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Rosemary extract as a natural antioxidant in structured lipid systems: a sustainable approach to enhance stability of perilla seed oil based omega-3 fatty acid rich margarine

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Introduction: Polyunsaturated fatty acids (PUFAs) are highly susceptible to oxidative deterioration, limiting their application in functional food systems. Synthetic antioxidants such as TBHQ are widely used to delay lipid oxidation, but their long-term consumption raises health concerns. This study investigated the use of rosemary extract (RE) as a natural antioxidant to enhance oxidative stability of omega-3 fatty acid rich margarine developed with structured lipids derived from perilla seed oil (PeO).

Methods: Structured lipid margarine (SLM) was developed using enzymatic interesterification of PeO, and RE was incorporated at concentrations of 500, 1000, 1500, and 2000 ppm. Samples were subjected to accelerated storage conditions using the Schaal oven test. Oxidative stability was evaluated by measuring peroxide value, acid value, p-Anisidine value, TOTOX index, conjugated dienes, conjugated trienes, and fatty acid composition. Antioxidant efficacy was compared with tert-butylhydroquinone (TBHQ, 200 ppm). Data were further analyzed by Principal Component Analysis (PCA).

Results: Incorporation of RE significantly delayed lipid oxidation in SLM, with reductions of about 40% in peroxide values compared to control samples at 1500 ppm. It reduced TOTOX index and the levels of conjugated dienes and trienes, indicating improved protection against primary and secondary oxidation. The p-Anisidine value showed reductions of 21.7%, 21.6%, 26.9%, 37.7%, and 29.3% for SLM and SLM with RE at 500, 1000, 1500, and 2000 ppm, respectively, compared to PeO. Antioxidant activity was concentration dependent, with 1500 ppm RE showing optimal stabilization. PCA confirmed that RE at 1500 ppm was comparable to TBHQ in enhancing stability.

Discussion and conclusion: The study demonstrates effectiveness of RE as natural antioxidant capable of stabilizing PUFA rich structured lipids systems. At 1500 ppm, RE provided oxidative protection equivalent to TBHQ, with the upside of being sustainable, plant-derived alternative. The combined effect of enzymatic interesterification and natural antioxidant supplementation offers a dual strategy to improve the stability of functional fat based products, supporting RE as a promising substitute for synthetic preservatives in the food industry.

KEYWORDS

PUFA, *Rosmarinus officinalis* L., preservation, shelf-life, enzymatic interesterification

1 Introduction

The oxidative stability of lipids is a significant challenge for food manufacturers, particularly in products containing unsaturated fatty acids. Lipid oxidation causes the degradation of lipids, thereby resulting in rancidity, off-flavors, and loss of nutritional value of the final product. To address this concern, techniques such as antioxidant addition along with physical modification technologies including blending and interesterification have been explored (Mishra et al., 2022). Antioxidants play a crucial role in mitigating oxidative deterioration by scavenging free radicals, chelating metal ions, and inhibiting the formation of peroxides. Traditionally, synthetic antioxidants, such as TBHQ (tert-butylhydroquinone), BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) have been used to extend the shelf life of lipids due to their high antioxidant potency and low cost. However, they are linked to negative health implications including cancer, mutations, and blood clotting when subjected to long term consumption (Rashid et al., 2022; Sharma et al., 2019). Furthermore, the growing consumer demand for natural food products has led to increased interest from industries and researchers towards plant-based antioxidants. Rosemary extract (RE) is one such antioxidant, which is known for its potent efficacy in inhibiting lipid oxidation (Kum et al., 2025). The extract contains a wide range of bioactive compounds, including phenolics like carnosol and carnosic acid, as well as flavonoids and rosmarinic acid, which exhibit potent free radical scavenging activity (Couto et al., 2012). Moreover, RE has shown notable health benefits including reduction in inflammation, memory improvement, and cardiovascular function (Veenstra and Johnson, 2021).

Margarine is a widely consumed fat that serves as a substitute to butter in various bakery and culinary applications. It is usually formulated by partial hydrogenation of plant-based oils to achieve the desirable textural and functional properties (Li et al., 2018). However, this process leads to the development of trans-fats, which have been associated to the risk of developing cardiovascular diseases, cancer, atherosclerosis and other fatal disorders (Marangoni et al., 2020; Nagpal et al., 2021; Shin et al., 2021). Additionally, it lacks or contains negligible amount of PUFA (polyunsaturated fatty acid), especially essential omega-3 fatty acid. The consumption of PUFAs and omega-3 fatty acids is associated with reduced risk of cardiovascular diseases, improved cognitive function, and anti-inflammatory effects. Thus, lipid modification technologies such as blending and interesterification have garnered attention in the recent years for the development of healthier margarine formulations (Dhiman et al., 2024; Li et al., 2018). These modification techniques have been employed to enhance the stability as well as applicability of PUFA rich oils in the food industry.

Oil blending is a widely employed technique for enhancing oxidative and thermal stability. It is also cost-effective and facilitates the improvement of nutritional properties. Nevertheless, oil blends exhibit non-uniformity and phase separation, which limits their applicability due to poor techno-functional properties (Dhiman et al., 2025). This can be addressed by interesterification, which is another lipid modification approach involving the redistribution of fatty acids within or between the triglyceride species. Interesterification is categorized into chemical and enzymatic process, depending on the type of catalyst used. Enzymatic interesterification is preferred due to its mild processing conditions, high substrate specificity, reduced formation of undesirable by-products, and improved environmental

sustainability (Jadhav and Annapure, 2021). This method has been extensively utilized for the development of structured lipids for applications in margarine, cocoa butter alternatives, and functional fat replacers (de Oliveira et al., 2021; Dhiman et al., 2024; Dong et al., 2023; Ma et al., 2019). It also facilitates the formulation of fats with an increased amount of PUFAs, which are found in negligible amounts in commercial margarines. The intake of PUFAs has been associated with various health benefits, including anti-inflammatory effects, cardiovascular protection, and neurological development (Wang et al., 2024). They aid in reduction of high-density lipoprotein (HDL) cholesterol while regulating triglyceride levels and blood pressure. Additionally, their regular consumption leads to a lower risk of non-communicable diseases such as cancer, osteoarthritis, and autoimmune disorders (Kapoor et al., 2021). They have also been reported to enhance metabolic health by improving insulin sensitivity and reducing the potential of metabolic disorders.

Perilla seed oil (PeO) is known for its high PUFA content (75%), of which about 60% is omega-3 fatty acid, ALA (Dhyani et al., 2022). It is extracted from the seeds of *Perilla frutescens*, a plant widely cultivated in East Asian countries, particularly China, Korea, and Japan (Kaur et al., 2024). Owing to its high ALA content, PeO has been extensively studied for its potential health benefits, including anti-inflammatory, neuroprotective, and cardioprotective effects. Despite its nutritional advantages, the application of perilla seed oil in food formulations poses significant challenges due to its oxidative instability (Aldamarany et al., 2023; Kaur et al., 2024).

In this study, the shelf life of trans-fat free omega-3 fatty acid rich margarine developed through the enzymatic interesterification of perilla seed oil was assessed. The oxidative stability of the formulated margarine was evaluated with and without the incorporation of RE at different concentrations. To assess the impact of RE on oxidative stability, an accelerated storage experiment was conducted using the Schaal oven test, a widely used method for determining lipid oxidation under elevated temperature conditions (Pokhrel et al., 2024). The study aims to determine the efficacy of RE in enhancing the oxidative stability of omega-3-rich margarine and its potential as a natural alternative to synthetic antioxidants. The present study introduces a novel approach by developing a trans-fat-free, omega-3-rich margarine using PeO, an underutilized but rich source of α -linolenic acid. The oil was incorporated into a structured lipid system by enzymatic interesterification, a sustainable alternative to chemical hydrogenation used to enhance the physical, chemical, and functional properties of the solid fat. The margarine was further stabilized using RE, a natural plant-based antioxidant, which provided oxidative stability comparable to synthetic additives. This combination of structured lipids and a natural antioxidant incorporates the innovation and distinguishes the formulation from existing commercial products.

2 Materials and methods

Perilla seeds utilized in the study were procured from Meghalaya farmers through Sudasha Foods Private Limited. All the chemicals, solvents, and gases used were of analytical grade. Standards for tocopherol, fatty acid and antioxidant analysis were procured from Sigma Aldrich. Rosemary extract was purchased from Veda oils, Delhi, India and the oil samples were stored at 4° C in tightly sealed amber bottles.

2.1 Preparation of margarine

The margarine was developed from the structured lipid (SL) using method given by Dhiman et al. (2024). Blended oil was initially prepared by blending the PeO and palm stearin (PS) in 50:50 ratio on a magnetic stirrer at 55° C and 150 rpm for 15 min. The SL was initially developed by incorporating immobilized TLIM at a concentration of 6.2% by weight. The interesterification reaction was carried out at 54° C for 5.4 h, after which the enzyme was recovered using a 0.45 µm PTFE membrane filter. This enzyme was subsequently rinsed with hexane, dried at 40° C for 6 h, and preserved for further use. Margarine was then formulated using these structured lipid systems. The lipid phase consisted of 80% SL and 0.5% soy lecithin. The aqueous phase contained 18% distilled water and 1.5% table salt. Both phases were heated to 60° C separately and then thoroughly mixed for 5 min to form an emulsion. Artificial butter flavour was added to the emulsion, which was then crystallized for 15 min using an ice-cream maker. The resulting margarine was refrigerated overnight. For tempering, the developed margarines were held at room temperature for 4 h and then vigorously blended to achieve a smooth, crystal-free consistency (Pande et al., 2013).

2.2 Rosemary extract incorporation

To evaluate the effect of RE on SLM, it was added at four distinct concentrations, i.e., 500, 1,000, 1,500, and 2000 ppm. A 200 ppm concentration of tertiary butylhydroquinone (TBHQ) was also included as a positive control, as it is the maximum permitted level for edible oils and fats as specified by the international and national safety standards, including regulations by FDA, European Food Safety Authority and FSSAI [Food Safety and Standards Authority of India (FSSAI), 2011; U.S. Food and Drug Administration (FDA), 2018; European Commission, 2008]. The chosen range for RE concentrations (500–2000 ppm) was based on relevant literature and pre-trial experiments confirming RE's effectiveness as an antioxidant (Cordeiro et al., 2013; Kum et al., 2025; Yeşilsu and Özyurt, 2019; Yıldırım and Yorulmaz, 2018). Also, RE is classified as Generally Recognized as Safe (GRAS), and its use in fats and oils is regulated by international food authorities. According to the European Food Safety Authority (EFSA) (2008) and the U.S. Food and Drug Administration (FDA) (2018), rosemary extract is permitted within specified limits, typically up to 2000 ppm depending on the application. For preparation, both RE and TBHQ were precisely weighed and dissolved into the lipid samples using a magnetic stirrer at 300 rpm for 30 min at 50° C.

2.3 Volatile organic profile of rosemary extract

Volatile organic compounds in RE were identified using Headspace Gas Chromatography–Mass Spectrometry (HSGC-MS) method on Agilent 7010B GC/TQ system equipped with HP-88 GC column. A precisely weighed 200 mg sample was incubated at 120° C for 20 min with agitation to collect volatiles. Nitrogen was used as a carrier gas at a 1 mL/min flow rate with a 10:1 split ratio. The GC oven temperature was ramped from 50 to 250° C at rate of 10° C/min, holding at 230° C for 10 min (Sharma et al., 2021). Compounds were

identified by comparing retention times and mass spectra to the NIST Mass Spectral Library.

2.4 Schaal oven test

The oxidative stability test of the samples was carried out at by keeping 300 mL of each sample at 60° C in an oven over a period of 21 days (Baştürk et al., 2018; Moczkowska et al., 2020; Wang et al., 2018). The physical and oxidative analysis of the samples was performed every 3 days while the fatty acids profile, and tocopherol content was determined at the start (0th day) and end (21st day) of the storage study.

2.5 Oxidative indices

2.5.1 Induction period

The oxidative stability index of the samples was evaluated on the initial day in terms of induction period (IP) at 110° C using Rancimat (Metrohm 892, Herisau, Switzerland). 3 g of oil sample was precisely weighed in the reaction vessel and subjected to heating with air flow of 20 L/h (Li et al., 2019).

2.5.2 Primary oxidation

The peroxide value (PV) and acid value (AV) of the samples were assessed every 3 days using AOCS methods Cd 9–53 and Ca 5a-40, respectively. The solid fat was melted and accurately weighed. In order to determine AV, 50 mL of neutralized ethyl alcohol was initially added to the sample, which was further subjected to boiling. This mixture was titrated against 0.1 N KOH solution after the addition of phenolphthalein reagent. For determining the PV, hydroperoxides formed in the fat sample due to oxidation were reacted with a saturated KI solution, which was further titrated against 0.01 M sodium thiosulfate solution.

2.5.3 Secondary oxidation

The p-Anisidine value (pAV), conjugated dienes (CD) and conjugated trienes (CT) of the samples were evaluated after every third day by spectrophotometric measurements at 530 nm, 232 nm and 268 nm, respectively, using a UV–Vis spectrophotometer (UV–Vis-1800 spectrophotometer). The analyses were performed in accordance with standard IS methods (Bureau of Indian Standards, 2021).

2.5.4 TOTOX

TOTOX value was used to determine the overall oxidative stability of the samples. It was computed by using the following equation combining PV and p-AV.

$$TOTOX = (2 \times PV) + pAV$$

2.6 Color

The color of the fat samples was evaluated in terms of L, a* and b* values, representing lightness, redness and yellowness, respectively,

using a CR400 Minolta colorimeter (Konica Minolta Inc., Tokyo, Japan). Triplicate value of all the samples were taken.

2.7 Fatty acid composition

The fatty acid profile of the lipid samples was determined in accordance with AOCS official method 996.06. They were initially trans esterified to FAMES using BF_3 and further subjected to gas chromatography (GC) coupled with a flame ionized detector (FID) using an Agilent 7890B. Separation was achieved using a DB capillary column (100 mm x 0.25 mm x 0.2 μm). The injection volume was set at 1 μL with a split ratio of 100:1. Nitrogen served as a carrier gas at a flow rate of 1.5 mL/min and column pressure of 20 psi. Both the injector and detector temperature were maintained at 280°C, while the hydrogen and oxygen flow rate were set at 30 mL/min and 300 mL/min, respectively. The oven temperature program began at 100°C for 4 min, followed by a ramp of 4°C/min to 240°C, which was held for 25 min. Identification and quantification of the fatty acids in the samples were performed by comparing the retention times to those of reference standard.

2.8 Antioxidant capacity

Polar compounds were extracted from the fat samples through liquid–liquid extraction using 80% (v/v) methanol solution. Briefly, 1 g of sample was diluted in 3 mL of methanolic solution and vortexed for a minute (Pan et al., 2019). Following this, the mixture was centrifuged at 6000 rpm for 5 min, and the supernatant was collected. The process was repeated thrice and the collected supernatants were combined and made up to a final volume of 10 mL by the addition of methanol solution.

2.8.1 DPPH radical scavenging activity

The free radical scavenging capacity of the samples was determined using the DPPH assay. A DPPH reagent solution (0.1 mM) was prepared by dissolving 4.9 mg of DPPH in 50 mL of 80% methanol in an amber volumetric flask. 0.5 mL of each sample extract was reacted with 2 mL of DPPH reagent and incubated in the dark at 25°C for 30 min followed by spectrophotometric measurements at 517 nm (Pointner et al., 2024; Samani, 2017). Results were quantified as μM TE (Trolox equivalent)/Kg using a Trolox standard curve (Brand-Williams et al., 1995).

2.8.2 Total phenolic compounds (TPC)

The TPC of the margarine samples was evaluated using the FC (Folic-Ciocalteu) method. Absorbance measurements were recorded at 765 nm with a UV–Visible spectrophotometer and the results were expressed as milligrams of gallic acid equivalent (mg GAE)/kg of the sample. The quantification was based on a standard curve with a gallic acid solution (0.02–0.1 mg/mL) (Polavarapu et al., 2011).

2.8.3 Total tocopherol content

Total tocopherol content (TTC) was determined following the methodology described by Singh et al. (2024). HPLC with a Shimadzu TF-10AXL fluorescence detector was used for detection, with excitation and emission wavelengths set at 294 nm and 329 nm,

respectively. Separation was carried out on a Spinco nucleosil C18 column (100–5, 5 μm , 4.6 mm x 250 mm) with a mobile phase of 3.85% tetrahydrofuran in n-heptane at a flow rate of 1 mL/min (Dhiman et al., 2024). 100 mg of precisely weighed sample was dissolved in n-heptane and 20 μL of aliquot was injected into the system. Data acquisition was performed using Lab Solution software.

2.9 Statistical analysis

All analyses were performed in triplicate. Mean values and standard deviations were calculated, and significant differences ($p < 0.05$) were identified using Tukey's test in SPSS. Furthermore, principal component analysis (PCA) was conducted using Origin Pro software to compare samples based on oxidative parameters.

3 Result and discussion

3.1 Volatile organic profile of rosemary extract

The HSGC-MS analysis of the RE demonstrated a complex profile of volatile organic compounds, with eucalyptol (1,8-cineole, 16.66%), 3-carene (15.67%), camphene (9.26%), D-limonene (8.75%), camphor [(+)-2-bornanone, 10.45%], and benzene, 1-ethyl-2,3-dimethyl (8.87%) as the predominant constituents (Table 1). Several other terpenoids and phenolic compounds, such as linalool (0.90%), α -terpineol (0.61%), terpinen-4-ol (0.58%), endo-borneol (0.64%), and eugenol (0.85%), were present in lower concentrations, contributing to the extract's overall potential bioactivity. This aligns with recent literature reporting 1,8-cineole, camphor, camphene, and borneol as major volatiles in rosemary essential oils and extracts. Minor compounds such as linalool, α -terpineol, and eugenol, though present in smaller amounts, have been detected in rosemary and are known for their antioxidant and antimicrobial properties (Luca et al., 2023; Mena et al., 2016). These compounds are not only responsible for the characteristic aroma of RE but also significantly contribute to its antioxidant and antimicrobial properties. The presence of both hydrocarbon and oxygenated volatiles suggests RE's synergistic role in stabilizing lipid systems by scavenging free radicals and delaying oxidative degradation. These volatiles suggest the functional potential of RE as a natural preservative in food applications, particularly in PUFA-rich formulations.

3.2 Oxidative indices

3.2.1 Induction period

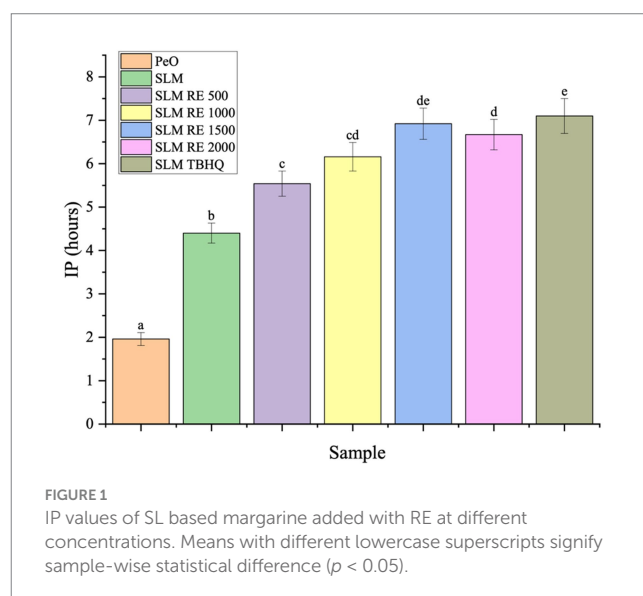
The initial oxidative stability of the developed lipid systems was evaluated using the Rancimat method, which provides an accelerated measure of the oxidative stability by determining the induction period (IP) or oxidative stability index (OSI) under standardized conditions of elevated temperature and constant air flow (Ghosh et al., 2019). The OSI is a widely used parameter to predict the relative shelf life of lipid-based products by assessing their resistance to thermo-oxidative degradation. The incorporation of both the natural (RE) and synthetic (TBHQ) antioxidants significantly enhanced the IP values (Figure 1).

TABLE 1 Volatile organic profile of rosemary extract.

Rt	Compound name	Cas number	Match factor	Component area	Area %
2.2588	3-Carene	13,466-78-9	94.3	11,397,458,881	15.67
2.5536	Camphene	79-92-5	96.8	6,735,556,190	9.26
2.7697	β -Pinene	18,172-67-3	95.9	2,998,822,854	4.12
3.0152	β -Pinene	18,172-67-3	94.3	598771609.8	0.82
3.2479	D-Limonene	5,989-27-5	97.7	6,366,055,731	8.75
3.9008	Eucalyptol	470-82-6	97.2	12,119,795,175	16.66
4.2123	Benzene, 1-ethyl-2,3-dimethyl-	933-98-2	91.2	6,449,607,489	8.87
5.5745	Copaene	3,856-25-5	94.6	408384139.6	0.56
6.7415	Linalool	78-70-6	95.7	652,407,198	0.9
7.2068	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	242,794-76-9	96.5	339451054.7	0.47
7.5933	(S)-(+)-1,2-Propanediol	4,254-15-3	91.5	2,355,446,866	3.24
7.6571	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	5,655-61-8	93.2	420820779.4	0.58
7.7134	Terpinen-4-ol	562-74-3	89.4	423244720.1	0.58
7.7735	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-, (1 α ,2 β ,5 α)-	15,358-88-0	82.4	319053417.9	0.44
8.0211	(+)-2-Bornanone (Camphor)	464-49-3	96.5	7,599,111,221	10.45
8.5653	α -Terpineol	98-55-5	95.6	445,695,199	0.61
8.7266	endo-Borneol	507-70-0	96.2	468,546,827	0.64
12.9446	Eugenol	97-53-0	99.1	618947730.5	0.85

PeO, characterized by high proportion of PUFAs exhibited lowest IP values (1.96 h). This can be attributed to the high susceptibility of PUFAs to oxidation resulting from the multiple double bonds in their structure, which are prone to attack by reactive oxygen species, leading to peroxidation (Machado et al., 2023). In contrast to this, the SLM possessed significantly higher stability with IP of 4.40 h. This observation aligns with previous studies suggesting that lipid modification by incorporation of saturated fat fractions like palm stearin, palm olein, and coconut oil through interesterification or blending significantly improves the oxidative stability of the lipid systems (Boukandoul et al., 2019; de Oliveira et al., 2021; Pang et al., 2019).

The incorporation of RE to SLM demonstrated a significant, concentration dependent increase on the oxidative stability. This can be attributed to the radical scavenging and metal chelating activity of the phenolic compounds present in RE, namely, carnosic acid and rosmarinic acid. The OSI values escalated from 5.54 h (500 ppm RE) to 6.16 h (1,000 ppm RE), reaching a maximum protective effect at 1500 ppm RE (6.92 h). Notably, a further increase to 2000 ppm RE (6.67 h) resulted in a non-significant decrease in the IP value as compared to the 1,500 ppm concentration. The highest IP values were observed for SLM incorporated with TBHQ. However, statistical analysis ($p < 0.05$) revealed no significant difference between the stability imparted by TBHQ (200 ppm) and that achieved with RE at 1500 ppm and 2000 ppm. This suggests that RE has promising potential as a natural antioxidant for preserving PUFA-rich oils.



3.2.2 Peroxide value

The degree of formation of primary oxidation products (hydroperoxides) was evaluated by monitoring the PV over the storage period of 21 days as illustrated in Figure 2. The PV of all the samples increased with storage time, indicating a progressive lipid oxidation, PeO exhibited the highest susceptibility to oxidation with PV values

rising from 3.46 to 26.74 meqO₂/Kg by day 21. In contrast to PeO, the increase observed in SLM was not as prominent, indicating higher oxidative stability of SLM, thereby suggesting the effectiveness of enzymatic interesterification technique for enhancing the oxidative stability and functional properties, and hence the applicability of PUFA rich oils. The SLM samples incorporated with RE had significantly lower PVs throughout the storage period, demonstrating improved oxidation stability. SLM with 500 ppm RE had final PV of 24.25 meqO₂/Kg, showing a 19.05% reduction from the control SLM without any antioxidant, which further decreased at higher RE concentrations. Overall, a 38.25, 43.32 and 42.9% decrease in PV was observed at 1000, 1500 and 2000 ppm RE, respectively. Notably, the lowest oxidative susceptibility was achieved at 1500 ppm RE suggesting it be the optimal concentration beyond which no further antioxidant effect was observed with RE addition. SLM added with TBHQ demonstrated no significant differences in the PV when compared to that with RE at 1500 and 2000 ppm. Shelf life of the margarine formulations was estimated using established oxidative quality limits, with peroxide value (PV) maintained below 10 meq O₂/kg and acid value (AV) below 4 mg KOH/g, in accordance with guidelines (European Commission, 2001; Food Safety and Standards Authority of India, 2021). The antioxidant effect of RE can be attributed to its high phenolic and bioactive concentration, which aids in scavenging free radicals and chelating pro-oxidant metals, thereby preventing lipid oxidation (Tohma and Turan, 2015; Veenstra and Johnson, 2021). However, no further increase in the protective effect was observed on increasing the concentration of RE above 1,500 ppm, which can be attributed to the pro-oxidant activity of antioxidants at higher concentrations, a phenomenon observed in other studies as well (Dhiman et al., 2025; Martin-Rubio et al., 2018).

3.2.3 Acid value

Acid value is one the most important parameter for evaluating the quality of fats and oils. The value is generally elevated on exposure to

heat, light or other harsh conditions during storage due to the hydrolysis reaction. This leads to the degradation in quality of oil, which is often accompanied by a decline in smoke point (Ramroudi et al., 2022). Similar to the trend observed for peroxide value, the highest AV was reported for PeO, owing to its high unsaturated fatty acid content which makes it susceptible to oxidation due to presence of multiple double bonds in its structure. As storage time increased, the AV rose across all samples. This increase can be attributed to the breakdown of TAG and a decrease in the extracts' ability to prevent hydrolysis. Notably, SLM exhibited significantly lower AV ($p < 0.05$) as compared to PeO (Tohma and Turan, 2015). The changes in acid value of SLM and SLM supplemented with varying levels of RE over 21 days of accelerated storage has been depicted in Figure 2b. The PeO control exhibited the highest increase in AV, rising from an initial value of about 3.5 mg KOH/kg to about 9 mg KOH/g. This significant rise ($p < 0.05$) suggests the high susceptibility of PeO to oxidative degradation under accelerated conditions. The SLM was found to be more stable than PeO, however, it showed a continuous increase in AV, reaching up to 7 mg KOH/g by the end of storage study. These results are consistent with previous studies, which report that structured lipids and polyunsaturated oils are particularly prone to hydrolytic and oxidative deterioration during storage (Golmakani et al., 2020; Kumari Singh et al., 2024). The addition of RE at increasing concentrations lead to a concentration dependent protective effect against rise in acid value. SLM incorporated with 1,500 ppm RE showed the highest improvement over that without antioxidant, resulting in significantly lower AV throughout the study, with final values of 2.5–2.8 mg KOH/g. Notably, lowest AV was reported for RE incorporated at 1500 ppm, suggesting it to be optimal concentration for oxidative stability. SLM RE 2000 ppm did not provide further significant benefit, indicating a stagnation in antioxidant efficacy at higher doses. The synthetic antioxidant TBHQ (SLM RE TBHQ) performed comparably to the higher concentrations of rosemary extract, maintaining AVs below 3 mg KOH/kg throughout the storage

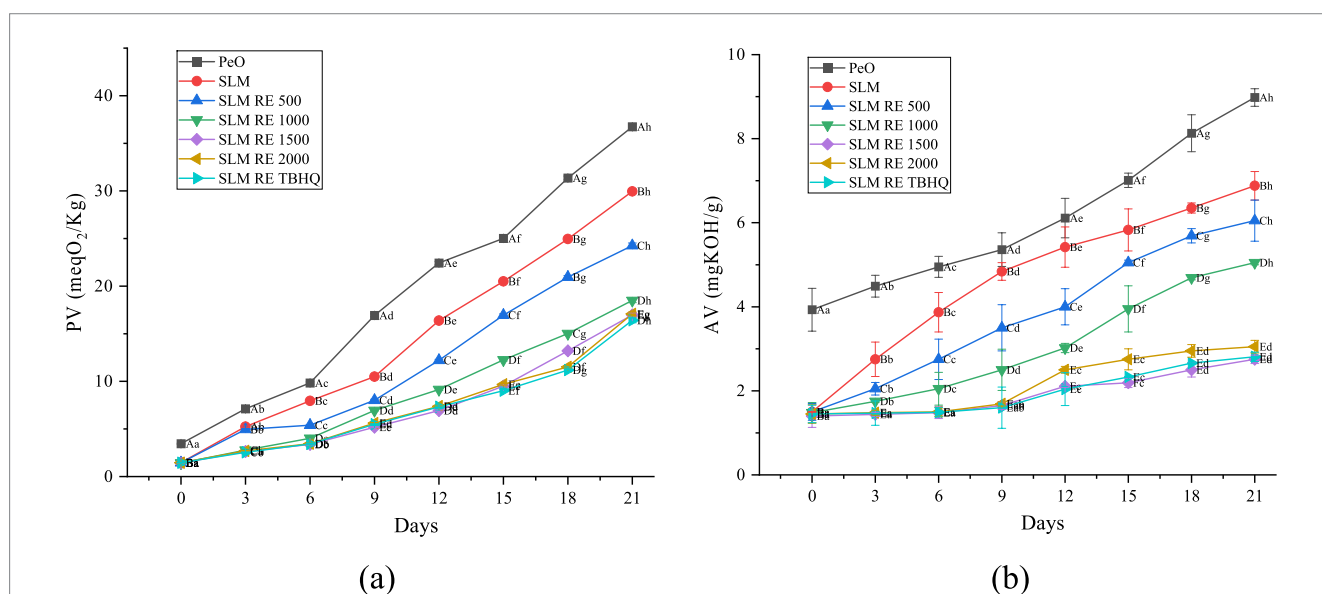


FIGURE 2

Changes in primary oxidation markers during the storage study. (a) PV; (b) AV. Mean values with different lowercase superscripts signify statistical difference sample-wise ($p < 0.05$) and means with different upper-case superscripts signify statistical difference day-wise ($p < 0.05$).

period. This demonstrates that RE, at appropriate concentrations, can be as effective as TBHQ in inhibiting oxidative degradation in structured lipids (see Figure 3).

3.3 Secondary oxidation

The degree of secondary oxidation in the samples was evaluated through p-AV, CD, and CT over 21 days of storage period under accelerated conditions. These parameters indicate the extent of oxidative degradation beyond primary oxidation, measuring the level of aldehydes and conjugated compounds formation, which in turn are responsible for imparting rancid flavours to food products. The p-AV showed a consistent increase across all samples with storage time, indicating the formation of aldehydes as secondary oxidation products. PeO exhibited most rapid and significant rise, with values reaching to a maximum of 52.55 by the end of the storage period. Conversely, SLM showed a comparatively lower increase, with maximum value of 44.11. This indicates the improvement of oxidative

stability resulting from the interesterification reaction. Amongst the samples treated with antioxidants, SLM TBHQ and SLM RE 1500 exhibited the best oxidative resistance, with final pAVs of 33.68 and 33.13, respectively. Notably, RE at 1500 ppm provided comparable protection to synthetic antioxidant TBHQ, indicating its efficacy as a natural alternative. Compared to PeO by the end of the storage period, p-AV was reduced by 21.7, 21.6, 26.9, 37.7, and 29.3%, in SLM, and SLM incorporated with RE at 500, 1000, 1500, and 2000 ppm, respectively. The addition of TBHQ resulted in a 36% reduction in p-AV compared to PeO at the end of study.

Both CD and CT values exhibited a rising trend throughout the storage study. PeO, due to high content of PUFAs and absence of antioxidant intervention, showed the highest degree of secondary oxidation with a value reaching 5.97 for CD and 1.95 for CT on day 21. In contrast, the SLM without added antioxidants displayed lower values, indicating a 14.2 and 24.4% reduction, respectively for CD and CT, which can be attributed to the oxidative stability imparted by enzymatic interesterification. SLM values containing antioxidant showed enhanced oxidative stability than both PeO and SLM in

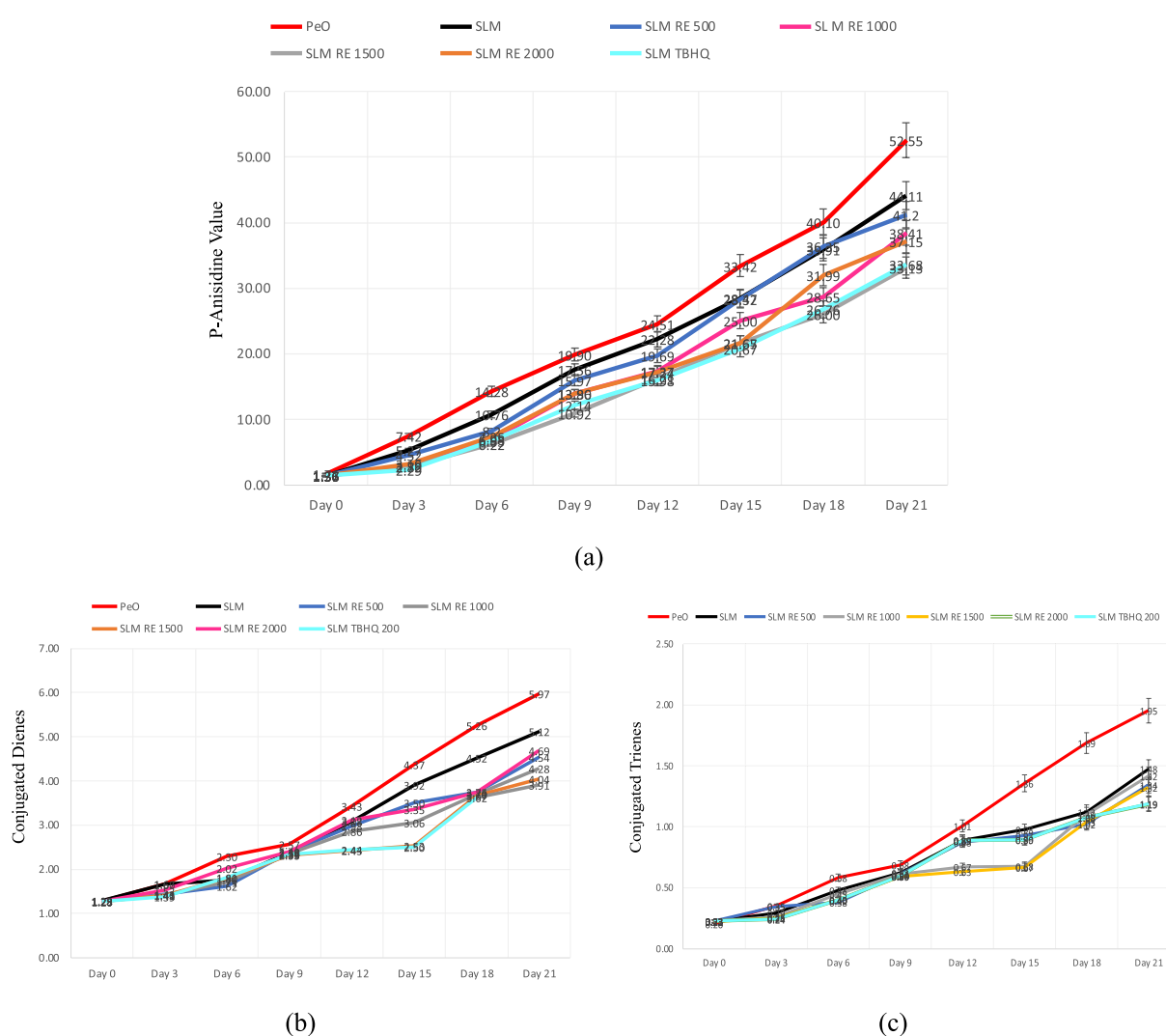


FIGURE 3

Changes in secondary oxidation markers of margarine throughout the accelerated storage period; (a–c) depict p-AV, CD and CT values, respectively.

mitigating oxidation. SLM with TBHQ (3.91 for CD and 1.19 for CT) and SLM RE 1500 (4.04 for CD and 1.321 for CT) were most effective, with CD reductions of 34.5 and 32.3%, and CT reductions of 39.1 and 32.4%, respectively, compared to PeO. Samples with RE at 1000 ppm (4.28 for CD, 1.418 for CT) and 2000 ppm (4.69 for CD, 1.186 for CT) also showed significant protection, though the marginal improvement between 1,500 and 2000 ppm suggests a stagnation beyond which the antioxidant effect does not increase. SLM RE 500 showed moderate protective effect reducing CD and CT by 23.9 and 31.2%, respectively.

3.4 TOTOX

The TOTOX value represents the combined effect of primary and secondary oxidation products. In this study a progressive increase in TOTOX values for all samples was observed during the Schaal oven accelerated storage period, indicating a continuous oxidative degradation over time (Table 2). PeO showed the highest values starting at 8.63 and reaching 126.03 by Day 21, thereby indicating its high susceptibility to oxidation due to its PUFA content. In contrast, the SLM and its antioxidant-enriched samples demonstrated significantly lower TOTOX values throughout the storage. On Day 21, SLM without antioxidants exhibited a value of 104.03, while incorporation of RE at increasing concentrations resulted in an

increased resistance to oxidation. SLM RE 1500 ppm showed the highest oxidative stability closely aligned to SL TBHQ. Notably, RE supplemented at 1000 ppm and 2000 ppm also provided significant protection, however, similar to p-AV no significant improvement was observed after 1,500 ppm, indicating a stagnation effect.

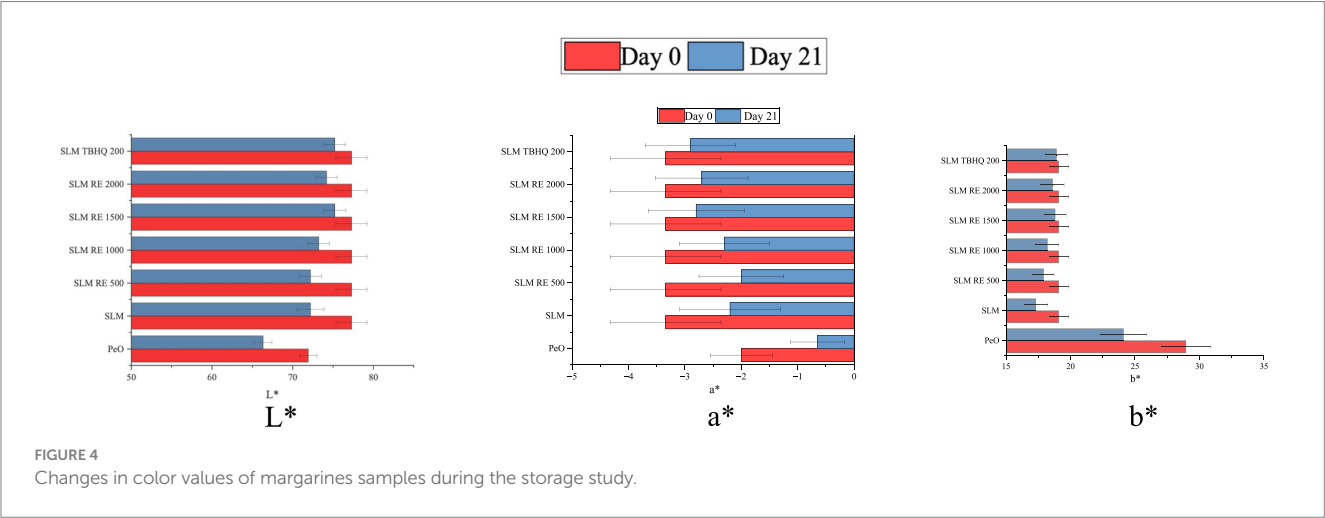
3.5 Colour

A constant decreasing trend was observed for the L* values of all samples during the accelerated storage study (Figure 4). Owing to the high susceptibility to oxidation due to the PeO's fatty acid composition, which is rich in PUFA, it showed the most significant darkening due to extensive oxidative degradation, with value reducing from 71.92 to 66.30. In contrast to this, the SLM samples incorporated with antioxidants exhibited a better colour retention, indicating a protective effect against pigment deterioration. The a* values on the other hand exhibited an increase during the storage period indicating reduced greenness, most notably in PeO. Antioxidant enriched samples, particularly SLM RE 1500 and TBHQ, maintained more stable colour values, suggesting slower pigment breakdown. A reducing trend in b* values was observed in all samples, demonstrating a decline in yellowness, due to the degradation of In carotenoids during storage. The reduction again was most significant in PeO, while SLM RE 1500,

TABLE 2 TOTOX values of margarines during the accelerated storage study.

Day	PeO	SLM	SLM RE 500	SLM RE 1000	SLM RE 1500	SLM RE 2000	SLM TBHQ 200
Day 0	8.63 ± 0.05 ^{ba}	4.29 ± 0.03 ^{aA}	4.26 ± 0.03 ^{aA}	4.22 ± 0.03 ^{aA}	4.22 ± 0.03 ^{aA}	4.27 ± 0.03 ^{aA}	4.26 ± 0.03 ^{aA}
Day 3	21.66 ± 0.08 ^{eb}	15.77 ± 0.07 ^{dB}	12.02 ± 0.06 ^{cB}	8.35 ± 0.04 ^{bB}	8.08 ± 0.03 ^{abB}	8.57 ± 0.04 ^{bB}	7.39 ± 0.04 ^{ab}
Day 6	33.94 ± 0.12 ^{ec}	26.66 ± 0.09 ^{dC}	19.8 ± 0.08 ^{cC}	15.02 ± 0.06 ^{abC}	12.98 ± 0.05 ^{aC}	14.25 ± 0.05 ^{abC}	13.51 ± 0.05 ^{cC}
Day 9	53.76 ± 0.15 ^{cd}	38.56 ± 0.11 ^{dD}	32.01 ± 0.10 ^{cD}	27.76 ± 0.07 ^{bdD}	21.3 ± 0.09 ^{aD}	25.14 ± 0.08 ^{abD}	23.14 ± 0.06 ^{dD}
Day 12	69.37 ± 0.17 ^{de}	55.04 ± 0.13 ^{dE}	44.09 ± 0.12 ^{cE}	35.67 ± 0.09 ^{abE}	30.07 ± 0.08 ^{aE}	32.02 ± 0.08 ^{aE}	30.64 ± 0.08 ^{dE}
Day 15	83.44 ± 0.18 ^{df}	69.47 ± 0.14 ^{cF}	62.22 ± 0.13 ^{cF}	49.5 ± 0.10 ^{bF}	40.65 ± 0.09 ^{aF}	41.05 ± 0.09 ^{aF}	38.71 ± 0.10 ^{dF}
Day 18	102.92 ± 0.20 ^g	85.81 ± 0.15 ^{dG}	78.25 ± 0.15 ^{dG}	58.69 ± 0.13 ^{bG}	52.4 ± 0.11 ^{aG}	55.03 ± 0.11 ^{bG}	49.12 ± 0.12 ^{dG}
Day 21	126.03 ± 0.30 ^h	104.03 ± 0.25 ^{cH}	89.7 ± 0.28 ^{dH}	75.41 ± 0.24 ^{cH}	67.09 ± 0.22 ^{aH}	71.31 ± 0.20 ^{abH}	66.5 ± 0.26 ^{aH}

Means with different lower-case superscripts in a row signify statistical difference sample-wise (*p* < 0.05) and means with different upper-case superscripts signify statistical difference day-wise (*p* < 0.05).



RE 2000, and TBHQ showed higher b^* values on Day 21 suggesting the protective effect of antioxidant addition.

3.6 Fatty acid profile

The accelerated storage conditions led to a significant changes in the fatty acids profile of the lipid samples. This change was based on both the lipid modification (enzymatic interesterification) as well as the antioxidant supplementation. The fatty acid composition of PeO, SLM, and SLM enriched with RE or TBHQ was analysed at day 0 and day 21 of accelerated oxidation study (Table 3). A substantial oxidative degradation of PUFA was observed in PeO, with about 7.22% reduction observed from initial to final day. These findings suggest the PUFA's susceptibility to oxidative degradation in the absence of structural modifications or antioxidants (Mane et al., 2024). Compared to SLM systems, PeO showed the highest PUFA loss, indicating the limitations of using it in functional foods without stabilization. This loss of PUFA is consistent with their high susceptibility to oxidative degradation during storage, as previously reported for edible oils rich in omega-3 and omega-6 FAs (Kum et al., 2025; Kumari Singh et al., 2024). The SLM sample without the addition of any antioxidants showed a higher omega-3 fatty acid degradation, corresponding to about 3.5% reduction in linolenic acid. These changes were accompanied by slight increase in palmitic and stearic acid, suggesting the early onset of lipid oxidation. The improved oxidative stability compared to PeO can be attributed to the modified physical structure of the lipid matrix. The addition of RE was found to be effective in mitigating the FA losses due to the Schaal oven storage conditions. The SLM added with RE at 1500, and 2000 ppm showed minimal ALA degradation by only 1.5 and 1.8%, respectively, over 21 days. This concentration dependent protective effect of RE aligns with recent findings demonstrating its efficacy as a natural antioxidant in preserving PUFA content and delaying lipid oxidation in edible oils (Wang et al., 2018). The synthetic antioxidant, TBHQ provided similar

protection, with minimal changes in FA composition, in line with recent comparative studies on the effectiveness of natural and synthetic antioxidants (Mishra et al., 2022).

3.7 Antioxidant capacity

The antioxidant activity demonstrated both a time and concentration dependent decrease in all samples (Figure 5). PeO exhibited a relatively high initial DPPH value owing to its inherent high concentration of bioactive compounds, however, it also showed a rapid decline in the absence of added antioxidants due to high PUFA content. In contrast, samples containing the RE at concentrations of 1,500 ppm and 2000 ppm along with the synthetic antioxidant, TBHQ exhibited significantly higher initial radical scavenging capacities compared to SLM. The addition of RE showed a concentration dependent effect on the preservation of radical scavenging activity; Notably, by Day 21, SLM RE 1500 exhibited statistically comparable radical scavenging activity to SLM TBHQ 200 verifying its efficiency as a natural antioxidant. Previous studies have reported the enhancement of antioxidant capacity and in turn the oxidative stability in oils and other products on addition of antioxidants (He et al., 2023; Kumari Singh et al., 2024; Wang et al., 2018).

The TPC analysis also showed a consistent decline in phenolic compounds across all samples over the 21 days of storage reflecting their utilization in preventing oxidative deterioration. Similar to DPPH, PeO was found to have a high initial value, indicating the presence of naturally occurring bioactive compounds but its sharp decline by Day 21 highlighted the degradation of these compounds in the absence of added antioxidants. In contrast, SLM RE 1500 and SLM RE 2000 had the highest initial TPC values, that can be attributed to the high content of phenolic compounds present in RE. Notably, by Day 21, SLM RE 1500 retained the highest phenolic content amongst these samples. In contrast, SLM TBHQ 200 exhibited a comparatively

TABLE 3 Fatty acid of margarines during the accelerated storage study with % increase decrease.

Sample	Day	Myristic (c14:0)	Palmitic (c16:0)	Stearic (c18:0)	Oleic (c18:1)	Linoleic (c18:2)	A-linolenic (c18:3)
PeO	0	nd	7.52 ± 0.02	2.68 ± 0.02	10.58 ± 0.03	15.26 ± 0.02	63.96 ± 0.02
	21	nd	8.19 ± 0.03	3.74 ± 0.01	9.93 ± 0.02	13.89 ± 0.02	59.61 ± 0.03
SLM	0	0.90 ± 0.02	38.10 ± 0.03	2.54 ± 0.02	19.08 ± 0.02	10.72 ± 0.02	28.61 ± 0.02
	21	0.98 ± 0.01	38.44 ± 0.02	2.64 ± 0.02	18.80 ± 0.01	10.51 ± 0.01	27.63 ± 0.02
SLMRE500	0	0.89 ± 0.01	38.09 ± 0.03	2.55 ± 0.02	19.10 ± 0.02	10.73 ± 0.02	28.63 ± 0.01
	21	0.92 ± 0.01	38.30 ± 0.02	2.60 ± 0.01	18.85 ± 0.01	10.57 ± 0.01	27.76 ± 0.02
SLMRE1000	0	0.91 ± 0.02	38.11 ± 0.02	2.53 ± 0.02	19.09 ± 0.02	10.74 ± 0.03	28.62 ± 0.02
	21	0.92 ± 0.01	38.24 ± 0.02	2.57 ± 0.02	18.65 ± 0.01	10.62 ± 0.02	27.99 ± 0.01
SLMRE1500	0	0.90 ± 0.01	38.08 ± 0.02	2.55 ± 0.02	19.00 ± 0.01	10.73 ± 0.02	28.63 ± 0.02
	21	0.91 ± 0.02	38.14 ± 0.02	2.56 ± 0.01	19.02 ± 0.01	10.67 ± 0.02	28.19 ± 0.01
SLMRE2000	0	0.90 ± 0.01	38.10 ± 0.02	2.54 ± 0.01	19.11 ± 0.02	10.72 ± 0.02	28.63 ± 0.01
	21	0.91 ± 0.01	38.13 ± 0.02	2.55 ± 0.02	19.06 ± 0.01	10.71 ± 0.01	28.12 ± 0.02
SLMTBHQ200	0	0.91 ± 0.02	38.09 ± 0.02	2.54 ± 0.02	19.10 ± 0.02	10.74 ± 0.01	28.62 ± 0.02
	21	0.92 ± 0.01	38.13 ± 0.03	2.55 ± 0.02	19.02 ± 0.01	10.70 ± 0.02	28.21 ± 0.01

Values expressed as mean ± SD ($n = 3$).

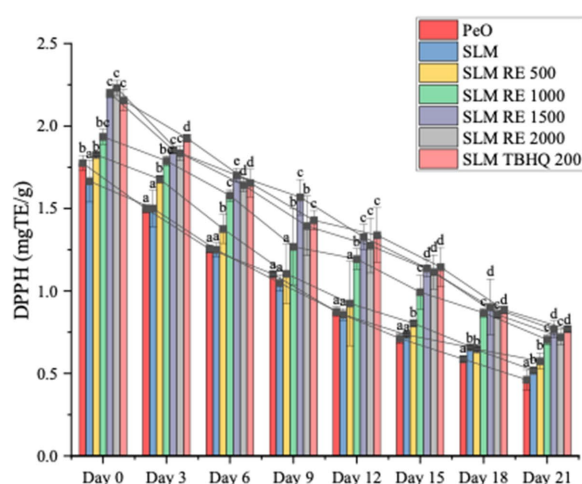
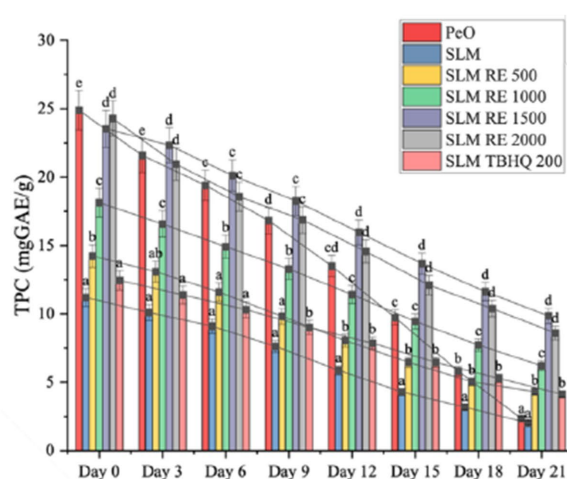


FIGURE 5

Antioxidant values of margarines during the accelerated storage study. Means different lowercase superscripts signify statistical difference sample-wise ($p < 0.05$).



low initial TPC and retained similarly low levels by Day 21. The TPC of TBHQ is relatively less due to absence of phenolic compounds in it, however it retained due to its potent antioxidant capacity, also observed in DPPH assay.

3.8 Total tocopherol content

Tocopherols are naturally found in most plant oils, and serve as important antioxidants that help prevent these oils from oxidizing (Guo et al., 2023; Martin-Rubio et al., 2018; Pan et al., 2019). The highest tocopherol content was observed in the PeO control on the initial day (482.67 ± 18.56 mg/kg), followed by significantly lower values in SLM and treated samples, indicating antioxidant loss due to enzymatic interesterification (Figure 6). This was also due to the addition of palm stearin, lacking bioactive compounds and antioxidants. Consequently, blending PeO with palm stearin for interesterification led to an overall decrease in tocopherol content in the SLM samples (Dhiman et al., 2024; Singh et al., 2023). Apart from this, the interesterification reaction conditions also contribute to this tocopherol loss. After 21 days, a significant decline in tocopherol levels was observed in the samples, with the most significant reduction in the PeO control corresponding to a 77.15% loss. This sharp decline indicates the high oxidative susceptibility of PeO rich in linolenic acid. In contrast, SLM samples treated with antioxidants exhibited significantly better tocopherol retention. Among them, SLM RE 1500, SLM RE 2000, and SLM 200 TBHQ showed the highest tocopherol content at the end of the study, with only about 45 to 50% reduction from their initial values. The effectiveness of natural antioxidant RE was concentration-dependent, with higher levels providing greater protection comparable to TBHQ. The sample with 1,500 ppm RE also demonstrated highest tocopherol retention, further supporting the potential of RE as a natural alternative to synthetic antioxidants. These findings indicate that antioxidant incorporation, especially at higher concentrations, plays a significant role in mitigating tocopherol degradation during

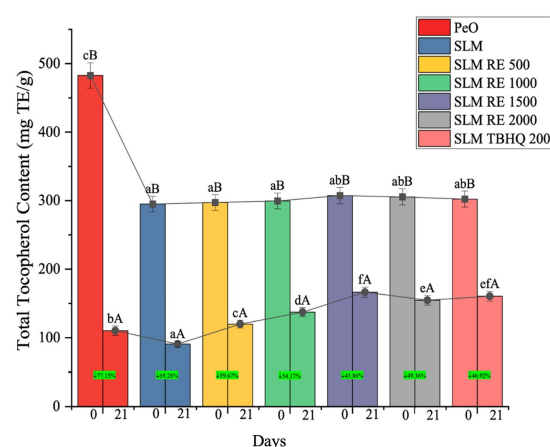


FIGURE 6

Changes in tocopherol values of margarines samples during the storage study with % reduction from day 0 to day 21 for each sample highlighted in green colour.

storage, thereby enhancing the oxidative stability of omega-3 rich lipid systems.

3.9 Principal component analysis

The Principal Component Analysis (PCA) biplot provided a comprehensive multivariate depiction of the oxidative stability and antioxidant properties of the lipid systems analyzed in the study, subjected to different treatments. The first two principal components, PC 1 and PC2 explained a cumulative of 95.79% of total variance (82.01% by PC1 and 13.78% by PC2), indicating that majority of the variability in the dataset was explained in two dimensions. Amongst the loading vectors for individual variables, a distinct separation was observed between oxidative deterioration markers and antioxidant attributes. Oxidation indices including PV, AV, p-AV, TOTOX value,

CD, and CT were clustered together in the positive quadrant of PC1, suggesting their mutual positive correlation and shared contribution to lipid oxidation. In contrast, variables representing antioxidant capacity, i.e., DPPH radical scavenging activity, TPC, and total tocopherol content were observed to be in the opposite direction, indicative of a negative correlation with oxidation parameters. The variable n-3 reduction, representing the loss of omega-3 fatty acids was aligned with oxidative parameters, thus validating that oxidative deterioration is directly linked to the degradation of PUFA. The position of the samples in the biplot further illustrates the effect of RE and TBHQ on the lipid systems. PeO, characterized by high unsaturation and absence of antioxidant or any lipid modification was found to be close to the oxidation parameters, indicating its high susceptibility to oxidative degradation. The control structured lipid, SLM, although slightly improved, remained on the oxidative side of the plot. However, with the addition of rosemary extract, a significant variation was observed based on the concentration of RE. The samples progressively shifted away from the oxidative markers and towards the antioxidant-based variables. Also, the minute shift between TBHQ and RE at 1500 and 2000 ppm is mainly due to low antioxidant capacity of TBHQ in terms of TPC and tocopherol content. SLM RE 1500 clustered closely with SLM RE 2000 and SLM TBHQ, suggesting that at RE at 1500 ppm concentration showed similar efficacy in enhancing oxidative stability and radical scavenging activity as the highest concentration (RE 2000 ppm) and TBHQ (Gurkan and Hayaloglu, 2023). These results validate the potential of RE as a sustainable and natural alternative for enhancing the shelf life and nutritional quality of PUFA-rich structured lipid-based margarine formulations (see Figure 7).

4 Conclusion

This study demonstrates the effectiveness of rosemary extract as a potential natural antioxidant for improving the oxidative stability and shelf life of omega-3 fatty acid rich margarine formulated using perilla seed oil based structured lipid systems. Among all the concentrations, rosemary extract at 1500 ppm exhibited significant reduction in the lipid oxidation and retention of polyunsaturated fatty acids, thereby maintaining the nutritional value of the PUFA rich margarine. The antioxidant performance of rosemary extract was comparable to that of the synthetic antioxidant (TBHQ), indicating its potential as a sustainable alternative for lipid stabilization in food products.

Additionally, lipid modification through enzymatic interesterification of perilla seed oil with palm stearin significantly enhanced the oxidative stability of PeO. The extrapolation of accelerated oxidation data indicates that the shelf life of the margarine formulations at ambient conditions is approximately three months for PeO, six months for the control SLM, seven to eight months for samples containing 500 and 1,000 ppm RE and about nine months for formulations enriched with 1,500 and 2000 ppm RE, with stability comparable to that of TBHQ. The combination of enzymatic interesterification and rosemary extract incorporation resulted in a synergistic effect, offering enhanced protection against the generation of primary and secondary oxidation products while minimizing the degradation of omega-3 fatty acids. This not only enhanced the physicochemical quality of the margarine but also aligned with the growing demand for natural, sustainable food preservation strategies. In conclusion, the findings of the study demonstrate the potential of green

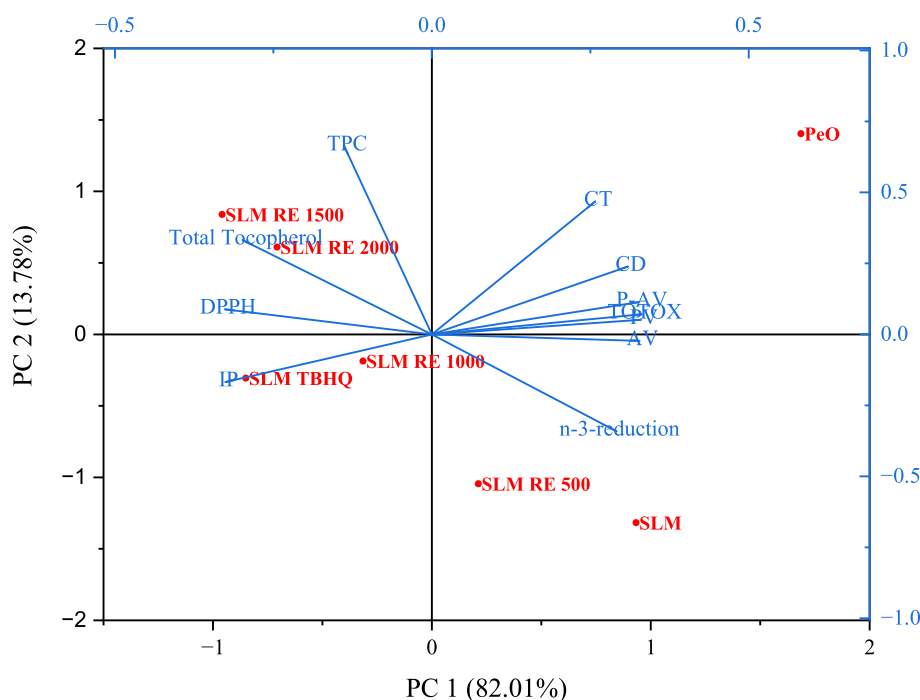


FIGURE 7
PCA biplot.

technologies like enzymatic interesterification and addition of plant-based antioxidants for developing functional lipid systems with extended shelf life and enhanced nutritional properties. Future studies can be aimed on the study of sensory attributes of the product.

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

AD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. RC: Conceptualization, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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