Effects of natural antioxidants and high-energy fabrication methods on physical properties and oxidative stability of flaxseed oil-in-water nanoemulsions

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ABSTRACT

The effects of high-energy fabrication methods, namely high-pressure homogenization (HPH) and ultrasonication (US), on physicochemical properties of flaxseed oil-in-water nanoemulsions (FNEs) containing clove essential oil (CEO) and/or pomegranate peel extract (PPE) were studied during storage at 4 and 25 °C. Nanoemulsions with relatively similar average droplet size were prepared by HPH and/or US. An increase in droplet size was observed over time. Lower storage temperature and fabrication by US increased Ostwald ripening rate. Higher storage temperature and fabrication by US decreased the centrifugal stability of nanoemulsions. CEO revealed better antioxidant properties than PPE. The oxidative stability was evaluated by determining secondary oxidation products, and fatty acids profile. The absence of antioxidant, fabrication by US, and higher storage temperature decreased the oxidative stability of nanoemulsions. The results of this study might be helpful in controlling the oxidation of FNEs during long-term storage and in designing functional foods and beverages.

1. Introduction

These days, there is an increasing interest in foods enriched with ω-3 fatty acids (FAs) due to beneficial effects on human body for instance decreasing the risk of colon and breast cancer, inflammatory, atherosclerosis, and mental disorders [1–4]. Fish oil is the main source of various ω-3 FAs. However, among vegetable oils, flaxseed oil has a high level of α-linolenic acid (ALA), which can convert into the more bioactive forms including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in the human body. The main challenges in the food industry to develop functional foods enriched with ω-3 FAs include high susceptibility to lipid oxidation and low solubility in water [5].

Several colloidal systems have been designed for the delivery of ω-3 FAs including those based on emulsions (like microemulsion, nanoemulsion, and conventional emulsion) as well as inclusion complexes [6]. One of the most suitable delivery systems is nanoemulsion. It is a kind of emulsion in which the diameter of dispersed oil droplets is<200 nm. The small droplet size results in kinetic stability, but not thermodynamic stability [7,8]. The optical clarity, and bioavailability of hydrophobic food ingredients delivered by nanoemulsions are better than those delivered by conventional emulsions [9].

Nanoemulsions are prepared by low-energy and high-energy methods. In the former, nanodroplets are formed spontaneously either under the controlled mixing of aqueous and oil phases (containing appropriate ingredients such as surfactants and co-solvents), or by phase inversion via changing the temperature and/or composition of previously prepared conventional emulsions [10,11]. In this approach, the energy consumption is very low. However, great quantity of surfactants is usually needed [7]. In the latter, tiny droplets are developed under the effect of strong disruptive forces by various techniques such as high-pressure homogenization (HPH), ultrasonication (US), and microfluidization (MF) [10,11]. Among high energy methods, HPH is the most common utilized technique for fabricating nanoemulsions in the food industry [10] because of the ease of utilization, high throughput and production repeatability [12]. In this technique, a coarse emulsion is required prior to the formation of nanoemulsion. In US technique, nanodroplets are developed either from the coarse emulsion or from the phase-separated oil–water mixture by means of high intensity ultrasonic waves [10]. High energy methods do not have any restrictions on the type of oils and surface-active agents. Also, the required surfactant to oil

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ratios (SORs) are usually less than those required in low energy methods.

Generally, emulsification facilitates the oxidative degradation of dispersed oil droplets mainly due to an increase in the specific surface area as well as the impurities present in the aqueous phase [13]. The lipid oxidation adversely affects the shelf life, safety, nutritional value, and sensorial properties of food products. In emulsion systems, lipid oxidation normally occurs at the oil–water interface because the reactions are catalyzed by the hydrophilic prooxidants in the aqueous phase. Due to the larger specific surface area of nanodroplets, nanoemulsions tend to be more sensitive to oxidation. Therefore, appropriate strategies are required to prevent nanodroplets from oxidation [14].

There are some ways to enhance the oxidative stability of nanoemulsions such as adding antioxidants and increasing the number (or thickness) of interfacial layers. Primary antioxidants increase the induction period thorough donating hydrogen atom to free radicals [15]. There are some safety concerns regarding the use of synthetic antioxidants. Natural antioxidants such as essential oils extracted from medicinal plants can be considered as a substitute for synthetic ones. These aromatic oily liquids are the steam distillate of flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots [16]. Among aromatic plants, clove contains high amounts of eugenol that has strong antioxidant properties. It has been reported that clove essential oil (CEO) imparts 97.3 % inhibition in lipid hydroperoxides formation in linoleic acid emulsion at a concentration of 15 mg/mL [17]. Pomegranate peel is another good source of antioxidants [18]. Pomegranate peel has the highest antioxidant activity among the peel, pulp and seed fractions of fruit [19]. It has significant amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid [20] and is utilized in the formulation of cosmetic, therapeutic and food products [21].

To our knowledge, there are some reported studies on the formation of (nano)emulsions from ω-3 rich oils (including fish and flaxseed oils) by high energy methods. Kuhn and Cunha [22] studied the influence of operating conditions such as homogenization pressure and number of cycles on the physicochemical properties of flaxseed oil emulsions stabilized by whey protein isolate. The droplet diameter was decreased by increasing the homogenization pressure and number of cycles. As compared to bulk oil, the evolution of primary oxidation products was enhanced in the emulsified samples particularly in those subjected to higher pressure. Nejadmansouri et al. [23] prepared fish oil nanoemulsions by ultrasonic method using different mixtures of Tween 80 and Span 80 as surfactant. Increasing storage temperature (from 4 to 25 °C) reduced Ostwald ripening rate and oxidative instability of nanoemulsions. The rise of oxidative stability at higher temperature was attributed to a decrease in the solubility of oxygen at higher temperature and temperature-dependent arrangement of triacylglycerols within nanodroplets. Kentish et al. [24] investigated the effect of sonication conditions on the properties of flaxseed oil-in-water nanoemulsions stabilized by Tween 40. In another study, Ma et al. [15] studied the formation of flaxseed oil-in-water nanoemulsions using transglutaminase-cross-linked sodium caseinate as emulsifier. The effect of whey protein concentration (5 %-12.5 %) on the physicochemical stability of flaxseed oil-in-water emulsion was studied by Goyal et al. [25]. In most samples, the peroxide value was increased after emulsification. It has been reported that eugenol served as an antioxidant in nanoemulsions stabilized by Purity Gum® Ultra [5]. Viuda-Martos et al. [26] considered the antioxidant activity of two types of bagasse (from aril and peel and only from aril) as byproducts obtained from pomegranate juice extraction. The bagasse composed of aril and peel had a higher antioxidant activity than that obtained from aril. Gull et al. [27] reported the successful application of nanoemulsion coatings containing pomegranate peel extract (PPE) on extending the shelf-life of apricot during storage.

The aim of present study was to compare the effect of HPH and US, as fabrication methods, as well as CEO and PPE, as natural antioxidants, on the characteristics of flaxseed oil-in-water nanoemulsions (FNEs). Tween 80 was used as emulsifier. Our focus was given to the physicochemical stability of nanoemulsions as a function of storage time and temperature. The emulsification conditions were selected in a manner to obtain relatively similar average droplet size (Z-average) by both methods in order to remove the effect of initial droplet size on physicochemical stability of nanoemulsions over time. The results of this work may assist in developing food-grade nanoemulsions for application in functional foods and beverages.

2. Materials and methods

2.1. Materials

Fresh flaxseed oil, extracted by the cold press method, was purchased from a local market (Shiraz, Iran) and stored in refrigerator in dark vials. During cold storage, the impurities were precipitated and the clear upper phase was used in nanoemulsion preparation. Tween 80, ethanol, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), malondialdehyde, methanol, hexane, TBHQ, and acetyl chloride were purchased from Merck Co. (Darmstadt, Germany). DPPH free radical was obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid, and sodium hydroxide were supplied from Kimia Exir (Tehran, Iran). Double distilled water (DDW) was utilized in the preparation of samples and solutions.

2.2. Preparation of natural antioxidants

Dried clove buds were obtained from a local market (Shiraz, Iran) and its essential oil (CEO) was extracted by hydrodistillation using a Clevenger apparatus. The pomegranate (*Punica grantum* var. Rabab) peel was separated from the arils and dried. The dried peel was ground and mixed with distilled water at ratio of 1:5. The mixture was stirred continuously at fixed temperature of 37 °C during 48 h. Then, the mixture was centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant (PPE) was filtered through Whatman No.41 filter paper and then stored at 4 °C in dark vials [26].

2.2.1. DPPH free radical-scavenging activity

Various concentrations of CEO (0–1000 ppm) and PPE (0–2500 ppm) were prepared in methanol containing 0.1 mM DPPH concentration. After mixing and incubation for 30 min in the dark, the absorbance of samples was recorded against methanol at 517 nm. The absorbance of control methanolic solution of DPPH (0.1 mM) at 517 nm was 0.8. The decrease in the absorbance (from purple to yellow color) was attributed to the free radical-scavenging activity of natural antioxidants. The scavenging activity was calculated using Eq. (1) [17].

$$Scavenging\ effect(\%) = \left(\frac{A_c - A_s}{A_c}\right) \times 100 \tag{1}$$

where, A_c and A_s are the absorbance of control and test samples, respectively. Finally, the IC_{50} (the concentration that resulted in 50 % reduction) was calculated from the constructed curve.

2.3. Preparation of flaxseed oil nanoemulsions

Tween 80 (5 % w/w) was dissolved in distilled water and stirred for 15 min. To fabricate the coarse emulsion, the oil phase (5 % w/w) was added to the aqueous phase (95 % w/w) and homogenized using a high-speed mixer (Ultra-Turrax T18 IKA, Germany) at 13000 rpm for 5 min at room temperature. Prior to emulsification, CEO and PPE were separately added to the oil and aqueous phases at 10 % w/w concentration of each phase, respectively. Nanoemulsions were then produced from the coarse emulsions by either high-pressure homogenization (FBF Italia, S.R.L. Parma-Italy operated at 600 Bar for 6 cycles) or ultrasonication (probe type TT13, Horn sonicator, Bandelin, Germany) working at the highest nominal power (150 W) for 10 min. The amplitude and on/off cycle of US were 80 % and 0.7, respectively. The rise of temperature during
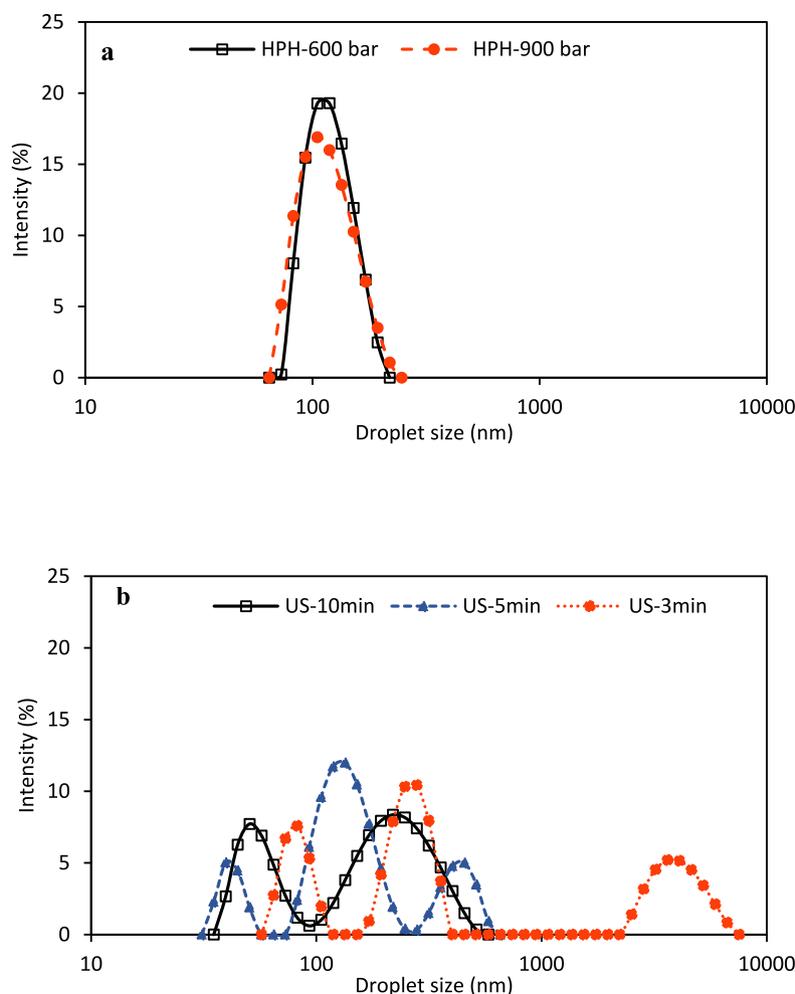
Fig. 1. Droplet size distribution of flaxseed oil nanoemulsions under the effect of homogenization pressure (a) and ultrasonication time (b); The US power and number of HPH cycle were kept constant at 150 W and 6, respectively.

sonication was controlled using an ice bath. The sonication conditions (e.g., probe type (or emitter surface area), the location of probe (in the center of the sample in a fixed depth of 1 cm), coarse emulsion volume and container type) remained constant to make the actual output power, acoustic intensity, and acoustic density delivered to each sample more uniform. Moreover, the maximum nominal power was utilized to increase the micro-agitation. According to Guimarães et al. [28] and Polachini et al. [29], the yield of power conversion is higher for the highest power level.

To reach relatively similar Z-averages in final nanoemulsions by both high-energy methods, a series of preliminary experiments was performed (Section 3.1). The required conditions for the preparation of nanoemulsions by US were firstly found. After that, the various conditions (i.e., applied pressure and number of cycles) of HPH were tested and then selected in a manner to reach relatively similar Z-average with that of US. Nanoemulsion samples were stored at two different temperatures (4 and 25 °C) in screw cap test tubes and dark conditions. The physical and chemical analyses were carried out in defined intervals. For chemical stability tests, a series of samples was prepared in the absence of natural antioxidants.

2.4. Physical properties

2.4.1. Droplet size distribution and zeta potential

The intensity-weighted mean diameter, polydispersity index (PI) and zeta-potential of nanoemulsions were evaluated by dynamic light scattering technique (DLS, SZ-100, Horiba, Japan) at 25 °C. The samples were diluted 20 times with DDW prior to the measurement. The pH of samples remained unchanged after dilution.

2.4.2. Absorbance

The absorbance was measured using a UV–vis spectrophotometer (UV9200, Rayleigh, China) at 600 nm against DDW. Prior to the measurement, the samples were diluted 20 times.

2.4.3. Creaming stability and Ostwald ripening rate

Nanoemulsions were centrifuged at 4724 *g* for 30 min at 25 °C (SW14R Froilabo, Lyon, France) and creaming stability was calculated using Eq. (2)

$$\% CreamingIndex = \left(\frac{H_c}{H_i}\right) \times 100 \qquad (2)$$

where, H_c is the height of the cream layer (mL) after centrifugation, and H_i is the initial height (mL) of nanoemulsions.

Table 1

Changes in the physical properties of flaxseed oil nanoemulsions containing two different natural antioxidants including clove essential oil (CEO) and pomegranate peel extract (PPE) prepared by either ultrasonication (US) or high-pressure homogenization (HPH) during storage at 4 and/or 25 °C; Nano-emulsions containing CEO were evaluated during 60 d, while, those containing PPE were evaluated during 28 d. Different uppercase letters indicate significant differences (P < 0.05) over storage time (in a same row). Different lowercase letters indicate significant differences (P < 0.05) between different samples at a same time of storage (in a same column).

	Sample	Time (day) 1	14	28	60
Z-average (nm)	HPH-CEO-4 °C	115.4 ± 0.25^{Dh}	146.6 ± 4.84^{Cf}	151.9 ± 5.58^{Be}	156.8 ± 1.07^{Ad}
	HPH-CEO-25 °C	115.7 ± 0.20^{Dg}	138.8 ± 0.20^{Cg}	150.2 ± 7.90^{Bf}	157.9 ± 0.61^{Ac}
	US-CEO-4 °C	148.6 ± 4.90^{Cb}	147.2 ± 11.76^{De}	208.0 ± 22.56^{Aa}	204.2 ± 0.68^{Ba}
	US-CEO-25 °C	151.7 ± 0.89^{Da}	186.9 ± 19.94^{Aa}	155.0 ± 25.53^{Cd}	158.2 ± 1.6^{Bb}
	HPH-PPE-4 °C	124.6 ± 1.97^{Cf}	149.2 ± 0.92^{Ad}	137.6 ± 1.97^{Bg}	
	HPH-PPE-25 °C	125.4 ± 1.98^{Ce}	158.2 ± 0.50^{Ab}	133.6 ± 2.65^{Bh}	
	US-PPE-4 °C	130.6 ± 3.45^{Cd}	150.9 ± 5.16^{Bc}	174.4 ± 17.61^{Ab}	
	US-PPE-25 °C	132.4 ± 2.26^{Cc}	134.8 ± 2.50^{Bh}	162.3 ± 5.86^{Ac}	
Polydispersity index	HPH-CEO-4 °C	0.35 ± 0.01^{Ad}	0.33 ± 0.04^{Bd}	0.36 ± 0.04^{Ab}	0.32 ± 0.01^{Bb}
	HPH-CEO-25 °C	0.36 ± 0.02^{Acd}	0.35 ± 0.01^{Ac}	0.33 ± 0.03^{Bc}	0.30 ± 0.03^{Cb}
	US-CEO-4 °C	0.38 ± 0.03^{Bab}	0.45 ± 0.03^{Aa}	0.33 ± 0.08^{Cc}	0.38 ± 0.00^{Ba}
	US-CEO-25 °C	0.37 ± 0.01^{Abc}	0.32 ± 0.05^{Cd}	0.35 ± 0.06^{Bb}	0.33 ± 0.03^{Cb}
	HPH-PPE-4 °C	0.26 ± 0.03^{Be}	0.36 ± 0.05^{Ac}	0.26 ± 0.01^{Bd}	
	HPH-PPE-25 °C	0.26 ± 0.03^{Be}	0.38 ± 0.01^{Ab}	0.24 ± 0.00^{Be}	
	US-PPE-4 °C	0.39 ± 0.06^{Aa}	0.33 ± 0.05^{Cd}	0.36 ± 0.11^{Bb}	
	US-PPE-25 °C	0.39 ± 0.06^{Ba}	0.39 ± 0.04^{Bb}	0.48 ± 0.03^{Aa}	
Zeta potential (mV)	HPH-CEO-4 °C	-42.2 ± 2.78^{Ba}	-47.3 ± 0.36^{Aa}	-39.4 ± 1.57^{Ca}	-39.2 ± 0.90^{Da}
	HPH-CEO-25 °C	-41.7 ± 1.12^{Ab}	-41.6 ± 1.34^{Ab}	-37.2 ± 3.20^{Bb}	-27.0 ± 4.66^{Cd}
	US-CEO-4 °C	-38.9 ± 1.16^{Ad}	-37.1 ± 1.94^{Bd}	-35.6 ± 1.30^{Cd}	-34.4 ± 2.00^{Dc}
	US-CEO-25 °C	-41.2 ± 2.05^{Ac}	-40.7 ± 0.83^{Bc}	-35.8 ± 1.00^{Cc}	-36.4 ± 2.70^{Db}
	HPH-PPE-4 °C	-26.7 ± 3.04^{Ag}	-23.8 ± 1.76^{Bg}	-26.6 ± 3.20^{Ag}	
	HPH-PPE-25 °C	-23.5 ± 1.41^{Bh}	-22.4 ± 1.56^{Ch}	-24.1 ± 3.12^{Ah}	
	US-PPE-4 °C	-27.5 ± 4.90^{Be}	-27.6 ± 4.40^{Be}	-28.5 ± 0.57^{Ae}	
	US-PPE-25 °C	-27.0 ± 0.57^{Bf}	-27.2 ± 2.05^{Bf}	-27.6 ± 0.91^{Af}	
Absorbance (1/cm)	HPH-CEO-4 °C	0.814 ± 0.01^{Ae}	0.804 ± 0.01^{Bc}	0.719 ± 0.00^{Ce}	0.564 ± 0.02^{Dd}
	HPH-CEO-25 °C	0.788 ± 0.01^{Ah}	0.701 ± 0.03^{Ce}	0.784 ± 0.01^{Bd}	0.651 ± 0.03^{Dc}

Table 1 (*continued*)

Sample	Time (day) 1	14	28	60
US-CEO-4 °C	0.888 ± 0.01^{Bb}	0.595 ± 0.02^{Ch}	0.518 ± 0.03^{Dh}	0.942 ± 0.03^{Ab}
US-CEO-25 °C	0.895 ± 0.01^{Ba}	0.615 ± 0.01^{Dg}	0.638 ± 0.02^{Cg}	0.975 ± 0.02^{Aa}
HPH-PPE-4 °C	0.861 ± 0.01^{Ad}	0.857 ± 0.02^{Bb}	0.842 ± 0.01^{Cc}	
HPH-PPE-25 °C	0.868 ± 0.00^{Bc}	0.866 ± 0.00^{Ba}	0.960 ± 0.02^{Ab}	
US-PPE-4 °C	0.811 ± 0.01^{Af}	0.790 ± 0.02^{Bd}	0.701 ± 0.01^{Cf}	
US-PPE-25 °C	0.796 ± 0.01^{Bg}	0.666 ± 0.01^{Cf}	1.137 ± 0.02^{Aa}	

The slope of the cube of droplet radius (r^3) versus storage time (t) was considered as the rate of Ostwald ripening (ω) [30].

2.4.4. Viscosity

A capillary viscometer (No. 518 10, Schott-Gerate GmbH, 0.43 mm inner capillary diameter, Mainz, Germany) was utilized to measure the flow time (t) of nanoemulsions under temperature control in a water bath (25 ± 0.1 °C). Then, the kinematic viscosity (ν mm^2/s) and dynamic viscosity (μ mPa.s) were calculated using Eq. (3) and Eq. (4), respectively [31].

$$\nu = Ct \tag{3}$$

$$\mu = \rho \times \nu \tag{4}$$

where, C is the constant (0.01 mm^2/s^2) and ρ is the density (kg/m^3) determined by pycnometer.

2.5. Chemical stability

2.5.1. Thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances (TBARS) were determined as a sign for the lipid oxidation secondary products according to the method described in our previous studies [31,32].

2.5.2. Fatty acids profile

Nanoemulsions (1 mL) were lyophilized. The changes in the profile of fatty acids over time were determined using gas chromatography coupled to flame ionization detector according to the method described in our previous studies [31].

2.6. Statistical analysis

The results were expressed as mean ± standard deviation of independent replicates. Statistical analysis of variance (ANOVA) was carried out using SPSS software (ver. 22, IBM, New York). Means were subjected to Duncan's multiple range tests at significance level of 5 %.

3. Results and discussion

3.1. Preliminary studies on the effect of homogenization pressure and sonication time on nanoemulsion formation

Long-term stability is an important factor for nanoemulsions as delivery system [33]. The final droplet size of nanoemulsions is dependent on the applied shear stress and emulsion rheology [34]. Nanodroplets are obtained when the shear stress is much greater than the Laplace pressure. In HPH and US, the pressure contrast and acoustic cavitation

are the main causes for the formation of tiny nanodroplets from microdroplets, respectively. A series of preliminary experiments was performed to find appropriate nanoemulsification conditions. At a constant sonication power (150 W), the average size of samples sonicated for 3, 5, and 10 min was 646.2, 346.3, and 148.6 nm, respectively. Moreover, at fixed 6 homogenization cycles, the rise of homogenization pressure from 600 to 900 bar shifted the average size of nanodroplets from 114.4 to 110.9 nm (Fig. 1). To remove the effect of initial droplet size on the characteristics of nanoemulsions over time, the following conditions were selected for the preparation of nanoemulsions by HPH and US: 600 bar for 6 cycles and 150 W for 10 min, respectively. Nejadmansouri et al. [23] similarly reported a decrease in average droplet size by increasing the US time. Moreover, Peng et al. [35] reported a droplet size reduction by increasing homogenization pressure from 400 to 1300 bar at fixed recirculation.

3.2. Effect of HPH and US on physical characteristics

Table 1 reports the changes in Z-average, polydispersity index (PI), zeta-potential, and absorbance of FNEs prepared by HPH and US during storage at two different temperatures (4 and 25 °C). The average droplet size of FNEs prepared by both methods increased during storage. However, this increment was higher in the samples prepared by US which could be attributed to the lower uniformity in the samples prepared by US. Fig. 2 illustrates the changes in the droplet size distribution of FNEs prepared by HPH and US as a function of storage time and temperature. Regardless of the added antioxidant, FNEs prepared by HPH showed monomodal size distribution, while, those fabricated by US presented bimodal distribution. A larger difference in the size of dispersed droplets could lead to a higher variation in the surface energy of (nano)droplets and thus result in a relatively higher instability over time. The stability of nanoemulsions depends not only on the average droplet size but also on the distribution width (e.g., PI and Span) [36]. PI values below 0.2 represent very high uniformity, while, values close to 1 indicate a heterogeneous structure [37,38]. The PI values of nanoemulsions prepared in this study ranged from 0.24 to 0.48. The relative increase in the average droplet size of FNEs stored at 4 °C was generally higher than 25 °C which was related to a higher Ostwald ripening rate at lower temperatures. The increase in the average size of FNEs containing PPE was less than that of nanoemulsions incorporated with CEO.

Zeta-potential is an indicator to predict the physical stability of colloidal systems [39]. The absolute values higher than 25 mV correspond to appropriate physical stability against destabilization processes such as flocculation and coalescence mainly due to the strong electrostatic repulsion among the droplets. Taking into account the non-ionic character of Tween 80 [40], the negative charge at the interface might be attributed to the anionic impurities (e.g., free fatty acids in the surfactant or those formed from the hydrolysis of triacylglycerols or surfactants molecules) [31,41,42]. The formation of hydrogen bonds between hydroxyl ions (OH⁻) in the continuous phase and ether-oxygen groups in the surfactant molecules could also contribute to the negative charge. Moreover, the natural phospholipids present in unrefined flaxseed oil were likely responsible for the anionic character of interface [43]. In this work, the zeta-potential values of FNEs were mainly influenced by the added antioxidant and not by the homogenization method. Lower absolute values were measured in the samples containing PPE in the aqueous phase. This reduction was attributed to the possible interactions between PPE constituents (like polyphenols) and the polar head of surfactant molecules. A decrease in the zeta-potential over time was observed in the samples containing CEO, while, FNEs containing PPE showed relatively constant values during storage. The relative decrease in the absolute values of zeta potential over time might be ascribed to the increase in the polarity of degradation products and thus diffusion into the aqueous phase [44].

The absorbance of nanoemulsions is the result of droplet concentration and droplet size. Generally, a decrease in the absorbance, with a

higher rate in the samples prepared by US than those prepared by HPH, was observed over time. Lower storage temperature led to a higher reduction likely due to an increase in the Ostwald ripening rate and thus a decrease in the droplet concentration.

3.3. Physical stability

Fig. S1 (supplementary data) shows the visual appearance of nanoemulsions prepared by HPH and US. As can be seen, the gravitational stability of nanoemulsions decreased during storage. The centrifugal stability of FNEs, reported as creaming index (CI), is shown in Fig. 3. Lower CI values indicate a higher centrifugal stability. Generally, the CI values of FNEs increased during storage indicating a lower physical stability. The CI values of FNEs containing CEO ranged between 3 and 20 % during 60-d storage at both temperatures (Fig. 3a). At the end of storage, higher instability (CI value) was observed in the samples prepared by US and kept at higher temperature. This observation was attributed to the lower uniformity in the droplet size distribution of FNEs prepared by US as well as the lower viscosity of continuous phase at higher temperature. The CI values of FNEs containing PPE remained relatively constant (~5%) for 7 d and then increased until the end of storage (28 d). A lower stability was similarly observed in PPE-incorporated samples prepared by US and then stored at higher temperature. Nanoemulsions are often more susceptible to Ostwald ripening than conventional emulsions. In O/W nanoemulsions, Ostwald ripening occurs due to the dissolution and then diffusion of the oil phase from small droplets to larger ones. Therefore, this phenomenon mostly occurs in nanoemulsions prepared from organic phases with higher water solubility (e.g., flavor oils). Previous studies have evidenced that Ostwald ripening can be retarded or inhibited by incorporating highly hydrophobic components such as long chain triacylglycerols (LCT; e.g., flaxseed oil) into the essential oil phase prior to homogenization, mainly via generating the mixing entropy to counterbalance the interfacial curvature effect [45,46]. Ostwald ripening rate can be estimated from the slope of the droplet radius cube (r^3) vs storage time (t). As seen in Fig. 4, FNEs prepared by US and then stored at lower temperature (4 °C) had a higher slope than other samples confirming a higher rate of Ostwald ripening. According to LSW equation, Ostwald ripening rate is inversely proportional with temperature. Therefore, lower storage temperature increased the ripening rate. Moreover, multimodal size distribution in the samples fabricated by US could accelerate the rate of Ostwald ripening likely due to the higher variations in the free energy of dispersed droplets. Taking into account the higher physical instability of FNEs stored at 25 °C (Fig. 3), it can be concluded that Ostwald ripening mostly happens in the initial stages of storage and other destabilizing mechanisms such as flocculation and coalescence had a higher involvement on the final stability of nanoemulsions specially at the end of storage. Similar results were reported by Nejadmansouri et al. and Zahi et al. [23,47].

3.4. Viscosity

Several factors including composition, droplet size, colloidal interactions, and droplet charge can influence the emulsions' viscosity [48,49]. Fig. 5 reports the changes in dynamic viscosity of nanoemulsions during time. Generally, the low viscosity values of FNEs were attributed to the low amount of dispersed phase volume fraction (φ = 5 %), and the absence of thickening agents in the aqueous phase [50]. The viscosity of nanoemulsions fabricated by US was slightly higher than that prepared by HPH, which could be related to the variations in droplet size distribution. Due to the increase in specific surface area, the viscosity of nanoemulsions is generally higher than that of conventional emulsions prepared at a same φ [51,52]. Physical instability mechanisms such as flocculation can also increase the viscosity of emulsions mainly via entrapping continuous phase within flocculated droplets [6]. As a result, the viscosity is considered as a function of droplet size and
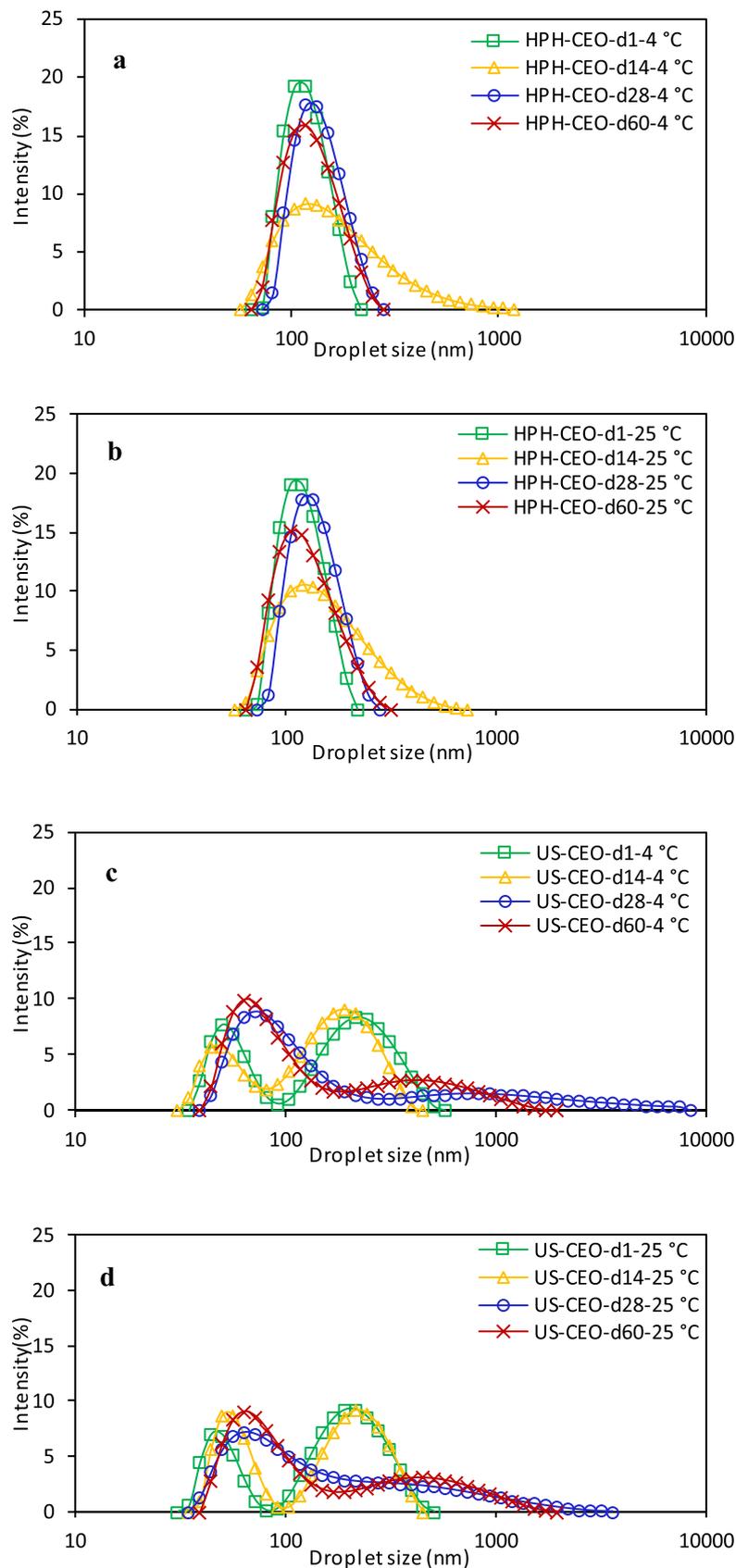
Fig. 2. Changes in the droplet size distribution of flaxseed oil nanoemulsions containing two different natural antioxidants (clove essential oil (CEO, a-d) and pomegranate peel extract (PPE, e-h)) prepared by either ultrasonication (US) or high-pressure homogenization (HPH) during storage at 4 and/or 25 °C; Nano-emulsions containing CEO were evaluated during 60 d, while, those containing PPE were evaluated during 28 d.
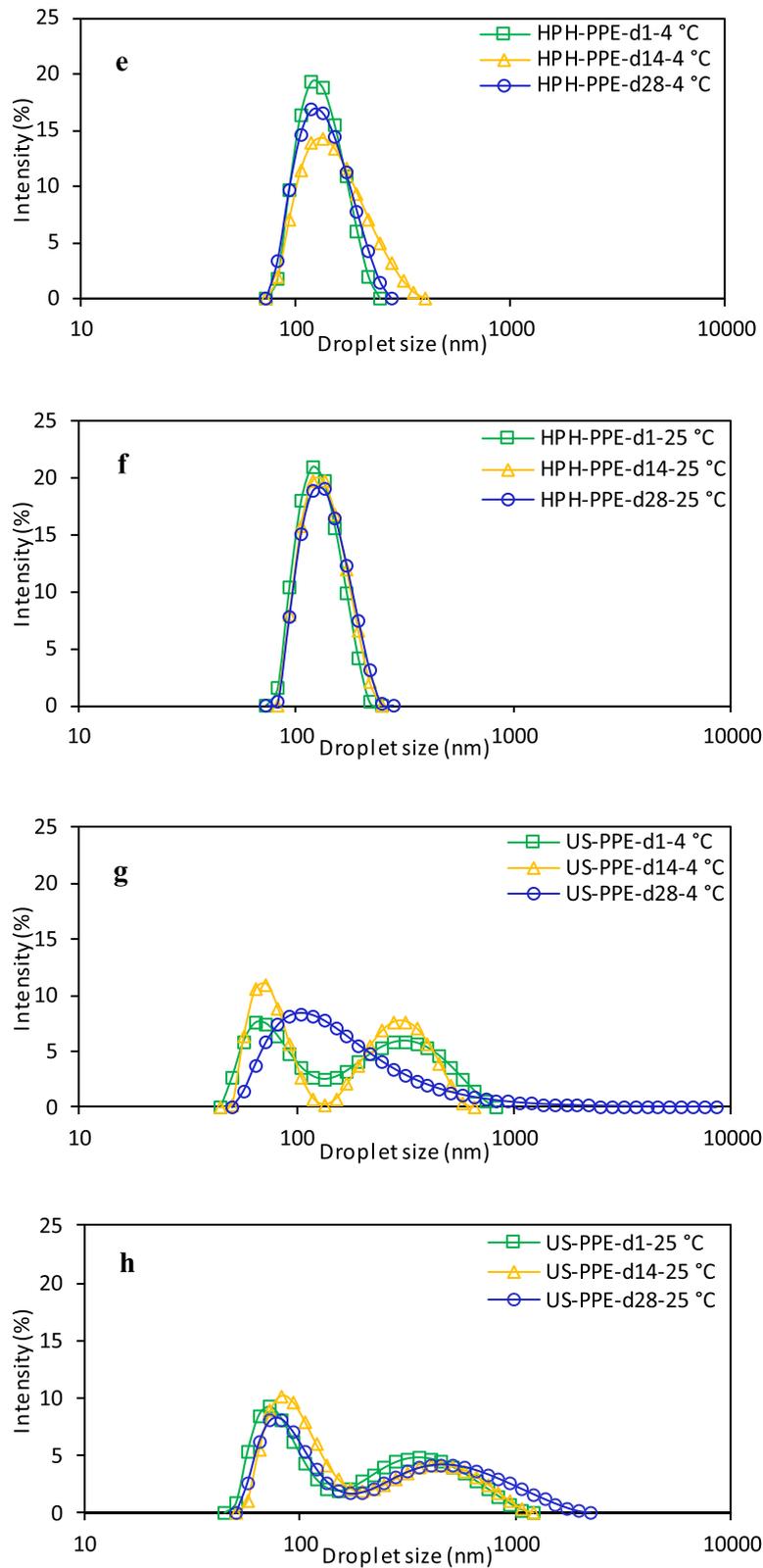
Fig. 2. (*continued*).
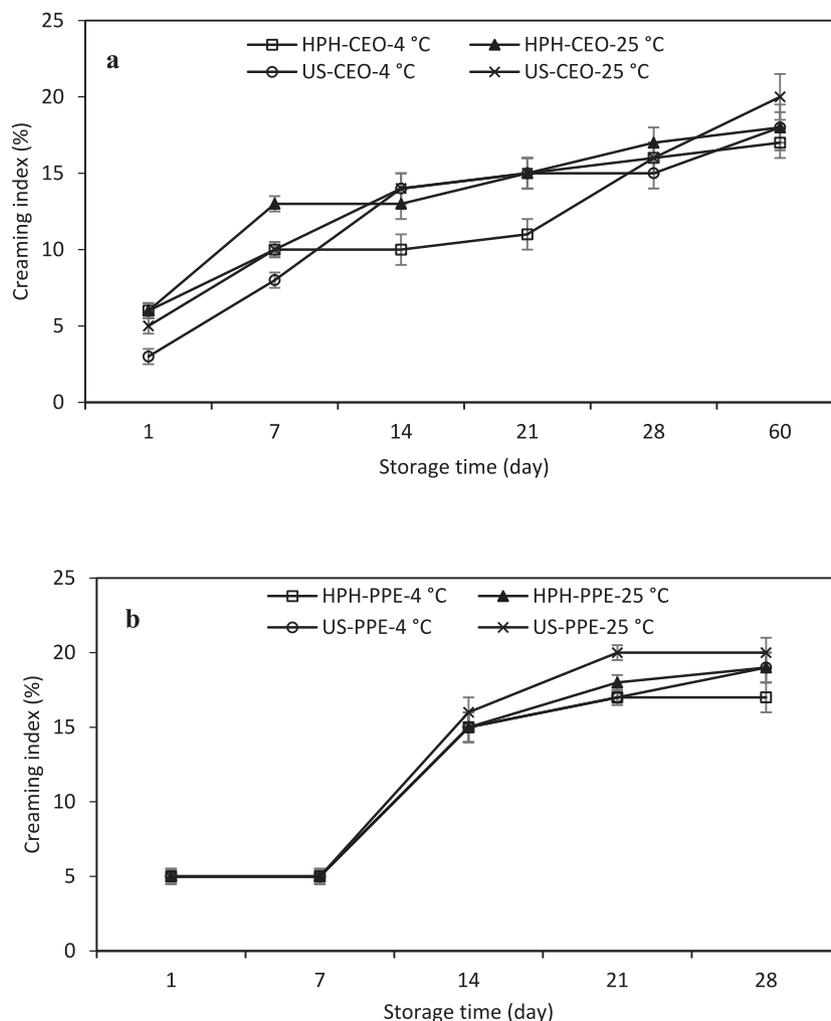
Fig. 3. Creaming index of flaxseed oil nanoemulsions containing two different natural antioxidants, namely clove essential oil (CEO, a) and pomegranate peel extract (PPE, b), prepared by either ultrasonication (US) or high-pressure homogenization (HPH) during storage at 4 and/or 25 °C. Nanoemulsions containing CEO were evaluated during 60 d, while, those containing PPE were evaluated during 28 d.

the number of flocculated particles. Changes in the viscosity of samples stored at 25 °C were slightly higher than those stored at 4 °C. However, these changes were negligible during storage. The viscosity of FNEs was not significantly affected by the type of added antioxidant.

3.5. Oxidative stability

3.5.1. DPPH free radical scavenging of natural antioxidants

In this study, the ability of natural antioxidants to scavenge DPPH free radicals was evaluated in terms of IC_{50} value (known as the concentration of test material for decreasing the absorbance of DPPH solution at 517 nm to half of its primary value). This value was extrapolated from the plot of percent inhibition vs concentration [17,53]. The IC_{50} values of CEO and PPE were around 50 and 91 ppm, respectively. Lower values indicate stronger antioxidant activity. Mahdi et al. [54] reported that the IC_{50} value of clove oil nanocapsules was 0.36 mg nanocapsules mL^{-1} (360 ppm). The IC_{50} value of CEO reported by Charfi et al. [55] was 96 ppm. Kanatt et al. [56] reported an IC_{50} value of 4.9 ppm for PPE. The variations in the results of this study and those reported by other researchers might be attributed to the initial composition of natural antioxidants resulting from botanical factors and growth conditions, as well as the extraction procedure (i.e., plant to water initial ratio, post-concentration, etc.).

3.5.2. Secondary oxidation products

Flaxseed oil contains high percentage of ALA (>50 %) as a fatty acid highly prone to lipid oxidation. Oxidative degradation of lipids leads to the formation of unpleasant off-flavors and detrimental compounds such as free radicals and reactive aldehydes. To provide a deeper insight into the effects of emulsification technique, antioxidant type, and storage temperature, some control samples including bulk (non-emulsified) flaxseed oil and antioxidant-free FNEs were similarly stored at two different temperatures. The results of TBARS formation (as an indicator of secondary oxidation products) are illustrated in Fig. 6. In bulk (non-emulsified) oils, oxidation greatly occurs at the surrounding air / oil interface, as well as dissolved air microbubbles / oil interface [13]. The oxidation of FNEs was generally higher than that of bulk flaxseed oil mainly due to the larger interfacial area between the dispersed nano-droplets and prooxidants present in the continuous phase [57]. It has also been reported that hydroperoxides are generated from the poly-ether head groups of Tweens after exposure to water-soluble prooxidants, which can accelerate the oxidation of emulsified PUFAs [58]. The parallel or perpendicular arrangement of fatty acids within the droplets at the interface affects the oxidation rate [59]. Except for the FNEs containing PPE, higher storage temperature reduced the oxidative stability of bulk flaxseed oil, antioxidant-free FNEs and those containing CEO. This observation could be attributed to the enhancing effect of temperature on the oxidative reactions of triacyclglycerols [60]. In
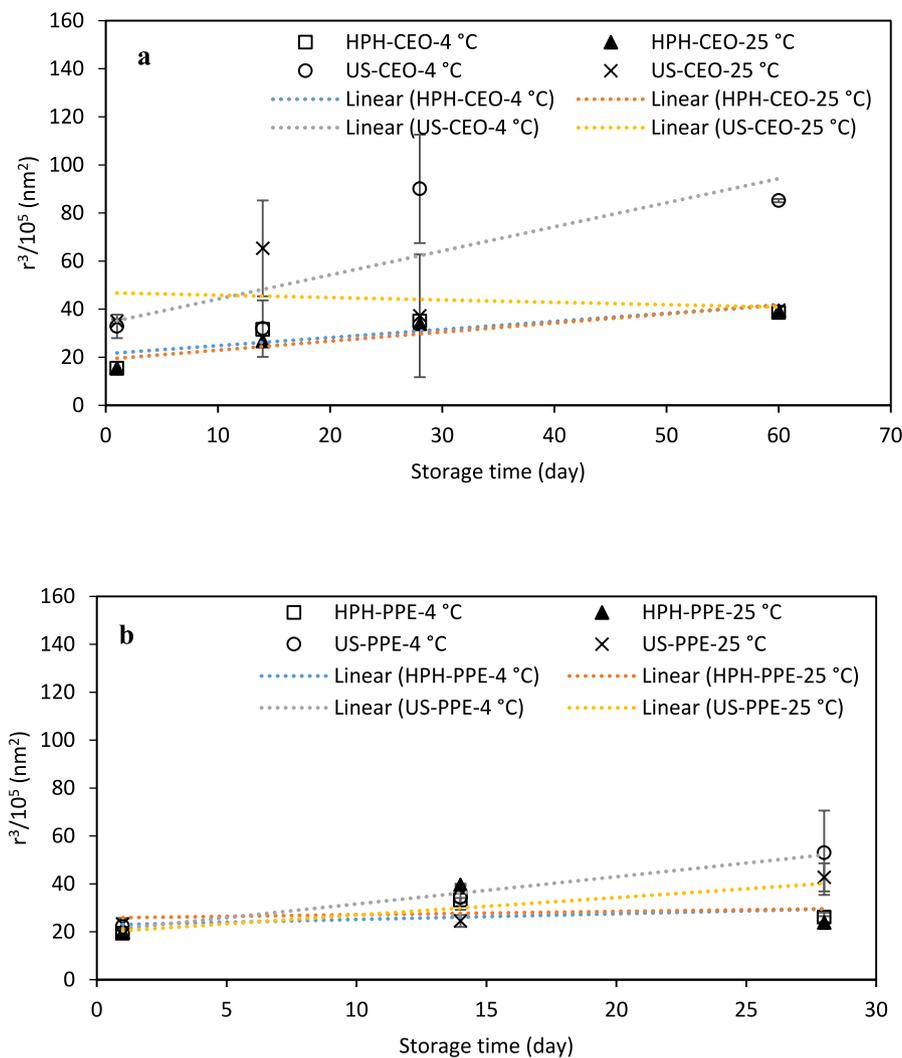
Fig. 4. Droplet radius cube (r^3) vs storage time (t) of flaxseed oil nanoemulsions containing two different natural antioxidants, namely clove essential oil (CEO, a) and pomegranate peel extract (PPE, b), prepared by either ultrasonication (US) or high-pressure homogenization (HPH) during storage at 4 and/or 25 °C. Nano-emulsions containing CEO were evaluated during 60 d, while, those containing PPE were evaluated during 28 d.

nanoemulsion samples containing PPE, a slightly higher oxidative stability was observed at the higher temperature which could be ascribed to the effect of temperature on the antioxidant characteristics of PPE constituents (e.g., a higher concentration of PPE constituents at the interface at higher temperature). Until day 21, antioxidant-free FNEs fabricated by HPH revealed a higher instability than those prepared by US. However, from day 28 and at the end of storage, the highest instability was measured in the antioxidant-free samples prepared by US and then kept at 25 °C. The reason for the lower initial stability of FNEs fabricated by HPH is not clear at this time. However, a higher concentration of oxygen in these samples (due to the nature of fabrication method) and applied high pressure could possibly accelerate the oxidative reactions. The lower final chemical stability of nanoemulsions prepared by US could likely be attributed to the sonochemical reactions resulting from intense local heating and high pressure during sonication [34]. The heat generation in the samples by the local heating effect of ultrasound and the high-intensity acoustic field in cavitation process are likely the key factors for the higher oxidative degradation of nanoemulsions prepared by US. During the cavitation process, rapid formation and collapse of micro-bubbles happens at the interface that accelerates the formation of shock waves and turbulence [61] which reduces the oxidative stability of resultant nanoemulsions as compared to those fabricated by HPH.

The presence of both antioxidants, either in the aqueous phase or in the lipid phase, could significantly retard the oxidation of FNEs. Taking into account the initial amount of each antioxidant (10 % of respective phase) as well as the IC_{50} values of CEO and PPE, it can be concluded that CEO was significantly more effective than PPE in retarding the oxidation of FNEs. The higher efficacy of CEO is in agreement with polar paradox theory, which states that non-polar antioxidants are more effective than polar ones in O/W emulsions [13]. Regardless of fabrication method, the relatively low chemical stability of FNEs containing CEO at 25 °C could be related to the volatile nature of essential oil at higher temperature. An improvement in the physical and oxidative stability of FNEs stabilized by modified starches was reported in the presence of eugenol (the main component of CEO) [5].
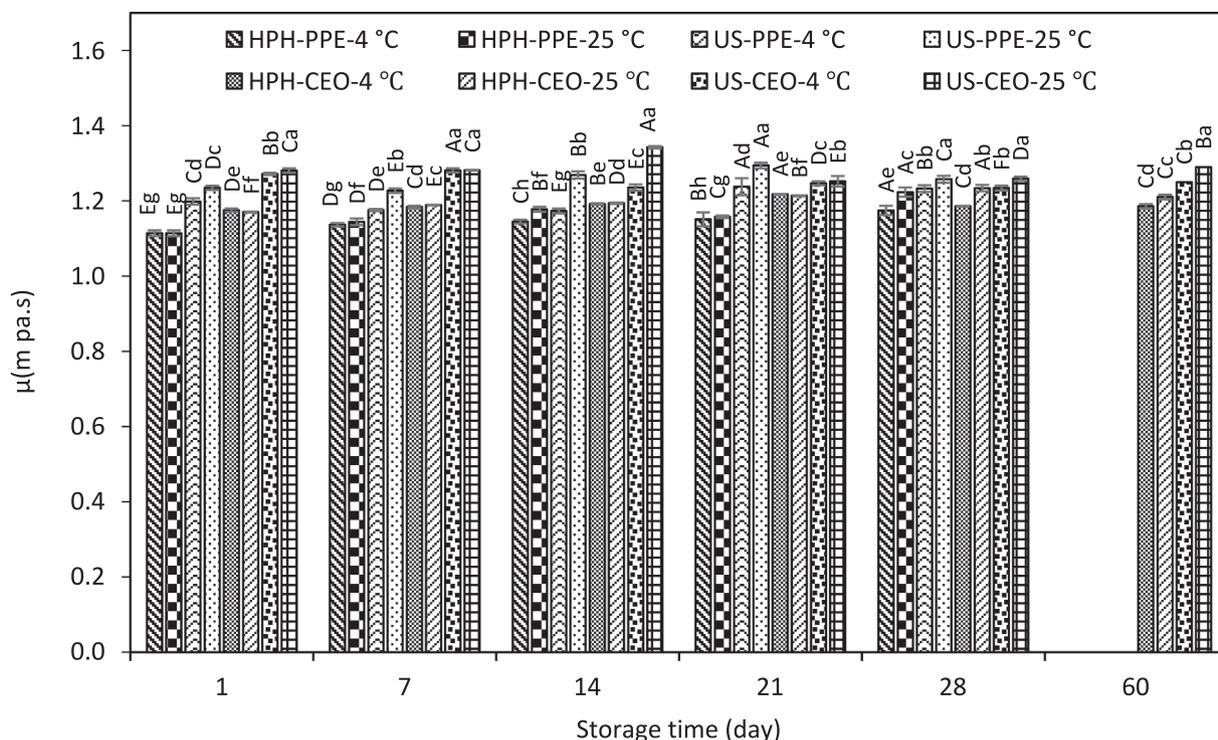
Fig. 5. Changes in the dynamic viscosity of flaxseed oil nanoemulsions containing two different natural antioxidants, namely clove essential oil (CEO) and pomegranate peel extract (PPE), prepared by either ultrasonication (US) or high-pressure homogenization (HPH) during storage at 4 and/or 25 °C. Nanoemulsions containing CEO were evaluated during 60 d, while, those containing PPE were evaluated during 28 d. Different uppercase letters indicate significant differences (P < 0.05) over storage time. Different lowercase letters indicate significant differences (P < 0.05) between different samples at a same time of storage.

3.5.3. Fatty acids profile

Studying the changes in the fatty acids profile (FAP) of nano-emulsions can provide valuable information regarding the oxidative stability. Table 2 reports the changes in the FAP of antioxidant-free FNEs and those containing CEO at different temperatures and storage times. Flaxseed oil was mainly composed of α-linolenic acid (54.1 %), followed by oleic (17.3 %) and linoleic (16.0 %) acids. Similar results were reported by Goyal et al. [62]. It is worth mentioning that in GC-FID analysis, the relative amount (%) of each fatty acid is reported as the relative peak area (The area under the peak of each fatty acid / Total peak area] × 100); therefore, the degradation of highly sensitive PUFAs resulted in an increase in the relative amount of other fatty acids. As expected, PUFAs were significantly more sensitive to oxidation than saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) due to a higher degree of unsaturation. The increase of free fatty acids in emulsions as a result of lipolysis enhances the lipid oxidation either via increasing the negative charge of interface and thus enhancing the prooxidant (e.g., transition metal cations)-lipid interactions [57] or through the higher oxidative instability of free fatty acids than esterified counterparts. Zeinalzadegan et al. [31] reported an increase in the amount of free fatty acids of flaxseed oil nanoemulsions during storage. Similar to the results of TBARS (section 3.5.2), the absence of antioxidant, higher storage temperature, and emulsification by US resulted in a higher degradation of PUFAs. At the end of storage, the highest reduction in the ratio of ω3/ω6 (as a nutritional index) was measured in the antioxidant-free FNEs fabricated by US and stored at 25 °C. The ratio of ΣPUFA/ΣSFA (known as polyene index) should be>0.45 to enhance healthy blood cholesterol levels [63]. Despite its reduction over time, this index was above its minimum level until the end of storage. Thus, FNEs can be considered as a good candidate for developing functional foods and beverages.

4. Conclusion

In this work, the effects of fabrication method (US vs HPH), antioxidant type (CEO vs PPE), and storage temperature (4 °C vs 25 °C) were studied on the physicochemical stability of Tween 80-stabilized FNEs over time. Totally, US led to bimodal size distribution of nanodroplets, while, HPH resulted in monomodal distribution and thus better physical stability (lower creaming index) over time. The rate of Ostwald ripening was influenced by the fabrication method (US > HPH) and storage temperature (4 °C > 25 °C). Higher storage temperature resulted in higher creaming indices over time. Changes in the viscosity were negligible during storage. Overall, the absence of antioxidant, higher storage temperature, and emulsification by US resulted in a higher oxidative instability. CEO was considerably more effective than PPE in retarding the oxidation of FNEs. The results showed that FNEs can be utilized as an ingredient in the formulation of functional foods and beverages. However, in future works, it would be instructive to replace the synthetic surfactant with a biosurfactant, since many consumers are demanding products with "clean" labels.

CRediT authorship contribution statement

Seyede Farnaz Sadeghian: Formal analysis, Investigation, Writing – original draft. **Marjan Majdinasab:** Validation. **Maryam Nejadman-souri:** Validation, Writing – original draft. **Seyed Mohammad Hashem Hosseini:** Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing.

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.
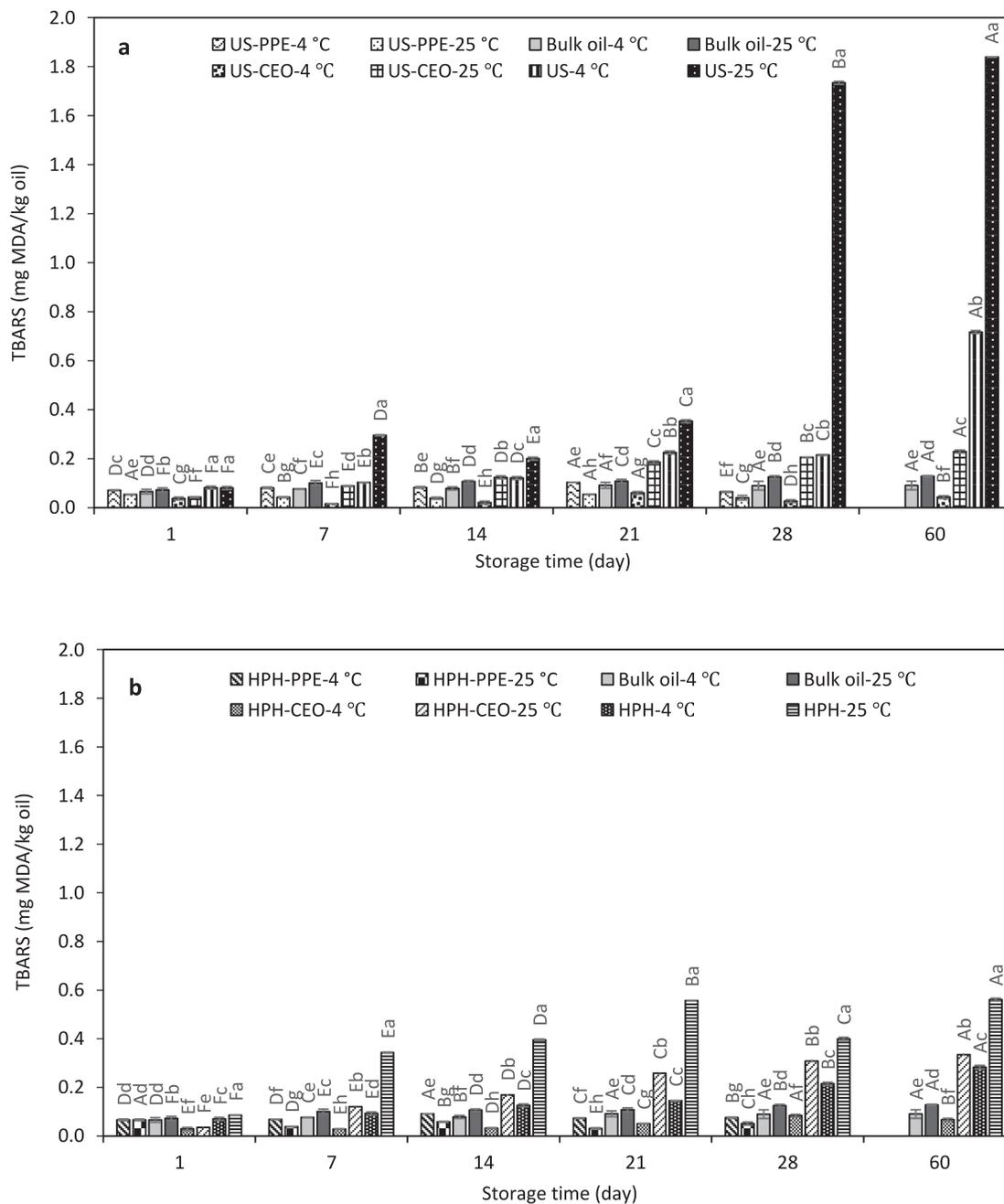
Fig. 6. Changes in the secondary oxidation products (TBARS) of bulk flaxseed oil and respective nanoemulsions containing two different natural antioxidants, namely clove essential oil (CEO) and pomegranate peel extract (PPE), prepared by either ultrasonication (US) (a) or high-pressure homogenization (HPH) (b) during storage time at 4 and/or 25 °C. Different uppercase letters indicate significant differences (P < 0.05) over storage time. Different lowercase letters indicate significant differences (P < 0.05) between different samples at a same time of storage.

Table 2

Changes in the fatty acids profile of flaxseed oil nanoemulsions prepared by HPH and/or US in the presence and absence of clove essential oil (CEO) during 60-d storage at 4 and 25 °C; Different uppercase letters indicate significant differences (P < 0.05) over storage time (in a same row). Different lowercase letters indicate significant differences (P < 0.05) between different samples at a same time of storage (in a same column).

		Time (day)					
	S.	1	7	14	21	28	60
ΣSFA (%)	HPH-4°C	21.5 ± 0.1^{Bc}	21.4 ± 0.3^{BCf}	21.6 ± 0.2^{ABe}	20.7 ± 0.4^{Cc}	22.5 ± 0.4^{Ad}	22.0 ± 0.2^{ABe}
	HPH-CEO-4°C	21.9 ± 0.1^{ABc}	22.1 ± 0.3^{ABef}	22.1 ± 0.4^{ABe}	21.5 ± 0.7^{Bbc}	23.0 ± 0.4^{Ad}	22.8 ± 0.4^{Ade}
	HPH-25°C	22.1 ± 0.3^{CDc}	21.6 ± 0.2^{Def}	23.1 ± 0.1^{ABd}	22.7 ± 0.4^{BCb}	23.3 ± 0.4^{ABd}	24.0 ± 0.6^{Ad}
	HPH-CEO-25°C	21.8 ± 0.1^{Cc}	23.0 ± 0.3^{Ae}	22.0 ± 0.3^{Be}	21.6 ± 0.4^{Cbc}	21.7 ± 0.7^{Cd}	22.6 ± 0.4^{Bde}
	US-4°C	25.1 ± 0.3^{Da}	27.6 ± 0.6^{Cc}	30.3 ± 0.4^{Bb}	28.6 ± 0.8^{Ca}	33.1 ± 0.8^{Ab}	32.1 ± 0.1^{Ab}
	US-CEO-4°C	24.2 ± 0.6^{Bb}	25.2 ± 0.5^{Bd}	29.2 ± 0.1^{Ac}	29.9 ± 1.3^{Aa}	29.4 ± 0.6^{Ac}	30.3 ± 0.4^{Ac}
	US-25°C	25.3 ± 0.4^{Ca}	31.0 ± 1.4^{Ba}	32.1 ± 0.7^{Ba}	30.1 ± 0.5^{Ba}	37.0 ± 1.4^{Aa}	36.7 ± 1.0^{Aa}
	US-CEO-25°C	25.5 ± 0.6^{Ba}	29.1 ± 0.3^{Ab}	29.5 ± 0.5^{Abc}	28.6 ± 0.8^{Aa}	28.8 ± 1.1^{Ac}	29.7 ± 1.0^{Ac}
ΣMUFA (%)	HPH-4°C	18.6 ± 0.3^{Cab}	18.8 ± 0.3^{Ca}	18.9 ± 0.1^{Ca}	19.4 ± 0.2^{BCa}	19.9 ± 0.4^{Ba}	21.2 ± 0.5^{Ab}
	HPH-CEO-4°C	18.6 ± 0.2^{Cab}	19.1 ± 0.3^{BCa}	19.0 ± 0.4^{Ca}	18.8 ± 0.2^{Ca}	20.1 ± 0.4^{Aa}	20.0 ± 0.6^{ABbc}
	HPH-25°C	18.3 ± 0.3^{Babc}	19.1 ± 0.1^{ABa}	18.9 ± 0.3^{ABa}	18.5 ± 0.7^{Ba}	19.0 ± 0.4^{ABab}	19.6 ± 0.3^{Abcd}
	HPH-CEO-25°C	19.2 ± 0.4^{ABa}	17.7 ± 0.3^{Cb}	18.5 ± 0.7^{BCa}	19.3 ± 0.4^{ABa}	20.0 ± 0.4^{Aa}	20.2 ± 0.3^{Abcd}
	US-4°C	18.0 ± 0.3^{ABbc}	17.3 ± 0.2^{Bbc}	18.6 ± 0.4^{Aa}	18.1 ± 0.6^{ABa}	18.1 ± 0.3^{ABb}	19.0 ± 0.7^{Acd}
	US-CEO-4°C	17.5 ± 0.7^{ABcd}	16.5 ± 0.7^{ABc}	18.5 ± 0.6^{Aa}	16.1 ± 1.4^{Bb}	18.1 ± 0.3^{ABb}	18.0 ± 0.5^{ABd}
	US-25°C	18.4 ± 0.4^{Babc}	17.7 ± 0.7^{Bb}	18.9 ± 0.7^{Ba}	19.8 ± 1.0^{Ba}	19.0 ± 1.3^{Bab}	23.5 ± 1.4^{Aa}
	US-CEO-25°C	17.0 ± 0.1^{CDd}	16.6 ± 0.3^{Dc}	17.7 ± 1.0^{BCDa}	19.5 ± 0.7^{Aa}	18.4 ± 0.6^{ABCb}	18.8 ± 0.4^{ABcd}
ΣPUFA (%)	HPH-4°C	59.9 ± 0.3^{Aa}	59.7 ± 0.2^{Aa}	59.5 ± 0.1^{Aa}	59.8 ± 0.6^{Aa}	57.6 ± 0.3^{Ba}	56.7 ± 0.4^{Ca}
	HPH-CEO-4°C	59.5 ± 0.4^{Aa}	58.8 ± 0.3^{Abc}	59.0 ± 0.5^{Aa}	59.7 ± 0.1^{Aa}	57.1 ± 0.8^{Ba}	57.2 ± 0.4^{Ba}
	HPH-25°C	59.6 ± 0.5^{Aa}	59.4 ± 0.1^{Aa}	58.0 ± 0.7^{Bb}	58.7 ± 0.4^{ABa}	57.6 ± 0.3^{Ba}	56.4 ± 0.4^{Ca}
	HPH-CEO-25°C	59.0 ± 0.7^{ABab}	59.3 ± 0.4^{Aab}	59.5 ± 0.3^{Aa}	59.1 ± 0.1^{ABab}	58.2 ± 0.5^{BCa}	57.1 ± 0.4^{Ca}
	US-4°C	57.0 ± 0.7^{Ade}	55.1 ± 0.4^{Bd}	51.2 ± 0.5^{Dd}	53.3 ± 0.4^{Cd}	48.8 ± 1.1^{Ec}	48.5 ± 0.9^{Ec}
	US-CEO-4°C	58.2 ± 0.2^{Abc}	58.3 ± 0.6^{Ac}	52.4 ± 0.1^{BCc}	54.1 ± 0.1^{Bc}	52.4 ± 1.6^{BCb}	51.7 ± 0.1^{Cb}
	US-25°C	56.3 ± 0.1^{Ae}	51.3 ± 0.2^{Be}	49.0 ± 0.2^{De}	50.1 ± 0.1^{Cf}	44.0 ± 0.3^{Ed}	39.9 ± 0.4^{Fd}
	US-CEO-25°C	57.5 ± 0.2^{Acd}	54.4 ± 0.1^{Bd}	52.8 ± 0.6^{Cc}	51.8 ± 0.4^{CDe}	52.7 ± 0.4^{Cb}	51.5 ± 0.6^{Db}
PUFA/SFA ratio	HPH-4°C	2.79 ± 0.03^{Aa}	2.79 ± 0.03^{Aa}	2.75 ± 0.03^{Aa}	2.89 ± 0.09^{Aa}	2.56 ± 0.04^{Bab}	2.58 ± 0.04^{Ba}
	HPH-CEO-4°C	2.72 ± 0.04^{Aa}	2.66 ± 0.02^{ABbc}	2.67 ± 0.07^{ABa}	2.78 ± 0.10^{Aab}	2.48 ± 0.08^{Cb}	2.51 ± 0.07^{BCa}
	HPH-25°C	2.70 ± 0.01^{ABa}	2.75 ± 0.03^{Aab}	2.51 ± 0.05^{Cb}	2.59 ± 0.07^{BCb}	2.47 ± 0.05^{Cb}	2.35 ± 0.07^{Db}
	HPH-CEO-25°C	2.71 ± 0.05^{Aa}	2.58 ± 0.05^{ABc}	2.70 ± 0.05^{Aa}	2.74 ± 0.06^{Aab}	2.68 ± 0.11^{ABa}	2.53 ± 0.06^{Ba}
	US-4°C	2.27 ± 0.05^{Ac}	2.00 ± 0.06^{Be}	1.69 ± 0.04^{Dc}	1.86 ± 0.07^{Cc}	1.47 ± 0.01^{Ed}	1.51 ± 0.02^{Ed}
	US-CEO-4°C	2.41 ± 0.04^{Ab}	2.32 ± 0.06^{Ad}	1.79 ± 0.01^{Bc}	1.81 ± 0.07^{Bc}	1.78 ± 0.09^{Bc}	1.71 ± 0.03^{Bc}
	US-25°C	2.23 ± 0.04^{Ac}	1.66 ± 0.07^{Bg}	1.53 ± 0.03^{Cd}	1.66 ± 0.03^{Bc}	1.19 ± 0.05^{De}	1.09 ± 0.04^{De}
	US-CEO-25°C	2.26 ± 0.05^{Ac}	1.87 ± 0.02^{Bf}	1.79 ± 0.05^{Bc}	1.81 ± 0.07^{Bc}	1.83 ± 0.09^{Bc}	1.74 ± 0.08^{Bc}
ΣUFA (%)	HPH-4°C	78.5 ± 0.6^{ABa}	78.5 ± 0.5^{ABa}	78.4 ± 0.0^{ABa}	79.2 ± 0.4^{Aa}	77.5 ± 0.1^{Ca}	77.9 ± 0.1^{BCa}
	HPH-CEO-4°C	78.1 ± 0.2^{Aa}	77.9 ± 0.0^{Aa}	78.0 ± 0.9^{Aab}	78.5 ± 0.3^{Aab}	77.1 ± 1.2^{Aa}	77.2 ± 1.0^{Aab}
	HPH-25°C	77.9 ± 0.2^{ABa}	78.5 ± 0.3^{Aa}	76.9 ± 0.4^{ABCb}	77.2 ± 1.1^{ABCb}	76.6 ± 0.7^{BCa}	76.0 ± 0.7^{Cb}
	HPH-CEO-25°C	78.2 ± 0.3^{Aa}	77.0 ± 0.1^{Bb}	78.0 ± 0.4^{Aab}	78.4 ± 0.3^{Aab}	78.2 ± 0.1^{Aa}	77.3 ± 0.1^{Bab}
	US-4°C	75.0 ± 0.4^{Abc}	72.4 ± 0.2^{Bd}	69.7 ± 0.1^{Cd}	71.4 ± 0.2^{Bc}	66.9 ± 0.8^{Dc}	67.5 ± 0.2^{Dd}
	US-CEO-4°C	75.7 ± 0.5^{Ab}	74.8 ± 0.1^{Ac}	70.9 ± 0.4^{Bc}	70.2 ± 1.6^{Bc}	70.5 ± 1.3^{Bb}	69.7 ± 0.4^{Bc}
	US-25°C	74.7 ± 0.3^{Ac}	69.0 ± 0.6^{Bf}	67.9 ± 0.6^{Be}	69.9 ± 1.1^{Bc}	63.0 ± 1.1^{Cd}	63.4 ± 1.0^{Ce}
	US-CEO-25°C	74.5 ± 0.4^{Ac}	71.0 ± 0.1^{BCe}	70.5 ± 0.4^{CDcd}	71.3 ± 0.3^{Bc}	71.1 ± 0.1^{BCb}	70.3 ± 0.1^{Dc}
UFA/SFA ratio	HPH-4°C	3.65 ± 0.05^{Ba}	3.67 ± 0.03^{Ba}	3.63 ± 0.04^{Ba}	3.83 ± 0.10^{Aa}	3.45 ± 0.07^{Cab}	3.54 ± 0.03^{BCa}
	HPH-CEO-4°C	3.57 ± 0.03^{Aab}	3.53 ± 0.05^{Ab}	3.53 ± 0.03^{Ab}	3.65 ± 0.11^{Aab}	3.35 ± 0.01^{Bb}	3.39 ± 0.02^{Bb}
	HPH-25°C	3.53 ± 0.03^{ABb}	3.63 ± 0.05^{Aab}	3.33 ± 0.04^{BCDc}	3.40 ± 0.11^{BCc}	3.29 ± 0.08^{CDb}	3.17 ± 0.10^{Dc}
	HPH-CEO-25°C	3.59 ± 0.04^{ABab}	3.35 ± 0.05^{Cc}	3.55 ± 0.03^{ABab}	3.63 ± 0.06^{Ab}	3.60 ± 0.12^{Aa}	3.42 ± 0.07^{BCab}
	US-4°C	2.99 ± 0.05^{Ad}	2.62 ± 0.06^{Be}	2.30 ± 0.04^{De}	2.50 ± 0.07^{Cd}	2.02 ± 0.03^{Ed}	2.10 ± 0.02^{Ee}
	US-CEO-4°C	3.13 ± 0.02^{Ac}	2.97 ± 0.05^{Bd}	2.43 ± 0.01^{Cd}	2.35 ± 0.05^{CDd}	2.40 ± 0.09^{CDc}	2.30 ± 0.02^{Dd}
	US-25°C	2.95 ± 0.04^{Ad}	2.23 ± 0.12^{BCg}	2.12 ± 0.06^{Cf}	2.32 ± 0.07^{Bd}	1.70 ± 0.03^{De}	1.73 ± 0.02^{Df}
	US-CEO-25°C	2.92 ± 0.06^{Ad}	2.44 ± 0.02^{Bf}	2.39 ± 0.03^{Bd}	2.49 ± 0.06^{Bd}	2.47 ± 0.09^{Bc}	2.37 ± 0.08^{Bd}
ω-3/ω-6 ratio	HPH-4°C	3.54 ± 0.07^{ABab}	3.49 ± 0.10^{ABab}	3.58 ± 0.04^{Aa}	3.50 ± 0.10^{ABa}	3.35 ± 0.05^{Ca}	3.36 ± 0.05^{Ca}
	HPH-CEO-4°C	3.54 ± 0.05^{Aa}	3.59 ± 0.10^{Aa}	3.47 ± 0.14^{Aab}	3.45 ± 0.10^{Aa}	3.37 ± 0.15^{Aa}	3.36 ± 0.02^{Aa}
	HPH-25°C	3.44 ± 0.05^{Aab}	3.45 ± 0.05^{Aab}	3.46 ± 0.13^{Aab}	3.32 ± 0.05^{Aab}	3.31 ± 0.03^{Aab}	3.32 ± 0.06^{Aa}
	HPH-CEO-25°C	3.54 ± 0.01^{Aab}	3.45 ± 0.10^{Aab}	3.47 ± 0.09^{Aab}	3.38 ± 0.17^{Aa}	3.22 ± 0.12^{Aab}	3.20 ± 0.20^{Aa}
	US-4°C	3.62 ± 0.06^{Aa}	3.42 ± 0.12^{ABab}	3.22 ± 0.12^{BCabc}	3.11 ± 0.11^{Cb}	3.07 ± 0.04^{Cab}	3.04 ± 0.06^{Ca}
	US-CEO-4°C	3.35 ± 0.11^{Ab}	3.45 ± 0.09^{Aab}	3.17 ± 0.29^{Abc}	3.33 ± 0.23^{Aa}	3.29 ± 0.23^{Aab}	3.28 ± 0.16^{Aa}
	US-25°C	3.57 ± 0.12^{Aa}	3.27 ± 0.06^{Bb}	2.96 ± 0.15^{BCc}	2.86 ± 0.16^{Cb}	2.94 ± 0.11^{Cb}	2.51 ± 0.13^{Db}
	US-CEO-25°C	3.54 ± 0.10^{Aa}	3.25 ± 0.15^{Ab}	3.26 ± 0.14^{Aabc}	3.06 ± 0.35^{Aab}	3.22 ± 0.27^{Aab}	3.16 ± 0.28^{Aa}

Acknowledgment

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2022.106277.

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