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Phenolic Compounds in *Hibiscus mutabilis* Seeds and Their Effects on the Oxidative Stability of DHA-Enriched Goat Milk Emulsion

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Abstract

Food emulsions undergo oxidative deterioration during production and storage, which is usually initiated from the unsaturated fatty acids. Synthetic antioxidants are frequently used to retard lipid oxidation in food emulsions. Most plants and their seeds are rich sources of natural antioxidants such as the carotenoids and polyphenols. The most abundant fatty acids found in the oil from the seeds of *Hibiscus mutabilis* (HM) are oleic acid (C18:1 n -9, 16.3%), linoleic acid, (C18:2 n -6, 64.7%), and palmitic acid (C16:0, 18.8%). The total tocopherols in HM seed oil were at an average concentration of 187.0 μ g/g, which included α -tocopherol (21.4%), γ -tocopherol (78.2%), and δ -tocopherol (0.4%). The HM seed oil can be incorporated into food emulsions such as in DHA-enriched goat milk emulsion to stabilize added oil from oxidation. The HM seed oil was mixed with algae oil, a rich source of omega-3 docosahexaenoic acid (DHA; C22:6 n -3), before emulsification and storage of goat milk. The addition of HM seed oil containing phenolics to algae oil at 1:1 ratio prior to goat milk emulsification significantly ($p < 0.05$) protected the goat milk emulsions against oxidative deterioration. In goat milk emulsions, the addition of ascorbyl palmitate retarded oxidation as was determined by the peroxide values and anisidine values.

Keywords: *Hibiscus mutabilis* seeds, phenolics, algae oil, milk emulsion, stability

1. Introduction

Human breast milk contains both docosahexaenoic acid (DHA, C22:6 n -3) and arachidonic acid (AA, C20:4 n -6) [1], which are essential for health. Studies in animals and humans indicate that DHA is essential for normal visual and brain function in the premature infants and possibly in the full term infants [2, 3]. In some breast-fed infants, colic has been related to the mother's consumption of cow milk [4, 5]. In older infants, the incidence of cow milk protein intolerance was encountered in 5–15% of cases [6]. A popular therapy among pediatricians is to change from cow milk to vegetable protein soy-based formula; however, infants with cow milk protein intolerance will also react adversely to soybean proteins [7]. When the problem is allergy to cow milk proteins (casein, whey), goat milk is a suitable substitute to cow milk [8].

The omega-3 fatty acids in the milk of grass-fed goats are predominantly linolenic acid (C18:3 n -3), or alpha (α)-linolenic acid (ALA). DHA can be synthesized from dietary ALA, but the human body can only make very small amounts of DHA from ALA [9]. Therefore, there is a need to supplement foods with DHA. The addition of DHA from algae oil in food emulsions such as in goat milk emulsion requires the need for antioxidants. Antioxidants increase the shelf life of emulsions, but a clean label ingredient is required when added to milk. Oxidation in oil-in-water emulsions is thought to occur at the interface region between the oil and the aqueous phases [10]. In the oil phase of the emulsions, fatty acids are the target of free radicals i.e., hydroxyl radicals, which stimulate lipid peroxidation. Nonpolar antioxidants such as tocopherols and ascorbyl palmitate have been shown to be highly effective in protecting oil-in-water emulsions [11]. Tocopherols are free-radical terminators thereby, interrupting the free-radical chain of oxidative reactions by contributing hydrogen from the phenolic hydroxyl groups [12]. Ascorbyl palmitate, a lipid-soluble antioxidant, exhibits antioxidant activities that include single oxygen quenching and free-radical scavenging [13–15]. Ascorbyl palmitate has been shown to work synergistically with tocopherols by donating a hydrogen to the tocopheroxyl radical, formed as a result of tocopherol donating a hydrogen to the lipid radical [16, 17].

The oxidative stability of DHA-enriched emulsion may also be accomplished by the addition of vegetable oil to algae oil. In this context, one of the strategies developed to protect fish oil in a cow milk emulsion against oxidation was the mixing of rapeseed oil with fish oil prior to emulsification of cow milk [18]. The authors found that tocopherol isomers in concentrations similar to those found in natural rapeseed oil, and added to rapeseed oil stripped of natural tocopherols, significantly inhibited oxidation in cow milk emulsions enriched with fish oil [18].

Hibiscus mutabilis (Malvaceae) are shrubs with peach color flowers and originally native of China. The seeds of *Hibiscus mutabilis*, which do not have economic applications yet, are a source of vegetable oil. Although not widely reported in the literature, a high content of phenolic compounds, tocopherols are found in *Hibiscus mutabilis* seed oil. The seeds of *Hibiscus mutabilis* are also a source of lectin. Lectin from the seeds of *Hibiscus mutabilis* has carbohydrate-binding specificity to galactonic acid, which potently inhibited HIV-1 reverse transcriptase [19]. HIV, the RNA virus that causes AIDS, gradually disrupts the immune system in humans. Since a recent study suggested that DHA in high DHA-concentrated fish oil positively contributed to certain aspects of immune function in middle-aged obese adults [20], DHA-enriched goat milk stabilized by *Hibiscus mutabilis* seed oil potentially can be used as immune stimulator for the adjunctive therapy of HIV.

In the present work the suitability of *Hibiscus mutabilis* seed oil for enhancing the oxidative stability of DHA-enriched goat milk emulsion was studied. Based on the potential synergistic effects of ascorbyl palmitate with tocopherols, it was assumed that the most efficient oxidative stabilization during homogenization and storage of DHA-enriched goat milk may be achieved by combining both of these lipophilic antioxidants.

2. Materials and methods

2.1 Materials

Raw milk from French-Alpine goats, raised at the International Goat Research Center, Prairie View A&M University, Prairie View, Texas, USA, was obtained. Raw milk with a fat content of 4.1% (wt/wt) that was determined according to the

method of Kleyn et al. [21] was collected during the early lactation period. Iron and copper contents in raw goat milk were determined by atomic absorption spectrometry using a Varian SpectrAA 55 (Varian Analytical Instruments, Inc., Walnut Creek, CA, USA). Raw goat milk was dry-ashed in a muffle furnace (Barnstead/Thermolyne Corp., Dubuque, IA, USA) at 550°C. Ashes were dissolved in 0.2% nitric acid solution. The concentrations of iron and copper in raw goat milk were determined from the calibration curves that were produced under the same experimental conditions with known standards.

Algae oil was provided by Nutrinova Inc. (Somerset, NJ, USA). Algae oil was subjected to chromatography to remove peroxides, carotenoids, tocopherols, and other antioxidants, as previously described [22]. The chromatographically purified algae oil has a DHA concentration of 42.9% (Table 1). The fatty acid composition of chromatographically purified algae oil was determined by preparation of methyl esters [23], which were analyzed by gas chromatography–mass spectrometry (GC–MS). For the fatty acid profile of raw goat milk, the samples were centrifuged at 10,000× g for 1 h to harvest milk fat. Fatty acids of milk fat (% wt/wt) were directly methylated by *in situ* transesterification as described [24] and analyzed by GC-MS (Agilent model 7890A GC system attached to an Agilent model 5975C mass detector; Agilent Technologies Inc., Santa Clara, CA, USA) on a 30 m × 0.25 mm internal diameter, 0.25 μm film thickness capillary column. Methyl ester of 10,

Fatty acid (% wt/wt)	GM	PAO	PHMO	NHMO
C4:0	1.2			
C6:0	1.5			
C8:0	2.1			
C10:0	7.7			
C11:0	0.1			
C12:0	3.2			
C13:0	0.1			
C14:0	8.2	2.8		
C14:1(n-5)	0.1			
C15:0	0.4	1.2		
C16:0	21.1	30.1	18.7	18.8
C16:1(n-7)	0.6			
C17:0	0.2	0.3		
C18:0	9.0	0.9		
C18:1 (n-9)	23.3		16.0	16.3
C18:2 (n-6)	4.1		64.6	64.7
C18:3 (n-6)	0.2			
C18:3 (n-3)	2.8			
C20:0	0.1			
C20:1 (n-9)	0.3			
C20:4 (n-6)	0.2			
C20:4(n-7)		0.9		
C20:4 (n-3)		0.9		
C22:1(n-9)				
C22:5(n-6)		10.5		
C22:5 (n-3)		0.7		
C22:6 (n-3)		42.9		
C24:1	0.1			
Others	13.4	8.8	0.7	0.2
Natural tocopherols (μg/g oil)				
α-tocopherol	17.8			40.0
β-tocopherol				
γ-tocopherol				146.2
δ-tocopherol				0.8
Peroxide value (meq/kg)	0.1			0.1
Anisidine value	0.2	0.01	0.01	0.1
Free fatty acids (% wt/wt)	0.1	0.01	0.01	0.01

Table 1. Chemical composition, peroxide value and anisidine value of goat milk (GM), chromatographically purified algae oil (PAO), chromatographically purified Hibiscus mutabilis seed oil (PHMO), and natural Hibiscus mutabilis seed oil (NHMO).

13-nonadecadienoate (Nu-Chek-Prep U-58M, Elysian, MN, USA) was used as an internal standard. The concentrations of tocopherols in the raw goat milk and the chromatographically purified algae oil were determined by reversed-phase high-pressure liquid chromatography (HPLC) [25], and the results were expressed as $\mu\text{g/g}$ of oil. The content of free fatty acids in the raw goat milk and the chromatographically purified algae oil was determined by AOAC method [26]. The fatty acid composition, the concentrations of tocopherols, the peroxide value (PV), the anisidine value (AV), and the content of free fatty acids in the raw goat milk and chromatographically purified algae oil samples are presented in **Table 1**.

The lipid-soluble antioxidant, ascorbyl palmitate, was purchased from DSM Nutritional Products, Inc. (Parsippany, NJ, USA). All reagents used were of analytical grade, ACS certified or HPLC grade, from Sigma-Aldrich (St. Louis, MS, USA). Deionized water was prepared by passing distilled water over a mixed bed of cation-anion exchanger and was used throughout this study.

2.2 Preparation of *Hibiscus mutabilis* seed samples

Fresh harvested seeds of *Hibiscus mutabilis* (**Figure 1**) were obtained from The Village Botanica, Inc. (Waller, TX, USA). The seeds were immediately frozen with liquid nitrogen until analysis. The frozen seeds were thawed, dried by air blower and then milled using a blender. The ground samples that passed through a 35-mesh sieve were used for oil extraction.

2.3 Oil extraction of *Hibiscus mutabilis* seeds

The ground fractions of *Hibiscus mutabilis* seeds were placed in a filter paper (Whatman No. 42) and introduced in a cartridge and they were extracted in a Soxhlet extractor (Southern Labware, Inc., Cumming, GA, USA) using hexane at 65–70°C during approximately 5 h, the time necessary to extract most of the oil from the seeds. The solvent was then evaporated by a vacuum dryer (Columbia International Tech, Irmo, SC, USA), and the oil yield was 9.0 g from 100 g of seeds. The extracted *Hibiscus mutabilis* seed oil was transferred into glass tubes, centrifuged at 12,000 \times g for 30 min at room temperature, and then stored at 4°C in the dark until analyses. This oil is referred to as the natural *Hibiscus mutabilis* seed oil (**Table 1**). The natural *Hibiscus mutabilis* seed oil was subjected to chromatography to remove naturally occurring antioxidants such as tocopherols and carotenoids [22]. Thus, this



Figure 1.
Image of seeds of *Hibiscus mutabilis*.

Hibiscus mutabilis seed oil is void of antioxidants and peroxides. The percent fatty acid composition of the chromatographically purified *Hibiscus mutabilis* seed oil and the natural *Hibiscus mutabilis* seed oil were determined according to procedures described [23] by GC–MS. The fatty acid composition of the chromatographically purified *Hibiscus mutabilis* seed oil was similar to the natural *Hibiscus mutabilis* seed oil (**Table 1**). The content of free fatty acids in the chromatographically purified *Hibiscus mutabilis* seed oil and the natural *Hibiscus mutabilis* seed oil was determined by AOAC method [26]. The concentrations of tocopherols, the PV, the AV, and the concentrations of free fatty acids of the natural and the chromatographically purified *Hibiscus mutabilis* seed oil samples are presented in **Table 1**.

2.4 Preparation of emulsions

Three liters of raw goat milk was pasteurized by heating at 72°C and holding milk at this temperature for 15 s. Chromatographically purified algae oil (0.25 wt%), chromatographically purified *Hibiscus mutabilis* seed oil (0.25 wt%) or natural *Hibiscus mutabilis* seed oil (0.25 wt%) with and without ascorbyl palmitate (200 µg/g of oil) were added to goat milk. Goat milk samples were then cooled to 50°C and immediately homogenized at 22.5 MPa (3263.35 psi) through a high-pressure TC5 homogenizer (Stansted Fluid Power, Harlow, UK). The goat milk emulsion samples were transferred to sterile 100-ml Pyrex dark brown glass bottles, which were flushed with nitrogen and then stored at 2°C in the dark for 14 days. The goat milk emulsions, with added oils, were labeled as follows: PAO = chromatographically purified algae oil, PHMO = chromatographically purified *Hibiscus mutabilis* seed oil, NHMO = natural *Hibiscus mutabilis* seed oil, and LAAP = lipid-soluble antioxidant ascorbyl palmitate.

2.5 Droplet size measurement

The particle size of the oil droplets in the goat milk emulsions was measured at day 1 and day 14 at 21 ± 1°C with a SALD-2101 laser diffraction particle size analyzer (Shimadzu Corporation, Columbia, MD, USA). The emulsion samples were diluted 100 times with double deionized water before they were transferred into the chamber of the instrument. Particle size measurements in µm were carried out in triplicate.

2.6 Measurement of peroxide value

Lipids from the DHA-enriched goat milk emulsions were extracted by chloroform:methanol (1:1 wt/wt), using a small volume of solvent [27, 28]. The PV was measured directly on the oils or fats extracted from the DHA-enriched goat milk emulsions by colorimetric determination of iron thiocyanate [29]. This method measures primary oxidation products of oils or fats i.e., hydroperoxides of oils and fats. The mean measurements in meq/kg of three replicates were reported.

2.7 Measurement of p-anisidine value

The para (*p*)-anisidine value was determined in the DHA-enriched goat milk emulsions by AOAC method [30]. This method determines the amount of aldehydes (principally 2-alkenals and 2,4-dienals) present in the emulsion samples. The mean measurements of three replicates were reported.

2.8 Statistical analysis

The results of triplicate analyses were expressed as means \pm standard deviations. The data were analyzed by ANOVA using PRO GLM procedure of SAS (version 8.2, SAS Institute, Cary, NC, USA). The least significant difference test was used to determine significant differences among treatment means at $p < 0.05$.

3. Results and discussion

The rate and extent of oxidation of marine oils i.e., algae oil, fish oil depends on the matrix of the food to be fortified. Milk relative to some other foods offers good protection against oxidation, since these marine oils are emulsified and stabilized by the casein micellar structure [18]. Casein adsorbs to the newly formed interface thereby, providing enhanced protection by forming a physical barrier [31, 32]. Although milk is stored in refrigerators (2–4°C) and has a relatively short life (21 days), it is still subject to overall stress due to UV and visible light, temperature fluctuations, and handling abuse.

Lipid oxidation proceeds from the interface to the oil droplet interior in oil-in-water emulsions i.e., goat milk emulsion, therefore, the susceptibility of lipids to oxidation at the interface is the most important factor affecting the oxidative stability of lipids in food and beverage emulsions. It is generally accepted that the attack of free radicals and trace metals on lipids at the interface increases with the increase in the area of interface. Thus, the oxidative stability of DHA in goat milk emulsions should decrease with decreasing droplet sizes. The results of the droplet size determinations (**Table 2**) showed that the droplets did not change in size from day 1 to day 14 of storage at 2°C, indicating that the goat milk emulsions were physically stable during the 2 weeks of storage. The average droplet size in all goat milk emulsions containing 0.5% oil was from 1.20 ± 0.01 to $1.25 \pm 0.03 \mu\text{m}$, while the droplet size in the original goat milk sample was $0.89 \pm 0.02 \mu\text{m}$. These results showed that the sizes of droplets in goat milk emulsions containing 0.5% added oil, irrespective of the oil type, were significantly ($p < 0.05$) larger than the droplets in the original goat milk sample. The decrease in the oil droplet size induces the increase in the droplet interface [33], from which the oxidation proceeds to the oil droplet interior.

Goat milk emulsion ³	Diameter (μm) ^{1,2}	
	Storage day 1	Storage day 14
PAO + PHMO	1.25 ± 0.03^a	1.23 ± 0.01^a
PAO + NHMO	1.21 ± 0.01^a	1.22 ± 0.03^a
PAO + NHMO + LAAP	1.20 ± 0.01^a	1.23 ± 0.03^a
Goat milk (no oil)	0.89 ± 0.02^b	0.86 ± 0.01^b

¹Mean \pm standard deviation.

²Means with different letters (a or b) are significantly different ($p < 0.05$).

³PAO = chromatographically purified algae oil, PHMO = chromatographically purified *Hibiscus mutabilis* seed oil, NHMO = natural *Hibiscus mutabilis* seed oil, and LAAP = lipid-soluble antioxidant ascorbyl palmitate.

Table 2.

Droplet sizes of goat milk emulsions prepared by mixing chromatographically purified algae oil (0.25 wt%), chromatographically purified *Hibiscus mutabilis* seed oil (0.25 wt%) or natural *Hibiscus mutabilis* seed oil (0.25 wt%) with and without added ascorbyl palmitate (200 $\mu\text{g/g}$ of oil) during 14-day storage at 2°C.

The results of **Table 2** suggest that DHA in goat milk emulsions containing 0.5% added oil is expected to be less oxidized in the droplet interior.

When supplementing foods with algae oil or fish oil, it is important to consider the initial quality of the raw material. In this work, we have ensured that all oils tested had similar variables such as age and storage temperature. The results of **Table 1** showed that all oils had low initial values of PV, and very low content of free fatty acids. Rancid flavors in goat milk is usually associated to the release of short-chain free fatty acids and is a persistent problem in dairy goat farms due to mishandling procedures starting from the farm until it reaches the consumers. The content of free fatty acids in the goat milk used in this study was very low (0.1%) and likewise, the PV content of goat milk was also low (0.1 meq/kg) suggesting that the quality of goat milk was acceptable from the sensory perspectives.

As to the goat milk emulsions, the chromatographically purified *Hibiscus mutabilis* seed oil (PHMO) together with the chromatographically purified algae oil (PAO) had a significantly ($p < 0.05$) higher PV than the other two goat milk emulsions at each storage time at 2°C (**Figure 2**). On the other hand, the goat milk emulsion with the natural *Hibiscus mutabilis* seed oil (NHMO) and the chromatographically purified algae oil (PAO) exhibited good oxidative stability as inferred from a low PV (**Figure 2**). The goat milk emulsion containing the chromatographically purified algae oil (PAO) and the natural *Hibiscus mutabilis* seed oil (NHMO) with added ascorbyl palmitate (LAAP) had the lowest PV during the study (**Figure 2**). These results suggest that the presence of antioxidants i.e., γ -tocopherol, ascorbyl palmitate improved the oxidative stability of goat milk emulsions under storage at 2°C for 14 days, which may contribute to the shelf life of goat milk emulsions.

The key challenge in formulating food products with marine oils is their sensitivity to iron and copper, catalysts to oxidation that exists in even the cleanest water, foods, and other ingredients. The goat milk used in this study, contained approximately 138 ppb iron and 27 ppb copper, and these values were not affected by the addition of oils to the goat milk. The presence of trace metals in goat milk is expected to accelerate the degradation of lipid hydroperoxides as

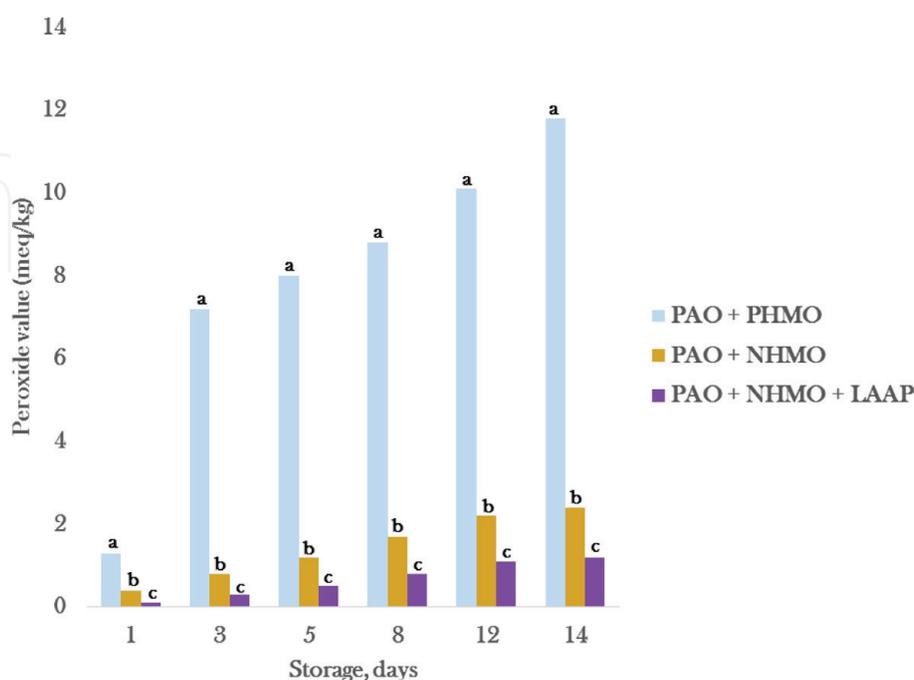


Figure 2. Peroxide values of goat milk emulsions containing the different oils with and without added ascorbyl palmitate during 14-day storage at 2°C. Means ($n = 3$) within each storage day with different letters (a–c) are significantly different ($p < 0.05$).

well as the degradation of the secondary oxidation products into shorter chain volatiles.

The results of **Figure 3** showed that the goat milk emulsions containing a mixture (1:1) of the chromatographically purified algae oil (PAO) and the chromatographically purified *Hibiscus mutabilis* seed oil (PHMO) were more oxidized than the goat milk emulsions containing a mixture (1:1) of the chromatographically purified algae oil (PAO) and the natural *Hibiscus mutabilis* seed oil (NHMO). The protective effect of the natural *Hibiscus mutabilis* seed oil (NHMO) may be partially ascribed to the high content of tocopherols, especially γ -tocopherol. As pointed out earlier, the tocopherols are free-radical terminators, which donate a hydrogen to the peroxy radical [12]. Goat milk contains citric acid [34], and citric acid is recognized as a metal chelator. The chelating properties of citric acid have been proposed to protect tocopherols during oxidation [35]. Therefore, citric acid in goat milk could enhance the antioxidant activity of tocopherols in the emulsions containing the natural *Hibiscus mutabilis* seed oil (NHMO) and the chromatographically purified algae oil (PAO) at 1:1 ratio (**Figure 3**).

Ascorbyl palmitate (LAAP), which was added (200 $\mu\text{g/g}$ of oil) to the natural *Hibiscus mutabilis* seed oil (NHMO) and the chromatographically purified algae oil (PAO) at 1:1 ratio, significantly reduced ($p < 0.05$) the extent of oxidation in this goat milk emulsion at 7-day and 14-day storage at 2°C (**Figure 3**). This protective effect of added ascorbyl palmitate (LAAP) was not observed in goat milk emulsions containing the chromatographically purified *Hibiscus mutabilis* seed oil (PHMO) and the chromatographically purified algae oil (PAO) at 1:1 ratio during 14-day storage at 2°C (data not shown). Ascorbyl palmitate (LAAP) had a more pronounced protective effect on the goat milk emulsion prepared with the chromatographically purified algae oil (PAO) and the natural *Hibiscus mutabilis* seed oil (NHMO) at 1:1 ratio by working synergistically with the γ -tocopherol isomer at 7-day and 14-day storage at 2°C (**Figure 3**). It is likely that ascorbyl palmitate retarded oxidation during storage of oil-in-water emulsions by direct scavenging of free radicals and tocopherol regeneration [18].

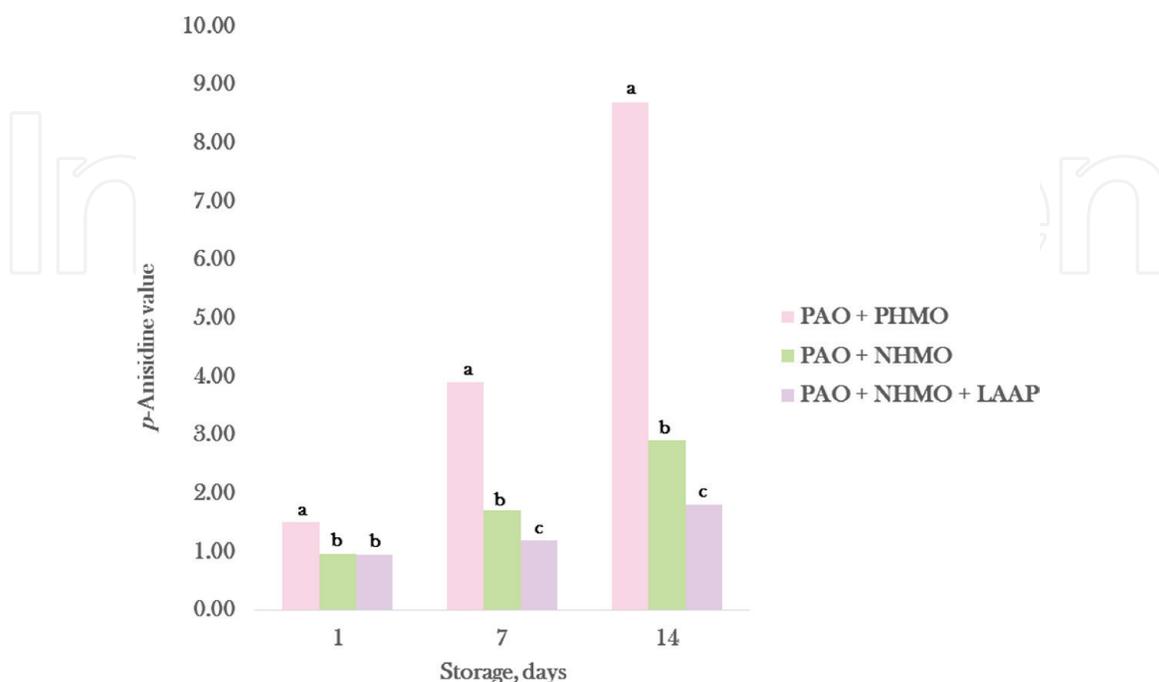


Figure 3. *p*-Anisidine values of goat milk emulsions containing the different oils with and without added ascorbyl palmitate during 14-day storage at 2°C. Means ($n = 3$) within each storage day with different letters (a–c) are significantly different ($p < 0.05$).

There is a direct relationship between the level of oxidation and sensory deterioration and even at times when there are no detectable oxidation parameters, the taste of the finished products could be displeasing. The decomposition of lipid hydroperoxides from marine derived *n*-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA, C22:6*n*-3) and eicosapentaenoic acid (EPA, C20:5*n*-3), produces undesirable rancid and fishy off-flavors. Future work will evaluate the sensory attributes of DHA-enriched goat milk emulsion stabilized by the natural *Hibiscus mutabilis* seed oil and ascorbyl palmitate.

4. Conclusions

This work showed the suitability of using *Hibiscus mutabilis* seed oil to protect marine-derived *n*-3 PUFAs in oil-in-water emulsions i.e., DHA-milk from oxidative degradation for 14 days at 2°C. The natural *Hibiscus mutabilis* seed oil efficiently protected the chromatographically purified algae oil from oxidation during emulsification and storage of DHA-enriched goat milk emulsion. The addition of ascorbyl palmitate to the natural *Hibiscus mutabilis* seed oil and the chromatographically purified algae oil prior to goat milk emulsification had a significant ($p < 0.05$) protective effect on DHA-enriched goat milk emulsion. The combination of differences in fatty acid composition and concentration of tocopherols for the natural *Hibiscus mutabilis* seed oil seems to affect the oxidative stability of the goat milk emulsions prepared with this oil. This study provides a useful precedent for understanding the antioxidant activity of *Hibiscus* seed oils in food and beverage emulsions containing marine *n*-3 PUFAs.

Complementary work is currently being performed in our laboratory to optimize the oxidative stability of DHA-enriched goat milk emulsions with added seed oils from *Hibiscus* species such as *Hibiscus moscheutos* and *Hibiscus dasycalyx* to be able to withstand the thermal and mechanical stresses of industrial processes.

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Conflict of interest

The authors declare that they have no conflict of interest.

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