃ (3 ng/ml) were added to 1 ml of urine sample. 100 μ L 2 M sodium potassium phosphate buffer pH 7, and 100 μ L β -glucuronidase (from *E. coli* type IXA Sigma-Aldrich, 1000 units) dissolved in 0.1 M phosphate buffer were added to the samples as well. Samples were placed in an incubator overnight at 37°C. (This step hydrolyzes glucuronide conjugates to the parent metabolite. This is done because a large percentage of NNAL is conjugated, (Carmella et al., 2002) and providing results as “total NNAL” improves sensitivity as well as reduces variability due to individual differences in the extent of conjugation. The amount of enzyme added is comparable to the amount previously shown to maximize deconjugation (Jacob et al., 2008)). To each sample 0.1 ml potassium carbonate (50% w/v), and 3 ml 70:30 toluene/1-butanol were added. The tubes were vortexed 5 min, centrifuged at 4,000 g for 5 min, and the aqueous phase frozen by immersion in a dry ice/acetone bath. The organic phase was poured to a new tube containing 0.5 ml 1 M sulfuric acid. The mixture was vortexed, centrifuged, and the upper layer was poured off and discarded after freezing the aqueous layer in dry ice/acetone. The acid phase containing the analytes washed with 2 ml of 1:2 ethyl acetate/toluene by vortexing, centrifuging and placing in a dry ice/acetone bath to freeze the lower aqueous layer. The upper layer was poured off

and discarded. The acid layer was made basic with 0.5 ml of 50% (w/v) potassium carbonate and 3 ml 2:1 toluene/ethyl acetate was added. The mixture was vortexed, centrifuged, and placed in a dry ice/acetone bath to freeze the lower aqueous layer. The organic phase poured into a tube containing 100 μ L 10% hydrochloric acid in methanol (to prevent evaporation of the analytes by converting them to non-volatile salts) before evaporating to dryness. The residues were derivatized by adding 50 μ L hexanoic anhydride and catalyst, 10 μ L of 50 mg/ml 4-dimethylaminopyridine (DMAP) in toluene and the tubes were capped and heated at 70 °C for 15 min. Saturated aqueous sodium bicarbonate (0.5 ml) and 3 ml of 10% ethyl acetate in pentane were added. The tubes were placed in a dry ice/acetone bath to freeze the lower aqueous layer, and the organic phase was poured into tubes containing 0.5 ml of 1 M sulfuric acid. The tubes were vortexed, centrifuged, and placed in a dry ice/acetone bath to freeze the aqueous layers. The organic layers were poured off and discarded. The acid layers were washed with 3 ml 10% ethyl acetate in pentane by vortexing, centrifuging, freezing the aqueous layers, pouring off and discarding the organic layers. The acid layers were made basic with 0.5 ml of 50% (w/v) potassium carbonate and then extracted with 3 ml of 10% ethyl acetate in pentane by vortexing, centrifuging, freezing the aqueous layer, and pouring organic layer to a new set tubes for evaporation. Evaporation to dryness was carried out using a SpeedVac. The residues were reconstituted in 200 μ L 20% methanol in 0.1% formic acid. Standards and QC samples were prepared by spiking pooled non-smokers' urine with the analytes, spanning the expected concentration ranges. QC sample concentrations were for anabasine, anatabine, and anattaline, in ng/mL: 30, 5, 0.25, 0.1, 0.03, and 0 = blank urine matrix. For NNAL they were, in ng/mL: 3, 0.5, 0.025, 0.01, 0.003, and 0 = blank urine matrix. Duplicate standards and QCs were extracted and analyzed with each sample run.

Chromatography and Mass Spectrometry, Method 2

A 20 μ L aliquot of the extract was injected *via* the autosampler into the Vantage LC-MS/MS system. Chromatography was performed on a Phenomenex Kinetex phenyl hexyl 100 A column (2.6 μ m particle size, 3 mm $\times$ 150 mm, Phenomenex, United States) at 50°C with a flow rate of 0.6 ml/min, applying a gradient consisting of 10 mM ammonium formate in 5% methanol (A) and methanol (B). Preparation of 1 L of mobile phase A involves mixing 0.625 g of ammonium formate, 50 ml methanol and HPLC grade water to volume. Gradient conditions were as follows: 0 min: 45% A, 0–3 min: 45% A, 3–4.5 min: 45–0% A, 4.5–5 min: 0% A, 5–5.5 min: 0–45% A, 5.5–8 min: 45% A. Positive electrospray ionization (ESI) was used. The spray voltage was 3500, the vaporizer temperature was 440°C, the capillary temperature was 395°C, the sheath gas Pressure was 45 psi, the auxiliary gas pressure was 5 psi, and the ion sweep gas pressure was 0 psi. The resolution of the first quadrupole, FWHM, was set at 0.5 amu, the resolution of the third quadrupole was set at 0.7 amu FWHM. The MS/MS system was run in the selected reaction monitoring (SRM) mode. Mass transitions for the analytes and internal standards are in **Table 1**.

Data Analysis Method 2

Calibration for quantitation was carried out as described for Method 1 above. Typical equations and correlation coefficients for representative standard curves are in the Supplementary Material document.

Methods Validation

Precision, accuracy, and limits of quantitation were determined by replicate analysis of spiked urine samples, at concentrations spanning the expected concentration ranges (**Tables 2, 3**) as described by Shah *et al.* (Shah *et al.*, 2000) and Viswanathan *et al.* (Viswanathan *et al.*, 2007). Briefly, the criteria are that the precision should be RSD less than 15%, except at the LOQ which should be less than 20%. The accuracy should be within $\pm$ 15% of the expected amount except at the LOQ in which $\pm$ 20% is acceptable. The LOQ was the lowest concentration meeting these criteria. Lack of carryover was verified by analysis of analytical blanks, extracts of non-smokers' urine described above. Blanks also served to identify potentially interfering substances derived from the sample matrix or from reagents and solvents used in extractions.

Human Urine Samples

Urine samples were available from previous studies. (Benowitz *et al.*, 2012; St Helen *et al.*, 2020b) All studies received the approval of the appropriate institutional review boards. Twenty urine samples from cigarette smokers were obtained at baseline in a longitudinal study of progressive reduction in the nicotine concentrations of cigarettes. (Benowitz *et al.*, 2012) Nineteen urine samples from non-smokers not exposed to SHS were obtained in San Francisco. Smoking status and SHS exposure was by self-report and/or the nicotine metabolite cotinine concentration below the established cutpoint of 40 ng/ml for distinguishing smokers from non-smokers. (Edwards *et al.*, 2021) Urine samples from 36 dual users of combusted cigarettes and e-cigarettes were 24 h collections in a crossover study of use of e-cigarettes and combusted cigarettes carried out on the Clinical Research Center at Zuckerberg San Francisco General Hospital. (St Helen *et al.*, 2020b) Urine samples were collected during 2 days of *ad libitum* vaping or cigarette smoking and 2 days of enforced abstinence.

RESULTS AND DISCUSSION

Two methods for minor tobacco alkaloids were developed, with the goal of simultaneously measuring urine concentrations of multiple analytes of interest. These include the established biomarkers anabasine and anatabine, and nicotelline, that we proposed as a biomarker for the particulate matter derived from tobacco smoke, (Jacob *et al.*, 2013) anattaline, a little-studied tobacco alkaloid that we are developing as a new biomarker, and NNAL, a well-established biomarker for the tobacco-specific nitrosamine NNK (Hecht, 2002).

Method 1 is based on an LC-MS/MS method we developed for nicotelline. Since little if any nicotelline is excreted in urine

TABLE 2 | Method 1 precision and accuracy for determination of anabasine, anatabine, anataline, anataline metabolite, and nicotelline in Urine. 6 replicate analyses.¹

Analyte	Added amount (ng/ml)	Measured mean (ng/ml)	Accuracy (percent of expected)	Precision CV (%)
Anabasine LLOQ = 0.1 ng/ml	30.0	30.1	102	1.1
	5.00	5.58	112	1.7
	0.250	0.225	90	10.2
	0.100	0.087	87	9.4
Anatabine LLOQ = 0.1 ng/ml	30.0	28.6	95	2.6
	5.00	5.28	106	1.9
	0.250	0.254	102	2.1
	0.100	0.101	101	2.4
Anataline LLOQ = 0.1 ng/ml	30.0	28.1	94	6.5
	5.00	5.36	107	5.8
	0.250	0.243	97	5.4
	0.100	0.080	80	2.2
Anataline Metabolite LLOQ = 0.5 ng/ml	3.00	3.25	108	2.4
	0.500	0.541	108	1.7
Nicotelline LLOQ = 0.01 ng/ml	3.00	2.73	91	1.8
	0.500	0.460	92	2.0
	0.025	0.022	89	7.5
	0.010	0.0090	90	3.5

¹Pooled non-smokers' urine was spiked with analytes to the specified concentrations. LLOQ = Lower Limit of Quantitation. Individual sample data are in the Supplementary Material document.

TABLE 3 | Method 2 precision and accuracy for determination of anabasine, anatabine, anataline, and NNAL in Urine. 6 replicate analyses.¹

Analyte	Added amount (ng/ml)	Measured mean (ng/ml)	Accuracy (percent of expected)	Precision CV (%)
Anabasine LLOQ = 0.030 ng/ml	30.0	30.3	101	3.7
	5.00	5.16	103	1.5
	0.250	0.260	104	2.7
	0.100	0.106	106	6.7
	0.030	0.027	91.5	8.7
	Smoker's Urine	5.35	NA	2.5
Anatabine LLOQ = 0.030 ng/ml	30.0	30.8	103	2.4
	5.00	4.79	95.9	2.5
	0.250	0.257	103	2.1
	0.100	0.105	105	3.3
	0.030	0.029	96.5	4.7
	Smoker's Urine	4.80	NA	1.1
Anataline LLOQ = 0.030 ng/ml	30.0	34.3	114	2.7
	5.00	5.62	112	3.3
	0.250	0.270	108	4.8
	0.100	0.108	108	5.5
	0.030	0.033	110	7.9
	Smoker's Urine	2.77	NA	0.9
NNAL LLOQ = 0.0030 ng/ml	3.00	3.25	108	2.4
	0.500	0.541	108	1.7
	0.025	0.028	113	3.2
	0.010	0.010	103	5.2
	0.003	0.0033	109	5.1
	Smoker's Urine	0.0479	NA	3.6

¹Pooled non-smokers' urine was spiked with analytes to the specified concentrations. LLOQ = Lower Limit of Quantitation. Individual sample data are in the Supplementary Material document.

TABLE 4 | Mean concentrations of nicotelline, anatalline, anabasine and anatabine, normalized to nicotine, in 70 e-liquids compared to the corresponding concentrations in a mainstream smoke of a reference cigarette. BLQ = Below the limit of quantitation.¹ Concentrations of nicotine and the other alkaloids were determined by the method of Jacob *et al.*, described in reference (Jacob *et al.*, 2020). The 70 e-liquids are also described in Supplementary Information for reference (Jacob *et al.*, 2020).

Product	N	Mean concentration (range) µg/mg nicotine			
		Nicotelline	Anatalline	Anabasine	Anatabine
E-Liquids	70	0.00016 (BLQ–0.0043) 91% BLQ	0.0042 (BLQ–0.081) 86% BLQ	0.41 (BLQ–2.80) 40% BLQ	1.00 (BLQ–8.89) 7% BLQ
Mainstream Smoke, 1R6F Reference Cigarette, HCl Regimen		1.55	1.92	1.71	6.14

¹LOQs were: nicotine, 1 µg/ml; nicotelline, 0.15 ng/ml; anatalline, 0.46 ng/ml; anabasine, 4.1 ng/ml; anatabine, 0.05 ng/ml.

unchanged, and the only metabolites characterized so far are N-oxides, the method involves treating urine with titanium trichloride, which reduces the N-oxides back to nicotelline that can be readily quantitated. (Jacob *et al.*, 2013) (**Figure 3**). We have modified this method to include anabasine, anatabine, anatalline, and a lactam metabolite of anatalline as analytes. Nicotelline is highly selective for tobacco, and was undetectable or present at very low concentrations in 70 e-liquids that we analyzed. (Jacob *et al.*, 2020) Therefore, we proposed that nicotelline could be used as a biomarker for combusted tobacco use in people using e-cigarettes. (Jacob *et al.*, 2020) Nicotelline has a short half-life, 2–3 h, and is useful for detecting recent tobacco use. (Jacob *et al.*, 2013) But nicotelline concentrations are undetectable in 12–24 h after tobacco use ceases. The tobacco-specific nitrosamine metabolite NNAL has also been used as a biomarker for tobacco use in ENDS users, but NNAL has a very long half-life, >10 days (Hecht *et al.*, 1999; Goniewicz *et al.*, 2009) and it can be measured in urine for several weeks after tobacco cessation. Consequently, biomarkers with half-lives longer than nicotelline, but shorter than NNAL, such as anabasine, anatabine, and anatalline would be also useful in studies of the short-term effects of switching from combusted cigarettes to e-cigarettes.

The minor alkaloids anabasine and anatabine have been used as biomarkers for tobacco use in people using nicotine-containing medications for tobacco cessation. (Jacob *et al.*, 2002; Suh-Lailam *et al.*, 2014; Sinclair *et al.*, 2020) They have also been used as biomarkers for tobacco use in people using ENDS. (Berlin *et al.*, 2019) Anabasine and anatabine have half-lives of about 16 and 10 h, respectively, and can detect tobacco use for a few days following tobacco cessation. (Jacob *et al.*, 1999) Therefore, they are complementary to nicotelline ($t_{1/2} = 2$ h) and NNAL ($t_{1/2} > 10$ days). (Benowitz *et al.*, 2020) However, in contrast to nicotelline, anabasine and anatabine have been found in e-liquids, sometimes in concentrations as high as in cigarette tobacco normalized to nicotine, (Palazzolo *et al.*, 2019; Jacob *et al.*, 2020) which may limit their utility as biomarkers selective for tobacco use. Anatalline is another minor alkaloid that we are developing as a biomarker. Like nicotelline, (Jacob *et al.*, 2020) and in contrast to anabasine and anatabine, anatalline was undetectable or present at very low concentrations in 70 e-liquids that we analyzed. (**Table 4**). Interestingly, nicotelline does not appear to be naturally occurring (probably

not biosynthesized) in the tobacco plant, and is mainly formed from anatalline by pyrolysis and oxidation in burning tobacco. This was demonstrated by adding anatalline to a non-tobacco plant material, oregano, preparing a “cigarette” from this, combusting, collecting and analyzing the smoke. In parallel, an oregano “cigarette” without anatalline was prepared and combusted. Nicotelline was detected in the smoke from the oregano “cigarette” spiked with anatalline, but not in the smoke from the “cigarette” without anatalline. (Jacob *et al.*, 2013) This demonstrated that nicotelline can be formed from anatalline during combustion conditions, and that neither nicotelline nor anatalline are likely to be formed by combustion/pyrolysis of organic materials. The half-life of anatalline *appears* to be similar to that of anabasine or somewhat longer, which is apparent from the data presented in **Figure 8**, but additional studies will be required to determine its half-life. Therefore, we propose that anatalline would be a more selective biomarker than anabasine and anatabine for tobacco use in ENDS users, but otherwise would have similar attributes.

Method 1 uses the same sample prep as our published LC-MS/MS method for nicotelline. (Jacob *et al.*, 2013) Chromatography and mass spectrometry parameters were modified to include anabasine, anatabine and anatalline. Data on precision, accuracy, and limits of quantitation for the method are presented in **Table 2**. Representative selected reaction monitoring (SRM) chromatograms are in **Figure 5**. This method was used to obtain data on concentrations of anabasine, anatabine, anatalline, an anatabine metabolite, and nicotelline in cigarette smokers and in non-smokers urine, summarized in **Table 5**. Concentrations of the widely used biomarkers for nicotine exposure, cotinine and *trans*-3'-hydroxycotinine are included for comparison, since concentrations, especially cotinine, have been used for many years as biomarkers of tobacco exposure, and can serve as an index of the extent of tobacco and/or nicotine product use. Applications of the minor alkaloids in studies of dual use of ENDS and conventional tobacco products would likely include cotinine concentrations as well to assess overall nicotine product use. We believe that this is the first published data on concentrations of anatalline and its metabolite in human urine.

Method 2 is based on a LC-MS/MS method we developed for the tobacco-specific nitrosamine metabolite NNAL. (Jacob *et al.*,

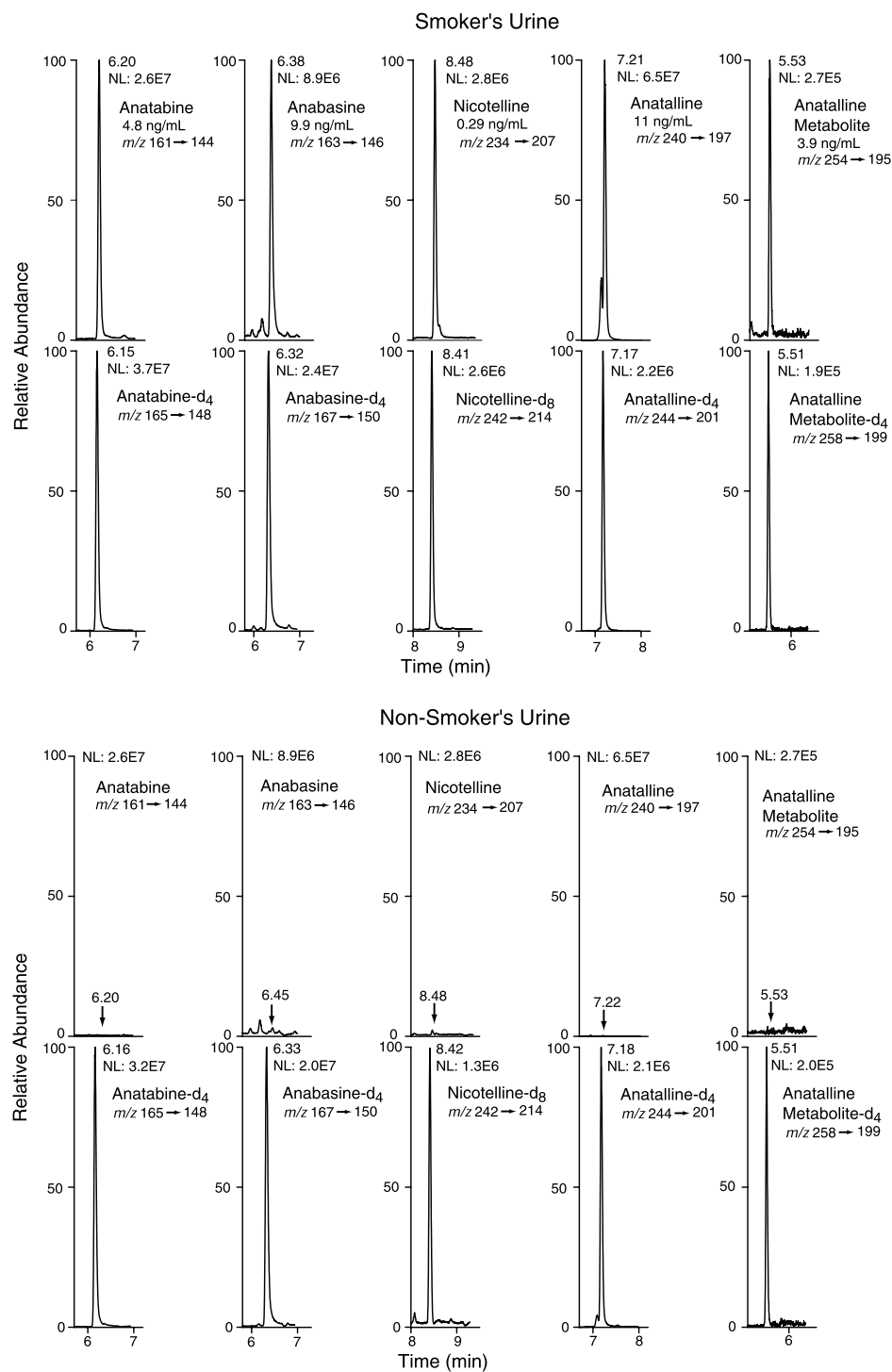


FIGURE 5 | Selected reaction monitoring (SRM) chromatograms from urine analyzed by Method 1. Analyte peaks in the non-smoker's chromatogram were scaled to match those of the smoker's urine chromatogram. The internal standard concentrations were anabasine-d₄ and anatabine d₄, 20 ng/ml; anataline-d₄, 0.8 ng/ml; anataline metabolite-d₄, 30 ng/ml; nicotelline-d₈, 2 ng/ml.

2008) It involves treating extracts with hexanoic anhydride to give an ester derivative, that facilitates sample clean up *via* extraction with non-polar solvents, resulting in increased sensitivity

compared to analyses with underivatized NNAL. Hexanoic anhydride converts anabasine, anatabine and anataline to hexanoic acid amides that likewise facilitates clean up of

TABLE 5 | Concentrations of biomarkers in urine of 20 cigarette smokers and 19 non-smokers¹.

Biomarker	Smokers ^{2,3}	Non-smokers ⁴
Anabasine, ng/mL		
Mean	14.1	0.346
Range	0.503–47.2	BLQ–5.23
SD	13.1	1.2
Detection Frequency (LLOQ = 0.100)	100%	32%
Anatabine, ng/mL		
Mean	11.7	BLQ
Range	0.508–33.7	BLQ
SD	10.5	
Detection Frequency (LLOQ = 0.100)	100%	0%
Anatalline, ng/mL		
Mean	14.3	BLQ
Range	0.595–78.1	BLQ
SD	17.4	
Detection Frequency (LLOQ = 0.050)	100%	0%
Anatalline Metabolite, ng/mL		
Mean	3.15	BLQ
Range	BLQ–9.07	BLQ
SD	2.5	
Detection Frequency (LLOQ = 0.500)	95%	0%
Nicotelline, ng/mL		
Mean	1.70	BLQ
Range	0.095–6.43	BLQ–0.059
SD	1.7	17
Detection Frequency (LLOQ = 0.010)	100%	26%
Cotinine, ng/mL		
Mean	1,557	0.20
Range	501–3,245	BLQ–2.6
SD	763	0.59
Detection Frequency (LLOQ = 10, 0.05) ⁵	100%	58%
3'-Hydroxycotinine, ng/mL		
Mean	6,458	0.88
Range	376–12,547	BLQ–9.8
SD	3,580	2.2
Detection Frequency (LLOQs = 10, 0.1) ⁵	100%	84%

¹Concentrations of anabasine, anatabine, anatalline, anatalline metabolite, and nicotelline were determined by Method 1. Concentrations of cotinine and 3'-hydroxycotinine were determined by the methods of Jacob et al. [Reference (Jacob et al., 2011)].

Concentrations of NNAL were determined by the method of Jacob et al. [Reference (Jacob et al., 2008)]. Individual sample data are in the Supplementary Material document.

²Smokers smoked an average of 18.9 cigarettes per day, 95% confidence interval = 15.5–22.3

³If below the limit of quantitation (BLQ), LLOQ/square root 2 was used.

⁴If below the limit of quantitation (BLQ), 0 was used.

⁵Two method variations were used. LLOQ is 10 for smokers' urine and lower for non-smokers' urine

BLQ = Below the limit of quantitation

extracts and allows simultaneous determination along with NNAL. (Figure 4) Data on precision, accuracy, and limits of quantitation for the method are presented in Table 3. Representative selected reaction monitoring (SRM) chromatograms are in Figure 6. We think that it is interesting to note that in all SRM chromatograms from smokers' urine extracts a peak with a retention time of about 0.45 min longer than the anatalline peak is observed, not found in chromatograms

from non-smokers urine spiked with the anatalline standard. In Method 1 chromatograms, SRM chromatograms from smokers' urine extracts a partially resolved peak with a shorter retention time than the anatalline is observed, not found in chromatograms from non-smokers spiked with the anatalline standard. (Figures 5–7). These peaks are clearly derived from a substance inhaled in cigarette smoke, possibly an isomeric alkaloid, and chromatographic separation is needed to accurately quantify anatalline. We also observed a peak partially resolved from anatalline in SRM chromatograms from a cigarette tobacco extract. (Jacob et al., 2013)

Method 2 has been used in a crossover study of dual users of e-cigarettes and combusted cigarettes. (St Helen et al., 2020b) The participants used e-cigarettes or smoked combusted cigarettes in separate 2-day study blocks, followed by a third 2-day block when they abstained from the use of any nicotine product, enforced by the study being carried out on a research ward with no access to nicotine-containing products. Urine samples were collected and analyzed for biomarker concentrations (Figure 8). These data are from a subset of participants ($n = 19$) in which the 2-day abstinence block immediately followed the e-cigarette block. For the five days prior to the e-cigarette block, participants were instructed to use their usual e-cigarette product and not use other tobacco or nicotine-containing products. However, since they were outpatients, compliance could not be enforced. We used data from this study, in which concentrations of anabasine, anatabine, anatalline, and NNAL were determined using Method 2, and nicotelline was available from previous analyses using a published method, (Jacob et al., 2013) to illustrate the attributes of the various biomarkers in terms of their different rates of elimination (Figure 8). Nicotelline, with a half-life of 2–3 h detects recent combusted tobacco use, and concentrations were near or below the limit of quantitation (LOQ) during the inpatient e-cigarette and abstinence blocks. Anatabine ($t_{1/2} = 10$ h) was measurable during the e-cigarette block but not the abstinence block. Anabasine, anatalline, and NNAL were measurable in both the e-cigarette and abstinence blocks because their half-lives are too long to fall below the LOQ during the course of this study. (Benowitz et al., 2020) NNAL, due to its very long (>10 days) half-life can be detected for several weeks following tobacco cessation. Depending on the goals of a particular study, confirmation of short-term or long-term tobacco cessation may be desirable. NNAL would be the most useful for population studies in which any tobacco use in the past 2–3 months is of interest. The minor alkaloids are more relevant for assessment of short-term cigarette smoking, such as looking for point prevalence of smoking in smoking cessation trials. Also, the high sensitivity of Method 2 extends the time frame of applicability of anabasine and anatabine in which these biomarkers can be measured following tobacco cessation, and makes low-level exposure assessment possible. The lower limits of quantitation for anabasine and anatabine in Method 2 are 0.03 ng/ml. For comparison, the LLOQs reported in Wei et al. for a method used in large, population-scale studies including the National Health and Nutrition Examination Survey (NHANES) and the Population Assessment of Tobacco and Health (PATH) study are an order of magnitude higher, 0.5 and 0.4 ng/ml,

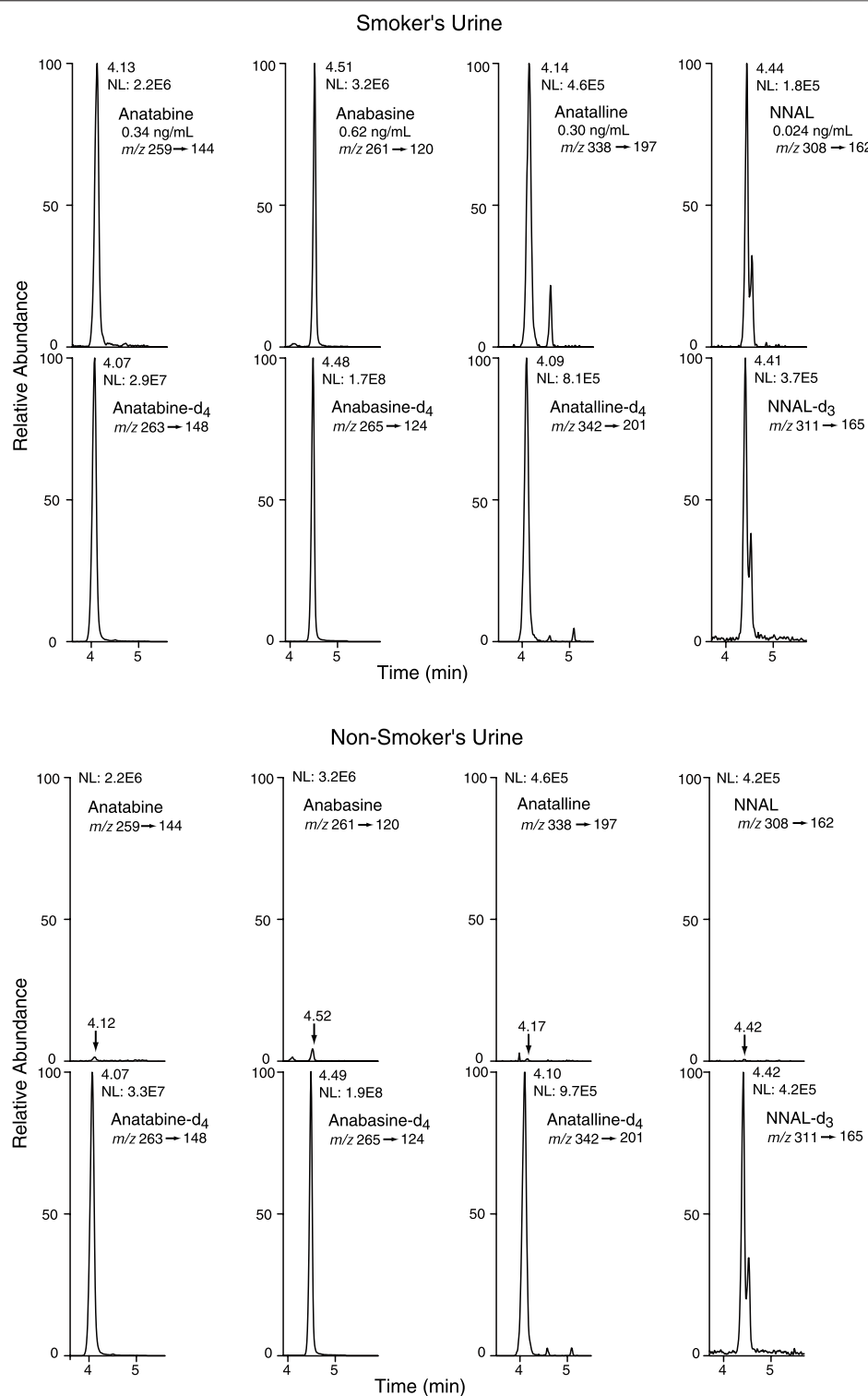
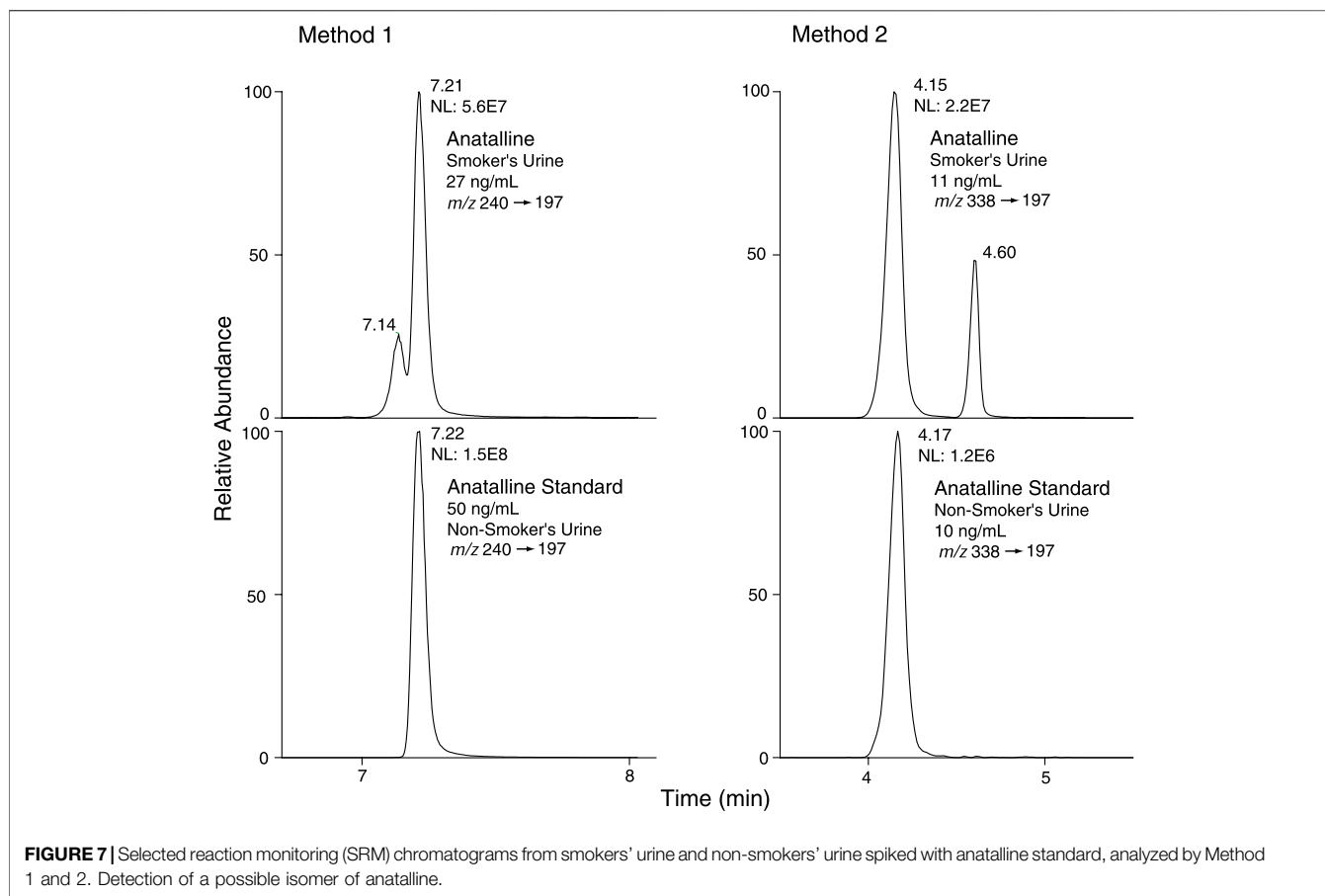


FIGURE 6 | Selected reaction monitoring (SRM) chromatograms from urine analyzed by Method 2. Analyte peaks in the non-smoker's chromatogram were scaled to match those of the smoker's urine chromatogram. The internal standard concentrations were anabasine-d₄ and anatabine d₄, 20 ng/ml; anattaline-d₄, 0.8 ng/ml; NNAL-d₃, 0.6 ng/ml. There are two partially resolved NNAL and NNAL-d₃ peaks, because NNAL exists as two slowly (on the timescale of the chromatography) equilibrating *syn* and *anti* isomers with respect to the N-N bond.



respectively. (Wei et al., 2014) The LLOQ of NNAL in Method 2, 0.003 ng/ml, is sufficient for measuring exposure in cigarette and cigar smokers and smokeless tobacco users, since this is well below the cutpoint of 0.010–0.040 ng/ml for distinguishing active use from passive exposure. (Benowitz et al., 2020) For low-levels of secondhand smoke exposure, a more sensitive method may be advantageous, such as the method we reported that uses a larger urine volume but the same sample prep as Method 2, and has a LLOQ of 0.00025 ng/ml. (Jacob et al., 2008)

SUMMARY AND CONCLUSION

The goal of our studies was to develop methods to simultaneously quantify multiple biomarkers useful in studies of dual use of ENDS and combusted cigarettes.

Both of the methods we describe include anabasine, anatabine, and anatalline as analytes. Anabasine and anatabine have been used for a number of years as biomarkers to distinguish the use of nicotine-containing medications from the use of conventional tobacco products, and continue to be used in large population studies, such as the National Health and Nutrition Examination Survey (NHANES) and the Population Assessment of Tobacco and Health (PATH) study. (Wei et al., 2014) They have also been used to distinguish e-cigarette use from combusted cigarette use.

(Berlin et al., 2019) In this report, we introduce anatalline as a new biomarker, with a rate of elimination similar to anabasine and anatabine, based on preliminary data, as illustrated in **Figure 8**. We suggest that anatalline has similar attributes to anabasine and anatabine, but unlike those two biomarkers it has not been found to any significant extent in e-liquids (**Table 4**) and therefore should be more selective for the use of conventional tobacco products.

The two methods differ in that Method 1 also measures nicotelline, and Method 2 also measures NNAL. Method 1 cannot measure NNAL because the titanium trichloride reagent used to reduce nicotelline N-oxides decomposes NNAL. Method 2 uses derivatization with hexanoic anhydride to enhance sensitivity for NNAL and also enhances the sensitivity for anabasine, anatabine, and anatalline. Nicotelline cannot be measured with Method 2, because little if any is excreted unchanged, and the only known metabolites are N-oxides, and reduction of these with titanium trichloride to nicotelline is required for sensitive quantitation. (Jacob et al., 2013) Another difference between the methods is that Method 2 employs a deconjugation step using β -glucuronidase, since about 50% of NNAL is excreted as glucuronides. Since nicotine and cotinine are N-conjugated (pyridine nitrogen), the possibility exists that anabasine, anatabine, and anatalline might likewise be N-conjugated. To address this possibility, we analyzed a

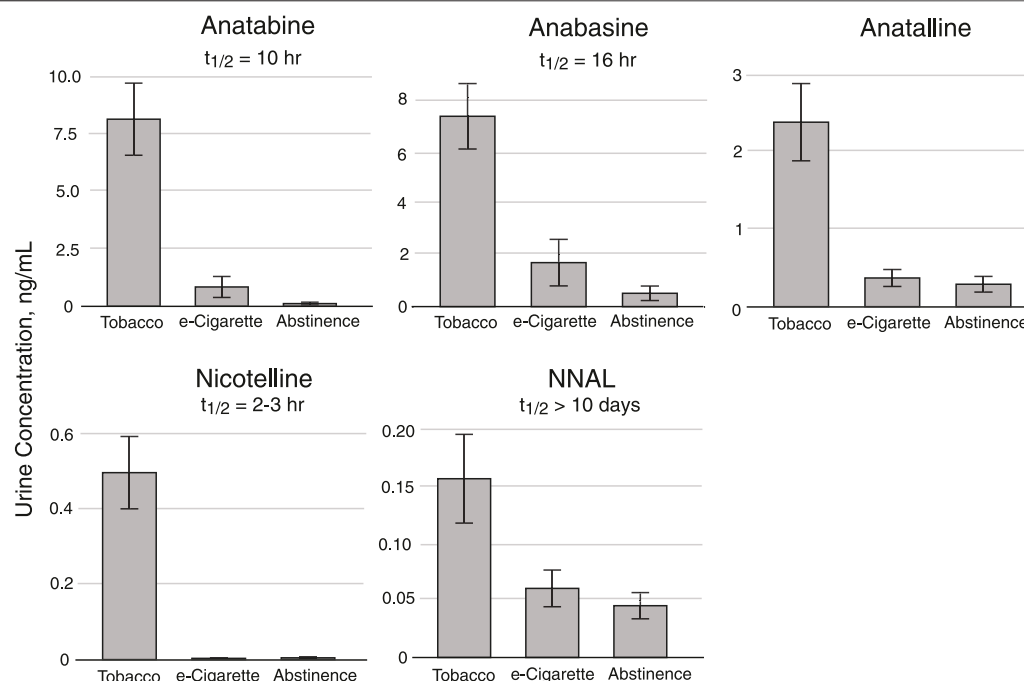


FIGURE 8 | Urine concentrations of Anatabine, anabasine, anattalline, nicotelline, and NNAL in dual users of combusted cigarettes and e-cigarettes enrolled in a crossover study carried out on a research ward. Participants were inpatients in three 2-day study blocks. For this subset of participants ($n = 19$), the 2-day abstinence block immediately followed the e-cigarette block. For the five days prior to the e-cigarette block, participants were instructed to use their usual e-cigarette product *ad libitum* and not use other tobacco or nicotine-containing products. However, since they were outpatients, compliance could not be enforced. Urine (24 h pool) collected during the second day of each 2-day study block was analyzed for the biomarkers. The “Tobacco” columns data were obtained during the 2-day cigarette smoking block. Individual sample data are in the Supplementary Material document.

TABLE 6 | Concentrations of anabasine, anatabine, anattalline, and NNAL in pooled smokers’ urine, with and without deconjugation using β -glucuronidase. Mean of 6 replicate analyses using Method 2.¹

Analyte	Total (Enzyme deconjugated) ng/mL (SD)	Free (No deconjugation) ng/mL (SD)	Percent conjugated (%)
Anabasine	5.9 (0.13)	4.7 (0.13)	20
Anatabine	5.5 (0.18)	3.8 (0.14)	31
Anattalline	2.9 (0.11)	2.9 (0.12)	0
NNAL	0.087 (0.0020)	0.044 (0.0013)	49

¹Individual sample data are in the Supplementary Material document.

pooled smokers’ urine with and without deconjugation using Method 2. The data are presented in **Table 6**. Anabasine and anatabine were excreted as glucuronides to the extent of 20 and 31%, respectively, but interestingly glucuronidation of anattalline was not detected. As expected, NNAL was about 50% conjugated.

Method 1 also measures a lactam metabolite (**Figure 1**) of anattalline, which is reported for the first time. The lactam metabolite of nicotine, cotinine is a valuable biomarker for various reasons, including a longer half-life than nicotine. (Benowitz et al., 2020) By analogy, the lactam metabolite of anattalline might likewise be a useful biomarker. Consequently, we postulated the existence of this metabolite, and found that it indeed it is excreted in urine of smokers (**Table 5**). But, it proved to be a difficult analyte, perhaps due to its polarity and inefficient extraction limiting method sensitivity. This is reflected in the relatively high

LOQ (0.5 ng/ml compared to the other analytes and the correlation coefficient of the standard curve. However, due to its potential attributes, further studies of this metabolite including efforts to develop a more sensitive method may be warranted.

Concerning the relative merits of these two new methods, the choice may depend on which analytes are most important for a particular study. Method 1 simultaneously measures nicotelline, anabasine, anatabine, and anattalline. Method 1 would be most appropriate if a measure of recent cigarette smoking, within 24 h, was desired, which would be provided by nicotelline, with a half-life of 2–3 h. This method also detects smoking occurring over the past several days, from anatabine, anabasine, and anattalline, with half-lives ranging from 10 h to 16 or more hours. Anabasine, anatabine, and anattalline may be useful for confirming tobacco cessation over this time frame, such as studies of the effects of short-term switching

from combusted cigarettes to e-cigarettes. Method 2 simultaneously measures anabasine, anatabine, anataline, and NNAL. Method 2 would be most appropriate if the goal were to detect, and measure the extent of smoking occurring over several weeks, which would be provided by NNAL, which has a half-life in excess of 10 days. Method 2 will also detect smoking occurring within several days, from concentrations of anabasine, anatabine, and anataline. Method 2 would also be most appropriate for low-level exposure assessment, because it is more sensitive than Method 1 by a factor of about 3 for anabasine, anatabine, anataline (Tables 2, 3).

The value of multiple tobacco biomarkers with a range of elimination rates is discussed in a recent publication, including a figure illustrating the time course for concentrations to fall below the LLOQ. (Benowitz et al., 2020)

In conclusion, two new methods for tobacco biomarkers have been developed, that can be applied to studies of dual users of ENDS and conventional tobacco products. Advantages include simultaneous determination of multiple analytes, and improved sensitivity compared to previous methods that could be useful for low-level exposures. We also introduce a new biomarker, anataline. Anataline has similar attributes to anabasine and anatabine, and may have greater specificity for tobacco than those two alkaloids.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Committee on Human Research, University of California, San Francisco. The patients/participants provided their written informed consent to participate in this study.

REFERENCES

- Benowitz, N. L., Bernert, J. T., Foulds, J., Hecht, S. S., Jacob, P., Jarvis, M. J., et al. (2020). Biochemical Verification of Tobacco Use and Abstinence: 2019 Update. *Nicotine Tob. Res.* 22 (7), 1086–1097. doi:10.1093/ntr/ntz132
- Benowitz, N. L., Dains, K. M., Hall, S. M., Stewart, S., Wilson, M., Dempsey, D., et al. (2012). Smoking Behavior and Exposure to Tobacco Toxicants during 6 Months of Smoking Progressively Reduced Nicotine Content Cigarettes. *Cancer Epidemiol. Biomarkers Prev.* 21 (5), 761–769. doi:10.1158/1055-9965.EPI-11-0644
- Berlin, I., Dautzenberg, B., Lehmann, B., Palmyre, J., Liégey, E., De Rycke, Y., et al. (2019). Randomised, Placebo-Controlled, Double-Blind, Double-Dummy, Multicentre Trial Comparing Electronic Cigarettes with Nicotine to Varenicline and to Electronic Cigarettes without Nicotine: the ECSMOKE Trial Protocol. *BMJ Open* 9 (5), e028832. doi:10.1136/bmjopen-2018-028832
- Borland, R., Murray, K., Gravely, S., Fong, G. T., Thompson, M. E., McNeill, A., et al. (2019). A New Classification System for Describing Concurrent Use of Nicotine Vaping Products Alongside Cigarettes (So-Called 'dual Use'): Findings from the ITC-4 Country Smoking and Vaping Wave 1 Survey. *Addiction* 114 (Suppl. 1), 24–34. doi:10.1111/add.14570

AUTHOR CONTRIBUTIONS

PJ, the laboratory director, carried out initial method development to provide proof of concept, and proposed the use of nicotelline and anataline as biomarkers. LC and PC contributed to method development by refining and validating the analytical methods, and analyzed the clinical study samples. KB and LY, laboratory managers, contributed to method development, supervised sample analysis, and carried out QC/QA for the analytical results. NB and GS conceived and directed the clinical studies that provided the urine samples and data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2022.749089/full#supplementary-material>

- Carmella, S. G., Le, K.-A., Upadhyaya, P., and Hecht, S. S. (2002). Analysis of N- and O-Glucuronides of 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol (NNAL) in Human Urine. *Chem. Res. Toxicol.* 15 (4), 545–550. doi:10.1021/tx015584c
- Edwards, K. C., Naz, T., Stanton, C. A., Goniewicz, M. L., Hatsukami, D. K., Smith, D. M., et al. (2021). Urinary Cotinine and Cotinine + Trans-3'-Hydroxycotinine (TNE-2) Cut-Points for Distinguishing Tobacco Use from Nonuse in the United States: PATH Study (2013-2014). *Cancer Epidemiol. Biomarkers Prev.* 30, 1175–1184. doi:10.1158/1055-9965.EPI-20-0997
- Goniewicz, M. L., Havel, C. M., Peng, M. W., Jacob, P., 3rd, Dempsey, D., Yu, L., et al. (2009). Elimination Kinetics of the Tobacco-Specific Biomarker and Lung Carcinogen 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol. *Cancer Epidemiol. Biomarkers Prev.* 18 (12), 3421–3425. doi:10.1158/1055-9965.EPI-09-0874
- Goniewicz, M. L., Smith, D. M., Edwards, K. C., Blount, B. C., Caldwell, K. L., Feng, J., et al. (2018). Comparison of Nicotine and Toxicant Exposure in Users of Electronic Cigarettes and Combustible Cigarettes. *JAMA Netw. Open* 1 (8), e185937. doi:10.1001/jamanetworkopen.2018.5937
- Hajek, P., Przulj, D., Phillips, A., Anderson, R., and McRobbie, H. (2017). Nicotine Delivery to Users from Cigarettes and from Different Types of E-Cigarettes. *Psychopharmacology* 234 (5), 773–779. doi:10.1007/s00213-016-4512-6

- Hecht, S. S., Carmella, S. G., Chen, M., Dor Koch, J. F., Miller, A. T., Murphy, S. E., et al. (1999). Quantitation of Urinary Metabolites of a Tobacco-Specific Lung Carcinogen after Smoking Cessation. *Cancer Res.* 59 (3), 590–596.
- Hecht, S. S. (2002). Human Urinary Carcinogen Metabolites: Biomarkers for Investigating Tobacco and Cancer. *Carcinogenesis* 23 (6), 907–922. doi:10.1093/carcin/23.6.907
- Jacob, P., 3rd, Hatsukami, D., Severson, H., Hall, S., Yu, L., and Benowitz, N. L. (2002). Anabasine and Anatabine as Biomarkers for Tobacco Use during Nicotine Replacement Therapy. *Cancer Epidemiol. Biomarkers Prev.* 11 (12), 1668–1673.
- Jacob, P., 3rd, Goniewicz, M. L., Havel, C. M., Schick, S. F., and Benowitz, N. L. (2013). Nicotelline: A Proposed Biomarker and Environmental Tracer for Particulate Matter Derived from Tobacco Smoke. *Chem. Res. Toxicol.* 26 (11), 1615–1631. doi:10.1021/tx400094y26
- Jacob, P., 3rd, Yu, L., Duan, M., Ramos, L., Yturralde, O., and Benowitz, N. L. (2011). Determination of the Nicotine Metabolites Cotinine and Trans-3'-Hydroxycotinine in Biologic Fluids of Smokers and Non-Smokers Using Liquid Chromatography-Tandem Mass Spectrometry: Biomarkers for Tobacco Smoke Exposure and for Phenotyping Cytochrome P450 2A6 Activity. *J. Chromatogr. B* 879 (3–4), 267–276. doi:10.1016/j.jchromb.2010.12.012
- Jacob, P., 3rd, Yu, L., Shulgin, A. T., and Benowitz, N. L. (1999). Minor Tobacco Alkaloids as Biomarkers for Tobacco Use: Comparison of Users of Cigarettes, Smokeless Tobacco, Cigars, and Pipes. *Am. J. Public Health* 89 (5), 731–736. doi:10.2105/ajph.89.5.731
- Jacob, P., Havel, C., Lee, D.-H., Yu, L., Eisner, M. D., and Benowitz, N. L. (2008). Subpicogram Per Milliliter Determination of the Tobacco-Specific Carcinogen Metabolite 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol in Human Urine Using Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Chem.* 80 (21), 8115–8121. doi:10.1021/ac8009005
- Jacob, P., St Helen, G., Yu, L., Nardone, N., Havel, C., Cheung, P., et al. (2020). Biomarkers of Exposure for Dual Use of Electronic Cigarettes and Combustible Cigarettes: Nicotelline, NNAL, and Total Nicotine Equivalents. *Nicotine Tob. Res.* 22 (7), 1107–1113. doi:10.1093/ntr/ntz235
- Lisko, J. G., Stanfill, S. B., Duncan, B. W., and Watson, C. H. (2013). Application of GC-MS/MS for the Analysis of Tobacco Alkaloids in Cigarette Filler and Various Tobacco Species. *Anal. Chem.* 85 (6), 3380–3384. doi:10.1021/ac400077e
- Palazzolo, D., Nelson, J. M., and Hudson, Z. (2019). The Use of HPLC-PDA in Determining Nicotine and Nicotine-Related Alkaloids from E-Liquids: A Comparison of Five E-Liquid Brands Purchased Locally. *Int. J. Environ. Res. Public Health* 16 (17), 3015. doi:10.3390/ijerph16173015
- Piper, M. E., Baker, T. B., Benowitz, N. L., Kobinsky, K. H., and Jorenby, D. E. (2019). Dual Users Compared to Smokers: Demographics, Dependence, and Biomarkers. *Nicotine Tob. Res.* 21 (9), 1279–1284. doi:10.1093/ntr/nty231
- Rodgman, A., and Perfetti, T. A. (2013). *The Chemical Components of Tobacco and Tobacco Smoke*. Second Edition. Boca Raton, FL, USA: CRC Press, Taylor and Francis Group.
- Schick, S. F., Blount, B. C., Jacob, P., Saliba, N. A., Bernert, J. T., El Hellani, A., et al. (2017). Biomarkers of Exposure to New and Emerging Tobacco Delivery Products. *Am. J. Physiol. Lung Cell Mol. Physiol.* 313 (3), L425–L452. doi:10.1152/ajplung.00343.2016
- Schmeltz, I., and Hoffmann, D. (1977). Nitrogen-Containing Compounds in Tobacco and Tobacco Smoke. *Chem. Rev.* 77 (3), 295–311. doi:10.1021/cr60307a001
- Shah, V. P., Midha, K. K., Findlay, J. W. A., Hill, H. M., Hulse, J. D., McGilverayMcKay, I. J. G., et al. (2000). Bioanalytical Method Validation-Aa Revisit with a Decade of Progress. *Pharm. Res.* 17, 1551–1557. doi:10.1023/a:1007669411738
- Sinclair, L., McFadden, M., McFadden, M., Tilbrook, H., Mitchell, A., Keding, A., et al. (2020). The Smoking Cessation in Pregnancy Incentives Trial (CPIT): Study Protocol for a Phase III Randomised Controlled Trial. *Trials* 21 (1), 183. doi:10.1186/s13063-019-4042-8
- Smith, D. M., Christensen, C., van Bommel, D., Borek, N., Ambrose, B., Erives, G., et al. (2021). Exposure to Nicotine and Toxicants Among Dual Users of Tobacco Cigarettes and E-Cigarettes: Population Assessment of Tobacco and Health (PATH) Study, 2013–2014. *Nicotine Tob. Res.* 23 (5), 790–797. doi:10.1093/ntr/ntaa252
- St Helen, G., Nardone, N., Addo, N., Dempsey, D., Havel, C., Jacob, P., 3rd, et al. (2020). Differences in Nicotine Intake and Effects from Electronic and Combustible Cigarettes Among Dual Users. *Addiction* 115 (4), 757–767. doi:10.1111/add.14884
- St Helen, G., Liakoni, E., Nardone, N., Addo, N., Jacob, P., 3rd, and Benowitz, N. L. (2020). Comparison of Systemic Exposure to Toxic and/or Carcinogenic Volatile Organic Compounds (VOC) during Vaping, Smoking, and Abstinence. *Cancer Prev. Res.* 13, 153–162. doi:10.1158/1940-6207.capr-19-0356
- Suh-Lailam, B. B., Haglock-Adler, C. J., Carlisle, H. J., Ohman, T., and McMillin, G. A. (2014). Reference Interval Determination for Anabasine: A Biomarker of Active Tobacco Use. *J. Anal. Toxicol.* 38 (7), 416–420. doi:10.1093/jat/bku059
- Surya Prakash Rao, H., Bharathi, B., and Jeyalakshmi, K. (1997). Total Synthesis of Anattaline. *Indian J. Chem. Sect. B Org. Chem. Incl. Med. Chem.* 36B (7), 557–561.
- Viswanathan, C. T., Bansal, S., Booth, B., DeStefano, A. J., Rose, M. J., Sailstad, J., et al. (2007). Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays. *Pharm. Res.* 24, 1962–1973. doi:10.1007/s11095-007-9291-7
- Wei, B., Feng, J., Rehmani, I. J., Miller, S., McGuffey, J. E., Blount, B. C., et al. (2014). A High-Throughput Robotic Sample Preparation System and HPLC-MS/MS for Measuring Urinary Anatabine, Anabasine, Nicotine and Major Nicotine Metabolites. *Clin. Chim. Acta* 436, 290–297. doi:10.1016/j.cca.2014.06.012

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