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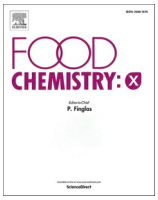

Improvement of margarine shelf-life using alginate-chitosan coated multiple W/O/W nanoemulsions containing sesamol and retinol

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ABSTRACT

Alginate-chitosan (Alg-CH) coated multiple water-in-oil-in-water (W/O/W) nanoemulsion loaded with sesamol and retinol was hypothesized to enhance the oxidative stability of margarine. Total phenol content (TPC), antioxidant activity, acid value (AV), peroxide value (PV), para-anisidine value (*p*AV), induction period (IP), and residual values of sesamol and retinol by HPLC were determined for 90 days. Margarine with tert-butylhydroquinone (TBHQ) (T3) had the higher TPC and antioxidant activity (8.05 mg gallic acid equivalent (GAE)/g), 53.1 %) than T1 (nanoemulsion, 7.39 mg GAE/g, 38.95 %), T2 (free-sesamol and -retinol, 6.98 mg GAE/g, 31.07 %), and T4 (no antioxidant, 6.46 mg GAE/g, and 14.45 %) while T1 had higher antioxidant activity and TPC than T2 and T4 after 90 days. On day 90, the highest residual values of sesamol (200.10 mg/100 g) and retinol (118.09 μg/100 g) obtained for T1. Overall, T1 contributed to the prolonged oxidative stability of margarine, potentially offering an alternative to synthetic antioxidants.

1. Introduction

Margarine (a water-in-oil emulsion, with tiny droplets of water dispersed uniformly throughout an oil phase in a stable solid form) is one of the most prevalent oils used in the food industry and like other oils need to be oxidatively stabilized. It is predominantly produced using vegetable oils (the dominant part) and emulsifiers. Hence, using antioxidants seems inevitable to increase the chemical and oxidative stability of oils (Chmykh & Nadeau, 2020; Mousavi et al., 2023; Sheybani et al., 2023a; Sheybani et al., 2023b). Antioxidants donate protons (hydrogen) and reduce the radicals and prooxidant agents, which lead to the reduction of oil oxidation intensity and increase the oxidative stability (Ahmadi et al., 2024; Mousavi, Javanmard Dakheli, et al., 2022; Mousavi, Nateghi, et al., 2022). There are synthetic and natural antioxidants which can take on the role donation and reduce the oxidation intensity (Mishra et al., 2021). Synthetic antioxidants (tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), etc) due to their low cost and high antioxidant activity are mostly used (Chen et al., 2021; Mousavi, Javanmard Dakheli, et al., 2022; Mousavi, Nateghi, et al., 2022; Rashidi et al., 2016). However, using synthetic antioxidants has been somehow suppressed, likely due to their unfavorable side and detrimental health effects such as mutations, tumors, impairments of blood clotting etc. Thus, the producers have developed foods with natural additives which have antioxidants and health promoting impacts (Bjerke et al., 2021). Natural antioxidants are inherently present in herbs, seeds, and plants (Fhaner et al., 2016; Mousavi et al., 2023). Sesamol is one of these antioxidants that is obtained from sesame seed oil (Fhaner et al., 2016). It has hydrophobic nature with a powerful antioxidant property, which is mostly present in roasted sesame seeds (Santos Basurto et al., 2018). Furthermore, antioxidant and anti-inflammation effects have been reported for sesamol (Yashaswini et al., 2017). Sesamol has been well-known as an antioxidant when used in nanoemulsions and compared to tocopherol (Guo et al., 2021; Wang et al., 2021). However, sesamol has low chemical-stability, and poor aqueous solubility (Yousefi et al., 2023). Retinoids are classified in a class of lipophilic vitamin A derivatives that have been used for decades to produce cosmetics and pharmaceuticals to treat acne, psoriasis, ichthyosis, and actinic keratosis (Bjerke et al., 2021). However, it is so liable to degradation by ambient condition likely due to presence of prooxidants, heavy metals, free radicals, and light (Pezeshki et al., 2014). Thus, it is necessary to use an antioxidant alongside the incorporation of retinol into foods or edible oils. Sesamol can be considered as a strong antioxidant to maintain retinol (Hadeel, Khalida, & Walsh, 2020; Hwang et al., 2013; Yashaswini

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et al., 2017). Development of encapsulation techniques has led to an increase in chemical stability of bioactive components (Gohari et al., 2024; Sheybani et al., 2023a; Sheybani et al., 2023b; Yousefi et al., 2023). Especially, nanoemulsions due to having high thermodynamic stability, high chemical stability, and also high bioavailability have been specifically focused (Yousefi et al., 2023). Multiple emulsions (MEs) are complex and heterogenous dispersion system so that the dispersed phase is itself an emulsion (Yousefi et al., 2023). However, W/O/W MEs suffer from an imbalance of osmotic pressure between the internal and external aqueous phases. The coating of W/OW ME with polyelectrolyte polymers such as polysaccharides has received great attention (Faghmous et al., 2020; Yousefi et al., 2023). These polysaccharides are alginate (Alg) and chitosan (CH) which can stabilize the MEs by balancing the osmotic pressure between two phases and form a rigid interfacial layer between the oil and internal aqueous phase. Combination of Alg with CH leads to occurrence of strong ionic interactions between the carbonyl residues of Alg and amino terminals of CH, forming a polyelectrolyte complex (Faghmous et al., 2020; Yousefi et al., 2023). In addition to applying the CH and Alg as carriers or wall materials, they can also have positive health impacts (Ta et al., 2021; Yousefi et al., 2023).

The objective of this study was to fortify and to increase the oxidative stability of margarine by incorporating MEs nanoemulsion containing sesamol (220 mg) and retinol (125 μg) and compared with margarines incorporated with free sesamol (220 mg) and retinol (125 μg). In this regard, two samples were prepared containing synthetic antioxidant (TBHQ, 75 ppm) and without antioxidant. For this purpose, total phenol content (TPC), antioxidant activity, acid, peroxide, and p-anisidine values, Rancimat analysis, residual values of sesamol and retinol were evaluated. This study was conducted to assess the effects of free- sesamol/retinol, encapsulated sesamol/retinol, and synthetic antioxidant (TBHQ) on the oxidative stability of edible margarine. Also, residuals of free- retinol/sesamol and sesamol/retinol encapsulated by MEs nanoemulsion in fortified margarines during 90 days of samples storage were studied. No study was found in this regard.

2. Materials and methods

2.1. Materials

The extra virgin olive oil (EVOO), retinol (VA, purity ≥98 %), sesamol, sorbitan monooleate (Span® 80), polysorbate (Tween® 80), sodium alginate (Alg) (medium molecular weight), chitosan (CH, medium molecular weight) with 74 % degree of deacetylation, monobasic potassium phosphate (KH_2PO_4), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were supplied by Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, Missouri, United States). Other chemicals used had all the analytical grade.

2.2. Fabrication of retinol and sesamol -loaded CH-Alg- coated W/O/W multiple emulsion

Accordingly, the coated W/O/W emulsion was produced based on a two-step emulsification protocol as reported by Yousefi et al. (2023).

First, the internal aqueous phase was produced by dissolving 400 mg of sesamol in phosphate buffer solution (pH 7.4) and agitated at 1000 ×g for 2 h. Next, oil phase was produced by dissolving 240 μg of vitamin A in 8 g of EVOO then it was mixed with the aqueous phase. The lipophilic Span 80® (optimized concentration of 10.84 % *w*/w) was incorporated to the obtained mixture and a W/O emulsion was developed by stirring at 1000 ×g for 15 min. The optimized water to oil ratio (W/O) was 37.70 % (Yousefi et al., 2023).

The produced W/O emulsion was used as a disperse phase for the second stage of emulsification. It was gently dropped into 18 g of aqueous solution composed of Tween 80 (optimized concentration of 6.24 % w/w based on the published data by Yousefi et al. (2023) and Alg solution (0.067 % *w/v*) which was adjusted at pH 4.9 using 0.1 M HCl. W/O/W nanoemulsion was generated by stirring at 800 ×g for 15 min.

W/O/W nanoemulsion was coated using CH through the polyelectrolyte complex formation. Briefly, 0.5 mL of $CaCl_2$ solution was dropped into 12 mL of W/O/W emulsion and stirred at 800 ×g for 30 min. Then, 2 mL of CH solution (0.05 % w/v) was dropped and agitated at 800 ×g for 90 min. The pH of CH solution was adjusted to 4.6 using 0.1 N NaOH solution. The produced coated-W/O/W nanoemulsion was kept at 4 °C for further studies. The Alg-CH coated multiple nanoemulsion containing sesamol and retinol was obtained with a total EE of 92.93 % (for retinol and sesamol), a particle size of 381.94 nm and a ζ-potential of −19.64 mV (Yousefi et al., 2023).

2.3. Incorporation of coated-W/O/W nanoemulsion into margarine

Margarine production was carried out according to the method of Panpipat et al. (2018) with slight modifications. Lipid phase (80 % *w*/w) was mixed with an aqueous phase (20 % w/w) using a blender. The lipid phase constituted of 79.3 % canola oil and hydrogenated sunflower oil along with 0.7 % of mixed emulsifier containing glyceryl monostearate, glyceryl distearate and lecithin. The aqueous phase was incorporated with 1 % (*w*/w) of sodium chloride. The obtained W/O/W nanoemulsion was incorporated into margarine based on the encapsulation efficiency of sesamol and vitamin A. Indeed, according to our previous research, encapsulation efficiency (EE) of vitamin A was 47.92 % and EE of sesamol was 45 % (Yousefi et al., 2023). Also, based on daily requirement of vitamin A (900 μg/day), 500 mg of nanoemulsion containing sesamol (220 mg) and vitamin A (125 μg) was added into 100 g of margarine and mixed (Yousefi et al., 2023). This sample was termed T1. Besides, a margarine sample was prepared with the mentioned concentrations of free-sesamol and free-vitamin A (without encapsulation) (220 mg of sesamol and 125 μg of retinol) (T2). The synthetic TBHQ was also incorporated at 75 ppm and the sample was termed T3. A blank margarine was also considered without the addition of antioxidants (T4). All margarine samples were kept in plastic containers and stored at 4 °C. The sampling was done at 0, 30, 60, 90 days for analysis.

2.4. Total phenol content (TPC) determination

The Folin-Ciocalteu method was used to determine TPC of the margarine samples (Mousavi, Javanmard Dakheli, et al., 2022; Mousavi, Nateghi, et al., 2022) incorporated with nanoemulsion (T1), free sesamol and retinol (T2), TBHQ (T3), and free margarine (T4). One gram of margarine was mixed with 5 mL of hexane and then centrifuged at 3420 ×*g* for 3 min. The extract was mixed with distilled water to solubilize the phenolic compounds. Also, the sedimented residue was distilled to elicit the internal phenolic compounds. The extracts were collected and about 100 μL of extract was dissolved in distilled water (6 mL) and mixed with Folin- Ciocalteu reagent (0.5 mL, 2 N). 1.5 mL of sodium carbonate (0.71 M) and 1.9 mL of distilled water were also incorporated to reach 10 mL volume. The obtained mixture was incubated in a dark place at an ambient temperature for 2 h. The absorbance value of samples was determined using UV–visible spectrophotometer at 760 nm. Distilled water was used as the control. Gallic acid was used as standard. The determined values were denoted as mg gallic acid equivalent per g of sample (mg GAE/g sample). The GA calibration curve was drawn in the range of 0 to 300 μg/mL($y = 0.0160\times\text{-}0.0362$, R^2 0.998) and was used to calculate TPC.

2.5. DPPH radical scavenging activity (RSA)

The antioxidant power was determined for margarine samples incorporated with encapsulated and free antioxidants (Ahmadi et al., 2024; Fhaner et al., 2016). Similar to the last section, the extracts were collected, and 1 mL of extracts was mixed with 2 mL of DPPH solution (0.2 mM). The obtained mixtures were vigorously shaken and kept in a

dark place for 30 min. The absorbances were determined at 517 nm. The DPPH RSA was computed using the equation below:

$$DPPH\ RSA\ (\%) = \frac{A_c - A_s}{A_c} \times 100 \quad (1)$$

Where: A_s and A_c indicate the absorbance values of sample and control, respectively.

1.1. Acid value (AV) measurement

The AV was determined for margarine samples by the method of Ahmadi et al. (2024) with brief modifications. Accordingly, the potentiometric titration was conducted in an automatic titrator model G20 (Mettler Toledo, Urdrof, Switzerland). 20 ± 0.05 g of margarine sample was mixed with 50 mL of hot ethanol that was made neutral (by adding a few drops of phenolphthalein solution in ethanol (1 % *w*/w)). The solution was titrated with NaOH (0.1 N) until a pink color was observed and stayed for 10 s. The results were expressed as % oleic acid. The AV was calculated using the following equation:

$$AV = (V \times C \times M)/10\ m \quad (2)$$

where: V is the volume of the KOH solution (in mL), C is the concentration of the KOH standard solution (in mol L^{-1}), M is the molar mass (in grams), and m is the mass of the sample (g).

2.6. Peroxide value (PV) measurement

The PV was determined using the ISO (2017) method. Briefly, 1 g of samples was dissolved in 30 mL of acetic acid and isooctane (3: 2) followed by addition of 0.5 mL of saturated potassium iodide (KI) solution (0.1 N) and keeping at darkness for 30 s. Then, the mixtures were mixed with 30 mL distilled water and titrated with $Na_2S_2O_3$ (0.01 N) after addition of 1 mL gelatinized starch (1 % (*w*/w)) as an indicator. PV was expressed in mg oxygen equivalent per kg of oil (meq O_2/Kg oil). The following equation was used for calculating PV:

$$PV = (V_2 - V_1) \times T/M \times 1000 \quad (3)$$

where: V_2 and V_1 are the volumes of $Na_2S_2O_3$ (mL) and blank, respectively; M is the mass of the oil (g), and T is the normality of $Na_2S_2O_3$ (0.01 N).

2.7. p-Anisidine value (pAV) measurement

The *p*AV was determined in the previous reported method by measuring absorbance at 350 nm using the UV–vis spectrophotometer (ISO 6885, 2016). First, *p*-anisidine (0.125 g) dissolved in glacial acetic acid (50 mL). The oil fraction of margarine samples (5 g) diluted by isooctane (25 mL) to obtain oil solutions (not-reacted with *p*-anisidine). Then, *p*-anisidine solution (1 mL) was added to the tubes which contained 5 mL of oil solutions, shaken, and kept in the dark place (10 min). Then, the absorbance of the prepared solutions (not- reacted and reacted) was determined at 350 nm. The reference sample had 5 mL of isooctane with 1 mL of *p*AV. The spectrophotometer was set while the absorbance of reference solution at 350 nm was zero. *p*AV was computed using Eq. (4).

$$pAV = (V \times [1.2 \times (A_1 - A_2 - A_0]/W \quad (4)$$

where V is the volume (mL) of sample dissolved, A_1 is the absorbance value of the reacted sample, A_2 is the absorbance value of not-reacted sample, A_0 is the absorbance value of reference, and W is the weight of oil fractions.

2.8. Oxidative stability

The oxidative stability of margarine samples was assessed by Rancimat apparatus (Metrohm 743; Metrohm Co., Herisau, Switzerland) (ISO 6886, 2016). About 2.5 ± 0.5 g of samples were exposed to heating temperature of 120 °C at an air velocity of 20 L/h. The process time was monitored until a sharp increase in the water conductivity appeared, which corresponded to the oil stability index, denoted induction period (IP). Thus, the oxidative stability of edible oils was expressed as a time (IP, h).

2.9. Residual value`

The residual value of sesamol was determined using the method of Santos Basurto et al. (2018). For extraction of sesamol and retinol, 500 mg of nanoemulsions was weighted and 20 mL methanol was added, then extracting was done with intermittent vortexing for 30 min. Extracts were centrifuged at 5000 ×*g* for 20 min at 25 °C. The supernatant was separated and filtered by the syringe filter (0.45 μm). After dilution with methanol, the amount of retinol or sesamol was determined using the HPLC as mentioned methods as follows.

Sesamol was measured by isocratic RP-HPLC equipped with the UV detector. A column type RP-C18 (Supelcosil™, 250 × 4.6 mm, 4.6 μm particles size, and pore size 12 nm) was applied. The mobile phase consisted of water: methanol (70: 30). The mobile phase was pumped at a flow rate of 1.0 mL/min and a detection wavelength of 290 nm was employed.

Retinol (vitamin A) content was determined according to the HPLC method described by Park et al., 2015). The HPLC system (L-2130; Hitachi, Tokyo, Japan) was used, which equipped to a reversed phase column (Supelcoil™ LC-18, 25 cm × 4.6 mm) that was applied for separation with a methanol: water (75: 25, *v*/v) solution as the mobile phase. The flow rate and fluorescence wavelength were 0.8 mL/min and excitation of 340 nm, emission of 460 nm, respectively. The injection volume was 10 μL. The retinol or sesamol concentration was measured based on the determination of peak areas and comparison with a known standard for each material.

2.10. Statistical analysis

All experiments were carried out at three replications and data were reported as means and standard deviation. Statistical analysis was performed by one way ANOVA and data mean difference was analyzed using Duncan test at the confidence level of 95 % ($p < 0.05$).

3. Results and discussion

3.1. TPC determination

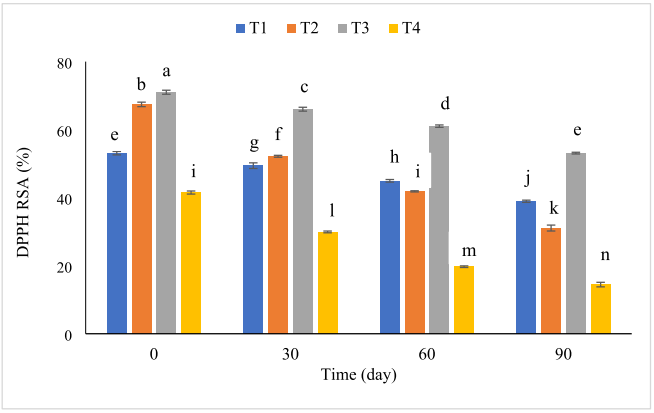
TPC results of margarine samples are presented in Table 1. Due to fortification of margarine samples with retinol (retinol may exhibit

Table 1
Total phenol content (TPC, mg GAE/g sample) of margarine samples containing W/O/W multiple nanoemulsion of sesamol and retinol (T1), free sesamol and retinol (T2), TBHQ (T3), and no antioxidant (T4). The samples were stored at 4 °C.

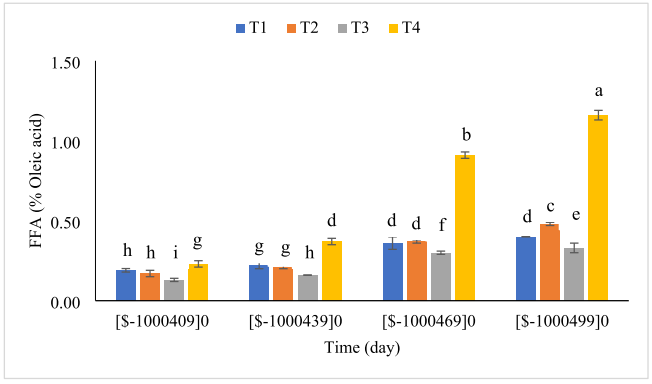
Sample	Storage time (day)			
	0	30	60	90
T1	8.82 ± 0.09^{Ab} *	8.40 ± 0.07^{Bb}	7.95 ± 0.16^{Cb}	7.39 ± 0.07^{Db}
T2	8.78 ± 0.06^{Abc}	8.38 ± 0.13^{Bb}	7.74 ± 0.13^{Cc}	6.98 ± 0.06^{Dc}
T3	9.04 ± 0.10^{Aa}	8.74 ± 0.10^{Ba}	8.40 ± 0.12^{Ca}	8.05 ± 0.08^{Da}
T4	8.67 ± 0.13^{Abc}	8.40 ± 0.08^{Bb}	7.52 ± 0.13^{Cc}	6.46 ± 0.19^{Dd}

* Different small and large superscripts indicate statistically significant difference between the columns and rows, respectively ($p < 0.05$).

direct antioxidant characteristics owing to the existence of hydrophobic chains made up of polyene units), sesamol and TBHQ (which have been known as derivative of phenols), the margarine samples were assessed for TPC during the storage time of 90 days at 4 °C. The highest values of TPC were obtained for T3 which was incorporated with TBHQ. On the other side, the lowest TPC values were achieved for T4 which was free-antioxidant. During the storage, the TPC values were significantly decreased ($p < 0.05$) which can be related to degradation of phenol



(a)



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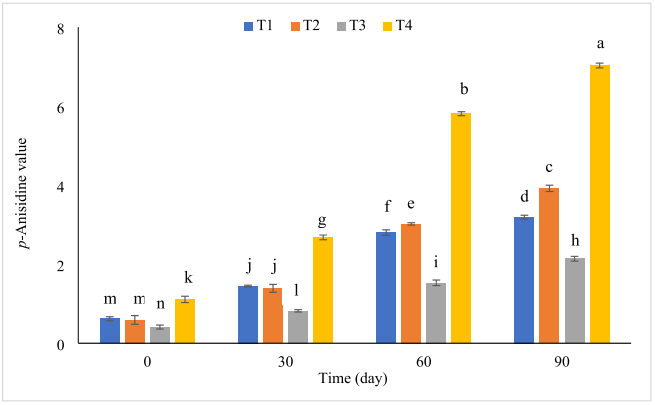

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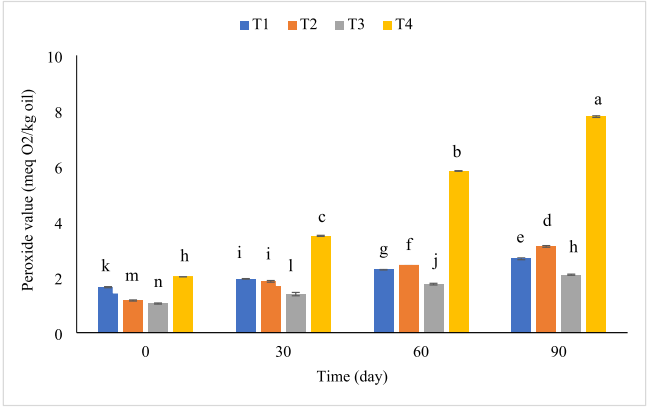

(c)

Fig. 1. (a) DPPH RSA of margarine samples containing W/O/W multiple nanoemulsion of sesamol and retinol (T1), free sesamol and retinol (T2), TBHQ (T3), and no antioxidant (T4).
(b) FFA of margarine samples containing W/O/W multiple nanoemulsion of sesamol and retinol (T1), free sesamol and retinol (T2), TBHQ (T3), and no antioxidant (T4).
(c) PV of margarine samples containing W/O/W multiple nanoemulsion of sesamol and retinol (T1), free sesamol and retinol (T2), TBHQ (T3), and no antioxidant (T4).
(d) pAV of margarine samples containing W/O/W multiple nanoemulsion of sesamol and retinol (T1), free sesamol and retinol (T2), TBHQ (T3), and no antioxidant (T4).

structures. Decomposition of phenolic compounds can be associated with lipid oxidation that not only changes the quality features of the oil, such as storage, appearance, odor, and taste, but also decreases its nutritional and biological values (Keivanfar et al., 2023; Sheybani et al., 2023a; Sheybani et al., 2023b). The oxidation originated from the oxygen invasion that can interact with phenolic compounds and make them oxidized (Gao et al., 2024; Rashidi et al., 2016). As much as incorporation of phenolic compounds is increased, their degradation is decreased due to improved antioxidant activity (Gao et al., 2024; Gohari et al., 2024). The most significant reduction was achieved for T4, which lacked an antioxidant. The phenolic compounds found in T4 are derived from canola and sunflower edible oils, which contain natural phenolics and a notable concentration of tocopherols. Following T4, T2 indicated a higher reduction of TPC due to incorporation of free sesamol and retinol. These natural compounds, especially sesamol in a free form can interact with oxidation inducing agents such as radicals, oxygen, light, etc., and donate protons, thus decreased during the time (Mishra et al., 2021; Park et al., 2015). The sample T1 also indicated reduction values during the storage but at a low rate compared to T2. T3 as a margarine sample with TBHQ indicated the highest TPC likely due to its high chemical stability (Chen et al., 2021; Gohari et al., 2024). Encapsulation showed if sesamol and retinol are protected by multiple layers of W/O/W and also polyelectrolyte complex (Alg-CH), they can be efficiently maintained during storage. Entrapment of natural antioxidants can increase their chemical stability and prolonged antioxidant activity can be conferred (Yousefi et al., 2023). Moreover, encapsulation can provide stable conditions to use natural antioxidants instead of synthetic ones. Although synthetic antioxidants can preserve the edible oils from oxidation, long consumption of them may lead to health problems. However, some natural antioxidants (α-tocopherol, caffeic acid, ferulic acid, and gallic acid) have been reported to have high thermal stability compared to synthetic antioxidants (PG, TBHQ, BHA, and BHT) (Sheybani et al., 2023a; Sheybani et al., 2023b). Hence, using an appropriate antioxidant can not only maintain the chemical properties of edible oils, but also increase their oxidative stability (Rashidi et al., 2016; Yousefi et al., 2023). The biopolymers used in the present study also can have maintaining effects on the sesamol and retinol. It was reported that chitosan had not only entrapment function but also can inherently show antioxidant activities (Atarian et al., 2019). It could be hypothesized that all amine groups of CH were not attached to the carboxylic acid groups of Alg, thus, there were some free amine groups. The charge of emulsion droplets could affect the absorption, and the removal of transition metals involved in lipid oxidation process. There are reports which demonstrated that lipid oxidation was decreased when transition metals (positive charge) were electrostatically repelled from the surface of the lipid droplets (positive charge) (Atarian et al., 2019).

3.2. Antioxidant activity measurement

DPPH RSA of margarine samples was assessed, and results are presented in Fig. 1a. Addition of both synthetic and natural antioxidants led to an increase in DPPH RSA of margarine samples. During the storage, DPPH RSA was decreased for all margarine samples likely due to the degradation of both synthetic and natural antioxidants ($p < 0.05$). The highest DPPH RSA was obtained for T3 so that it was decreased from 71 % to 53.1 %, on day 0 and day 90, respectively. The lowest DPPH RSA was obtained for T4 on all storage days (41.51 % to 14.45 %). T1 and T2 indicated higher DPPH RSA than T4 and less than T3. Encapsulation of sesamol and retinol maintained their antioxidant activities especially the significant effect was observed on day 60 and 90. The margarine sample with free sesamol and retinol had higher DPPH RSA up to day 30 while it was decreased due to the chemical degradation and reduction of donating activity. Although addition of TBHQ showed higher DPPH RSA than addition of natural antioxidants, due to unfavorable health effects, the usage of synthetic antioxidants is limited (Fhaner et al., 2016). Moreover, natural antioxidants had lower DPPH RSA than TBHQ while if they are added at higher concentrations, they can show stronger antioxidant activity (Fhaner et al., 2016). It has been reported that higher concentration of sesamol provided better antioxidant effects indicating that no prooxidant activity occurred in soybean oil (Fhaner et al., 2016; Hadeel et al., 2020). On the other side, encapsulation of these natural antioxidants through W/O/W production and electrolyte complex formation approach caused an increase in the chemical stability of incorporated antioxidants and thus, higher DPPH RSA was achieved on day 90. Several studies have reported that encapsulation of bioactive components is a good technique to increase their retaining stability (Pezeshki et al., 2014). Encapsulation provides maintenance effects on the natural antioxidant compounds (Sheybani et al., 2023a; Sheybani et al., 2023b). Also, encapsulation protects the sensitive antioxidant compounds against harsh conditions (Sheybani et al., 2023a; Sheybani et al., 2023b). Especially, when multiple layer nanoemulsions are applied for an encapsulation approach, the protectivity is considerably increased (Faghmous et al., 2020). Indeed, Alg-CH coated multiple nanoemulsion provided high protection circumstances for sesamol and retinol. These components can be maintained during storage while they can be also released in a sustained state and increase the antioxidant potential of margarine (Faghmous et al., 2020; Yashaswini et al., 2017; Yousefi et al., 2023). Sesamol is a potential hydrophobic antioxidant that can strongly interact with hydrophobic DPPH radicals. When it is used in free form, it exerts antioxidant activity; however, the intensity of antioxidant activity is affected by oxidation accelerating factors such as oxygen, and thereby, the antioxidant activity is diminished during the storage. Therefore, when antioxidant compounds are incorporated in a nanoemulsion form, especially those covered by electrolyte complex generated by carbohydrates or proteins, the antioxidant intensity is maintained during storage (Faghmous et al., 2020; Yousefi et al., 2023). Also, the positive charges of CH can support the decreasing of oxidation intensity. Indeed, CH was electrostatically attached to the Alg through positive and negative charge attractions. However, there were some positive charges of CH that were not involved in electrostatic attractions. These positive charges repelled the transition metals with positive charges; thus, the oxidation was reduced (Atarian et al., 2019). The reduction of oxidation intensity can be associated with reduction of natural antioxidants (sesamol and retinol) with free radicals and transition metals. Therefore, the antioxidant activity of these natural antioxidants was maintained.

3.3. AV analysis

AV indicates the chemical quality of lipids and oils. When the lipids are exposed to hydrolytic conditions, AV is increased. The higher AV leads to the higher free fatty acid percentage (FFA%), which implies that oils can be promptly oxidized. The increase of FFA % values during 90 days of storage of margarine samples are presented in Fig. 1b. T4 as the margarine samples without antioxidant did show a higher FFA % during the storage and reached 1.16 % based on the oleic acid. All margarine samples with antioxidants indicated lower FFA % than T4. Especially, T3 due to containing TBHQ had lower FFA % during the storage. T1 and T2 did not show remarkable difference up to day 60 while on day 90, T1 had lower FFA % than T2 which was attributed to encapsulation effects. Entrapment of antioxidants maintained the antioxidant activity of sesamol and thus, lower FFAs were formed. It could be due to controlling the hydrolytic conditions by the antioxidants (Sharma et al., 2019). A comparison between T1 and T2 revealed that the encapsulation of sesamol and retinol resulted in significant chemical stability in the margarine sample. Consequently, by day 90, T2 sample showed a higher AV, suggesting that the initial antioxidant activity of the unencapsulated sesamol and retinol had diminished. In contrast, T1 due to having Alg-CH coated-W/O/W nanoemulsion of sesamol and retinol exhibited lower AV than T2. Similar results were reported in previous studies (Sharma et al., 2019). Regarding the maintenance effects of Alg and CH, there are some reports that CH with free amine groups can form positive

charges surrounding the oil droplets (Atarian et al., 2019). These positive charges can repel the transition metals with positive charges and prevent from approaching the oxidation factors to the antioxidants and oils (Atarian et al., 2019). In an accurate explanation, the addition of Alg-CH coated multiple nanoemulsion incorporated with sesamol and retinol into margarine controlled the formation of FFAs. Indeed, Alg and CH first protected the entrapped bioactive components and second, they individually acted as antioxidant and protective agents. Alg with its hydroxyl groups can donate hydrogen to the free radicals and transition metals. On another side, CH with its mentioned function increases the spaces with the oxidation factors and encapsulated cores. By these, Alg can have not only antioxidant effects on its surrounding media (margarine) but also can maintain the antioxidant activity of entrapped sesamol and retinol. Furthermore, CH contributes to the stability of the encapsulated cores through its positively charged surface, thereby facilitating the establishment of significant antioxidant activity within the medium (Atarian et al., 2019). All these can support the reduction of FFA formation.

3.4. Primary oxidation analysis (PV measurement)

Oxidation of fats occurs in multiple stages and the primary oxidation is related to production of peroxides and hydroperoxides (Keivanfar et al., 2023; Sheybani et al., 2023b). These products are determined by PV which has been known as unstable index of oxidation, and colorless, tasteless, and odorless (Ahmadi et al., 2024; Mousavi, Javanmard Dakheli, et al., 2022; Mousavi, Nateghi, et al., 2022). Since primary oxidation products are easily degraded into secondary oxidation products including ketones and aldehydes which have been realized as stable index of oxidations. Despite this, PV can show the temporary oxidative stability of edible oils and higher values (>5 meq O_2/kg oil) indicate the lower oxidation quality due to presence of carcinogenic radicals (Ahmadi et al., 2024;Sheybani et al., 2023a; Sheybani et al., 2023b).

The PV was determined for margarine samples during the storage time of 90 days and results are presented in Fig. 1c. Addition of antioxidants increased the oxidative stability of margarine and lower PVs were obtained. T4 indicated the highest PVs during the storage time. Indeed, deficiency of antioxidant in margarine decreased its chemical stability and oxidation inducing factors such as light, metals, ions, and oxygen propagated the oxidation and PV was significantly increased ($p < 0.05$). On the other side, the addition of synthetic and natural antioxidants retained the oxidation quality of margarine samples, and the lower PVs were obtained. TBHQ as a synthetic antioxidant efficiently controlled the primary oxidation and the lowest PVs were obtained for margarine sample (T3). TBHQ has been known as the strong donating and quenching antioxidant (Ahmadi et al., 2024). Addition of natural antioxidants caused significant lower PVs compared to non-addition ($p < 0.05$). The incorporation of free sesamol and retinol in margarine (T2) indicated slightly lower PVs than those were added in the encapsulated form up to day 30. It could be related to the direct contact of antioxidants with oxidation inducing agents (Atarian et al., 2019). Encapsulation of antioxidants created multiple layers surrounding them; thus, their contact was reduced (Yousefi et al., 2023). However, PVs of T1 were lower than T2 on day 60 and 90, which could be associated with the maintaining impacts of multiple layers of nanoemulsion and also complex electrolyte created by Alg-CH on natural antioxidants (Atarian et al., 2019). In summary, when antioxidants are protected from oxidative factors, their ability to act as antioxidants is preserved, resulting in an extended duration of their beneficial activity. Addition of natural antioxidants such as sesamol has been reported as a good alternative for synthetic antioxidants (Azizkhani & Zandi, 2009; Park et al., 2015). In addition, using multiple nanoemulsions in susceptible oils has shown enhanced oxidative stability in terms of lower PV, *p*AV, and also the formation of marker of oxidation of oils, indicating the enhanced oxidative stability (Sheybani et al., 2023a; Sheybani et al., 2023b). The green coffee oil emulsions stabilized by lecithin and chitosan through the electrostatic layer-by-layer deposition technique have shown higher oxidative stability (Carvalho et al., 2014). The double-layer nanoemulsions of capsaicin incorporated with alginate and chitosan improved the stability of nanoemulsion (Choi et al., 2011). Using natural antioxidants along with retinol stabilized in phosphatidylcholine vesicles has shown an improvement effect on the oxidative stability of retinol (Chmykh & Nadeau, 2020).

Based on the present study, Alg and CH were used as encapsulating agents to coat the multiple nanoemulsion. These biopolymers individually might act as antioxidants associated with their chemical structures (Yousefi et al., 2023). Indeed, Alg with its hydroxyl groups has high potential in scavenging the radicals and donating hydrogen. On another side, CH with its positive charges can repel the transition metals with positive charges; thereby, the oxidation intensity is decreased (Atarian et al., 2019).

3.5. Secondary oxidation analysis (pAV measurement)

When the unstable primary oxidation products (peroxides and hydroperoxides) are degraded, the stable ketones and aldehydes are produced which are responsible for off-flavor and off-odor of edible oils (Ahmadi et al., 2024; Keivanfar et al., 2023). When the oxidation intensively occurs, PV cannot show the real chemical quality of edible oils. Since primary oxidation products are degradable at higher temperature, exposure to light, and oxygen, thus, a more reliable oxidation stability index should be determined (Mousavi et al., 2023). *p*AV is an oxidative stability index which determines the aldehyde compounds such as hexanal, propanal, 2-alkenals, 2-hexenal, and 2-nonenal which have been well-known for off-flavor (odor) (Sheybani et al., 2023a; Sheybani et al., 2023b).

The results of *p*AVs are presented in Fig. 1d. Accordingly, addition of antioxidants controlled the secondary oxidation intensity so that T4 indicated the highest *p*AVs. The lowest *p*AVs were obtained when TBHQ was added (T3). TBHQ lowered the oxidation intensity and lower secondary oxidation products were generated. Addition of free sesamol and retinol (T2) or encapsulated form (T1) did not differ the *p*AVs significantly up to day 30 ($p > 0.05$) while *p*AVs were increased for T2 on day 60 and 90. It could be likely due to the protection of multiple layers of nanoemulsion exerted on the maintaining of natural antioxidants (Yousefi et al., 2023). The higher contact with oxidation factors, the lower antioxidant activity was obtained. Encapsulation retained the sesamol and retinol antioxidant activity and lower *p*AVs were obtained for T1. Several studies have shown that using natural antioxidants can efficiently preserve the oil molecular integrity (Fruehwirth et al., 2021). Also, using the multiple nanoemulsion systems has caused maintaining of oil oxidative quality by controlling PV and *p*AV (Guo et al., 2021). The multilayer nanoemulsion of *Heracleum Lasiopetalum* extract coated with whey protein concentrate enhanced the oxidative stability of sunflower oil, indicating by lower PV, *p*AV, and thiobarbituric acid value (TBAV), comparatively to TBHQ (Yazdan-Bakhsh et al., 2021). In our previous study, using nanoemulsion-loaded with sesamol and retinol and coated with Alg-CH led to control the release of entrapped antioxidants in the simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF) (Yousefi et al., 2023). In the present study, it was hypothesized that this delivery system led to a sustained and controlled release of sesamol and retinol which maintained the oxidative stability of margarine, and also the lower secondary oxidation products were produced during the storage time.

3.6. Rancimat analysis

The oxidative stability of edible margarine oils incorporated with synthetic and natural antioxidants was assessed by Rancimat method and the results are presented in Table 2 and Fig. 2**.** The oxidative stability was denoted by IP which implied that the chemical stability depends on the time of oxidation. If the oxidation process and changes in

Table 2
Induction period (IP, h) of margarine samples containing W/O/W multiple nanoemulsion of sesamol and retinol (T1), free sesamol and retinol (T2), TBHQ (T3), and no antioxidant (T4). The samples were stored at 4 °C.

Sample	Storage time (day)			
	0	30	60	90
	IP (h)			
T1	8.58 ± 0.05^{Ac} *	8.31 ± 0.05^{Bb}	7.70 ± 0.01^{Cb}	7.39 ± 0.02^{Db}
T2	8.82 ± 0.03^{Ab}	7.68 ± 0.02^{Bc}	6.81 ± 0.02^{Cc}	6.60 ± 0.03^{Dc}
T3	9.33 ± 0.02^{Aa}	9.11 ± 0.06^{Ba}	8.61 ± 0.03^{Ca}	8.13 ± 0.04^{Da}
T4	7.71 ± 0.03^{Ad}	7.29 ± 0.04^{Bd}	6.55 ± 0.04^{Cd}	6.15 ± 0.02^{Dd}

* Different small and large superscripts indicate statistically significant difference between columns and rows, respectively ($p < 0.05$).

electric conductivity of water occurred in low time, it means the edible oil has low chemical stability (Amadi et al., 2024). In contrast, if the process time of oxidation needed more time, it means the edible oil has high chemical, subsequently high oxidative stability. It was observed that during the storage, IP values were decreased for all samples. The highest IP values were obtained on day 0 and the lowest values were obtained on day 90. The sample T3 indicated higher IP values while T4 indicated lower IP values compared to others. On day 0, T2 had higher IP (8.82 h) than that obtained for T1 (8.58 h). It could be related to direct contact of antioxidants with the oxidation radicals produced during the heating and retarded the oxidation. Indeed, encapsulation created a multi-layer system which protected the antioxidants against the inducing oxidation parameters. This reduced the contact of antioxidants with the surrounding oxidation factors. However, the encapsulation effects were revealed on day 30, 60, and 90. T1 exhibited higher IP values than T2. Indeed, the protection of multi-layer system led to maintaining the antioxidant activity of natural antioxidants and thus, controlled release of antioxidants from the nanoemulsion vehicle to the surrounding oil retarded the oxidation by increasing IP. Indeed, generation of multiple-layered nanoemulsion provide a controlled and sustained release which can increase the oil oxidative stability during the storage. Several studies have shown that incorporation of natural antioxidants obtained from herbs and plants can increase the oxidative stability of susceptible oils especially when exposed to accelerating conditions. It was reported that the mixtures of natural antioxidants (tocopherol, ascorbyl palmitate, and rosemary extract) provided a good oxidative stability when exposed to oven test (60 °C), Rancimat test (110 °C), and storage at 4 °C (Azizkhani & Zandi, 2009). The influence of different ingredients of margarine including emulsifiers, antioxidants, citric acid, β-carotene, and NaCl was studied on the oxidative stability of margarine, heated at 80 °C for 1 h to accelerate lipid oxidation, which was analyzed by peroxide value and oxidation IP. It was found that monoglycerides influenced lipid oxidation depending on their fatty acyl chain. The α-tocopherol acetate promoted lipid oxidation, while rosemary and green tea extract led to the opposite. The green tea extract lonely showed the most prominent antioxidant effect, while combinations of green tea extract with citric acid, β-carotene or NaCl increased lipid oxidation in margarine (Fruehwirth et al., 2021). Regarding the effects of monoglycerides on lipid oxidation, the hypothesis is that when molecular weight of lipids is increased, the lipid oxidation process is slowed down. Indeed, the chains of triglycerides are larger than monoglycerides. Thus, triglycerides can build up thick interfacial layers that act as physical barrier from approaching metals, such as iron (Fruehwirth et al., 2021). On this basis, as we used glyceryl monostearate, glyceryl distearate and lecithin along with canola oil as dominant part of margarine, the concentration of these emulsifiers and glycerides are so vital in oxidation controlling.

3.7. Residual values

The chromatograms of residual values of sesamol and retinol which were determined by HPLC, are represented in Fig. 3. Based on Fig. 3a, sesamol concentration after storage time (90 days) reached 118.09 mg/100 g margarine. Regarding the retinol (Fig. 3b), the residual value was obtained 80.09 μg/100 g.

The residual values of sesamol and retinol on days 1 and 90 are represented in Table 3. Accordingly, sesamol concentrations were 215.80, and 209.79 mg/100 g for T1, T2 on day 1, respectively ($p > 0.05$). The values were significantly decreased on day 90 while the most reduction was observed for T2 (178.03 mg/ 100 g) which contained sesamol in free state. It was also found that T1 which had Alg-CH-coated nanoemulsion of sesamol remarkably maintained the sesamol and retinol content which was so comparable with T3 which had TBHQ. The respective values were 200.10, and 178.03 mg/100 g for T1, and T2 on day 90, respectively.

Regarding the retinol (Table 3), the residual values were obtained 120.40 and 112.30 μg/100 g for T1, and T2 on day 0 ($p > 0.05$). After 90 days, the respective values were significantly decreased for T1 (118.09 μg/100 g) and T2 (80.09 μg/100 g) ($p < 0.05$). However, the reduction of retinol content was negligible for T1 which showed that incorporation of bioactive components into edible oils at multiple nanoemulsion form can significantly maintain their residual values. Indeed, nanoemulsion delivery systems provide high stabilized conditions for bioactive components which retain their residual values during storage. Also, the nanoemulsion based delivery systems cause sustained and controlled release of bioactive components which can increase the oxidative stability of labile products. Combination of sesamol with retinol also efficiently supported the maintenance of retinol due to high antioxidant activity of sesamol (Yousefi et al., 2023).

It was reported that Kenaf seed oil-in-water nanoemulsion stabilized by sodium caseinate, beta-cyclodextrin, and Tween 20, which contained vitamin E and phytosterols had maintained half of its initial concentration until week 4 and week 2 of storage at the accelerated storage condition (40 °C), which was equivalent to 16 weeks and 8 weeks of storage at room temperature (Cheong et al., 2018). Hence, nanoemulsion formation of bioactive components can increase their chemical stability (Cheong et al., 2018). However, it has been also reported that emulsion systems have higher lipid surface areas exposed to the aqueous phase and light easily can penetrate through the emulsions than the bulk oil. According to the Park et al. (2019) reported that within the emulsions, retinol is dispersed on the surface of the oil, suggesting that degradation of retinol occurs at the water-oil interface. It was also reported that high oil concentration (>10 %) can increase the oxidative stability of retinol when compared to WPI-stabilized nanoemulsions (Park et al., 2019). Same results were reported by Liu et al. (2019) and Yazdan-Bakhsh et al. (2021).

4. Conclusion

Incorporation of synthetic and natural antioxidants (either in free or nanoemulsion form) increased the total phenol content and antioxidant activity of margarines. The margarine incorporated with Alg-CH coated multiple nanoemulsion containing sesamol and retinol indicated higher antioxidant activity compared to those had free or non-added natural antioxidants on day 90. The margarine incorporated with TBHQ indicated higher oxidative stability as determined by peroxide, acid, para-anisidine values, and IP. The margarine that had encapsulated sesamol and retinol indicated comparable results with the former. Using Alg-CH coated multiple nanoemulsion also provided higher sesamol and retinol residual values. Encapsulation of retinol with sesamol increased maintenance of retinol. Adding Alg-CH coated multiple nanoemulsion containing sesamol and retinol in the formulation of margarine led to fortify and increase of oxidative stability in the final product. The encapsulation system led to a sustained and maintained antioxidant activity during the storage of 90 days, implying that sesamol and retinol can be potentially replaced with TBHQ. As a result, the use of Alg-CH coated multiple nanoemulsions containing sesamol and retinol is suggested to

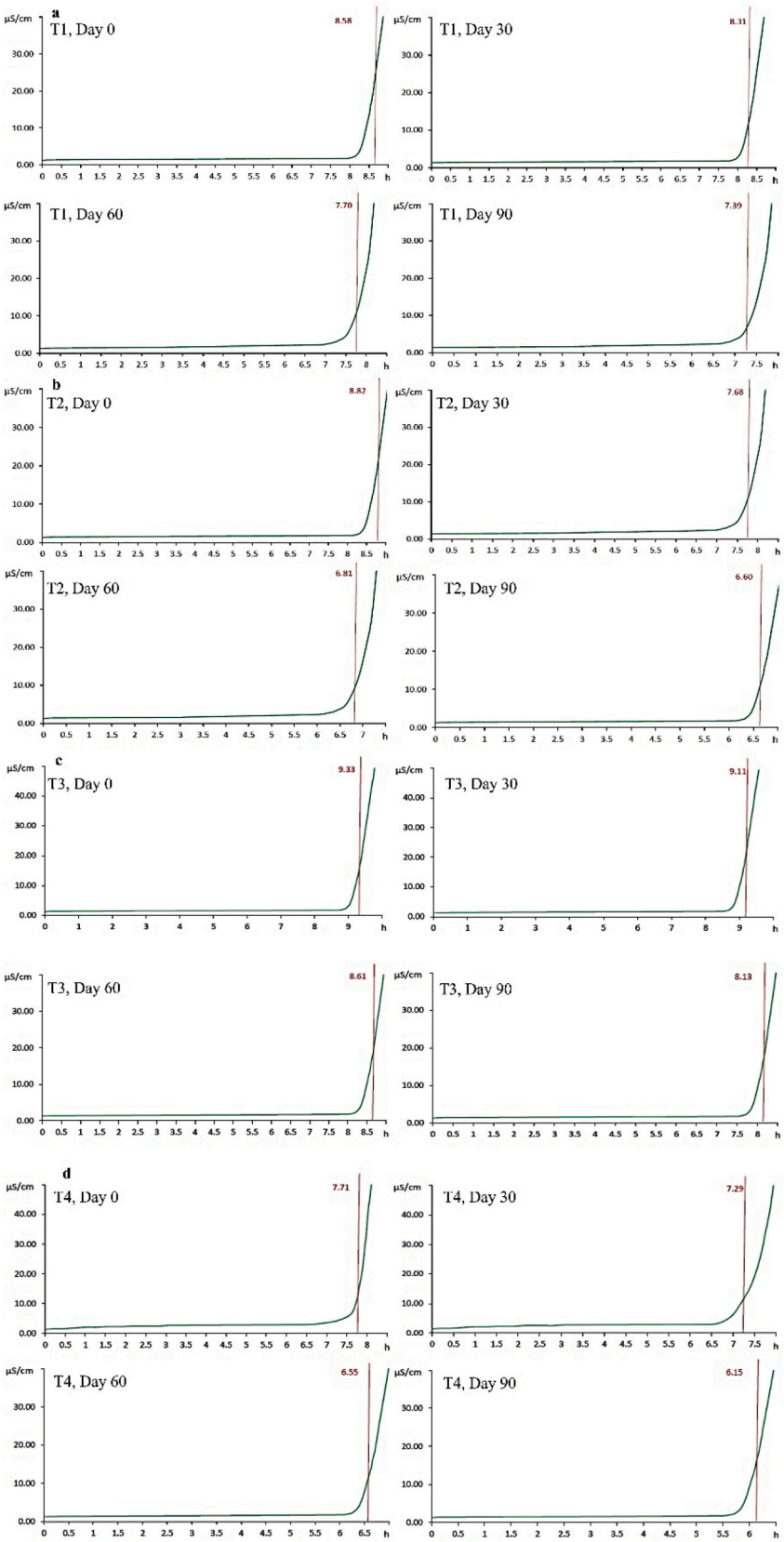

Fig. 2. Rancimat analysis of margarine samples incorporated with synthetic (TBHQ) and natural sesamol antioxidants and retinol. T1, T2, T3, and T4 denote margarine incorporated with sesamol and retinol Alg-CH-coated nanoemulsion (T1), margarine with free sesamol and retinol (T2), margarine with TBHQ (T3), and margarine without addition of antioxidants (T4).

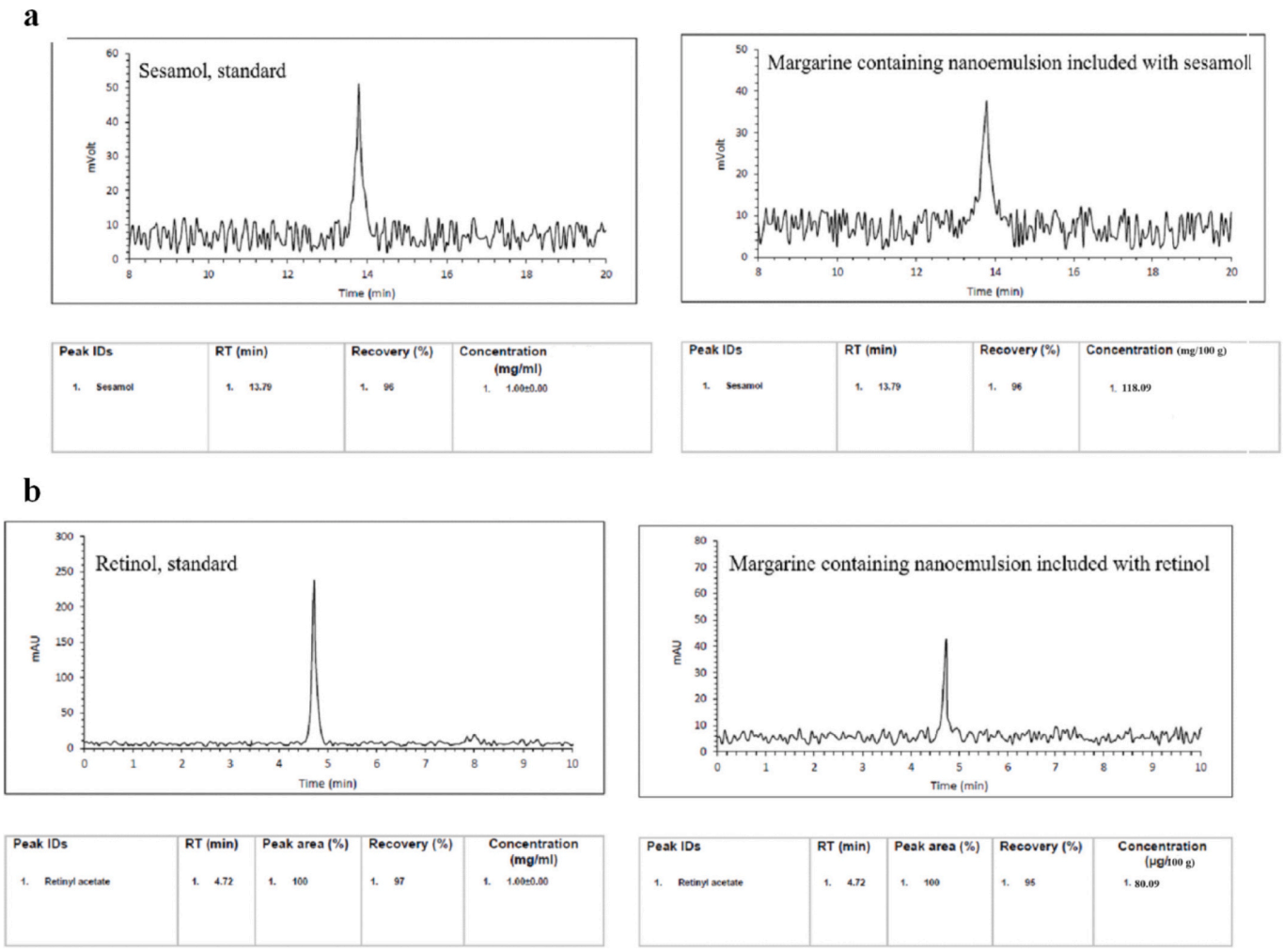

Fig. 3. Residual values of sesamol (a) and retinol (b) entrapped within the Alg-CH coated nanoemulsion incorporated to the margarine and standard solution each of them.

Table 3
The residual values of sesamol and retinol for margarine samples containing W/O/W multiple nanoemulsion of sesamol and retinol (T1), free sesamol and retinol (T2), TBHQ (T3), and no antioxidant (T4). The results were determined by HPLC during day 1 and day 90. The samples were stored at 4 °C.*

Sample	Sesamol residual value (mg/100 g)		Retinol residual value (μg/100 g)	
	On day 1	On day 90	On day 1	On day 90
T1	215.80 ± 0.02^{Aa}*	200.10 ± 0.02^{Ba}	120.40 ± 0.04^{Aa}	118.09 ± 0.03^{Bb}
T2	209.79 ± 0.03^{Aa}	178.03 ± 0.01^{Bb}	112.30 ± 0.02^{Aa}	80.09 ± 0.02^{Bc}
T4	–	–	–	–

* Different small and large superscripts indicate statistically significant difference between the columns and rows, respectively ($p < 0.05$).

produce margarine, aimed at fortifying the product and extending its shelf life for consumer use.

Also, the Alg-CH coated multiple nanoemulsion, which functions as a secure nanocarrier for sesamol and retinol, is suggested for application in edible fats and oils, along with various other products, to improve their nutritional value and extend their shelf life.

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CRediT authorship contribution statement

Shahryar Yousefi: Writing – original draft, Software, Investigation. **Leila Nateghi:** Writing – review & editing, Visualization, Methodology. **Ladan Rashidi:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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