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Optimization of solid phase extraction for simultaneous quantification of efavirenz and levonorgestrel in wastewater using HPLC

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This study aims to optimize the SPE parameters for purification and preconcentration of EFV and LVG to enable optimum detection and quantification by LC-20 Prominence High-Performance Liquid Chromatography (HPLC) system. The gradient elution method was used to profile and quantify efavirenz (EFA) and levonorgestrel (LVG). The optimized parameters were solution pH, solvent type and concentration, and elution volume. The 60 mg/3 mL Hydrophilic-lipophilic balance (HLB) was used to extract the target pharmaceutical contaminants. The percentage recoveries of EFA and LVG ranged from 67% to 83% and 70% to 94.61%, respectively at an optimal pH of 2, solvent concentration and type 100% Methanol and an elution volume of 4 mL using HLB cartridges. The method's accuracy was validated by obtaining a correlation coefficient (R^2) > 0.98 from the respective calibration curves of the target contaminants. The limit of detection (LOD) and limit of quantification (LOQ) for efavirenz were 0.705 µg/L and 0.14 µg/L, respectively, and for levonorgestrel, they were 0.061 µg/L and 0.199 µg/L. The optimized SPE method was used to extract wastewater samples, and the yield results showed that the method could be applied for the simultaneous detection of efavirenz and levonorgestrel, demonstrating its potential applications in environmental research. The concentration of EFA ranged from 0.36 to 8.10 µg/L in influent samples and 2.88 to 8.11 µg/L in effluent samples. Conversely, the concentration of levonorgestrel ranged from 2.64 to 32.31 µg/L in influent samples and 2.32 to 12.35 µg/L in effluent samples. The obtained results were validated by analyzing these samples using ultra-high-performance liquid chromatography. Based on the results, the optimized Solid Phase Extraction (SPE) method can be used to pre-concentrate EFA and LVG in wastewater samples, inspiring future research.

KEYWORDS

wastewater, solid phase, efavirenz, levonorgestrel, high-performance liquid chromatography

1 Introduction

Contraceptives and antiviral drugs are among the different pharmaceutical compounds used for birth control and treating viral infections (Quirke, 2017; Tariq et al., 2019). The release of synthetic progestogens such as levonorgestrel into aquatic environments has significantly increased due to continuous human population growth over the past decades (King et al., 2016). Notably, the discharge of raw and treated wastewater has been identified as the primary source of these pharmaceutical compounds in wastewater due to their inadequacy in removing them in aqueous solution. Prolonged exposure to antiviral and contraceptive drugs such as efavirenz and levonorgestrel can disrupt the endocrine system even at low concentrations, which could affect aquatic organisms' development, growth, and reproduction (Narváez et al., 2019; Kloas et al., 2009; O et al., 2014). The occurrence of efavirenz and levonorgestrel in wastewater and surface water has been reported in wastewater with a concentration range of ng/L to µg/L. Recently, an average concentration of 3.81–11.9 µg/L (influent) and 0.69–6.3 µg/L (effluent) and 6.2–8.09 µg/L (influent) and 4.25–20.9 µg/L (effluent) of efavirenz and levonorgestrel was reported in wastewater within the Vhembe and Mopani district, Limpopo, South Africa (Munzhelele et al., 2024; Schoeman et al., 2017). Although these compounds have been reported in surface water and wastewater streams (Schoeman et al., 2017; Golovko et al., 2018). There is data scarcity about their occurrence, mainly in African countries, due to a lack of advanced resources and analytical techniques to detect these compounds at trace levels. Furthermore, our prior investigation (Munzhelele et al., 2025) revealed that these compounds exhibit similar retention times, complicating their simultaneous profiling and quantification. Consequently, optimizing the solid-phase extraction (SPE) methodology to enhance the purification and preconcentration of these compounds would facilitate their effective separation and enable simultaneous detection. Thus, improving the detection of pharmaceutical compounds remains paramount (Fick et al., 2010; Campos et al., 2019; Oro et al., 2020; Madikizela et al., 2020).

Extraction and/or pre-concentration of analytes from different matrixes plays a crucial role in enhancing their detection by analytical equipment (Madikizela et al., 2020; Hawthorne et al., 1994; Namieśnik et al., 2005). Solid phase extraction (SPE) method is the most commonly used for pre-concentration of the analytes due to its ability to clean up, isolate the analyte, and remove interfering matrices from the extract (Furey et al., 2013; Muhammad et al., 2017). Hydrophilic and Lipophilic balance (HLB) cartridges are commonly used to extract pharmaceutical residues and other organic compounds due to the better retention capabilities towards both polar and non-polar compounds (Fatoki et al., 2018; Giebułtowicz et al., 2016). Parameters such as the pH of the solution, concentration, the type of solvent, and the volume of the eluent generally impact the extraction efficiency of the cartridge and, subsequently, the detection of the analyte. This study, therefore, is designed to optimize the solid phase extraction parameters for the simultaneous detection and quantification of efavirenz and levonorgestrel in wastewater using high-performance liquid chromatography equipped with a photodiode array detector.

2 Experimental protocol

2.1 Chemical reagents and materials

Efavirenz (EFA: $C_{14}H_9ClF_3NO_2$), levonorgestrel (LVG: $C_{21}H_{28}O_2$), acetonitrile (C_2H_3N), methanol (CH_3OH) (HPLC grade), HPLC water, and isopropanol of HPLC grade were purchased from Sigma-Aldrich, South Africa. Sodium chloride, hydrochloric acid, nylon syringe filters (0.22 µm), 1 mm syringes, insets, amber vials (2 mL), and Oasis Hydrophilic-Lipophilic Balance (HLB: 223170-AC (60 mg/3 mL)) were purchased from Stargate, South Africa. An individual stock solution of 1,000 ppm was prepared by dissolving the analyte (efavirenz and levonorgestrel) into 100 mL of 80% Methanol. The dilution method was used to prepare the respective mixed working standards of the analytes (500, 1,000, 5,000, 10,000, 15,000, 20,000, and 30,000 µg/L). All the stock solutions were prepared in amber bottles to avoid sample degradation. All chemicals used were of analytical grade. EFA and LVG chemical structures are shown in Figure 1.

2.2 Methods

2.2.1 Optimization of solid phase extraction conditions

The optimum pH, solvent concentration, and elution volume for sample extraction were determined using a synthetic solution containing 1 ppm of efavirenz and levonorgestrel. The extraction was performed using an Oasis HLB cartridge. Before extraction, the cartridges were preconditioned by adding 5 mL of 10% methanol and then rinsed with 5 mL of ultra-pure water at a 1 mL/min flow rate (Abafe et al., 2018). The optimization was performed by varying one parameter and keeping others constant. Briefly, the effect of solution pH in the extraction of efavirenz and levonorgestrel was accomplished by changing the solution pH from 2 to 12 using 0.1 M of NaOH and HCl. A volume of 100 mL of the synthetic solution containing 1 ppm of the EFA and LVG was then loaded into the HLB cartridges under vacuum. After extraction, the cartridges were rinsed with 5 mL of 10% Methanol and 5 mL ultra-pure water to remove the untargeted compounds from the surface of the cartridge. Thereafter, the adsorbed analytes were eluted with 6 mL of 80% Methanol. The eluted samples were then dried under nitrogen at 50°C and reconstituted using 1 mL of Methanol. Before analysis, the samples were filtered using nylon syringe filters (0.22 µm). To elucidate the effect of elution solvent and solvent concentration, acetonitrile and Methanol were used at concentrations varying from 50, 80, and 100% to elute efavirenz and levonorgestrel. The above procedure was repeated to evaluate the effect of elution volume, except that the volume of eluent solvent varied from 3, 4, 5, and 6 mL using 100% Methanol. The summary of the method is shown in Figure 2.

2.2.2 Instrumentation and chromatography conditions

LC-20 Prominence HPLC system with a photodiode array detector (Shimadzu, Japan) was used for analysis. The column type, size, and analytical parameters are summarized in Table 1.

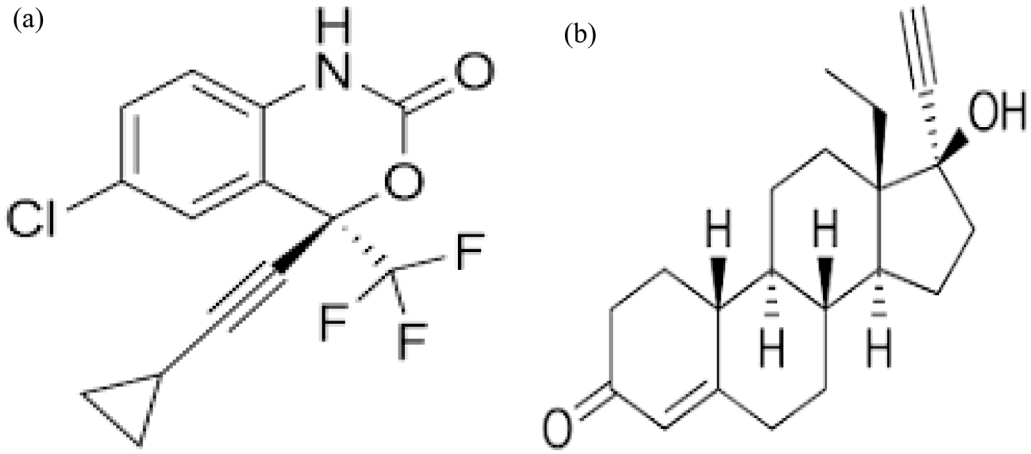

FIGURE 1
Chemical structures of **(a)** efavirenz and **(b)** levonorgestrel.

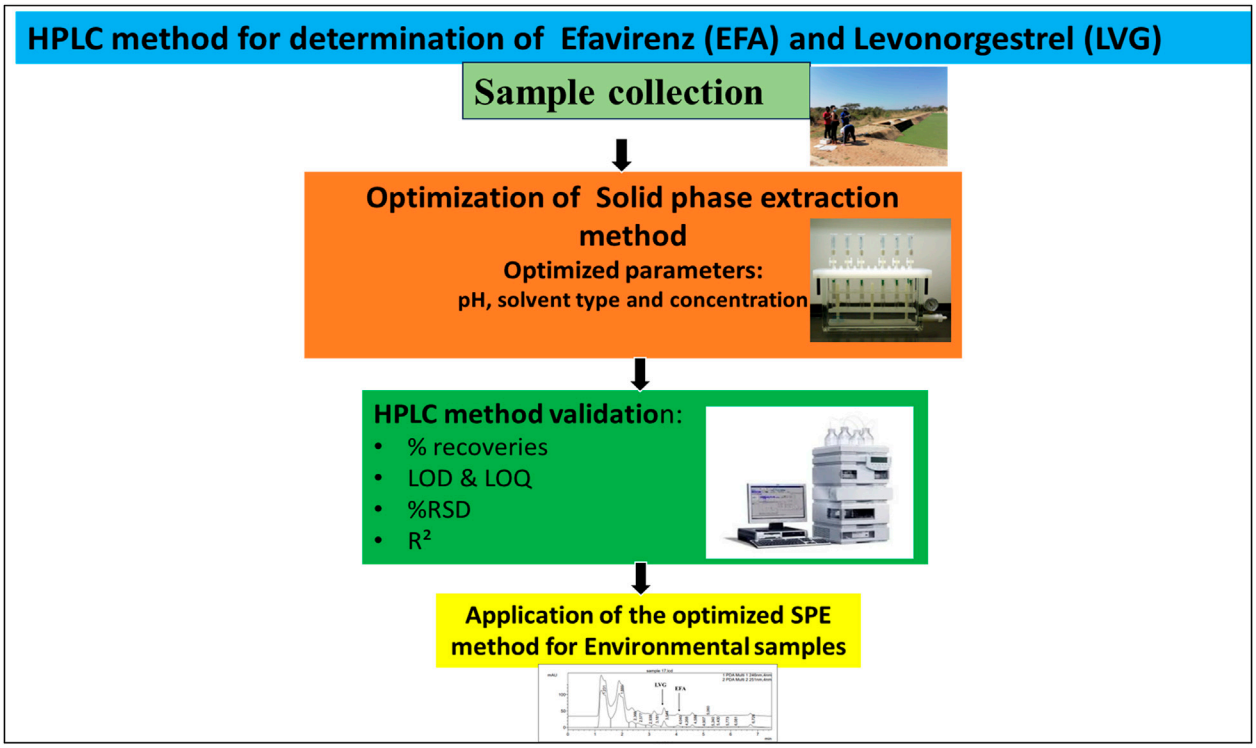

FIGURE 2
Summarized methodology for sample collection and analysis.

2.2.3 Instrument and method validation

Seven-point calibration curves were constructed for EFA and LVG development to determine the linear working range, with concentrations ranging from 0.5 to 30 ppm. The calibration curve was plotted using the linear regression peak area vs. concentrations. The respective standards were analysed in triplicates. The calibration curve's correlation coefficient (R^2) values were >0.98, implying method precision. The limit of detection and quantification were calculated using Equations 1, 2 (Nicolay et al., 2011) to evaluate the linear working range, recoveries, and method sensitivity. The lowest and highest known concentrations (0.5 and 30 ppm) and the blank matrix were analysed to evaluate the method's accuracy.

$$LOD = 3.3^{*}\sigma/S \quad (1)$$

where σ is the standard deviation of the calibration curve, and *S* is the slope of the calibration curve.

TABLE 1 Analytical parameters were employed to analyze LVG and EFA.

Analytical parameters	
Instrument name	LC-20 Prominence HPLC
Column	A 5 µm C18 column (4.6*150 mm (HSS))
Method	Gradient elution method
Temperature	Ambient
Eluent	Acetonitrile/water (70:30 v/v)
Flow rate	1 mL/min
Injection volume	20 µL

$$LOQ = 10^{*}\sigma/_{S} \quad (2)$$

The analytes recoveries were calculated using Equation 3:

$$\%\,Recovery = \frac{Sample\ peak\ area}{Standard\ peak\ area}\,100\% \quad (3)$$

The chromatographic variations on the RSD, peak area, resolutions, tailing factor, and theoretical plates of the mixed standard working solution were used to assess the method's robustness. The validated method was employed to quantify EFA and LVG in environmental samples. The concentration (C_f) of the efavirenz and levonorgestrel were calculated using the following expression (Equation 4) (Pindihama and Gitari, 2020):

$$C_f = \frac{Measured\ concentration^{*}volume\ of\ extract\ used}{Extracted\ volume} \quad (4)$$

where, C_f is the final concentration (µg/L), measured concentration (µg/L), volume extracted in (L), extracted volume in (L).

2.2.4 Applicability of method in wastewater

The method was applied for the quantification of EFV and LVG in wastewater collected from Thohoyaṉdou, Malamulele, Giyani, Makhado, Nkowankowa, Tzaneen, Kgapane, and Siloam wastewater treatment plants located in Limpopo Province, South Africa (Supplementary Figure S1). For sample collection, bottles were soaked in 10% Methanol, rinsed with de-ionized water, and oven-dried for 2 h at 80°C to avoid contamination. The samples were kept in ice and transported to the laboratory. In the laboratory, samples were subjected to a solid phase extraction process using optimized conditions at pH 2, 100% Methanol, and an elution volume of 4 mL. The eluted samples were dried under nitrogen flow at 50°C. Thereafter, the samples were reconstituted with 1 mL of 100% Methanol and analysed.

2.2.5 Quality assurance

To validate the obtained results from HPLC, the EFA and LVG were analysed using liquid chromatography–quadrupole time-of-flight tandem MS instrument (LCMS-9030 qTOF, Shimadzu Corporation, Kyoto, Japan). Chromatographic separation of the analytes was done using a Shim-pack Velox C18 column (100 × 2.1 mm, 2.7 µm) (Shimadzu Corporation, Kyoto, Japan) maintained at 40°C. An injection volume of 10 µL was used. The analytes were separated using a mobile phase gradient elution method at a flow rate of 0.4 mL min^{-1}. The mobile phase composition of A and B was 0.1% (v/v) formic acid in ultrahigh purity water and acetonitrile, respectively. The mobile phase composition was 5% mobile B from 0 to 1.5 min. At 1.5–4 min, the composition of mobile B was increased to 95% and kept constant until 4 min. The gradient was changed to 5% of mobile phase B at 5 min and maintained at this composition until 6 min. Mass spectral analysis was performed using a QqQ mass spectrometer with an electrospray interface (ESI) in positive (LVG) and negative (EFA) modes. Parameters were set as follows: nebulization 3 L min^{-1}, heating gas, and drying gas flow 10 L/min, interface voltage of 4.0 kV, interface temperature of 300°C, dissolving temperature 526°C, DL temperature of 250°C, heat block temperature of 400°C, detector voltage of 1.8 kV and the flight tube temperature at 42°C.

3 Results and discussion

3.1 Quality assurance parameters of the method

The chromatogram of the target compounds and their respective calibration curves are presented in Figure 3. The obtained chromatogram of LVG and EFA indicated that these compounds eluted after 3.55 and 4.08 min of retention times, respectively. The two compounds are quantified at 246 and 251 nm wavelengths using HPLC with a photodiode array detector. Two distinct peaks were identified in the sample to validate the optimized SPE method's applicability for analyte detection using HPLC, corresponding closely to those observed in the standard chromatogram. The method accuracy was further validated by a correlation (R^2) of 0.99 obtained from the fitted standard calibration curve of the respective target compounds (Table 3). The instrument's detection limit was calculated as the limit of detection (LOD) and limits of quantification (LOQ) using a statistical Equations 1, 2. The summary of the results is shown in Table 2. It was observed that the sample sensitivity for EFA and LVG increased as the detection limit was reduced. Specifically, the sensitivity for EFA decreased from 0.705 to 2.138 µg/L, while the sensitivity for LVG increased from 0.199 to 0.061 µg/L. High RSD% % further validated the accuracy and quality assurance of the method, with an accuracy of >79.9% for all the target compounds since they were within the acceptable range of >80%.

Standards and samples were further analysed for quality assurance using UHPLC-QqQ-MS/MS. The chromatograms of both EFV and LVG are presented in Figure 4. The linear concentration range was 25 µg/L to 750 µg/L. The coefficient of determination (R^2) was 0.99 for all analytes. The retention times for EFA and LVG were 3.50 and 3.79 min, respectively. The chromatograms of the blank samples did not show any peak of the analytes. The chromatograms of the standard were distinct from those of the sample. However, a similar resemblance between the standard and sample chromatograms and retention times was observed. EFA and LVG were profiled and quantified using 314.67 and 312.92 m/z. The product *m/z* for levonorgestrel 109. 91 and 245 *m/z* were similar to the one reported by Theron et al. (2004). Meanwhile, for EFA products, *m/z* were 245.1, 68.95, and 242.

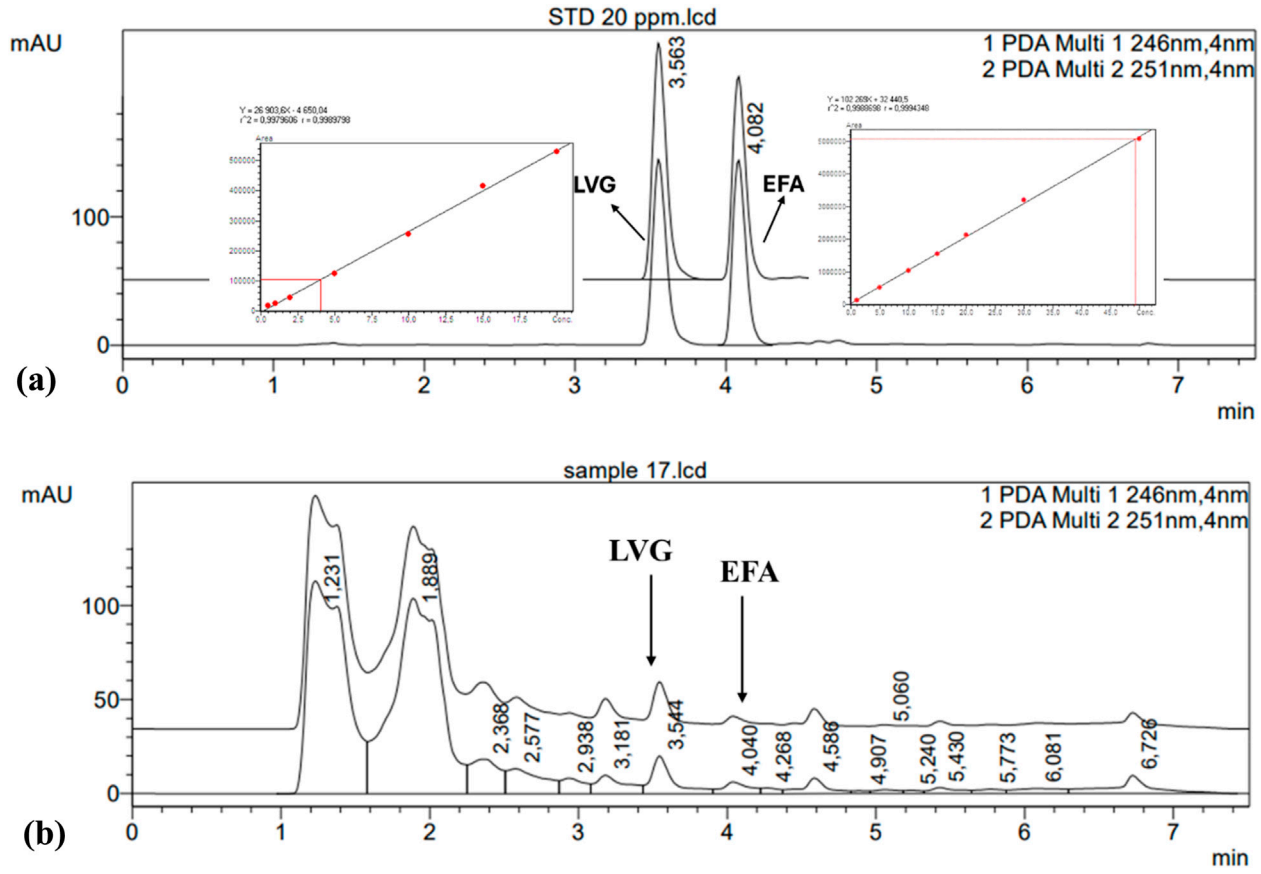

FIGURE 3
The chromatogram of levonorgestrel and efavirenz standards **(a)** with their respective calibration curves and sample **(b)**.

TABLE 2 The performance metrics the HPLC method for the detection and quantification of LVG and EFA.

Compound	R_t	R^2	RSD (%)	Accuracy (%)	LOD (µg/L)	LOQ (µg/L)
EVA	4.019	0.99	92.99	92.33	0.705	0.14
LVG	3.526	0.99	79.99	92.34	0.061	0.199

3.2 Optimization of solid phase extraction method

3.2.1 Effect of sample pH

Figure 5 shows the variation of % recoveries for LVG and EFA with the change in solution pH. The highest percentage recoveries for EFV and LVG were observed at pH of 2 (67.3% and 70.82%), respectively. A decrease in the respective analytes recoveries was observed with increasing pH. LVG and EFA are weak acid compounds that are easily protonated and become soluble at low pH, hence their substantial recoveries at low pH. At alkaline pH, they form strong bonds with HLB carboxylic, divinyl benzene, and N-vinylpyrrolidone, reducing their mobility. Hence, low EFA and LVG recoveries with increasing pH. Based on the obtained results, it was observed that sample pH can significantly affect the recoveries of EFA and LVG. pH of 2 was further used for subsequent experiments.

3.2.2 Solvent type and concentration

Figure 6 depicts the effect of solvent type and concentration on the recovery of efavirenz and levonorgestrel. Methanol gave higher recoveries for EFV (71%) and LVG (84.89%) compared to acetonitrile (50.17% and 73.63%). High recoveries for Methanol could be attributed to the introduction of methoxy groups on the surface of the HLB cartridges, enhancing their reactivity (Cortés-Lagunes et al., 2024). Comparatively, methanol is a proactive solvent with high polarity compared to acetonitrile. Thus, the OH functional group dissociate from methanol through hydrogen transfer (Jordaan and Shapi, 2017; Ngwenya and Mahlambi, 2023) and infused into the HLB matrix, improving the solubility of the analytes and enhancing their leaching efficiency. Due to its strong nitrile group, acetonitrile is a non-aprotic solvent that suggests lower polarity than methanol, affecting its reactivity due to its nitrile functional group, which undergoes hydrolysis in acid pH to form carboxylic acid through protonation of nitrogen taking time to

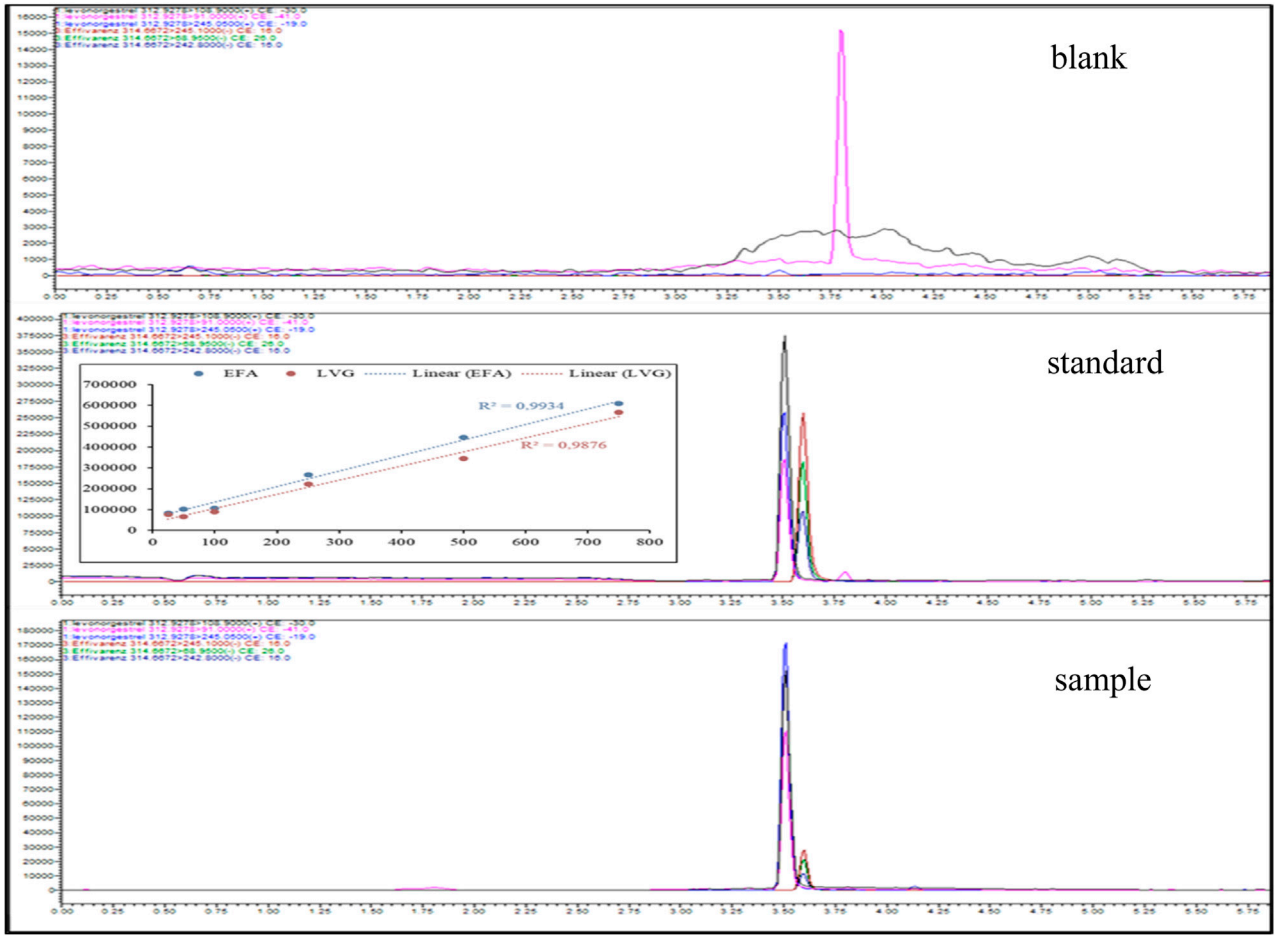

FIGURE 4
Multiple retention monitoring chromatograms of the blank sample, calibration standard, and environmental sample.

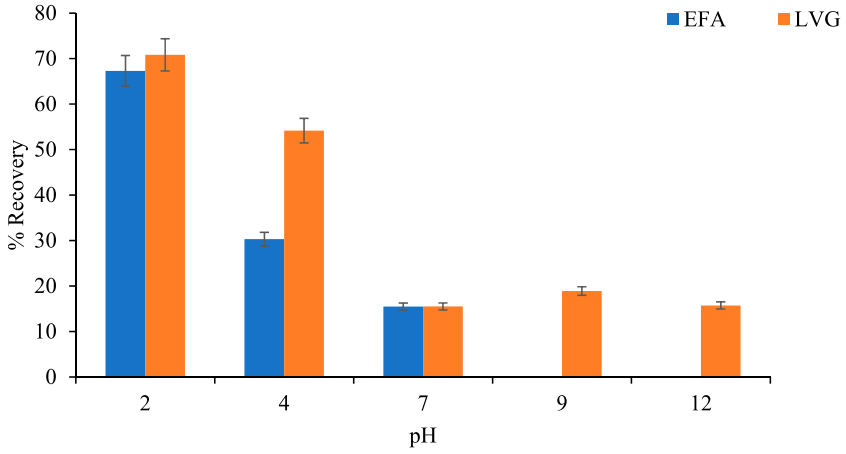

FIGURE 5
Variation of % recoveries for EFV and LVG with pH (extracted volume:100 mL, elution volume: 5 mL, and elution solvent MeOH: 80%).

infuse the HLB matrix (Zarzycki et al., 2010; Gooßen et al., 2008). An increase in recoveries of the target compounds was observed as the solvent concentration increased for both solvents. This could be attributed to an improved reactivity of the analytes and increased solvent purity (Hu et al., 2009; Jing et al., 2020). Low recoveries at low concentrations might be attributed to reduced reactivity due to

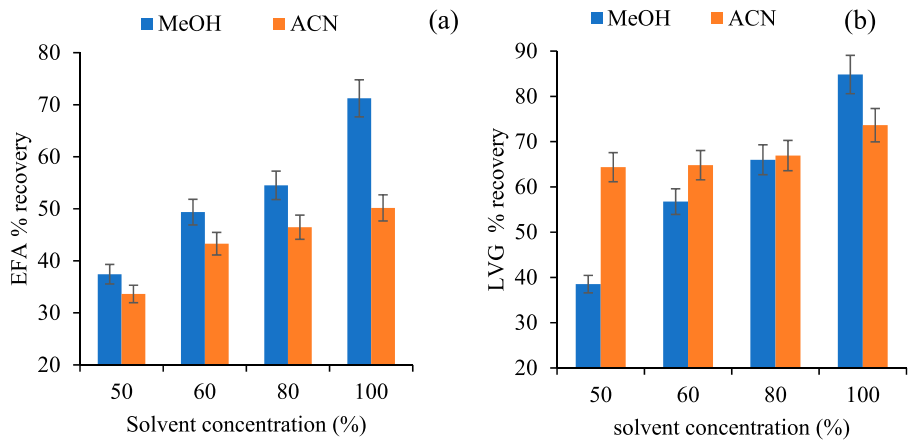

FIGURE 6
Effect of solvent type and concentration of EFA **(a)** and LVG **(b)** recoveries (extracted volume 100 mL, solution pH: 2, elution volume 5 mL).

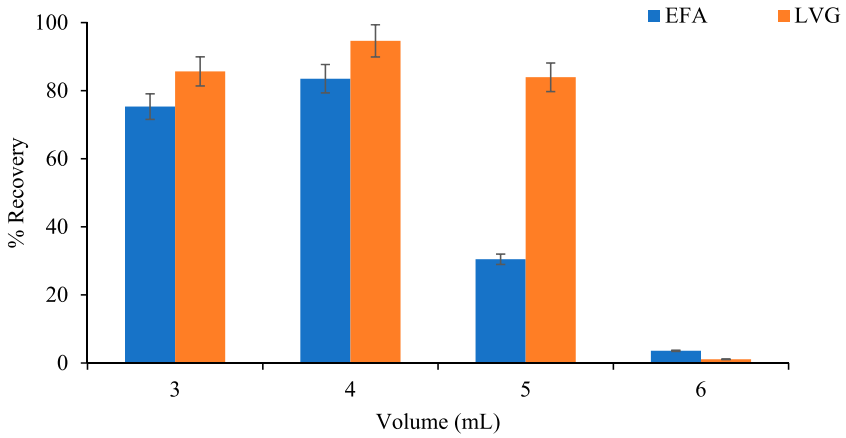

FIGURE 7
Effect of volume elution on recovery of LVG and EFA in wastewater (Solvent: 100% MeOH, pH of 2, Sample volume 100 mL).

increased solvent impurity. Based on the results, 100% of Methanol achieved high EFA and LVG recoveries and was used for subsequent experiments.

3.2.3 Elution volume

Figure 7 shows the effect of elution volume towards efavirenz and levonorgestrel recoveries. EFA and LVG increased from 3.5% to 83% and 1.08% to 94.61% with an increasing volume from 3 mL to 6 mL, respectively. The higher EFA and LVG recoveries were observed with an elution volume of 3–4 mL. This could be attributed to improved dissociation from surface charges, possibly due to increased methoxy ions (Koekemoer, 2016; Sturm, 2005). This phenomenon effectively returns the pollutants to their original state. A slight decrease in the recovery of LVG with increasing elution volume could be due to the degradation of these compounds during prolonged drying. A significant reduction in EFA recoveries was observed when using an elution volume of 5–6 mL, which could be linked to the degradation of EFAs during prolonged drying periods (Yao et al., 2021). Furthermore, since methanol was used as an elution solvent, its increased volume increases the methoxy ions in a solution, which initiates the EFA leaching process from the HLB sorbent. This further increases the solution's electronegativity, reducing the solute's leaching activity (EFA) since it mainly comprises electronegative charges, implying less EFA recoveries.

3.3 Application of the method in field water samples

Wastewater samples were analysed using optimized solid-phase extraction (SPE) parameters, including a pH of 2, 100% methanol (Methanol), and an elution volume of 4 mL. This method was employed to analyze the effects of the sample matrix on the recovery of wastewater samples from the Vhembe and Mopani districts. Table 3 depicts the concentration of LVG and EFA obtained following SPE at optimized conditions. The concentrations of efavirenz and levonorgestrel ranged from LOD-33.675 µg/L and LOD- 7.195 µg/L, respectively, with Nkowankowa showing the

TABLE 3 Concentrations of levonorgestrel and efavirenz obtained using high-performance liquid chromatography (HPLC) at optimized SPE conditions.

HPLC results								
Influent	Malamulele	Giyani	Nkowankowa	Tzaneen	Kgapane	Makhado	Siloam	Thohoyandou
LVG (μg/L)	6.55	6.86	7.08	5.78	0	7.035	5.71	4.945
EVA (μg/L)	5.245	10.42	33,675	7.135	12.205	2.75	10.375	29.51
Effluent								
LVG (μg/L)	0	0	7.195	7.02	0	7	5.81	5.045
EVA (μg/L)	10.82	12.355	3.115	0	11.91	13.825	9.705	7.4

highest concentrations of LVG and EFA. A slight increase in LVG was observed in effluent samples in most WWTP samples, which could be ascribed to immobilization in different stages of the purification process. A similar trend was observed with EFA. All the target compounds were detected in all sampling sites except LVG at Kgapane. Based on the obtained results, the optimized SPE conditions could be used for simultaneous pre-concentration to improve the detection of EFA and LVG in wastewater using HPLC. For quality assurance, samples were analysed using the UHPLC-QqQ-MS/MS technique, and the results are presented in Supplementary Table S2. The results of the UHPLC-QqQ-MS/MS were in the same range as those obtained from HPLC with less than 10% difference, validating that the conditions developed effectively enhance the simultaneous detection of EFA and LVG.

4 Conclusion

This study successfully determined solid-phase extraction conditions for the simultaneous quantification of efavirenz and levonorgestrel by HPLC-PDA. The established conditions were a pH of 2, 100% Methanol, and an elution volume of 4 mL. Under these conditions, maximum recoveries of 83% for efavirenz and 94% for levonorgestrel were obtained. The optimized SPE method was successfully applied in the preconcentration of EFA and LVG in wastewater samples. In influent samples, the EFA concentration ranged from 0.36 to 8.10 μg/L, while in effluent samples, it ranged from 2.88 to 8.11 μg/L. The levonorgestrel concentration in influent samples ranged from 2.64 to 32.31 μg/L; in effluent samples, it ranged from 2.32 to 12.35 μg/L. The optimized SPE method for preconcentration and simultaneous analysis of EFA and LVG using HPLC was validated by UHPLC results, which showed no significant differences.

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

EM: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. WA: Conceptualization, Supervision, Validation, Visualization, Writing – review and editing. WG: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Visualization, Writing – review and editing. RM: Conceptualization, Funding acquisition, Resources, Supervision, Visualization, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of 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/fenvs.2025.1539932/full#supplementary-material

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