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Enzyme extraction of cupuassu (*Theobroma grandiflorum* S.) fat seeds

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SUMMARY: Enzyme-assisted extraction is considered an environmentally friendly technique. Cellulase, pectinase and protease were tested for cupuassu seeds fat extraction. The best fat efficiency (81.66%) was obtained for the solute:solvent 1:5 (m:w), orbital shaker at 120 rpm, 60 °C, for 8 hours and enzyme concentrations (cellulase, pectinase and protease) of 1.0%. The fat was characterized for physicochemical properties, fatty acid profile, phenolic compounds, antioxidant activities and oxidative stability. The fat showed good thermal stability (14.26 h) and high contents of monounsaturated (42.42%) and saturated (43.47%) fatty acids with higher concentrations of oleic and stearic acids, respectively, and a high content of phenolic compounds (141.84 µg EAG·g^{-1}) in the fat, and in the aqueous extract (926.47 µg EAG·g^{-1}). The results indicated that the cupuassu seed fat obtained by enzymatic extraction showed superior properties to cupuassu fat obtained by cold pressing, in addition to generating an aqueous fraction which is rich in bioactive compounds that can be used as ingredients in the food and pharmaceutical sectors.

KEYWORDS: *Amazon seeds; By-products properties; Green extraction;* Theobroma grandiflorum.

RESUMEN: ***Extracción enzimática de semillas grasas de cupuaçu (*****Theobroma grandiflorum** ***S.).*** La extracción asistida por enzimas se considera una técnica respetuosa con el medio ambiente. Se probaron celulasa, pectinasa y proteasa para la extracción de grasa de semillas de cupuaçu. La mejor eficiencia grasa (81,66%) se obtuvo para la relación soluto:solvente 1:5 (m:w), agitador orbital a 120 rpm, 60 °C, durante 8 horas y concentraciones de enzimas (celulasa, pectinasa y proteasa) 1,0%. La grasa se caracterizó por sus propiedades fisicoquímicas, perfil de ácidos grasos, compuestos fenólicos, actividades antioxidantes y estabilidad oxidativa. La grasa mostró buena estabilidad térmica (14,26 h) y alto contenido de ácidos grasos monoinsaturados (42,42%) y saturados (43,47%) con mayores concentraciones de ácido oleico y esteárico, respectivamente, y alto contenido de compuestos fenólicos (141,84 µg EAG·g^{-1}) en la grasa y en el extracto acuoso (926,47 µg EAG·g^{-1}). Los resultados indicaron que la grasa de semilla de cupuaçu obtenida por extracción enzimática mostró propiedades superiores a la grasa de cupuaçu obtenida por prensado en frío, además de generar una fracción acuosa rica en compuestos bioactivos que puede ser utilizada como ingrediente en los sectores alimentario y farmacéutico.

PALABRAS CLAVE: *Extracción verde; Propiedades de los subproductos; Semillas Amazónicas;* Theobroma grandiflorum.

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1. INTRODUCTION

The cupuassu tree is found in the forests of the Amazon region, mainly in the states of Pará, Amazonas, Rondônia and Acre. Its fruit is composed of approximately 38% pulp, 17% seeds, 2% placenta and 43% bark (Pereira *et al*., 2018). Cupuassu pulp is appreciated for its characteristic exotic flavor and odor; the seeds are rich in lipids, representing 60% of its total dry weight and promising sources of phenolic compounds (Contreras-Calderón *et al*., 2011).

The conventional processes used for extracting oils and fats from seeds and pulps are pressing, solvent extraction or a combination of both. Other methods such as extraction by super-critical fluids, microwaves, enzymatic and ultrasonic extraction are used, but they present obstacles to large-scale production in efficiency which is compatible with conventional methods (Tang *et al*., 2011). The cupuassu seed fat solid is traditionally extracted by the cold pressing process, with a characteristic composition of fatty acids, and its main use is in the preparation of chocolates as a substitute for cocoa butter (Bezerra *et al*., 2017).

Enzyme fat extraction is considered an environmentally-friendly technology, which employs enzymes which hydrolyze the constituents of the material's cell walls, according to the structural nature of each raw material, where the lipid fraction is associated, making the structure more permeable and exposing the fat. One of the advantages of this process is the use of mild temperatures which reduce the degradation of thermo-sensitive compounds such as pigments and antioxidants, ensuring higher quality to the final product, meeting the demand for quality and safety based on alternative technologies, eliminating the use of solvents and substantially reducing energy consumption compared to conventional methods (Yusoff *et al*., 2017).

The enzymes used for the extraction of oils and fats frequently reported in the literature are protease, α-amylase, cellulase and pectinase, which act on the main constituents of the cell wall and are directly responsible for the oil efficiency of the extraction. In addition to the enzyme, parameters such as pH, temperature, particle size and agitation can contribute to the extraction efficiency. Several researchers have used the enzymatic extraction process to obtain oils and fats in various vegetable matrices (Silva *et al*., 2019). In some of these studies they obtained oil efficiency greater than 80% (Teixeira *et al*., 2013; Hu *et al.,* 2019).

Cupuassu seeds constitute waste material from the production of fruit pulp, and could be used for fat extraction, in view of the greater demand for oils and fats, and considering that the characterization of the quality attributes of cupuassu fat obtained by aqueous-enzymatic extraction has not yet been reported, the present study aimed to use a more sustainable technological process to obtain fat from cupuassu with physicochemical characteristics, technological properties and nutritional quality superior to those obtained by traditional methods.

2. MATERIALS AND METHODS

2.1. Characterization of raw material

The cupuassu seeds (20 kg) from the 2018 harvest were donated by the *Cooperativa dos Fruticultores de Abaetetuba* (COFRUTA), located in the city of Abaetetuba-Pa, and transported to the Food Analysis Laboratory of the Federal University of Pará (Belém – Pará, Brazil).

Fresh seeds were characterized for moisture, lipids, antioxidant capacity (ABTS) and total phenolics.

2.1.1. Moisture and lipids

Moisture content (method no. 925.10) and total lipids (method no. 922.06) were determined according to the official methodologies of AOAC (2002). The value of total lipids found in the lipid extraction with solvent was considered as the maximum extraction efficiency for the purpose of comparison with the value of lipids found in aqueous enzymatic extraction.

2.1.2. Antioxidant activity (ABTS)

The quantification of antioxidant activity was performed by the ABTS radical method as described by Re *et al*. (1999). Results were expressed as μMol of Trolox equivalents per g of sample.

2.1.3. Total phenolic compounds

The concentration of total phenolic compounds was determined by the Folin-Ciocalteu method described by Singleton and Rossi (1965). The colorimetric method is based on the ability to reduce phosphomolybdic and phosphotungstic acid by the hydroxyl groups of phenols, producing a blue color. Results were expressed as µg of gallic acid equivalent/g of sample (µg EAG g^{-1}).

2.2. Enzymes

The enzymes Celluclast® 1.5L, (700 U g^{-1} of EGU activity), Pectinex® Ultra SP-L (3800 U g^{-1} of PGNU activity) and Alcalase® 2.4L FG (2.4 U g^{-1} of AU-A activity), were kindly donated by a commercial representative of Novozymes® enzymes (Bento Gonçalves, Rio Grande do Sul, Brazil)

2.3. Enzymatic aqueous extraction

2.3.1. Pre-treatment of raw material

To improve the efficiency in the extraction of fat from the cupuassu seed, the skin was removed and then the almond underwent three pre-treatments: drying in an oven with air circulation (Ethik Technology®, 400-2^{ND}, Vargem Grande Paulista, São Paulo, Brazil) (60 °C;18 hours), autoclaving (121 °C; 1 minute), autoclaving (121 °C; 1 minute) and dry (60 ºC; 18 hours) and fresh. The pre-treatment which showed the highest fat extraction efficiency was autoclaved and drying, and thus was the one used in studies on enzymatic extraction conditions. (Figure 1).

After pre-treatment, the dried almonds were ground in a domestic blender (Walita) and sieved in a Tyler series sieves with 20-mesh granulometry (selected based on previous laboratory work) to standardize the particle size and facilitate the extraction process. The crushed product was packed in transparent plastic bags with a capacity of 300 g and stored at room temperature (25 ºC) until the tests were carried out.

2.3.2. Enzymatic aqueous extraction process

The enzymatic extraction process was performed according to the method proposed by Teixeira *et al.* (2013). The conditions used for the extraction study were selected according to published works (Rosenthal *et al.*, 2001), solute:solvent 1:5 (m:w), orbital shaker (Lucadema, Brazil) at 120 rpm, 60 °C, for 8 hours and enzyme concentrations (cellulase, pectinase and protease) 1.0% according to the manufacturer's recommendation. After 8 h of extraction the enzymes were inactivated at 80 °C for 5 minutes and the mixture was centrifuged for 20 minutes at 10,000 g, to separate the aqueous phase from the oil phase. The fat extraction efficiency was calculated according to Equation 1.

$$\text{Efficiency } \% = \frac{Wo\,(g)/\,Wp\,(g)}{Wt\,(g/g)}.100 \quad (1)$$

Where: *W*o is the mass of fat extracted by the enzymatic method (g), *W*p is the total mass of the sample used in the enzymatic extraction process (g) and *W*t is the mass of fat present in the sample extracted by solvent (g). After separating the phases of the mixture, the oily (fat) and aqueous (aqueous extract) fractions were characterized.

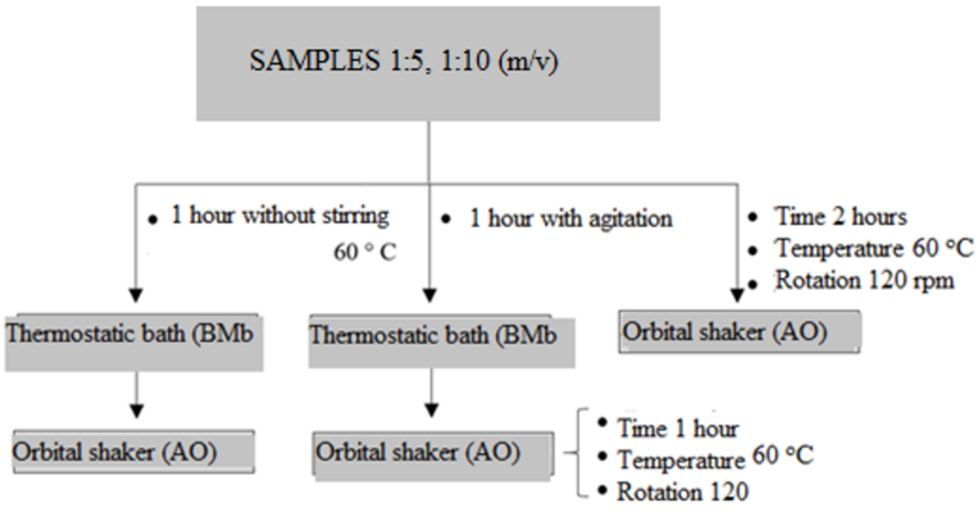

Figure 1. Evaluation of different incubation systems as pre-treatment for the enzymatic aqueous extraction of fat from cupuassu seeds.

2.3.3. Cupuassu seed fat characterization

Fatty acid composition. Fatty acid composition was determined by converting fatty acids into methyl esters (FAMEs) based on the method proposed by Rodrigues *et al.* (2010) and identified using a gas chromatograph (Varian model CP 3380, Texas City, USA) equipped with a flame ionization detector and CP-Sil 88 capillary column (length 60 m, internal diameter 0.25 mm, thickness 0.25 mm). The results were expressed as a relative percentage of the total fatty acids.

Physical chemical Indexes. Acidity was determined according to the Cd 3d-63 method (AOCS, 2004), peroxide value was determined according to the Cd 8-53 method (AOCS, 2004), iodine value was determined by the indirect method Cd 1c-85 (AOCS, 2004), saponification was determined by the indirect method Cd 3b-76 (AOCS 2004).

Oxidative stability. Oxidative stability was determined using the Rancimat equipment (Rancimat Metrohm model 873, USA) according to the method Cd 12b-92 (AOCS, 2004) at a temperature of 130 °C and an air flow of 20 $L \cdot h^{-1}$.

Nutritional parameters. Atherogenicity (*AI*) and thrombogenicity (*TI*) indexes were calculated based on the fatty acid profile, Equations 2 and 3, respectively, according to the methodology proposed by Ulbricht and Southgate (2001).

$$AI = \frac{C12{:}0 + 4\ x\ C14{:}0 + C16{:}0}{\Sigma MUFA + \Sigma FA\omega 6 + \Sigma FA\omega 3} \quad (2)$$

$$TI = \frac{C14{:}0 + C16{:}0 + C18{:}0}{(0.5\ x\ \Sigma MUFA) + (0.5\ x\ \Sigma FA\omega 6) + (3\ x\ \Sigma FA\omega 3) + (\Sigma FA\omega 3 / \Sigma FA\omega 6)} \quad (3)$$

Where C12:0, C14:0, C16:0, and C18:0 are relative percentage masses of lauric, myristic, palmitic, and stearic acids, respectively; *MUFA* is the relative percentage mass of monounsaturated fatty acids; *FAω6* and *FAω3* are the relative percentage mass of omega-3 fatty acids and omega-6 fatty acids, respectively.

Solid fat content. Solid fat content was determined by nuclear magnetic resonance (NMR) (Bruker pc120 Minispec, German) by the direct method, at temperatures of 10, 20, 25, 30, 35, 40 and 45 °C, according to the Cd 16b-93 method (AOCS, 2004).

Thermal analysis. The evaluation of the thermal decomposition profile, TG and DTG, of the fat was carried out by thermogravimetric analysis, under the following conditions: heating rate of 10 $°C \cdot min^{-1}$, temperature range from 27 to 600 °C and flow of 50 mL $nitrogen \cdot min^{-1}$.

Fourier transform infrared spectrophotometry (FTIR). For the ATR-FTIR analyses, Shimadzu Corporation IR Prestige 21 Cat. No. 206-73600-36-Kyoto-Japan was used. All spectra were obtained in the range of 4000 – 600 cm^{-1}, with a resolution of 4 cm^{-1} and 32 scans. Origin Pro v8.0 software was used to graphically plot the extracted spectra.

Table 1. Preliminary tests for cupuassu seed fat extraction efficiency (2 h extraction time, cellulase enzyme)

		Efficiency (%) ± SD			
Conditions	**Dilution**	**Dry**	**Autoclaved**	**Autoclaved + Dry**	**Control**
BMa	1:5	28.02b ± 0.38	16.10c ± 0.15	57.44a ± 0.26	11.62d ± 0.65
	1:10	28.18b ± 0.17	11.57c ± 0.13	55.15a ± 0.27	10.33c ± 0.58
BMb	1:5	36.56b ± 0.24	13.81c ± 0.86	63.86a ± 0.10	11.12d ± 0.37
	1:10	28.18b ± 0.17	11.36c ± 0.19	56.93a ± 0.25	10.74c ± 0.38
AO	1:5	42.90b ± 0.15	28.24c ± 0.20	69.95a ± 0.11	17.35d ± 0.25
	1:10	32.77b ± 0.16	12.33c ± 0.14	61.74a ± 0.75	10.11d ± 0.20

Different letters on the same line indicate a statistical difference at 5% significance. SD: Standard deviation; BMa: Thermostatic bain without stirring. BMb: Thermostatic bain with agitation. AO: Orbital shaker. SE: Dry. AU: Autoclaved. AU+SE: Autoclaved + Dry. CO: Control.

Antioxidant activity. Antioxidant activity of the cupuassu seed fat and aqueous extract were determined by the ABTS radical method as described by Re *et al.* (1999). Results were expressed in µMol of Trolox equivalents per g of sample. The concentration of total phenols was determined by the Folin-Ciocalteu method described by Singleton and Rossi (1965).

2.4. Statistical analysis

All analyses were performed in triplicate. The results were statistically evaluated by analysis of variance (ANOVA) and Tukey's test for comparison of means, at the level of 5% probability, using the STATISTICA 12.0® software.

3. RESULTS AND DISCUSSIONS

3.1. Physicochemical characteristics of cupuassu seed fats

The cupuassu seeds studied in this work had a high moisture content (68.25 ± 0.49%) and the lipid content was 45.03 ± 0.58% (d.b.), consistent with data found in the literature (Silva et *al.*, 2018). The concentration of phenolic compounds and the antioxidant capacity observed in fresh cupuassu seeds was 1,503.07 ± 22.38 µg EAG·g^{-1} and 320.42 ± 9.48 µmol Trolox·g^{-1} of sample, respectively. The content of phenolic compounds in cupuassu seeds was close to that reported for cocoa beans (1,441 µg EAG·g^{-1}) in the study conducted by Hu *et al.* (2016). Matrices rich in lipid content and bioactive compounds were present, in addition to other benefits, such as the potential antioxidant capacity, a characteristic directly related to the stability and quality of vegetable oils or fats in general (Hu *et al.*, 2016).

3.2. Cupuassu seed oil aqueous enzymatic extraction

Based on preliminary tests, the test with the highest extraction yield (69.95 ± 0.11) was the one in which the raw material was subjected to autoclaving followed by drying, confirming that the heat applied during this step helps to break the wall of the plant cell and facilitates the extraction of fat. Along with this, the most advantageous incubation condition was found when the orbital shaker was used. Efficiency values were maximized when using the lowest solution dilution value (1:5).

Under these previously defined conditions (Table 1), the results of oil extraction efficiency at different times for the three enzymes studied are shown in Table 2. It was observed that as the time increased, the greater the fat extraction efficiency was, until it reached 6 hours, and was kept constant for up to 8 hours of extraction. This behavior was expected, as the longer the enzyme-substrate contact time, the greater the fat extraction (Rosenthal *et al.*, 2001). The fat extraction efficiency values found in the processes for the studied enzymes ranged from 31.61 to 81.66%, indicating that the the enzymatic process may be a viable alternative for obtaining fat from cupuassu seeds, resulting in a product obtained from an environmentally-friendly process which is suitable for the Amazon biome.

The protease enzyme was the one that obtained the highest values for fat extraction efficiency from cupuassu seeds (81.66%), an expected behavior, because protein is one of the main components of this seed (15.9%, d.b) (Silva *et al.*, 2018). The efficiency values found under this condition are comparable to those obtained by traditional methods, indicating once again the feasibility of its use in commercial

Table 2. Kinetics of enzymatic extraction (cellulase, pectinase and protease) from cupuassu seed fat.

Enzymes	**Enzyme extraction efficiency (%)***				
	Time (h)				
	0	**2**	**4**	**6**	**8**
Protease	36.83 ± 0.28e	58.24 ± 0.23d	69.70 ± 0.41c	80.86 ± 0.21b	81.66 ± 0.16a
Pectinase	33.07 ± 0.53e	53.72 ± 0.27d	64.36 ± 0.33c	70.03 ± 0.23b	75.07 ± 0.32a
Cellulase	31.61 ± 0.51e	54.65 ± 0.18d	56.21 ± 0.17c	68.50 ± 0.19b	77.60 ± 0.29a

*: Calculated as a function of fat content determined by solvent extraction - Soxhlet (45.03 ± 0.58%) (Equation 1). The result presented is the triplicate mean ± standard deviation. Means followed by different letters in the same row are significantly different by Tukey's test (p ≤ 0.05.

processes (Teixeira *et al*., 2013; Hu *et al*., 2019). It is important to highlight that the use of enzymes, in addition to minimizing environmental impacts, produces oils/fats in milder conditions and with their original characteristics.

Similar oil efficiency results were found by Teixeira *et al.* (2013) in the study of enzymatic extraction from palm pulp (82.35%), using different combinations of enzyme blends (pectinase, cellulase and tannase) and by Santos *et al.* (2022), with the enzymatic extraction of oil from tucumã-i-da-várzea pulp, with pectinase, reaching an extraction efficiency of 81.51%.

Compared to the conventional processes of extraction of oils and fats usually employed, such as pressing and solvent extraction, which have efficiency of between 50 and 80%, the enzymatic extraction proved to be effective in the recovery of fat from the cupuassu seed, and also with the advantage of not presenting a risk of contamination with chemical residues and the use of relatively low extraction temperature, which is considered to be important for the quality of the final product (Daukšas *et al*., 2012; Jiao *et al*., 2014; Hu *et al*., 2019).

In addition to the advantages presented in relation to the extraction process is the quality of the fat obtained, another advantage presented in the aqueous extraction is the generation of the aqueous extract, a by-product which is rich in antioxidants, and can expand the possibilities of use in the industry for the development of new products and for adding value to the products obtained from the Amazon's oilseeds.

3.3. Cupuassu seed fat characterization

Cupuassu seed fat samples obtained from the extraction of 8 hours, were characterized for three enzymes.

3.3.1. Physicochemical characterization

Table 3 presents the characterization of cupuassu fat obtained by the enzymatic process (cellulase,

Table 3. Physicochemical composition and properties of cupuassu seed fat enzymatic extraction (cellulase, pectinase and protease) (CFE).

Fatty acids (%)	**Celulase**	**Pectinase**	**Protease**	**Bezerra *et al.* (2017)**[*]
Palmitic acid (C16:0)	8.02 ± 0.13^{a}	7.92 ± 0.20^{a}	7.44 ± 0.11^{a}	7.46 ± 0.36^{a}
Stearic acid (C18:0)	33.34 ± 0.25^{b}	36.62 ± 0.41^{a}	33.92 ± 0.34^{b}	31.45 ± 0.35^{c}
Oleic acid (C18:1, ω-9)	41.84 ± 0.36^{ab}	40.78 ± 0.28^{b}	42.5 ± 0.24^{a}	41.91 ± 0.91^{ab}
Linoleic acid (C18:2, ω-6)	3.56 ± 0.14^{a}	3.71 ± 0.30^{a}	3.58 ± 0.01^{a}	2.95 ± 0.09^{b}
Linolenic acid (C18:3, ω-3)	10.49 ± 0.31^{b}	10.98 ± 0.19^{ab}	10.59 ± 0.05^{b}	11.59 ± 0.32^{a}
Araquidic acid (C20:0)	0.47 ± 0.01^{a}	0.45 ± 0.00^{b}	0.45 ± 0.00^{b}	-
Behenic acid (C22:0)	1.53 ± 0.01^{a}	1.64 ± 0.02^{a}	1.67 ± 0.02^{a}	1.68 ± 0.16^{a}
∑ SFA	43.28	46.60	43.42	40.59
∑ MUFA	41.92	40.80	42.41	41.91
∑ PUFA	14.06	14.70	14.17	14.54
AI	0.14	0.14	0.13	0.13
TI	0.72	0.76	0.71	0.64
Iodine index (g I_2·100 g^{-1})	69.68	70.26	70.38	64.1
Saponification index (mg KOH·g^{-1})	190.61	190.69	190.61	191.12
Acidity index (mg KOH·g^{-1})	1.26 ± 0.05^{b}	1.14 ± 0.18^{b}	1.07 ± 0.03^{b}	11.46 ± 0.87^{a}
Peroxide value (meq O_2·kg^{-1})	5.69 ± 0.21^{b}	5.77 ± 0.19^{b}	5.46 ± 0.11^{b}	14.28 ± 0.95^{a}
Oxidactive stability (h)	14.15 ± 0.29^{a}	14.20 ± 0.11^{a}	14.26 ± 0.17^{a}	2.38 ± 0.17^{b}
Bioactive compounds				
Oil phase				Fresh seed
ABTS (µmol Trolox·g^{-1})	18.29 ± 1.40^{b}	18.74 ± 1.74^{b}	22.09 ± 1.08^{b}	320.42 ± 22.38^{a}
TPC (µg EAG·g^{-1})	134.50 ± 12.57^{b}	125.54 ± 10.36^{b}	141.84 ± 12.57^{b}	$1{,}503.07 \pm 9.48^{a}$
Aqueous phase				
ABTS (µmol Trolox·g^{-1})	106.26 ± 4.26^{b}	110.27 ± 3.94^{b}	122.76 ± 4.70^{b}	320.42 ± 22.38^{a}
TPC (µg EAG·g^{-1})	897.06 ± 15.31^{b}	838.24 ± 15.72^{c}	926.47 ± 22.61^{b}	$1{,}503.07 \pm 9.48^{a}$

The result presented is the mean of triplicate ± standard deviation. *SFA*: saturated fatty acids; *MUFA*: monounsaturated fatty acids; *PUFA*: polyunsaturated fatty acids; *AI*: atherogenicity index; *TI*: thrombogenicity index.
Means followed by different letters in the same row are significantly different by Tukey's test ($p \leq 0.05$).

pectinase and protease) (CFE) and of the commercial fat (CCF) obtained from the literature, in work carried out with cupuassu fat obtained by cold extraction. The main fatty acids found in cupuassu seed fat extracted by the enzymatic method were oleic acid (42.35%), stearic acid (33.72%) and linolenic acid (10.59%), a composition that gives greater plasticity to cupuassu seed fat. These fatty acids and most of the others are in accordance with the profile found by Bezerra *et al.* (2017).

Enzymes can influence the concentration of fatty acids, which can be attributed to the action on different tissues of the cupuassu seed according to the way the enzyme works, as well as to the bonds of fats in the seed. However, despite these differences, the general fat profile is not significantly altered for all enzymes. Cupuassu fat extracted with pectinase showed the highest concentration of stearic acid ($p < 0.05$), and the lowest concentration of oleic acid, which presented the highest concentration in the fat extracted by protease.

Cupuassu seed fat has higher levels of unsaturated fatty acids in relation to cocoa and murumuru fats, and can be considered high quality nutritional fat due to the high content of monounsaturated fatty acids, such as oleic acid which play an important role in the prevention of retinal and cardiovascular diseases and in the formation of nerve cells, among other benefits (Rodrigues *et al.*, 2010; Bhattacharjee *et al.*, 2020).

Oleic acid (ω-9) is the most important in the group of monounsaturated fatty acids, and is present in high concentrations in cupuassu seed fat, and traditionally found in vegetable oils, such as olive, canola, avocado and oil seeds, nuts, walnuts and almonds, which makes it another source of lipids in the diet (Garcia-Aloy *et al.*, 2019).

In addition, cupuassu fat extracted by enzymes presented high saturated fatty acids (42.43%), which has a beneficial effect on thermal stability, suggesting that this fat may be useful in the food industry as a frying fat. These characteristics show the versatility of cupuassu seed fat for the food industry (production of cupulate, fat for frying, source of ω-9) in addition to its consolidated use by the cosmetic industry (Connor, 2000; Rodrigues *et al.*, 2010).

The iodine index of cupuassu seed fat was 70.38 g $I_2 \cdot 100$ g^{-1}, close to the value found by Bezerra *et al.* (2017) of 64.1 g $I_2 \cdot 100$ g^{-1}. These values are in agreement with the fatty acid compositions, raw materials with high contents of unsaturated fatty acids (Dubois *et al.*, 2007; García-González *et al.*, 2013).

The Codex Alimentarius Commission (1999) established the limits for maximum acidity and peroxide values for cold-pressed and unrefined oils and fats, as 4.0 mg KOH·g^{-1} and 15 meq $O_2 \cdot$kg^{-1}, respectively. Ther acidity index (1.07 mg KOH·g^{-1}) and peroxide value (5.46 meq $O_2 \cdot$kg^{-1}), of cupuassu seed fat showed values within the established standard indicating that the extraction method used as well as the fat storage was adequate.

Oxidative stability is an important property of oils and fats and is expressed as the time required for the formation of oxidation by-products which are detected under different conditions (expressed by the induction period - *IP*) (Pardauil *et al.*, 2011). Cupuassu seed fat extracted by the enzymatic process presented high oxidative stability (14.26 h), indicating that it has a high temperature resistance which is compatible with refined fats such as palm (Teixeira *et al.*, 2013) and coconut (Mohammed *et al.*, 2021). In addition to the fatty acid profile, oxidative stability may also be related to the concentration of antioxidants present in the fat (Pardauil *et al.*, 2011).

In general, the oxidative stability of vegetable oils such as cotton (1.50 h), canola (1.85 h), soybean (1.51 h) and sunflower oils (0.88 h) is lower due to the predominance of unsaturated fatty acids in their composition (Anwar *et al.*, 2003).

3.3.2. Nutritional quality

Atherogenicity (*AI*) and thrombogenicity (*TI*) indexes are directly related to the potential to stimulate platelet aggregation. Lipids with lower *AI* and *TI* values have a greater potential to prevent coronary heart disease; values lower than 1.0 and 0.5, respectively, are recommended in terms of human health (Fernandes *et al.*, 2014). Cupuassu fat has a value for *AI* (0.13) which indicates that these samples have high levels and equivalents of anti-thrombogenic fatty acids and *TI* results presented a value of 0.71, higher than indicated for this parameter, due to the saturated fatty acid composition of these products, especially stearic acid (Table 3).

Bezerra *et al.* (2017), analyzed oils and fats from some Amazonian raw materials, including cupuassu seed fat, and found *AI* and *TI* indexes, which ranged from 0.02 to 1.03 and 0.14 to 2.01, respectively.

Santos *et al.* (2022), found values of *AI* (0.09) and *TI* (0.6) in tucumã-i-da-várzea pulp oil. The results indicate the nutritional potential of these Amazonian oil and fat sources.

3.3.3. Antioxidant activity

Cupuassu seed fat showed potential antioxidant activity of 22.09 and 122.76 µmol Trolox·g^{-1} and total phenol values of 141.84 µg EAG·g^{-1} (Table 3) and in aqueous phase (926.47 µg EAG·g^{-1}).

These results suggest that the fat obtained by enzymatic extraction has technological properties which are superior to the fat extracted by the conventional method, and can be better explored and absorbed by the industrial sector of the pharmaceutical, cosmetic and food areas, in addition to representing a sustainable friendly technique that collaborates in the preservation of the environment and encourages the use of residues from the agro-industry.

The determination of phenolic compounds in oils and fats is considered necessary, as it is one of the important indicators of oil quality. These compounds are responsible for the ability to scavenge free radicals and lipid peroxidation.

Phenolic compounds are water soluble, that is, soluble in water, a fact that can be proven by the concentration of phenolics found in the oil phase (fat) 141.84 µg EAG·g^{-1} and in the aqueous phase (extract) 926.57 µg EAG·g^{-1} (Hayouni *et al.*, 2007). The enzymatic extraction process makes it possible to potentiate the use of antioxidant compounds, since the fat-soluble compounds present in the raw material remain in the fat and the water-soluble compounds are extracted by water, thus producing two extracts. Teixeira *et al.* (2013), characterized palm oil in terms of the content of phenolic compounds and found values between 14.76 and 26.43 µg EAG·g^{-1} oil. Jiao *et al.* (2014), used the enzymatic aqueous extraction to obtain pumpkin seed oil and found that this extraction (128.8 µg EAG·g^{-1}oil) had a greater effect on phenolic compounds than the Soxhlet extraction (73.3 µg EAG·g^{-1} oil), confirming the efficiency of this extraction technique for the preservation of bioactive compounds.

According with the physicochemical properties of the cupuassu seed fat obtained by the enzymatic extraction process, it can be verified that for all properties, the values found are close (fatty acid profile and *AI* and *TI*) or higher (oxidative stability, acidity, peroxide) than presented by commercial fat, with emphasis on the quality and antioxidant compounds.

3.3.4. ATR-FTIR

The ATR-FTIR spectra, obtained in the region from 4,000 to 500 cm^{-1}, of the cupuassu seed fat samples (extracted by cellulase, pectinase and protease) are shown in Figure 2. Table 4 shows the wave number (cm^{-1}) of the peaks identified in the spectra for the cupuassu fat samples extracted with

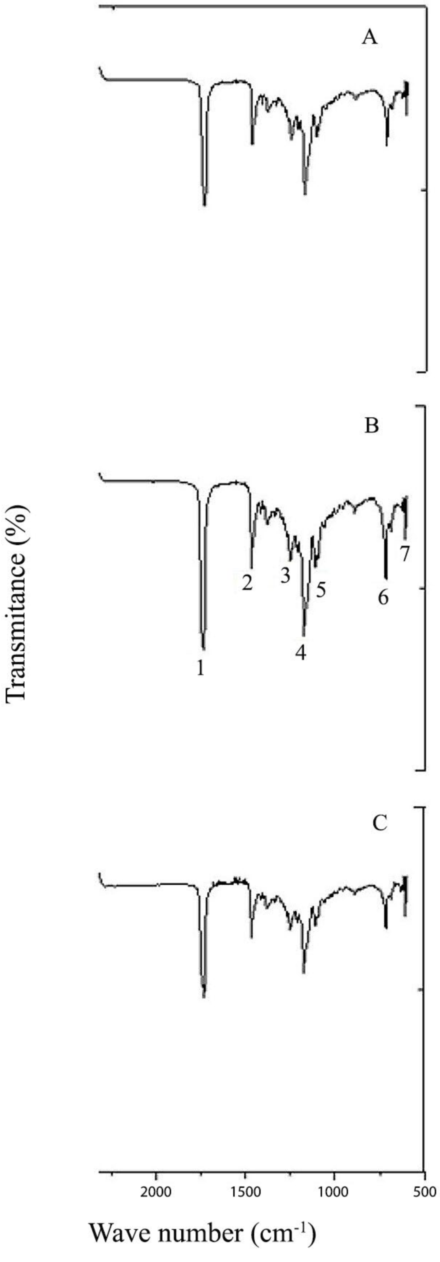

Figure 2. Infrared vibrational spectra of cupuassu seed fat extracted by enzymatic process (CFE), celulase (A), pectinase (B), and protease (C).

Table 4. Wave number (cm^{-1}) of the peaks identified in the FTIR spectra of cupuassu seed fat samples extracted by cellulase, pectinase and protease.

Identification (Band)	Cellulase	Pectinase	Protease
		cm^{-1}	
1	2918	2914	2920
2	2851	2849	2850
3	1734	1744	1736
4	1468	1470	1466
5	1179	1177	1179
6	717	717	719
7	609	608	608

cellulase, pectinase and protease, where it can be observed that there is great similarity between the extracted fats.

The spectra obtained by FTIR show vibrational modes and combinations of functional groups of fatty acids present in the chemical composition of the cupuassu seed fat, with higher intensity absorption bands in the region of 3,000 to 2,800 cm^{-1}, which can be attributed to vibrations of axial deformation of the *CH* bonds of the methyl (CH_3), methylene (CH_2) groups and of the double bonds ($=C–H$) .

The bands with intermediate intensity, which appear in the region of 1,500 to 1,300 cm^{-1}, originate from the angular deformation vibrations of the *C-H* bonds of the methyl and methylene groups. The band that appears approximately in the region of 1730 cm^{-1} is related to the axial deformation vibrations of the carbonyl group ($C=O$) present in the constituent ester groups of triacylglycerides. In the region from 1,300 to 900 cm^{-1}, which contains part of the "fingerprint" region of the compounds, are the absorption bands referring to the axial deformation vibrations of the *CO* bond of the constituent esters of the triacylglycerides (García-González *et al.,* 2013). Finally, the peak at 719 and 609 cm^{-1} can be attributed to the benzene ring.

It can be observed that enzymatic extraction with the three enzymes tested did not cause changes in the chemical characteristics of cupuassu seed fat. The properties found in this work for cupuassu seed fat support future work because of the industrial interest in this material and because the results found offer important data for the elaboration of programs to control the composition of Amazonian fats as ingredients.

3.3.5. Solid fat content

In addition to physicochemical properties, other specific attributes of fats define their applications, such as physical properties, and among them, solid fat content is one of the most important. An analysis of the solid fat content (*SFC*) at 20 °C provides information about the resistance to oil exidation of a lipid matrix, which should not be below 10% (Bezerra *et al*., 2017).

Table 5 illustrates the solid fat content of cupuassu fat extracted by the enzyme process (CFE), cupussu fat extracted with solvent and cocoa fat (CF) (Zarringhalami, 2021), at temperatures of 10, 15, 20, 25, 30, 35, 40 and 45 ºC.

The information from the solid fat content curve as a function of temperature is used to predict the applicability of a fat. It is responsible for many product characteristics such as margarines, shortenings and spreads, including their general appearance, ease of packaging, oil exudation, sensory and melting properties and consistency (Rao *et al*., 2001).

Table 5. Solid fat content in cupuassu seed fat extracted by the aqueous enzymatic process and for cacao fat, as a function of temperature.

Samples	Content of solid fat (%)						
	ºC						
	10	20	25	30	35	40	45
CFE	68.28 ± 0.31^{b}	49.11 ± 0.38^{b}	33.06 ± 0.46^{b}	2.33 ± 0.08^{b}	0.11± 0.00^{b}	0.27± 0.00	0.07± 0.00
CCF	63.12 ± 0.28^{c}	39.85 ± 0.36^{c}	19.23 ± 0.31^{c}	1.08 ± 0.19^{c}	0.02 ± 0.00^{c}	0.00	0.00
CF*	94.80 ± 0.10^{a}	78.60 ± 0.30^{a}	26.90 ± 0.30^{a}	8.49 ± 0.02^{a}	0.76± 0.03^{a}	0.00	0.00

CF: Cocoa fat; * Zarringhalami (2021). The result presented is the mean of triplicate ± standard deviation. Means followed by different letters in the same column, are significantly different by Tukey's test ($p \leq 0.05$).

It can be observed that the enzyme-extracted cupuassu fat differs statistically from the commercial cupuassu fat, which is significantly higher ($p < 0.05$) in terms of solid contents at all temperatures tested. This is another important difference between the extraction methods and an additional proof that the enzymatic process preserves the identity of the extracted fat as well as the oxidative stability, the peroxide index and iodine value, which showed differences between the commercial sample and the one extracted in an enzymatic process. Although the fatty acid general profile did not show differences, the cupuassu fat obtained by the enzymatic process is different from the same commercial fat.

Cupuassu fat has been used in the preparation of cupulate. The solid contents in cocoa fat (Table 5) exceed that of the cupuassu fat sample extracted by enzymes up to 35 °C, while at 40 and 45 °C, it resembles commercial cupuassu fat. Although the solid contents in cocoa fat show this behavior, studies have been conducted using blends and modifications in order to achieve the ideal properties in these fats (Zarringhalami *et al.*, 2021), which contributes to improving the properties of fats and expands opportunities for applications of these materials.

3.3.6. Thermal analysis

The thermogravimetric profile indicates the effect of temperature on fat degradation. To evaluate the thermal behavior in a nitrogen atmosphere, the fat was heated to a temperature of 600 °C at a heating rate of 10 °C·min^{-1} (Figure 3).

The *TG* analyses indicated stability in the sample up to 345 °C, with great mass loss at around 440 °C, until complete decomposition at around 480 °C. This loss increases with the rise in temperature, which is higher than the operating temperature normally used in most activities involved in food preparation. This event can be better visualized by the decay of the baseline, as shown by the *DTG* curve, confirming the decomposition of the samples at temperatures above 345 °C.

Cupuassu seed fat was degraded at very high temperatures, due to the fact that it has a relatively high proportion of saturated compounds, making it a thermally stable fat, which is the second indication of fat in frying with the generation of few degradation compounds, which may be associated with safety in consumption and for the environment in the cooking process, considering the stability at the temperatures practiced, which are below that determined as the degradation temperature of this fat.

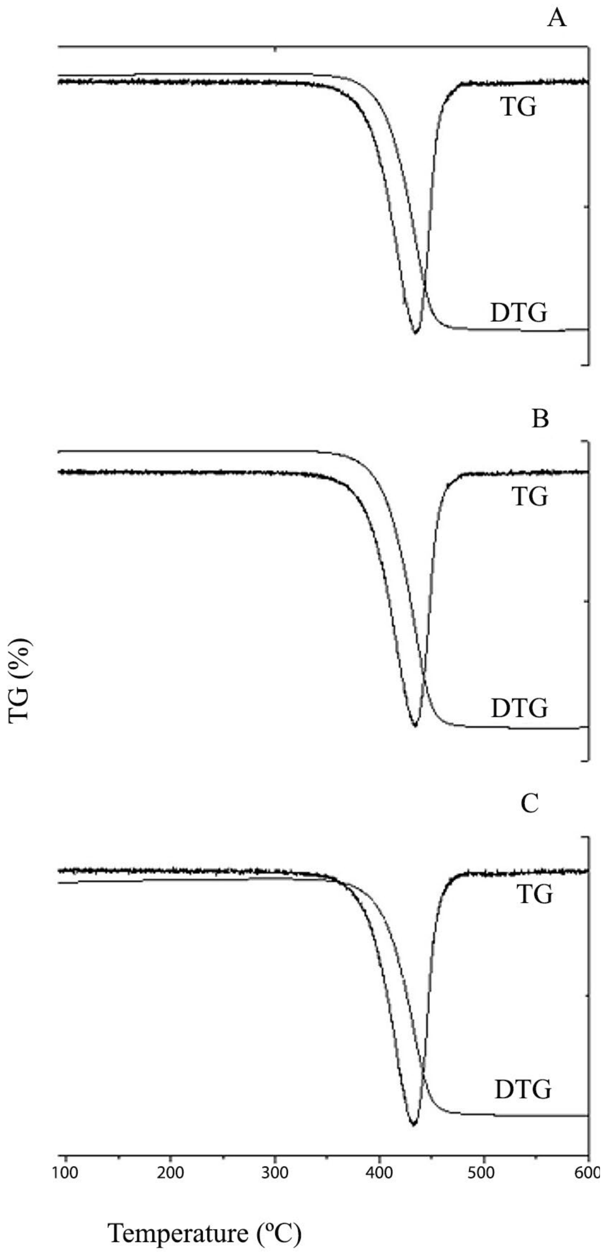

FIGURE 3. TG – DTG curves of cupuassu seed fat samples extracted by celulase (A), pectinase (B), and protease (C), in a nitrogen atmosphere, heated to 600 °C at a heating rate of 10 °C min^{-1}.

The cupuassu fats extracted by different enzymes showed similar thermal behavior (Table 6) regarding the *on-set* and *end-set* of temperature. However, regarding the mass loss and residue, the cupuassu fat sample obtained by protease showed the lowest value for mass loss and the highest value for waste. This

Table 6. Thermal behavior by thermogravimetry of cupuassu fat samples extracted by cellulase, pectinase, and protease.

Enzymes	Temperature *On-set* (°C)	Temperature *End-set* (°C)	Lost of mass (%)	Residue (%)
Cellulase	405.94	450.73	97.14	2.86
Pectinase	405.55	450.28	98.21	1.79
Protease	406.77	449.16	95.95	4.05

response may be related to the characteristics of the fat, as confirmed by the data shown in Table 3, with slightly lower concentrations of monounsaturated fatty acids.

These results suggest the need for future works with more detailed analyses regarding the specific characteristics of cupuassu seed fat extracted with enzymes and by pressing which can contribute to indicate the viability of the enzymatic process, as well as studies for the valorization of the aqueous extract obtained as a by-product with antioxidant properties.

4. CONCLUSION

The enzymatic aqueous extraction process presented results for fat extraction efficiency similar to those obtained by the conventional extraction method.

The extracted fat showed better quality parameters (fat solid content, acidity, iodine index and oxidative stability) and a higher concentration of bioactive compounds than the fat obtained by the pressing extraction method currently adopted by the industry.

The cupuassu seed fat extracted by the aqueous enzymatic process showed several important properties that justify its potential for application in the food industry.

The results presented in this work contribute to the understanding of the enzymatic extraction process as an alternative to the conventional methodology for extracting fat from cupuassu seeds.

The aqueous enzymatic extraction process produced a superior quality fat, in addition to the aqueous extract, a by-product that presents prospects with bioactive properties, and is, therefore, an environmentally-friendly and sustainable technology.

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Conflict of Interest

The authors declare no conflict of interest.

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GC/MS quantification of individual fatty acids of selected green leafy vegetable foliage and their biodiesel attributes

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SUMMARY: The current demand for edible vegetable oil is increasing worldwide, and the development of new sources of high-quality vegetable edible oil is an essential task. There is also a huge demand for biodiesel in domestic and industrial applications, and foliage oils could be a good source for diesel applications. The current study aimed at the identification and quantification of fatty acids from commonly consumed green leafy vegetables (GLVs) viz., *Hibiscus cannabinus, Hibiscus sabdariffa, Basella alba, Basella rubra,* and *Rumex vesicarius* and to calculate the biodiesel attributes of the oil. The total oil content was ascertained as the highest in *R. vesicarius* foliage (3.91 ± 0.27 g/100 g dry leaf powder). GC/MS chromatographic investigation identified 9,12,15-octadecatrienoic acid as a significant compound followed by hexadecanoic acid. In *Hibiscus* spp. C18:3 (49.3 µmol % and 50.4 µmol %) was recorded to be the most noteworthy followed by C16:0 (23.2 µmol % and 21 µmol %) in *H. cannabinus* and *H. sabdariffa,* respectively. The GLVs foliage-fatty acid biodiesel attributes were additionally assessed through an empirical formula. Consequently, the overall examined results will be helpful for the investigation of these oils as vegetable oil for human consumption and biodiesel applications.

KEYWORDS: *Biodiesel properties; Fatty acids; Green leafy vegetables; Total oil*

RESUMEN: ***Cuantificación por GC/MS de la composición de ácidos grasos del follaje de hortalizas de hoja verde seleccionadas y sus atributos para biodiesel.*** La demanda actual de aceite vegetal comestible está aumentando en todo el mundo, y el desarrollo de nuevas fuentes de aceite vegetal comestible de alta calidad es una tarea esencial. También existe una gran demanda de biodiesel para aplicaciones domésticas e industriales, así, los aceites de follaje podrían ser una buena fuente para estas aplicaciones. El presente estudio tuvo como objetivo la identificación y cuantificación de ácidos grasos de vegetales de hoja verde (GLV) de consumo común, como, *Hibiscus cannabinus*, *Hibiscus sabdariffa*, *Basella alba*, *Basella rubra* y *Rumex vesicarius*, y determinar los atributos para el biodiesel de los aceite. El contenido total de aceite más alto se obtuvo para el follaje de *R. vesicarius* (3,91 ± 0,27 g/100 g de polvo de hojas secas). La determinación cromatográfica, GC/MS, identificó al ácido 9,12,15-octadecatrienoico como el ácido mayoritario, seguido del ácido hexadecanoico. En *Hibiscus* spp. el ácido C18:3 fue el mayoritario (49,3 µmol % y 50,4 µmol %), seguido de C16:0 (23,2 µmol % y 21 µmol %) en *H. cannabinus* y *H. sabdariffa*, respectivamente. Los características para el biodiesel de los ácidos grasos de follaje de GLV también se evaluaron empíricamente. En consecuencia, los resultados generales obtenidos serán útiles para investigaciones de estos aceites como aceites vegetales para uso humano y aplicaciones de biodiesel.

PALABRAS CLAVE: *Aceite total; Ácidos grasos; Hortalizas de hoja verde; Propiedades del biodiésel*

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1. INTRODUCTION

The current demand for edible vegetable oil is increasing worldwide (Wu *et al*., 2020). An enormous part of oils and fats are obtained from plants (leaves, seeds, and fruits), and most of them are used for human or industrial use (Diemeleou *et al*., 2014). Overall, 75% of vegetable oils are in a liquid state which is utilized in the food industry for frying, canning, and the preparation of margarine and emulsions (Salas *et al*., 2009). There are notable, well-known vegetable oils, for example, palm, peanut, coconut, and sunflower oils and so on (Gunstone, 2011).

Sustainable and affordable plant sources as alternate sources of renewable energy are gaining greater significance from an environmental perspective (Valenga *et al*., 2019). The post-harvest agricultural residues and seeds of a good number of plants have been explored in this regard (Kumar *et al*., 2020). However, few reports show the production of bio-oils from vegetative parts especially the foliage of plants which can be generated without difficulty (Hoseini *et al*., 2019). Biodiesel is a renewable fuel that is a sustainable option to meet energy demands compared to petroleum-derived diesel. Biodiesel will reduce the environmental effects of burning fossil fuels (Valenga *et al*., 2019).

Fatty acids are one of the main ingredients in edible oil, and many nutritional properties are related to them. The fatty acid composition, trans-esterification, long-chain saturated factor, and the degree of unsaturation provide information about the biodiesel properties of oils (de Freitas *et al*., 2019). These parameters could be used to estimate the cetane number, cold filter plugging point, iodine value, and other parameters of biodiesel (de Freitas *et al*., 2019). As these selected unexplored edible GLVs foliage oils are more affordable compared to exorbitant vegetable oils which are accessible commercially. This could be conceived as promising selective post-harvest foliage feedstock for biodiesel production because these GLVs are richly accessible all over the globe. In order to fulfil this expanding need for oil-yielding vegetable sources, we chose GLVs such as *Hibiscus cannabinus*, *Hibiscus sabdariffa*, *Basella alba*, *Basella rubra* and *Rumex vesicarius* for the GC/MS characterization of fatty acid composition. In addition, based on the fatty acid profiling, the biodiesel properties of the GLVs were calculated using empirical formulas for commercial exploration. To our knowledge, this is the first report on the forecast of biodiesel properties for the five chosen GLV foliage.

The genus Hibiscus contains more than 300 species of angiosperms (Kim *et al*., 2019). *Hibiscus cannabinus* and *Hibiscus sabdariffa* (Malvaceae), commonly known as kenaf and roselle are the two most common species. They are industrially important fibrous plants and are also known worldwide in the food and nutraceutical industries (Jin *et al*., 2013). In Ayurveda, the leaves are utilized for bilious, blood, diabetes, hacks, and throat issues (Jin *et al*., 2013). They are used in processed foods, as a flavoring agent, or in cold or hot beverages as an herbal remedy for hyperlipidemia, and hypertension (Paraíso *et al*., 2020). The oil extracted from seeds of *Hibiscus* spp. showed two unusual fatty acids such as di-hydro sterculic acid, and vernolic acids (Wang *et al*., 2012). The seeds of the plant contain omega-3-fatty acids, phenolics, sterols, and so forth (Wang *et al*., 2012).

In *Basella* spp. there are two popular varieties such as *Basella rubra* (red leaf, stem, and fruits) and *Basella alba* (green leaf and stem) belonging to Basellaceae (Kumar *et al*., 2018). The nutritional and antioxidant potentials of the leaf extracts of *Basella* spp have been reported (Kumar *et al*., 2015a). In addition, the nutraceutical possibilities of *B. rubra* fruit extracts on human cervical cancer cells (SiHa) have likewise been illustrated (Kumar *et al*., 2015b). Recently, the *B. rubra* seeds have been reported to contain 33% total oil with a good amount of nutraceutical compounds (phytosterols, tocopherols, polyphenols, and oryzanol) and 1% squalene has been reported (Kumar *et al*., 2020). The biochemical profile of *B. alba* seed oils relates to yield, specific gravity, color, and fatty acid composition (Diemeleou *et al*., 2014).

Rumex vesicarius L. (Polygonaceae) is referred to as blister sorrel, and rosy dock in English, and in Indian vernacular dialects it is well-known as chooka, chukka Kura, or chukki soppu. The leaves of the herb contain carotenoids, vitamin C, proteins, lipids, organic acids and minerals (Alfawaz *et al*., 2006). In folklore medicine, it is used for pain-relieving, as an astringent, hepatoprotective agent, remedy for tumours, and scurvy (Mostafa, 2014). Nonetheless, in all the chosen GLVs the information on the oil content, GC/MS fatty acid profiles, and biodiesel properties of the foliage had not been reported to date.

2. MATERIALS AND METHODS

2.1. Chemicals

HPLC grade hexane and anhydrous sodium sulfate were obtained from Sisco Research Laboratory (Mumbai, India) and Boron trifluoride solution (BF_3) was procured from Sigma Aldrich, Bangalore, India. All other chemicals used were of analytical grade.

2.2. Source of the fresh foliage material

The seeds of all five selected GLVs such as *Hibiscus cannabinus* L*, Hibiscus sabdariffa* L*, Basella alba* L.*, Basella rubra* L., and *Rumex vesicarius* L., were collected from the local markets of Tirupati, Andhra Pradesh, India. The seeds were sown in micropots containing sand:soil:compost (1:1:1) for seed germination, maintained at a controlled temperature and relative humidity under greenhouse conditions (Figure 1). The botanical confirmation of the selected plant was confirmed, and herbarium specimen was deposited at the Herbarium deposition center of the Department of Botany, University of Mysore, Mysore (Ref. No.02.08.13). The leaves of the mature plants were collected separately and dried in a hot air oven set at 55 °C for complete dryness. The dried leaf samples were made to a coarse powder using a mixer grinder (Maharaja Whiteline Perfect W&R 500 Mixer grinder) for 3 ± 0.2 min at high speed to maintain a uniform particle size and stored in polythene airtight bags until further use.

2.3. Determination of total oil content

The total oil was extracted from the above-prepared leaf powder samples with hexane in a Soxhlet extractor, wherein the samples were subjected to heating a round-bottom flask containing boiling chips on a water bath set at 60 ± 5 °C for about 8 h. The obtained hexane-oil mixture was filtered using Whatman filter paper no. 1 containing anhydrous sodium sulfate and the filtrate was evaporated under reduced pressure in a pre-weighed round-bottom flask in a rotary evaporator (Hei-VAP Advantage, Heidolph Instrument GmbH & Co. KG, Schwabach, Germany). The flasks were kept for one min in a hot air oven set at 100 °C to evaporate the leftover hexane residue, cooled in a desiccator, and the difference in weight of the flask was measured using a sensitive balance to determine the total oil content (AOCS, 2003). The obtained oil samples were stored at -20 °C in screw-cap vials until further use.

Figure 1. The five selected GLVs cultivated under greenhouse conditions a. *H. sabdariffa*, b. *H. cannabinus*, c. *B. rubra*, d. *B. alba,* and e. *R. vesicarius*. Scale bar is 20 cm.

2.4. Fatty acid analysis by GC/MS

The fatty acid methyl esters (FAME) were prepared for the five selected foliage oil samples in triplicate by trans-esterification (Figure 2), according to the AOCS Official Method (AOCS, 2003). Briefly, for 100 mg of oil sample, 1 mL of BF_3 methanol was added and kept in a water bath for 30 min at 60 °C. The tubes were immediately transferred into the ice bath for 5 min, and 1 mL hexane was added, followed by 1 mL distilled water and the tubes were vortexed for complete mixing. The reaction mixture tubes were kept aside for layer separation, and the upper layer was collected in a tube containing anhydrous sodium sulfate for the removal of moisture. Finally, the undisturbed top methyl ester layer free of water moieties and residual particles was transferred to GC vials for GC/MS analyses. 1 mg/mL heptadecanoic acid (C17:0) was added as the internal standard. Before GC/MS analysis, 0.2 µm nylon membrane filtered samples were used for analysis.

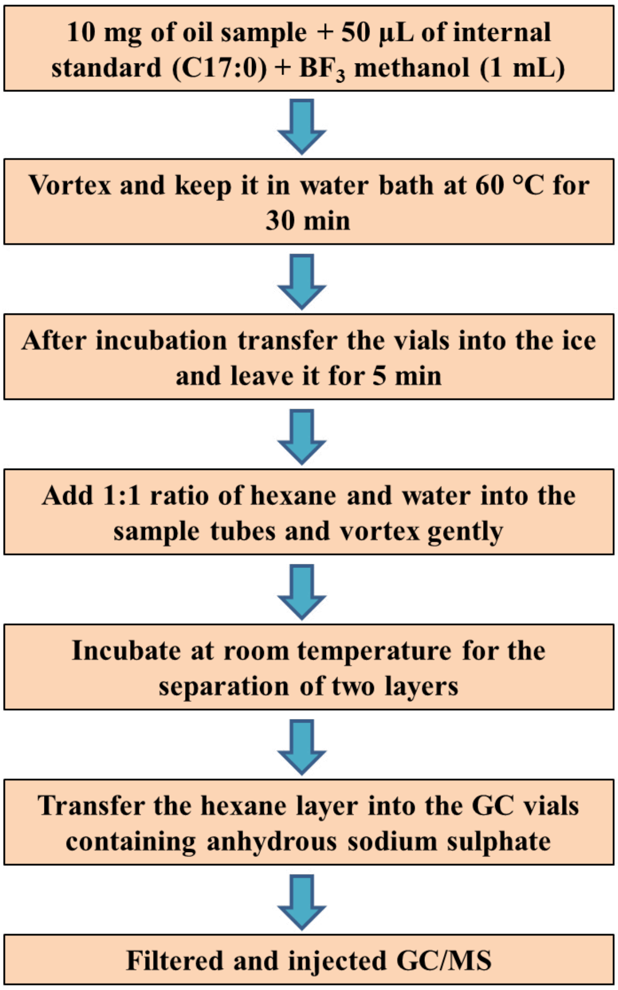

Figure 2. Block diagram representing the trans-esterification process of fatty acids. BF_3 – Boron trifluoride.

The GC/MS analysis was performed using an Agilent HP-7890B chromatograph connected directly to a 5977 inert mass spectrometer (Agilent Technologies, Milan, Italy), with GC column, DB-23 (60 m 0.25 mm I.D 0.25 mm film thickness). The analyses were performed in splitless mode (0.5 min), at 25 °C inlet temperature, with helium as carrier gas at a flow rate of 1 mL/min. The temperature was programmed at 10 °C/min to 300 °C, and then isothermal at 300 °C for 5 min. The MS detector was operated in electron ionization (EI) mode (70 eV, 200 mA), in full-scan mode (*m/z* 40-400), and also in selected–ion monitoring (SIM) mode (ions at *m/z* 127, 140, and 256 for heptadecanoic acid as the internal standard). The transfer line was set at 290 °C, and the solvent delay was set at 3 min.

2.5. Determination of biodiesel characteristics of the five selected foliage oils

2.5.1. Thermo-physical properties

Thermo-physical properties of the extracted oils for biodiesel properties such as density, kinematic viscosity and higher heating values were determined by using the modelled empirical equations (Srinivasan and Jambulingam, 2019):

Density (kg/m^3) = 881.86 - (0.07 × M) + (11.91 × D)
Kinematic viscosity ($\times 10^{-6}\, m^2/s$) = -5.59 + (0.04 × M) - (0.78 × D)
Higher heating value (MJ/kg) = 25.7+ (0.057 × M)- (3.16 × D)

Where, D = Number of Double bonds and M = Molecular Mass.

2.5.2. Viscosity determination

The absolute viscosity of the oils was determined by using the following relationship as reported by (Igbum *et al*., 2013):

$$\text{Viscosity } (\ln\eta) = -4.80 + 2525.93 \times \left(\frac{1}{T}\right) + 1.61 \times \left(\frac{(SV^2)}{T^2}\right) - (101.06 \times (IV^2) \times 10^{(-7)})$$

Where, T=Temperature, SV=Saponification value, IV=Iodine value, η=Viscosity and all the vales are constants.

2.5.3. Determination of ester content

The ester content of the oils was determined experimentally, and by using empirical correlations, the major biodiesel properties such as saponification value (SV), cetane number (CN), iodine value (IV) and degree of unsaturation (DU) were calculated (Madhubalaji *et al*., 2020):

$$\mathrm{SV} = \sum \frac{(560 \times \mathrm{N})}{\mathrm{M}}$$

$$\mathrm{CN} = 46.3 + \left(\frac{5458}{\mathrm{SV}}\right) - (0.225 \times \mathrm{IV})$$

$$\mathrm{IV} = \sum \frac{(254 \times \mathrm{D} \times \mathrm{N})}{\mathrm{M}}$$

$$\mathrm{DU} = \mathrm{MUFA} + (2 \times \mathrm{PUFA})$$

Where, SV – saponification value, CN – cetane number, IV – iodine value, D – number of double bonds, M – molecular mass, N – Percentage of each fatty acid, MUFA – monounsaturated fatty acids and PUFA – polyunsaturated fatty acids.

2.5.4. Biodiesel cold flow properties

Long-Chain Saturated Factor (LCSF) and Cold-Filter Plugging Point (CFPP) were also calculated using empirical correlations (Madhubalaji *et al*., 2020):

LCSF (°C) = (0.1 × C16:0 + 0.5 × C18:0 + 1 × C20:0) + 1.5 × (C22:0 + 2 × C24:0)
CFPP (°C) = 3.1417 × LCSF – 16.477

2.5.5. Heat of combustion

The gross calorific value H or heat content of vegetable oils is the quantity of heat evolved when one mole of oil is burnt to carbon dioxide (CO_2) and water (H_2O) may be obtained from its structure indices using the relationship:

H = 47.645 – [4.187(IV) + 38.31(SV)] kJ/kg

2.6. Carbon, Hydrogen, Nitrogen and Sulphur (CHNS) contents

The selected five oil samples were analyzed for CHNS content using an Elemental Analyzer (Vario EL III, Germany) where sulphanilic acid was used as a standard compound. Briefly, 5.0-10.0 mg of oil sample were filled in a tin capsule and weighed using an electronic balance (Sartorius). The percentage of CHNS content was determined (Sekhar *et al*., 2018).

2.7. Statistical analysis

All the results are presented in the form of mean ± S.D of three replicates. Data were subjected to one-way ANOVA followed by post hoc Duncan's Multiple Range Test (DMRT) using SPSS 17 (SPSS Inc., Chicago, IL, USA) for determining significance at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Total oil content

The *R. vesicarius* showed the highest (3.91 ± 0.27) oil content; whereas the least was seen in *B. alba* (1.10 ± 0.08) and *B. rubra* (1.40 ± 0.10) in g/100 g of dry weight of leaves (DW). There was not much difference in the oil content found in *Hibiscus* spp. and *H. sabdariffa* contained 1.61 ± 0.14, and *H. cannabinus* contained 1.40 ± 0.12 g/100 g oil on DW. The reported 19% oil content in *H. sabdariffa* seeds contained a good amount of lipid-soluble antioxidant compounds such as γ-tocopherols at 0.2% (Mohamed *et al*., 2007). Similarly, the fatty acid profile of the *B. rubra* fruit pulp showed a total oil content of 1.38% with palmitic acid as the major fatty acid (Kumar *et al*., 2016).

3.2. Fatty acid profile

GC/MS chromatographic analysis revealed different compounds from the FAME prepared from the selected GLVs. Based on the retention times, molecular formulas, and molecular weights, the area percentage of each compound was presented (Table 1). Among the identified compounds, 9,12,15-octadecatrienoic acid (z,z,z) methyl ester followed by hexadecanoic acid methyl ester with 38.61 and 14.11% peak area of *H. cannabinus* and *H. sabdariffa* foliage were recorded, respectively. In *B. alba* samples, 9,12-octadecadienoic acid (z,z) methyl ester, followed by hexadecanoic acid methyl ester at 23.55 and 21.16%, respectively, were prominent. Nonetheless, in *B. rubra*, the hexadecanoic acid methyl ester was discovered to be the most elevated, followed by 9-Octadecadienoic acid (z) methyl ester with 39.73%. Essentially, in *R. vesicarius*, the 9,12,15-octadecatrienoic acid (z,z,z) methyl ester was the most

Table 1. Various compounds detected by GC/MS in the five selected GLV foliage oil samples.

Compound name	Molecular Formula	Molecular weight	*H. cannabinus*		*H. sabdariffa*		*B alba*		*B. rubra*		*R. vesicarius*	
			+/-	Area (%)	+/-	Area (%)	+/-	Area (%)	+/-	Area (%)	+/-	Area (%)
Dodecanoic acid methyl ester	$C_{13}H_{26}O_2$	214	-	-	-	-	+	0.25 ± 0.01	+	0.79 ± 0.04	-	-
Phytol acetate	$C_{22}H_{42}O_2$	338	+	0.53 ± 0.02	+	0.40 ± 0.01	+	0.47 ± 0.02	-	-	+	0.53 ± 0.02
Phytol acetate	$C_{22}H_{42}O_2$	338	+	0.81 ± 0.06	+	0.62 ± 0.04	+	0.64 ± 0.02	-	-	+	0.77 ± 0.03
2(3H)-Furanone-dihydro-5-pentyl	$C_9H_{16}O_2$	156	+	1.69 ± 0.14	+	1.34 ± 0.08	+	1.31 ± 0.05	-	-	+	1.45 ± 0.07
Phytol acetate	$C_{22}H_{42}O_2$	338	+	0.40 ± 0.02	-	-	+	0.40 ± 0.02	+	1.74 ± 0.03	+	0.40 ± 0.01
Phosphoric acid monododecyl ester	$C_{12}H_{27}O_4$	266	+	0.67 ± 0.04	+	0.46 ± 0.05	+	0.50 ± 0.04	-	-	+	0.60 ± 0.02
Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	+	0.84 ± 0.06	+	0.52 ± 0.04	+	0.58 ± 0.02	+	1.48 ± 0.11	+	0.70 ± 0.02
Phytol acetate	$C_{22}H_{42}O_2$	338	+	4.10 ± 0.168	+	3.15 ± 0.21	+	2.97 ± 0.38	+	1.47 ± 0.12	+	3.43 ± 0.12
3,3,5,5-Tetramethyl cyclohexanol	$C_{10}H_{20}O$	156	+	9.11 ± 0.34	+	7.28 ± 0.43	+	6.65 ± 0.24	+	5.15 ± 0.24	+	7.22 ± 0.22
Hexadecanoic acid methyl ester	$C_{17}H_{32}O_2$	270	+	16.81 ± 0.53	+	14.11 ± 1.32	+	21.16 ± 1.68	+	39.73 ± 2.68	+	18.22 ± 1.21
9-Hexadecenoic acid methyl ester	$C_{17}H_{32}O_2$	268	+	2.58 ± 0.15	+	2.48 ± 0.30	+	0.67 ± 0.04	+	1.44 ± 0.12	+	1.79 ± 0.14
Heptadecanoic acid methyl ester	$C_{18}H_{36}O_2$	284	+	3.68 ± 0.28	+	3.17 ± 0.38	+	3.06 ± 0.12	+	4.05 ± 0.21	+	1.25 ± 0.11
Nonanedioxic acid dimethyl ester	$C_{11}H_{20}O_4$	216	-	-	-	-	-	-	+	1.13 ± 0.02	-	-
2-Ethyl butyric acid heptadecyl ester	$C_{23}H_{46}O_2$	354	+	1.19 ± 0.24	+	0.89 ± 0.04	+	0.82 ± 0.08	-	-	+	1.07 ± 0.05
Methyl stearate	$C_{19}H_{38}O_2$	298	+	2.07 ± 0.11	+	1.82 ± 0.22	+	3.41 ± 0.26	+	6.04 ± 0.25	+	1.64 ± 0.06
9-Octadecadienoic acid(z) methyl ester	$C_{19}H_{36}O_2$	296	+	2.71 ± 0.22	+	2.45 ± 0.13	+	8.79 ± 0.89	+	12.80 ± 0.62	+	2.74 ± 0.11
11-Octadecanoic acid methyl ester	$C_{19}H_{36}O_2$	296	-	-	-	-	-	-	+	0.60 ± 0.02	-	-
12-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	-	-	-	-	-	-	-	-	+	0.47 ± 0.02
9,12-Octadecadienoic acid(z,z) methyl ester	$C_{19}H_{34}O_2$	296	+	12.16 ± 0.86	+	12.04 ± 1.02	+	23.55 ± 2.43	+	8.03 ± 0.21	+	15.65 ± 0.21
9,12,15-Octadecatrienoic acid(z,z,z) methyl ester	$C_{19}H_{32}O_2$	292	+	38.61 ± 2.58	+	36.59 ± 2.39	+	20.44 ± 1.68	+	7.52 ± 0.14	+	38.90 ± 2.04
Docosanoic acid methyl ester	$C_{22}H_{44}O_2$	340	+	0.53 ± 0.02	-	-	+	0.47 ± 0.02	+	0.99 ± 0.09	+	0.98 ± 0.09
Nonacosane	$C_{29}H_{60}$	408	+	1.10 ± 0.04	+	0.71 ± 0.04	-	-	-	-	-	-
Ethyl isoallocholate	$C_{22}H_{44}O_2$	436	-	-	-	-	-	-	+	0.89 ± 0.06	+	0.57 ± 0.01
Tetracosanoic acid methyl ester	$C_{22}H_{50}O_2$	382	+	0.42 ± 0.03	-	-	+	0.92 ± 0.11	+	1.84 ± 0.11	-	-
Squalene	$C_{30}H_{50}$	410	-	-	+	1.83 ± 0.14	+	1.20 ± 0.08	-	-	-	-
Octacosane	$C_{28}H_{58}$	394	-	-	+	10.15 ± 1.03	-	-	-	-	-	-
2,2,4-Trimethyl-1,3-(3,3,12,16-Tetramethyl heptadeca-3,7,14,15-tetraenyl)-cyclohexanol	$C_{30}H_{52}O$	428	-	-	-	-	-	-	+	1.00 ± 0.04	-	-
6,9,12-Octadecatrienoic acid methyl ester	$C_{19}H_{32}O_2$	292	-	-	-	-	-	-	+	1.16 ± 0.05	-	-
Octadecane, 3-ethyl-5-(2-ethyl butyl)	$C_{26}H_{54}$	366	-	-	-	-	+	1.74 ± 0.22	+	2.18 ± 0.12	+	1.61 ± 0.08

All values represented are mean ± SD of three replicates analysed. +/- indicate the presence / absence of compounds analyzed by GC/MS.

noteworthy (38.90%), followed by hexadecenoic acid methyl ester (18.22%) individually.

The quantification profile of the fatty acids of the selected foliage showed that in *Hibiscus* spp. C18:3 (49.3 µmol % and 50.4 µmol %) was recorded to be the highest, followed by C16:0 (23.2 µmol % and 21 µmol %) in *H. cannabinus* and *H. sabdariffa,* respectively. However, C18:2 (28.2 µmol %) was recorded to be the highest followed by C16:0 (27.5 µmol %) in *B. alba;* whereas *B. rubra* recorded C16:0 (49.9 µmol %) as the highest fatty acid followed by C18:1 Cis 12 (15.3 µmol %). Similarly, C18:3 was the major fatty acid (47.2 µmol %) followed by C16:0 (24 µmol %) in *R. vesicari-*

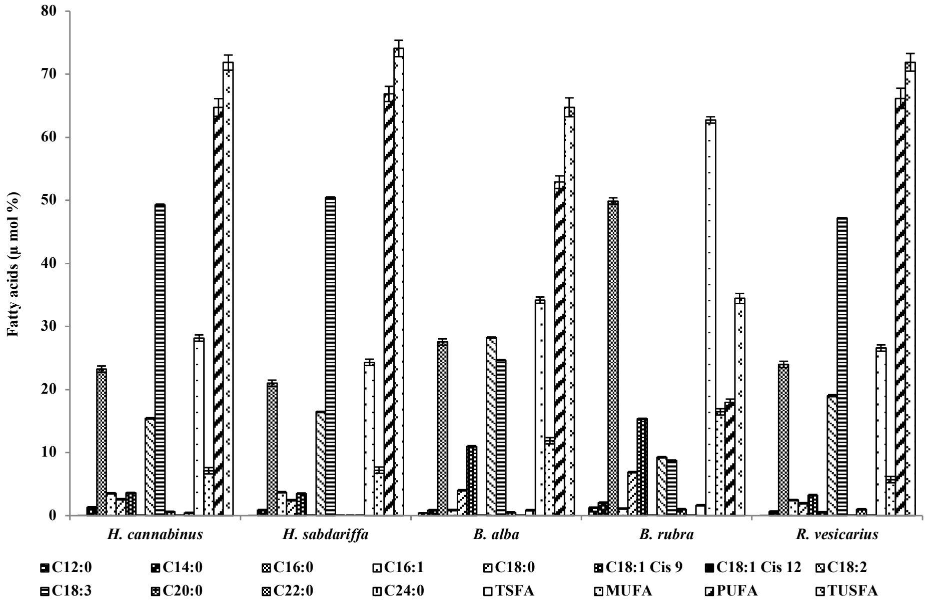

FIGURE 3. Fatty acid profiles of the five selected GLVs. Values are mean ± SD of three replicates analyzed. TSFA - Total saturated fatty acid, MUFA - Mono unsaturated fatty acid, PUFA - Poly unsaturated fatty acid and TUSFA - Total unsaturated fatty acid

us (Figure 3). With the exception of *B. rubra,* every single other extracted sample showed the highest total unsaturated fatty acid composition (TUSFA) above 65% with the significant composition of polyunsaturated fatty acids (PUFA) above 52% individually (Figure 3). This higher PUFA may add to the flexibility, fluidity and selective permeability of cellular membranes which will beneficially reduce cardiovascular ailments (Das, 2006). The structure of rice grain oil and its higher free fatty acid values were in connection with the investigations on vegetable oils (Gopala Krishna *et al.*, 2006). The higher content of C18:3 in *Hibiscus* spp and *R. vesicarius* oil could be beneficial for the use of these oils in the cosmetic industry for the dehydration of skin tissue and reduction of scaly lesions which are deficient in essential fatty acids (Aburjai and Natsheh, 2003).

3.3. Biodiesel characteristics of the five selected foliage oils

Investigations have now been carried out on the fuel properties of different vegetable oils. Therefore, the current work is focused on assessing the vegetable oils' suitability to biodiesel. The five chosen vegetable oils were described in terms of thermo-physical/biodiesel properties through empirical connections.

3.3.1. Thermo-physical properties

Depending on the properties of esters present in the vegetable oils, the thermo-physical properties are considered one of the deciding properties of biodiesel (Montero and Stoytcheva, 2011). The theoretical evaluation of five vegetable oils' thermo-physical properties was carried out.

3.3.2. Density

Density is the critical parameter that directly influences many fuel properties of biodiesel such as cetane number, heating value, combustion and atomization characteristics. *R. vesicarius* showed the highest density (956.76 kg/m^3) among all the experimental samples**.** Other samples, such as *B. rubra*, *B. alba*, *H. sabdariffa*, and *H. cannabinus* have shown good density in the extracted oils in the range of 870 to 875 kg/m^3, which falls in the range of the biodiesel standards (860-900 kg/m^3). In general, an engine's output power depends on the density of the biodiesel. Hence it could be concluded that all the selected samples come close to the international standards for biodiesel. A study on the evaluation of waste and the efficiency of refined cooking oil to be used as biodiesel was also carried out. Waste cooking oil showed 916 kg/m^3 and refined cooking oil showed 913 kg/m^{3,} slightly lower than pure water at 998 kg/m^3 (Chuah *et al.*, 2017).

The present data is comparable to several vegetable oils such as peanut, soybean, babassu, palm, and sunflower in the range of 860 to 883 kg/m^3 (Shereena and Thangaraj, 2009).

3.3.3. Kinematic viscosity

As shown in Table 2, the kinematic viscosity of the oils varied between 3.8-5.66 ×10^{-6} m^2/s. The highest kinematic viscosity was observed in *R. vesicarius* (5.66×10^{-6} m^2/s), which may be due to the presence of large molecular mass, followed by *B. rubra* (4.84×10^{-6} m^2/s), *B. alba* (4.84×10^{-6} m^2/s), *H. sabdariffa* (4.28×10^{-6} m^2/s), and *H. cannabinus* (3.81×10^{-6} m^2/s). Shereena and Thangaraj reported that palm oil showed the highest (5.7×10^{-6} m^2/s) and babassu oil showed the least (3.6×10^{-6} m^2/s) kinematic viscosity compared to other vegetable oils and commercial diesel (3.06×10^{-6} m^2/s) (Shereena and Thangaraj, 2009).

3.3.4. Higher heating value

The higher heating value is the determination of heat released when a one-unit volume of fuel is combusted. Among the experimental vegetable oils analyzed, the maximum heating value was observed in *R. vesicarius* (43.65 MJ/kg) as compared to the requirement of biodiesel fuels (> 45 MJ/kg). *B. rubra* (39.14 MJ/kg) and *B. alba*, *H. cannabinus* showed similar values (39.14 MJ/kg). *H. sabdariffa* lower highest heating value (37.72 MJ/kg) compared to other vegetable oils extracted. This variation in the highest heating values is due to the presence of chemically-bound oxygen atoms. These values are higher compared to the reported commercial vegetable oils where babassu oil showed a lower value (31.8 MJ/kg) than peanut and soybean (33.5 MJ/kg) (Shereena and Thangaraj, 2009). The heat of combustion of all the selected GLVs samples was recorded in the range of 54659 to 55239 kJ/kg.

3.3.5. Viscosity

Viscosity is one of the important physical properties which provides information about the resistance of the fluid in biodiesel. The qualities of biodiesel such as the size of the fuel drop, jet penetrations, and atomization depended on viscosity.

Table 2. Predicted biodiesel properties in the five selected GLV foliage oils.

Parameter	ASTM D6751 Standards	*H. cannabinus*	*H. sabdariffa*	*B. alba*	*B. rubra*	*R. vesicarius*
Density (kg/m^3)	860-900	871.31 ± 0.00	875.11 ± 0.00	870.96 ± 0.00	870.96 ± 0.00	956.76 ± 0.00
Kinematic viscosity (m^2/s) (×10^{-6})	1.9-6.0	3.81 ± 0.01	4.28 ± 0.01	4.84 ± 0.01	4.84 ± 0.01	5.66 ± 0.01
Higher heating value (MJ/kg)	*	39.36 ± 0.00	37.72 ± 0.03	39.14 ± 0.05	39.14 ± 0.05	43.65 ± 0.00
Viscosity (N.m^{-2}. s)	*	104.58 ± 14.62	103.64 ± 14.19	104.04 ± 14.27	104.34 ± 14.31	104.51 ± 14.50
Saponification Value (SV)	164-220	205.62 ± 0.02	202.18 ± 0.33	203.17 ± 0.06	204.29 ± 0.03	205.34 ± 0.01
Cetane Number (CN)	>47	34.67 ± 0.009	33.99 ± 0.12	44.05 ± 0.005	60.51 ± 0.003	34.75 ± 0.009
Iodine Value (g I_2/100 g)	<140	169.63 ± 0.03	174.67 ± 0.34	129.38 ± 0.05	55.55 ± 0.01	169.44 ± 0.03
Degree of unsaturation (DU)	*	136.57 ± 0.03	140.96 ± 0.26	117.64 ± 0.06	52.41 ± 0.01	138.66 ± 0.16
Long chain saturated factor (°C)	*	5.56 ± 0.03	3.32 ± 0.01	7.83 ± 0.01	14.34 ± 0.04	4.84 ± 0.01
Cold filter plugging point (°C)	*	-0.88 ± 0.10	-6.04 ± 0.01	8.13 ± 0.01	28.60 ± 0.12	-1.24 ± 0.05
Heat of combustion (kJ/kg)	*	54812.28 ± 0.73	54659.29 ± 11.20	54886.83 ± 2.58	55239.04 ± 1.22	54802.16 ± 0.86
Elemental composition (%)						
Carbon (C)	86.5	72.74 ± 1.30	66.38 ± 1.26	75.51 ± 0.17	76.86 ± 0.12	71.24 ± 1.50
Hydrogen (H)	13.5	14.20 ± 0.49	10.78 ± 1.08	13.89 ± 0.12	16.24 ± 0.09	15.60 ± 0.29
Nitrogen (N)	*	3.00 ± 0.21	3.13 ± 0.14	1.58 ± 0.03	0.71 ± 0.01	2.79 ± 0.41
Sulphur (S)	*	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.08 ± 0.00	0.00 ± 0.00
Carbon/Hydrogen (C/H)	6.24	5.12 ± 0.08	6.15 ± 0.51	5.43 ± 0.04	4.73 ± 0.02	4.56 ± 0.06

All values represented are mean ± SD of three replicates analyzed. * American Society for Testing and Materials (ASTM) standard values were not available for these parameters. Biodiesel values are based on standard conversion factor and empirical formulas. CHNS (Carbon, Hydrogen, Nitrogen, Sulphur) values.

Fuel viscosity has both upper and lower limitations (3.5-5.0 N·m^{-2}·s). Low viscosity causes leakage problems and higher viscosity causes poor fuel atomization. The experimental vegetable oils contained almost the same amount in all the samples, in the range of 103.6-104.5 N·m^{-2}.s.

3.3.6. Cetane number (CN)

CN is a key indicator of fuel quality, and it characterizes the fuel's ease of combustion. The higher cetane values indicate the smooth running of the engine, and according to the ASTM standards, it should be greater than 47 (Montero and Stoytcheva, 2011). In the present study, a higher CN 60.51 was observed in *B. rubra* followed by *B. alba* (44.05), *H. sabdariffa* (34.99), *R. vesicarius* (34.75), and *H. cannabinus* (34.67) (Table 2). Compared to the standard CN 28% more was observed in *B. rubra,* and the other four showed lower CN values than the standard. The CN of *B. rubra* oil (60.51) is comparable to the CN of palm oil (62). Similarly, the commercial diesel was recorded to show a CN of 50 as reported by (Shereena and Thangaraj, 2009).

3.3.7. Iodine value

Iodine value is the standard marker for biodiesel quality, which reveals the biodiesel's stability to oxidation. It is estimated by the nearness of unsaturated fatty acids and ester composition. Biodiesel with a higher iodine value provides ease in oxidation when in contact with air. Among the experimental samples, the most elevated was seen in *H. sabdariffa* (174.67 g I_2/100 g). *H. cannabinus* and *R. vesicarius* presented practically comparable iodine levels (169 g I_2/100 g). The lowest iodine value was seen in *B. rubra* (55 g I_2/100 g). Biodiesel with a higher iodine value causes polymerization and deposits on piston rings and injector nozzles. The standard iodine value detailed in different nations indicates that in Japan and Europe it is 120, and in South Africa 140; while in India and Australia iodine values were not considered to assess the biodiesel nature of the fuel.

3.3.8. Biodiesel cold-flow properties

At cold temperatures, the performance of the oil/biodiesel will vary and worsen fuel flow. It is important to characterize the biodiesel's cold properties. Mostly, these properties depend on the fatty acid composition of the triacylglycerol. Higher molecular weight triacylglycerol is responsible for poor cold-flow characteristics of biodiesel.

3.3.9. Cold-filter plugging point (CFPP)

CFPP is the indicator of flow performance of biodiesel at lower temperatures. The CFPP of vegetable oils produced from various feedstock is shown in Table 2. The results showed that the highest was observed in *B. rubra* (28.60 °C), which is due to a higher long-chain saturated factor of 14.34 °C, indicating the presence of higher long-chain fatty acids. *B. alba* (8.13 °C), *H. cannabinus* (-0.88 °C), *R. vesicarius* (-1.24 °C), and *H. sabdariffa* (-6.04 °C) were observed due to present lower LCSF values at 7.83, 5.56, 4.84, 3.32, respectively, indicating the presence of lower long-chain fatty acids. The presence of long-chain fatty acids decreases the properties of biodiesel at cold temperatures. Due to the presence of Arachidic acid and Lignoceric acid in *B. rubra*, the oil showed higher CFPP values. CFPP values are climate dependent, and for temperate climate conditions they are reported to be in the range of -20 to 5 °C, except for *B. rubra* and *B. alba,* which are comparatively within the range for temperate conditions (Montero and Stoytcheva, 2011).

3.4. Carbon, Hydrogen, Nitrogen and Sulphur (CHNS) contents

Table 2 shows the elemental (CHNS) composition of oil extracted from selected GLV foliage. In the present study, the carbon and hydrogen components were seen as the most noteworthy in all the oil samples with 66 to 76% carbon and 10 to 16% hydrogen. In any case, there was less substance of nitrogen with 0.71 to 3%. There was an immaterial amount of sulfur in all the oil samples chosen. A comparable pattern in elemental composition was additionally detailed in *Pithecellobium dulce* seed oil with carbon as most noteworthy (76%) and no sulfur detected (Sekhar *et al*., 2018). It was determined that this synthesis is practically identical and that commercial diesel properties include 85% carbon and 0.15% sulfur. A comparative pattern of bio-diesel attributes was recorded for all the chosen GLVs.

4. CONCLUSIONS

The information on total oil and fatty acid profiles uncovered the distinctions among the chosen GLV foliage. Despite the fact that the significant fatty acids distinguished were the same in four foliage powders aside from *B. rubra*, which was affirmed by MS. The major fatty acids of the selected foliage showed that in *Hibiscus* spp. C18:3 (49.3 µmol % and 50.4 µmol %) were recorded to be the highest, followed by C16:0 (23.2 µmol % and 21 µmol %) in *H. cannabinus* and *H. sabdariffa,* respectively. However, *B. rubra* showed the highest TUSFA at above 65% with the significant composition of PUFA above 52%. The inferences from the bio-diesel properties showed that aside from *R. vesicarius,* every single vegetable oil was within the scope of bio-diesel measurements. Kinematic viscosity and saponification values for all the oils were inside the range of (3.81 to 5.66×10^{-6} m^2/s and 202 to 206 N.m^{-2}.s), which lie within the ASTM standard range (164-220 N.m^{-2}.s). However, as for cetane number (60.51), *B. rubra* indicated values in the scope of bio-diesel properties (> 47). In cold flow properties, for example, LCSF and CFPP demonstrated that *H. cannabinus, H. sabdariffa* and *R. vesicarius* could be utilized in temperate conditions. It is important to carry out a comparative study on the fatty acid profile and bio-diesel qualities of the chosen foliage which could be helpful for these oils as vegetable oil for human consumption and in bio-diesel applications.

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Effect of phosphatidylethanolamine and phosphatidylserine on antioxidant capacity, oxidative stability and color reversion of camellia seed oil

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SUMMARY: Non-hydratable phospholipids as pro-oxidants are likely to cause a decrease in the quality of vegetable oils. The influence of phosphatidylethanolamine (PE) and phosphatidylserine (PS) on the oxidative stability, antioxidant capacity and color reversion of refined camellia seed oil (RCSO) was evaluated in this work. The PE/PS addition could improve the oxidative stability and antioxidant capacity, but was not a key factor in the color reversion of RCSO. The results clearly showed that PE and PS were not prooxidants but antioxidants in camellia seed oil, and the findings of the present study would be useful for extending the shelf-life of camellia seed oil and for retaining phospholipids during moderate refining.

KEYWORDS: *Antioxidant capacity; Color reversion; Oxidative stability; Phosphatidylethanolamine (PE); Phosphatidylserine (PS); Refined camellia seed oil (RCSO).*

RESUMEN: ***Efecto de la fosfatidiletanolamina y la fosfatidilserina sobre la capacidad antioxidante, la estabilidad oxidativa y la reversión del color del aceite de semilla de camelia.*** Es probable que los fosfolípidos no hidratables, como prooxidantes, causen una disminución en la calidad de los aceites vegetales. En este trabajo se ha evaluado la influencia de la fosfatidiletanolamina (PE) y la fosfatidilserina (PS) sobre la estabilidad oxidativa, la capacidad antioxidante y la reversión del color del aceite de semilla de camelia refinado (RCSO). La adición de PE/PS pudo mejorar la estabilidad a la oxidación y la capacidad antioxidante, pero no fue un factor clave en la reversión del color de RCSO. Los resultados mostraron claramente que PE y PS no eran prooxidantes sino antioxidantes en el aceite de semilla de camelia, y los resultados obtenidos en el presente estudio serán útiles para extender la vida útil del aceite de semilla de camelia y para retener los fosfolípidos, tanto como sea posible, durante el refinado moderado.

PALABRAS CLAVE: *Aceite de semilla de camelia refinado (RCSO); Capacidad antioxidante; Estabilidad oxidativa; Fosfatidiletanolamina (PE); Fosfatidilserina (PS); Reversión del color.*

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1. INTRODUCTION

Phospholipids, a low-level compound in vegetable oil, are usually present in the form of hydratable phospholipids (HP) and non-hydratable phospholipids (NHP). HP, mainly including phosphatidylcholine (PC) and phosphatidylinositol (PI), are removed after hydration and degumming, and what remains in the vegetable oil is NHP, including phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidic acid (PA) (Oybek *et al*., 2009). The concentration and composition of phospholipids which are endogenous to foods are dependent on the origin of the food and how it is processed. At the same time, phospholipids have a very significant effect on the oxidative stability of lipids as antioxidant, prooxidant, or oxidation substrates themselves (Cui and Decker, 2016; Rajesh, *et al*., 2021). Previous studies focused on the adverse effects of phospholipids as prooxidant on the quality of bulk edible oil, including changes in the oil's appearance such as color reversion and apparent turbidity (Zamora *et al*., 2004), producing bad flavor (likely rancid) and a large amount of foam and black deposits during cooking (Hafidi *et al*., 2005), and the negative effects on the storage stability of oil (Bo *et al*., 2006). However, more and more studies have demonstrated that phospholipids, as an antioxidant or antioxidant synergists, can maintain or improve the quality of canola oil (Jiyeun and Eunok, 2009) and Virgin olive oil (VOO) (Olivera *et al*., 2008), decreasing the intensity of VOO bitterness (Olivera *et al*., 2009), and interfere with the extraction of hydrophilic phenols in VOO (Olivera *et al*., 2010). The high-value phospholipid products used as functional food and nutraceutical ingredients have been exploitated from waste coming from the seed oil refining industry (Chiara *et al*., 2021; Christine *et al*., 2020).

Camellia seed oil (CSO), which is extracted from the seeds of *Camellia oleifera* Abel, has been used extensively for over two thousand years as edible oil and medicine in China, and has been labeled "Oriental Olive Oil" due to the more than 90% unsaturated fatty acids (mainly oleic acid and linoleic acid) and high levels of endogenous biophenols which are rich in quantity and diversity (Haiyan *et al*., 2007). Based on Chinese eating habits for the pursuit of characteristic flavor and nutrition, the current production of CSO in China is based on a physical pressing process after oilseed pre-treatment (including roasting or sun-dried), and then physical degumming (hydration or winterization degumming). Therefore, the prepared CSO contains a certain amount of NHP. To the best of our knowledge, few literature reports focused on the effects of NHP on the quality of CSO, which leads to a lack of practical theoretical basis for the practice of physical degumming of CSO. Therefore, research about NHP (PE and PS) and their effects on the antioxidant capacity, oxidative stability and color reversion of CSO has been carried out, and the results will have a very important theoretical impact on the development of pressing technology of CSO to preserve flavor and nutrition.

2. MATERIAL AND METHODS

2.1. Chemicals

The authentic standards (≥ 98.5%) and chromatographic grade organic solvent (≥ 99.9%) used in this work were all obtained from Sigma-Aldrich. Other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China.

2.2. Preparation of refined camellia seed oil and phospholipids

Refined camellia seed oil (RCSO) was obtained from pressed camellia seed oil by a laboratory-scale refining processes according to our previous report (Bo *et al*., 2016).

One thousand grams of the physically- pressed CSO were twice stirred with 5000 mL of *n*-hexane to collect crude phospholipids. The resulting *n*-hexane in crude phospholipids was removed by a rotary evaporation (RV 10 digital, 104IKA, Germany). About 5 g of crude phospholipids were used to separate PE and PS on a silica gel chromatographic column (3.0 × 50 cm). A solvent of light petroleum (65-75 °C)/isopropanol/water (1:1:0.175, v:v:v) was selected to elute and PE and PS was collected, evaporated and stored at -20 °C (Zheng *et al.*, 2005).

The phospholipids were spotted onto prepararred silica gel TLC plates (TLC, silica gel GF UV-254, thickness 2 mm, 10 × 20 cm) and developed in the solvent chloroform:methanol:water (42:22:3, v/v/v). PL bands were identified by comparison with authentic standards [PE (Rf = 0.62) and PS (Rf = 0.43)] which were run in parallel. The band only containing PE or PS was scraped off and extracted three time with chloroform:methanol (2:1, v/v). The solvents were removed by nitrogen gas.

2.3. Analysis of fatty acid composition, phosphorus contents, total phenols and moisture in RCSO and phospholipids

An analysis of fatty acid composition in RCSO was carried out, and purified PE and PS were methylated and analyzed by the GC-FID according to our previous reports (Bo *et al.*, 2016). The analysis of total phenols, phosphorus content and moisture in RCSO was conducted according to our previous report (Haiyan *et al.*, 2007).

2.4. Accelerated oxidation experiment

0.2 g, 0.5 g, 1.0 g, 1.5 g, and 2.0 g of PE and PS were weighed and put in a test tube filled with 100 g RCSO, Then the tubes were placed in a water bath at 50°C with constant stirring to dissolve phospholipids, and the oil sample was cooled to 4°C. Then the Schaal oven method was used to heat continuously at 63°C for 20 d, and the oil samples were collected by taking out three separate test tubes every 4 d. The collected oil samples were stored in a refrigerator at 4°C for later use. Three replicates for each sample and RCSO without phospholipids were used as the control group.

2.5. Determination of oxidative stability of RCSO

Acid value (AV, expressed as mg KOH/g of oil), peroxide value (POV, expressed as the mass fraction of peroxide equivalent to iodine with g/100g), *p*-anisidine value (*p*-AV), and the induction period (IP) of RCSO were determined according to our previous report (Bo *et al.*, 2018).

The Totox value was calculated as twice POV plus *p*-AV (Bo *et al.*, 2018).

In order to evaluate the rate of changes in the oxidative stability of RCSO, the ΔAV, ΔPOV or Δ*p*-AV was calculated as follows:

$$\Delta = \frac{(X_T - X_C)}{X_C}\%$$

Where, and represents the data (AV, POV and *p*-AV) of samples with and without phospholipid addition on the same heating days.

2.6. Antioxidant capacity test of RCSO

The free-radical scavenging capacity (FRSC), including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and oxygen radical absorbance capacity (ORAC), were detrmined according to our previous report (Bo *et al.*, 2018). The results of the DPPH, ABTS and ORAC tests were expressed as μmol of Trolox equivalent g^{-1} oil (μmol TE/g).

2.7. Color determination

The color value for RCSO was assayed using a colorimeter (Minolta CR-10 Plus, Konica Minolta (China) Investment Ltd.) according to the manufacturer's instructions.

$$\Delta E = \sqrt{(\triangle L^*)^2 + (\triangle a^*)^2 + (\triangle b^*)^2}$$

Where, ΔE: total color difference; ΔL*: brightness difference between the treated and control samples; Δa*: red / green difference between the treated and control samples; Δb*: yellow / blue difference between the treated and control samples.

2.8. Statistical analysis

All data were evaluated using analysis of variance (ANOVA) and significant differences among the means of three replicates ($p < 0.05$) were determined by Turkey's test using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). All figures were drawn with OriginPro 8.0 (OriginLab Corporation, Northampton, MA 01060, USA).

3. RESULTS AND DISCUSSION

3.1. Chemical composition analysis of RCSO, purified PE and PS

In present work, the fatty acid profile of RCSO was palmitic acid (PMA, C16:0) at 8.7%, stearic acid (SA, C18:0) at 1.52%, oleic acid (OA, C18:1) at 71.31%, linoleic acid (LA, C18:2) at 10.25%, and linolenic acid (LLA, C18:3) at 0.86% (Table 1), and the unsaturated fatty acid (UFA) content in RCSO was 89.87 (Table 1). The fatty acid profile and content in RCSO was consistent with our previous reports (Haiyan *et al.*, 2007). The fatty acid profiles of PE and PS from CSO were as follows: PMA (20.87, 19.81%), SA (2.39, 3.15%), OA (48.88, 50.69%), LA (27.56%, 25.72%), and LLA (1.16 and 0.96%) (Table 1). The UFA contents in PE and PS were 77.28 and 76.41%, respectively (Table 1). The proportions and profiles of fatty acids in PE and PS were con-

Table 1. Analysis of chemical profiles of RCSO, purified PE and PS

Samples	C16:0	C18:0	C18:1	C18:2	C18:3	∑SFA	∑UFA	∑PUFA	Phosphorus Contents (mg/kg oil)	Total phenols (µg/g caffeic acid)	Moisture (%)
PS	19.81±2.21	3.15±0.39	50.69±6.13	25.72±4.92	0.96±0.3	22.87±2.08	76.41±2.01	26.68±1.94			
PE	20.87±0.04	2.39±0.03	48.88±0.07	27.56±0.04	1.16±0.04	23.26±0.08	77.28±0.08	28.72±1.33			
RCSO	8.70±1.03	1.52±0.52	71.31±8.02	10.25±2.01	0.86±0.22	10.21±0.23	89.87±1.36	12.57±0.15	ND	ND	≤0.05

Note: ND, not detected. Values are means ± SD of triplicate determinations.

sistent with that of RCSO. The phosphorus and total phenols were not detected in RCSO; the moisture of RCSO was less than 0.05% (data not shown).

The above results indicated that the main fatty acid profiles of RCSO, PE and PS were PMA, SA, OA, LA, and LLA. The proportions and profiles of fatty acids in PE and PS were consistent with that of RCSO. Refining had no significant influence on the fatty acid profiles or contents of CSO and polyphenols were removed very effectively.

3.2. Antioxidant capacity analysis of purified PE and PS

As expected, the key factor in determining whether phospholipids play a role in prooxidants or antioxidants in foods is closely related to their physical environment (Cui and Decker, 2016). The results in the present study indicated that PE and PS both have a certain DPPH scavenging capacity, and the scavenging capacity was positively related to the added concentration of PE and PS (Figure 1). For example, when the addition amount was 2.0%, the DPPH scavenging rate of PE and PS was 15 and 14%, respectively (Figure 1). Our results also matched the findings of Espín *et al.* (2000).

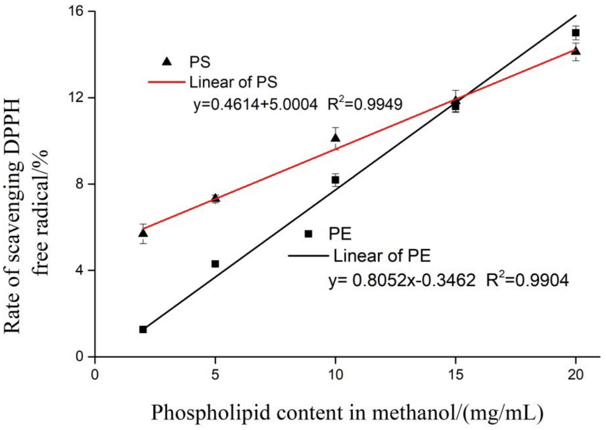

Figure 1. DPPH scavenging effect of PE and PS methanol chloroform solution (means ± SD of triplicate determinations)

The above results indicated that PE and PS obtained from CSO themselves played a role in antioxidants, and the antioxidant capacity of PS and PE depended on the concentration added.

3.3. Effect of PE/PS addition on antioxidant capacity of RCSO

DPPH, ABTS, and ORAC were used to evaluate the antioxidant capacity of RCSO, PE and PS in the present work. The values of DPPH, ABTS, and ORAC for RCSO without phospholipid (PE and PS) addition were 52.23 µmol TE/g, 63.45µmol TE/g, and 146.87µmol TE/g, respectively (Table 2). The results indicated that RCSO itself has a certain antioxidant capacity, which may be due to the more than 70% oleic acid in the RCSO (Haiyan *et al.*, 2007).

The DPPH and ABTS changes in RCSO with PE addition showed an initial y decrease followed by a slightly increasing pattern (Table 2). Compared to RCSO without PE addition, the DPPH and ABTS was first decreased by 35 and 23% (0.2% addition), 17 and 9% (0.5% addition), 24 and 22% (1.0% addition), 9 and 1% (1.5% addition), and then increased by 11 and 5% (2.0% addition), respectively. Interesting, the ORAC changes in RCSO with PE addition always showed an upward trend, which was increased from 165.61µmol TE/g (0.2% addition) to 245.8 µmol TE/g (2.0% addition) (Table 2). DPPH, ABTS and ORAC changes with PS addition showed an upward trend (Table 2). DPPH, ABTS and ORAC were increased by 37, 79, and 29% (0.2% addition), and 54, 114, and 176% (2.0% addition), respectively, compared to RCSO without PS addition (Table 2). The results showed that PE and PS could both significantly improve the antioxidant capacity of RCSO, which was similar to the

Table 2. Antioxidant capacity and oxidative stability of PE and PS on RCSO

Phospholipid	Adding amount (w/v, %)	DPPH (µmol TE/g)	ABTS (µmol TE/g)	ORAC (µmol TE/g)	IP (h)
PE	0.0	52.23 ± 0.47^{e}	63.45 ± 0.69^{b}	146.87 ± 2.96^{a}	3.72 ± 0.02^{a}
	0.2	33.23 ± 0.17^{a}	48.64 ± 0.17^{a}	165.61 ± 3.92^{b}	6.13 ± 0.05^{b}
	0.5	43.37 ± 0.25^{c}	57.56 ± 0.25^{c}	207.84 ± 2.50^{c}	9.11 ± 0.01^{c}
	1.0	39.56 ± 0.67^{b}	49.36 ± 0.67^{c}	194.60 ± 2.90^{c}	10.57 ± 0.04^{c}
	1.5	47.45 ± 0.91^{d}	62.74 ± 0.91^{d}	223.24 ± 4.54^{d}	17.70 ± 0.02^{d}
	2.0	58.04 ± 0.66^{f}	66.32 ± 0.66^{e}	245.80 ± 4.72^{d}	20.05 ± 0.04^{e}
PS	0.0	52.23 ± 0.47^{a}	63.45 ± 0.69^{a}	146.87 ± 2.96^{a}	3.72 ± 0.02^{a}
	0.2	71.73 ± 2.96^{bc}	113.36 ± 2.96^{b}	189.35 ± 6.35^{b}	5.35 ± 0.02^{b}
	0.5	71.84 ± 1.61^{bc}	108.71 ± 1.61^{b}	235.56 ± 4.75^{c}	7.92 ± 0.03^{c}
	1.0	75.94 ± 2.37^{cd}	117.54 ± 2.37^{b}	308.23 ± 6.88^{d}	10.06 ± 0.02^{d}
	1.5	67.97 ± 0.53^{b}	116.79 ± 0.53^{b}	348.07 ± 7.01^{e}	13.20 ± 0.07^{e}
	2.0	80.68 ± 1.57^{d}	135.52 ± 1.57^{c}	405.22 ± 6.28^{f}	16.22 ± 0.05^{f}

Note: Values are means ± SD of triplicate determinations. Different letters in superscript within the same column indicate significant differences among the oil samples (Tukey's test, $p < 0.05$).

results reported for refined olive oil (Hidalgo *et al.*, 2006), but contrary to results on perilla oil (Minoru e*t al.*, 1991) and virgin olive oil (Olivera *et al.*, 2008; Olivera *et al.*, 2010).

As expected, the DPPH scavenging capacity of phospholipids was the cause of the changes in the DPPH scavenging capacity of the oil (Jiyeun and Eunok, 2009). The key factor to determine whether phospholipids have a certain DPPH scavenging capacity in RCSO depends on the polar groups of phospholipids and their content (Reis and Spickett, 2012). The one key factor that affects the antioxidant capacity of RCSO with PE/PS addition may be attributed to the stronger hydrophilicity of PS than that of PE in this paper, which could not only enhance the antioxidant activity of some hydrophilic primary oxidation products (likely peroxyl, alkane, alkene and aldehyde derivatives) derived from lipid oxidation (Zheng *et al.*, 2005), but also promote their production (Reis and Spickett, 2012). Therefore, the addition of PE to improve the antioxidant capacity of RCSO was mainly attributed to the ORAC of PE, but PS addition to improve the antioxidant capacity of RCSO was attributed to not only ORAC but also to the FRSC of PS in the present work.

The above results in the present study indicated the RCSO itself has a certain antioxidant capacity, and phospholipids (PE and PS) could also significantly improve the antioxidant capacity of RCSO.

3.4. Effect of PE/PS addition on oxidative stability of RCSO

3.4.1. Induction period

The induction period (IP) of RCSO with PE and PS addition has been extended by 2.41 and 1.63 h (0.2%), 5.39 and 4.2 h (0.5%), 6.85 and 6.34 h (1.0%), 13.98 and 9.48 h (1.5%), 16.33 and 12.5 h (2.0%), respectively (Table 2). Our findings were compatible to some previous reports that a high concentration (0.5–2.0%) of lecithin showed obvious auto-oxidation inhibitory activity on VOO (Olivera *et al.*, 2008), although contrary to the results reported for perilla oil (Minoru e*t al.*, 1991), which may be related to the fatty acid composition and contents of phospholipids themselves (Cui and Decker, 2016). All the results in the present study indicated that PE and PS could improve the IP of RCSO.

3.4.2. Acid value

Acid value (AV) is used to measure the production of free fatty acids in RCSO in the present work. The AV of RCSO without PE/PS addition increased from 0.32 mg/g (0 d) to 0.71 mg/g (20 d), and the significant changes in the AV of RCSO mainly occurred after 8 days (Figure 2A, 2B, 2a, 2b). Simultaneously, although PE/PS addition could promote an increase in the AV of RCSO, the increase rate of the AV in RCSO was inhibited (Figure 2B, 2b). For example, the AV of RCSO without PE/PS addition increased by 122% on the 20th day, and the AV of RCSO with the addition of PE/PS increased by 84/60.44% (0.2%), 95/47% (0.5%), 71/67% (1.0%), 38/36% (1.5%), and 33/26% (2.0%) compared to no added PE/PS (Figures 2A, 2a), respectively.

In general, thermal processing could result in hydroperoxides producing in primary oxidation processes and increasing the level of free fatty acid in heat-treated oils (Fozia *et al.*, 2006). The results from this work demonstrated that the AV of RCSO did not change significantly before heating for 8 days due to the high-stability oleic acid with a content of more than 70% in RCSO (Table 1). The addition of PE and PS could inhibit the hydrolysis of RCSO, reduce the generation of free fatty acids and then slow down the rise of its AV, which may be ascribe to the antioxidant capacity of PE/PS (McDonnell *et al.*, 1995). The inhibitory effect of PS on the AV of RCSO was more effective than that of PE, which was consistent with the report of Peng *et al.* (2020). The reason may be attributed to the FRSC of PS, which is stronger than that of PE (Figure 1 and Table 2), or the speed of antioxidant (including peroxyl, alkane, alkene, and aldehyde derivatives) production from the oxidation of PE, which is slower than that of PS (Reis and Spickett, 2012).

The results in the present work indicated PE and PS could inhibit the hydrolysis of RCSO to generate free fatty acids, and play a role in delaying the increase in the acid value (AV) of RCSO.

3.4.3. Peroxide value

The peroxide value (POV) of RCSO without PS/PE addition significantly increased from 0.11 g/100 g (4 d) to 1.02 g/100 g (20 d) (Figures 2C, 2D, 2c, 2d), respectively. The PE/PS addition could inhibit the POV increase in RCSO. For example, the POV of RCSO with 2.0% PS/PE addition increased by 0/200% (4 d), 100/500% (8 d), 167/733% (12 d), 333/ 1767% (16 d), 467/3300% (20 d) compared to no added PE/PS (Figure 2D, 2d), respectively. At the same time, PE/PS addition could significantly inhibit the increase rate of POV in RCSO. The rate of POV in RCSO with PE/ PS addition was increased by 49/-11% (0.2%), 25/-36% (0.5%), 15/-75% (1.0%), 5/-78% (1.5%), 0/-83% (2.0%) compared to without PE/PS addition (Figures 2C,2c), respectively.

The generation of oxidative free radicals occurred in the induction period (early period), and then a series of hydroperoxides and new free radicals were generated in the propagation period (late storage period) (McDonnell *et al.*, 1995). So, the POV increase mainly occurred in the late heating period due to the automatic oxidation of RCSO (Figures 3A and 3C). The inhibition of PE/PS addition on the increase of POV in RCSO attributed to the improvement in the ORAC in RCSO in the present work (Table 2). At the same time, the inhibitory effect of PE was weaker than that of PS, which was contrary to previous reports (Jiyeun and Eunok, 2009), maybe due to the fact that the antioxidant capacity of PS was stronger than that of PE (Figure1 and Table 2). Other possible reasons included the following: the speed of antioxidant (including peroxyl, alkane, alkene, and aldehyde derivatives) production from the oxidation of PE, which is slower than that of PS (Reis and Spickett, 2012), or/and the hydrophilicity of PS, which is greater than that of PE (Reis and Spickett, 2012).

The results in the present work indicated that PE/ PS could delay the POV increase in RCSO as they inhibited the oxidation of RCSO, the inhibitory effect of PS was significantly stronger than that of PE.

3.4.4. p-*Anisidine value*

The *p*-anisidine value (*p*-AV) increased slowly before 12 days in the present work. PE/PS had an inhibitory effect on the increase in *p*-AV, and it was positively correlated with the amount of PE/PS addition (Figures 2E, 2F, 2e, 2f). In terms of PE addition, when the amount of PE addition was less than 1.0%, the inhibition of *p*-AV was not obvious, but the *p*-AV of RCSO with PE addition (1.0, 1.5 and 2.0%) showed a decreasing-increasing trend. For example, the *p*-AV of RCSO with a 0.2% PE addition increased by 18 (16 d) and 19% (20 d) compared to without PE addition, respectively (Figures 2E, 2F). As far as PS addition was concerned, the *p*-AV of RCSO with PS addition (0.2 and 0.5%) increased slowly before 12 days, then there was a downward trend with the increase in PS addition, such as the *p*-AV of RCSO with PS addition of 1.0, 1.5 and 2.0 decreased by 29, 224, and 268% on the 4th day, and

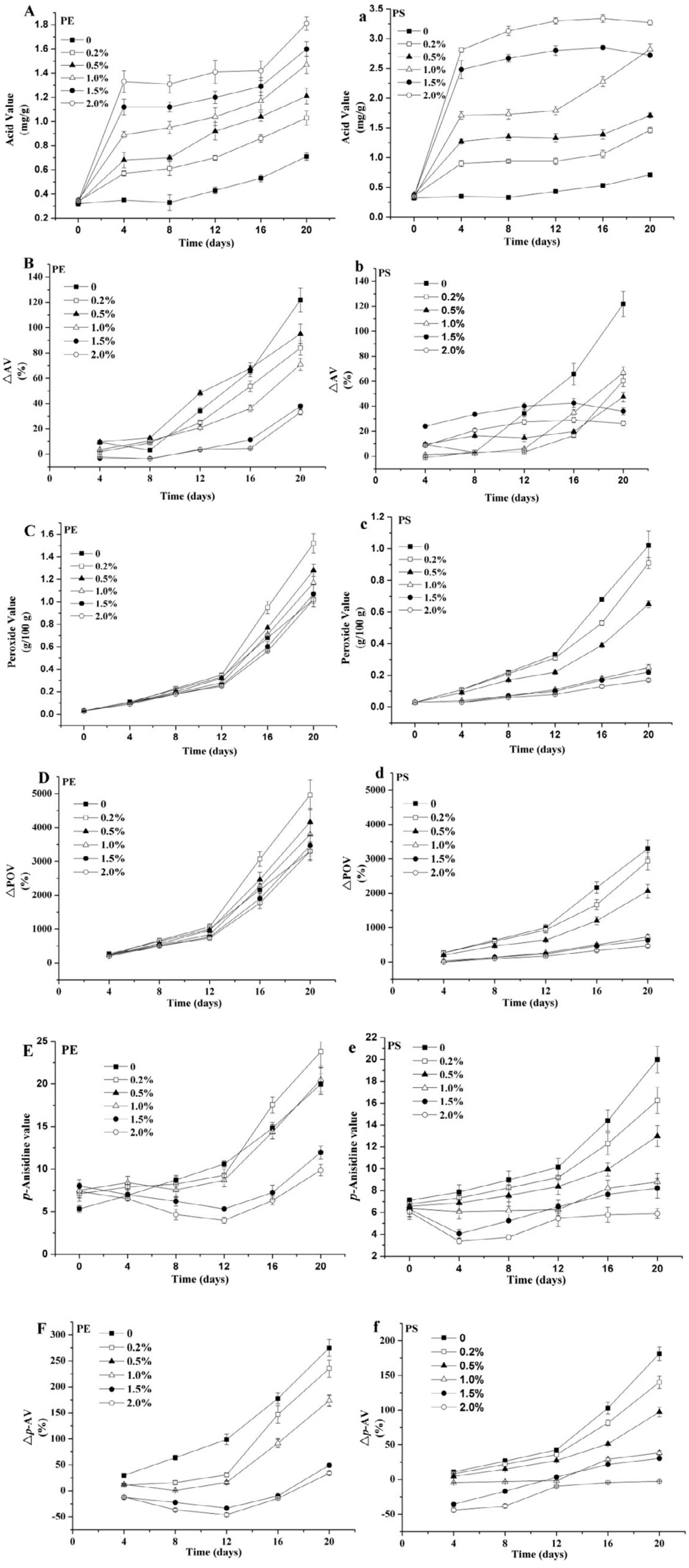

FIGURE 2. Effect of PE and PS on oxidative stability of RCSO during heating (means ± SD of triplicate determinations): (A, B, a, b) AV (mg/g), (C, D, c, d) POV (g/100 g), (E, F, e, f) *p*- AV

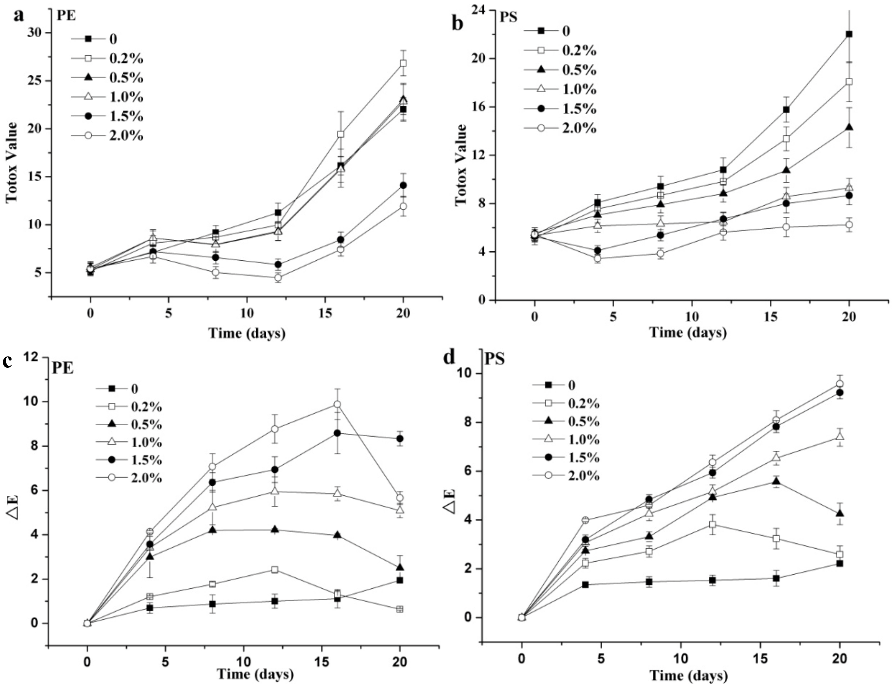

Figure 3. Effect of PE (a, c) and PS (b, d) on total oxidation value and total color difference (△E) of RCSO during heating (means ± SD of triplicate determinations)

then increased by 29, 22, and -4% on the 16th day compared to no addition of PS (Figures 2e, 2f), respectively.

As expected, with prolonging oxidation, hydrogen peroxide will decompose, and most of the decomposed products cannot react with potassium iodide, so POV is not able to evaluate the oil quality accurately. Some other complementary indicators must be used to measure oil rancidity such as *p*-AV, which reflects the amount of unsaturated aldehydes (secondary oxidation products, including aldehydes, ketones, and quinones) of oils and fats (Seung *et al.*, 2010). The more unsaturated the aldehydes, the easier it is to produce small molecules of aldehydes and ketones. In the present work, an increasing trend in *p*-AV in the early stage of heating (Figures 2E, 2F, 2e, 2f) may be attributed to the accumulation of primary oxidation products (Gökhan *et al.*, 2010) and products of phospholipid degradation, which have a strong ability to convert primary oxidation products to the corresponding hydroxyl lipids (Xiangqing *et al.*, 2010). The results of positive correlation between the inhibitory effect on the increase of *p*-AV and PE/PS addition amount was consistent with the report of (Peng *et al.*, 2020). The reason may be due to the strong antioxidant capacity of PE/PS (Figure 1 and Table 2), and/or many antioxidants (including peroxyl, alkane, alkene, and aldehyde derivatives) produced from PE/PS oxidation (Reis and Spickett, 2012).

The results in the present work indicated that PS/PE could inhibit the formation of the secondary metabolites of carbonyl compounds, and the inhibitory effect of PS addition was far better than that of PE.

3.4.5. Totox value

PE addition could slow down the increase in the Totox value in RCSO.The Totox value of RCSO was increased by 22, 5, 4, -36 and -46% on the 20th day compared to no addition of PE (Figure 3a). Regardless of the amount of addition or the duration of heating, PS addition could dramatically decrease the Totox value of RCSO. The otox value of RCSO was decreased by 7% and 18% (0.2%), 13 and 35% (0.5%), 24 and 56% (1.0%), 49 and 61% (1.5%), 57 and 72% (2.0%) on the 4th and 20th days compared to no addition of PS (Figure 3b), respectively.

Besides *p*-AV, Totox value is also an important indicator to measure the oil rancidity acidity and can indicate an oil's overall oxidation state, which means the lower the Totox value, the better the quality of oil (Seung *et al.*, 2010). The PE/PS addition could inhibit the increase in the Totox value of RCSO, which indicated that PE/PS could maintain the quality of RCSO (Figure 3a, 3b). The reasons for the Totox value change in RCSO may be due to the antioxidant capacity of PE/PS (Figure 1 and Table 2) to inhibit the formation of primary and secondary oxidation products (Figure 3b).

The results regarding changes in the Totox value of RCSO further indicated that PS/ PE addition could improve the oxidative stability of RCSO.

3.4.6. Color value

As shown in Figures 3c and 3d, Tables 3 and 4, the L*, a*, b*, c* and h* indicate the brightness, red-green, yellow-blue, chroma (the degree of color saturation or purity), and hue angle, respectively. In terms of PE addition to RCSO, except for the L* and h*, a*, b* and c* changed significantly. The more PE addition, the greater the a*, b* and c* of RCSO (Table 3). The PE addition caused a trend of first

Table 3. Effect of PE addition on color value of RCSO during heating

Heating (d)	PE (w/v,%)	L*	a*	b*	c*	h*
0	0	47.20±0.17^{a}	-0.57±0.12^{c}	14.27±0.38^{a}	14.28±0.38^{a}	92.28±0.49^{a}
	0.2	47.57±0.29^{a}	-0.54±0.06^{c}	14.77±0.75^{a}	14.78±0.75^{a}	92.09±0.32^{a}
	0.5	47.73±0.06^{a}	-0.54±0.06^{c}	16.07±0.06^{b}	16.08±0.06^{b}	91.91±0.20^{a}
	1.0	47.67±0.15^{a}	-0.44±0.06^{b}	16.67±0.38^{b}	16.68±0.38^{b}	91.50±0.21^{a}
	1.5	46.70±0.17^{a}	-0.40±0.06^{ab}	18.00±0.12^{c}	18.01±0.11^{c}	91.28±0.19^{a}
	2.0	46.63±0.61^{a}	-0.34±0.15^{a}	18.1±0.31^{c}	18.11±0.30^{c}	91.07±0.50^{a}
4	0	46.60±0.17^{b}	-0.57±0.06^{c}	13.93±0.21^{a}	13.94±0.21^{a}	92.33±0.21^{a}
	0.2	46.43±0.00^{b}	-0.53±0.06^{c}	15.20±0.06^{b}	15.21±0.06^{b}	92.15±0.21^{a}
	0.5	46.80±0.46^{b}	-0.40±0.10^{ab}	17.20±0.98^{c}	17.20±0.98^{c}	91.47±0.40^{a}
	1.0	46.43±0.00^{b}	-0.43±0.06^{b}	17.60±0.06^{cd}	17.60±0.06^{c}	91.53±0.19^{a}
	1.5	46.00±0.06^{a}	-0.43±0.06^{b}	17.63±0.10^{cd}	17.64±0.10^{c}	91.53±0.19^{a}
	2.0	45.83±0.00^{a}	-0.37±0.06^{a}	18.16±0.06^{d}	18.17±0.06^{d}	91.27±0.18^{a}
8	0	46.60±0.26^{a}	-0.57±0.06^{f}	13.63±0.32^{a}	13.65±0.32^{a}	92.38±0.23^{b}
	0.2	47.03±0.06^{a}	-0.66±0.06^{e}	16.03±0.10^{b}	16.04±0.10^{b}	92.37±0.22^{b}
	0.5	47.10±0.00^{a}	-0.46±0.06^{d}	18.46±0.06^{c}	18.47±0.06^{c}	91.44±0.18^{ab}
	1.0	46.77±0.32^{a}	-0.36±0.12^{c}	19.46±0.81^{cd}	19.47±0.81^{cd}	91.08±0.39^{ab}
	1.5	46.63±0.25^{a}	-0.26±0.06^{b}	20.60±0.46^{d}	20.60±0.46^{de}	90.74±0.17^{a}
	2.0	46.17±0.47^{a}	-0.10±0.17^{a}	21.23±1.65^{e}	21.23±1.65^{e}	90.29±0.47^{a}
12	0	46.60±0.20^{b}	-0.57±0.06^{c}	13.47±0.25^{a}	13.48±0.25^{a}	92.41±0.26^{b}
	0.2	47.03±0.21^{b}	-0.74±0.12^{d}	16.67±0.26^{b}	16.69±0.26^{b}	92.53±0.43^{b}
	0.5	46.37±0.00^{ab}	-0.70±0.00^{d}	18.40±0.06^{c}	18.42±0.06^{c}	92.19±0.01^{b}
	1.0	45.97±0.30^{a}	-0.54±0.06^{c}	20.07±1.15^{d}	20.08±1.15^{d}	91.54±0.25^{ab}
	1.5	45.43±0.21^{a}	-0.27±0.12^{b}	20.97±0.61^{d}	20.97±0.61^{d}	90.74±0.34^{a}
	2.0	45.57±0.26^{a}	-0.14±0.15^{a}	22.87±0.70^{e}	22.87±0.70^{e}	90.35±0.40^{a}
16	0	47.13±0.21^{bc}	-0.57±0.06^{b}	13.17±0.40^{a}	13.18±0.40^{a}	92.47±0.30^{b}
	0.2	47.26±0.31^{c}	-0.74±0.06^{c}	15.54±0.47^{b}	15.55±0.47^{b}	92.71±0.13^{b}
	0.5	46.96±0.38^{b}	-0.87±0.06^{cd}	18.20±0.72^{c}	18.22±0.72^{c}	92.74±0.26^{b}
	1.0	46.23±0.20^{ab}	-0.67±0.06^{bc}	20.04±0.35^{d}	20.05 ±0.35^{d}	91.92 ±0.19^{ab}
	1.5	46.36±0.21^{ab}	-0.27±0.15^{a}	22.80±0.95^{e}	22.81±0.94^{e}	90.69±0.41^{a}
	2.0	45.60±0.21^{a}	0.00±0.20^{d}	24.00±1.11^{f}	24.00±1.11^{f}	90.02±0.48^{a}
20	0	47.13±0.12^{c}	-0.50±0.00^{a}	12.33±0.06^{a}	12.34±0.06^{a}	92.32±0.01^{b}
	0.2	46.86±0.38^{b}	-0.73±0.06^{b}	13.80±0.40^{b}	13.82±0.40^{b}	93.04±0.17^{c}
	0.5	47.00±0.23^{c}	-0.87±0.10^{bc}	16.73±0.60^{c}	16.76±0.60^{c}	92.98±0.45^{b}
	1.0	46.30±0.15^{b}	-0.93±0.06^{c}	19.27±0.35^{d}	19.29±0.35^{d}	92.77±0.13^{b}
	1.5	45.73±0.35^{a}	-0.50±0.06^{a}	22.47±0.40^{e}	22.47±0.40^{e}	91.28±0.17^{a}
	2.0	49.70±0.15^{d}	-0.97±0.10^{c}	19.33±0.71^{d}	19.36±0.71^{d}	92.87±0.35^{bc}

Note: Values are means ± SD of triplicate determinations. Different letters indicate that there are significant differences between columns (Tukey's test, $p < 0.05$). L*: brightness; a*: red / green; b*: yellow / blue; c*: chroma; h*: hue angle.

increasing and then decreasing in RCSO ΔE during the heating process, and the higher amount of PE addition, the greater the ΔE of RCSO (Figure 3c). Regarding PS addition in RCSO, the brightness (L*) of RCSO decreased, but the a*, b* and c* of RCSO increased with the increase in heating time and PS addition (Table 4). The changing trend in RCSO ΔE with PS addition was similar to that of PE addition, but the effect of PS addition on RCSO ΔE was more obvious than that of PE addition (Figures 3c, 3d).

Table 4. Effect of PS addition on color value of RCSO during heating

Heating (d)	PE (w/v, %)	L*	a*	b*	c*	h*
0	0	47.20±0.17^{a}	-0.57±0.12^{b}	14.27±0.38^{a}	14.27±0.38^{a}	92.23±0.42^{a}
	0.2	46.67±0.42^{a}	-0.47±0.12^{ab}	14.70±0.35^{a}	14.70±0.35^{a}	91.87±0.38^{a}
	0.5	46.20±0.35^{a}	-0.43±0.12^{a}	14.53±0.38^{a}	14.53±0.38^{a}	91.63±0.38^{a}
	1.0	46.47±0.29^{a}	-0.53±0.06^{b}	14.60±0.35^{a}	14.60±0.35^{a}	92.13±0.25^{a}
	1.5	46.93±0.38^{a}	-0.40±0.10^{a}	15.40±0.52^{b}	15.40±0.52^{b}	91.60±0.30^{a}
	2.0	46.50±0.10^{a}	-0.50±0.10^{ab}	15.47±0.06^{b}	15.47±0.06^{b}	91.70±0.36^{a}
4	0	46.60±0.17^{b}	-0.57±0.06^{c}	13.93±0.21^{a}	13.2±0.00^{a}	92.30±0.20^{b}
	0.2	45.63±0.12^{ab}	-0.40±0.10^{b}	15.37±0.76^{b}	15.13±0.15^{b}	91.47±0.38^{ab}
	0.5	45.07±0.06^{a}	-0.30±0.00^{ab}	15.73±0.21^{bc}	15.30±0.10^{b}	91.13±0.12^{ab}
	1.0	44.90±0.17^{a}	-0.37±0.06^{ab}	16.03±0.42^{bc}	15.00±0.00^{b}	91.20±0.17^{ab}
	1.5	44.93±0.15^{a}	-0.30±0.00^{ab}	16.33±0.29^{c}	16.23±0.06^{c}	91.07±0.06^{ab}
	2.0	44.9±0.10^{a}	-0.23±0.12^{a}	17.40±0.66^{d}	17.40±0.66^{d}	90.83±0.38^{a}
8	0	46.60±0.26^{b}	-0.57±0.06^{d}	13.63±0.32^{a}	13.63±0.32^{a}	92.43±0.23^{bc}
	0.2	45.67±0.06^{ab}	-0.47±0.12^{c}	16.23±0.35^{b}	16.23±0.35^{b}	91.60±0.36^{b}
	0.5	45.27±0.12^{ab}	-0.43±0.12^{c}	16.73±0.65^{b}	16.73±0.65^{b}	91.40±0.44^{b}
	1.0	45.53±0.31^{ab}	-0.07±0.06^{b}	18.10±0.82^{c}	18.10±0.82^{c}	90.17±0.25^{a}
	1.5	45.30±0.30^{ab}	0.03±0.21^{a}	18.63±1.19^{c}	18.63±1.19^{c}	89.90±0.72^{a}
	2.0	44.87±0.12^{a}	0.00±0.10^{a}	18.20±0.20^{c}	18.20±0.20^{c}	90.03±0.21^{a}
12	0	46.60±0.20^{c}	-0.57±0.06^{d}	13.47±0.25^{a}	13.47±0.25^{a}	92.33±0.31^{b}
	0.2	46.00±0.17^{bc}	-0.53±0.06^{d}	17.70±0.52^{b}	17.70±0.52^{b}	91.77±0.31^{b}
	0.5	45.37±0.40^{b}	-0.33±0.06^{c}	18.73±0.15^{c}	18.73±0.15^{c}	90.97±0.15^{ab}
	1.0	45.07±0.15^{ab}	0.00±0.00^{b}	18.93±0.31^{c}	18.93±0.31^{c}	90.00±0.10^{a}
	1.5	44.77±0.21^{a}	0.23±0.06^{a}	19.67±0.51^{cd}	19.67±0.51^{cd}	89.33±0.21^{a}
	2.0	44.57±0.12^{a}	0.33±0.23^{a}	20.03±1.12^{d}	20.03±1.12^{d}	89.03±0.64^{a}
16	0	47.13±0.21^{c}	-0.57±0.06^{d}	13.17±0.40^{a}	13.17±0.40^{a}	92.40±0.35^{bc}
	0.2	46.20±0.20^{bc}	-0.87±0.06^{e}	16.97±0.60^{b}	16.97±0.60^{b}	92.90±0.17^{bc}
	0.5	45.90±0.10^{b}	-0.53±0.06^{d}	19.57±0.25^{c}	19.57±0.25^{c}	91.57±0.15^{b}
	1.0	44.97±0.12^{a}	-0.07±0.21^{c}	20.37±0.86^{c}	20.37±0.86^{cd}	89.63±0.45^{ab}
	1.5	44.73±0.06^{a}	0.50±0.10^{b}	21.70±0.26^{d}	21.70±0.26^{d}	88.70±0.20^{a}
	2.0	44.17±0.06^{a}	0.73±0.25^{a}	21.77±0.93^{d}	21.73±0.87^{d}	88.20±0.61^{a}
20	0	47.13±0.12^{e}	-0.50±0.00^{d}	12.33±0.06^{a}	12.33±0.06^{a}	92.43±0.06^{c}
	0.2	46.13±0.15^{d}	-0.90±0.00^{e}	16.07±0.57^{b}	16.07±0.57^{b}	93.17±0.06^{c}
	0.5	45.57±0.21^{cd}	-0.87±0.06^{e}	17.90±0.62^{c}	17.93±0.61^{c}	92.73±0.21^{c}
	1.0	44.90±0.20^{c}	0.17±0.15^{c}	21.27±1.07^{d}	21.27±1.07^{d}	89.63±0.42^{bc}
	1.5	43.57±0.15^{b}	0.80±0.17^{b}	22.73±0.55^{e}	22.73±0.55^{e}	88.00±0.35^{b}
	2.0	42.27±0.84^{a}	1.23±0.25^{a}	22.43±0.25^{e}	22.47±0.25^{e}	86.90±0.56^{a}

Note: Values are means ± SD of triplicate determinations. Different letters indicate that there are significant differences between the columns (Tukey's test, $p < 0.05$). L*: brightness; a*: red / green; b*: yellow / blue; c*: chroma; h*: hue angle.

For example, the ΔE of RCSO reached 3.81 (12th day) and 5.57 (16th day) for 0.2 and 0.5% PS addition (Figure 3d), respectively; while the RCSO ΔE reached 7.39, 9.22, and 9.58 for 1.0, 1.5, and 2.0 PE addition on the 20th day (Figure 3c), respectively.

As expected, refined edible vegetable oil appears light yellow and pale amber, but the color reversion of oils (especially for refined soybean, cottonseed and corn oils) often makes them darker and develop into deep yellow and light red during the transportation, storage and use (Mostafa *et al.*, 2014). So far, it has been recognized that precursors to colored substances (such as chroman-5, 6-quinone, γ-tocopherol, γ-tocopherol dimer). Their degradation products, and the oxidation of oils (such as oxidized unsaturated fatty acids) were said to be responsible for the color reversion of oils (Ming-Tain *et al.*, 1989). It is worth noting that precursors promote the dark color of oils at the same time, which also promotes or inhibits the oxidation of oils (František *et al.*, 2016). The color changes with inconspicuous darkening, reddening, and yellowing in the present work indicated that PE and PS could improve a slightly dark color in RCSO (Figure 3, Tables 3 and 4), which was similar to what was reported by (František *et al.*, 2016). PE and PS are amino phospholipids and prone to Maillard reaction to produce a small amount of colored substances (likely pyrroles) during the temperature acceleration process (Reis and Spickett, 2012), which may be a factor to cause some color reversion RCSO.

The results in the present study indicated that PS/PE addition could cause some color reversion in RCSO due to the formation of colored products from the Maillard reaction or/and hydrolysis and oxidation of lipids.

3.5. Correlation of oxidative stability, antioxidant capacity and color reversion of RCSO in terms of addition of PE and PS

3.5.1 Correlation of antioxidant capacity of RCSO in terms of addition of PE and PS

PE addition has an extremely significant correlation with ORAC (0.967, $p < 0.01$) and significant correlations with DPPH (0.787, $p < 0.05$), and ABTS (0.886, $p < 0.05$) (Table 5). However, PS addition has extremely significant correlations with ORAC, DPPH and ABTS, and the Pearson correlation coefficients are 0.931 ($p < 0.01$), 0.897 ($p < 0.01$) and 0.843 ($p < 0.01$), respectively (Table 5). The above results further showed that PS/PE addition can improve the antioxidant capacity of RCSO (Table 3 and 5). PE mainly improved the ORAC in RCSO; while PS not only improved the ORAC of RCSO, but also improved its FRSC (Tables 3 and 5, and Figure 2).

3.5.2 Correlation of oxidative stability of RCSO in terms of addition of PE and PS

As far as the oxidative stability (including AV, POV and *p*-AV) of RCSO was concerned, apart from an extremely significant correlation between PE addition and AV (0.860, $p < 0.01$), an unobvious and negatively significant correlation between PE addition and POV (-0.096), and *p*-AV (-0.434, $p < 0.01$), respectively. These results indicated that PE addition has no obvious inhibitory effect on the primary oxidation of RCSO, leading to an increase in *p*-AV. The positive correlation between PS addition and the AV of RCSO, POV and *p*-AV were extremely significant, and the Pearson correlation coefficients were 0.951 ($p < 0.01$), 0.676 ($p < 0.01$) and 0.629 ($p < 0.01$), respectively (Table 5). These results indicated that PS could inhibit primary and secondary oxidation reactions, and then result in a decrease in the production of primary and secondary oxidation products (Figure 2). An extremely significant correlation between PE/PS addition and the IP of RCSO (0.958, $p < 0.01$)/ (0.966, $p < 0.01$) further indicated that PE and PS could improve the oxidation stability of RCSO (Table 5).

3.5.3 Correlation of color reversion of RCSO in terms of addition of PE and PS

PE/PS addition had significant negative correlations with L*, the Pearson's correlation coefficients were -0.302 ($p < 0.01$) and -0.668 ($p < 0.01$) (Table 5), respectively. However, there was a significant positive correlation between PE/PS addition and color value (a*, b* and ΔE), the Pearson's correlation coefficients between PE/PS addition and a*, b* and ΔE were 0.420/0.506 ($p < 0.05$), 0.323/0.436($p < 0.05$) and 0.417/0.408 ($p < 0.05$) (Table 5), respectively. The above results from the correlation analysis further showed that PE/PS addition can cause some color reversion (Tables 3, 4 and Figure 3).

Based on the analysis of the correlation between color change and oxidative stability indexes (including AV, POV and *p*-AV) of RCSO in terms of PE/PS addition, the POV of RCSO had no correlation with L*, b*, and ΔE, and a significant negative correlation with a*

Table 5. Pearson correlation analysis between PE, PS, and oxidative stability of RCSO

	IP	**ORAC**	**ABTS**	**DPPH**	**AV**	**POV**	**p-AV**	**L***	**a***	**b***	**ΔE**
IP	1	.932**	.635*	.542*	.765**	.886**	.753**	.235*	.156*	.266*	.324*
ORAC		1	0.007	-0.012	.851**	.810**	.798**	.123	.105	0.089	.111
ABTS			1	-0.207	.567**	.154*	.235*	-0.365	-0.214	.156	.212
DPPH				1	.862**	-0.097	-.433**	-0.307	.509**	.836**	.827**
AV					1	.386*	0.008	-0.184	0.131	.781**	.790**
POV						1	.846**	0.142	-.516**	0.005	0.093
p-AV							1	0.246	-.624**	-.432**	-.362*
L*								1	-.536**	-.442**	-.463**
a*									1	.469**	.510**
b*										1	.969**
ΔE											1
PE	.958**	.967**	.886*	.787*	.860**	-0.096	-.434**	-0.302**	.420*	.323*	.417*
IP	1	.932**	753**	.798**	.813**	.857**	.798**	.324*	.361*	.231*	.278*
ORAC		1	0.036	-0.035	.836**	.887**	.813**	.089	.134	0.116	.098
ABTS			1	-0.026	.812**	.668**	.735**	.165	.105	.116	.097
DPPH				1	.936**	-.482**	-.633**	-.709**	.633**	.705**	.633**
AV					1	-0.326	-.497**	-.764**	.753**	.781**	.761**
POV						1	.961**	0.282	-.349*	-0.189	-0.057
p-AV							1	.440**	-.436**	-0.324	-0.196
L*								1	-.837**	-.847**	-.873**
a*									1	.787**	.846**
b*										1	.963**
ΔE											1
PS	.966**	.931**	.843**	.897**	.951**	.676**	.629**	-.668**	.506*	.436*	.408*

Notes: **0.01 level (bilateral) extremely significant.* 0.05 level (bilateral) significant. L*: brightness; a*: red / green; b*: yellow / blue; ΔE: total color difference.

(-0.516, $p < 0.01$) and (-0.349, $p < 0.05$) for PE and PS addition, respectively. In term of PE addition, AV had no correlation with L* (-0.184) and a* (0.013), and extremely significant positive correlation with b* (0.781, $p < 0.01$) and ΔE (0.790, $p < 0.01$). *p*-AV had no correlation with L* (0.246), significant negative correlation with a* (-0.624, $p < 0.01$), b* (-0.432, $p < 0.01$), and ΔE (-0.362, $p < 0.05$). As far as PS addition was concerned, AV had an extremely significant negative correlation with L* (-0.764, $p < 0.01$), and extremely significant positive correlation with a* (0.753, $p < 0.01$), b* (0.781, $p < 0.01$), and ΔE (0.761, $p < 0.01$). *p*-AV had no correlation with b* (-0.324) and ΔE (-0.196), extremely significant positive correlation with L* (0.440, $p < 0.01$), and extremely significant negative correlation with a* (-0.436, $p < 0.01$). These results indicated that the key reason for the color reversion of RCSO added with PE/PS may have been due to the AV changes in RCSO. As expected, the prerequisite for the color reversion is the free fatty acid formation by lipid hydrolysis or further oxidation of the PE/PS to produce some colored substances (causing the oil's yellow value to deepen) (Yuquan *et al.*, 2013). In the present work, the possible reasons for the color reversion caused by PE/PS addition may be attributed to the free fatty acid formation or the color of the phospholipid itself (Reis and Spickett, 2012).

4. CONCLUSION

This work demonstrated that PE/PS addition could improve the oxidative stability and antioxidant capacity of RCSO, and the effect of PS addition was far better than that of PE. The possible reason for PE addition to improve the oxidative stability and antioxidant capacity of RCSO is the inhibiting of the generation of free fatty acids, while there was a comprehensive result (including the inhibition of free fatty acid production, and the generation of primary and secondary oxidation products) for PS addition. Therefore, moderate refining to keep the non-hydratable phospholipids as much as possible can not only improve the functional and nutritional value but also extend the shelf-life of the CSO.

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Conflict of Interest

The authors declare no competing financial interest.

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3-MCPD and glycidol levels in edible oils and fats obtained from local markets in Türkiye

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SUMMARY: In this study, it was aimed to determine the 3-MCPD and glycidol levels in 9 types (46 brands) of edible fat and oil offered for sale in markets located in Türkiye. 3-MCPD and glycidol levels were determined by making some modifications to the DGF C VI 18 (10) method. The highest levels of 3-MCPD and glycidol levels were detected in hazelnut oils, riviera olive oils, margarines, and shortenings. As expected, these contaminants were not observed in extra-virgin olive oils, while they were detected at low levels in fish oils. The highest 3-MCPD levels were found in the range of 0.06-2.12 mg·kg^{-1} in hazelnut oil, 0.16-1.69 mg·kg^{-1} in riviera olive oils, and 0.17-1.17 mg·kg^{-1} in margarines. The highest glycidol levels were found in the shortenings in the range of 1.98-6.46 mg·kg^{-1}, followed by hazelnut oil (0.54-2.63 mg·kg^{-1}) and riviera olive oil (0.19-3.53 mg·kg^{-1}).

KEYWORDS: *3-MCPD; Edible oils; Glycidol; Margarine; Olive oil.*

RESUMEN: ***Niveles de 3-MCPD y glicidol en aceites y grasas comestibles obtenidos de mercados locales en Turquía.*** En este estudio, el objetivo fue determinar los niveles de 3-MCPD y glicidol en 9 tipos (46 marcas) de grasas y aceites comestibles ofrecidos a la venta en mercados ubicados en Turquía. Los niveles de 3-MCPD y glicidol se determinaron haciendo algunas modificaciones al método DGF C VI 18 (10). Los niveles más altos de 3-MCPD y glicidol se detectaron en aceites de avellana, aceites de oliva riviera, margarinas y mantecas. Como era de esperar, estos contaminantes no se observaron en los aceites de oliva virgen extra, mientras que se detectaron en niveles bajos en los aceites de pescado. Los niveles más altos de 3-MCPD se encontraron en el rango de 0,06-2,12 mg·kg^{-1} en aceite de avellana, 0,16-1,69 mg·kg^{-1} en aceites de oliva riviera y 0,17-1,17 mg·kg^{-1} en margarinas. Los niveles más altos de glicidol se encontraron en las mantecas en el rango de 1,98-6,46 mg·kg^{-1}. Le siguieron el aceite de avellana (0,54-2,63 mg·kg^{-1}) y el aceite de oliva riviera (0,19-3,53 mg·kg^{-1}).

PALABRAS CLAVE: *3-MCPD; Aceites comestibles; Aceite de oliva; Glicidol; Margarina.*

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1. INTRODUCTION

Most of the edible oils on the market shelves are made ready for consumption by refining. With the help of the refining process, sensory characteristics such as taste, smell, appearance, and shelf stability are provided (Pudel *et al.,* 2011). Due to the application of high temperatures, process contaminants may be formed as a result of some reactions (hydrolysis, oxidation) (Shahidi, 2005). 3-monochloropropane-1,2-diol (3-MCPD) and glycidyl esters (GE) are formed during several processes including frying, cooking, distillation, and refining (Zhou *et al.,* 2014). 3-MCPD and GE could be formed during the refining of crude oils, especially in the deodorization stage where high temperatures are applied (230-250 °C) (Özdikicierler *et al.,* 2016). These researchers stated that the formation of contaminants accelerated with the increase in monoglyceride, diglyceride, and chloride ion concentrations in the environment. Cyclic acyloxonium ions and glycidyl esters are formed when mono and diglycerides are formed as a result of triglyceride hydrolysis when edible fats and oils are exposed to high temperatures. These compounds are converted into 3-MCPD with the presence of chloride ions (Hamlet *et al.,* 2011). In the presence of water and at high temperatures, triglyceride is hydrolyzed to diglyceride and monoglyceride. In glycerol and glycerides, 3-MCPD ester formation increases with increasing salt concentration and reaches its maximum level with approximately 15% water content (EFSA, 2016). 3-MCPD are formed as a result of the replacement of the acyl or hydroxyl groups in the acylglycerol molecule with the chloride ion (Svejkovska *et al.,* 2006). In the absence of water in the environment, pre-hydrolysis of the acyl groups cannot occur, but the hydroxyl group of the glycerol molecule and the chlorine are directly replaced (EFSA, 2016). It has been stated that precursors such as monoglycerides, diglycerides, and chloride ions which cause 3-MCPD formation, as well as parameters such as processing temperature and duration, play a significant role in 3-MCPD formation (Sevindirici *et al.,* 2018). In addition, it was observed by Shimizu *et al.* (2012) that glycidol was formed from di- and monoacyl glycerols in the absence of chlorine ions at temperatures of 240 °C and above. However, Destaillats *et al.* (2012) determined that the majority of MCPD diesters are formed above 200 °C by the reaction of organochlorines with triacylglycerols. Additional experiments by these researchers confirmed that this reaction could be initiated during palm oil deodorization with hydrogen chloride (HCl) gas released through the thermal degradation of the organochlorines present in the oil. Since toxicological studies have shown that the free form of 3-MCPD is carcinogenic (Cho *et al.,* 2008), 3-MCPD has possible health risks. Similar to 3-MCPD, glycidol is also highly risky for health because of its epoxy ring structure (IARC, 2013). The International Agency for Research on Cancer has classified 3-MCPD as a “potential carcinogen to humans” in group 2B. Glycidyl, on the other hand, was stated to have mutagenic and carcinogenic properties and classified in group 2A, which means that it was “probably carcinogenic to humans” (IARC 2013). According to the report published in 2018, the tolerable daily intake of 3-MCPD was recommended as 2 $\mu g \cdot kg^{-1}$ body weight (EFSA 2018).

Regulation EU 2020/1322, amending Regulation EC No. 1881/2006, applies as of January 1st 2021 with regard to foods in the EU. This specifies maximum quantities for free 3-MCPD in hydrolyzed plant protein and soya sauce as well as maximum quantities for glycidyl fatty acid esters, expressed as glycidol, in vegetable oils and fats, fish oils and other marine oils which are placed on the market for consumers or for use as an ingredient in foods (maximum quantity 1 $mg \cdot kg^{-1}$) (EU, 2020). In addition, EU regulation set the maximum level for GE at 1 $mg \cdot kg^{-1}$ in vegetable oils and fats which are aimed for the final consumer or as an ingredient in food. In addition, the level of GE for vegetable oils and fats destined for baby food and processed cereal-based food is set at 0.5 $mg \cdot kg^{-1}$ (EU, 2020). With the same regulation, maximum quantities for the sum of free and fatty acid bound 3-MCPD (analyte group) are set for the same foods for which maximum levels for GE are established. Due to the low 3-MCPD formation potentials, a stricter maximum level (1.25 $mg \cdot kg^{-1}$) for the sum of 3-MCPD and 3-MCPD fatty acid esters, expressed as 3-MCPD is set for oils and fats from coconut, maize, rapeseed, sunflower, soybean, palm kernel and olive oils and mixtures of oils and fats with oils and fats only from this category. A higher maximum level of 2.5 $mg \cdot kg^{-1}$ applies to other vegetable oils (including olive pomace oils), fish oils and oils from other marine organisms and mixtures of oils and fats with oils and fats only from this category. For oil mixtures from both categories with quantitatively known ingredients, the category-related maximum levels apply to single ingredients. In the case of oil and fat mixtures of unknown composition, the higher maximum level of 2.5 $mg·kg^{-1}$ applies. For vegetable oils and fats, fish oils and other marine oils which are destined for the production of baby food and processed cereal-based food for infants and young children the maximum level for the sum of 3-MCPD and 3-MCPD fatty acid esters, expressed as 3-MCPD is set to 0.75 $mg·kg^{-1}$. Finally, for infant formula, follow-on formula and foods for special medicinal purposes for infants and young children as powders the maximum level is addressed as 0.125 $mg·kg^{-1}$ for powders and as 15 $\mu g·kg^{-1}$ for liquids. These limits set by the EU are valid for European countries.

Although there are many studies in the literature on the detection of 3-MCPD and glycidol in different food products, few studies have been conducted in Türkiye on the determination of 3-MCPD and glycidol in potato chips (Önal *et al.,* 2016), in steam distillation of olive oils (Özdikicierler *et al.,* 2016), in baking in biscuit making (Mogol 2014), or in frying stages (Deniz Şirinyıldız *et al.,* 2019). These processes applied to the oils mentioned above may differ from region to region. In this respect, we think that it is important to examine the edible fats and oils offered for sale in the country's markets in terms of these process contaminants. Considering the results of this study, the daily intake limits determined by the relevant authorities for consumers may not be exceeded. In addition, manufacturers can make efforts to limit these compounds. Within the scope of this study, sunflower oil (7 brands), hazelnut oil (4 brands), corn oil (6 brands), natural extra virgin olive oil (7 brands), riviera olive oil (7 brands), margarine and shortening (9 brands), peanut oil (2 brands), and fish capsules (4 brands) which are sold and consumed widely in the markets of Türkiye were investigated. In total, 3-MCPD and glycidol levels were detected in 46 samples.

2. MATERIALS AND METHODS

2.1. Materials

The different cooking oils, fish oils, margarines and shortenings used in the study were obtained from local markets in Türkiye in 2019. Their names and number of brands are shown in Table 1. All of these oils, except for virgin olive oil and fish oils, are refined oils. Virgin olive oil is obtained by cold pressing., that is, it is not refined. Riviera oil consists of 20% virgin olive oil and 80% refined olive oil. 3-MCPD and 3-chloro-1,2-propane-1,1,2,3,3-d5-diol (3-MCPD-d5), glycidyl stearate, diethyl ether, methanol, sodium hydroxide, sodium bromide, ethyl acetate, phenylboronic acid (PBA), acetone and toluene were obtained from Sigma-Aldrich (Steinheim, Germany). The purity of the chemicals was ensured.

Table 1. The sample names and number of brands.

Sample names	Number of brands
Sunflower oil (Refined)	7
Hazelnut oil (Refined)	4
Peanut oil (Refined)	2
Corn oil (Refined)	6
Virgin Olive Oil (Cold-pressed)	7
Riviera olive oil (Blend from refined and cold pressed)	7
Pastry oil (Shortening) (Refined)	3
Margarine (Refined)	6
Fish oil (capsule) (Native-Refined)	4
Total number of samples	46

2.2. Methods

2.2.1. Preparation of samples

The lipid fractions of margarine and fats were obtained by centrifugation of the molten product, then filtered through anhydrous sodium sulfate by removing the upper oil phase. In margarines and shortenings, analyses were performed only for the lipid phase. Encapsulated fish oils were removed from the capsules with a sterile needle. Bottled edible oils were taken with a straw directly from the bottle in which they were kept in the market. All oil samples were stored at 4 °C in a dark environment until the experiments.

2.2.2. Analysis of 3-MCPD and glycidol

3-MCPD and glycidol levels were determined based on the standard method of DGF C VI 18 (10) (DGF, 2011) with some modifications. In part A of

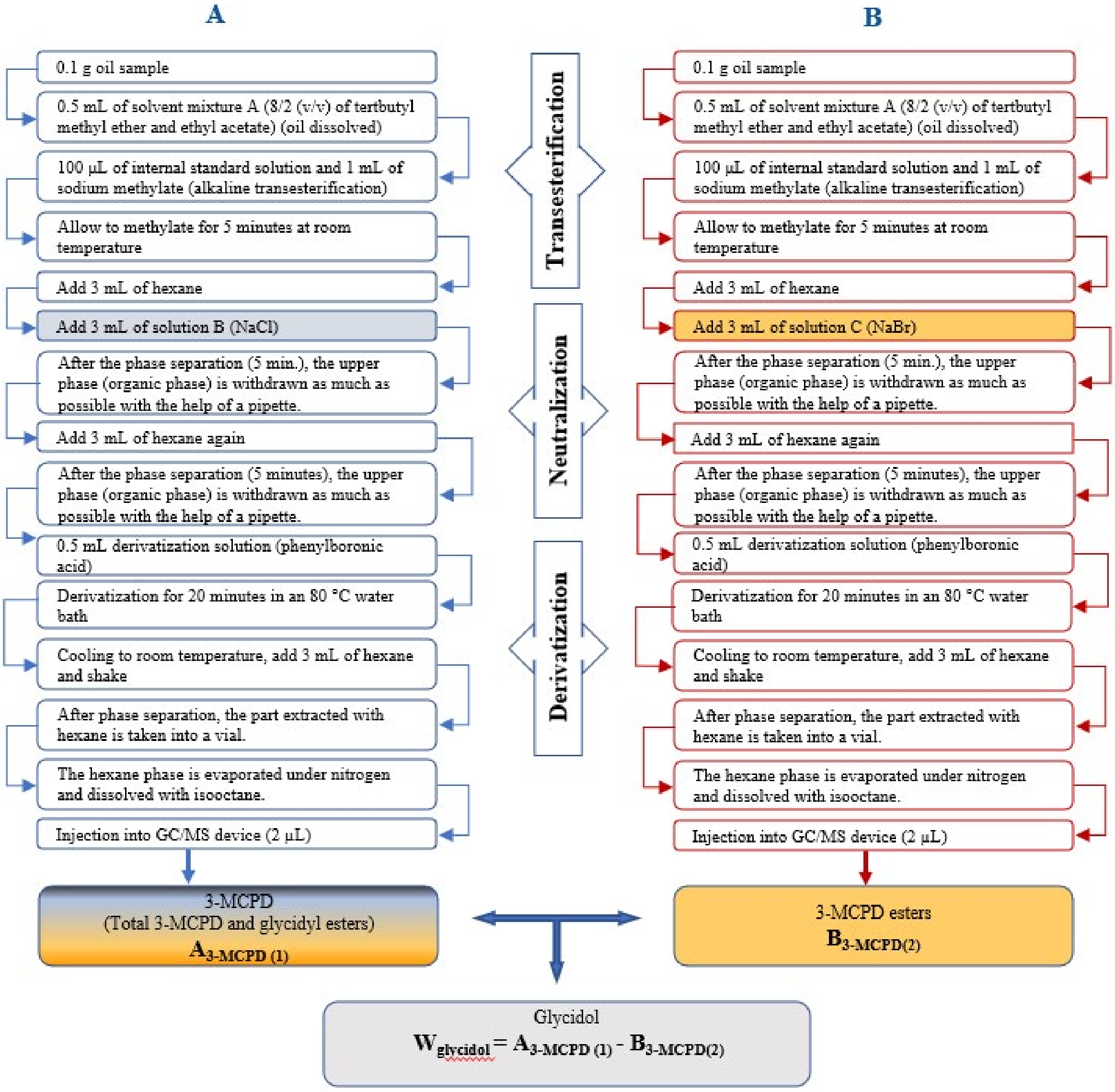

FIGURE 1. 3-MCPD and Glycidol analysis flow chart.

this method, 3-MCPD, corresponding to the sum of bound 3-MCPD and bound glycidol was determined, as in the DGF C VI 17 (10) method. We modified the DGF C VI 18 (10) method by applying the DGF C VI 17 (10) method in part A. We modified the DGF C VI 18 (10) method part B so that only the amount of ester-bound 3-MCPD was determined. We did not intend to determine 2-MCPD in this part B. Therefore, the relevant standard was not used. Both methods which form the basis of our method, namely C-VI 17 (10) and C-VI 18 (10), have been developed and approved only for the analysis of edible oils and fats. The flow chart of processing steps is shown in Figure 1. According to this method, isotope-labeled 3-MCPD-d5 was used as an internal standard. All oil samples were derivatized after esterification and neutralization processes and injected into the GC/MS (Shimadzu GC-2010 Gas Chromatography-QP-2010 Ultra Mass Spectrometry System, Shimadzu Corporation, Kyoto, Japan) device. The operating conditions of the GC/MS device are provided in Table 2.

Solutions used in esterification, neutralization, and derivatization in analysis. A sodium methylate solution with methanol ($NaOCH_3$): 0.5 molar methanol solution of sodium methylate (27 grams of sodium methylate tart) was dissolved in 1 liter of methanol.

Table 2. Operating conditions of the GC/MS device.

Device:	Shimadzu GC-2010
Detector:	EI+, SIM Mode
Internal standard Mass:	m/z = 201 or 150 (3-MCPD- d5) m/z = 196 or 147 (3-MCPD)
Column:	TRB - 5MS, 30m × 0.25mm × 0.25µm
Gases:	Helium 2.1 mL/min.
Temperature:	Oven Program: 60 °C (1 min), 6 °C·min^{-1} to 190 °C 190 °C - 280 °C to 20 °C /min (10–30 min)
Split:	20 mL·min^{-1}.
Splitless time:	1.5 min.

Solvent mixture A: 8/2 (v/v) mixture of tertbutyl methyl ether and ethyl acetate

Sodium chloride (NaCl) solution: 20% (m/v) aqueous solution of sodium chloride

Sodium bromide (NaBr) solution: 60% (m/v) aqueous solution of sodium bromide

Solvent mixture B: A mixture of 30 mL of sodium chloride solution and 1 mL of acetic acid (v/v) (prepared daily)

Solvent mixture C: A mixture of 30 mL of sodium bromide solution and 1 mL of acetic acid (v/v) (prepared daily)

Derivatization solution: 2.5 grams of phenylboronic acid dissolved in a mixture of 19 mL of acetone and 1 mL (m/v) distilled water

Internal standard stock solution: 2000 mg·L^{-1} (m/v) of 3-MCPD-d5 dissolved in ethanol

Internal standard solution: Prepared from the internal standard stock solution by dissolving in tert-butyl methyl ether (20 mg·L^{-1}).

The first step in the determination of 3-MCPD and glycidol based on the indirect DGF C-VI 18 (10) method was to evaluate the efficiency of the conversion from glycidol to 3-MCPD following the method used for Assay A. Figure 2 shows the amount of 3-MCPD formed as a function of the amount of glycidol (in the form of glycidyl stearate) in a spiked blank oil (olive oil) at seven different levels (0.05; 0.1; 0.25; 0.5; 1; 2.5 and 5 mg·kg^{-1}). A linear regression of the type y = mx + b was performed, the reciprocal slope (1/m) provided the conversion factor (t) (Lucas *et al.,* 2017).

Two-stage analysis was performed. In the first step (A), the total amount of 3-MCPD and glycidol in the sample, expressed as 3-MCPD, was calculated according to Eq.1. Here, 3-MCPD and glycidol were not separated. Therefore, the solvent was prepared with sodium bromide instead of sodium chloride in the second step (B) of the analysis in order to prevent the conversion of glycidol to 3-MCPD (Karl *et al.,* 2016). In the second step, pure or bound 3-MCPD was determined according to the Eq.2. The glycidol levels were calculated by subtracting the result from the second stage from the result from the first stage and multiplying the conversion rate of glycidol to 3-MCPD (Eq.3).

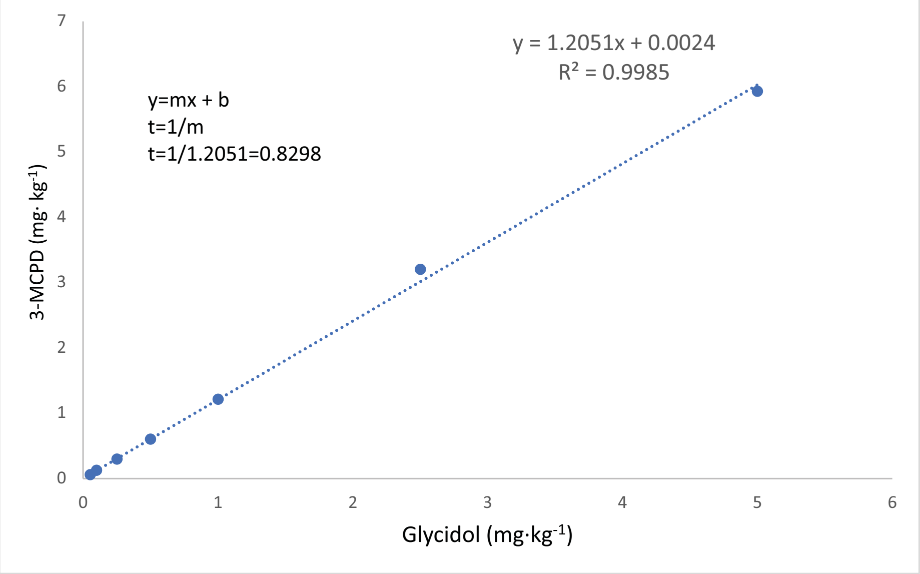

Figure 2. The amount of 3-MCPD formed as a function of the amount of Glycidol at seven different levels. A linear regression of the type y = mx + b was performed, the reciprocal value of the slope (1/m) provides the conversion factor (t).

$$A_{3\text{-}MCPD\,(1)} = [Q\,(147)\ x\ C_{d5-3-MCPD\,(1)}] \,/\, Q\,(150) \qquad \text{(Eq. 1)}$$

$A_{3\text{-}MCPD\,(1)}$ = Mass fraction of 3-MCPD (mg·kg^{-1}) detected in the first step
Q (147) = Peak area of 3-MCPD determined in the first step
Q (150) = Peak area of 3-MCPD – d5 determined in the first step
$C_{d5-3-MCPD\,(1)}$ = Concentration of internal standard (mg·kg^{-1}) used in the first step

$$B_{3\text{-}MCPD\,(2)} = [Q\,(147)\ x\ C_{d5-3-MCPD\,(2)}] \,/\, Q\,(150) \qquad \text{(Eq. 2)}$$

$B_{3\text{-}MCPD\,(2)}$ = Mass fraction of 3-MCPD (mg·kg^{-1}) detected in the second step
Q (147) = Peak area of 3-MCPD determined in the second step
Q (150) = Peak area of 3-MCPD-d5 determined in the second step
$C_{d5-3-MCPD\,(2)}$ = Concentration of internal standard (mg·kg^{-1}) used in the second step

$$W_{Glycidol} = t\ x\ (A_{3\text{-}MCPD\,(1)} - B_{3\text{-}MCPD\,(2)}) \qquad \text{(Eq. 3)}$$

$W_{Glycidol}$ = Mass fraction of glycidol in samples (mg·kg^{-1})
t = In the equation in the created calibration graph (Figure 2), the ratio of 1/m (y=1.2051x+0.0024, R^2=0.9984).
$A_{3\text{-}MCPD\,(1)}$ = Mass fraction calculated in the first step
$B_{3\text{-}MCPD\,(2)}$ = Mass fraction calculated in the second step

2.3. Statistical analysis

All data were statistically analyzed using SPSS (version 20.0 for Windows, SPSS Inc., Chicago, Illinois) package program by conducting one-way analysis of variance (ANOVA), and defining a significant difference at $P < 0.05$ by Duncan's test. All measurements were performed with triplicate fresh samples, and values were expressed as means ± SD of triplicates from each independent experiment.

3. RESULTS AND DISCUSSION

3.1. The levels of 3-MCPD and glycidol in sunflower oils

The levels of 3-MCPD and glycidol determined in the sunflower oil samples are presented in Figure 3A. The amounts of 3-MCPD in the sunflower oil samples belonging to 7 different brands were determined in the range of 0.02-0.44 mg·kg^{-1}. The highest 3-MCPD were determined in SO6, the lowest 3-MCPD were obtained from the SO2 sample. On the other hand, while the lowest glycidol was found in the SO5 sample, the highest glycidol was detected in the SO4 sample. It is noteworthy that the amount of glycidol was higher compared to 3-MCPD (Figure 3A). The levels of 3-MCPD in the sunflower oil samples were found to be SO6 > SO7 > SO3 > SO1 > SO4 > SO5 >SO2. On the other hand, the levels of glycidol were found to be SO4 > SO1 > SO7 > SO6 > SO2 > SO3 < SO5. Kuhlmann (2011) found 3-MCPD values in sunflower oils in the range of 0.1-2.1 mg·kg^{-1} and glycidol values in the range of 0.1-0.4 mg·kg^{-1}. The same author found 3-MCPD levels in

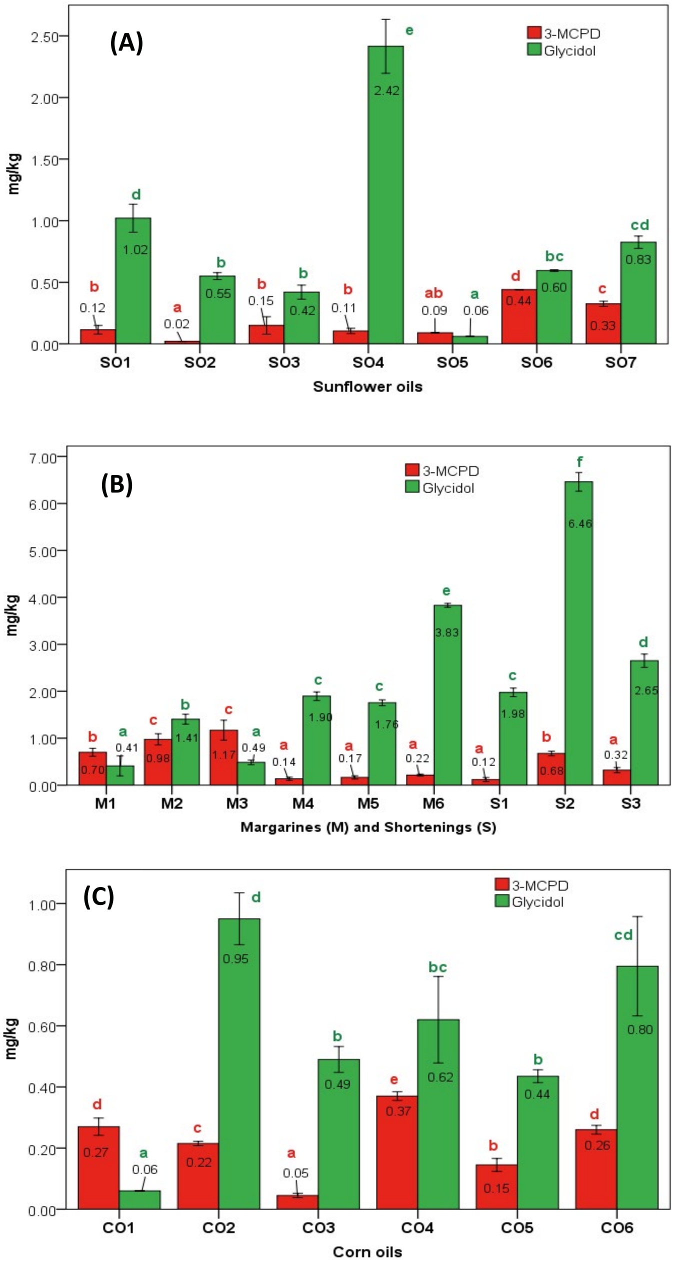

Figure 3. 3-MCPD and glycidol levels in sunflower oils (A), margarines and shortenings (B), corn oils (C). Means followed by similar letters of the same color on the bars are not significantly different at 5% probability level by the Duncan test. All treatments were performed in three replicates.

the range of 0.08-0.96 mg·kg^{-1} and glycidol levels in the range of 0.02-0.90 mg·kg^{-1} in sunflower oil in another study (Kuhlmann 2016). Zelinkova *et al.* (2006) determined 3-MCPD values of less than 0.1 and 0.3 mg·kg^{-1} in two types of sunflower oil. Weißhaar and Perz (2010) found 3-MCPD values for sunflower oils of less than 1.0 mg·kg^{-1} and glycidol values of less than 0.4 mg·kg^{-1}. The results of the current study partially overlap with these literature findings. The majority of our glycidol values were higher than those. The possible reason for this variation in glycidol levels may be the difference in parameters such as temperature and time applied in refining. The glycidol in SO1 and SO4 samples were determined as 1.02 and 2.42 mg·kg^{-1}, respectively. They were determined in the range of 0.06-0.83 mg·kg^{-1} in other samples. Similarly, Kalkan *et al.* (2021) determined glycidol in the range of 0.06-0.72 mg·kg^{-1} in sunflower oil during the frying of french fries in their research.

3.2. The levels of 3-MCPD and glycidol in margarines and shortenings

The levels of 3-MCPD and glycidol determined in different kinds of margarine and shortenings are presented in Figure 3B. The 3-MCPD concentration was found in the range of 0.14-1.17 mg·kg^{-1}, and the glycidol concentration in the range of 0.41-3.83 mg·kg^{-1} for 6 different kinds of margarine. The results obtained for margarine and shortening are valid only for the oil phase. The concentrations of 3-MCPD and glycidol in three shortenings were in the range of 0.12-0.68 mg·kg^{-1} and 1.98-6.46 mg·kg^{-1}, respectively. It was observed that the glycidol level was higher than 3-MCPD in all kinds of margarine except for M1 and M3 samples (Figure 3B). The glycidol level was found significantly higher in M6 samples compared to the other samples ($p < 0.05$). No significant changes were observed among the M4, M5, and M6 samples in terms of 3-MCPD values. Similarly, the 3-MCPD values in M2 and M3 samples were not statistically different. In terms of glycidol, there was no significant difference between M1 and M3, or M4 and M5. By looking at the shortenings, the amounts of glycidol were found significantly higher than the 3-MCPD ($p < 0.05$). The amount of glycidol in S2 shortening samples was approximately 2.5-3 times higher than the other samples. Custodio-Mendoza *et al.* (2019) determined that the total bound 3-MCPD concentrations in the lipid fractions of margarines were in the range of 0.11-2.61 mg·kg^{-1}. The 3-MCPD values were found in the range of 0.79-1.60 mg·kg^{-1} in five samples (Li *et al.,* 2015), 0.4-4.5 mg·kg^{-1} in 37 samples (Weißhaar 2011), and 0.09-0.43 mg·kg^{-1} in four kinds of margarine (fat portion) and 0.50 mg·kg^{-1} in a vegetable shortening (Becalski *et al.,* 2015). In a report published in the Netherlands (Boon and te Biesebeek 2016), 3-MCPD concentrations were reported as 0.16-1.8 mg·kg^{-1} in seven margarines and shortenings. These findings are in agreement with the current results. The glycidol concentrations (0.15-5.5 mg·kg^{-1}) determined by Weißhaar (2011) in 22 margarines (fat portion) were similar to our findings. Deniz Şirinyıldız *et al.* (2019) found 3-MCPD concentrations in the range of 0.57-4.54 mg·kg^{-1} in 14 margarines obtained from the market in Türkiye. The possible reason why these values were higher in margarines compared to many other oils may be salt and water contents. As known, palm oil is generally used to produce margarine. Palm oil is a fruit oil and contains more water than seed oils. Therefore, it is more sensitive to hydrolysis reactions. The high levels of monoglyceride and diglyceride formed as a result of these reactions cause the formation of 3-MCPD and glycidol (Shahidi and Zhong, 2005). On the other hand, the presence of organochlorine compounds in palm oil has been implicated as a potential source of chlorine for the formation of 3-MCPD (Nagy *et al.,* 2011). While the palm tree is growing, it absorbs chloride ions from the soil and water, which paves the way for the formation of 3-MCPD (Anonymous, 2018). In addition, the formation of 3-MCPD in palm oil is directly related to the oil's exposure to high temperatures during refining (Franke *et al.,* 2009; Weißhaar, 2008).

3.3. The levels of 3-MCPD and glycidol in corn oils

The levels of 3-MCPD and glycidol determined in corn oils are presented in Figure 3C. The 3-MCPD and glycidol values were determined to be in the range of 0.05-0.37 mg·kg^{-1}, and 0.06-0.95 mg·kg^{-1}, respectively in six corn oils. The glycidol levels were found higher than 3-MCPD in corn oil samples except for CO1 samples.

While the mean value of 3-MCPD in corn oils was 0.22 mg·kg^{-1}, the mean glycidol value was 0.56 mg·kg^{-1}. The difference between the values of the samples was found statistically significant ($p < 0.05$). Among the samples, the CO1 sample appears to be more stable in terms of these process contaminants. Kuhlmann (2011) found that the 3-MCPD value in corn oil was 0.2 mg·kg^{-1} and the glycidol value was 0.7 mg·kg^{-1}. The result of the current study was similar to these findings. In another study, the amounts of 3-MCPD and glycidol levels in corn oils were found to be < 1.7 and < 0.6 mg·kg^{-1}, respectively (Weißhaar 2011). Zelinkova *et al.* (2006) reported 3-MCPD values of less than 0.3 and 0.372 mg·kg^{-1} in crude and refined corn oils, respectively. These results are also in good agreement with our findings. Deniz Şirinyıldız *et al.* (2019) found that the 3-MCPD concentration was 0.51-2.49 mg·kg^{-1} in five corn oil samples. The values determined in the current study were lower than those findings.

3.4. The levels of 3-MCPD and glycidol in hazelnut and peanut oils

The amount of 3-MCPD and glycidol levels in hazelnut and peanut oils are exhibited in Figure 4A. While the 3-MCPD concentration was found as 0.06 mg·kg^{-1} in HO4 samples, it was found as 1.13-2.12 mg·kg^{-1} in the other three hazelnut oils (HO1, HO2, and HO3). Deniz Şirinyıldız *et al.* (2019) found that the 3-MCPD concentration in three hazelnut oils was in the range of 0.24-0.45 mg·kg^{-1}. Our results (except HO4) were higher than these values. The possible reason for this variation may be the difference in parameters such as temperature and time applied in refining. The amounts of glycidol in hazelnut oil samples were determined to be in the range of 0.54-2.63 mg·kg^{-1}. While there was no significant difference between the 3-MCPD values of HO2 and HO3 samples, the difference between 3-MCPD amounts in these samples and others was found statistically significant ($p < 0.05$). The same trend was followed for the glycidol amounts in the samples. The order of 3-MCPD and glycidol levels determined in hazelnut oil samples were found to be HO2 > HO3 > HO1 > HO4. Kuhlmann (2011) found the 3-MCPD value at 19 mg·kg^{-1} and the glycidol value at 0.5 mg·kg^{-1} for hazelnut oil. Zelinkova *et al.* (2006) reported that the amount of 3-MCPD in unrefined crude hazelnut oil was less than 0.1 mg·kg^{-1}. According to the results obtained in the current study, it was important to indicate that these compounds were formed in higher concentrations in hazelnut oils compared to the other oil samples analyzed in the study. It is well known

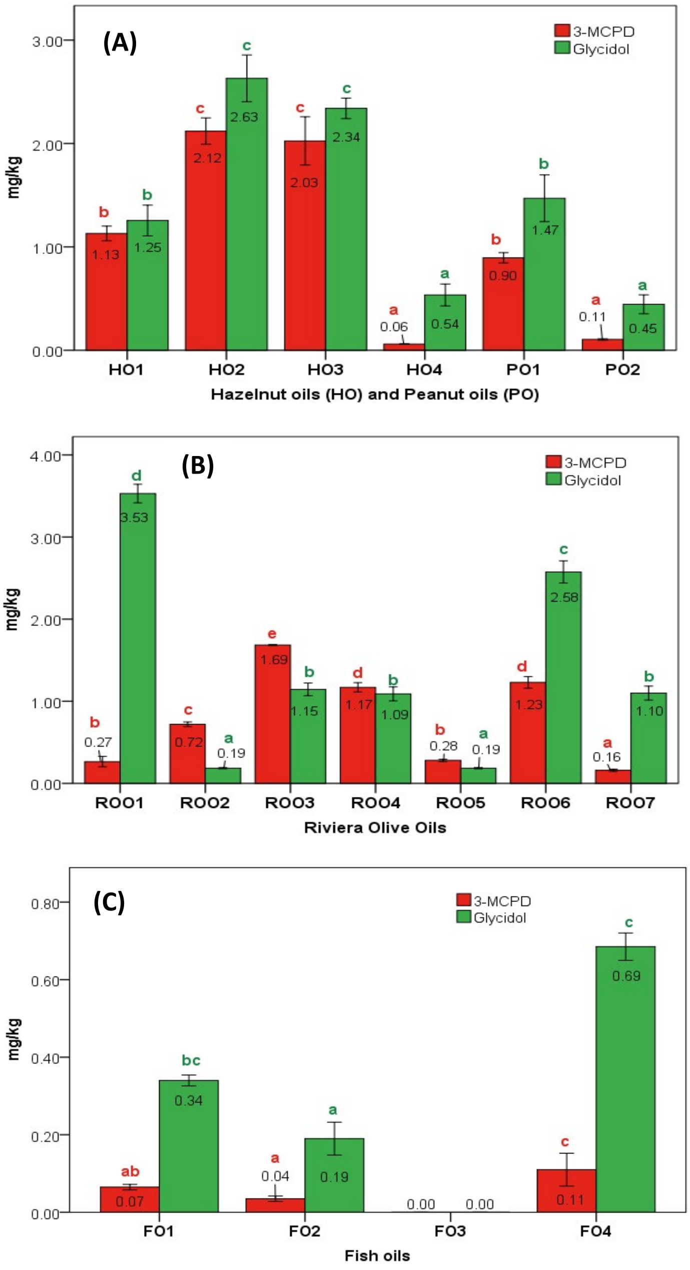

Figure 4. 3-MCPD and glycidol levels in hazelnut and peanut oils (A), riviera olive oils (B), fish oils (C). Means followed by similar letters of the same color on the bars are not significantly different at 5% probability level by the Duncan test. All treatments were performed in three replicates.

that fruit oils such as hazelnut oil are more prone to hydrolysis because of their high water content compared to seeds. It has been determined that the risk of 3-MCPD formation during the refining of these oils is quite high because of the formation of mono and diglycerides as a result of the hydrolysis reaction and the chlorine ion in the structure of the fruits which pass into the oil (Xu *et al.,* 2016).

In two peanut oils, 3-MCPD concentrations were found to be 0.90 and 0.11 mg·kg^{-1}, and glycidol concentrations were found at 1.47 and 0.45 mg·kg^{-1} (Figure 4A). Li *et al.* (2016) found that the 3-MCPD contents in 3 different peanut oils after deodorization to be 0.43-0.62 mg·kg^{-1}. Kuhlmann (2011) determined that the 3-MCPD content was 0.1-0.9 mg·kg^{-1} and glycidol content was 0.4-1.1 mg·kg^{-1} in peanut oils. Similar results were found for the 3-MCPD level by Zelinkova *et al.* (2006) at < 0.1 mg·kg^{-1} and Li *et al.* (2015) at 0.45-1.18 mg·kg^{-1}. The results of the studies given above are similar to our observations. The amounts of 3-MCPD and glycidol levels determined in these peanut oils we used in our study showed a significant difference ($p < 0.05$). The amount of 3-MCPD in PO1 oil was 8 times higher than that of PO2, and the amount of glycidol was approximately 3 times higher.

3.5. The levels of 3-MCPD and glycidol in extra virgin olive oils

The 3-MCPD and glycidol levels were not detected in 7 different extra virgin olive oils used in the study. It was expected that extra virgin olive oil does not contain any traces of 3-MCPD or glycidol since it is mechanically extracted without any heat treatments. The 3-MCPD in edible oils were studied by Jedrkiewicz *et al.* (2016) and according to the obtained results from that study, MCPD were not detected in cold-pressed or unrefined edible oils. Similarly, the 3-MCPD and glycidol in extra virgin olive oils were not determined in the study by Custodio-Mendoza *et al.* (2019). On the other hand, Zelinkova *et al.* (2006) determined the amount of 3-MCPD to be less than 0.1 mg·kg^{-1} in virgin olive oils and in the range of 0.3-2.4 mg·kg^{-1} in refined olive oils.

3.6. The levels of 3-MCPD and glycidol in riviera olive oils

According to the International Olive Council classification, riviera olive oil is an oil consisting of a mixture of refined olive oil and extra virgin olive oil, and is suitable for consumption. It has a free acidity, expressed as oleic acid, of not more than 1.00 gram per 100 grams and its other physico-chemical and organoleptic characteristics correspond to those fixed for this category in this standard (IOOC, 2021). Riviera olive oil is the oil whose properties are improved by mixing natural olive oil in different proportions ranging from 5 to 20% with refined olive oil (Türkoğlu *et al.,* 2012). The amounts of 3-MCPD and glycidol levels determined in riviera olive oil samples are shown in Figure 4B. The 3-MCPD values varied between 0.16-1.69 mg·kg^{-1} ($p < 0.05$). The highest level was determined in ROO3 oil, the lowest level was determined for ROO7 oil. The amounts of glycidol were found in the range of 0.19-3.53 mg·kg^{-1} ($p < 0.05$). It is noteworthy that the variation in glycidol concentration was higher than the variation in 3-MCPD concentration. Kuhlmann (2011) found 3-MCPD amounts in the range of 0.3-1.2 mg·kg^{-1} and glycidol amounts in the range of 0.1-0.4 mg·kg^{-1} in riviera olive oils. While the amounts of 3-MCPD in this study were similar to our findings, the amounts of glycidol only partially matched our results. Weißhaar and Perz (2010) determined that the average 3-MCPD value was 1.2 mg·kg^{-1} and glycidol was 0.3 mg·kg^{-1} in six riviera olive oil samples.

3.7. The levels of 3-MCPD and glycidol in fish oils

The 3-MCPD and glycidol contents detected in four different brands of fish oil are shown in Figure 4C. The 3-MCPD and glycidol values were not determined in the FO3 sample. The highest amount of 3-MCPD was obtained for the FO4 sample at 0.11 mg·kg^{-1}. While the glycidol could not be detected in the FO3 sample, the highest amount was found in the FO4 sample at 0.69 mg·kg^{-1}. Kuhlmann (2011) reported that the amounts of 3-MCPD and glycidol levels in different fish oils were less than 0.05 and 0.025 mg·kg^{-1}, respectively. Jedrkiewicz *et al.* (2016) determined that the 3-MCPD content in refined fish oils was in the range of 1.5-5.5 mg·kg^{-1}. The results of the present study were lower than these findings. The reason for this might be that the refining processes changed according to the fish type.

4. CONCLUSIONS

This study surveyed the contamination levels of 3-MCPD and glycidol in edible oils collected in 2019 in Türkiye. The results for different oils and fats showed that 3-MCPD and glycidol levels did not occur in crude or natural oils and fats. 3-MCPD and glycidol were commonly detected in different amounts in refined oils and fats. The highest 3-MCPD amounts were found in some hazelnut and riviera olive oils. Glycidol was found above the limit values in most margarine, shortening, riviera and hazelnut oils. It is known that fruit oils such as hazelnut oil, olive oil and palm oil are more prone to hydrolysis because of their highwater content compared to seeds. It is thought that 3-MCPD and glycidol formation may be higher during the refining of these oils because of the hydrolysis reaction of triglycerides to form mono and diglycerides and the chlorine ion in the structure of the fruits, which pass into the oil. It was observed that the amounts of 3-MCPD did not exceed a certain level while the glycidol presented significantly higher maximum amounts. As expected, 3-MCPD and glycidol levels were not found in extra virgin olive oils as they were not subjected to refining processes. While these compounds were not detected in one brand in fish oils, 3-MCPD and glycidol were determined at 0.12 $mg \cdot kg^{-1}$ and less than 0.69 $mg \cdot kg^{-1}$, respectively in other fish oil samples. 3-MCPD and glycidol limit values in Türkiye are generally compatible with European regulations. When evaluated in this respect, the majority of margarine, shortening, hazelnut and riviera oils are above the glycidol limit value. 3-MCPD was above the limit in most of the hazelnut oils. The formation of 3-MCPD and glycidol can be limited by reducing their precursors such as monoglycerides and diglycerides in hazelnut, olive and palm oils before high temperature processing, as well as by reducing the palm oil content in margarine and shortening. A dietary risk assessment for 3-MCPD and glycidol was not performed in this study. In future studies, in the light of these data, the risk assessments of edible oils and fat-containing foods should be made.

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A survey of phthalates in flavored olive oils from Turkey

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SUMMARY. Phthalates are organic contaminants that are used as plasticizers in many plastic food packaging materials. Because of their lipophilic character, oils are the primary source of human exposure to phthalates. In this study, the presence of five phthalate esters; diethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP) in olive oils flavored with different materials and marketed in Turkey in 2020 was determined. The samples included BBP, DINP, DIDP at lower concentrations than their LOQ. DEHP was the most abundant phthalate in all samples at varying concentrations between < LOQ to 1.81 mg/kg. The highest amount of DEHP was found in the oil sample flavored with bergamot (1.81 mg/kg). DBP was detected in 9 of the 25 samples at concentrations from 0.11 to 0.27 mg/kg.

KEYWORDS: *Contaminants; Diethylhexyl phthalate (DEHP); Flavored olive oil; Phthalates; Turkey.*

RESUMEN. Estudios sobre ftalatos en aceites de oliva aromatizados de Turquía. Los ftalatos son contaminantes orgánicos que se utilizan como plastificantes en muchos materiales plásticos de envasado de alimentos. Debido a su carácter lipofílico, los aceites son la principal fuente de exposición humana a los ftalatos. En este estudio se determinó, la presencia de cinco ésteres ftalatos, ftalato de dietilhexilo (DEHP), ftalato de dibutilo (DBP), ftalato de butilbencilo (BBP), ftalato de diisononilo (DINP) y ftalato de diisodecilo (DIDP) en aceites de oliva aromatizados con diferentes materiales comercializados en Turquía en 2020. Las muestras incluían BBP, DINP, DIDP en concentraciones más bajas que su LOQ. El DEHP fue el ftalato más abundante en todas las muestras en concentraciones variables entre <LOQ y 1,81 mg/kg. La mayor cantidad de DEHP se encontró en la muestra de aceite con sabor a bergamota (1,81 mg/kg). Se detectó DBP en 9 de las 25 muestras en concentraciones de 0,11 a 0,27 mg/kg.

PALABRAS CLAVE: *Aceite de oliva aromatizado; Contaminantes; Ftalato de dietilhexilo (DEHP); Ftalatos; Turquía.*

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1. INTRODUCTION

Virgin olive oil (VOO) is considered a high-value vegetable oil which possesses a characteristic aroma, taste and color. Olive oil is produced by only mechanical methods, thus there is no use of chemicals during its production. Besides, unlike the other vegetable oils, olive oil can be consumed directly without further refining (Fitó *et al.,* 2000). Virgin olive oil contains some minor compounds including tocopherols, sterols, carotenoids, squalene and phenolic compounds which have health-promoting attributes. A high consumption of virgin olive oil could reduce oxidative stress, prevent some cancer types, heart disease, and ageing (Nath and Nath 2000; Owen *et al.,* 2000). Barbagallo *et al.* (2017) reported that olive oil has many phytochemicals including phenolic components and secoiridoids that exert antioxidant activities.

Innovative and healthy products attract consumers' attention. One innovative product is flavored olive oils. Flavored olive oils are improved with healthy ingredients which make them preferable to consumers. Several materials are used to make flavored olive oils. These materials are essential oils, fruits, herbs, mushrooms, nuts, spices and vegetables (Sousa *et al.,* 2015). These innovative oils are generally produced via the maceration method. In this method, flavoring materials are mixed with virgin olive oil and the mixture is left for a certain time, enabling the increase in some constituents such as aroma compounds and phenolics which exhibit antioxidant and antimicrobial properties (Moldão-Martins *et al.,* 2004; Ayadi *et al.,* 2009; Baiano *et al.,* 2010; Jović *et al.,* 2018). Beside aroma, these flavoring materials may contribute to a longer shelf-life for virgin olive oils due to their preventive effect from oxidation. In addition, some constituents, especially phenolics, contribute to human health (Baiano *et al.,* 2010).

Phthalates are synthesized by a double-esterification mechanism of 1,2-benzenecarboxylic acid, and they are reported to have branched, apolar and linear components (Notardonato *et al.,* 2018). The Environmental Protection Agency (EPA) classified phthalates as priority pollutants which endanger human health by carcinogenic effects, adverse reproductive effects, and by altering endocrine function (Cadogan, 2002; Rios *et al.,* 2010). Phthalates are used in different materials such as polyvinyl chloride, polyvinyl acetate and polyester materials (Rios *et al.,* 2010). In many previous studies associated with foods and phthalates, di (2-ethylhexyl) phthalate (DEHP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP) benzylbutyl phthalate (BBP) were determined to be the most commonly identified phthalates (Tsumura *et al.,* 2002; Jarošová *et al.,* 2006; Fierens *et al.,* 2012).

Phthalates have higher solubility in vegetable oils than in water, which makes them a risky food product. Therefore, the determination of the presence and amount of phthalates in vegetable oils is very important for human health and confidence (Cavaliere *et al.,* 2008). Several studies have been conducted on the presence of phthalates in olive oil. DEHP, DnBP and BBP were identified in extra virgin olive oils, olive oils and olive pomace oils from the Italian market. Olive pomace oils contained higher concentration of phthalates, while lower amounts were detected in extra virgin oils (Cavaliere *et al.,* 2008). DiBP, BBP and DEHP were determined as the most abundant phthalates in Sicilian virgin olive oils, while DiNP and DiDP were the most abundant in Molise olive oils (Mo Dugo *et al.,* 2011). In another study by Nanni *et al.* (2011), the researchers demonstrated that DEHP and DINP were found at the highest levels among phthalates in extra virgin olive oils in Italy.

According to the legislation in this field, five phthalates are listed due to their potential health effects on humans including dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), the sum of diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP) with their specific migration limits as 0.3, 30, 1.5 and 9 mg/kg, respectively (EFSA 2005a; 2005b, 2005c, 2005d, 2005e; European Commission 2011). To the best of our knowledge, there is no information on phthalates in flavored olive oils. The aim of this work is to evaluate the presence of phthalates in flavored olive oils sold in Turkish markets, and to determine whether phthalate concentrations depend on the type of flavoring materials. The results will be useful for future studies regarding specific phthalate exposure from flavored olive oils.

2. MATERİALS AND METHODS

2.1. Sample collection

A total of 25 flavored olive oil samples contained in glass bottles were purchased from a local firm in Turkey and analyzed in October 2020. Each sample was stored at -20 ℃ until analyses. Olive oils were flavored with Chili Black Pepper, Aniseed, Bergamot, Rosemary, Tee, Bay, Basil, Thyme, Black Pepper, Lemon, Mandarin, Orange, Garlic, Sumac, Green Pepper, Chili Pepper, Gentian and Mix (rosemary, black pepper, garlic, sun-dried tomato). The details of the flavoring material for olive oil and their codings are listed in Table 1.

Table 1. Codes and names of flavored olive oils

Codes	Flavoring material
FOO1	Chili Black Pepper
FOO2	Aniseed
FOO3	Bergamot
FOO4	Rosemary
FOO5	Tee
FOO6	Bay
FOO7	Basil
FOO8	Thyme
FOO9	Black Pepper
FOO10	Lemon
FOO11	Mandarin
FOO12	Orange
FOO13	Garlic
FOO14	Sumac
FOO15	Green Pepper
FOO16	Basil
FOO17	Chili Pepper
FOO18	Gentian
FOO19	Gentian
FOO20	Lemon
FOO21	Rosemary
FOO22	Thyme
FOO23	Mix (rosemary, black pepper, garlic, sun-dried tomato)
FOO24	Basil
FOO25	Rosemary

2.2. Reagents and materials

Benzyl butyl phthalate (BBP), diisodecyl phthalate (DIDP), diisononyl phthalate (DINP), 2,6-Di-tert-butyl-4-methylphenol (BHT), and dibutyl phthalate (DBP) as phthalate standards were purchased from Dr. Ehrenstorfer GmbH (Germany). The purities of all standards were 99% except for DINP (98%). Acetonitrile and *n*-hexane were obtained from Sigma – Aldrich (Milwaukee, WI, USA) at HPLC grade.

2.3. Preparation of standards

1000 mg/L of stock standard solutions were prepared by dissolving appropriate amounts of the phthalates in hexane. DBP, DEHP, DINP, DIDP, and BBP from stock solution were transferred to 50-mL volumetric flasks at concentrations of 50 µL, 250 µL, 1500 µL, 1500 µL and 2500 µL, respectively. The intermediate standard mixtures (ISM) were then prepared by diluting with *n*-hexane. 40, 80, 160, 320, 480, 640 µL of the ISM for each phthalate were then transferred to 10-mL glass tubes, and 1 g of reference vegetable oil and 10 µL of BHT (1000 ppm) were added. Then the mixture was centrifuged at 2500 rpm at 10-15 °C immediately after vortexing for 2 minutes.

2.4. Sample preparation

2.4.1. Quality assurance and quality control of phthalate analyses

Only glass laboratory equipment was used for sample preparation in order to prevent cross contamination. The sample flow line was purged with *n*-hexane to avoid potential contamination with samples. A special sample preparation laboratory was used for analysis of phthalates.

2.4.2. Oil sample preparation

The previous method reported by Ierapetritis *et al.* (2014) was followed with some modifications. 1 g oil was weighed into 10-mL glass tubes, and 10 µL of the internal standard (BHT) were added. Approximately 10 mL of acetonitrile were used for the completion of the final volume. The samples were centrifuged at 2500 rpm for 10 min after a primary vortexing. The upper phase was transferred to another glassware and left to dry under nitrogen at 40 °C until a final 1 mL remained at the bottom. The extract was then injected into the GC-MS after a settling time of 1 hour.

2.5. Chromatographic analysis by GC–MS

GC–MS was operated in the electron impact mode because ionization by this technique is the most sensitive and reproducible. A 6890GC-5973MS (Agilent Technologies, Palo Alto, CA, USA) device was used. The colum used was a HP-5MS capillary column (30 m × 0.25 mm I. D. × 0.25 µm film thick-

Table 2. GC parameters and conditions

Parameter	Condition
Carrier gas	Helium (purity: ≥ 99.999%)
Flow rate (mL/min)	1.0 mL/min
Injection volume (µL) / mode	1 µL / splitless
Injection temperature (°C)	280
Temperature program	80 °C for 1 min, Ramp to 280 °C, rate: 15 °C/min. Hold at 280 °C for 15 min
Electron impact energy (eV)	70
Source temperature (°C)	230

Table 3. CAS identifiers, retention times, SIM ions and time windows for the phthalates and internal standard (BHT) in SIM mode

Compounds	CAS #	Retention time (min)	SIM ion (m/z)	Time window (min)
BHT (IS)	128-37-0	8.099	205[1], 145, 177, 220	4.00-10.00
DBP	84-74-2	11.354	223[1], 150, 205	10.00-12.50
BBP	85-68-7	13.748	238[1], 91, 150, 206	12.50-14.00
DINP	68515-48-0	16.074	307[1], 150, 167	15.50-18.00
DIDP	68515-49-1	17.909	293[1], 150, 167	15.50-18.00
DEHP	117-81-7	14.750	279[a], 150, 167	14.00-15.50

[1] Quantitative ion; DEHP: diethylhexyl phthalate; DBP: dibutyl phthalate; BBP: butyl benzyl phthalate; DINP: diisononyl phthalate; DIDP: diisodecyl phthalate; BHT: 2,6-Di-tert-butyl-4-methylphenol

ness). All other operation conditions are listed in Table 2. Selected-ion monitoring (SIM) was used for data acquisition. The compound names, CAS identifiers, retention times, SIM ions and time windows of the five phthalates and internal standard (BHT) are shown in Table 3.

2.6. Validation parameters

The validation procedure was performed according to the specifications established in the Turkish "TS EN 14372:2004; Child use and care articles – Cutlery and feeding utensils- Safety requirements and tests".

The method validation was performed by assessing recovery, linearity, specificity, and precision of peak areas. Limit of detection (LOD) and limit of quantification (LOQ) were determined by multiplying the signal from experimental noise ratios by 3 and 10 times, respectively. Calibration curves prepared from internal standards were used for evaluating the data. The linearities in all cases were satisfactory with correlation coefficients higher than 0.990. The method performance parameters are given in Table 4. The average recoveries of the five phthalates were between 87 and 100% and the RSD values were less than 20% for overall concentrations.

3. RESULTS AND DISCUSSION

Table 5 shows the phthalates found in flavored olive oils. In all analyzed samples BBP, DINP and DIDP were less than LOQ. In previous reports, DBP, BBP, DINP and DIDP were identified in olive oil samples marketed in Italy (Fusari and Rovellini, 2009; Mo Dugo *et al.,* 2011).

The current study revealed that DEHP was present in 18 of 25 samples with a mean value ranging from < LOQ to 1.81±0.025 mg/kg. The oil sample flavored with bergamot (FOO3) included the highest level of DEHP at 1.81±0.025 mg/kg, followed by the oil flavored with mandarin (FOO11, 1.61±0.045 mg/kg). In this study, the concentration of DEHP in 5 of the 25 samples was past the substance migration limit (SML) (1.5 mg/kg) as determined by EU 2011/10 for this phthalate. In a study by Mo Dugo *et al.* (2011), the DEHP levels in all olive oil samples from 3 years were detected with mean values of 1.171, 1.935 and

Table 4. Method performance parameters (n=3)

Phthalates	Linear Equation	R^2	LOD (mg/kg)	LOQ (mg/kg)	RSD (%)	Recovery (%)
DBP	y =1300x-1593	0.95	0.06	0.09	11	94
BBP	y=4137x-4493	0.97	1.97	2.28	10	87
DINP	y=6823x-8488	0.95	1.37	1.75	4	100
DIDP	y=6849x-8525	0.95	1.20	1.40	3	92
DEHP	y=3006x-3097	0.99	0.10	0.23	7	87

DEHP: diethylhexyl phthalate; DBP: dibutyl phthalate; BBP: butyl benzyl phthalate; DINP: diisononyl phthalate; DIDP: diisodecyl phthalate; LOD: limit of detection; LOQ: limit of quantification.

Table 5. Concentrations of phthalate esters in flavored olive oils (mg /kg) (n=2)

Sample	DBP	BBP	DEHP	DINP	DIDP
FOO1	0.27±0.015	<LOQ	1.16±0.035	<LOQ	<LOQ
FOO2	<LOQ	<LOQ	0.85±0.03	<LOQ	<LOQ
FOO3	<LOQ	<LOQ	1.81±0.025	<LOQ	<LOQ
FOO4	<LOQ	<LOQ	1.59±0.065	<LOQ	<LOQ
FOO5	0.13±0.02	<LOQ	0.92±0.03	<LOQ	<LOQ
FOO6	<LOQ	<LOQ	1.29±0.035	<LOQ	<LOQ
FOO7	<LOQ	<LOQ	1.34±0.01	<LOQ	<LOQ
FOO8	<LOQ	<LOQ	1.45±0.03	<LOQ	<LOQ
FOO9	0.25±0.025	<LOQ	1.59±0.03	<LOQ	<LOQ
FOO10	0.19±0.01	<LOQ	1.23±0.01	<LOQ	<LOQ
FOO11	0.25±0.025	<LOQ	1.61±0.045	<LOQ	<LOQ
FOO12	0.12±0.01	<LOQ	1.41±0.045	<LOQ	<LOQ
FOO13	<LOQ	<LOQ	1.51±0.145	<LOQ	<LOQ
FOO14	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
FOO15	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
FOO16	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
FOO17	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
FOO18	<LOQ	<LOQ	0.68±0	<LOQ	<LOQ
FOO19	<LOQ	<LOQ	0.53±0.02	<LOQ	<LOQ
FOO20	0.11±0.01	<LOQ	0.81±0.015	<LOQ	<LOQ
FOO21	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
FOO22	0.14±0.01	<LOQ	0.59±0.03	<LOQ	<LOQ
FOO23	0.18±0.015	<LOQ	0.4±0.02	<LOQ	<LOQ
FOO24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
FOO25	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

DEHP: diethylhexyl phthalate; DBP: dibutyl phthalate; BBP: butyl benzyl phthalate;
DINP: diisononyl phthalate; DIDP: diisodecyl phthalate; LOQ: limit of quantification.

0.445 mg/kg, respectively (Mo Dugo *et al.,* 2011). The DEHP concentrations were also lower than extra virgin olive oils in glass and in tinplate (Nanni *et al.,* 2011). No phthalates were detected in sample FOO14 (flavored with sumac), FOO15 (flavored with green pepper), FOO16 (flavored with mandarin basil), FOO17 (chili pepper), FOO21 (rosemary), FOO24 (basil) or FOO25 (rosemary).

As shown in Table 5, DBP was present in nine of 25 samples with a mean value ranging from < LOQ to 0.27±0.015 mg/kg. In a study by Sungur *et al.* (2015), DBP concentrations were 0.117-1.418 mg/kg for olive oils and 0.102-1.048 mg/kg for virgin olive oils. In our study, the concentration of DBP did not exceed the substance migration limit (SML) (0.3 mg/kg) as set by EU 2011/10. The DBP concentrations in virgin olive oils in the current study were similar to those reported by Sungur *et al.* (2015).

The results obtained showed that a relation could be found between phthalate concentrations and flavoring materials. In a survey work by Cao *et al.* (2015), the phthalate contents in different food stuffs were investigated and DEHP was detected at the highest levels in the herbs and spices samples in almost all food stuffs. In another study by Ning *et al.* (2017), the concentration of DEHP in raw green pepper ranged from 1.83 to 5.95 mg/kg, which exceeded the legislated limit by EU 2011/10. The findings in the previous reports are considered useful to explain the high DEHP concentrations in flavored olive oils with herbs as observed in our study. In our study, the DEHP concentrations were 1.81 mg/kg for olive oil flavored with bergamod, 1.59 mg/kg for olive oil flavored with rosemary, and 1.59 mg/kg for olive oil flavored with black pepper. In a study by Di Bella *et al.* (2018), in which phthalates were determined in Tunisian culinary spices and herbs, DEHP concentrations in spices and herbs including caraway, black pepper, mint, oregano, coriander, thyme, rosemary, fennel, verbena, and laurel were found to range from 0.6 to 1.18 µg/kg.

4. CONCLUSIONS

The results from our study regarding phthalates in flavored oils indicated that 6 of 25 virgin olive oil samples flavored with different materials included DEHP (1.45±0.03 – 1.81±0.025 mg/kg), and these concentrations exceeded the SML set by EU 2011/10 regulation (1.5 mg/kg). In this study, DBP concentrations in all analyzed samples did not exceed the migration limit (SML) (0.3 mg/kg) of EU 2011/10, while BBP, DINP and DIDP were found to be less than their LOQ in all analyzed samples. However, from a food safety perspective for phthalates, seven flavored olive oil samples contained no residue of phthalates. The findings of this study are useful for the flavored olive oil producers and oil industry and will be a guide for a phthalate-free flavored olive oil production.

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Conflict of Interest

The authors declared that there are no conflicts of interest.

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Modulation of essential fatty acid levels in coconut oil with flaxseed oil

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SUMMARY: Coconut oil (CO) is a popular cooking medium but its lack of essential fatty acids (FA) is a health concern. Therefore, the aim of this work was to improve the FA profile of CO by blending with flaxseed oil (FO). Blends with various percentages of FO were prepared and studied for physicochemical characterization, thermal and long-term storage stability. The results indicated that the blends made favorable alterations in FA composition without adverse effects to the oxidative stability of the fatty acids and they resisted secondary thermal deterioration up to two hours at 180 ºC. The blend with the highest percentage of FO was stable for nine months. THP-1 cell line studies showed that ω-3 FA from the blend was incorporated into the cells with no adverse effect on cell viability but the inflammatory markers studied remained unaltered. Thus, CO and FO blends could be stored for at least nine months and could be used as cooking medium when prolonged heating is not involved.

KEYWORDS: *Blending; Coconut oil; Essential fatty acids; Flaxseed oil; ω-3 fatty acids; ω-6:ω-3 ratio*

RESUMEN***: Modulación de los niveles de ácidos grasos esenciales en aceite de coco con aceite de linaza.*** El aceite de coco (CO) es un medio popular para cocinar, pero su falta de ácidos grasos esenciales (AG) supone un problema para la salud. Por tanto, el objetivo de este trabajo fue mejorar el perfil de AG de CO mediante la mezcla con aceite de linaza (AL). Se prepararon y estudiaron mezclas con diversos porcentajes de AL para su caracterización fisicoquímica, estabilidad térmica y almacenamiento a largo plazo. Los resultados indicaron que las mezclas tuvieron una alteración favorable en la composición de AG, sin efectos adversos en su estabilidad oxidativa y resistieron el deterioro térmico secundario hasta dos horas a 180 °C. La mezcla con el mayor porcentaje de AL se mantuvo estable durante nueve meses. Los estudios de la línea celular THP-1 mostraron que los ácidos grasos ω-3 de la mezcla, se incorporaron en las células sin efectos adversos sobre la viabilidad celular, pero los marcadores inflamatorios estudiados permanecieron inalterados. Por lo tanto, las mezclas de CO y AL podrían almacenarse al menos durante nueve meses y podrían usarse como medio de cocción donde no se requiere un calentamiento prolongado.

PALABRAS CLAVE: *Aceite de coco; Aceite de linaza; Ácido graso ω-3; Ácidos grasos esenciales; Mezclas; Relación ω-6:ω-3*

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1. INTRODUCTION

In the tropical countries coconut oil (CO) has been used as cooking oil since long ago. In recent years, CO has become popular globally. CO is available as virgin coconut oil (VCO) and copra oil. The FA compositions of these two varieties are almost the same but the former contains higher levels of biologically active compounds (Wallace, 2019). CO is dominant in medium-chain saturated fatty acids (MC-SFA). CO belongs to the lauric oil group in which oils rich in lauric acid are included. CO has a long shelf-life and is very stable against atmospheric oxidation (Bhatnagar *et al*., 2009). However, regulatory authorities in many countries are concerned about the adverse health effects of CO mainly because of its high SFA content and lack of essential FA (omega-3 (ω-3) and omega-6 (ω-6)) (Lima and Block, 2019). Dietary intake of Linoleic acid (ω-6 FA, LA) and Alpha linolenic acid (ω-3 FA, ALA) is required as the human body cannot synthesize these FA. Unfortunately, today, the human diet is low in ω-3 FA (anti-inflammatory nature) and loaded with ω-6 FA (inflammatory nature) (Kaur *et al*., 2014). Therefore, improving the consumption of ω-3 FA and simultaneously reducing the consumption of ω-6 FA is important. Additionally, Bhatanagar *et al*. (2009) have suggested that prolonged use of CO may result in diets deficient in MUFA and PUFA.

Edible oils serve as an excellent source of FA including essential FA. They provide FA in the form of triacylglycerols. (Kaur *et al.,* 2014). It is possible to alter CO's nutritional value (i.e. FA composition) by blending it with an oil rich in MUFA/PUFA (Chandrashekar *et al*., 2010). ALA is known to possess many health benefits and flaxseed oil (FO) is a major plant source of ALA. It also contains a good amount of ω-6 FA; LA (Kaur *et al*., 2014). Because of its high PUFA content, FO is oxidatively and thermally very unstable (Symoniuk, 2016).

Therefore, the development of oxidatively and thermally stable CO blends to provide essential ω-3 and ω-6 FA was the objective of this study. CO blends containing various percentages of FO were prepared to achieve different ω-6 and ω-3 FA percentages. Here, we report changes in the FA composition (modulation of nutritive parameter), oxidative and thermal stability of the developed CO blends. The storage stability of ω-3 FA contained in the blend (with FO 20%) was studied for up to nine months. THP-1 cell line was used to study the effects of the CO blends at the cellular level.

2. MATERIALS AND METHODS

2.1. Materials used

The Real World Nutrition Laboratory Foundation, Pune (India) had supplied cold-pressed FO and cold-pressed CO was bought from local market. Analytical grade reagents were procured from SRL Laboratories. Fine chemicals (like MTT and LPS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). ELISA kits for Human TNFα and IL-6 were bought from eBiosciences (Vienna, Austria). Cell culture grade reagents like RPMI 1640 medium (Cell culture medium), fetal bovine serum (FBS), DMSO and other reagents required for THP-1 cell line studies were obtained from Hi-Media (India).

2.2. Preparation of CO blends

Three blends containing either 20, 10 or 5% FO (v/v) were prepared using CO as the base oil. For proper blending of the two oils, a mechanical homogenizer (Remi Elektrotechnik Ltd., India) was used (15-20 minutes, three cycles of five minutes, mixing with two-minute breaks). Blending was done at room temperature and no rise in temperature was detected in any of the cases.

2.3. Characterization of CO blends: Study of initial physicochemical parameters

2.3.1. Determination of FA composition of CO blends

The Ichihara and Fukubayashi (2010) method with some modifications was used to determine the FA compositions of the oils and blends. To summarize, 50 mg oil/blend were used for the preparation of fatty acid methyl esters (FAME) by adding methanolic HCl containing BHT (antioxidant, 50 mg/mL) and subjecting the mixture to 80 ºC (in a water bath) for 2 h. n-hexane was used to extract the formed FAME. After drying the FAME (using nitrogen gas), they were reconstituted in n-hexane and subjected to FA analysis by gas chromatography (GC) (7820A, Agilent, Santa Clara, CA, USA), which was coupled with a Flame Ionization Detector (FID). The stationary phase was HP88 capillary column; 30 m long with i.d. 0.25 mm and the thickness was 0.2 µm. The injector and FID temperature was set at 250 ºC. Oven temperature was set at 140 ˚C for an initial 5 min and with 4 ºC/min rise, until reaching 230 ºC and held at 230 ºC for 12.5 min. 1:25 was the split ratio and nitrogen was the carrier gas at a flow rate of 1.1 mL/min. The FA composition is expressed as % FA.

2.3.2. Physico-chemical parameter determination

The acid value (AV, expressed as mg KOH/ g oil), % free fatty acid (% FFA, expressed as % FFA (as lauric acid)) and peroxide value (PV, expressed as milliequivalent O_2/ kg oil) were determined using AOAC official Method 940.28, Ca 5a-40 and Cd 8b-90, respectively (Jagtap *et al*., 2021, Joshi *et al*., 2022 and De Boer *et al*., 2018). Smoke point (SP) was determined according to a method reported by Das *et al*. (2013).

2.4. Effect of heat on various chemical parameters of CO blends

2.4.1. Evaluation of K232 and K268 of oils/blends

The CO blends were subjected to heat at 180 ºC for 240 minute (four hours). Samples were collected at 0, 60, 120 and 240 minutes of heating. Using UV/VIS spectrophotometer (UV 3000$^+$LABINDIA ANALYTICAL, India), the absorbance of the oils/blends (dissolved in iso-octane) was measured at 232 nm and 268 nm. Values for K_{232} and K_{268} were estimated using the formula cited by Malvis *et al*. (2019).

2.4.2. Evaluation of oxidation status of oils/blends

The oxidation status of CO blends after four hours of heating was analyzed by estimating the PV, the para-anisidine Value (*p*-AV) and total oxidation value (TOTOX Value) using AOCS official methods.

2.5. Determination of storage stability of CO blends at room temperature

A storage stability study was conducted for the blend containing the highest level of oxidatively susceptible FO (20 %). During this study, the oils and blends were stored at room temperature. The study was continued up to nine months by evaluating the PV, AV and FA composition of the blends at specified time points.

2.6. Assessment of effects of CO blends in THP-1 cell line

The human monocytic leukemia cell line, THP-1 was cultured in 10% FBS containing a RPMI 1640 medium under standard incubation conditions. The oils or blends were dissolved in DMSO (50 mg/mL). DMSO stocks were diluted in FBS under sterile conditions and pre-incubated (before addition to cells) at 37 ºC for one hour. When the FBS stocks were added to the cells, the final FBS concentration was maintained at 10 % and the final DMSO concentration was 0.5% in all the studies. Only DMSO pre-incubated with FBS served as the control (Joshi *et al*., 2022).

2.6.1. Effect of CO blends on THP-1 cell viability

THP-1 cells were seeded at the density of 1×10^5 cells/well on the transparent 96W plate. The concentrations of oils or CO blends used to treat the cells were 125, 62.5 and 31.25 µg/mL. A MTT assay was performed to assess cell viability at the end of 24, 48 and 72 h treatment. In brief, 1 mg/mL MTT stock was added to the cells (200 µL/well) after careful removal of the culture medium. The plates were incubated in the CO_2 incubator for three hours followed by careful removal of the MTT solution from all the wells. DMSO was added to all the cells (100 µL/well) and the plates were kept at room temperature for 20 minute to allow dissolution of formazan crystals. Absorbance was measured at 570 nm. Viability percentage was calculated, assuming the viability of control cells (cells treated with DMSO) to be 100%.

2.6.2. Effect of CO blends on fatty acid composition of THP-1 cells

At the end of 48 h treatment with the CO blends (125 µg/mL), THP-1 cells were harvested followed by two, 1X PBS washes. As per Folch *et al*. (1957), the total lipid extraction was done and the Ichihara and Fukubayashi (2010) method was used for the esterification of the lipids extracted from the cells. The THP-1 FA composition was determined by performing GC on the esterified lipids. The GC conditions as mentioned in section ‘2.3.1’ were used. The data is presented as % FA of the total lipids extracted from the cells.

2.6.3. Effect of the CO blends on TNFα and IL-6

THP-1 cells (1×10^5/well in 24 W plate) were treated with individual oils or CO blends (125 µg/

mL) for 48 h. For TNFα and IL-6 estimation, oil or blend treated cells were LPS (25 ng/mL) stimulated. The cell supernatant was collected (after LPS stimulation) at 6 h for TNFα and at 24 h for IL-6. ELISAs were performed as per manufacturer's instructions to determine cytokine levels. The data is graphically represented as fold change compared to +LPS (only LPS stimulation).

2.7. Statistical analysis

Each measurement was taken in triplicate. Data is represented as Mean ± SD (n=3). GraphPad Prism (version 5.02) software was used for statistical analysis. Details of the statistical analyses and tests used are reported in the footnote and caption for each data.

3. RESULTS AND DISCUSSION

3.1. Preparation of CO blends

The aim of this study was to improve ω-3 and ω-6 levels in CO through blending with FO. Blends of CO with soybean, safflower, rice bran oil, tiger nut oil, and groundnut oil have been prepared and studied in various animal models (Chandrashekar *et al.*, 2010). As the richest plant source of ALA, FO (5-52 %) was used in the blends (Hintze *et al.*, 2016). Here, 20, 10 and 5 FO (v/v) were used to prepare blends with CO to achieve various percentages of essential FA. Depending on the FO percentage in the blend, they were labeled C20, C10 and C5.

3.2. Characterization of CO blends: Study of initial physicochemical parameters

3.2.1. Fatty acid composition of CO blends

GC-FID analysis was used to confirm altered essential FA levels in the blends. The FA composition of the individual oils and CO blends is presented in Table 1. The FA compositions of CO and blends were statistically different than FO which was also reflected in the total SFA, MUFA and PUFA contents (#; $p < 0.001$ versus FO, $; $p < 0.05$ versus FO). FO had the highest PUFA content while CO had the highest saturated FA content with lauric acid as the major FA. The reported percentages in lauric acid are in the range of 45.9-52.6 (Kumar *et al.*, 2015; Bhatnagar *et al.,* 2009). CO had meager amounts of both MUFA and PUFA, especially essential FA and LA. CO had no ALA. A similar FA composition for CO has been reported in the literature (Kumar *et al.*, 2015; Bhatnagar *et al.*, 2009; Guillaume *et al.*, 2018,

Table 1. Fatty acid composition of CO blends

Fatty acid	FO	CO	C5	C10	C20
Caprillic acid	0.00 ± 0.00	$6.69 \pm 0.03^{\#}$	$5.78 \pm 0.04^{\#,\omega}$	$4.89 \pm 0.01^{\#,\omega,\mu}$	$4.28 \pm 0.04^{\#,\omega,\mu}$
Capric acid	0.00 ± 0.00	$5.5 \pm 0.04^{\#}$	$4.99 \pm 0.06^{\#,\Delta}$	$4.4 \pm 0.01^{\#,\omega,\mu}$	$3.98 \pm 0.02^{\#,\omega,\mu,*}$
Lauric acid	0.00 ± 0.00	$47.94 \pm 0.07^{\#}$	$45.2 \pm 0.07^{\#,\omega}$	$42.26 \pm 0.2^{\#,\omega,\mu}$	$36.36 \pm 0.02^{\#,\omega,\mu,*}$
Myristic acid	0.00 ± 0.00	$20.64 \pm 0.04^{\#}$	$19.7 \pm 0.04^{\#,\omega}$	$19.21 \pm 0.03^{\#,\omega,\text{¨}}$	$16.05 \pm 0.08^{\#,\omega,\mu,*}$
Palmitic acid	6.61 ± 0.08	$8.7 \pm 0.23^{\#}$	$8.72 \pm 0.06^{\#}$	$8.98 \pm 0.04^{\#,\text{¨}}$	$8.7 \pm 0.01^{\#}$
Stearic acid	6.59 ± 0.07	$3.76 \pm 0.02^{\#}$	$3.91 \pm 0.03^{\#}$	$4.22 \pm 0.01^{\#,\Delta,\text{¨}}$	$4.68 \pm 0.04^{\#,\omega,\mu,\alpha}$
Oleic acid	21.61 ± 0.01	$5.54 \pm 0.04^{\#}$	$6.61 \pm 0.02^{\#,\omega}$	$7.39 \pm 0.22^{\#,\omega,\mu}$	$9.09 \pm 0.11^{\#,\omega,\mu,*}$
Linoleic acid (LA)	14.47 ± 0.1	$0.87 \pm 0.03^{\#}$	$1.65 \pm 0.08^{\#,\omega}$	$2.37 \pm 0.02^{\#,\omega,\mu}$	$3.97 \pm 0.02^{\#,\omega,\mu,*}$
Alpha Linolenic acid (ALA)	50.72 ± 0.23	$0.00 \pm 0.00^{\#}$	$3.14 \pm 0.01^{\#,\omega}$	$5.9 \pm 0.01^{\#,\omega,\mu}$	$12.1 \pm 0.02^{\#,\omega,\mu,*}$
ΣS	13.2 ± 0.16	$93.22 \pm 0.42^{\#}$	$88.29 \pm 0.3^{\#,\omega}$	$83.95 \pm 0.25^{\#,\omega,\mu}$	$74.03 \pm 0.09^{\#,\omega,\mu,*}$
ΣM	21.61 ± 0.01	$5.54 \pm 0.04^{\#}$	$6.61 \pm 0.02^{\#,\omega}$	$7.39 \pm 0.22^{\#,\omega,\mu}$	$9.09 \pm 0.11^{\#,\omega,\mu,*}$
ΣP	65.19 ± 0.13	$0.87 \pm 0.03^{\#}$	$4.24 \pm 0.83^{\#,\omega}$	$8.26 \pm 0.03^{\#,\omega,\mu}$	$16.06 \pm 0.04^{\#,\omega,\mu,*}$
LA: ALA	0.29 ± 0.00	ND	$0.52 \pm 0.03^{\#}$	$0.40 \pm 0.00^{\#,\beta}$	$0.33 \pm 0.00^{\$,\mu,\&}$

The FA compositions of the individual oils and CO blends were estimated by GC-FID. Data are presented as Mean ± SD (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil, C20: Coconut oil blend containing 20% flaxseed oil, ΣS: total saturated fatty acids, ΣM: total monounsaturated fatty acids and ΣP: total unsaturated fatty acids. Statistically significant differences were determined by Two Way ANOVA and Bonferroni posttests. #; $p < 0.001$ versus FO, $; $p < 0.05$ versus FO, ω; $p < 0.001$ versus CO, Δ; $p < 0.05$ versus CO, μ; $p < 0.001$ versus C5, ¨; $p < 0.05$ versus C5, β; $p < 0.01$ versus C5, * ; $p < 0.001$ versus C10, α; $p < 0.05$ versus C10, &; $p < 0.01$ versus C10

Maurikaa *et al*., 2020). FO showed ALA as the dominant FA and OA and LA were the next abundant FA. When compared to CO, the blends had significantly modified FA composition except for PA content. As the FO percentage in the CO blends increased, ALA content also increased, resulting in significantly lower LA:ALA. Therefore, from Table 1, it is clear that by blending FO and CO, the FA profile, especially ALA and LA contents and LA:ALA ratio were significantly altered. In addition, total SFA was significantly lower with a significant increase in total MUFA and PUFA contents just by adding 5% FO into the blend.

3.2.2. Physico-chemical characterization of CO blends

The physico-chemical parameters of the prepared blends were estimated immediately after blend preparation. These parameters (AV, % FFA, PV and SP) are represented in Table 2. The quality of any oil is indicated by its AV and % FFA (Mahesar *et al.,* 2014). The AV for FO was significantly higher than CO and the blends. Maurikaa *et al.* (2020) reported the AV of CO at 0.9 mg KOH/g oil. For FO, the reported AV range from 0.21 (Symoniuk *et al*., 2016) to 0.53 (Bhardwaj *et al*., 2015). There were no significant differences between and among the AV of the CO and the blends. % FFA values were similar for FO, CO and the blends except for C20, which had a significantly higher value than FO. In the case of CO, reported % FFA values were in the range of 0.53 - 0.65 (Kumar *et al.,* 2015 and Perera *et al*., 2020) while 0.32 was the % FFA reported for FO by Bhardwaj *et al.* (2015). PV represents oxidative deterioration of the oil (Perera *et al*., 2020). No peroxides were detected in CO but blending with FO resulted in significant increase in PV for the blends compared to CO. Among the blends, there were statistically significant differences. The PV for CO was reported in the range of 0.00 -3.9 (Kumar *et al*., 2009; Moigradean *et al*., 2012; Kumar *et al*., 2015; Perera *et al*., 2020), while the reported values for the PV of FO were 0.98 (Bhardwaj *et al*., 2015) and 2.34 (Symoniuk *et al*., 2016). A temperature at which various components of oil undergo breakdown and are noticed in the form of fumes is known as the SP (Guillaume *et al.,* 2018). The SP of FO was significantly lower than CO and its blends. Compared to CO, the blends had significantly lower SP. But compared to the SP of FO, the SP for the blends were significantly higher ($p < 0.01$ versus FO). The literature reports the SP of CO at 191±3.6 ºC (Guillaume *et al*., 2018) and 193±3.0 ˚C by Perera *et al*., (2020). The observed differences in the values of the studied parameters may be due to differences in the raw material, handling, processing and storage conditions and duration **(**Mahesar *et al*., 2014)**.**

For the blends, AV, % FFA and PV increased as the contribution (percentage) of FO in the blend increased, although the SP of the blends decreased. The observed increase in the studied parameters may be because of the presence of pro-oxidants in the oil (Nering 2016). It is important to note that, though the SP for the blends were lower than CO, they were still higher than the routinely used cooking temperatures. Thus, the data from Table 2, indicates that the CO blends containing FO at up to 20% can be used as cooking oils without disturbing their physicochemical characteristics adversely.

Table 2. Physico-chemical parameters of FO, CO and their blends determined immediately after the blend preparation.

Oil/Blend	**AV (mg KOH/ g oil)**	**% FFA (as Lauric acid)**	**PV (meq O_2/kg oil)**	**SP (ºC)**
FO	2.52 ± 0.48	0.49 ± 0.01	0.80 ± 0.00	104.0 ± 1.41
CO	$0.84 \pm 0.08^{**}$	0.59 ± 0.09	$0.00 \pm 0.00^{***}$	$205.0 \pm 1.41^{\mu}$
C5	$0.90 \pm 0.00^{**}$	0.67 ± 0.06	$0.65 \pm 0.07^{\#}$	$198.5 \pm 0.71^{\mu,\pi}$
C10	$0.90 \pm 0.00^{**}$	0.73 ± 0.11	$0.60 \pm 0.00^{*,\#}$	$198.5 \pm 0.71^{\mu,\pi}$
C20	$1.09 \pm 0.04^{**}$	$0.85 \pm 0.00^{*}$	$0.85 \pm 0.07^{\#,@,\%}$	$196.5 \pm 0.71^{\mu,\pi}$

Data are Mean ± SD (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil. Statistically significant differences were determined by One-way ANOVA and Tukey's Multiple Comparison Test. *and **and ***; $p < 0.05$ versus FO, #; $p < 0.05$ versus CO, @; $p < 0.05$ versus C5, %; $p < 0.05$ versus C10, μ; $p < 0.01$ versus FO and π; $p < 0.01$ versus CO

3.3. Effect of heat on various chemical parameters of CO blends

Heating oil results in the formation and accumulation of various primary and secondary oxidation products. This results in declined oil quality. K_{232} and K_{268} are used to determine the levels of conjugated dienes and conjugated trienes formed during the heating of oil (Malvis *et al*., 2019). PV indicates initial oxidation status of the oil while *para*-anisidine (*p*-AV) represents secondary oxidation of the oil. Additionally, TOTOX value gives overall oxidation status of the oil.

3.3.1. Effect on K_{232} and K_{268} of oils/blends

Figure 1 represents the changes in the K_{232} and K_{268} values for the oils and blends heated up to 240 min (four hour) at 180 ºC. As shown in Figure 1a, before heating (0 min) as well as after heating, FO had a significantly higher K_{232} value when compared to CO and all the blends (*; $p < 0.001$) at respective time points. No statistically significant differences were observed between or among CO and all the blends at 0 min. FO, CO and all the blends started to show a significant rise in K_{232} right from 60 min (#; $p < 0.001$ versus 0 min). Though the fold rises (0 and 240 min) for CO and the blends were higher than FO, FO had higher values than the blends. It is significant to note that when compared to CO, C10 and C20 showed significant deterioration after 120 minutes; whereas in the case of C5, it was at 180 minutes (μ; $p < 0.001$ and β; $p < 0.05$ versus CO at respective time points).

Figure 1b represents the effect of heating on K_{268}. Before heating (0 min), no significant differences for K_{268} were detected among the individual oils or the CO blends. FO started showing a significant rise in K_{268} right from 60 min (&; $p < 0.001$ versus 0 min and previous time point). CO did not show any rise in K_{268} at any of the time points of heating. Although the blends did show resistance for significant rise (at 60 min), the significance level rise at later time points (#; $p < 0.001$ versus 0 min). FO K_{268} values were significantly high at all the time points of heat-

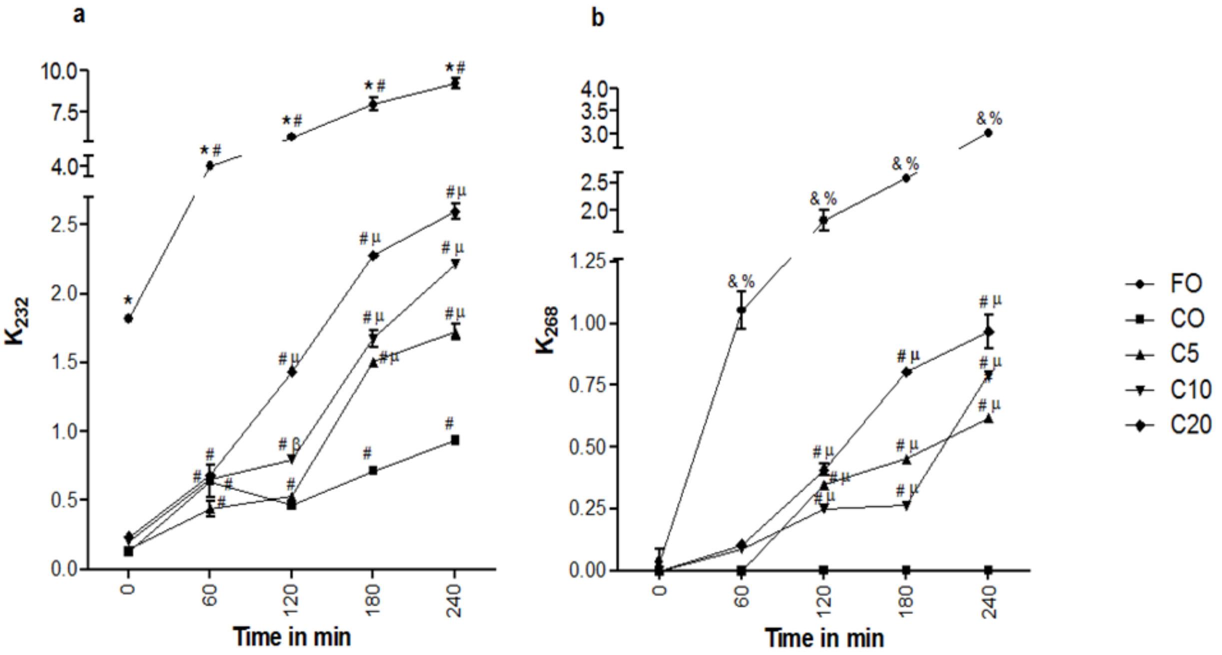

Figure 1. Effect of heat (180 ºC for four hours) on K_{232} and k_{268} of the oils and blends. K_{232} (a) and K_{268} (b) were estimated for individual oils or CO blends when heated at 180 ºC for up to 240 mins for above specified time points (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil. Statistically significant differences were determined by Two-Way ANOVA and Bonferroni posttests. *; $p < 0.001$ versus CO and all blends 0 min and respective time points, #; $p < 0.001$ versus 0 min, μ; $p < 0.001$ versus CO at respective time points, β; $p < 0.05$ versus CO at respective time points, &; $p < 0.001$ versus 0 min and previous time point, %; $p < 0.001$ versus CO and blends at respective time points

Table 3. Effect of heat (180 ˚C for four hour) on oxidative status of CO blends

Oil / Blend	PV (meq O_2/kg oil)		*p*-AV		TOTOX value	
	Before heating	After heating	Before heating	After heating	Before heating	After heating
FO	0.80 ± 0.00	9.13 0.81$^{\#}$	0.338 ± 0.04	9.586 ± 0.05$^{\#}$	1.94 ± 0.04	27.85 ± 1.63$^{\#}$
CO	0.00 ± 0.00	6.33 ± 0.12$^{\#}$	0.074 ± 0.01	1.65 ± 0.00	0.07 ± 0.01$^{@}$	14.31 ± 0.23$^{\#}$
C5	0.65 ± 0.07	9.07 0.61$^{\#}$	0.082 ± 0.01	5.31 ± 0.39$^{\#}$	1.38 ± 0.1	21.67 ± 3.2$^{\#}$
C10	0.60 ± 0.00	18.6 ± 0.2$^{\#}$	0.064 ± 0.02	6.16 ± 0.19$^{\#}$	1.26 ± 0.02	43.36 ± 0.48$^{\#}$
C20	0.85 ± 0.07	18.63 0.95$^{\#}$	0.096 ± 0.02	6.29 ± 0.02$^{\#}$	1.8 ± 0.09	43.55 ± 1.91$^{\#}$

Peroxide value (PV), *para*-anisidine value (*p*-AV) and total oxidation value (TOTOX value) for oils/blends were determined before and after heating at 180 ˚C for four hours (240 min). Data is presented as Mean ± SD (n=3).
FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil
Statistically significant differences were confirmed by Two Way ANOVA and Bonferroni posttests.
#; $p < 0.001$ versus 0 h, @; $p < 0.05$ versus FO and C20 before heating

ing (except 0 min) when compared to CO and all the blends for respective time points (%; $p < 0.001$). It is also important to note that, when compared to CO, all the blends showed significant deterioration from 120 min (µ; $p < 0.001$ versus CO at respective time points). It is clear that though there was no statistical difference at 0 min for K_{268} values, the rise at 240 min was in the order FO > C20 > C10 > C5 > CO, indicating that as the FO levels increased in the blend, K_{268} also increased upon heating. Thus, from Figure1, it is clear that though CO and the blends displayed significant rises in both K_{232} and K_{268}, their values were always lower than FO. In addition, the blends resisted these rises up to a certain time period compared to CO.

3.3.2. Effect on oxidation status of oils/blends

Table 3 represents the PV, *p*-AV and TOTOX values for the individual oils and CO blends initially and at the end of four hours heating at 180 ºC. It is clear that all three parameters after four hours heating were very significantly high for all the oils and blends except the *p*-AV for CO when compared to the before-heating values. Although the fold rise in TOTOX value was the highest for CO, it had the lowest actual value. It is important to note that fold rise for all these three parameters in the blends were higher than FO. This is probably due to the fact that thermo-oxidative deterioration products in the blends are further attacking oxidatively and thermally susceptible components present in the blends. In another study, when CO was heated to 180 ºC, the oxidative stability significantly dropped within the first hour of heating, indicating thermo-oxidative deterioration (Guillaume *et al.*, 2018). The same authors evaluated the performance of coconut oil under two temperature conditions. In the first one, they heated the oil to 180 ºC for six hours and in the second, oil was heated up to 240 ºC. Samples were collected at specific time points or temperatures. In both the cases, CO showed a rise in K_{232} at later time points or temperatures after initial resistance to rise but there was no rise in K_{268} in both cases. Thus, our data is in line with Guillaume *et al.* (2018), where we also see a rise in K_{232} but no change in K_{268}. A significant rise in the primary oxidation but no significant deterioration measured as secondary oxidation products was also well supported by the before and after heating PV and the *p*-AV values in the case of CO.

From Table 3 and Figure 1 it is clear that though CO alone resisted secondary oxidation, it showed significant deterioration upon heating (PV and K_{232}). The thermo-oxidative deterioration reflected by the TOTOX value was highest for the CO blends. In one study, Subramanian (2019) found that CO was the least thermally and thermo-oxidatively stable oil when compared to sunflower oil, groundnut oil and gingelly oil. The thermal and oxidative susceptibility were attributed to factors other than the unsaturation level of the oil. Bhatnagar *et al.* (2009) experimentally showed that CO had low levels of natural anti-oxidants, especially total tocopherols. Researchers could successfully lower the oxidative deterioration of CO during heating by adding essential oils which were rich in antioxidants. (Perera *et al.*, 2020).

Thus, various chemical parameters were used to determine and confirm the thermal stability of the CO blends. As the FO contribution (percentage) in the CO blends increased, thermal degradation also increased. K_{232} and K_{268} indicated that the blends tolerated different heating durations before demonstrating significant deterioration. The extent of deterioration was indicated either as the level of significance and or the time required to reach that level of significance. Eyres *et al.* (2016) suggested the use of CO as a cooking oil where intense heat for long duration (like continuous deep frying) was not used but mild or medium heat for short duration (like single shallow fry) was applied.

3.4. Determination of storage stability of the CO blend at room temperature

At room temperature, CO is known to have a long shelf-life, while FO is known to suffer significant oxidative deterioration. Therefore, the effect of blending FO with CO on long-term storage stability was studied for the blend containing the highest percentage of FO (i.e. C20). Table 4 presents the effects of storage on PV and AV for up to nine months. As the data indicates, there was a significant rise in both the parameters right from third month to the ninth month for CO when compared to the 0-month values. A similar trend was seen in the case of C20 for PV but for AV, a significant change was noticed from the sixth month. At the end of nine months, there were 3-fold and 4.4-fold rises in PV for CO and C20, respectively, while 2.4 and 1.1-fold rises in AV were observed for CO and C20, respectively. Moigradean *et al.* (2012) conducted a similar study on CO and noticed an approximate two-fold rise in the PV at the end of nine months when compared to the initial value. We also observed similar trend. Pazzoti *et al.* (2018) studied the performance of CO, FO and their blend (1: 1 v/v) under accelerated storage conditions (60 ºC for 20 days). They found that FO showed the least oxidative and thermal stability followed by the blend. Here, we observed that though there were significant rises in PV and AV, these values were significantly lower than CODEX (1999). According to Codex Stan 19-1981 for refined oils, PV and AV should be lower than 10 milliequivalents of active oxygen/kg oil and 0.6 mg KOH/g fat or oil, respectively. The values for these parameters in cold-pressed oils are 15 milliequivalents of active oxygen/kg oil and 4 mg KOH/g fat or oil respectively.

Thus, from Table 4, it is clear that both CO and C20 are stable at room temperature for at least up to nine months, as indicated by their PV and AV.

A FA analysis was done for CO and C20 at specified time points. We did not see any significant changes in FA composition at these time points compared to the initial (0 month) data (data not shown). Srivastava *et al.* (2017) also reported no significant change in the FA profile of VCO and refined soybean oil blend or VCO and safflower oil blend which were stored at room temperature for one year. Thus,

Table 4. Effect of storage at room temperature on peroxide value and acid value for CO and C20

Oil/Blend	0 Month	3rd Month	6th Month	9th Month
		Peroxide value (PV, meq O_2/ kg oil)		
CO	0.3±0.0	0.46±0.06$^{\mu}$	0.74 ± 0.06$^{\mu}$	0.9 ±0.0$^{\mu}$
C20	0.5±0.0$^{\beta}$	0.82±0.06$^{\mu\beta}$	0.98 ± 0.01$^{\mu\beta}$	2.2 ±0.1$^{\mu\beta}$
		Acid value (AV, mg KOH/g oil)		
CO	0.24±0.00	0.37± 0.01$^{\mu}$	0.46 ± 0.06$^{\mu}$	0.58 ±0.01$^{\mu}$
C20	0.48±0.00$^{\beta}$	0.49 ± 0.01$^{\beta}$	0.46 ±0.06*	0.55 ± 0.00$^{\mu\beta}$

Peroxide value and acid value were evaluated for CO and C20 when stored at room temperature for up to nine months at specified time intervals. Data are presented as Mean ± SD (n=3).
CO: Coconut oil alone and C20: Coconut oil blend containing 20% flaxseed oil
Statistical significance was confirmed by Two-way ANOVA and Bonferroni posttests.
µ; $p < 0.001$ versus 0 Month, *; $p < 0.05$ versus 0 Month, β; $p < 0.001$ versus CO respective time points

Table 4 indicates that CO and C20 were oxidatively stable at least for nine months when stored at room temperature without significant alteration in their FA composition.

3.5. Assessment of the effects of CO blends on the THP-1 cell line

As CO and blends showed good physicochemical characterization and storage stability, we assessed the biological effects of the CO blends in the THP-1 cell line. The THP-1 cell line is widely used for lipo-toxicity studies and the induction of inflammation studies. Few studies have evaluated effect of VCO, lauric acid and ALA on the viability and inflammation of THP-1 cells (Varma *et al*., 2019; Tham *et al.,* 2020), but to our knowledge there are no reports on CO and FO blends with the percentages of FO we used.

3.5.1. Effect of CO blends on THP-1 cell viability

Varma *et al*. (2019) used VCO to demonstrate anti-inflammatory and skin protective effects. They found that 200 µg/mL and lower concentrations of VCO were non-toxic in THP-1 cells (24 h treatment). Here, we used commercially available CO. We used three doses of oils and the blends (i.e. 125, 62.5 and 32.25 µg/mL) for three different treatment periods (24 h, 48 h and 72 h). Here, Figure 2 represents data for 125 µg/mL. For the other two concentrations, there were no statistically significant differences wrt Control (data not shown). From Figure 2, it is clear that at 24 h, CO and blends showed higher % viability, which was significant for C10 and C20. But for CO, blends and FO, at later time points, % viability was not significantly different than their respective Controls. Thus, at early time points we observed increased viability, which came down to the level of control cells at later time points. Similar rapid response (after 24 h treatment with CO) for elevated mitochondrial functioning was observed by Gil-Villarino *et al*. (1999) in hepatic tissue. The authors showed that in chicks fed with CO oil, up-regulation in the mitochondrial function was seen after 24 h but the up-regulated mitochondrial functioning returned back to the level of controls at longer treatment time points of CO feeding. Indeed, lauric acid from CO has been shown to improve mitochondrial functioning and biogenesis in insulin resistant THP-1 derived macrophages (Tham *et al*., 2020). From Figure 2, it is clear that oils and their blends do not adversely affect cell viability.

3.5.2. Effect of CO blends on FA composition of THP-1 cells

The enrichment of FA within the cells is possible by the external supply of that particular FA to the cells. Here, the FA analysis of THP-1 cells was done after treating the cells with individual oils or CO

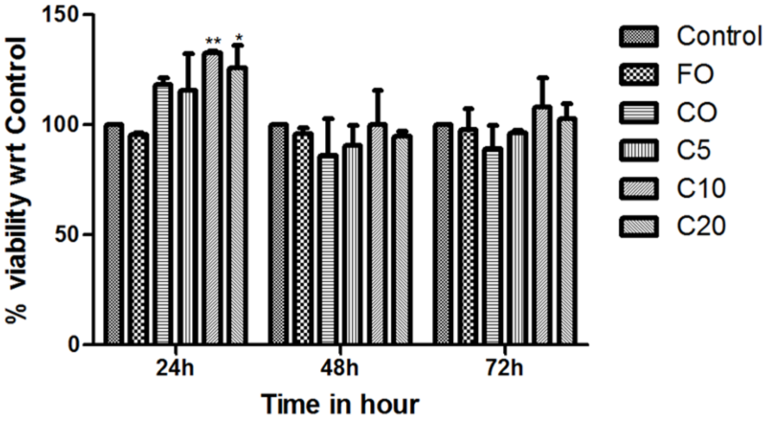

Figure 2. Effect of CO blends on THP-1 cell viability determined by MTT assay after treating the cells with oils/blends (125 µg/mL) for specified time points. Data are presented as % viability wrt Control (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil and C20: Coconut oil blend containing 20% flaxseed oil. Statistical analysis was done by Two-way ANOVA and Bonferroni posttests. *; $p < 0.05$ versus Control, **; $p < 0.01$ versus Control

Table 5. Effect of CO blends on fatty acid composition of THP-1 cells

FA	Control	FO	CO	C5	C10	C20
Lauric acid	8.02 ± 1.43	7.47 ± 1.65	8.94 ± 1.51	8.8 0.95	8.43 ± 2.08	8.50 ± 1.01
Myristic acid	0.96 ± 0.46	0.91 ± 0.36	1.11 ± 0.63	2.39 ± 0.19	2.40 ± 1.11	1.97 ± 0.24
Palmitic acid	23.38 ± 3.17	20.71 ± 1.51	24.33 ± 0.21*	24.93 ± 1.06$^{@}$	25.29 ± 1.34$^{@}$	23.87 ± 0.23
Stearic acid	15.86 ± 1.53	14.21 ± 1.74	16.91 ± 4.83	15.58 ± 1.53	15.67 ± 2.93	15.04 ± 3.37
Oleic acid	4.52 ± 0.04	7.15 ± 2.36	4.03 ± 0.20	4.62 ± 1.81	4.8 ± 1.09	6.7 0.64
Linoleic acid (LA)	0.23 ± 0.05	2.00 ± 0.92	0.21 ± 0.06	0.4 ± 0.17	0.34 ± 0.04	0.45 ± 0.1
Alpha linolenic acid (ALA)	0.00 ± 0.00	4.48 ± 1.49$^{\#}$	0.00 ± 0.00	0.11 ± 0.08$^{@}$	0.125 ± 0.05$^{@}$	0.47 ± 0.1*
Arachidonic acid	0.9 ± 0.33	1.18 ± 0.62	0.36 ± 0.16	0.90 ± 0.56	0.87 ± 0.17	0.77 ± 0.17
Eicosapentaenoic acid	0.00 ± 0.00	0.62 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.48 ± 0.09
Docosahexanoic acid	0.86 ± 0.3	0.54 ± 0.19	0.21 ± 0.08	0.53 ± 0.06	0.54 ± 0.14	0.71 ± 0.33
LA: ALA	ND	0.46 ± 0.05	ND	4.33 ± 1.80	3.02 ± 1.54	1.19 ± 0.65
Σ ω-6	1.13 ± 0.28	3.18 ± 1.53	0.56 ± 0.23	1.30 ± 0.73	1.21 ± 0.21	1.22 ± 0.27
Σ ω-3	0.86 ± 0.30	5.63 ± 2.84$^{\#}$	0.21 ± 0.29$^{\&}$	0.64 ± 0.15$^{\&}$	0.67 ± 0.09$^{\&}$	1.66 ± 0.57*
ω-6:ω-3	1.34 ± 0.16	0.57 ± 0.01	2.73 ± 0.78	1.96 ± 0.69	1.81 ± 0.07	0.75 ± 0.1

Total lipids extracted from THP-1 cells were subjected to FAME preparation after 48 h treatment of cells with the individual oils and CO blends. FA analysis of the FAME was done by GC-FID. Data are presented as Mean ± SD (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil, C20: Coconut oil blend containing 20% flaxseed oil, Σ ω-6: total omega-6 fatty acids, Σ ω-3: total omega-3 fatty acids. Statistical significance was confirmed by Two-way ANOVA and Bonferroni. #; $p < 0.01$ versus Control, *; $p < 0.05$ versus FO, @; $p < 0.01$ versus FO, &; $p < 0.001$ versus FO

blends. Total lipids were extracted from the treated cells, esterified and subjected to GC-FID. Table 5 presents FA levels represented as a % FA of the total extracted lipids. Here, the major FA available in the individual oils and ALA and LA derived major FA were identified and presented. The FA analysis of the Control and CO-treated cells indicated that ALA was absent in these cells. Compared to these cells, FO-treated cells had significant incorporation of ALA within the cells. It was also reflected in the total ω-3 FA content. CO was the major contributor in the blends with lauric acid as major FA. In CO or blend-treated cells, no alteration in the FA related to CO was observed. In addition, as the FO percentage in the blend increased, there was a rise in the ALA (0.11, 0.13 and 0.47% for C5, C10 and C20, respectively) and total ω-3 FA content (0.64, 0.67 and 1.66% for C5, C10 and C20, respectively). An increase in ALA and total ω-3 FA content was also reflected in the LA: ALA ratio (from 4.3 for C5 to 1.2 for C20), although these changes were not statistically significant. This might be because of the preferential uptake and utilization of MC-SFA present in the CO for energy generation as seen in the liver. It is important to note that percentages of ω-6 FA remained relatively constant in all treatments.

Therefore, it is clear that the external addition of ALA in the form of CO and FO blend can result in a dose-dependent incorporation and rise in ω-3 FA content with a simultaneous lowering of ω-6:ω-3 ratio in THP-1 cells.

3.5.3. Effect of the CO blends on TNFα and IL-6

ALA (present in FO) is a precursor for Eicosapentaenoic acid and Docosahexaenoic acid. These ω-3 FA are known for their anti-inflammatory potential (Zhao *et al.*, 2007). From the data presented in Table 5, it is clear that externally added ALA as (alone FO or as a CO blend) could alter LA: ALA as well as ω-6:ω-3 ratio in THP-1 cells. Therefore, it was interesting to investigate the effect of CO blends on the release of TNFα and IL-6 inflammatory markers.

As shown in Figures 3a and 3b, when THP-1 cells were stimulated by LPS, there were significant rises in the release of TNFα and IL-6 in the supernatant (denoted as +LPS). For FO pre-treated (before LPS addition) THP-1 cells, there was a significant decrease in the TNFα level in the supernatant but there was no significant alteration in IL-6 level. The blends did not affect either inflammatory marker. This might be because

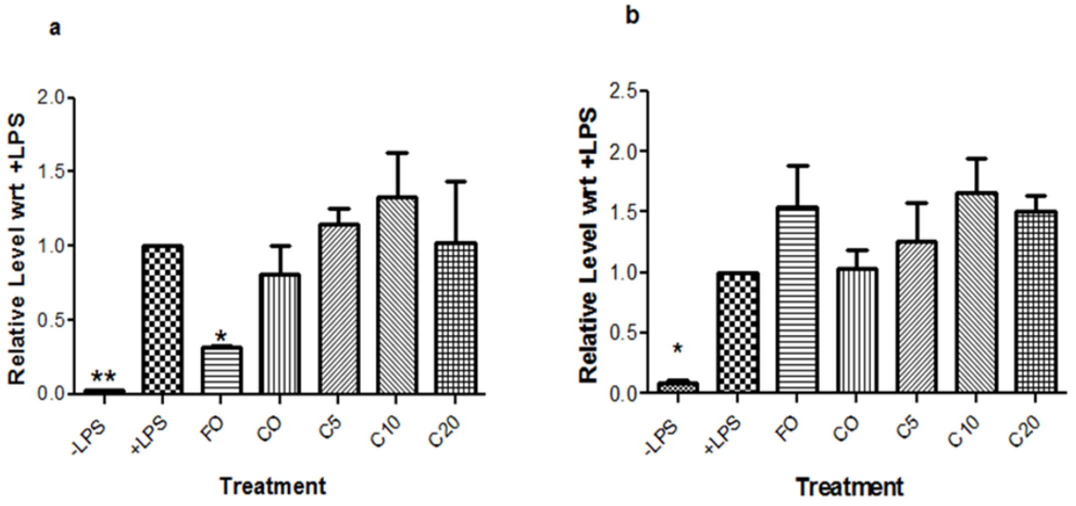

Figure 3. Effect of CO blends on inflammatory markers: TNFα and IL-6 in THP-1 cells. THP-1 cells pre-treated with the individual oils or CO blends (125 µg/mL) for 48 h were stimulated by co-incubation with LPS (25 ng/mL). Cell supernatants were collected at 6 h for TNFα and at 24 h for IL-6 estimation. ELISA was performed for quantitative analysis of these inflammatory markers. Data are presented as fold change wrt +LPS (n=3). FO: Flaxseed oil alone, CO: Coconut oil alone, C5: Coconut oil blend containing 5% flaxseed oil, C10: Coconut oil blend containing 10% flaxseed oil, C20: Coconut oil blend containing 20% flaxseed oil, -LPS: without LPS stimulation and +LPS: with LPS stimulation only. Statistically significant differences were determined by One-way ANOVA and Dunnett's Multiple Comparison Test.*,** ; $p < 0.01$ versus+LPS

the ω-3 FA levels achieved in the cells are not sufficient to down-regulate LPS-induced inflammation. Indeed, Hintze *et al*. (2016) have shown that not only ω-6:ω-3 ratio but concentrations of individual FA also regulate cytokine production. In addition, when VCO-treated THP-1 cells were LPS-stimulated, VCO displayed anti-inflammatory potential at the transcriptional and translational levels (Varma *et al*., 2019). It is known that CO has lower levels of biologically active compounds compared to VCO (Lima and Block, 2019). Thus, the observed differences in the results might be due to the availability of anti-inflammatory bioactive compounds in the commercially-available CO compared to VCO. Here, we observed that although the ω-6:ω-3 ratios were favorably altered in the case of the blends, the concentrations of individual FA were probably not high enough to show anti-inflammatory effects.

4. CONCLUSIONS

The aim of this study was to improve the ω-3 and ω-6 levels in CO using the blending technique. We selected FO as a rich source of essential FA for blending. The physico-chemical characteristics of edible oil govern the observed health effects of cooking oil. The blending of FO and CO resulted in significantly improved ALA and LA levels, which is also reflected in LA: ALA ratios. SFA, MUFA and PUFA levels were also favorably altered. The storage stability study of C20 (blend with highest FO percentage) showed good oxidative stability. The thermal stability study indicated that the blends could resist thermal deterioration up to a certain time point only, probably indicating that the blends can be used for cooking which does not involve intense heating for a long time. The THP-1 cell line study indicated that it was possible to improve the LA: ALA ratio without adverse effects on cell viability. Still, future studies warrant that not only the FA profile but also the bioactive compound profile of the blends need to be studied and improved to ensure thermo-oxidative stability. In additionally, the uptake of essential FA from the blends by the cells needs to be increased in order to observe biological effects.

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Elucidation of key odorants and sensory properties of five different extra virgin olive oils from Turkey by GC-MS-Olfactometry

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SUMMARY: The present study investigates the aroma, key odorants and sensory profile of extra virgin olive oils from five well-known Turkish cultivars. The aromatic extract obtained by the purge and trap extraction system, according to a sensory analysis, resembled the odor of olive oil. A total of 22, 21, 18, 22 and 21 aroma-active compounds were detected in the extracts of Ayvalık, Memecik, Gemlik, Sarı Ulak and Beylik olive oils, respectively. The results show that Ayvalık has the highest flavor dilution (FD) value of 1024 with hexanal, *(E)*-2-hexenal and *α*-farnesene. Memecik has the highest FD value at 2048 with (E)-2-hexenal. Gemlik has the highest FD value of 1024 with *(Z)*-3-hexenyl acetate, (E)-2-hexen-1-ol and *α*-farnesene. Sarı Ulak has the highest FD value at 2048 with *(E)*-2-hexenal. Beylik has the highest FD value of 2048 with *(E)*-2-hexenal and hexanal. All cultivars represent the characteristic green, cut-grass, fruity odor notes.

KEYWORDS: *AEDA; Aroma-Active Compounds; EVOO; GC-MS-O; Olive oils.*

RESUMEN: ***Interpretación de olores clave y propiedades sensoriales de cinco aceites de oliva virgen extra diferentes de Turquía utilizando GC-MS-Olfatometría.*** El presente estudio investiga el aroma, los olores clave y el perfil sensorial de los aceites de oliva virgen extra de cinco cultivares turcos bien conocidos. El extracto aromático obtenido por el sistema de extracción mediante purga y trampa, según el análisis sensorial, se asemejaba al olor del aceite de oliva. Se detectaron un total de 22, 21, 18, 22 y 21 compuestos aromáticos activos en los extractos de los aceites de oliva Ayvalık, Memecik, Gemlik, Sarı Ulak y Beylik, respectivamente. Los resultados muestran que Ayvalık tiene el valor de dilución de sabor (FD) más alto de 1024 con hexanal, (E)-2-hexenal y α-farnesene, Memecik tiene el valor de FD más alto para 2048 con (E)-2-hexenal, Gemlik tiene el valor de FD más alto de 1024 con acetato de (Z)-3-hexenilo, (E)-2-hexen-1-ol y α-farneseno, Sarı Ulak tiene el valor de FD más alto de 2048 con (E)-2-hexenal, Beylik tiene el valor de FD más alto de 2048 con (E)-2-hexenal y hexanal, todos ellos representando las características de notas de olor afrutado, hierba cortada y verde.

PALABRAS CLAVE: *Aceites de oliva; AEDA; AOVE; Compuestos Aromáticos-Activos; GC-MS-O.*

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1. INTRODUCTION

Virgin olive oil (VOO) is different from other vegetable oils because only mechanical and/or physical procedures are used to obtained VOO from the olive fruit of the olive tree, *Olea europaea* L. Since it is not subjected to additional refining, there is no change in the volatile and non-volatile components. Therefore, the sensory and nutritional properties of VOO are also protected (Perestrelo *et al.,* 2017). Its composition depends on several factors such as production area, degree of fruit ripening and quality of olives, cultivar, climate conditions of regions, and the process systems (Ozturk *et al.,* 2021; Perestrelo *et al.,* 2017). Fresh and good-quality Extra Virgin Olive Oils (EVOOs) are distinguished by consumers and differentiated by their flavor and aroma (Kesen *et al.,* 2014). The aroma properties of VOOs, and especially the positive features of green and fruity, depend on many volatile components in VOO which are produced by enzymatic processes. The lipoxygenase pathway, which involves several volatile components resulting from the breakdown of polyunsaturated fatty acids, is a well-known enzymatic method for creating attractive aromatic volatiles in VOOs (Amanpour *et al.,* 2016).

Aroma components are among the most crucial agents which affect the quality of VOOs and play a vital role in consumer preference. The volatile composition of VOO is known to consist of hundreds of volatile compounds including unsaturated aldehydes, ketones, alcohols, esters, furans and terpene compounds present in low concentrations, from a few ppm or even less (Perestrelo *et al.,* 2017). Gas chromatography-olfactometry (GC-O) can be used to detect these compounds, which are typically classified as odor-active or non-odor-active, based on their current quantity (Ben Brahim *et al*., 2018). The aroma-active compounds (AAC) in Turkish VOOs have been the subject little research. Kesen *et al.* (2013) utilized the aroma extract dilution analysis (AEDA) for the first time in Turkish VOOs and found that guaiacol, 1-penten-3-ol, hexanal, octanal and (Z)-3-hexenyl acetate were the aroma actives with the highest FD factors in VOOs.

The most important feature which distinguishes EVOO from other oils is its smell and special taste. Its characteristic aroma shows green and fruity properties due to volatile components, some of which come directly from the fruit and some which are due to the degradation of polyunsaturated fatty acids as a result of lipoxygenase (LOX) enzyme activity (Guclu *et al.,* 2016). The sensory characteristics of olive oil primarily depend on the concentration and nature of the volatile compounds found in olives (Genovese *et al.,* 2021). Therefore, olive oils are evaluated in a sensory analysis, for positive (fruity, bitter, pungent) and negative (warming-muddy residue, moldy-moist, vinous-vinegar, metallic, stinking (old-stale), heated or burnt, straw-woody, coarse, machinery. oil, black water, salt water, whitish, earthy, wormy, cucumber, wet wood) properties (IOC, 2018).

In Turkey, Ayvalık, Memecik and Gemlik are the most dominant and important olive varieties with distribution of 20.66%, 19.11% and 48.71%, respectively. Also, Beylik and Sarı Ulak varieties are among the important varieties of West and East Mediterranean Regions, respectively. The main aims of this investigation were: i) to identify the volatile compounds in VOOs obtained from the economically important olives of Ayvalık, Memecik, Gemlik, Sarı Ulak and Beylik with the three-phase extraction system, ii) to detect the ACCs with the AEDA approach and GC-MS-Olfactometry and (iii) to determine the sensory profiles of the samples.

2. MATERIALS AND METHODS

2.1. Sampling

EVOOs were used in this work. The EVOOs from the Ayvalık (AY), Memecik (ME), Gemlik (GE), Sarı Ulak (SU) and Beylik (BE) cultivars were collected from the South Aegean, North Aegean, South Marmara, West and East Mediterranean Regions in Turkey, respectively. All olives were harvested during the yellowish-purplish period, which we call the ideal harvest time. All the EVOOs provided by the producers were obtained under a three-phase extraction system during the 2014/15 and 2015/16 crop seasons. The olives were crushed with a hammer crusher after leaf separation and washing. They were then subjected to malaxation at 30-35 °C for 30-45 minutes. Then, olive oil, pomace and olive oil mill wastewater were separated from the olive paste with the help of a decanter, and the olive oil obtained was purified from remaining impurities by passing it through a separator with 200 L of water per hour. 500 mL were taken from each sample and then stored in bottles at 4 °C. Quality parameters (QP), volatile compounds and GC-MS-O, aroma extract dilution, and sensory analyses were performed for both crop seasons.

2.2. Quality parameters of samples

The QP detected were free fatty acidity (FFA) (represented as an oleic acid percentage) (IOC, 2017a), peroxide value (PV) (represented as meqO_2/kg of oil) (IOC, 2017b) and characteristics of ultraviolet absorption at 232 and 270 wavelengths (K_{232} and K_{270}) (IOC, 2019). The samples were analyzed in triplicate.

2.3. Sensory assessment of samples

The sensorial evaluation of the EVOO was performed by an IOC (International Olive Council)-approved panel of fully qualified judges. The samples were sensory analyzed according to the parameters outlined in the IOC approved technique COI/T.20/Doc. no 15 (IOC, 2018). A 15-ml sample was placed in a blue tasting glass. The temperature of the sample was maintained at 28±2 °C. The sensory assessment of the sample was defined using the median of panelists' scores obtained via sensory analysis.

2.4. Analysis of volatile compounds and GC-MS-Olfactometry conditions

The aroma substances in the EVOO samples were analyzed using the purge and trap extraction technique. Representative tests were performed on the aromatic extract to determine the extraction method's reliability. The aroma compounds of the EVOOs were extracted according to the method of Amanpour *et al.* (2016). The extract was passed through sodium sulfate and concentrated to 0.5 mL at 40 °C in a "Vigreux" distillation column. The concentrated extract was directly injected into GC-FID (Flame Ionization Detector), GC-MS and GC-MS-O systems and the AACs were determined. The extractions were performed in three replicates (Kesen *et al.*, 2013). The GC with a flame ionization and a mass selective detector (Agilent 5973, USA), and a sniffing port (Gerstel ODP-2, USA) were used in the GC system at 250 ºC. A capillary column (DB-WAX 0.25 mm x 0.4 m x 60 m) was used for the separation of the aroma components. Chemical standards, retention index, and a mass spectral database were used to identify aroma compounds (NIST 98, Wiley 11). The injector and temperature were set to the same parameters as the GC. The substances were quantified in scan mode using a mass range of 29-350 amu and mass spectra were acquired in electron impact mode (energy voltage: 70 eV). Peaks were identified using standard solutions. The internal standard ((4-nonanol) method was used to calculate the volatile concentrations.

2.5. Aroma active compounds of samples

Three qualified sniffers used GC–MS–O to evaluate the AACs. The extract sniffing process was completed in three stages (25 min each). Based on previous research, the AEDA technique was used to determine the FD factors of the AAC (Ozkara *et al.,* 2019). CH_2Cl_2 was used to dilute the EVOOs: 1:1, 1:2, 1:4, … 1:4096. Sniffing continued until no odorant was detected.

2.6. Statistical analysis

Analysis of variance (ANOVA) and Principal component analysis (PCA) were carried out on the Minitab® 17 program (Minitab Inc., State College, PA, USA) to reveal the discrimination pattern of the EVOO samples.

3. RESULTS AND DISCUSSION

3.1. Quality parameters of samples

The general QP of the samples are shown in Table 1. As seen, the FFA, PV, K_{232} and K_{270} values of the VOOs did not exceed the limit defined for EVOO by IOC (IOC, 2021). The percentage of FFA in the oils ranged from 0.30 to 0.59 for the 2014 harvest year and from 0.22 and 0.77 for the 2015 harvest year. FFA is known to be the main criterion for classifying VOO. All the samples were determined to contain less than the maximum legal limit of 0.8 (oleic acid %) for EVOO (IOC, 2021). The PV of the oils ranged from 5.07 to 9.73 meqO_2/kg oil and from 4.59 to 12.33 meqO_2/kg oil, for 2014 and 2015 harvest years, respectively. The results showed that the PV of the samples were below the limit of 20 meqO_2/kg oil as established by the IOC (IOC, 2021) for classifying EVOO. According to the IOC limit, K_{232} and K_{270} values must be less than 2.50 and 0.22 for EVOO, respectively. The K_{232} and K_{270} values of the oils were determined to be below this legal limit.

Table 1. Quality parameters of samples

Year	Sample	FFA (oleic acid %)	PV (meq O_2/kg oil)	K_{232}	K_{270}
2014	**ME**	0.30 ± 0.01	6.88 ± 0.06	1.74 ± 0.02	0.15 ± 0.05
	AY	0.59 ± 0.00	8.68 ± 0.08	1.71 ± 0.05	0.11 ± 0.04
	GE	0.52 ± 0.03	5.75 ± 0.05	1.68 ± 0.04	0.12 ± 0.02
	SU	0.48 ± 0.01	9.73 ± 0.04	1.62 ± 0.06	0.12 ± 0.03
	BE	0.32 ± 0.03	5.07 ± 0.03	2.12 ± 0.05	0.18 ± 0.06
2015	**ME**	0.22 ± 0.00	8.24 ± 0.04	1.97 ± 0.05	0.17 ± 0.04
	AY	0.66 ± 0.02	9.83 ± 0.07	1.84 ± 0.03	0.16 ± 0.05
	GE	0.52 ± 0.01	4.59 ± 0.04	1.79 ± 0.04	0.14 ± 0.03
	SU	0.36 ± 0.01	7.61 ± 0.06	1.67 ± 0.06	0.13 ± 0.02
	BE	0.77 ± 0.03	12.33 ± 0.05	2.37 ± 0.05	0.20 ± 0.05

Values expressed as mean ± standard deviation AY: Ayvalık, ME: Memecik, GE: Gemlik, SU: Sarı Ulak and BE: Beylik. Experiments were conducted 3 times.

The results agree with previous studies carried out with cv. AY, MY and GE (Kesen *et al.,* 2013), AY and ME (Karagoz *et al*., 2017), ME and GE (Köseoğlu *et al.,* 2016). ANOVA analysis was performed to determine the significance of difference according to the varieties of olive oil samples. The difference among the means of the quality parameter results was not found significant at the 95% confidence level.

3.2. Sensory assessment of samples

Sensory analysis is a quality criterion in VOO standards, and evaluating the sensory quality of VOOs comprises assessing positive and negative properties. The results of the sensory assessment of the samples are shown in Figure 1. According to the IOC standard (IOC, 2021), samples are classified as EVOOs if the median of defects is “0” and the median of fruity is greater than “0”. Sensory assessment showed that the studied oils had no defects and therefore considered as EVOOs. The highest fruity medians were found at 5.3 and 4.95 for the SU EVOO, from 2014 and 2015, respectively. With regards to the bitterness value, the highest medians were detected in the BE EVOO at 4 and 4.4 from 2014 and 2015, respectively. The pungent values were determined at the highest values in the AY (4.0), ME (4.0) and BE (4.0) EVOO from 2014 and in the BE (4.6) EVOO from 2015. According to the IOC standard (IOC, 2018) the term, “robust” can be used when the median of the positive attribute is more than “6.0”, “medium” can be used when the median of the attribute is between “3.0” and “6.0” and “delicate” can be used when the median of attribute is less than “3.0”. In this research, all samples were characterized as medium for fruity properties from both harvest years. Regarding the the bitterness value, the BE EVOO was classified as medium from 2014 and the AY, ME and BE EVOOs were classified as medium from 2015. The other samples were characterized as delicate. When we look at the results of pungent values, we can characterize the samples as medium for both years, except for the SU EVOO. SU EVOO was classified as delicate.

3.3. Volatile compounds of samples

Table 2 shows the identified and classified (µg/kg) volatile components in the samples. As shown in Table 2, a total of 52, 57, 51, 57 and 54 compounds, including aldehydes, alcohols, terpenes, acids, volatile phenols, ketones, esters, lactones, hydrocarbons and furans, were qualitatively and quantitatively identified in AY, ME, GE, SU and BE EVOO, respectively. GC–MS chromatograms of EVOOs are shown in Figure 2. As seen from these chromatograms, the most volatile components were found in BE EVOO. The highest amount of total volatile compounds (45364 and 31990 µg/kg) was determined in the BE EVOO, for 2014 and 2015, respectively. It was followed by the AY EVOO (34890.1 µg/kg) from 2014 and the SU EVOO (15282 µg/kg) from 2015. The lowest volatile compounds were found in the GE

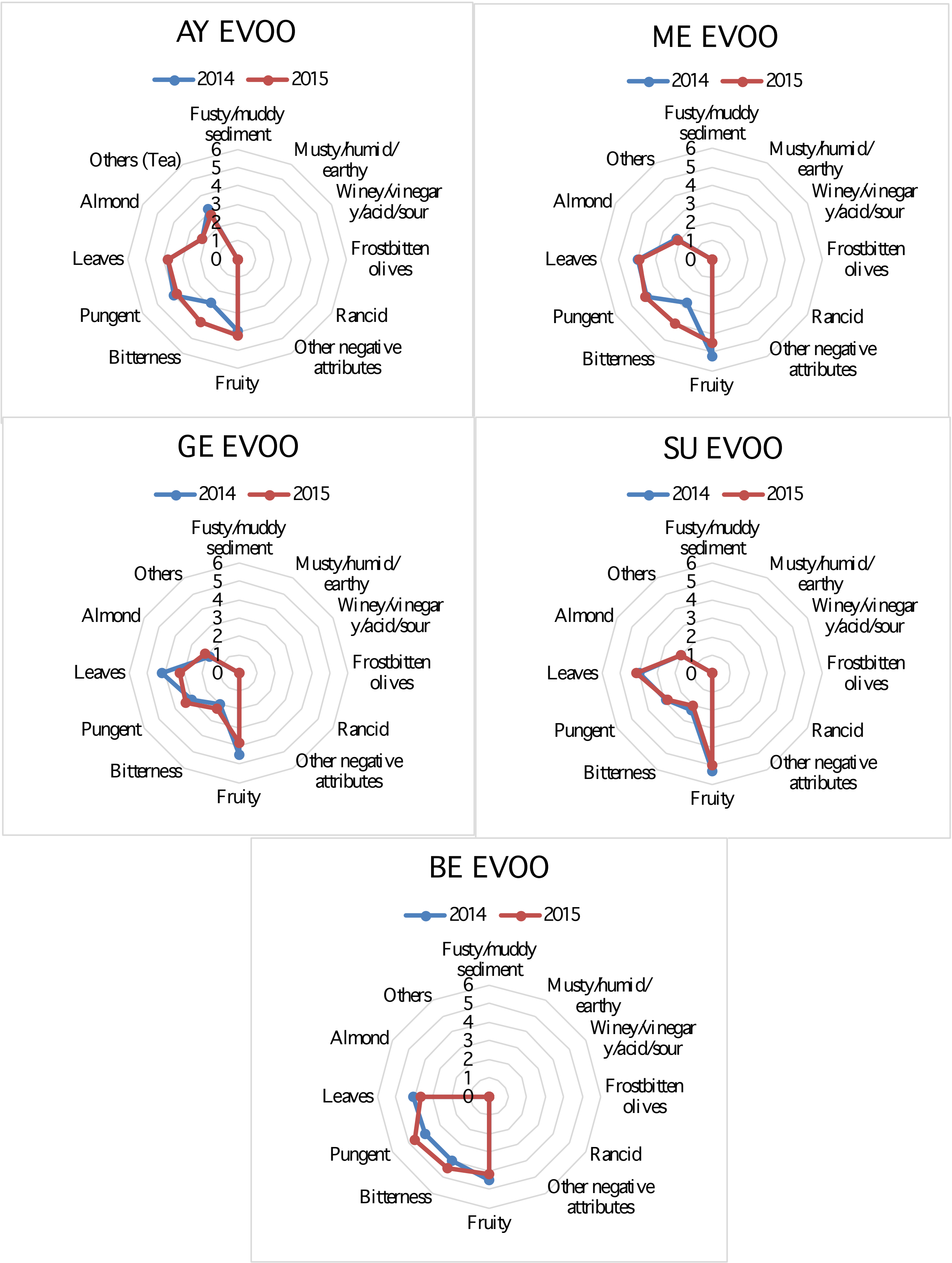

FIGURE 1. Sensory spider plot of Ayvalık, Memecik, Gemlik, Sarı Ulak and Beylik Extra Virgin Olive Oils. AY: Ayvalık, ME: Memecik, GE: Gemlik, SU: Sarı Ulak and BE: Beylik

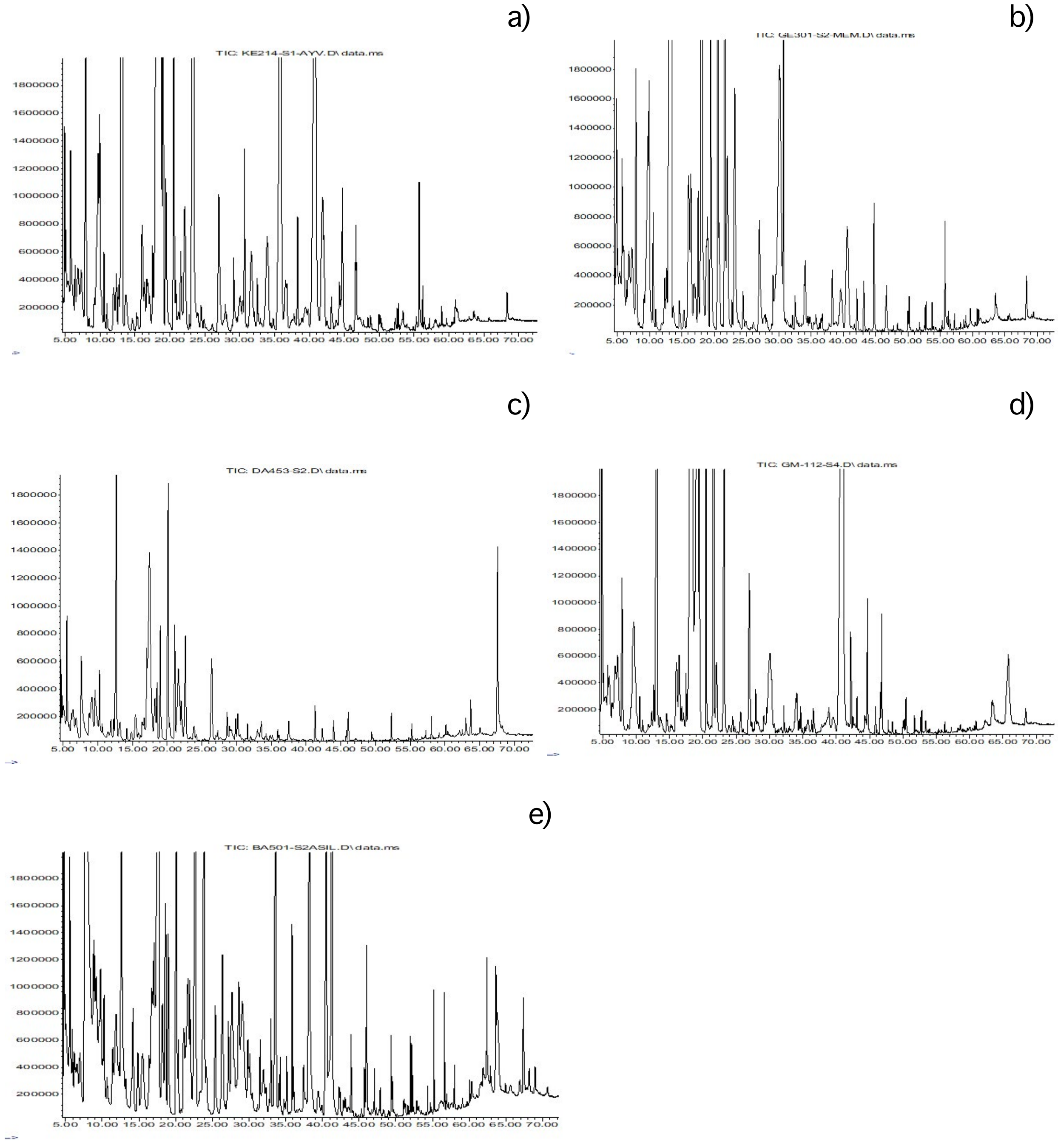

Figure 2. Volatile compound chromatogram of Ayvalık (a), Memecik (b), Sarı Ulak (c), Gemlik (d) and Beylik (e) Extra Virgin Olive Oils.

EVOO (20947.8 and 8677.8 μg/kg) from 2014 and 2015, respectively. The majority of the volatile components found in this research had been previously identified in the VOOs of the same and different olive cultivars (Aparicio and Morales, 1998; Vichi *et al*., 2007; Baccouri *et al*., 2008; Giuffrè *et al*., 2019; Žanetić *et al*., 2021). Among the volatile compounds detected in different EVOOs, aldehydes were identified and quantified as the major components with regard to the volatile part, followed by alcohols. The lipoxygenase pathway is activated during the olive oil extraction process, resulting in the release of enzymes. Aldehyde compounds are produced by the hydroperoxide lyase enzyme, which is then reduced into alcohols by the alcohol dehydrogenase enzyme in VOOs (Amanpour *et al.,* 2016). C5 and C6 aldehydes and alcohols are the four most common chemical groups of the 18 components which contribute positively to the aroma composition of VOOs from the positive sensory properties (Procida *et al*., 2016). As previously stated by Issaoui *et al.* (2015), Caporaso *et al.* (2016) and Žanetić *et al.* (2021) the volatile component profile of VOOs is affected by the region where it is grown, the geographical origin, the pedoclimatic conditions, the variety, the extraction systems and VOO storage conditions. It was also seen in the study that the volatile component profile changed according to year, variety and region.

Aldehydes. A total of 7, 8, 4, 11 and 12 aldehydes and 7, 8, 3, 10 and 12 aldehydes were identified in AY, ME, GE, SU and BE EVOO from 2014 and 2015, respectively. The primary aldehyde compounds in EVOO were (E)-2-hexenal and hexanal (Table 2). Total concentrations of aldehydes were found in the AY EVOO at 14055.7 and 5440.1 μg/kg, in the ME EVOO at 14722.2 and 6277.5 μg/kg, in the GE EVOO at 3465.8 and 993 μg/kg, in the SU EVOO at 20894.8 and 8194.4 μg/kg and in the BE EVOO at 27598.9 and 17346 μg/kg, for the 2014 and 2015 seasons, respectively. According to the results, the aldehyde compounds were higher in 2014 than in 2015. In the SU EVOO (E)-2-hexenal was determined to be the highest aldehyde compound with 14280 and 5265 μg/kg, and it was followed by the ME EVOO with 12363 and 5145 μg/kg, for 2014 and 2015, respectively. In the BE EVOO hexanal was found to be the highest aldehyde compound with 8753 and 6312 μg/kg, and it was followed by the AY EVOO with 4790 and 1091 μg/kg, and ME EVOO 886 and 468 μg/kg, for 2014 and 2015, respectively. It was reported by other authors (Kesen *et al*., 2014; Sacchi *et al.,* 2015; Ben Brahim *et al*., 2018; Giuffrè *et al*., 2019; Žanetić *et al.,* 2021) that (E)-2-hexenal and hexanal are common aldehydes in many VOOs, including AY, GE, ME, Halhalı, Nizip Yağlık, and Kilis Yağlık from Turkey, Mari from Iran, Jemri, Touffehi and Fakhari from Tunisian, Arbequina, Cornicabra, Morisca, Picolimon, Picudo and Picual from Spain, and Ravece from Italy. According to studies, the percentage of C6 aldehydes, particularly (E)-2-hexenal, increased during olive ripening, which was primarily detected when the olive fruit skin color changed from yellow-green to purple (Ben Brahim *et al.,* 2018). The amount of hexanal. which was the second major aldehyde in the samples, mostly decreased with maturation. In this study, the results are in agreement with these reports, that our samples were harvested during the yellowish-purplish period, which we consider the ideal harvest time. Among the aldehydes, (E)-2-hexenal and hexanal are responsible for the positive green sensory attributes in EVOO. The results showed that our samples' positive sensory attributes are in accordance with this criterion.

Alcohols. Alcohols are associated with positive sensory properties such as green, bitter, fruity aromatic, and they present weaker sensory attributes than aldehydes (Žanetić *et al.,* 2021). Alcohols produced by the ADH enzyme, are found in plants and are responsible for the production of volatile alcohols which contribute to the aroma of VOO (Kesen *et al*., 2014). A total of 12, 16, 14, 16 and 15 alcohols and 16, 16, 11, 14 and 15 alcohols were determined in the AY, ME, GE, SU and BE EVOOs in the 2014 and 2015 seasons, respectively. In all EVOO samples (Table 2) alcohols were determined to be the second main group of volatile compounds, as confirmed in previous studies (Kesen *et al.,* 2014; Karagoz *et al*., 2017; Žanetić *et al.* 2021). The highest amount of alcohols was found in the first year, which was likely due to increased ADH enzyme activity, and determined in the ME EVOO at 6767.1 μg/kg, followed by the SU EVOO at 5820.9 μg/kg, and the GE EVOO at 5310.5 μg/kg, the BE EVOO at 5193 μg/kg and the AY EVOO at 5094.0 μg/kg. The second year, the highest total amount of alcohols was found in the BE EVOO at 4108 μg/kg, followed by SU EVOO at 3678 μg/kg, the ME EVOO at 3294.8 μg/kg, the GE EVOO at 3038 μg/kg and the AY EVOO at 2587.5 μg/kg. The results showed that (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol and 1-hexenol were the dominant C6 alcohols in all the analyzed samples. These results are in agreement with other researchers (Baccouri *et al.,* 2008; Karagoz *et al.,* 2017). The contents of (Z)-3-hexen-1-ol main alcohols in the ME EVOO were 1625 μg/kg and 1115 μg/kg in the BE EVOO from 2014 and 2015, respectively. (Z)-3-hexen-1-ol is the most prominent green note, and in our study this chemical appears to have a green-leaf characteristic similar to freshly cut grass. (E)-2-hexen-1-ol were determined to be the predom-

Table 2. Volatile compounds in samples (µg/kg)

	LRI[a]	Compounds	Concentration (µg/kg)[b]									
			SU		ME		AY		GE		BE	
Aldehydes			**2014**	**2015**	**2014**	**2015**	**2014**	**2015**	**2014**	**2015**	**2014**	**2015**
1	8.006	Hexanal	2379	1083	886	468	4790	1091	475	125	8753	6312
2	9.187	*(E)*-2-Pentenal	148	139	87.4	82.0	73.8	72.6	ND	ND	188	237
3	9.923	*(Z)*-3-Hexenal	ND	ND	623	131	ND	ND	ND	ND	ND	ND
4	9.982	3-Hexenal	996	350	ND	ND	ND	ND	ND	ND	3243	1041
5	12.344	Heptanal	177	95.0	ND	ND	ND	ND	ND	ND	1188	1065
6	13.134	*(E)*-2-Hexenal	14280	5265	12363	5145	7752	3642	2780	769	10038	4023
7	17.015	Octanal	267	134	113	67.6	245	70.7	ND	ND	168	157
8	17.057	*(Z)*-2-Heptanal	ND	ND	ND	ND	ND	ND	ND	ND	2574	3168
9	20.939	*(E,E)*-2.4-Hexadienal	101	39.0	ND	ND	157	39.7	ND	ND	340	218
10	22.256	Nonanal	2261	907	477	256	893	454	179	98.5	562	595
11	24.636	*(E,E)*-2.4-Heptadienal	159	99.4	87.4	56.2	145	70.1	31.8	ND	173	124
12	33.528	*(E)*-2-Decenal	96.9	83.3	85.4	71.6	ND	ND	ND	ND	317	347
13	41.451	*(E,E)*-2.4-Decadienal	29.8	ND	ND	ND	ND	ND	ND	ND	55.1	58.9
		Total	**20894.8**	**8194.4**	**14722.2**	**6277.5**	**14055.7**	**5440.1**	**3465.8**	**993**	**27598.9**	**17346**
Alcohols												
1	6.451	2-Methyl-3-buten-2-ol	518	222	242	175	ND	ND	ND	ND	229	202
2	10.599	1-Penten-3-ol	662	455	304	145	416	121	191	95.0	596	576
3	11.039	3-Penten-2-ol	242	121	183	84.1	486	84.1	151	74.0	178	164
4	12.724	Isoamyl alcohol	226	167	127	64.4	292	131	164	90.0	190	143
5	14.659	1-Pentanol	62.7	52.7	62.1	48.7	ND	22.7	52.4	30.0	156	105
6	16.380	2-Hexanol	ND	90.3	114	86.0	203	143	ND	95.3	246	169
7	17.205	*(E)*-2-Penten-1-ol	148	84.0	95.1	60.0	ND	ND	ND	ND	ND	ND
8	17.573	*(Z)*-2-Penten-1-ol	603	410	312	181.0	125	86.0	147	109	820	613
9	19.520	1-Hexanol	695	443	1156	472	946	527	1618	1033	557	530
10	20.600	*(Z)*-3-Hexen-1-ol	1389	851	1625	602	1528	826	816	545	1447	1115
11	21.651	*(E)*-2-Hexen-1-ol	736	559	2318	1199	630	353	1957	859	264	183
12	24.993	1-Heptanol	64.7	ND	24.4	16.6	ND	22.2	24.0	ND	68.6	65.0
13	30.518	1-Octanol	172	100	ND	57.8	105	55.0	ND	ND	ND	ND
14	34.922	1-Nonanol	ND	ND	ND	ND	ND	15.3	8.6	ND	ND	ND
15	35.462	*(Z)*-3-Nonen-1-ol	40.7	ND	ND	ND	ND	24.3	11.8	ND	85.0	43.4
16	43.303	Benzyl alcohol	107	56.0	61.4	26.1	125	51.6	35.8	17.6	142	59.2
17	44.828	Phenylethyl Alcohol	136	66.4	119	69.5	141	89.1	122	89.8	190	121
18	46.591	3-Octanol	ND	ND	12.5	ND	96.0	37.1	ND	ND	ND	ND
19	51.862	2-Phenoxyethanol	19.2	ND	11.1	7.3	ND	ND	10.4	ND	25.8	20.4
		Total	**5820.9**	**3678**	**6767.1**	**3294.8**	**5094.0**	**2587.5**	**5310.5**	**3038**	**5193**	**4108**
Terpenes												
1	13.787	dl-Limonene	128	77.3	109	73.2	337	143	59.7	35.0	127	59.0
2	14.813	Styrene	86.5	ND	31.3	ND	223	24.0	23.1	ND	ND	ND
3	16.137	*β*-Ocimene	2654	239	471	247	644	315	230	151	3645	570
4	30.162	*α*-Copaene	180	112	894	744	140	138	351	286	3022	3259
5	34.014	*(E)*-*α*-Bergamotene	138	86.2	ND	ND	124	80.1	9.2	ND	ND	ND
6	34.180	*(Z,E)*-*α*-Farnesene	179	ND	ND	ND	ND	62.0	111	ND	ND	ND
7	35.991	*β*-Sesquiphellandrene	104	73.2	125	76.9	143	114	44.6	ND	64.4	ND
8	39.670	*α*-Muurolene	ND	ND	121	67.1	ND	ND	ND	ND	402	272
9	40.680	*α*-Farnesene	387	98.8	841	230	4525	1675	4200	1240	451	138
		Total	**3857.2**	**686.7**	**2593.2**	**1438.5**	**6136**	**2551.8**	**5029.4**	**1711.3**	**7711.7**	**4297.3**
Acids												
1	22.891	Acetic acid	58.3	120	27.6	57.3	132	48.3	9.2	25.1	ND	257
2	28.055	Propanoic acid	67.7	57.3	23.1	64.7	305	55.2	43.3	ND	ND	ND
3	32.240	Butanoic acid	136	48.3	13.5	ND	186	14.0	38.8	12.3	73.3	60.8
4	36.649	Pentanoic acid	59.4	36.5	14.8	26.7	268	27.8	31.3	16.3	37.3	336

			Concentration (μg/kg)[b]									
	LRI[a]	Compounds	SU		ME		AY		GE		BE	
Acids			**2014**	**2015**	**2014**	**2015**	**2014**	**2015**	**2014**	**2015**	**2014**	**2015**
5	41.315	Hexanoic acid	152	127	31.6	116	305	98.2	106	ND	178	2522
6	46.472	*(E)*-3-Hexenoic acid	ND	ND	4.7	ND	ND	ND	10.2	ND	106	86.2
7	46.977	*(E)*-2-Hexenoic acid	ND	ND	ND	ND	ND	ND	110	69.0	ND	ND
8	50.099	Octanoic acid	41.3	28.4	21.5	23.1	ND	33.4	18.1	ND	45.8	96.7
9	52.912	Nonanoic acid	80.6	73.8	23.9	60.0	266	74.2	28.4	ND	27.9	75.7
10	55.405	Decanoic acid	48.8	ND	12.2	17.6	ND	ND	5.9	ND	33.0	20.8
11	58.212	Benzoic acid	78.6	40.0	19.5	ND	ND	ND	13.4	ND	65.4	17.9
12	59.672	Dodecanoic acid	ND	ND	31.2	19.5	ND	ND	22.5	ND	83.4	19.6
13	62.984	Tetradecanoic acid	ND	121.8	ND	45.5	ND	ND	ND	ND	141	56.6
14	68.587	Hexadecanoic acid	309	734	145	143	1104	234	153	248	375	259
		Total	**1031.6**	**1388**	**368.9**	**574.1**	**2564**	585.2	588.9	371.1	1166.4	3809.1
Volatile Phenols												
1	42.389	Guaiacol	43.5	ND	8.9	ND	46.0	29.8	55.8	ND	ND	ND
2	47.903	Phenol	24.0	ND	6.7	6.6	33.3	12.3	11.6	ND	25.7	17.0
3	50.128	*p*-Cresol	ND	ND	ND	10.8	27.6	ND	ND	ND	ND	13.2
4	52.746	4-Ethyl-phenol	21.4	ND	13.0	11.6	ND	22.1	10.6	ND	96.4	84.6
5	56.367	2.4-Di-tert-butylphenol	40.8	ND	16.8	14.9	86.1	60.6	9.1	ND	40.6	ND
		Total	**129.7**	**0.0**	**45.4**	**43.8**	**192.9**	124.8	87.2	0.0	162.7	114.8
Ketones												
1	4.897	3-Methyl-3-buten-2-one	586	139	127	72	391	152	191	88.0	ND	ND
2	4.919	2-Pentanone	ND	ND	ND	ND	ND	ND	2149	1200	ND	ND
3	5.827	1-Penten-3-one	620	401	659	407	701	220	ND	ND	1484	1090
4	6.373	2-Methyl-3-buten-2-one	ND	ND	ND	ND	284	184	ND	ND	ND	ND
5	33.047	Acetophenone	30.3	13.0	14.0	ND	40.1	15.3	7.1	ND	ND	ND
		Total	**1236**	**553**	**800**	**479**	**1415**	**571**	**2347**	**1288**	**1484**	**1090**
Esters												
1	15.852	Hexyl acetate	122	62.2	407	214	234	166	204	144	ND	ND
2	18.078	*(Z)*-3-Hexenyl acetate	ND	ND	2382	598	3540	1295	3443	875	ND	ND
3	38.442	Methyl salicylate	118	68.3	61.4	32.0	268	39.3	ND	ND	183.0	137.0
		Total	**240.2**	**130.6**	**2850.7**	**844.3**	**4042.1**	**1500.7**	**3646.7**	**1019.1**	**183.0**	**137.0**
Lactones												
	34.655	*γ*-Caprolactone	ND	ND	13.51	ND	ND	ND	25.9	18.4	148	70.0
Hydrocarbons												
	6.860	3-Ethyl-1.5-octadiene	1359	652	937	567	1309	665	446	240	1659	958
Furans												
	14.635	2-Pentylfuran	ND	ND	ND	ND	ND	ND	ND	ND	57.9	59.4
	General Total		**34569.5**	**15282**	**29097.7**	**13519.0**	**34809.1**	**14026.0**	**20947.8**	**8677.8**	**45364**	**31990**

[a] LRI: Linear retention index calculated on DB-WAX capillary column; [b]Concentration. Results are the means of three repetitions as μg/kg Identification. Standardt deviation of all aroma compounds was below 10%. AY: Ayvalık, ME: Memecik, GE: Gemlik, SU: Sarı Ulak and BE: Beylik. Experiments were conducted 3 times.

inant alcohols in the ME EVOO (2318 and 1199 μg/kg); while 1-hexenol was found to be the most abundant alcohol in the GE EVOO (1618 and 1033 μg/kg) for both years.

Terpenes. Terpenes (dl-Limonene, styrene, β-ocimene, α-copaene, (E)-α-bergamotene, (Z,E)-α-farnesene, β-sesquiphellandrene, α-muurolene and α-farnesene) were found to be the third most abundant group of volatile compounds in the samples, with total amounts of 6136 μg/kg, 2593.2 μg/kg, 5029.4 μg/kg, 3857.2 μg/kg and 7711.7 μg/kg in 2014, and 2551.8 μg/kg, 1438.5 μg/kg, 1711.3 μg/kg, 686.7 μg/kg and 4297.3 μg/kg in the AY, ME, GE, SU and BE EVOO from 2015, respectively. A total of 7, 7, 8, 8 and 6 terpenes and 8, 6, 4, 6 and 5 terpenes were found in the AY, ME, GE, SU and BE EVOO from 2014 and 2015, respectively. The highest amounts of terpenes were determined in the BE EVOO from both years. In the AY (4525 and 1675 μg/kg) and GE (4200 and 1240 μg/kg) EVOO α-farnesene was determined to be the prominent terpene from both years. α-Copaene was found as the highest terpene for the ME (894 and 744 μg/kg) and for the BE (3022 and 3259 μg/kg) EVOO from 2014 and 2015. β-ocimene was identified as the highest terpene for the SU EVOO (2654 and 239 μg/kg) from both years. These terpenes were also detected in Turkish VOOs (Kaftan and Elmaci, 2011; Kesen *et al.,* 2013; Guclu *et al.,* 2016), Greek VOOs (Issaoui *et al.,* 2015), Tunisian VOOs (Ben Brahim *et al.,* 2018) and Iranian VOO (Amanpour *et al.,* 2016). Kelebek *et al.* (2015) reported that terpenes mostly affected the varieties of VOOs. The results of our study support this assertion.

Acids. Fourteen acid components were found in the studied samples. A total of 7, 11, 13, 10 and 11 acids and 8, 10, 6, 11 and 12 acids were detected in the AY, ME, GE, SU and BE EVOO from 2014 and 2015, respectively. Kesen *et al.* (2013) determined acetic acid, nonanoic acid, and decanoic acid as the major acids in AY, GE, ME VOOs, respectively. Acetic acid was found in the highest concentration in Mari VOO by Amanpour *et al.* (2016), and acetic acid was identified as a major acid in Tunisian and Sicilian VOOs by Baccouri *et al.* (2008). In our study, the most representative acid was hexadecanoic acid with 1104 and 234 μg/kg, 145 and 143 μg/kg, 153 and 248 μg/kg and 309 and 734 μg/kg, in the AY, ME, GE and SU EVOO from 2014 and 2015, respectively. Hexadecanoic acid (375 μg/kg) and hexanoic acid (2522 μg/kg) were the highest acids in the BE EVOO from 2014 and 2015, respectively.

Volatile Phenols. Five volatile phenols, namely guaiacol, phenol, p-cresol, 4-ethyl-phenol and 2,4-di-tert-butylphenol, were identified in the studied EVOOs. They are generally responsible for the bitter and pungent attributes of VOOs (Amanpour *et al.,* 2016). A total of 4, 4, 4, 4 and 3 volatile phenols and 4, 4, 0, 0 and 3 volatile phenols were determined in the AY, ME, GE, SU and BE EVOO from 2014 and 2015, respectively. The highest total volatile phenols were found in the AY EVOO (192.9 and 124.8 μg/kg), followed by the BE EVOO (162.7 and 114.8 μg/kg), from 2014 and 2015, respectively. Guaiacol, phenol and 4-ethyl-phenol were detected in Turkish VOOs (Kesen *et al.,* 2013) and Iranian VOO (Amanpour *et al.,* 2016).

Ketones. A total of 4, 3, 3, 3 and 1 ketones and 4, 2, 2, 3 and 1 ketones were identified in the AY (1415 and 571 μg/kg), ME (800 and 479 μg/kg), GE (2347 and 1288 μg/kg), SU (1236 and 553 μg/kg) and BE (1484 and 1090 μg/kg) EVOO from 2014 and 2015, respectively. 3-methyl-3-buten-2-one was detected in the AY, ME, GE and SU EVOO from both years. 2-pentanone was found only in the GE EVOO from both years. 1-penten-3-one was determined in the AY, ME, SU and BE EVOO from both years. 2-methyl-3-buten-2-one was identified only in the AY EVOO from both years. Acetophenone were detected in the AY and SU EVOO from both years and in the ME and GE EVOO from 2014. It was reported by Kalua *et al.* (2007) that especially the short ketones are responsible for the positive sensory attributes in VOOs.

Esters. A total of 3, 3, 2, 2 and 1 esters were determined in the AY, ME, GE, SU and BE EVOOs from both years. Hexyl acetate, (Z)-3-hexenyl acetate and methyl salicylate esters were determined in the studied EVOOs. The highest total esters were found in the AY EVOO with 4042.1 and 1500.7 μg/kg, followed by the GE EVOO with 3646.7 and 1019.1 μg/kg and the ME EVOO with 2850.7 and 844.3 μg/kg, from 2014 and 2015, respectively. Esters are accountable for the pleasant fruity and flowery odor of the olive fruits (Kelebek *et al.,* 2015). These compounds were also detected in the AY, ME, GE VOOs in previous studies by Kesen *et al.* (2013), Karagoz *et al.* (2017), Guclu *et al.* (2016) and in Jemri, Touffehi and Fakhari OOs by Ben Brahim *et al.* (2018). Our results are in agreement with these studies.

Lactones, hydrocarbons, furans. The other minor volatile compounds in the samples were lactones, hydrocarbons and furans. Lactone (γ-caprolactone) was identified in the ME, GE and BE EVOO; hydrocarbon (3-ethyl-1,5-octadiene) was detected in the all EVOO samples and furan (2-pentylfuran) was determined only in the BE EVOO. Kesen *et al.* (2014) reported that lactones contribute to the characteristic fruity odors of VOOs.

3.4. Aroma active compounds of samples

Table 3 shows the results of AACs detected using AEDA, as well as their FD values and odor descriptions. AAC odor intensities were measured as FD factors and ranged from 4 to 2048. Aromatic extracts of the samples revealed a total of 29 AACs. Aromatic extracts of AY, ME, GE, SU, and BE EVOOs contained a total of 22, 21, 18, 22 and 21 AACs, respectively.

One of the most important AACs which affects the overall composition of VOO is aldehydes. Ten odorants were defined as aroma active aldehydes

Table 3. Aroma-active compounds in Ayvalık, Memecik, Gemlik, Sarı Ulak and Beylik Extra Virgin Olive Oils from 2014 and 2015 harvest years

Year	No	Compound	RT[a]	Odor description[b]	FD factor				
					AY	ME	GE	SU	BE
2014	1	*α*-Pinene	7.30	Plant	-	-	16	-	-
2015					-	-	16	-	-
2014	2	Hexanal	9.47	Green-cut grass	1024	512	256	1024	2048
2015					512	256	32	512	2048
2014	3	*(E)*-2-Pentenal	11.20	Fresh-plant	-	8	-	4	4
2015					-	16	-	4	8
2014	4	(*Z*)-3-Hexenal	11.69	Fresh-cut grass	-	128	-	-	1024
2015					-	32	-	128	16
2014	5	3-Hexenal	11.90	Pleasant-cut grass	-	128	-	256	1024
2015					-	32	-	4	16
2014	6	1-Penten-3-ol	12.80	Herbal-green	128	128	32	256	128
2015					-	32	32	128	128
2014	7	3-Penten-2-ol	13.18	Herbal-fruity	-	16	8	32	-
2015					16	16	128	32	-
2014	8	Heptanal	13.72	Green-oily	-	-	-	32	-
2015					-	-	-	-	16
2014	9	dl-Limonene	14.21	Floral-citrusy	64	-	-	-	-
2015					32	-	32	32	-
2014	10	*(E)*-2-Hexenal	15.25	Cut grass-green	1024	2048	512	2048	2048
2015					1024	512	128	512	512
2014	11	*β*-Ocimene	16.93	Fruity-leafy	32	64	32	256	512
2015					16	32	32	32	64
2014	12	Hexyl acetate	17.92	Fruity-plant	32	64	32	-	-
2015					16	32	64	-	-
2014	13	Octanal	18.50	Oily-floral	-	8	-	64	-
2015					16	-	-	32	32
2014	14	Unknown	19.45	Oily-fruity	-	-	-	-	-
2015					128	-	-	-	-
2014	15	*(Z)*-3-Hexenyl acetate	19.98	Fruity-green	-	1024	1024	-	-
2015					-	512	512	-	-
2014	16	*(Z)*-2-Penten-1-ol	20.33	Green-oily	32	32	8	64	64
2015					-	-	8	64	-
2014	17	1-Hexanol	21.99	Floral-herbal	128	256	512	64	64
2015					64	64	512	128	64
2014	18	*(Z)*-3-Hexen-1-ol	23.62	Herbal-cut grass	512	512	128	512	256
2015					128	128	64	512	1024
2014	19	Nonanal	23.89	Oily-citrusy	64	-	-	128	64
2015					32	-	-	64	64
2014	20	*(E,E)*-2.4-Hexadienal	23.96	Oily	4	-	-	-	16
2015					-	-	-	-	16
2014	21	*(E)*-2-Hexen-1-ol	24.99	Grassy-cool	64	1024	1024	512	128
2015					128	32	512	512	64
2014	22	*(E,E)*-2.4-Heptadienal	27.89	Oily	16	-	-	-	16
2015					8	8	-	-	16
2014	23	*α*-Copaene	29.25	Sweet-fruity	-	256	128	-	256
2015					-	-	64	-	256
2014	24	1-Octanol	32.70	Fruity-green	32	16	-	32	-
2015					16	-	-	32	-
2014	25	*α*-Farnesene	41.46	Floral-green plant	1024	512	1024	64	16
2015					128	128	256	-	-
2014	26	Hexanoic acid	45.90	Buttery-cheesy	64	-		-	32
2015					-	-	-	32	-
2014	27	Guaiacol	46.20	Olive paste	-	-	32	32	
2015					16	-	-	-	-
2014	28	Benzyl alcohol	47.03	Floral	64	32	16	64	64
2015					32	16	16	32	-
2014	29	Phenylethyl alcohol	48.28	Floral	64	64	64	64	128
2015					32	32	32	32	-

[a]RT: Retention Time on DB-WAX capillary column; [b]Odor description as perceived by panelists during olfactometry. AY: Ayvalık, ME: Memecik, GE: Gemlik, SU: Sarı Ulak and BE: Beylik. Experiments were conducted 3 times.

(Table 3). The most dominant was (E)-2-hexenal, which had a cut grass-green odor and an FD factor ranging from 128 to 2048. The first year, FD factor was determined at the highest level with 2048 in ME, SU and BE EVOOs. AY EVOO followed it with an FD factor of 1024. The second year, it was determined in AY EVOO with the highest 1024 FD factor. The FD factor was determined as 512 in ME, SU and BE EVOO s. (E)-2-hexenal was followed by hexanal with a green-cut grass odor and an FD factor ranging from 128 to 2048. In 2014 and 2015, the FD factor was determined at the highest level with 2048 in BE EVOO. Guth *et al*. (1991) states that (E)-2-hexenal contributes to the aroma of VOOs with its strong odor. Solinas *et al*. (1988) also suggests that (E)-2-hexenal can be used for distinguishing a monovariatel VOO. Other aldehydes are (E)-2-pentenal, (Z)-3-hexenal, 3-hexenal, heptanal, octanal, nonanal. (E,E)-2.4-hexadienal and (E,E)-2.4-heptadienal were determined to impart fresh-plant, fresh-cut grass, pleasant-cut grass, green-oily, oily-floral, oily-citrusy, oily and oily odors, respectively. The detection threshold of aldehydes is low. It is known that aldehydes have a significant effect which can change the general properties of VOOs, even at low detection thresholds and low concentrations (Kesen *et al.,* 2013). It can be seen from previous studies that the detected aldehydes are commonly found in many VOOs (Guth *et al.,* 1991). The results are consistent with the studies performed.

Alcohols are the second most important aroma active compounds which influence the VOO's overall composition. Aldehydes have a higher sensory value than alcohols. The FD factors of the samples varied from 8 to 1024. As aroma active alcohols, 10 odorants were detected in the samples (Table 3). Among them, (E)-2-hexen-1-ol, (Z)-3-hexen-1-ol and 1-hexanol were the most dominant with grassy-cool, herbal-cut grass and floral-herbal odors and an FD factor ranging from 32 to 1024. In 2014, (E)-2-hexen-1-ol was determined at the highest level in ME and GE EVOOs with an FD factor of 1024. It was followed by SU EVOO with an FD factor of 512. In 2015, the highest FD value was determined for GE and SU EVOOs with an FD factor of 512. The (Z)-3-hexen-1-ol aroma-active compound was determined at the highest level with 512 FD factor in AY, ME and SU EVOOs from 2014. In 2015, it was determined at the highest level in BE EVOO with an FD factor of 1024. The results are in accordance with other studies (Kesen *et al*., 2014; Amanpour *et al.,* 2016).

Aldehydes and alcohols are affected according to the region where the olive is grown, especially *cis*-3-hexenal, *cis*-3-hexenol, hexanal, hexanol, *trans*-2-hexenal, *trans*-3-hexenol and *trans*-2-hexenol. (Vicchi *et al*., 2003; Žanetić *et al.*, 2021).

Five terpenes were determined in the study: α-farnesene, β-ocimene, α-copaene, dl-Limonene and α-pinene (Table 3). α-farnesene (floral, green-plant odor) was detected as the highest aroma-active terpene with an FD factor of 1024. The first year, it was found to be the highest in AY and GE EVOOs; the second year it was found at its highest in GE EVOO. β-ocimene was determined to have a fruity-leafy odor with an FD factor of ≤ 512. In 2014, all the samples were determined to have an FD factor which ranged from 32 to 512. In 2015, the FD factor decreased, and ranged from 16 to 64. α-Farnesene aroma-active compound was previously determined in Kilis Yağlık Turkish VOO by Kesen *et al.* (2014), and has also been determined as a key odorant in Moroccan green olives (Iraqi *et al.,* 2005).

Esters are associated with sweet and fruity sensory properties. Two esters, hexyl acetate with fruity-plant odor and (Z)-3-hexenyl acetate with fruity-green odor, were identified. (Z)-3-hexenyl acetate was detected only in ME and GE EVOOs. The FD factor was ≤ 1024 in 2014, and ≤ 512 in 2015. Hexyl acetate was detected in AY, ME and GE EVOOs (Table 3). Žanetić *et al.* (2021) stated that hexyl acetate caused significant differences in the differentiation of Dalmatian monovariatel EVOO.

Hexanoic acid was detected as a butter-cheesy odor in AY and BE EVOOs with an FD factor of ≤ 64 and only in 2015 (Table 3).

The most powerful AACs in the extracts were identified using the FD factor for AY EVOO hexanal, (E)-2-hexenal and α-farnesene (FD:1024) in 2014 and (E)-2-hexenal in 2015, for ME OO (E)-2-hexenal (FD:2048) in 2014 and (E)-2-hexenal (FD: 512) in 2015, for GE EVOO (Z)-3-hexenyl acetate, (E)-2-hexen-1-ol and α-farnesene (FD:1024) in 2014 and (Z)-3-hexenyl acetate, (E)-2-hexen-1-ol and 1-hexanol (FD: 512) in 2015, for SU EVOO (E)-2-hexenal (FD:2048) in 2014 and hexanal, (E)-2-hexenal, (Z)-3-hexen-1-ol and (E)-2- hexen-1-ol (FD:512) in 2015, for BE EVOO hexanal and (E)-2-hexenal (FD:2048) in 2014 and hexanal in 2015.

3.5. Principal component analysis

Total volatile compounds were used to construct the PCA models for the EVOOs of from different varieties of olives. The PCA model was formed with 4 components. PCA score plot and biplot are illustrated in Figure 3 based on 2 main components. The first major component explains 46.7% of the total variance, and the second major component explains 21.9% of the total variance. When the classification pattern of EVOO samples was examined, it was seen that EVOOs obtained in the first crop season were grouped and separated from the 2nd crop season except for the BE variety of EVOOs. The PCA biplot was used to establish the relationship between the varieties and total volatiles in the EVOOs.

Total ES is negatively correlated on PC1, while ALC, TER, PHE, KET, ES and HYD are positively correlated on PC2. SU1, ME1, GE1 and AY1 were characterized by T-ALC, T-ES, T-TER, T-PHE and T-KET groups of volatile compounds. T-ALD, T-TER, T-LAC and T-HYD characterized BE1 EVOOs, while T-AC and T-FU characterized BE2 EVOOs. Total volatile compounds were not found to be effective for the characterization of SU2, GE2 or AY2 EVOOs.

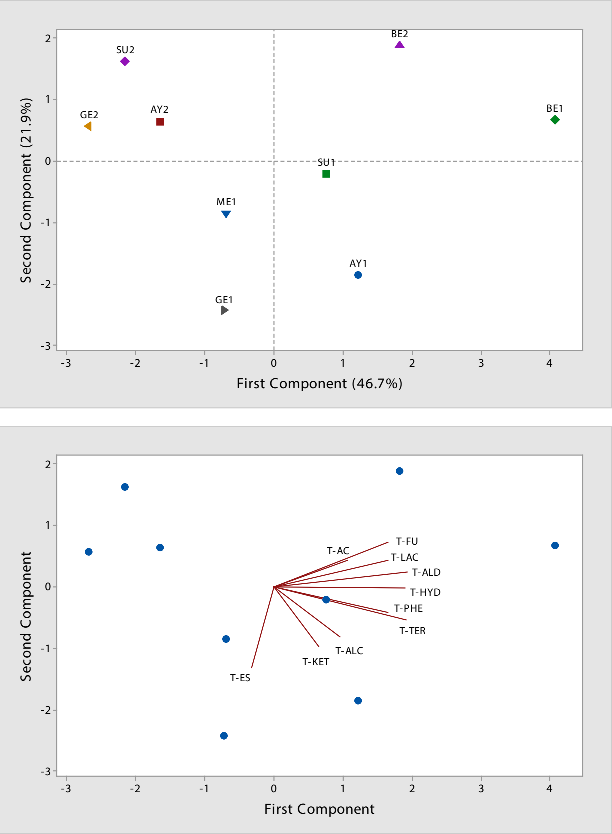

Figure 3. Principal component analysis score and biplot of Ayvalık, Memecik, Gemlik, Sarı Ulak and Beylik Extra Virgin Olive Oils according to total volatiles. AY: Ayvalık, ME: Memecik, GE: Gemlik, SU: Sarı Ulak and BE: Beylik

4. CONCLUSIONS

In the present study, the key odorants of EVOOs obtained from five different varieties grown in three different regions in Turkey were investigated. This work is the first study in which the aroma composition, key odorants and sensory properties of Turkish EVOOs were investigated in detail in terms of two different harvest seasons with a three-phase centrifuge system. All samples were classified as EVOO based on the results of the quality parameters. According to the ANOVA results, the difference between the averages of the quality parameter results was not significant at the 95% confidence level. A total of 52, 57, 51, 57 and 54 volatile compounds were identified and characterized in the studied EVOOs. Alcohols and aldehydes were determined to be the most dominant volatile compounds both qualitatively and quantitatively in the samples. According to the AEDA results, based on the FD factor, the strongest aroma-active compounds detected in the extracts were hexanal, with the cut green-grass odor, *(E)*-2-hexenal, with cut green-grass notes, and *(E)*-2-hexen-1-ol, which was associated with the odor of grassy-cool. Although the abundant compounds were similar and included mostly aldehydes, their FD factors varied for each cultivar and displayed differences in the aroma of the investigated Turkish EVOOs. The results show that AY has the highest FD value of 1024 with hexanal, *(E)*-2-hexenal and α-farnesene. ME has the highest FD value for 2048 with *(E)*-2-hexenal. GE has the highest FD value of 1024 with *(Z)*-3-hexenyl acetate, *(E)*-2-hexen-1-ol and α-farnesene. SU has the highest FD value of 2048 with *(E)*-2-hexenal. BE has the highest FD value of 2048 with *(E)*-2-hexenal and hexanal. The sensory and principal component analyses displayed clear discrimination of samples and according to the spider graphs, none of the samples had off-flavor attributes. The sensory aspects of EVOOs studied in the current work differed slightly according to harvest year, especially in bitterness, leaves and pungent parameters.

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ABBREVIATIONS

Flavor Dilution (FD), Virgin Olive Oil (VOO), Extra Virgin Olive Oil (EVOO), Gas Chromatography-Olfactometry (GC-O), Aroma-Active Compound (AAC), Aroma Extract Dilution Analysis (AEDA), Ayvalık (AY), Memecik (ME), Gemlik (GE), Sarı Ulak (SU), Beylik (BE), QP (Quality Parameter), Free Fatty Acidity (FFA), Peroxide Value (PV), FID (Flame Ionization Detector).

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Effects of oleuropein-rich olive leaf extract on the oxidative stability of refined sunflower oil

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SUMMARY: The aim of this study is to investigate the ability of oleuropein-rich olive leaf extract (OLE) to improve the quality and oxidative stability of sunflower oil subjected to accelerated thermal oxidation. Oleuropein was the major phenolic compound determined by HPLC-DAD with a content ranging from 20.81 to 32.56 mg·g^{-1} of dry extract (DE). The evaluation of the *in vitro* antioxidant capacity of OLE showed good scavenging capacity of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and hydrogen peroxide (H_2O_2) (1.01 and 0.96 mmol Trolox equivalents (ET·g^{-1} DE, respectively). The enrichment of sunflower oil with 0.1, 0.25 and 0.5% OLE (w/v) significantly inhibited thermal-induced peroxidation in a dose-dependent fashion. 0.25% OLE was the most effective concentration and showed a significant reduction in peroxide value and conjugated dienes by 61.4 and 17.4%. These results indicate that OLE can be considered a good natural alternative for extending the shelf-life of polyunsaturated vegetable oils.

KEYWORDS: *Antioxidants; Oils oxidative stability; Oleuropein; Olive leaf extract*

RESUMEN: ***Efectos del extracto de hojas de olivo rico en oleuropeína sobre la estabilidad oxidativa del aceite de girasol refinado.*** El objetivo de este estudio es investigar la capacidad del extracto de hoja de olivo (EHO) rico en oleuropeína para mejorar la calidad y la estabilidad oxidativa del aceite de girasol sometido a oxidación térmica acelerada. La oleuropeína fue el compuesto fenólico mayoritario determinado por HPLC-DAD con un contenido que varió de 20,81 a 32,56 mg·g^{-1} de extracto seco (ES). La evaluación de la capacidad antioxidante in vitro de EHO mostró una buena capacidad de captura del radical 2,2-difenil-1-picrilhidrazilo (DPPH) y peróxido de hidrógeno (H_2O_2) (1,01 y 0,96 mmol Trolox equivalentes (ET·g^{-1} ES, respectivamente). El enriquecimiento de aceite de girasol con 0,1, 0,25 y 0,5 % de EHO (p/v) inhibió significativamente la peroxidación inducida térmicamente de forma dependiente de la dosis. El EHO al 0,25 % fue la concentración más eficaz que mostró una reducción significativa del índice de peróxido y dienos conjugados en un 61,4 % y un 17,4 %. Estos resultados indican que el EHO puede considerarse una buena alternativa natural para prolongar la vida útil de los aceites vegetales poliinsaturados.

PALABRAS CLAVE: *Antioxidantes; Estabilidad oxidativa de los aceites; Extracto de hojas de olivo; Oleuropeína.*

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1. INTRODUCTION

The oxidation of lipids is among the main causes of nutritional quality deterioration and alteration of sensory attributes of commercial oils and fats. Dietary lipid hydroperoxides, the unstable end products of the peroxidation of polyunsaturated fatty acids (PUFA), are considered harmful and are involved in various diseases including atherosclerosis, rheumatoid arthritis, neurodegenerative disorders and aging (Miyazawa, 2021). Consequently, the use of antioxidants to prevent or delay lipid peroxidation has received a great deal of attention. In this context, various additives such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisol (BHA), tert-butylhydroquinone (tBHQ) and propyl gallate (PG) have been widely used as autoxidation inhibitors to improve the stability of oils and fats. Recent studies have raised concerns over the safety and side effects of, supposedly harmless, synthetic preservatives which are possibly involved in serious disorders and diseases (Naidenko *et al.*, 2021). Seeking novel sources of natural antioxidants is a challenging task which has received considerable attention from the scientific community (Blasi and Cossignani, 2020). Previous studies involving namely, polyphenols from pomegranate peel (El-Hadary and Taha, 2020) and essential oils from common aromatic herbs (Benkhoud *et al.*, 2022) showed to effectively prevent the oxidative deterioration of refined soybean and sunflower oils.

Olive (*Olea europaea* L.) leaves (OL) are common by-products from the olive oil industry and tree-pruning in Mediterranean countries. They were traditionally recycled to provide raw material for cattle food, fertilizers, soap making and energy generation. Nevertheless, OL represent an excellent source of valuable bioactive compounds including secoiridoids (oleuropein, oleuropein aglycone, and metoxyoleuropein), flavonoids (rutin, taxifoline, diosmetin, diosmin, quercetin, luteolin and its glycosylated isomers, apigenin and its glycosylated isomers,) phenyl alcohol (tyrosol and hydroxytyrosol), phenolic acids (quinic, gallic, protocatechuic, vanillic, *p*-coumaric, ferulic and syringic acids), triterpenoids (oleanolic acid and its glucosidic isomers, uvaol), tocopherols (α-tocopherol), tannins and pigments (chlorophylls and carotenoids) (Taâmalli *et al.,* 2012; Tarchoune *et al.,* 2019), which are suitable for many applications such as cosmetics, pharmaceuticals, nutraceutical and functional foods. Integrating olive leaf extract (OLE) as an antioxidant and antimicrobial agent is an advantageous alternative to synthetic food preservatives, contributing to a circular model economy. Thereby, OLE (leaf juice) has been proposed as a cheap, natural and efficient solution which improves oxidative stability and extends the shelf-life of various oils (Blasi and Cossignani, 2020).

The "Chétoui" cultivar is the second most common variety of *O. europaea*, in Tunisia. Growing mainly in northern territories, it generally produces bitter and highly pigmented olive oil with relatively important amounts of secoiridoids and good oxidative stability (Hassine *et al.*, 2015). Consonantly, the leaves of this variety were also characterized, among others, as having higher contents of phenolic acids, hydroxytyrosol, oleuropein and oleuroside (Ben Salah *et al.*, 2012). The supplementation of extra-virgin olive oil with "Chétoui" fresh leaves was reported to increase its phenolic content with higher loading on oleuropein and ligstroside aglycones (Ammar *et al.*, 2017).

Therefore, this study was devoted to examining the effect of the addition of a food-compatible aqueous-ethanol extract obtained by ultrasound-assisted extraction (UAE) of the "Chétoui" cultivar leaves on the quality and oxidative stability of sunflower oil subjected to accelerated oxidation. The extraction of bioactive ingredients from olive leaves using UAE has been optimized for some cultivars like Istrska Belica (Cifá *et al.,* 2018), Arbequina, Arbosana, Changlot Real, Frantoio, Picual, Koroneiki, and Sikitita (Martín-García *et al.,* 2022). The UAE of olive leaves has been described in the enrichment process and the incorporation of active extracts into oils (Samli *et al.,* 2020; Sousa *et al.,* 2022). This emerging, low-cost, and environmentally-friendly method shows added advantages in minimizing the time of extraction and volume of solvent and improving the extraction yield. (Cifá *et al.*, 2018).

It is anticipated that the results will contribute to the sustainable use of this agro-industrial waste, provide a safe alternative for preventing the oxidative deterioration of edible oils and thus minimize loss in their nutritional and economic values.

2. MATERIALS AND METHODS

2.1. Standards and reagents

Gallic acid (≥ 97%), syringic acid (≥ 98%), quercetin hydrate (≥ 95%), oleuropein (≥ 97%), tyrosol (98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Rutin (≥ 90%) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), 3-hydroxytyrosol (≥ 98%) from TCI America (OR, USA). Reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) from Sigma-Aldrich, iron (III) chloride (FeCl3), aluminum chloride (AlCl3), Folin-Ciocalteu's phenol reagent from Loba Chemie (Mumbai, India). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was provided by Acros Organics (NJ, USA). All solvents used were HPLC grade and provided by VWR International (Darmstadt, Germany). Sunflower oil was obtained from a local market in Sidi Thabet (Ariana, Tunisia).

2.2. Olive leaf material

Olive leaves were obtained from "Chétoui" variety olive groves in Raf-Raf during February 2020. Raf-Raf is in the North-East region of Tunis (latitude of 37°11' N; longitude of 10°11' E) at an altitude of 136 m above sea level. A representative sample of old leaves (1 kg) was randomly collected from ten individuals (1 kg/batch). The olive leaves were washed thoroughly with tap water and then dried for 48 h at 40 °C in a Venticell oven (MMM Medcenter Einrichtungen GmbH, Munich, Germany). The dried leaves were ground using a Polymix MFC 90D mill (Kinematica, Couëron, France) to a fine powder and passed through a sieve of 1mm mesh size.

2.3. Preparation of OLE

Powdered olive leaves (1 g) were dispersed in 20 mL of a 50% ethanol-water solution (v/v) at a solid/liquid ratio of 1:20 (w/v). Aqueous ethanol was chosen as the extracting solvent due to its efficient recovery of bioactive compounds from olive leaves (Cifá *et al.*, 2018). The suspension was sonicated for 20 min using an ultrasonic probe (Sonics & Materials Inc., Kolkata, India) at 20 kHz with a power of 500 W. During sonication, the dispersion was held in a thermostatic bath at 20 °C. The resulting suspension was centrifuged for 15 min at 2000 rpm. The supernatant was filtered using a 0.45 µm syringe filter and concentrated in a rotary evaporator (Schwabach, Germany). The obtained OLE was freeze-dried (Christ-Alpha, Osterode, Germany) and stored in amber glass bottles at -20 °C until use. The extraction yield of OLE was expressed as % of dry olive leaf weight.

2.4. Characterization of OLE

2.4.1. Determination of the total phenolic and total flavonoid contents

The total phenolic content (TPC) of OLE was determined according to the method described by (Ghasemi *et al.*, 2018). An aliquot of 0.5 mL of the OLE diluted in ethanol (1 mg·mL^{-1}) sample was mixed with 2.5 mL of Folin-Ciocalteu reagent (10%) followed by 2 mL of a sodium carbonate solution (7.5 g·L^{-1}). Afterward, the samples were held at room temperature for 1 hour for incubation. The absorbance of samples incubated for 1 h at room temperature was measured at 765 nm by a JASCO V-630 UV/visible spectrophotometer (Tokyo, Japan). Gallic acid was used for calibration (0-300 µg·mL^{-1}; R^2 = 0,9943) and the TPC is expressed as mg of Gallic acid equivalent per g of freeze-dried extract (mg GAE·g^{-1}).

The total flavonoid content (TFC) was measured according to the aluminum trichloride colorimetric method (Miliauskas *et al.*, 2004). 0.1 mL of OLE solution was mixed with 100 µL $AlCl_3$ (10%), 100 µL of sodium acetate solution (5%) and 1.8 mL of distilled water. After 30 min incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used as a calibration standard (0-250 µg·mL^{-1}; R^2 = 0,9969) and TFC is expressed as mg of Quercetin equivalent·g^{-1} of freeze-dried extract (mg QE·g^{-1}).

2.4.2. Identification and quantification of phenolic compounds by HPLC-DAD

The analysis of OLE phenolic composition was performed according to the International Olive Council method (COI/T.20/ Doc. No. 29) for the determination of biophenols by HPLC. Briefly, OLE (5 mg) and standards were dissolved in 1 mL of methanol:water 80:20 (v/v), sonicated for 10 min, filtered through a 0.45 µm syringe filter and then 20 µL were injected into an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a diode array detector (DAD) (G1315B, Agilent). The separation was achieved on a reversed-phase C18 Spherisorb ODS-2 column (5 µm, 250 x 4.6 mm; Kromasil Co., Sweden) with a flow rate of 1mL·min^{-1} at room temperature. Compounds were eluted with a ternary mobile phase gradient consisting of degassed aqueous 0.2% H_3PO_4 (v/v) (A), methanol (B) and acetonitrile (C). Elution was performed in the following steps: 0-40 min: 96-50% A, 2-25 % B and 2-25% C; 45 min: 40% A, 30% B and 30% C; 60-70 min: 0% A, 50% B and 50% C; followed by column re-equilibration with initial conditions for 4 min. Chromatograms were recorded at 230, 280 and 350 nm. All data were acquired and processed using Agilent Chemstation software.

The quantification of phenolic compounds, including oleuropein, hydroxytyrosol, tyrosol, rutin and quercetin was achieved using 5 point external calibration solutions of 10, 20, 40, 60, 100 μg·mL^{-1} analyzed under the same conditions. The determination coefficient (R^2) of calibration curves relating integrated areas at 280 nm and concentrations was between 0.9996 and 0.9998. The quantification of phenolic acids (caffeic acid and *p*-coumaric acid) was carried out according to the COI method using syringic acid as internal standard (IS) added to OLE solution with a final concentration of 15 μg·mL^{-1}. The response factor of IS was used in the calculation of phenolic acid concentrations, as well as an additional criterion for peak identification using the relative retention time calculated with respect to the IS retention time. The results were expressed as mg of phenolic compound per g of freeze-dried OLE.

2.4.3. Antioxidant activity

The antioxidant activity of OLE was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and hydrogen peroxide (H_2O_2) assays. For the DPPH assay (Edziri *et al.*, 2019), 1 mL of an ethanol solution incorporating different OLE concentrations (0.2 – 1 mg·mL^{-1}) was added to 2 mL of DPPH methanolic solution (0.1 mM). The obtained mixture was thoroughly homogenized and kept in the dark for 20 min before measuring its absorption at 517 nm. A FRAP assay was carried out according to the method of (Benzie and Strain, 1996) with some modifications. The FRAP reagent was prepared by mixing 25 mL of acetate buffer 300 mM (pH 3.6), 2.5 mL of TPTZ (10 mM), and 2.5 mL 10 mM of $FeCl_3$ (20 mM) and incubating in a water bath at 37 °C for 30 min. OLE (150 μL) was mixed with 2850 μL of FRAP solution in the dark for 30 min before measuring the absorbance at 593 nm. Hydrogen peroxide (H_2O_2) scavenging capacity was performed according to the method of (Ruch *et al.*, 1989). 100 μL of olive leaf extract (200-1000 μg·mL^{-1}) were mixed with 300 μL of 50 mM phosphate buffer (pH 7.4) and 600 μL of 2 mM hydrogen peroxide solution. After incubation for 10 min, the absorbance at 230 nm was measured against a blank (phosphate buffer solution).

The potential to scavenge DPPH and H_2O_2 was determined using the following equation:

$$\% \text{ scavenging} = (1 - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the blank and A_{sample} is the absorbance of the extract.

The positive control Trolox was used to construct calibration curves for all assays, and the results were expressed as mmol TE·g^{-1} of freeze-dried extract.

2.5. Application of OLE for controlling sunflower oil oxidation

Three different concentrations of OLE (0.1, 0.25, and 0.5%, w/v) were mixed with 25 mL sunflower oil. The sunflower oil initially showed (at T0, time corresponding to its production) 0.59 mg KOH·kg^{-1} acid value, 2.1 meq O_2·kg^{-1} peroxide value, conjugated dienes (K_{232}) and conjugated trienes (K_{270}) at 0.93 and 0.3, respectively. It was a linoleic acid-rich oil with 8.2% C16:0; 3.4% C18:0, 27.6% C18:1 and 59.3% C18:2 as main fatty acids. It also contained α-tocopherol and carotenoids with average values of 39.6 mg·100g^{-1} oil and 0.34 mg·100g^{-1} oil, respectively (data not shown). The different mixtures were homogenized for 20 min at 13500 rpm with an Ultra-Turrax T25 (IKA, Labortechnik GmbH, Munich, Germany). Then, the obtained samples of sunflower oil incorporating OLE were incubated in a forced air circulation oven (Venticell) to accelerate their oxidation at 70 °C for 1 week according to the Schaal oven test. The oxidative stability of treated sunflower oil was evaluated in terms of quality indices and fatty acid composition. Sunflower oil without OLE was used as a control.

2.6. Quality indices

Acid value (AV), peroxide value (PV) and ultraviolet spectrophotometric indices at 232 nm (K_{232} value) and 270 nm (K_{270} value) were determined according to the standard protocols of the association of official analytical chemists (AOAC, 2003).

2.7. Fatty acid composition of sunflower oil (GC-FID)

Fatty acid methyl esters (FAME) of sunflower oil fatty acids were obtained by cold transesterification in alkaline conditions. Briefly, 0.1 g of sample dissolved in 2 mL of n-hexane were mixed with 0.5 mL of sodium methoxide in methanol (3 %), followed by methanolic H_2SO_4 (1 N). The hexane layer containing fatty acid methyl esters (FAMES) was washed with 10% NaCl solution and concentrated under a nitrogen steam before analysis. FAME analysis was carried out on a Hewlett-Packard HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and polar TR-FAMEs (Thermo Fisher Scientific Inc., Bordeaux, France) capillary column (60 m × 0.25 mm, 0.25 µm film thickness). The column temperature was initially held at 100 °C for 5 min, raised to 240 °C (4 $°C \cdot min^{-1}$), and then kept isothermal for 15 min. The temperatures of the injector and detector were held at 240 °C and 260 °C, respectively. Fatty acids were identified by comparison of their retention times with those of standard FAME mixtures injected in the same conditions (Supelco, 37 Component FAME Mix, PA, USA). The area of each fatty acid peak was integrated by Agilent. ChemStation software and the result is expressed as a percentage of total peak areas.

The Iodine value (IV) and oxidative susceptibility (OS) were determined from the percentages of fatty acids using the following equations (Torres and Maestri, 2006):

$$IV = (\%\ \text{palmitoleic acid} \times 1.001) + (\%\ \text{oleic acid} \times 0.899) + (\%\ \text{linoleic acid} \times 1.814) + (\%\ \text{linolenic acid} \times 2.737)$$

$$OS = \%MUFA + (\%\ \text{linoleic acid} \times 45) + (\%\ \text{linolenic acid} \times 100)$$

Where MUFA is the % of monounsaturated fatty acids.

2.8. Statistical analysis

All experiments were performed in triplicate and results are reported as mean ± standard deviation (SD). One-way ANOVA with Tukey's HSD (Honestly Significant Difference) test was used to assess significant differences between means with a significance level of $p < 0.05$. Data were treated using SYSTAT (Systat Software, San Jose, CA).

3. RESULTS AND DISCUSSION

3.1. OLE extraction yield

Olive leaves, which can be obtained during olive tree pruning or harvesting as well as from olive mills, are a low-cost source of active compounds. An OLE extraction yield of 25 ± 1.7% was obtained using UAE for 20 min at room temperature and a solid/liquid ratio of 1/20 (w/v). Under similar conditions (same extraction procedure (UAE), 50% ethanol and 1:20 solid/liquid ratio) an average extract yield ranging from 16.2-20.12% was recorded by Şahin and Şamli (2013) for olive leaves from an unidentified variety. The observed discrepancy might be attributed to varietal differences as has been reported by Martín-García *et al.* (2022). Variable extraction yields depending on olive variety and extraction procedure have also been described for Tunisian olives (Taâmalli *et al.,* 2012). Extraction yields ranging from 5.2 to 22.4% were obtained for the varieties Jarboui and Chemchali, using supercritical fluid extraction and pressurized liquid extraction, respectively. Regarding the variety Chétoui, a maximum extract yield (19.5 %) was achieved with pressurized liquid extraction using ethanol as extracting solvent and a temperature of 150 °C (Taâmalli *et al.,* 2012). At this point, we can postulate that UAE (operating at low temperature) was more suitable for the extraction of bioactive compounds from olive leaves without possible thermal alterations, a fact that could justify the orientation of most of the research teams to the use of UAE as a method of choice for extracting useful nutraceuticals from olive leaves (Cifá *et al.*, 2018). Indeed, the acoustic cavitation generated by ultrasound treatment induces cuticle erosion and fragmentation of leaf surface protrusion (hair), the polyphenol-rich structures of olive leaves lead to an increased release of phenolic compounds.

3.2. TPC, TFC, main phenolic compounds and antioxidant activities of OLE

The TPC, TFC and antioxidant activities of OLE are summarized in Table 1. In this study, The TPC of 50% (v/v) ethanol OLE obtained by UAE was 135 mg

GAE·g^{-1} extract (Table 1). TPC values ranging from 73 to 144 mg GAE·g^{-1} extract have been reported for OLE obtained with 70% (v/v) ethanol from eight Tunisian olive leaf varieties (Ben Salah *et al.*, 2012). This value is higher than that reported by Edziri *et al.* (2019) for the methanol OLE of the "Chétoui" cultivar (47.5 mg GAE·g^{-1} extract) obtained by regular maceration. Higher TPC was achieved with MAE by Mohammadi *et al.* (2016) for "Mission" variety (206.8 mg GAE·g^{-1} extract) but using methanol as extraction solvent. The TFC value for the "Chétoui" variety OLE is highly variable and fluctuates between 7.29 – 94 catechin equivalent CE·g^{-1} extract (Edziri *et al.*, 2019; Ben Salah *et al.*, 2012). The difference between the obtained value (Table 1) could be attributed to the type of standard used for calibration (CE vs. QE in this study) and to the known limits of this method (Shraim *et al.*, 2021). In fact, Shraim *et al.* (2021) showed significant variation in the $AlCl_3$-flavonoid complex response according to the flavonoid type used and the composition of the extract, leading to an under or an overestimation of this parameter.

Table 1. Phytochemical and antioxidant properties of ethanolic olive leaf extract.

Parameters	**Values (g^{-1} of extract)**
Phenolic content (g^{-1} DE)	
TPC (mg GAE)	134.73 ± 1.05
TFC (mg QE)	62.48 ± 0.43
Antioxidant activities (mmol TE·g^{-1} DE)	
DPPH scavenging	1.01 ± 0.01
FRAP	1.36 ± 0.15
H_2O_2 scavenging	0.96 ± 0.04
Phenolic compouds (mg·kg^{-1} DE)	
Hydroxytyrosol	2406.02 ± 245.21
Tyrosol	797.05 ± 135.50
Caffeic Acid	915.58 ± 121.03
p-coumaric acid	537.70 ± 156.12
Rutin	2033.95 ± 247.83
Oleuropein	32568.31 ± 143.17
Quercetin	1272.09 ± 252.05

Results expressed as mean ± SD (n = 3); DE: dry weight extract, TE: Trolox equivalent, GAE: Gallic acid equivalent, QE: Quercetin equivalent, 1-7: compounds identified by HPLC-DAD and quantified using external standard calibration curves.

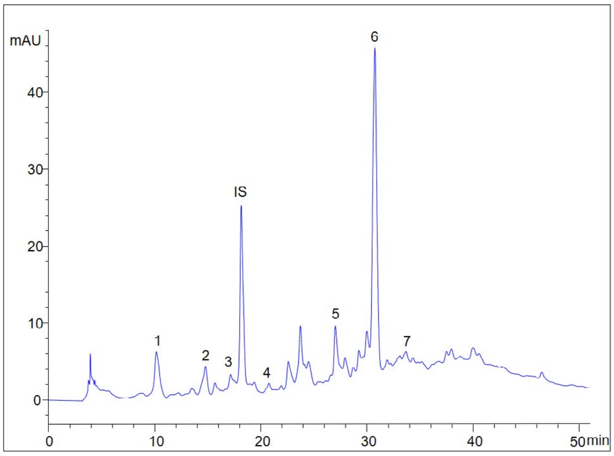

Figure 1. Chromatographic profile of the OLE (280 nm). Identification of compounds: (1) Hydroxytyrosol, (2) Tyrosol, (3) Caffeic acid, (IS) Internal standard, (4) *p*-Coumaric acid, (5) Rutin, (6) Oleuropein, (7) Quercetin.

The profiling of phenolic compounds by HPLC-DAD resulted in the identification of 7 compounds by comparison of retention times and UV-visible spectra with those of standards analyzed under the same conditions (Figure 1). These compounds have been previously reported in the literature (Edziri *et al.*, 2019; Quirantes-Piné *et al.*, 2013) and can be grouped, as shown in Table 1, into simple phenols (hydroxytyrosol, tyrosol), secoiridoids (oleuropein), flavonols (quercetin; rutin) and phenolic acids (caffeic acid; *p*-coumaric acid). The OLE obtained by UAE is rich in secoiridoids with oleuropein being the major phenolic compound (87.9% of total identified compounds) (Figure 1). This agrees with studies that demonstrated that oleuropein is the main phenolic compound in ethanolic extracts from olive leaves; while hydroxytyrosol and phenolic acids are the most prevalent phenolic compounds in aqueous extracts (Quirantes-Piné *et al.*, 2013). The oleuropein content (32.56 mg·g^{-1} freeze-dried OLE) is in the range of the oleuropein contents reported for OLE obtained by maceration in ethanol:water (70:30 v/v) (27.3 mg oleuropein·g^{-1} extract) and those obtained by UAE using aqueous ethanol as a solvent (35.6 – 39.2 mg oleuropein·g^{-1} extract) (Cifá *et al.*, 2018). Other phenolic compounds were identified, such as caffeic acid (3.0%), hydroxytyrosol (2.4%), tyrosol (2.2%), rutin (2%), *p*-coumaric acid (1.4%) and quercetin (1.2%). However, due to the lack of analytical standards for confirmation, other known compounds previously identified in olive leaves: luteolin, apigenin, verbascoside and ligstroside derivatives were not assigned (Quirantes-Piné *et al.*, 2013). The differences observed in the phenolic composition of OLE may be related to many factors including variety, cultivar, geographic origin, solvent type and polarity, solvent-solid ratio, extraction method and parameters (Ghasemi *et al.*, 2018).

The antioxidant activity of OLE was evaluated by three complementary tests. As shown in Table 1, average values of 1.01, 1.4 and 0.96 mmol TE·mg^{-1} for freeze-dried extract have been observed for DPPH, FRAP and H_2O_2 assays, respectively. The ability of OLE to scavenge DPPH radicals and reduce Fe^{3+} to Fe^{2+} suggests that its antioxidant activity involves a single electron transfer (SET) mechanism (Ivanova *et al.,* 2020). Moreover, OLE also has an effective H_2O_2 scavenging activity. Being a hydrogen atom transfer-based (HAT) mechanism (Ivanova *et al.,* 2020), the result of the H_2O_2 assay indicates that OLE might act as a hydrogen donor. Collectively, the antioxidant activity of OLE could be attributed to the simultaneous presence of electron and hydrogen donors.

The antioxidant activity was found to be correlated with TPC and TFC values (Miliauskas *et al.*, 2004). The phenolic compounds of OLE, mainly oleuropein, may directly contribute to its antioxidant capacity. In a comparative study among four Tunisian olive varieties, the OLE from “Chétoui” was the most effective DPPH radical scavenger (Edziri *et al.,* 2019). Moreover, the ability of OLE to scavenge H_2O_2 and reduce Fe^{3+} has also been evidenced (Martín-García *et al.,* 2022), confirming our findings. The antioxidant activity of OLE appears to be intimately linked to the presence of putative antioxidants which have strong electron and hydrogen donating ability. Among the identified components, some have received particular attention because of their well-known antioxidant properties, including oleuropein, hydroxytyrosol, rutin, gallic and caffeic acids, tyrosol, elenolic acid, luteolin, apigenin and their glycosylated forms (Taâmalli *et al.,* 2012; Tarchoune *et al.,* 2019; Martín-García *et al.,* 2022). In addition to phenolic components, other fat-soluble components such as pigments (carotenoids and chlorophylls) and tocopherols have also been reported as strong radical scavengers and reducing agents, thereby conferring a potent antioxidant activity to OLE (Tarchoune *et al.,* 2019). It should be noted that the overall antioxidant activity resulted from synergistic interactions between the active ingredients present in OLE (Lee *et al.,* 2009).

3.3. Oxidative stability of sunflower oil samples incorporated with OLE

In this study, the ability of OLE to improve the oxidative stability of sunflower oil was assessed through the determination of some analytical indices and fatty acid composition of sunflower oil. At the beginning of the experiments (before thermal treatment), the acid value, peroxide value, K_{232} and K_{270} of sunflower oil were 1.16 ± 0.07 mg KOH·g^{-1}, 11.67 ± 0.58 meq O_2·Kg^{-1}, 2.14 ± 0.18 and 1.28 ± 0.24, respectively (Table 2). The thermal treatment of sunflower oil for 7 days resulted in a 2.9-fold increase in acid value compared to the untreated oil sample at the start of the experiment. This may be attributed to extended lipid peroxidation leading to excessive production of hydroperoxide. The incorporation of OLE significantly decreased ($p < 0.05$) the acid value with 0.25 and 0.5% concentrations being the most effective in reducing the content of free fatty acids. The ability of OLE to delay the thermal-induced production of free radicals and lipid peroxidation could be due to its antioxidant capacity (Şahin *et al.*, 2017). So, the presence of putative antioxidants in the OLE (e.g. tyrosol, hydroxytyrosol, oleuropein, quercetin, and rutin) could effectively inhibit the formation of free radicals at the initial stage of lipid peroxidation, break the chain reaction during the propagation stage, and/or scavenge the free radicals in the oil by donating a hydrogen atom (Blasi and Cossignani, 2020). Peroxide value (PV) as a key parameter reflecting the early stage of lipid oxidation was determined in all sunflower oil samples. As shown in Table 2, the PV of thermally treated-control oil was increased significantly owing to an extended autoxidation of the oil and its subsequent increase in hydroperoxides. In contrast, the incorporation of OLE reduced peroxide formation and delayed the thermal oxidation of sunflower oil in the early stage, thereby delaying the onset of oil autoxidation. Sunflower oil incorporated with 0.25 and 0.5% OLE showed the lowest PV value among the enriched oil samples. As the PV is closely related to the polyunsaturated fatty acid (PUFA) content, it was clear that the decrease in the PV in sunflower oil incorporated with 0.25 and 0.5% OLE was mainly

Table 2. Quality indices and fatty acid composition (%) of sunflower oil incorporating different concentrations of OLE.

Parameter	**Sunflower oil before heat treatment**	**Thermally-treated sunflower oil (70 °C, 1 week)**			
		Sunflower oil control (without OLE)	**+ OLE 0.1%**	**+ OLE 0.25%**	**+ OLE 0.5%**
Acid value (mg KOH·g^{-1} oil)	1.16 ± 0.07^{c}	3.31 ± 0.08^{a}	2.22 ± 0.04^{b}	1.24 ± 0.16^{c}	1.49 ± 0.12^{c}
Peroxide value (meq O_2·Kg^{-1})	11.6 ± 0.5^{d}	33.3 ± 0.5^{a}	22.7 ± 0.3^{b}	12.8 ± 0.1^{d}	14.1 ± 0.5^{c}
K_{232}	2.1 ± 0.1^{a}	2.6 ± 0.2^{a}	2.2 ± 0.2^{a}	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}
K_{270}	1.3 ± 0.3^{a}	1.4 ± 0.2^{a}	1.3 ± 0.1^{a}	1.3 ± 0.1^{a}	1.2 ± 0.2^{a}
Palmitic acid (C16:0)	9.09 ± 0.06^{b}	8.76 ± 0.07^{c}	9.05 ± 0.07^{b}	8.39 ± 0.11^{d}	9.49 ± 0.07^{a}
Palmitoleic acid (C16:1)	0.63 ± 0.02^{a}	0.77 ± 0.06^{b}	0.67 ± 0.06^{b}	1.05 ± 0.05^{a}	0.98 ± 0.05^{a}
Stearic acid (C18:0)	3.43 ± 0.04^{c}	3.68 ± 0.04^{b}	3.29 ± 0.04^{c}	4.05 ± 0.07^{a}	3.76 ± 0.04^{b}
Oleic acid (C18:1)	32.1 ± 0.3^{a}	30.1 ± 0.3^{b}	29.1 ± 0.4^{c}	30.9 ± 0.5^{a}	31.3 ± 0.4^{a}
Linoleic acid (C18:2)	53.2 ± 0.7^{b}	55.6 ± 0.3^{a}	56.2 ± 0.6^{a}	53.7 ± 1.4^{b}	53.3 ± 0.4^{b}
Linolenic acid (C18:3)	0.46 ± 0.04^{a}	0.17 ± 0.02^{c}	0.32 ± 0.06^{b}	0.36 ± 0.03^{ab}	0.30 ± 0.08^{b}
Arachidic acid (C20:0)	0.072 ± 0.061^{b}	0.290 ± 0.06^{a}	0.092 ± 0.007^{b}	0.093 ± 0.006^{b}	0.095 ± 0.001^{b}
Gadoleic acid (C20:1)	0.17 ± 0.02^{c}	0.083 ± 0.001^{c}	0.65 ± 0.07^{a}	0.61 ± 0.06^{a}	0.25 ± 0.01^{b}
Behenic acid (C22:0)	0.73 ± 0.45^{a}	0.42 ± 0.01^{a}	0.51 ± 0.04^{a}	0.72 ± 0.08^{a}	0.40 ± 0.03^{a}
SFA	12.0 ± 0.4^{c}	13.1 ± 0.1^{b}	12.9 ± 0.06^{ab}	13.25 ± 0.3^{a}	13.74 ± 0.08^{a}
MUFA	27.4 ± 0.3^{c}	31.0 ± 0.4^{b}	30.5 ± 0.3^{b}	32.6 ± 0.6^{a}	32.6 ± 0.5^{a}
PUFA	60.6 ± 0.7^{a}	55.8 ± 0.3^{ab}	56.5 ± 0.7^{a}	54.2 ± 1.3^{bc}	53.6 ± 0.5^{c}
MUFA/PUFA	0.456 ± 0.002^{c}	0.561 ± 0.011^{b}	0.543 ± 0.002^{b}	0.602 ± 0.028^{a}	0.619 ± 0.003^{a}
Oxidative Susceptibility	2475 ± 14^{c}	2552 ± 17^{ab}	2594 ± 36^{a}	2485 ± 60^{bc}	2465 ± 28^{c}
Iodine value	127 ± 1^{a}	129 ± 0.3^{a}	129 ± 0.5^{a}	127 ± 1.9^{a}	126 ± 1.4^{a}

Results expressed as mean ± SD (n = 3); OLE: Olive leaf extract; OLP: Olive leaf powder; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; Different lowercase letters in the same row indicate significant differences (Tukey's HSD; $p < 0.05$).

due to the extended degradation of hydroperoxides to secondary oxidation products such as volatile aldehydes and ketones (Benkhoud *et al.*, 2022). The specific extinction coefficients are indicative of the presence of conjugated dienes (CD) and trienes (CT) (K_{232} and K_{270}, respectively). CD as a measure of the oxidative state of the oil is considered a reliable indicator of the effectiveness of antioxidants to prevent lipid oxidation. The results from Table 2 show that the highest CD contents were found in the thermally treated control owing to their high content in polyunsaturated fatty acids, namely linoleic. In contrast, samples incorporated with OLE showed CD values close to the non-treated control, suggesting the protective function of OLE against thermal-induced lipid peroxidation. The CT contents in all samples did not differ significantly ($p > 0.05$), indicating a similar rate of oxidation. This may be due to the inhibition of the production of CT through the dehydration of CD hydroperoxides and/or the absence of secondary oxidation products (Zhang *et al.*, 2020). Regardless of thermal treatment, Iodine values (IV) were similar in all sunflower oil samples, suggesting that this parameter could not be considered a reliable parameter for estimating the rate of oxidation of sunflower oil. Moreover, the implication of native antioxidants like tocopherols could, at least in part, explain the steady oxidation state of the untreated control and the oil samples incorporated with OLE.

The fatty acid profiles of control untreated and thermally treated sunflower oil samples are presented in Table 2. PUFA made up the highest contribution (53.6-60.6%) with linoleic (53.2-56.2%) and linolenic (0.17-0.46%) acids as the main components. The monounsaturated fatty acids (MUFA) represent the second-most abundant class with percentage contributions ranging from 27.4 to 32.6%. The MUFA fraction was dominated by oleic (29.1-32.1%), followed by palmitoleic (0.63-1.05%) and gadoleic (0.08-0.65%) acids. The saturated fatty acids (SFA) made up the lowest contribution (12.0-13.74%) and the main fatty acids were palmitic (8.39-9.49%), followed by stearic (3.29-4.05%) and behenic (0.40-0.73%). Despite their high nutritional quality, the oil samples were particularly prone to oxidation as revealed by their high oxidative susceptibility (OS) (2465-2594) in comparison with other edible oils like olive oil (453-922) (Benkhoud *et al.*, 2022). At this point, the incorporation of 0.25 or 0.5% OLE confers better resistance to thermal oxidation of sunflower oil and improves its oxidative stability.

The protective effect of OLE against the thermal oxidation of sunflower oil may be attributed to the presence of putative antioxidants such as polyphenols (Samli *et al.,* 2020) and fat-soluble antioxidants, including tocopherols (Jaski *et al.,* 2022), carotenoids and chlorophylls (Şahin *et al.*, 2017; Sousa *et al.,* 2022). In a previous study, it has been reported that the enrichment of olive oil with OLE containing 272 ppm oleuropein resulted in a 2.5-fold increase in the free-radical scavenging ability, thus resulting in enhanced oil stability (Şahin *et al.*, 2017). In another study, hydroxytyrosol was found to be the most effective in inhibiting the formation of conjugated hydroperoxides in bulk fish oil and fish oil-in-water emulsions at a concentration of 100 ppm, mainly due to its electron-donating and ferrous-chelating properties (Pazos *et al.*, 2008). Using antioxidant activity guided-fractionation, Lee *et al.* (2009) showed that the protective effect of OLE against lipid oxidation was linked to oleuropein, caffeic acid, vanillin, rutin and catechin. More recently, Sousa *et al.* (2022) showed that the incorporation of chlorophyll-rich extract from *Crithmum maritimum* markedly enhanced the oxidative stability of sunflower oil. Another point to be considered is that the incorporation of OLE into sunflower oil might not only improve its oxidative stability through its direct action on retarding the lipid peroxidation process, and/or scavenging free radicals, but also indirectly through the protection of α-tocopherol and carotenoids, the natural antioxidants of sunflower oil from thermal degradation empowering thus its oxidative stability. Further studies are needed to confirm this assumption.

4. CONCLUSIONS

OLE obtained by a green process from olive leaves which represent an abundant, inexpensive, and underutilized by-product, is revealed as a rich source of antioxidant compounds including the valuable oleuropein. The evaluation of its antioxidant activities showed potent anti-radical, ferric reducing and H_2O_2 scavenging capacities. Therefore, the incorporation of OLE into sunflower oil significantly enhanced its oxidative stability. OLE at 0.25 and 0.5% were effective against the development of rancidity induced by thermal oxidation. This beneficial effect could be attributed to the synergistic effect of antioxidant components (secoiridoids, flavonoids, and phenolic compounds) identified and quantified in OLE. Based on these findings, OLE could substitute conventional synthetic antioxidants associated with many environmental and health risks, adding value to olive leaf by-products obtained after harvesting and offering an economic and environmental solution to the management of this agricultural waste. The search for the adequate harvesting stage which provides the highest OLE yield deserves further investigation.

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DATA AVAILABILITY

Data available on request from the authors.

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Fatty acids and mineral composition of seed oils extracted from different *Rosa* L. taxa

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SUMMARY: In the study, the macro and micro elements and fatty acid contents in rose seeds, which are generally evaluated in the waste category, were determined. Among the plants belonging to these taxa, only the registered varieties belonging to four taxa were used, while the others were genotypes. The plant materials of the study include rosehip (*R. canina* L.), Yildiz variety of rosehip (*R. canina* L. cv 'Yildiz'), Syrup rose (*R. heckeliana* Tratt. subsp. *vanheurckiana*), Austrian briar (*R. foetida* Herrm.), lax rootstock [*R. caesia* Sm. (Syn: *R. laxa* Retz.)], wild rose [*R. montana* subsp. *woronovii* Chaix subsp. *woronovii* (Lonacz) Ö. Nilsson L.], hybrid landscape roses (*R.* x *hybrida*) and Hosap rose [*R. pisiformis* (Christ) D.] taxa. It was determined that the contents of nitrogen, potassium, phosphorus, magnesium and calcium of the macro elements examined in the seed samples differed statistically from each other. The seeds of different *Rosa* L. species examined within the scope of the research can be considered as one of the fruit oil sources with its 3.71-10.01% oil content. The fatty acid contents were determined as follows: linoleic acid (ω6) contents in the taxa ranged from 41.63 to 50.11% with an average of 44.88%; oleic acid (ω9) ranged from 20.80 to 30.27% with an average of 24.95%; linolenic acid (ω3) varied between 14.00-28.51% with an average of 19.20%; arachidic acid ranged from 0.75-1.63% and the average was 1.97%; eicosenoic acid ranged between 0.13-0.65% and averaged 0.33%; palmitoleic acid contents ranged from 0.08-0.60; behenic acid varied between 0.08-0.19% with 0.11% average. It was observed that the (ω3/ω6 ratio of the hybrid rose, which is especially used as a landscape rose and whose fruits are not evaluated, had an average value. *R. canina* 'Yildiz' cultivar showed a high (ω3/ω6 ratio, which is important in health terms. The high oleic acid contents found in these taxa are important results.

KEYWORDS: *Element contents; Fatty acids; Oil extraction;* Rosa *L.; Rosehip oil.*

RESUMEN: ***Composición en ácidos grasos y minerales de aceites de semillas extraídos de diferentes taxones de* Rosa L**. En este estudio se determinaron los contenidos de macro y microelementos y ácidos grasos de las semillas de *Rosas*, que generalmente se clasifican en la categoría de residuos. Entre las plantas pertenecientes a estos taxones, solo se utilizaron las variedades registradas pertenecientes a cuatro taxones, mientras que las demás fueron genotipos. Los materiales vegetales del estudio incluyen rosa mosqueta (*R. canina* L.), variedad de rosa mosqueta Yildiz (*R. canina* L. cv 'Yildiz'), rosa de jarabe (*R. heckeliana* Tratt. subsp. *vanheurckiana*), brezo austríaco (*R. foetida* Herrm.), patrón laxo [*R. caesia* Sm. (Syn: *R. laxa* Retz.)], rosal silvestre [*R. montana* subesp. *woronovii* Chaix subsp. *woronovii* (Lonacz) Ö. Nilsson L.], rosas híbridas de paisaje (*R.* x *hybrida*) y rosa Hosap [*R. pisiformis* (Cristo) D.] taxones. Se determinó que los contenidos de nitrógeno, potasio, fósforo, magnesio y calcio entre los elementos examinados en las semillas diferían estadísticamente entre sí. Las semillas de diferentes especies de *Rosa* L. pueden considerarse como una de las fuentes de aceite de frutas con un contenido de 3,71-10,01%. El contenido de los ácidos linoleico (ω6) de los taxones de las rosas varió de 41,63 a 50,11 % con un promedio de 44,88 %, el oleico (ω9) varió de 20,80 a 30,27 % con un promedio de 24,95 %, el linolénico (ω3) varió entre 14,00 y 28,51 % con un promedio de 19,20 %, el araquídico osciló entre 0,75 y 1,63 % y 1,97 % de promedio, el eicosenoico osciló entre 0,13 y 0,65 % y promedio de 0,33, el behénico varió entre 0,08-0,19% con un promedio de 0,11%. Se ha observado que la relación ω3/ω6 de la rosa híbrida, que se utiliza especialmente como rosa de paisaje y cuyos frutos no se han evaluado, tienen un valor medio. El cultivar de *R. canina* 'Yildiz' tiene un alto valor de omega ω3/ω6 importante en términos de salud. El alto contenido de ácido oleico de estos taxones son unos resultados muy importantes.

PALABRAS CLAVE: *Aceite de rosa mosqueta; Ácidos grasos; Contenido de elementos; Extracción de aceite;* Rosa *L.*

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1. INTRODUCTION

The Rosales order includes nine families of economic and ecological importance. The Rosaceae family, which generally consists of trees and shrubs for landscaping and fruit growing, includes 90 genera and 3000 species. The members of the family have worldwide distribution but are more concentrated in the northern temperate regions. The family is extremely economically important as a source of many fruits, essential oils, and countless varieties of landscape and ornamental plants (Simpson, 2019).

Horticulture is basically a discipline concerned with the cultivation of plant materials by humans for food supply, medicinal use, or functional and aesthetic purposes. Roses are a genetically diverse group and play an important role in the economy of modern society but are also central to the healthy diets of urban populations. From this point of view, they are an extremely important garden plant because they contain these features. *R. canina*, *R. rugosa* and *R. montana* taxa are the most commonly used species in industry and are known as 'rosehip'.

The most important characteristics affecting the quality of the fruits of these species are taste and aroma. The soluble solid content of fruits is directly related to taste, and sugars make up a large part of the water-soluble dry matter. Rosehip has always been appreciated as a cheap source of vitamin C in times of war and economic depression due to its biological and nutritional values. Rosehip fruits contain vitamins A, B1, B2, E, C, P, and K (Roman *et al.*, 2013; Oz *et al.*, 2018; Fascella *et al.*, 2019; Fetni *et al.*, 2020). In this context, the rosehip (*Rosa* L.) plant has maintained its importance in folk medicine and medicine since historical times. Rosehip seed oil is a valuable raw material for the development of herbal cosmetics and skin care products such as lotions and creams.

The seeds of many products containing bioactive compounds such as rosehip are classified as waste during the processes in the factory, and it has been stated that the effective use of these waste products is limited (Szentmihályi *et al.*, 2002; Vasic *et al.,* 2020; Saygi, 2021). The seeds in question contain unsaturated fatty acids such as linoleic acid (C18:2n6c), oleic acid (C18:1n9c) and α-linolenic acid (C18:3n3) (McGaw *et al.*, 2002; Nowak, 2005; Ercisli, 2007; Machmudah *et al.*, 2007; Kazaz *et al.*, 2009; Kizil *et al.*, 2018; Vasic *et al.*, 2020; Mannozzi *et al.*, 2020).

The originality of this article is that many of the taxa examined have not been studied in this respect before. It can be said that the reasons for this are that it is difficult to reach the regions where it is located, the number of seeds varies and most of them are collected as *R. canina*. As a result of the research, it was determined that the species that were not evaluated before (with waste seeds) also had important characteristics. Zero waste is an important phenomenon for today. In the research, it was determined that different properties of rose oils, which are used in landscape architecture studies and whose fruits (seeds) are in the waste category, are also important.

The fatty acids extracted from *Rosa* L. seeds show significant antibacterial, antioxidant, anti-inflammatory, and anti-cyanobacterial activity (McGaw *et al.*, 2002). Considering both the phytochemical contents of these oils for human health from research in medicine pharmacy and chemistry (Guven *et al.*, 2021) and the positive effects of using them as feed in agricultural production and aquaculture studies (Dyck and Evans, 2021), there are many promising factors. Therefore, their separation from the waste category is an important phenomenon. Within the scope of the study, fixed oil contents and compositions and macro and micro element contents of seeds belonging to 8 different *Rosa* L. taxa were determined.

2. MATERIALS AND METHODS

2.1. Materials

The plant materials in the study include rosehip (*R. canina* L.), Yildiz variety of rosehip (*R. canina* L. cv 'Yildiz'), Syrup rose (*R. heckeliana* Tratt. subsp. *vanheurckiana*), Austrian briar (*R. foetida* Herrm.), lax rootstock [*R. caesia* Sm. (Syn: *R. laxa* Retz.)], wild rose [*R. montana* subsp. *woronovii* Chaix subsp. *woronovii* (Lonacz) Ö. Nilsson L.], hybrid landscape roses (*R.* x *hybrida*) and Hosap rose [*R. pisiformis* (Christ) D.] taxa. The taxa names are written in full at this stage, and in other parts of the research, they are given in a short form without authorization.

Cuttings belonging to the taxa were taken from Van Yüzüncü Yıl University Faculty of Architecture and Design, Landscape Architecture Department Research Fields and Tokat Gaziosmanpaşa University Faculty of Agriculture Department of Horticulture

Research Field. The literature of Brummitt and Powell (1992) was taken as a reference for the standard spelling of scientific names of species and subspecies, including the authors (abbreviations), and was examined by considering the UPOV (2010) criteria.

Among the plants belonging to these taxa, only the registered varieties belonging to four taxa were used, while the others were genotypes. In the study carried out under greenhouse conditions, the greenhouse temperature was measured with an Onset Computer H21-001 HOBO brand thermometer and the average annual temperature was determined as 24.73 °C. This value was determined as a result of measurements made for 24 months. It was determined that the pH of the growing medium in which the study was carried out was 7.14 and had a clay/loamy structure. The lime content was determined as 6.14% and the salt content was 0.04 %.

2.2. Methods

2.2.1. Determination of oil yield from seeds

The seeds of the specified taxa were dried in an oven at 65 °C for 72 hours. The experiment was established with 3 replications and 12 grams of seed in each replication. The seeds were ground in a coffee grinder (Sinbo SCM-2934 110 W). The cartridge was placed on the hangers in the extraction beaker of the instrument and programmed to extract for 4 hours in the Soxhlet extractor (Gerhardt®) by adding 150 ml of solvent (ether with a boiling point of 40-60 °C) into the extraction beaker. At the end of the program, the hanging cartridges were taken and the extraction beakers were dried at 103 °C for 1 hour and cooled in the desiccator, then weighed with an accuracy of 0.001 g. The drying-cooling and weighing process continued until the difference between the two weights was less than 0.1%. The amount of oil in the sample after weighing was calculated as a weight percent (Gokturk Baydar and Akkurt, 2007; Sabir *et al.*, 2012; Canbay and Bardakci, 2011). At the end of the process, the flasks were kept in an oven at 60 °C for 24 hours to evaporate the hexane from the mixture, and the fixed oil content was determined as the weight of the oil yield (%w/w) (Ercisli, 2007; Celik *et al.*, 2010; Lachmann *et al.*, 2015). 130 ml of hexane were added to the ground rose seeds (35 g) and kept in a circular shaker at 180 rpm for 2 hours. At the end of the period, the content was filtered coarsely and the hexane in the filtrate was removed in a rotary evaporator at 40 °C to obtain oil.

2.2.2. Determination of fatty acid content and composition

Fatty acid methyl esters were formed as indicated for the determination of fatty acid quantities. For this, 4 g of the oil sample were weighed and kept in the dark for 6 minutes with isooctane and methanolic KOH. Then, 2-3 drops of methyl orange and 1 N HCl were added, and phase separation was observed. At the end of the process, 1 ml of the clear phase formed in the phase separation was taken and injected into the device. The oils extracted in the Soxhlet extractor were stored in 10 mL hexane in screw-capped tubes at 4 °C until the fatty acids were determined. Before analysis, the hexane in the tubes was separated from the oils in an oven at 60 °C for 4 hours (Yakar *et al.*, 2021). In order to determine the fatty acid composition, a Thermo brand, TraceGC Ultra model, FID (Flame Ion Dedector) detector gas chromatography device was used. A 60 m HP-88 column was used for the separation of fatty acids. The temperature of the injector block was set at 280 and 250 °C. It was adjusted to reach 180 °C with an increase of 20 °C/min after waiting for 2 minutes at 50 °C. With an increase of 5 °C/min from this value, it reached 230 °C and was held at this temperature for 5.5 minutes. The split ratio was 1/50. Hydrogen was used as the carrier gas, and the determination was made with 3 replications.

2.2.3. Determination of elements in seeds

Rose seeds, which were dried in an oven at 65 °C for 72 hours, were ground in a coffee grinder until they turned into powder. The core samples were then pulverized in 3 replications, with 0.5 g of sample burnt in each replication using the wet burning method. The % of N was determined according to the Khejdal method (Kacar, 1972). Plant elements such as P, K, Ca, Mg, Fe, Mn, Cu, Zn, B, Na were determined in the ICP device by dissolving them in a 1 N HCl acid solution (Ryan *et al.*, 2001).

2.3. Statistical analysis

The data from analysis and measurements were subjected to Analysis of Variance (ANOVA) in the Minitab 18 computer package program. Differences between means were expressed by grouping according to ANOVA and compared among themselves according to the LSD 5% level.

3. RESULTS AND DISCUSSION

3.1. Oil contents of Rosa seeds

In this study, the seed fatty acid compositions of *Rosa* L. taxa (*R. montana* subsp. *woronovii*, *R. foetida*, *R. laxa*) which had not been studied before were determined. In addition to these taxa, the seed oil contents of previously studied taxa *R. canina*, *R. heckeliana*, *R. pisiformis* and hybrid landscape rose seeds were also investigated (Szentmihalyi *et al.,* 2000; Nowak 2005; Ercisli 2007; Machmudah *et al.,* 2007; Kazaz *et al.*, 2009; Celik *et al.,* 2010; Prescha *et al.,* 2014, İlyasoğlu, 2014; Turan *et al.,* 2018; Kizil *et al.,* 2018; Vasic *et al.,* 2020). As a result of the study, the different values for *R. canina* and *R. canina* 'Yildiz' seeds obtained under the same ecological conditions and from the same species show that the fatty acid compositions may vary not only on the basis of species, but also according to varieties. In this context, the oil content in the seeds of the *Rosa* L. taxa, which is the subject of this research, was determined and statistical evaluations were made for the years 2020, 2021 and the average of the two years (Table 1).

According to the average values of the two-year data, although the seed oil contents varied according to the species and subspecies, the difference between the years was found to be statistically insignificant ($p > 0.05$).

Considering the descriptive statistics, the seed oil contents from 2020 varied between 3.79 and 10.06% and the average value of the investigated taxa was 6.83%. The seed oil contents from 2021 ranged between 3.64 and 9.96% and the average value of the investigated taxa was 6.78%. According to the data obtained, *R. montana* subsp. *woronovii* seeds had the lowest seed oil content in 2020 and 2021, and the *R. heckeliana* species had the highest seed oil content.

Ercisli (2007) studied six taxa and found that the oil content was between 4.60 and 5.37%. In this context, when the seed oil contents of the *Rosa* L. taxa used in this research were taken into account, it was determined that the data obtained were comparable to the literature data. When the relevant literature is examined, it is possible that the differences in fatty acid composition may be related to environmental conditions (climate and altitude, etc.), which are known to have an effect on fatty acid composition (Ilyasoglu, 2014). The subspecies were determined to have a value above the average (10.01%).

In recent years, research on different fruit oils in the cosmetic and food industries has increased and alternative products have begun to be examined in more detail. The seeds from different rose species examined within the scope of this research can be considered as a fruit oil source with its 3.71-10.01% oil content.

3.2. Fatty acid composition of seed oil

According to the analyses made on the eight *Rosa* L. taxa investigated, these taxa contain an

TABLE 1. Total oil contents in Rosa seeds (%)

Taxa	**2020**	**2021**	**Average**
R. canina	8.48±0.07^{c}	8.40±0.04^{c}	8.44±0.05
R. canina 'Yildiz'	9.07±0.05^{b}	9.04±0.07^{b}	9.05±0.05
R. foetida	3.99±0.02^{h}	3.84±0.10^{h}	3.91±0.03
R. heckeliana subsp. *vanheurckiona*	10.06±0.05^{a}	9.96±0.05^{a}	10.01±0.08
R. laxa	8.14±0.02^{d}	8.22±0.02^{d}	8.18±0.06
*R. montana*subsp.*woronovii*	3.79±0.11^{i}	3.64±0.03^{i}	3.71±0.04
R. pisiformis	7.78±0.02^{e}	7.69±0.03^{e}	7.73±0.05
R. x hybrida	4.70±0.02^{g}	4.80±0.03^{g}	4.75±0.04
LSD (%5)	0.090[a]	0.095[a]	1.375[b]

[a] At the 5% level, taxa were found to be statistically significant. (The data obtained were compared among themselves according to the LSD 5% level)

[b] The difference between years at the level of 5% was found to be statistically significant. Each application was analyzed in triplicate.

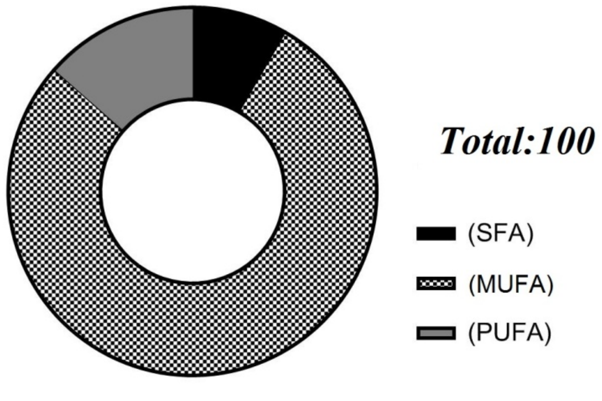

Figure 1. Percent oil content in Rosa L. seeds

average of 91.62% unsaturated fatty acids and 8.38% saturated fatty acids (Figure 1). These rates are 96.17 - 3.83%, respectively, in the studies by Nowak (2005), 85% and 15% according to Macmudah *et al.* (2007), and in the studies by Kazaz *et al.* (2009), they were determined as 91.1% and 8.90%. Therefore, it has been concluded that the results obtained in this research are comparable to the literature data in question.

Unlike data from the literature (McGaw *et al.*, 2002; Nowak, 2005; Ercisli, 2007; Machmudah *et al.*, 2007; Kazaz *et al.*, 2009; Kizil *et al.*, 2018; Vasic *et al.*, 2020), it was determined that the *Rosa* L. taxa contained low levels of lauric acid. In accordance with the results from the statistical analysis of the saturated fatty acid contents found, the saturated fatty acids varied according to the taxa and the difference between the taxa was statistically significant at the 5% level. When the descriptive statistics of the palmitic acid contents in the seeds of *Rosa* L. taxa were considered, the content of palmitic acid varied between 4.44-7.28% with an average of 5.50%, the stearic acid contents ranged between 1.15-4.80% and averaged at 2.70% (Table 2).

It was determined that *R. pisiformis* has the lowest palmitic acid content; while the *R. montana* subsp. *woronovii* taxa has the highest palmitic acid content. *R. foetida* has the lowest stearic acid content, and hybrid tea rose seeds have the highest stearic acid content.

In some studies on *Rosa* L. taxa, the palmitic acid values for seeds were found by Szentmihalyi *et al.* (2002) to be 7.87%, by Nowak (2005) at 3.05%, Machmudah *et al.* (2007) found 5.01%, and Kazaz *et al.* (2009) at 3.66%. The average palmitic acid values for the *Rosa* L. taxa in this study were found to be 5.50%, which is comparable to the literature findings. Considering the palmitic acid values of the rose taxa used in this research, it was found that they were high for the roses used for landscaping and in *R. foetida* species.

In some studies on *Rosa* L. taxa, the stearic acid values for the seeds were found by Szentmihalyi *et al.* (2002) at 3.18%, by Nowak (2005) at 1.84%, by Machmudah *et al.* (2007) at 2.72%, by Prescha *et al.* (2014) at 1.80%, and Turan *et al.* (2018) at 2.19% stearic acid. The average stearic acid values for the *Rosa* L. taxa examined in this study were found to be 2.70%, which is comparable to the literature.

Table 2. Saturated fatty acid (SFA) contents in seeds of *Rosa* L. taxa (%)

Taxa	**C12:0(%)**	**C14:0(%)**	**C15:0(%)**	**C16:0(%)**	**C17:0(%)**	**C18:0(%)**	**C20:0(%)**	**C22:0(%)**
R. canina	0.03 ± 0.01^{de}	0.03 ± 0.01^{e}	0.03 ± 0.01^{c}	4.53 ± 0.89^{e}	0.04 ± 0.01^{e}	1.73 ± 0.18^{e}	0.04 ± 0.02^{bcd}	0.12 ± 0.03^{bc}
R. canina 'Yildiz'	0.05 ± 0.02^{de}	0.03 ± 0.01^{e}	0.04 ± 0.00^{c}	4.45 ± 0.22^{e}	0.09 ± 0.01^{a}	3.92 ± 0.30^{b}	0.06 ± 0.02^{abc}	0.19 ± 0.06^{a}
R. foetida	0.27 ± 0.08^{a}	0.08 ± 0.01^{b}	0.06 ± 0.01^{bc}	6.48 ± 0.39^{bc}	0.08 ± 0.02^{ab}	1.54 ± 0.01^{e}	0.02 ± 0.01^{d}	0.08 ± 0.01^{c}
R. heckeliana subsp. *van-heurckiona*	0.07 ± 0.00^{cd}	0.05 ± 0.01^{cd}	0.06 ± 0.01^{bc}	3.59 ± 0.06^{f}	0.03 ± 0.01^{e}	1.15 ± 0.16^{f}	0.04 ± 0.00^{cd}	0.11 ± 0.01^{c}
R. laxa	0.01 ± 0.00^{e}	0.06 ± 0.01^{c}	0.04 ± 0.01^{c}	5.88 ± 0.16^{cd}	0.07 ± 0.01^{ab}	3.37 ± 0.03^{c}	0.04 ± 0.01^{cd}	0.11 ± 0.01^{c}
R. montana subsp.*woronovii*	0.10 ± 0.01^{bc}	0.12 ± 0.01^{a}	0.08 ± 0.01^{ab}	5.81 ± 0.06^{d}	0.07 ± 0.01^{abc}	2.47 ± 0.06^{d}	0.04 ± 0.01^{cd}	0.09 ± 0.04^{c}
R. pisiformis	0.13 ± 0.01^{b}	0.08 ± 0.01^{b}	0.09 ± 0.03^{a}	4.44 ± 0.36^{e}	0.05 ± 0.01^{cd}	1.19 ± 0.03^{f}	0.08 ± 0.02^{a}	0.18 ± 0.06^{ab}
R. x hybrida	0.05 ± 0.01^{de}	0.06 ± 0.01^{c}	0.08 ± 0.01^{ab}	7.28 ± 0.01^{a}	0.08 ± 0.01^{ab}	4.80 ± 0.04^{a}	0.03 ± 0.01^{d}	0.09 ± 0.01c
LSD (%5)	0.051^{a}	0.015^{a}	0.027^{a}	0.622^{a}	0.023^{a}	0.242^{a}	0.030^{a}	0.058^{a}

[a] Significant at the 5% level. (The data obtained were compared among themselves according to the LSD 5% level) (C12:0: Lauric acid; C14:0: Myristic acid; C15:0: Pentadecanoic acid; C16:0: Palmitic acid; C17:0: Heptadecanoic acid; C18:0: Stearic acid; C22:0: Behenic acid). Each application was analyzed in triplicate.

Table 3. Polyunsaturated fatty acid (PUFA) contents in Rosa L. taxa (%)

Taxa	**C18:2n6c (%)**	**C18:3n3 (%)**	**C20:5n3 (%)**
R. canina	50.11±0.92^{a}	19.90±1.12^{cd}	0.04±0.02^{bcd}
R. canina 'Yildiz'	44.78±0.63^{b}	14.00±1.29^{g}	0.06±0.02^{abc}
R. foetida	43.96±0.49^{bc}	16.40±0.90^{e}	0.02±0.01^{d}
R. heckeliana subsp. *vanheurckiona*	41.64±0.16^{c}	28.51±0.36^{a}	0.04±0.01^{cd}
R. laxa	45.18±0.02^{b}	19.03±0.04^{d}	0.04±0.01^{cd}
R. montana subsp. *woronovii*	41.63±0.17^{c}	20.82±0.20^{c}	0.04±0.01^{cd}
R. pisiformis	43.69±0.14^{bc}	23.34±0.18^{b}	0.08±0.02^{a}
R. x hybrida	49.20±0.34^{a}	15.20±0.06^{f}	0.03±0.01^{d}
LSD (%5)	2.468^{a}	1.156^{a}	0.030^{a}

^{a}Significant at the 5% level. (The data obtained were compared among themselves according to the LSD 5% level) (C18:2n6c: Linoleic acid; C18:3n3: alpha-Linolenic acid; C20:5n3: cis-5,8,11,14,17-Eicosapentaenoic acid). Each application was analyzed in triplicate.

Table 4. Monounsaturated fatty acid (MUFA) contents in Rosa L. taxa (%)

Taxa	**C16:1(%)**	**C17:1(%)**	**C20:1n9(%)**	**C18:1n9c(%)**
R. canina	50.11±0.92^{a}	19.90±1.12^{cd}	0.37±0.15^{b}	22.07±1.34^{e}
R. canina 'Yildiz'	44.78±0.63^{b}	14.00±1.29^{g}	0.35±0.02^{b}	30.27±1.41^{a}
R. foetida	43.96±0.49^{bc}	16.40±0.90^{e}	0.63±0.09^{a}	29.16±0.62^{a}
R. heckeliana subsp. *vanheurckiona*	41.64±0.16^{c}	28.51±0.36^{a}	0.26±0.23^{bc}	23.53±0.04^{d}
R. laxa	45.18±0.02^{b}	19.03±0.04^{d}	0.24±0.02^{bc}	24.66±0.16^{cd}
R. montana subsp. *woronovii*	41.63±0.17^{c}	20.82±0.20^{c}	0.29±0.01^{bc}	27.00±0.14^{b}
R. pisiformis	43.69±0.14^{bc}	23.34±0.18^{b}	0.65±0.16^{a}	25.05±0.27^{c}
R. x hybrida	49.20±0.34^{a}	15.20±0.06^{f}	0.13±0.04^{c}	22.04±0.63^{e}
LSD (%5)	2.468^{a}	1.156^{a}	0.194^{a}	1.258^{a}

^{a} Significant at the 5% level. (The data obtained were compared among themselves according to the LSD 5% level). (C16:1: Palmitoleic acid; C17:1: cis-10-Heptadecanoic acid; C20:0: Arachidic acid; C20:1n9: cis-11-Eicosenoic acid; C18:1n9c: Oleic acid). Each application was analyzed in triplicate.

It was determined that there are saturated fatty acids such as lauric acid, mystric acid, pentadecanoic acid, heptadecanoic acid, and behenic acid in the seeds of the taxa in question. A high amount of lauric acid content (0.27%) was found in the *R. foetida* taxa compared to other taxa, and the mystric acid content was found at a higher level (0.12%) in *R.montana* taxa compared to other taxa.

According to the results from the analysis of the unsaturated fatty acid contents in the seeds of the Rosa L. taxa with the data from 2021, oleic, linoleic, linolenic, palmitoleic, heptadecanoic, arachidic, eicosenoic and eicosapentanoic acid contents varied according to the taxa and the difference between the taxa was statistically significant (Table 3).

Linoleic acid (ω6) contents in the investigated rose taxa ranged from 41.63 to 50.11% with an average of 44.88%. Oleic acid (ω9) contents ranged from 20.80 to 30.27% with an average of 24.95%. Linolenic acid (ω3) contents in eicosenoic acid varied between 14.00 and 28.51%, with an average of 19.20%. Arachidic acid contents ranged from 0.75 to 1.63% and the average was 1.97%. Eicosenoic acid contents ranged between 0.13 and 0.65% with an average of 0.33%. Palmitoleic acid contents ranged from 0.08 to 0.60. The content of behenic acid was found to vary between 0.08 and 0.19%, which is 0.11% on average.

According to the data obtained, the lowest oleic acid content was determined in *R.* x *hybrida*, and the highest oleic acid content was found in *R. foetida* and 'Yildiz' seeds from 2021 (Table 4). As a result of the analysis, the lowest linoleic acid content was found in *R. montana* subsp. *woronovii* and *R. heckeliana*, and the highest linoleic acid content was found in *R.*

canina and hybrid rose seeds. It was determined that *R. canina* ‘Yildiz’ contained the highest alpha-linolenic acid content and *R. heckeliana* species had the highest linolenic acid content.

Szentmihalyi *et al.* (2000), Nowak (2005), Ercisli (2007), Machmudah *et al.* (2007), Kazaz *et al.* (2009), Celik *et al.* (2010), Prescha *et al.* (2014), İlyasoğlu (2014), Turan *et al.* (2018), Kizil *et al.* (2018), Vasic *et al.* (2020) stated that the distribution of fatty acids is more important than the oil content in the seeds, and that rose/rosehip seeds are rich in unsaturated fatty acids and their fatty acid compositions consists of major linoleic, oleic, and alpha-linolenic acids. Considering the fatty acid values for the rose taxa used in the research, it was determined that the data obtained and the literature data were generally comparable.

In Aydın’s (2010) study, it was stated that the ideal omega-3/omega-6 ratio should be between 1 and 0.25, and that among these ratios, the anti-inflammatory, analgesic and blood thinning effects of the herbal product would be more effective. The ω-3/ω-6 values for olive oil and grape seed oils were found to be in these ranges. It was determined that *R. canina* ‘Yildiz’ and *R.* x *hybrid* taxa of *Rosa* L. taxa were close to this oil yield. The hybrid Chinese rose, which is used as a landscape rose and whose fruits were not evaluated, and the *R. canina* ‘Yildiz’ cultivar, were found to contain high rates of oleic acid, known as omega-9 fatty acid. These are promising results.

3.3. Macro and micro element contents in Rosa seeds

The contents of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) in the macro elements examined in the seed samples differed statistically from one another. Accordingly, the highest nitrogen content was found in the *R. pisiformis s*taxa (Table 5).

It was determined that the iron (Fe), copper (Cu), zinc (Zn), manganese (Mn) and boron (B) contents in the micro elements examined in the seed samples statistically differed from one another by 5%. Accordingly, the highest iron content (73.26 ppm) was found in *R. montana* subsp. *woronovii*; the lowest iron content (30.70 ppm) was detected in the seeds of the *R. canina* ‘Yildiz’ taxa (Table 6).

Kazaz *et al.* (2009) determined the macro elements in *R. canina* seeds as 0.12% P, 0.32% K, 0.38% Ca, 0.09% Mg. According to the findings obtained in the study, the mean P values of 11 *Rosa* L. taxa were determined as 0.12% P and 0.31% K, and these two elements were found to be similar to the relevant literature. On the other hand, Ca and Mg values were found to be higher in the study. The iron, copper, zinc, manganese and boron contents in the seeds of *R. canina* were averaged and the taxa was also examined. They reached similar findings in the order of the mentioned micro elements and reported that there were higher mineral contents compared to the seeds of *R.* x *damascena*.

Ercisli (2007) and Kazaz *et al.* (2009) also found low Cu contents in the species. It was determined that the ‘Yildiz’ rosehip cultivar, which was examined within the scope of the research, contained higher levels of copper compared to these studies and other taxa. Szentmihályi *et al.* (2002) determined the average Fe content in the seeds of the

Table 5. Macro element content values in seeds of *Rosa* L. taxa (%)

Taxa	**N (%)**	**P (%)**	**K (%)**	**Mg (%)**	**Ca (%)**
R. canina	1.34 ± 0.02^{f}	0.06 ± 0.01^{ef}	0.23 ± 0.01^{cd}	0.13 ± 0.01^{g}	0.45 ± 0.04^{i}
R. canina ‘Yildiz’	1.47 ± 0.01^{d}	0.10 ± 0.01^{d}	0.25 ± 0.02^{cd}	0.16 ± 0.01^{e}	0.59 ± 0.03^{f}
R. foetida	1.55 ± 0.01^{b}	0.07 ± 0.02^{ef}	0.34 ± 0.01^{a}	0.15 ± 0.01^{ef}	0.62 ± 0.04^{cd}
R. heckeliana subsp. *vanheurckiona*	1.51 ± 0.02^{c}	0.08 ± 0.01^{de}	0.33 ± 0.01^{ab}	0.19 ± 0.01^{b}	0.83 ± 0.02^{a}
R. laxa	1.15 ± 0.01^{h}	0.08 ± 0.01^{de}	0.28 ± 0.00^{bc}	0.18 ± 0.00^{d}	0.75 ± 0.02^{b}
*R. montana*subsp.*woronovii*	1.40 ± 0.01^{e}	0.18 ± 0.01^{b}	0.24 ± 0.01^{cd}	0.13 ± 0.01^{g}	0.48 ± 0.02^{h}
R. pisiformis	0.98 ± 0.01^{i}	0.15 ± 0.00^{c}	0.28 ± 0.02^{bc}	0.13 ± 0.00^{g}	0.60 ± 0.03^{ef}
R. x hybrida	1.61 ± 0.01^{a}	0.21 ± 0.04^{a}	0.34 ± 0.02^{a}	0.21 ± 0.00^{a}	0.64 ± 0.04^{c}
LSD (%5)	0.019^{a}	0.023^{a}	0.081^{a}	0.011^{a}	0.064^{a}

[a]Significant at the 5% level. (The data obtained were compared among themselves according to the LSD 5% level). Each application was analyzed in triplicate.

Table 6. Micro element content values in seeds of *Rosa* L. taxa (ppm)

Taxa	**Fe (ppm)**	**Cu (ppm)**	**Zn (ppm)**	**Mn (ppm)**	**B (ppm)**
R. canina	32.23 ± 0.18^{g}	8.28 ± 0.11^{f}	20.80 ± 0.11^{f}	34.04 ± 0.03^{de}	7.39 ± 0.04^{f}
R. canina 'Yildiz'	30.70 ± 0.09^{h}	18.19 ± 0.30^{a}	17.52 ± 0.12^{h}	43.06 ± 0.03^{b}	7.21 ± 0.03^{g}
R. foetida	38.10 ± 0.22^{d}	9.87 ± 0.14^{c}	22.02 ± 0.11^{cd}	18.80 ± 0.02^{j}	7.85 ± 0.04^{e}
R. heckeliana subsp. *vanheurckiona*	35.36 ± 0.14^{e}	9.57 ± 0.18^{d}	20.76 ± 0.11^{f}	27.73 ± 0.03^{f}	10.05 ± 0.02^{b}
R. laxa	47.25 ± 0.28^{c}	10.93 ± 0.21^{b}	26.17 ± 0.10^{b}	19.49 ± 0.04^{i}	6.83 ± 0.02^{h}
R. montana subsp. *woronovii*	34.99 ± 0.11^{e}	9.02 ± 0.09^{e}	20.75 ± 0.21^{f}	50.50 ± 0.04^{a}	5.57 ± 0.02^{i}
R. pisiformis	73.26 ± 0.12^{a}	8.36 ± 0.09^{f}	18.15 ± 0.22^{g}	41.17 ± 0.03^{c}	5.79 ± 0.03^{i}
R. x hybrida	52.98 ± 0.24^{b}	10.06 ± 0.21^{c}	30.92 ± 0.22^{a}	21.45 ± 0.04^{g}	10.72 ± 0.04^{a}
LSD (%5)	0.872[a]	0.199[a]	0.090[a]	0.091[a]	0.062[a]

[a] Significant at the 5% level. (The data obtained were compared among themselves according to the LSD 5% level). Each application was analyzed in triplicate.

R. canina taxa to be 20.15 ppm in their research. Ercişli (2007), Szentmihályi *et al.* (2002) and Kazaz *et al.* (2009) found zinc values between 3.69 and 14 ppm in their studies conducted on different rosehip genotypes. These researchers generally examined the taxa of *R. canina* and *R.* x *damascena*. It was concluded that they may be different species with high element contents apart from the commonly known taxa due to the different reasons for the genotype effect. For example, *R. pisiformis* seeds contain higher amounts of zinc and potassium. As a result of the research, it was found that the taxa examined had higher iron and zinc contents than this value. In light of this information, it can be said that the presence of some mineral substances in the seeds of *Rosa* L. taxa grown under similar greenhouse conditions is directly related to the genotypes, and the seed of the said taxa contains significant amounts of macro and micro nutrients.

The differences between the taxa in terms of the macro and micro element contents in both leaves and seeds show that the element intake of the taxa is under genotype control. As a matter of fact, there are findings in the literature that the plant element contents in the seeds of *Rosa* L. taxa differ from each other (Ercisli, 2007; Szentmihályi *et al.*, 2002; Kazaz *et al.*, 2009). The findings obtained for the seeds of the taxa also bring to mind the effect of antagonism, which is very important in plant nutrition. As a matter of fact, K and Ca and Mg often show antagonism. In this context, it can be said that the elements that are absorbed in high amounts from the soil accumulate in the seeds and that the antagonistic effects can also be seen in the seeds. As a result, it is thought that it is possible to use rose seeds in food and food additive sectors, as well as rosehip fruits, and to use the seeds in different production areas.

4. CONCLUSIONS

Difficulties are often encountered in the identification of *Rosa* L. taxa due to the diversity and variability of morphological features. In addition, these difficulties arise in establishing genetic links between different taxa. Clarity in the diagnostic role of a particular or a group of morphological characters is not only of theoretical but also of practical importance. Nowadays, more attention is paid to vegetable raw materials as a source of biologically active substances. As a result of this widespread scientific interest, *R. canina*, a widely distributed species of the Rosaceae family, is commonly used as a vitamin source and food raw material. Species of the genus *Rosa* have rich vitamin contents and different chemical structures.

The healing and protective health effects of rose/rosehip seeds were known in ancient times and have been supported by current studies. Nowadays, it can be said that the positive effects of extracts obtained from rose seeds such as delaying aging (anti-aging) increase the interest in rose seeds. As a result of this research, it was determined that the taxa examined had higher iron and zinc contents than the values specified in the references. In light of this information, it can be said that the presence of some mineral substances in the seeds of *Rosa* L. taxa grown under similar greenhouse conditions is directly related to the genotypes, and the seeds of the said taxa contain significant amounts of macro and micro elements.

In this study, the seed fatty acid compositions of the *R. montana*, *R. foetida* and *R. laxa* taxa were determined for the first time. The seed oil contents in rosehip seeds were also investigated. The total oil content in the seeds of *R. heckeliana* was above average for the species and subspecies examined, *R. laxa*, *R. canina* cv. Values above average were determined for taxa such as 'Yildiz'. In addition, the different values for *R. canina* and *R. canina* 'Yildiz' seeds obtained under the same ecological conditions and of the same species showed that the fatty acid compositions may vary not only on the basis of species, but also according to variett.

In recent years, alternative oil sources have gained importance in the cosmetic and food industries (Hammond, 2003; Taylor *et al*., 2011). It is thought that it can be considered as a fruit oil sources with 3.71-10.01% oil content in the seeds of different rose taxa examined within the scope of this research. The results obtained from *R. canina* and *R. montana* taxa, which are widely used in food and industry and known as rose hips, and the oil parameters in the seeds of other taxa, show differences.

As a result of the analysis of the fixed fatty acid values in the seeds of the *Rosa* L. taxa, which are the subject of this research, the lowest palmitic acid content was determined in *R. pisiformis*, and the highest content was determined in *R. montana* subsp. *woronovii*taxa, *R. foetida* showed the lowest stearic acid content, and hybrid tea rose seeds had the highest stearic acid content. Higher palmitic and stearic acid contents were determined in hybrid landscape rose compared to other taxa and species examined in the literature. In addition, as a result of this research, it was determined that the related taxa contain low levels of lauric acid, unlike the literature.

The fact that rose seed oil is rich in unsaturated fatty acids is a very important result. Especially its rich contents in linoleic and oleic acids is one of the main reasons for the increasing interest in rose seed oil. Although studies to determine the fatty acid composition of rose seed oil have been carried out by different researchers before, the seed fatty acid compositions of landscape roses and species that have not been studied before, were determined.

It was concluded that the fatty acid contents in the taxa examined within the scope of this research contain higher levels of linolenic acid compared to grape and pomegranate seeds. It has also been determined that rose seeds have a high level of linolenic acid, similar to oils such as canola and soybean oils. It has been stated that rose seed oil contains high linoleic acid and linolenic acid levels, which is an important result, and higher unsaturated fatty acid content compared to other oils consumed today. Based on these findings it can be said that rose seed oil may be beneficial to human health.

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Effect of antioxidant-enriched microcrystalline cellulose from almond residue on physicochemical and textural characteristics of mayonnaise

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SUMMARY: The purpose of this study was to investigate whether antioxidant-enriched microcrystalline cellulose from almond residue **(**AE-MCC-AS) affects the physicochemical and textural characteristics of mayonnaise during 56 days of storage at 25 °C. The L^* value of the mayonnaise decreased by increasing the AE-MCC-AS ratio; whereas the redness and yellowness values increased. The emulsion stability and viscosity increased by increasing the AE-MCC-AS ratio from 0.2% to 0.4%; however, they decreased with an increase in the AE-MCC-AS ratio from 0.4% to 0.6%. The largest oil droplets were observed in the micrographs of the control, 0.2% AE-MCC-AS-M and 0.6% AE-MCC-AS-M; while the smallest ones were observed in the micrographs of α-tocopherol-M, BHT-M and 0.4% AE-MCC-AS-M. During the storage period, the total MUFA and PUFA showed a declining trend in all treatments with a higher decrease in the control; while total SFA showed an upward trend with a higher increase in the control. In terms of textural characteristics, a significant declining trend ($P < 0.01$) was observed in firmness and consistency; whereas an upward trend was observed in cohesiveness during the storage in all treatments.

KEYWORDS: *Color; Mayonnaise; Microstructure; Textural characteristics; Viscosity*

RESUMEN: ***Efecto de la celulosa microcristalina enriquecida en antioxidantes de residuos de almendras sobre las características fisicoquímicas y texturales de mayonesas.*** El propósito de este estudio fue investigar si la celulosa microcristalina enriquecida con antioxidantes del residuo de almendras (CM-EA-RA) afecta a las características fisicoquímicas y texturales de la mayonesa durante 56 días de almacenamiento a 25 °C. El valor L^* de la mayonesa disminuyó al aumentar la relación CM-EA-RA, mientras que los valores de rojo y amarillo aumentaron. La estabilidad y la viscosidad de la emulsión aumentaron al aumentar la relación CM-EA-RA de 0,2 % a 0,4 %; sin embargo, disminuyeron al aumentar la relación CM-EA-RA de 0,4 % a 0,6 %. Las gotas de aceite más grandes se observaron en las micrografías del control, 0,2 % CM-EA-RA-M y 0,6 % CM-EA-RA-M, mientras que las más pequeñas se observaron en las micrografías de α-tocoferol-M, BHT-M y 0,4 % CM-EA-RA-M. Durante el almacenamiento, los MUFA y PUFA totales mostraron una tendencia decreciente en todos los tratamientos con una mayor disminución en el control, mientras que los SFA totales mostraron una tendencia ascendente con un mayor aumento en el control. En cuanto a las características texturales, se observó una tendencia significativamente decreciente ($P<0.01$) en la firmeza y consistencia, mientras que se observó una tendencia ascendente en la cohesividad durante el almacenamiento en todos los tratamientos.

PALABRAS CLAVE: *Características texturales; Color; Mayonesa; Microestructura****;*** *Viscosidad.*

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1. INTRODUCTION

Mayonnaise is a semi-solid sauce which is well-known all over the world. It is presumed that the origin of mayonnaise is based in Port Mahon, Spain, in 1756. Commercial mayonnaise was first produced in the early 1900s, and since then it has gradually gained popularity in America, Japan and other nations (Mirzanajafi-Zanjani *et al.,* 2019). Mayonnaise is an oil-in-water (O/W) emulsion which generally consists of oil, egg yolk, salt, vinegar, and flavoring materials. The emulsion is formed by slowly blending oil into the water phase which contains egg yolk, salt, and other ingredients. The oil content varies from 65 to 80% in the full-fat mayonnaise formula, and sunflower, corn and/or soybean oil are chosen generally for mayonnaise production. The fatty acid composition of these vegetable oils consists of mostly polyunsaturated fatty acids (PUFA), which increases the possibility of lipid oxidation and reduces the shelf-life of mayonnaise (Gorji *et al.,* 2019).

To inhibit or retard oil oxidation in food emulsions, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ) and propyl-gallate are widely used in the food industry. The low cost, chemical stability and availability of synthetic antioxidants are the main reasons for this preference. However, the toxicity suspicion of the synthetic antioxidants and the consumer demand for natural food ingredients have directed both the industry and researchers to find natural antioxidant sources. Much research has been focused on the use of natural antioxidants extracted from different plants such as fruit, vegetables and their wastes for inhibiting or retarding oil oxidation in mayonnaise (Mihov *et al.,* 2012; Altunkaya *et al.,* 2013; Li *et al.,* 2014; Chatterjee and Bhattacharjee, 2015; Shabbir *et al.,* 2015). However, natural antioxidants also have some disadvantages compared to synthetic antioxidants. Natural antioxidants should be added to food formulas in larger amounts to provide the same oxidative stability as synthetic antioxidants since they are less effective compared to synthetic analogues (Pokorný, 2007). There is no restriction or safety limits of natural antioxidants; however, it should be considered that natural antioxidants may pose a nutritional and health risk as a result of their interactions with proteins when consumed in large quantities (Pokorný, 2007). The long-term stability of natural antioxidants is limited because they are sensitive to temperature, light and oxygen. To reduce the effect of these environmental conditions, we proposed the binding method of phenolic extract to microcrystalline cellulose (MCC) in our previous study (Ünver and Çelik, 2021). We obtained a natural food additive that exhibited good thermal stability, crystallinity, antioxidant, and flow characteristics by means of this method. Besides, AE-MCC-AS at the concentration of 0.6% showed better activity in preventing oil oxidation in mayonnaise than BHT and α-tocopherol.

MCC, listed as GRAS by the FDA, is a white, odorless, renewable, and biodegradable powder. In emulsions, MCC has the ability to form a viscoelastic network which entraps oil droplets (Meirelles *et al.,* 2020). This viscoelastic network can reduce the rate of lipid oxidation in the emulsion by slowing down the mobility of pro-oxidants (Kargar *et al.,* 2012). Most of the studies related to mayonnaise which contained natural antioxidants have focused on their antioxidant effect, the detection and observation of hydroperoxides and their degradation products such as aldehydes, ketones, and volatile compounds (Vahidyan *et al.,* 2012; Altunkaya *et al.,* 2013; Li *et al.,* 2014; Hermund *et al.,* 2015; Alizadeh *et al.,* 2019; Gorji *et al.,* 2019). Few studies have focused on the effect of natural antioxidants on the physicochemical and textural characteristics of mayonnaise (Chatterjee and Bhattacharjee, 2015; Shabbir *et al.,* 2015; Raikos *et al.,* 2016).

The possible interactions between mayonnaise ingredients and antioxidants can also affect the texture, color, rheological and emulsion characteristics, which are important factors for sensory perception and consumer satisfaction. The knowledge about the interactions and effect mechanism is still insufficient due to the complexity of these interactions. Therefore, the purpose of this study was to investigate whether AE-MCC-AS affects the physicochemical and textural characteristics of mayonnaise during 56 days of storage at 25 °C. The investigated parameters of the mayonnaises that contained AE-MCC-AS at three different ratios (0.2, 0.4 and 0.6%) were compared to the negative control (without antioxidant) and two different positive controls (mayonnaise containing 0.02% BHT; mayonnaise containing 0.02% α-tocopherol).

2. MATERIAL AND METHODS

2.1. Material

Almond shell (AS) and almond hull (AH) were supplied from local farmers in Şanlıurfa, Turkey for the production of AE-MCC-AS. Sunflower oil, vinegar, sugar and salt were supplied from a local market in Şanlıurfa for the production of mayonnaise. The egg yolk was supplied from Alfasol® (Gaziantep-Turkey). BHT, α-tocopherol, and FFA standards were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Production of AE-MCC-AS

The production of AE-MCC-AS was carried out according to the method suggested by (Ünver and Çelik, 2021). In brief, the phenolic extract obtained from the almond hull (PE-AH) with ultrasonic-assisted extraction was bound to microcrystalline cellulose obtained from the almond shell (MCC-AS) with acid hydrolysis in a rotary evaporator flask under vacuum at 40 °C. Afterwards, the obtained AE-MCC-AS was dried in an air oven at 40 °C until constant weight.

Green almond hull was extracted with 90% ethanol in an ultrasonic bath to obtain PE-AH. The extraction parameters were as follows: extraction time, 60 min; extraction temperature, 65 °C; and solvent-sample ratio, 50:1, at a frequency of 40 kHz, 100% full power. At the end of the extraction time, PE-AH was separated from the residue by centrifugation at 4000 rpm for 5 min. The extraction procedure was repeated, and the supernatants were combined (Ünver and Çelik, 2021).

2.3. Production of mayonnaise

All mayonnaise samples were formulated from the following ingredients in the percentage (w/w): sunflower oil (65%), egg yolk powder (4%), water (17%), vinegar (8%), sugar (5%) and salt (1%) with a mixer (Tefal Mastermix, İstanbul, Turkey). BHT, α-tocopherol and AE-MCC-AS were used in different samples according to the experimental design shown in Table 1. The mayonnaise was filled into glass jars (80 mL) and stored at 25 °C in the dark for 56 days. The productions were carried out in duplicate.

TABLE 1. Experimental design of mayonnaise production

Antioxidant	Concentration (%)	Sample code
AE-MCC-AS	0.2	0.2% AE-MCC-AS-M
	0.4	0.4% AE-MCC-AS-M
	0.6	0.6% AE-MCC-AS-M
BHT	0.02	BHT-M
α-tocopherol	0.02	α-tocopherol-M
-	-	Control

2.4. Some physicochemical characteristics of mayonnaise samples

The pH value of mayonnaise samples was measured using a laboratory pH meter (Model HQ40d, Hach Company) at a temperature of 20±0.5 °C (Khalil and Mansour, 1998).

The color values for the mayonnaise samples were measured using a Hunterlab Color Quest Instrument (Hunter Associates Laboratory, Inc., Reston, VA 22090, USA). The instrument calculates the results according to the CIE Lab color space coordinates, as L^*, a^* and b^* values. L^* stands for the axis of lightness, a^* and b^* represent two scales of opponent color pairs (i.e., (-) greenness/ (+) redness–and (-) blueness/ (+) yellowness, respectively).

The viscosity of mayonnaise was measured at 25 °C using a Viscometer (Brookfield DV-II +, USA) with a spindle (No. 5) rotation of 50 rpm. The readings were recorded at the 10th second of the measurement.

The fatty acid composition of the samples was determined in the oil phase of the mayonnaise according to the procedure of ISO 12966–2:2011. Separation of the oil phase from the mayonnaise was performed in freezing-thawing cycles. The samples were subdivided into polypropylene centrifuge tubes (50 mL), and they were frozen at -24 °C for 24 h to break the emulsion. Afterwards, the samples were thawed at 25 °C for 2 h, and they were centrifuged at 10000 rpm for 10 min at room temperature. After the second freezing-thawing cycle, the oil phase (upper phase) was separated and stored at -24 °C for further analysis. Before the analysis, the samples were esterified to their corresponding fatty acid methyl esters. The fatty acid composition was analyzed using a gas chromatography (Thermo Quest Trace GC 2000 Series; Oshawa, Canada) with a flame ionization detector (FID) equipped with a Hewlett Packard (HP-88) capillary column of 60 m×0.25 mm×0.20 µm film thickness purchased from Agilents Technologies Ltd., Santa Clara, CA, USA. The temperatures of the injector and detector were set at 250 and 280 °C, respectively. The carrier gas was helium at a constant flow of 1.6 ml/min. Operating conditions for GC were as follows: Injection mode/volume: Split (1/50)/1 µL at 250 °C, Flow rates: H_2:Air: N_2 = 33:370:30 mL/min. The oven temperature was 100 °C for 1 min, increased to 180 °C at a rate of 10 °C/min, then increased to 220 °C at a rate of 5 °C/min, and held for 5 min.

2.5. Textural characteristics of mayonnaise samples

Texture measurements were carried out using a TA-XT2 Texture Analyzer (Stable Micro System Ltd., UK) based on the back extrusion method at 25 ± 2 °C. A disc probe (diameter: 30 mm, TA-30A) using a 50 kg load cell attached to the instrument was compressed onto a 30 mm depth of the sample with a test speed of 1 $mm \cdot s^{-1}$. The pre-test speed and post-test speed were set at 10 $mm \cdot s^{-1}$ and the trigger force was 10 g. The force-time curves were analyzed for hardness (g), consistency (g·sec), cohesiveness (g), and work of cohesion (g·sec). The tests were carried out in standard glass jars (50 mm diameter). When the surface trigger of 10 g was reached (i.e. the point at which the disc's lower surface is in full contact with the product), the mayonnaise samples were subjected to compressive force by probe up to the distance of 30 mm, afterwards, the probe returned to its original position.

2.6. Emulsion microstructure

The mayonnaise samples were examined under an optical microscope (Leica Microsystems, Wetzlar, Germany) at a magnification of 40×. First, one drop of mayonnaise sample was placed on the slide and covered with a coverslip. After uniform thickness was attained, the images were captured using LAS-EZ software (Leica Microsystems, Wetzlar, Germany).

2.7. Emulsion stability

The emulsion stability (ES) of the mayonnaise was determined according to the method suggested by Phuah *et al.* (2016), with a slight modification. Briefly, 5.0±0.5 g of each sample were filled into 15 mL centrifuge tubes, and the tubes were incubated in a water bath at 50 °C for 24 hours. At the end of the incubation time, the tubes were centrifuged at 4000 rpm for 20 min. The upward phase was removed, and the remaining phase was weighed to calculate the emulsion stability according to the Equation:

$$ES\,(\%) = 100\ x\ m_1/m_2$$

where m_1 is the weight of the remaining phase, and m_2 is the initial weight of the mayonnaise.

2.8. Statistical analysis

The data obtained from the study were analyzed using one-way ANOVA, and the difference between the significant averages was tested using Tukey's multiple comparison test. Statistical analysis was performed using Minitab software (Minitab, State College, Pa). All experiments were performed in triplicate.

3. RESULT AND DISCUSSION

3.1. Effect of AE-MCC-AS on some physicochemical characteristics of mayonnaise

The physicochemical characteristics of the mayonnaise samples are summarized in Table 2. The pH of mayonnaise plays an important role in emulsion stability (Depree and Savage, 2001; Martillanes *et al.,* 2020). Therefore, the pH value of the mayonnaise samples was monitored during storage. The initial pH value of the mayonnaise samples was in the range of 3.81-3.86. Afterwards, the pH value increased significantly ($P < 0.01$) on the 28th day of storage and then decreased significantly ($P < 0.01$) on the 56th day of storage. We had observed a similar trend in the TBARs value of mayonnaise samples during storage at 25 °C in our previously published study (Ünver and Çelik, 2021). As known, TBARs value is an indicator of the amount of malonaldehyde, which is a major secondary product of the lipid oxidation process. During lipid oxidation, carboxylic acids could form due to the oxidation of the aldehydes (Mohammadi *et al.,* 2016). The various oxidation products such as these carboxylic acids could be responsible for the fluctuation in the pH value during storage. It was reported that the risk of microbial growth of foodborne pathogens in mayonnaise was low since the pH value of mayonnaise was approximately 4.00 (Khalil and Mansour, 1998). However, some species of microorganisms, such as lactic acid bacteria, can grow in the pH range of 4.00-5.00. Another reason for the pH decrease during storage could be related to the activity of these bacteria in the mayonnaise (Kishk and Elsheshetawy, 2013). On the other hand, Depree and Savage (2001) reported that the stability and viscoelasticity of mayonnaise were expected to be at their highest value when a pH value was close to the average isoelectric point of the egg yolk proteins. The mean pH value of the mayonnaise samples containing AE-MCC-AS was found to be significantly ($P < 0.01$) lower than the negative control and positive control samples. This situation might be due to the microbial load of the samples. Martillanes *et al.* (2020), who studied the antioxidant and antimicrobial evaluation of rice bran extracts in a mayonnaise-type emulsion, reported that pH values increased with time and temperature during the storage period of 7 days. However, these differences were not significant in all treatments including a rice bran extract-added mayonnaise-type emulsion, BHT-added mayonnaise-type emulsion or the control sample without antioxidant. Compared to our results, Rasmy *et al.* (2012), who studied the effect of sage extracts on the shelf-life of mayonnaise, found that the pH values of the control and samples treated with BHA and sage extracts at different concentrations increased slightly during the storage period of 4 months.

According to the color results presented in Table 2, there was no significant difference in the lightness index (L^*) of all the treatments on the first day of storage, except for 0.6% AE-MCC-AS-M. The lightness index of the mayonnaise samples containing AE-MCC-AS decreased by increasing the AE-MCC-AS ratio. This case might be due to the pigments in the PE-AH. In our previously published study (Ünver and Çelik, 2021), the L, a^* and b^* values of AE-MCC-AS were found to 71.46, 8.65 and 32.9, respectively. At the end of storage, the highest lightness index was observed in the control, while the lowest lightness index was observed in the 0.6% AE-MCC-AS-M. The lightness value plays a key role in determining the visual acceptability of the mayonnaise (Flamminii *et al.*, 2020). For this reason, the visual acceptability of the mayonnaise samples containing AE-MCC-AS was expected to decrease with the increase in the AE-MCC-AS ratio. In our previous study, the decrease in the lightness index due to increasing the AE-MCC-AS ratio was observed by the sensory panel, and 0.6% AE-MCC-AS-M received the lowest appearance score at the end of storage, followed by 0.4% AE-MCC-AS-M and 0.2% AE-MCC-AS-M (Ünver and Çelik, 2021). The positive a^* and b^* values were the sign of redness and yellowness in the color of mayonnaise. The redness (a^*) and yellowness (b^*) values decreased during storage in all treatments except for 0.6% AE-MCC-AS-M. A slight increase in the redness of 0.6% AE-MCC-AS-M was observed on the 56th day of storage. The redness and yellowness values increased with increasing levels of AE-MCC-AS. This case might be due to the pigments in PE-AH, which had a reddish and yellowish color. Generally, the pale yellowness of mayonnaise is sourced from the main ingredients such as egg yolk and oil. However, adding an unusual ingredient to the mayonnaise formulation could change the color values of the final product (Flamminii *et al.*, 2020). Researchers who studied the effect of several phenolic extracts on the physical characteristics of mayonnaise (Gorji *et al.,* 2019; Abd El-Rahman *et al.,* 2020), reported similar differences in the color attributes of mayonnaise. Nevertheless, a minimal color change was tolerable for consumer acceptability.

The emulsion stability could not be determined on the 1st or the 28th days of storage because all the mayonnaise samples showed high stability (Table 2). However, α-tocopherol-M exhibited the highest emulsion stability at the end of storage, followed by 0.4% AE-MCC-AS-M, 0.2% AE-MCC-AS-M, 0.6% AE-MCC-AS-M, BHT-M and the control. This result showed that the AE-MCC-AS ratio was an important parameter for the emulsion stability of the mayonnaise samples; however, a direct relationship was not determined between the AE-MCC-AS ratio and emulsion stability. The emulsion stability of the mayonnaise increased with the increase in the AE-MCC-AS ratio from 0.2 to 0.4%. However, it decreased as a result of the addition of 0.6% AE-MCC-AS. This effect may be due to the interaction between egg yolk proteins, which are mainly responsible for the emulsion structure, and AE-MCC-AS. The addition of AE-MCC-AS at up to 0.4% in the mayonnaise formulation may have empowered the shielding effect of the egg yolk proteins against the coalescence of lipid droplets. However, the decrease in the emulsion stability with the addition of 0.6% AE-MCC-AS showed the importance of the

Table 2. Results of some physicochemical characteristics of mayonnaise samples

		TREATMENTS					
	Time (Day)	**Control**	**BHT-M**	**α-tocopherol-M**	**0.2% AE-MCC-AS-M**	**0.4% AE-MCC-AS-M**	**0.6% AE-MCC-AS-M**
pH	**1**	3.86 ± 0.02^{Ca}	3.86 ± 0.02^{Ba}	3.81 ± 0.06^{Ba}	3.83 ± 0.05^{Ba}	3.85 ± 0.05^{Ba}	3.84 ± 0.05^{Ba}
	28	4.02 ± 0.02^{Aa}	4.01 ± 0.03^{Aa}	3.99 ± 0.03^{Aab}	3.94 ± 0.01^{Ac}	3.95 ± 0.01^{Abc}	3.95 ± 0.02^{Abc}
	56	3.90 ± 0.04^{Ba}	3.78 ± 0.03^{Cbc}	3.81 ± 0.04^{Bb}	3.71 ± 0.05^{Cd}	3.71 ± 0.02^{Cd}	3.74 ± 0.03^{Ccd}
L*	**1**	80.24 ± 1.44^{Aa}	80.35 ± 0.13^{Aa}	79.36 ± 0.67^{Ba}	79.38 ± 0.14^{Aa}	80.08 ± 0.59^{Aa}	77.40 ± 0.48^{Ab}
	28	80.69 ± 0.34^{Aa}	79.19 ± 0.34^{Cb}	80.77 ± 0.14^{Aa}	79.62 ± 0.02^{Ab}	79.18 ± 0.11^{Bb}	77.75 ± 0.21^{Ac}
	56	80.15 ± 0.30^{Aa}	79.99 ± 0.11^{Ba}	79.69 ± 0.49^{Ba}	78.75 ± 0.35^{Bb}	78.27 ± 0.68^{Cb}	76.51 ± 0.15^{Bc}
a*	**1**	2.26 ± 0.31^{Ac}	3.18 ± 0.07^{Ab}	3.12 ± 0.09^{Ab}	3.25 ± 0.03^{Ab}	3.11 ± 0.02^{Ab}	3.60 ± 0.07^{Aa}
	28	1.23 ± 0.05^{Be}	1.93 ± 0.08^{Bc}	1.72 ± 0.03^{Bd}	$2.78\pm0.0.2^{Bb}$	2.87 ± 0.02^{Bb}	3.37 ± 0.06^{Ba}
	56	0.92 ± 0.10^{Ce}	1.40 ± 0.09^{Cd}	1.03 ± 0.13^{Ce}	2.37 ± 0.02^{Cc}	2.70 ± 0.03^{Cb}	3.45 ± 0.02^{Aa}
b*	**1**	17.31 ± 2.03^{Ab}	20.71 ± 0.07^{Aa}	19.64 ± 0.26^{Aa}	20.27 ± 0.08^{Aa}	19.97 ± 0.32^{Aa}	20.87 ± 0.18^{Aa}
	28	17.26 ± 0.12^{Ad}	17.74 ± 1.13^{Bcd}	18.09 ± 0.04^{Bbcd}	18.73 ± 0.11^{Babc}	19.20 ± 0.01^{Bab}	19.48 ± 0.72^{Ba}
	56	16.48 ± 0.21^{Ad}	17.65 ± 0.17^{Bc}	16.70 ± 0.12^{Cd}	18.01 ± 0.24^{Cb}	18.13 ± 0.06^{Cb}	19.50 ± 0.10^{Ba}
ES (%)	**1**	nd.	nd.	nd.	nd.	nd.	nd.
	28	nd.	nd.	nd.	nd.	nd.	nd.
	56	87.51 ± 2.38^{b}	90.76 ± 0.95^{ab}	94.26 ± 2.06^{b}	91.76 ± 1.26^{ab}	92.26 ± 2.63^{ab}	91.50 ± 3.12^{ab}

All data are presented as mean value ± standard deviation (three repetitions, n=3). Means in the same column with different capital letters are significantly different (by Tukey's test at $P < 0.01$). Means in the same row with different small letters are significantly different (by Tukey's test at $P < 0.01$). ES: Emulsion stability. nd: Not detected .

AE-MCC-AS ratio in emulsion stability. For this reason, in future studies, it is recommended to determine the most appropriate AE-MCC-AS ratio, which provides the best emulsion stability, using an optimization model. Xu *et al.* (2016) studied the effect of MCC on the physical stability of soybean protein hydrolysate stabilized curcumin emulsion through microfluidization. Similarly, they reported that the emulsion stability improved with the addition of MCC and claimed that the proper addition of polysaccharides and emulsifiers could improve emulsion properties.

The viscosity values of the mayonnaise samples are presented in Figure 1. At the beginning of storage, the highest viscosity was observed in the α-tocopherol-M, followed by BHT-M, control, 0.4% AE-MCC-AS-M, 0.2% AE-MCC-AS-M and 0.6% AE-MCC-AS-M, respectively. A significant ($P < 0.01$) declining trend was observed in the viscosity behavior of the mayonnaise samples during the storage of 56 days. At the end of storage, 0.4% AE-MCC-AS-M showed the most viscous structure, followed by α-tocopherol-M, BHT-M, 0.2% AE-MCC-AS-M, 0.6% AE-MCC-AS-M and the control, respectively. These findings were partially in good agreement with the firmness and consistency results of the mayonnaise samples (Figures 3a and 3b). Liu *et al.* (2007) reported that the viscosity of mayonnaise might partially reflect texture analysis parameters, but not all of them. The increase in the AE-MCC-AS ratio from 0.2 to 0.4% caused an increase in the viscosity of mayonnaise since adding AE-MCC-AS at the mentioned ratio may have contributed to the formation of a gel-like structure that trapped oil droplets. Therefore, the movement of oil droplets may have slowed down, which caused an increasing effect on the viscosity. However, the addition of AE-MCC-AS higher than 0.4% might cause a decreasing effect on the viscosity of mayonnaise. 0.6% AE-MCC-AS-M showed the lowest viscosity among the treatments of AE-MCC-AS. It can be seen as a reflection of the change in emulsion stability due to the increase in AE-MCC-AS concentration. Therefore, the AE-MCC-AS ratio played a crucial role in the formation of interfacial membranes and the prevention of partial coalescence of oil droplets. Similarly, Raikos *et al.* (2016), who studied the effect of beetroot supplementation on the physical stability, textural and sensory properties of mayonnaise, reported that the mean value of viscosity of mayonnaise supplement-

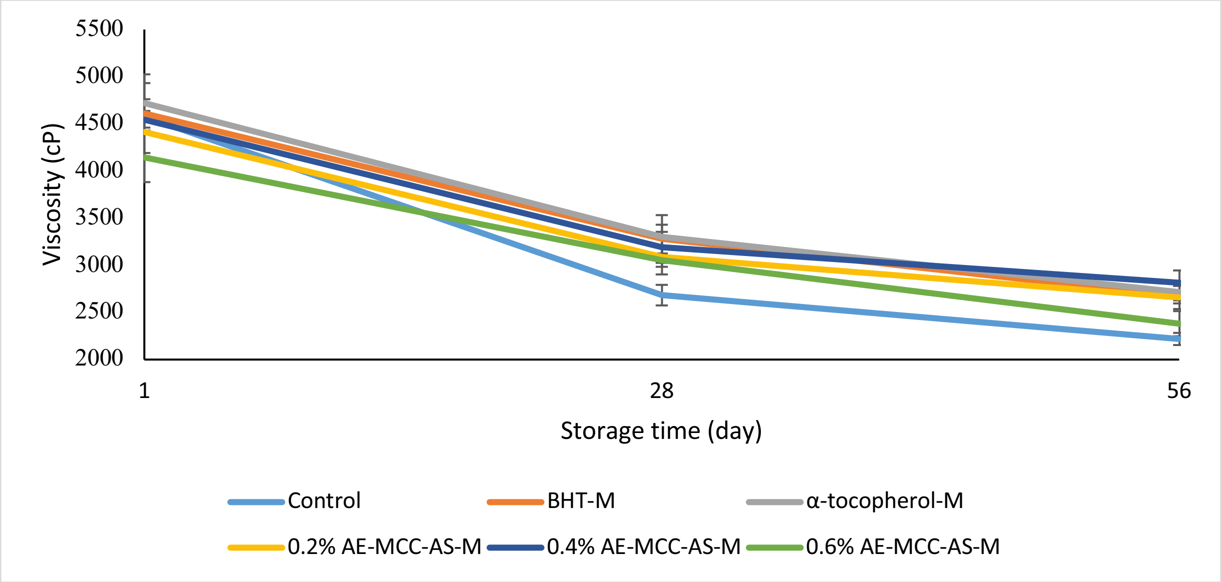

Figure 1. Viscosity results of the mayonnaise samples during storage at 25 °C (Mean ± SD; n=3). The data were analyzed using one-way ANOVA, and groups were compared with Tukey's multiple comparison test ($P \leq 0.01$).

ed with beetroot was higher than the control (without antioxidant) and the commercial control. Kishk and Elsheshetawy (2013) studied the effect of ginger powder on the oxidative stability, rheological, and sensory characteristics of mayonnaise. In contrast to our results, the researchers reported that adding ginger powder at different concentrations (0, 0.5, 0.75 and 1.25%) to the mayonnaise formula showed no significant effect on the apparent viscosity. Xu *et al.* (2016) reported that the viscosity of soybean protein isolate stabilized curcumin emulsion increased with the addition of MCC.

The microstructure of the mayonnaise samples is shown in Figure 2. Larger oil droplets were observed in the micrographs of the control, 0.2% AE-MCC-AS-M and 0.6% AE-MCC-AS-M; while the smallest oil droplets were observed in the micrograph of α-tocopherol-M, followed by BHT-M and 0.4% AE-MCC-AS-M. These observations were consistent with the findings of the viscosity results on the 28th day of storage. Larger oil droplets lower the level of uniformity of the samples, while smaller oil droplets create a larger contact surface, which causes frictional forces opposing the free flow of the emulsion in a shear field (Golchoobi *et al.,* 2016). Ultimately, the decrease in oil droplet size caused an increase in viscosity. Chatterjee and Bhattacharjee (2015), who studied the effect of eugenol-lean clove extract on the physicochemical characteristics of mayonnaise, reported that the microstructure of mayonnaise containing eugenol-lean clove extract exhibited a more homogenous and compact microstructure compared to mayonnaise formulated with mustard. Furthermore, the researchers claimed that eugenol-lean clove extract positively affected the emulsification of the mayonnaise.

The saturated fatty acid composition of the mayonnaise samples is presented in Table 3. In addition to the saturated fatty acids (SFA) presented in Table 3, myristic acid (C14:0) and heptadecanoic acid (C17:0) were also determined; however, their concentration was lower than 0.1%. While the total SFA of mayonnaise was 9.88-10.79% at the beginning of storage, it increased to 11.35-12.18% at the end of storage. 0.4% AE-MCC-AS-M showed the highest SFA content, followed by α-tocopherol-M, control, 0.2% AE-MCC-AS-M, 0.6% AE-MCC-AS-M and BHT-M at the beginning of storage. The mean of the total SFA ratio increased in all treatments during storage. The increase in the total SFA is probably due to the degradation of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), which increases the ratio of the total SFA (Kunyaboon *et al.,* 2021). At the end of storage, the highest SFA ratio was observed in the control, followed by 0.4% AE-MCC-AS-M, BHT-M, 0.2% AE-MCC-AS-M, α-tocopherol-M, and 0.6% AE-MCC-AS-M. Among the SFA, palmitic acid (C16:0) and

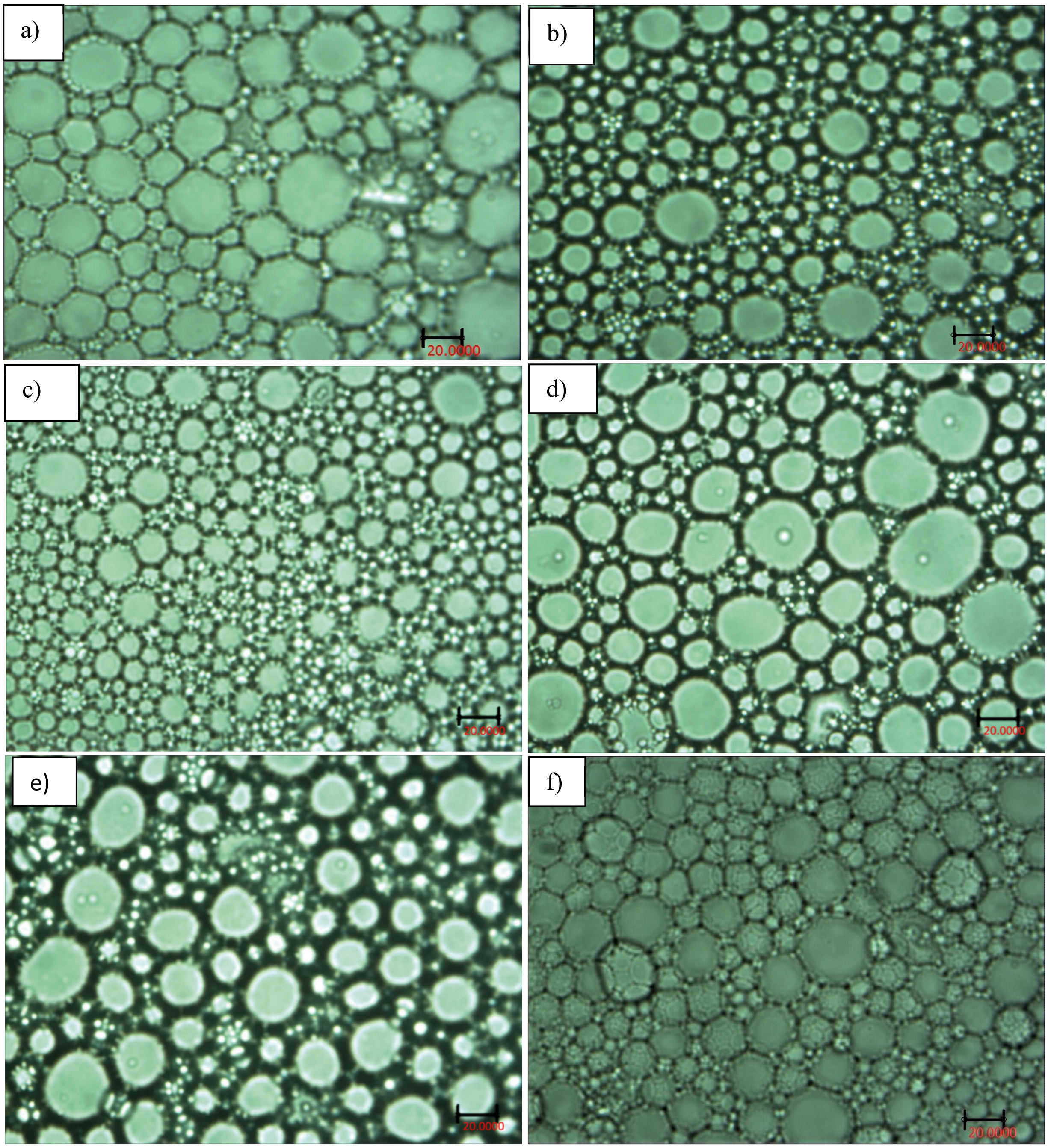

Figure 2. Micrographs of mayonnaise samples taken by optical microscope on the 28th day of storage. (a) Control; (b) BHT-M; (c) α-tocopherol-M; (d) 0.2% AE-MCC-AS-M; (e) 0.4% AE-MCC-AS-M; (f) 0.6% AE-MCC-AS-M

stearic acid (C18:0) had the highest ratio in all treatments. Similarly, Turan (2018) reported that the SFA content in hazelnut oil during 12 months of storage showed fluctuations and variability. However, the researcher reported that total SFA increased slightly at the end of storage compared to the beginning.

The MUFA and PUFA compositions of the mayonnaise samples are presented in Table 4. In addition to the unsaturated fatty acids presented in Table 4, heptadecenoic (C17:1), erucic acid (C22:1n9), nervonic acid (C24:1n9) and γ-Linolenic acid (C18:3n3) were also determined in all treatments, although their concentration was lower than 0.1%. While the total MUFA of mayonnaise was 25.84-26.25% at the beginning of storage, it decreased to 25.03-25.80% at the end of

Table 3. Saturated fatty acid composition of mayonnaise samples

		Treatments					
Fatty acid	**Time (Day)**	**Control**	**BHT-M**	**α-tocopherol-M**	**0.2% AE-MCC-AS-M**	**0.4% AE-MCC-AS-M**	**0.6% AE-MCC-AS-M**
C16:0	1	6.70 ± 0.24^{Ba}	6.63 ± 0.52^{Aa}	6.77 ± 0.21^{Aa}	7.13 ± 0.01^{Aa}	6.39 ± 0.15^{Aa}	6.33 ± 0.01^{Ba}
	28	7.27 ± 0.13^{ABab}	7.37 ± 0.06^{Aa}	6.83 ± 0.42^{Aab}	6.70 ± 0.00^{Aab}	6.92 ± 0.11^{Aab}	6.61 ± 0.03^{Bb}
	56	7.45 ± 0.14^{Aa}	7.59 ± 0.26^{Aa}	7.39 ± 0.42^{Aa}	7.49 ± 0.44^{Aa}	7.86 ± 0.19^{Aa}	7.15 ± 0.13^{Aa}
C18:0	1	3.39 ± 0.23^{Aa}	2.72 ± 1.00^{Aa}	3.32 ± 0.02^{Aa}	2.54 ± 0.38^{Aa}	3.75 ± 0.00^{Aa}	3.31 ± 0.33^{Aa}
	28	3.59 ± 0.04^{Aa}	2.93 ± 1.21^{Aa}	3.75 ± 0.20^{Aa}	3.31 ± 0.42^{Aa}	3.48 ± 0.04^{Aa}	3.61 ± 0.08^{Aa}
	56	3.95 ± 0.34^{Aa}	3.40 ± 0.01^{Aa}	3.66 ± 0.21^{Aa}	3.53 ± 0.00^{Aa}	3.62 ± 0.13^{Aa}	3.76 ± 0.20^{Aa}
C20:0	1	0.12 ± 0.02^{Ac}	0.22 ± 0.01^{Aab}	0.18 ± 0.02^{Ab}	0.20 ± 0.01^{Bab}	0.25 ± 0.00^{Aa}	0.22 ± 0.01^{Aab}
	28	0.17 ± 0.00^{Aa}	0.20 ± 0.00^{Aa}	0.19 ± 0.01^{Aa}	0.23 ± 0.01^{Aa}	0.21 ± 0.04^{Aa}	0.20 ± 0.01^{Aa}
	56	0.21 ± 0.06^{A}	0.13 ± 0.04^{A}	0.17 ± 0.01^{A}	0.18 ± 0.01^{B}	0.14 ± 0.05^{A}	0.16 ± 0.05^{A}
C24:0	1	0.16 ± 0.04^{Aab}	0.20 ± 0.03^{Aa}	0.11 ± 0.01^{Bb}	0.10 ± 0.01^{Bb}	0.21 ± 0.00^{Aa}	0.15 ± 0.01^{Aab}
	28	0.12 ± 0.00^{Ac}	0.19 ± 0.03^{Aab}	0.24 ± 0.00^{Aa}	0.18 ± 0.01^{Ab}	0.21 ± 0.00^{Aab}	0.16 ± 0.01^{Abc}
	56	0.17 ± 0.06^{Aa}	0.15 ± 0.08^{Aa}	0.25 ± 0.03^{Aa}	0.12 ± 0.02^{Ba}	0.12 ± 0.08^{Aa}	0.18 ± 0.01^{Aa}
Total SFA	1	10.46 ± 0.48^{Aa}	9.88 ± 1.53^{Aa}	10.48 ± 0.21^{Aa}	10.06 ± 0.42^{Aa}	10.79 ± 0.15^{Aa}	10.00 ± 0.41^{Ba}
	28	11.25 ± 0.09^{Aa}	10.80 ± 1.30^{Aa}	11.11 ± 0.70^{Aa}	10.52 ± 0.45^{Aa}	10.92 ± 0.03^{Aa}	10.67 ± 0.09^{ABa}
	56	12.18 ± 1.03^{Aa}	11.38 ± 0.23^{Aa}	11.57 ± 0.23^{Aa}	11.43 ± 0.46^{Aa}	11.85 ± 0.55^{Aa}	11.35 ± 0.27^{Aa}

All data are presented as mean value ± standard deviation (three repetitions, n=3). Means in the same column with different capital letters are significantly different (by Tukey's test at $P < 0.01$). Means in the same row with different small letters are significantly different (by Tukey's test at $P < 0.01$). SFA: Saturated fatty acid.

Table 4. Unsaturated fatty acid composition of mayonnaise samples

		Treatments					
Fatty acid	**Time (Day)**	**Control**	**BHT-M**	**α-tocopherol-M**	**0.2% AE-MCC-AS-M**	**0.4% AE-MCC-AS-M**	**0.6% AE-MCC-AS-M**
C16:1	1	0.10 ± 0.01^{Aab}	0.10 ± 0.00^{Aab}	0.09 ± 0.01^{Ab}	0.10 ± 0.00^{Aab}	0.11 ± 0.00^{Aa}	0.10 ± 0.00^{Aab}
	28	0.10 ± 0.01^{Aa}	0.10 ± 0.00^{Aa}	0.09 ± 0.02^{Aa}	0.10 ± 0.00^{Aa}	0.09 ± 0.00^{Ba}	0.09 ± 0.00^{Aa}
	56	0.10 ± 0.01^{Aa}	0.10 ± 0.00^{Aa}	0.09 ± 0.00^{Aa}	0.11 ± 0.01^{Aa}	0.10 ± 0.01^{ABa}	0.10 ± 0.01^{Aa}
C18:1n9c	1	25.59 ± 0.01^{Aa}	25.86 ± 0.21^{Aa}	25.55 ± 0.24^{Aa}	25.94 ± 0.06^{Aa}	25.71 ± 0.01^{Aa}	25.80 ± 0.24^{Aa}
	28	25.30 ± 0.27^{Aa}	25.22 ± 0.56^{Aa}	25.38 ± 0.24^{Aa}	25.65 ± 0.36^{Aa}	25.68 ± 0.15^{Aa}	25.68 ± 0.33^{Aa}
	56	24.75 ± 0.35^{Aa}	25.24 ± 0.21^{Aa}	25.21 ± 1.04^{Aa}	25.53 ± 0.03^{Aa}	25.38 ± 0.18^{Aa}	25.31 ± 0.41^{Aa}
C20:1n9	1	0.14 ± 0.01^{Aa}	0.13 ± 0.00^{Aa}	0.13 ± 0.00^{Aa}	0.13 ± 0.02^{Aa}	0.15 ± 0.00^{Aa}	0.14 ± 0.02^{Aa}
	28	0.12 ± 0.00^{Aa}	0.14 ± 0.00^{Aa}	0.12 ± 0.02^{Aa}	0.13 ± 0.01^{Aa}	0.11 ± 0.00^{Aa}	0.14 ± 0.01^{Aa}
	56	0.12 ± 0.00^{Aa}	0.14 ± 0.00^{Aa}	0.13 ± 0.01^{Aa}	0.12 ± 0.02^{Aa}	0.12 ± 0.02^{Aa}	0.13 ± 0.01^{Aa}
Total MUFA	1	25.90 ± 0.04^{Aa}	26.16 ± 0.20^{Aa}	25.84 ± 0.22^{Aa}	26.25 ± 0.06^{Aa}	26.09 ± 0.07^{Aa}	26.12 ± 0.26^{Aa}
	28	25.42 ± 0.27^{Aa}	25.51 ± 0.57^{Aa}	25.66 ± 0.22^{Aa}	26.04 ± 0.23^{Aa}	25.91 ± 0.23^{Aa}	25.98 ± 0.32^{Aa}
	56	25.03 ± 0.33^{Aa}	25.47 ± 0.32^{Aa}	25.50 ± 1.05^{Aa}	25.80 ± 0.05^{Aa}	25.65 ± 0.21^{Aa}	25.59 ± 0.42^{Aa}
C18:2n6c	1	62.55 ± 0.24^{Aa}	63.23 ± 1.31^{Aa}	62.94 ± 0.09^{Aa}	62.96 ± 0.22^{Aa}	62.41 ± 0.18^{Aa}	62.53 ± 0.40^{Aa}
	28	62.53 ± 0.41^{Aa}	62.98 ± 0.76^{Aa}	62.58 ± 0.37^{Aa}	62.65 ± 0.16^{Aa}	62.35 ± 0.00^{Aa}	62.25 ± 0.57^{Aa}
	56	61.57 ± 0.74^{Aa}	62.54 ± 0.23^{Aa}	61.99 ± 0.74^{Aa}	62.25 ± 0.19^{Aa}	61.94 ± 0.13^{Aa}	62.23 ± 0.33^{Aa}
C20:2	1	0.71 ± 0.01^{Aa}	0.65 ± 0.01^{Aa}	0.68 ± 0.11^{Aa}	0.67 ± 0.13^{Aa}	0.62 ± 0.02^{Aa}	0.61 ± 0.03^{Aa}
	28	0.51 ± 0.02^{Bab}	0.64 ± 0.04^{Aa}	0.59 ± 0.11^{Aa}	0.69 ± 0.01^{Aa}	0.31 ± 0.00^{Ab}	0.70 ± 0.01^{Aa}
	56	0.63 ± 0.06^{ABa}	0.54 ± 0.16^{Aa}	0.68 ± 0.01^{Aa}	0.42 ± 0.16^{Aa}	0.51 ± 0.18^{Aa}	0.54 ± 0.11^{Aa}
Total PUFA	1	63.33 ± 0.26^{Aa}	63.95 ± 1.32^{Aa}	63.69 ± 0.02^{Aa}	63.69 ± 0.36^{Aa}	63.07 ± 0.13^{Aa}	63.24 ± 0.46^{Aa}
	28	63.09 ± 0.39^{Aa}	63.68 ± 0.73^{Aa}	63.24 ± 0.68^{Aa}	63.40 ± 0.16^{Aa}	62.73 ± 0.02^{Aa}	62.99 ± 0.59^{Aa}
	56	62.26 ± 0.69^{Aa}	63.15 ± 0.09^{Aa}	62.74 ± 0.73^{Aa}	62.73 ± 0.35^{Aa}	62.50 ± 0.33^{Aa}	62.84 ± 0.43^{Aa}

All data are presented as mean value ± standard deviation (three repetitions, n=3). Means in the same column with different capital letters are significantly different (by Tukey's test at $P < 0.01$).

Means in the same row with different small letters are significantly different (by Tukey's test at $P < 0.01$). MUFA: Monounsaturated fatty acid. PUFA: Polyunsaturated fatty acid.

storage. The total MUFA ratios showed a decreasing trend in all treatments during the storage period. Although there was no significant difference among the treatments during storage, the lowest total MUFA was observed in the control, and the highest MUFA was observed in 0.2% AE-MCC-AS-M. Turan (2018) reported a similar declining trend in the MUFA content of hazelnut oil at the end of storage. Total PUFA was 63.07-63.95% at the beginning of storage, and it decreased to 62.26-63.15% at the end of storage. Similar to total MUFA, a declining trend was observed in total PUFA during storage. The lowest total PUFA was observed in the control, followed by 0.4% AE-MCC-AS-M, 0.2% AE-MCC-AS-M, α-tocopherol-M, 0.6% AE-MCC-AS-M, and BHT-M. While oleic acid (C18:1n9c) had the highest ratio among the MUFA, linoleic acid (C18:2n6c) was the dominant fatty acid among the PUFAs. This phenomenon could be originated from the sunflower oil used in the production of mayonnaise.

It is worth noting that the total MUFA and PUFA of mayonnaise showed a declining trend in all treatments with a higher decrease in the control; whereas the total SFA showed an increasing trend with a higher increase in the control during storage. The result of the oxidation analysis (peroxide value, TBARs value and induction time) that had been determined in our previous study (Ünver and Çelik, 2021) corroborated with these findings for the fatty acid composition. During oxidation, free radicals can easily react with long-chain unsaturated fatty acids, which cause off-flavors and taste in food products (Uçar, 2020). Exposure to temperature, air trapped in the headspace of the jars, and iron cations released from egg yolk during the storage of mayonnaise can cause rapid deterioration of long-chain unsaturated fatty acids. Therefore, the use of synthetic and natural antioxidants in mayonnaise can retard or inhibit oil oxidation. Furthermore, packaging mayonnaise under vacuum or changing the air in the headspace with nitrogen is beneficial to retard the oxidation reaction in mayonnaise.

3.2. Effect of AE-MCC-AS on the textural characteristics of mayonnaise

The textural characteristics were determined as firmness, consistency, and cohesiveness on the basis of force-time curves. Firmness, which indicates the strength of the emulsion, is the maximum force or peak of the force-time curve, and the recorded area of the positive region of the force-time curve represents the consistency in the back extrusion method. As shown in Figure 3, α-tocopherol-M showed the highest firmness, followed by the control, BHT-M, 0.2% AE-MCC-AS-M, 0.4% AE-MCC-AS-M and 0.6% AE-MCC-AS-M at the beginning of storage, and the difference between the firmness values of the treatments was found to be statistically significant ($P < 0.01$). The mean value for firmness showed a significant ($P < 0.01$) declining trend during the storage in all treatments. At the end of storage, α-tocopherol-M showed the highest firmness, followed by 0.4% AE-MCC-AS-M, BHT-M, 0.2% AE-MCC-AS-M, 0.6% AE-MCC-AS-M and the control. The same declining trend and order were observed in the consistency values of the mayonnaise samples. Cohesiveness (g) is the maximum negative force of the force-time curve obtained during the return of the probe. In other words, cohesiveness indicates the level of deforming before breaking when extended (Rojas *et al.*, 2019). At the beginning of storage, 0.4% AE-MCC-AS-M exhibited the most cohesive structure; while α-tocopherol-M exhibited the least cohesive structure, and the difference between the cohesiveness values of the treatments was found to be statistically significant ($P < 0.01$). The mean of cohesiveness showed a significant ($P < 0.01$) upward trend during storage in all treatments. At the end of storage, the highest cohesiveness was observed in the control, followed by 0.2% AE-MCC-AS-M, 0.4% AE-MCC-AS-M, BHT-M, 0.6% AE-MCC-AS-M and α-tocopherol-M, and the difference between the treatments was found to be statistically significant ($P < 0.05$). Raikos *et al.* (2016) reported that hardness, work done, adhesive force and adhesiveness of mayonnaise supplemented with beetroot were higher than the control sample (without antioxidant) and the commercial control.

4. CONCLUSIONS

The addition of AE-MCC-AS contributed to the emulsion stability, viscosity and some textural characteristics of mayonnaise compared to the control. Furthermore, the findings of fatty acid composition in this study supported the results of the oxidation analysis that had been determined in our previous study (Ünver and Çelik, 2021). In conclusion, the

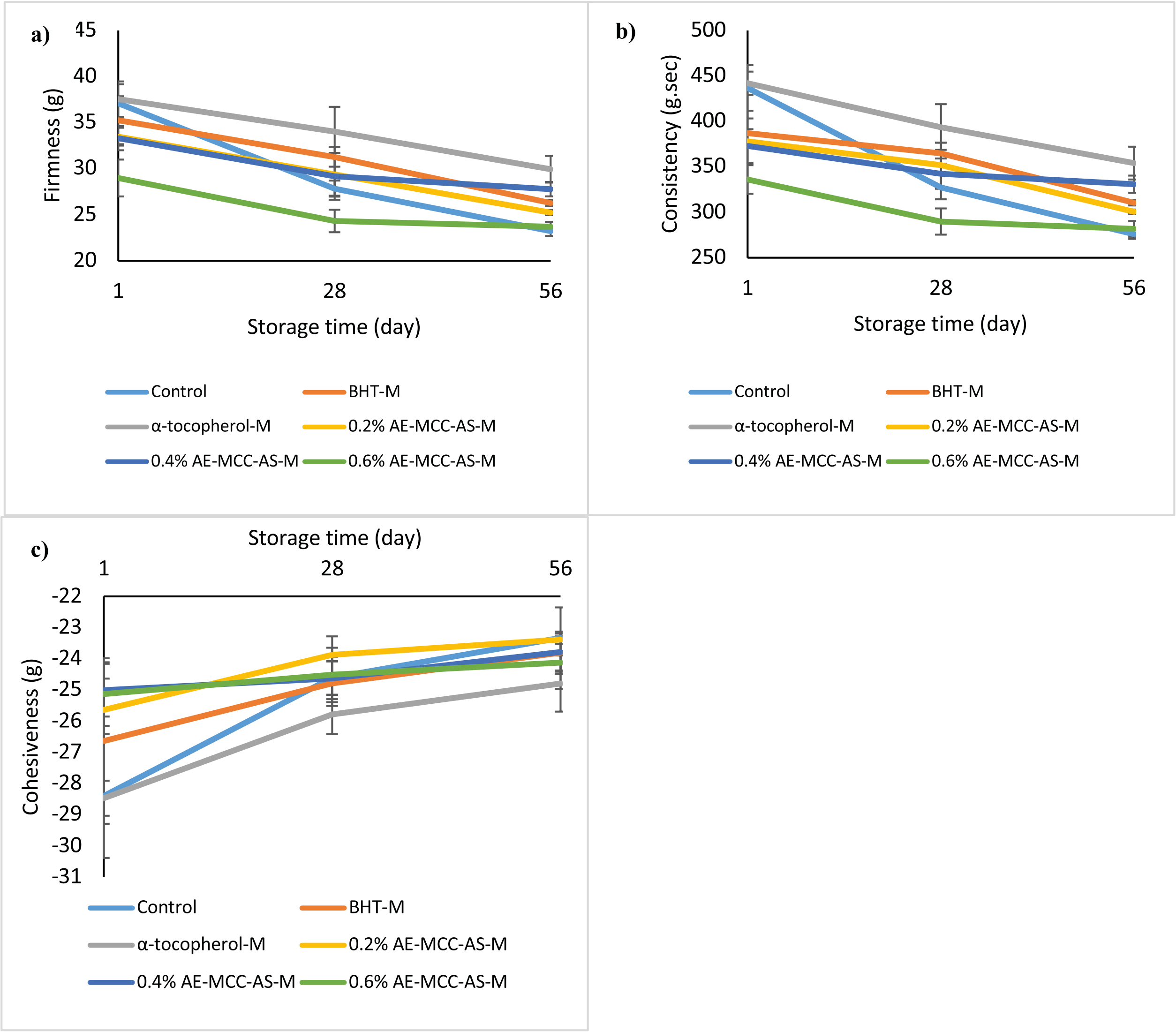

Figure 3. Results of firmness (a), consistency (b) and cohesiveness (c) of the mayonnaise samples during storage at 25 °C (Mean ± SD; n=3). The data were analyzed using one-way ANOVA, and groups were compared with Tukey's multiple comparison test (P ≤ 0.01).

study revealed that 0.4% AE-MCC-AS-M showed the highest emulsion stability, viscosity, firmness, and consistency among the samples that contained AE-MCC-AS. Furthermore, this study revealed that the AE-MCC-AS ratio was an important factor in the color characteristics of mayonnaise. Therefore, we suggest determining the effect AE-MCC-AS ratio on the physical, textural and emulsion characteristics of mayonnaise in detail with an optimization study.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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Factors affecting nutritional quality in terms of the fatty acid composition of *Cyprinion macrostomus*

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SUMMARY: This study aimed to evaluate the effect of different factors (season, gender, location, total lipid, weight and length) on the fatty acid composition and nutritional quality of *Cyprinion macrostomus.* The results were evaluated through PERMANOVA, principal coordinates (PCO), and cluster analysis for similarity ranges. An analysis of similarity (ANOSIM) was performed on the distance matrix using multiple permutations within a significant fixed effect ($p < 0.05$). C18:1ω9, EPA and DHA were the most important fatty acids which had an effect on the nutritional quality in all the factor groups. Total lipid amount, season and length factors were the most influential on the fatty acid compositions of *C. macrostomus*. Summer and Spring were the best the periods for the good nutritional quality of *C. macrostomus* in terms of AI (Atherogenicity index), TI (Thrombogenicity index) and h/H (Σhypocholesterolemic/Σhypercholesterolemic fatty acid index). In addition, station, gender and weight had no effect on nutritional quality. The study indicated that *C. macrostomus* is a potential fish meat for human nutrition with high nutritional value in terms of fatty acid composition.

KEYWORDS: *AI;* Cyprinion macrostomus*; EPA; Fatty acids; h/H; PERMANOVA*

RESUMEN: ***Factores que afectan la calidad nutricional en términos de composición de ácidos grasos de* Cyprinion macrostomus.** El estudio tuvo como objetivo evaluar el efecto de diferentes factores (estación, género, ubicación, lípidos totales, peso y talla) que afectan la composición de ácidos grasos sobre la calidad nutricional de *Cyprinion macrostomus*. Los resultados se evaluaron mediante PERMANOVA, coordenadas principales (COP) y análisis de cluster para rangos de similitud. Se realizó un análisis de similitud (ANDSIM) en la matriz de distancias utilizando múltiples permutaciones dentro de un efecto fijo significativo ($p<0,05$). C18:1ω9, EPA y DHA fueron los ácidos grasos más importantes que tuvieron efecto sobre la calidad nutricional para todos los grupos de factores. Los factores más influyentes fueron la cantidad total de lípidos, la estación y la longitud, en la composición de ácidos grasos de *C. macrostomus*. El verano y la primavera fueron los mejores períodos para la buena calidad nutricional de *C. macrostomus* en términos de IA (Índice de aterogenicidad), IT (Índice de trombogenicidad) y h/H (Índice de ácidos grasos Σhipocolesterolémico/Σhipercolesterolémico). Asimismo, la estación, el sexo y el peso de los grupos de factores no tuvieron efecto sobre la calidad nutricional. El estudio indicó que *C. macrostomus* es una carne de pescado potencial en la nutrición humana con un alto valor nutricional en términos de composición de ácidos grasos.

PALABRAS CLAVE: *Ácidos grasos;* Cyprinion macrostomo*; EPA; h/S; AI; PERMANOVA*

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1. INTRODUCTION

Cyprinion macrostomus (HECKEL, 1843), belonging to Cyprinidae family, is a widely distributed fish species between the Tigris Euphrates Rivers and the Asi Basin (Coad, 1995). The Murat River is between the Tigris Euphrates Rivers and Asi Basin in Turkey. Despite the aquaculture fishery of *C. macrostomus* in some inland regions such as Northern Iraq (Langroudi and Mousavi, 2018), its catch is very limited in countries such as Turkey, where it is commonly found in its natural waters, yet has not been farmed for consumption.

Marine fishery is more common than freshwater fishery around the World for both aquaculture and captured fish. Carp species are in the highest percentage and include Grass carp, Silver carp, and Common carp (29%, 2016), among the freshwater fish. Every day, natural stocks are decreasing and cultural fisheries are increasing (FAO, 2018). Although its importance as a commercial species is well-known, its ecological and biochemical characteristics have not been fully investigated. It is critical to gain a better understanding of its energy storage, diet and nutrition quality of lipids, especially fatty acids (FAs) as they are considered the most important energy source in aquatic ecosystems. FA precursors of anti-inflamatory eicosanoids have vital functions in a living metabolism for physiological processes, including maintenance of cell membranes and their functions, as well as energy storage (Parrish, 2009).

Aquatic ecosystems are the primary source of ω3 FAs in the environment, thus supporting both aquatic and terrestrial heterotrophs through trophic transfer of these important essential fatty acids (EFAs) through food webs (Gladyshev *et al.,* 2013). EFAs support multiple physiological processes and cannot be synthesized at all or in sufficient proportions to meet the demand. Therefore, these compounds must be obtained through diet by aquatic organisms for optimal growth, reproduction and survival (Parrish, 2009). There is a large body of literature reporting that the reproduction and development of various consumers in aquatic ecosystems is limited by certain essential fatty acids. Therefore, fatty acids are promising biochemical components to be used in determining the food quality of organisms in an ecosystem basin (Galloway and Winder, 2015). Moreover, certain sources of lipids, such as some long-chain polyunsaturated fatty acids (LC-PUFAs), provide consumers with essential nutrients. LC-PUFAs such as 20:5ω3 (eicosapentaenoic acid, EPA), 22:6ω3 (docosahexaenoic, DHA) and 20:4ω6 (arachidonic, ARA) are the most important nutrients in aquatic ecosystems (Parrish, 2009). Specific FAs are also considered dietary biomarkers (Napolitano, 1999) and consumers' fatty acid composition typically reflects that of the fish's diet (Parrish, 2009) and feeding habitat (Parzanini *et al.*, 2020). Over the past 150 years, an increased intake of ω6 LC-PUFA has been associated with an increase in heart disease, which has contributed to the development of a healthy diet concept that balances ω3 to ω6 LC-PUFA (Simopoulos, 2008). To date, multiple lines of scientific evidence have confirmed the beneficial effects of dietary ω3 LC-PUFA, EPA and DHA on human health (Calder, 2018). It is known that marine fish species have more quality lipid and fatty acid contents than freshwater fish species. However, it is more difficult to reach marine fish in interior regions where freshwater resources are more abundant. In the inner regions where *C. macrostom*us is found, it is preferable and often consumed by local people as it is more flavorful and boneless than other Cyprinidae species. *C. macrostomus* can be added to cyprinidae species as an alternative nutrition source in addition to species such as Common carp, Grass carp, and Silver carp, which play important roles in freshwater fish. If *C. macrostomus* has a high omega fatty acid content in its natural environment, we think that it would be appropriate to culture it under suitable conditions all over the world. Thus, a quality food source would be provided for human consumption. Firstly, we need first know the food quality in its natural environment if it is to be consumed as a cultured fish or by hunting. Nutrition quality indicators such as omega 3 (ω3), DHA/EPA, ω6/ω3, which reveal food quality should be investigated. Consumers prefer natural products rather than synthetic products, and the market demand for natural ω3 LC-PUFA, especially from the natural environment is increasing. Therefore, there is an urgent need to find and to extend cultured alternative sources of natural ω3 LC-PUFA. In this study, the differences in fatty acid composition of the edible muscle of *C. macrostomus* collected from the Murat River, Bingöl province, Turkey were investigated by quantifying variations in fatty acid composition according to season, location, gender, weight and length. In particular, two different stations with hotter and colder water temperatures were selected. The objective of the study is to reveal the effects of different factors (season, gender, location, total lipid, station, weight and length) on the nutritional quality of *C. macrostomus* in terms of fatty acids.

2. MATERIALS AND METHODS

2.1. Sampling area and samplings

Fish samples were procured alive by hunting from the Murat River, Turkey. Wild fish samples were collected monthly from the stations (Garip and Ilıcalar). Nets with different eye apertures were used for catching the fish. Two stations on the Garip Stream of the Murat River 111 were determined in the Bingöl Province, Turkey. One of the stations was chosen from the Ilıcalar location (Ilıcalar Station; 36º59'01.5" N, 40º40"58.9"E), which has water temperatures above seasonal norms. The other station was the Garip location (Garip Station; 30º47'10.7"N, 40º32'58.7"E), which has water with colder temperature. Water temperature was measured randomly at the same location in both stations between March 2017-February 2018. Nets with different eye apertures were used for catching the fish. Individual fish weight ranged from 8 to 87 g, while length ranged from 8.5 to 18.5 cm.

2.2. Laboratory studies

2.2.1. Preliminary preparations

Wild-caught fish were kept on ice and delivered to the laboratory in 1 h. Wild *C. macrostomus* samples were kept on ice before being slaughtered, since ice has an anesthetic effect on small fish. 30 fish samples from the Garip Station and 17 fish samples from the Ilıcalar Station were used in the study. Length and weight measurements were made for each fish sample. Fish samples were divided into groups according to length and weight. Length was divided into two groups: Group 1, 8.5 -14.5 cm and Group 2, 15 -18.5. Weight was divided into three groups: Group 1, 8-40 g; Group 2, 42.5-58 g; and Group 3, 60.5-87 g. The samples were chosen from sexually mature fish. Transport and slaughter procedures were applied as established in the European Commission (EU) report on the welfare of fish and a mechanism was applied for using effective stunning and slaughter equipment, in accordance with the European Food Safety Authority (EFSA), 2013 guidelines. The mechanism was established in accordance with the Bingol University Animal Experiments Local Ethics Committee Directive (2016/06-5). Fish samples were cut following the butterfly fillet technique. The edible muscle tissue (raw form) of the fish samples was separated from the inedible parts of the fish. The internal organs were removed by hand. Gender determination was made macroscopically from the gonads. This procedure was performed to evaluate to total lipid and fatty acid in the muscle tissue of the fish according to gender discrimination. Every fish muscle was cut into uniform pieces of (2.0 131 × 2 × 1 cm; ~1-2 g) using a scalpel from the non-posterior part. Also, every sample was sealed in plastic bags. All the fish muscle samples were stored at -80 °C for further analysis.

2.2.2. Lipid extraction and fatty acid derivatization

Lipid extraction was performed on the separated muscle tissue samples. The weight of each sample was determined with a precision of 0.001 mg wet weight (WW). A hexane/isopropanol mixture (3/2) was used for lipid extraction. The homogenate was centrifuged (5000 rpm, 5 min, 4 ºC) and the supernatant phase was used for the fatty acid analysis (Hara and Radin, 1978).

20 g methanolic sulfuric acid were mixed into 1000 mL of pure water and a 2% methanolic sulfuric acid solution was prepared and a 5 mL methanolic sulfuric acid solution (20%) was added. The mixture was left to be methylated in an oven at 55 ºC for 15 hours. At the end the period, 5 mL of 5% NaCl were added. 5 mL hexane was added to the fatty acid methyl esters (FAMEs) formed in the tubes and the tubes were turned over. After waiting for 3 hours at room temperature, the hexane phase formed was taken from the top, and 5 mL of 2% $KHCO_3$ solution were added to the tubes, and the nitrogen (N_2) was left to evaporate with the help of the nitrogen evaporator (Allsheng WD-12). To determine the amount of dry lipid remaining after voiding occurred, the samples were weighed on a precision scale and the average total lipid amount (%) per individual was calculated as given in the formula below. After adding 1 mL of hexane to the dry lipid layer, they were vortexed (Christie, 1992) and the samples were taken

into 2 mL capped autosampler vials and analyzed in a mass spectrometer gas chromatograph (GC/MS).

Total Lipid (%) = (Wet Weight/ Dry Weight) x100

Wet Weight = Weight of wet fish sample (g)

Dry Weight = Weight of lipid remaining after evaporation (g)

2.3. GC-MS analysis

GC-MS (7890A-Agilent 5975C) was used for the FAME analysis. MS and FID detectors were used simultaneously. The injection volume was 1 µL and the splitless mode was selected. The GC column was a BPx90 capillary column. The column length was 100 m with an internal diameter 0.25 mm. The column temperatures started from 120 °C and reached 252 °C at a rate of 3 °C/min and was held there for 8 minutes. The injector temperature started at 150 ºC and was ramped up to a final temperature of 250 ºC at a rate of 120 ºC/min. The detector temperature was held at 260 °C. He (1 mL/min) was used as carrier gas. Total analysis time was 52 minutes. FAME analysis of the samples was made by injecting a standard of fatty acid methyl esters (Supelco component FAME Mix) and the retention times of each fatty acid were determined.

After the analysis, wsearch32 software (Wsearch 2008; version 1.6 2005, Sidney, Australia) was used for integration of the peaks of each fatty acid. Quantification was done by interpolation of peak areas with a calibration curve of the fatty acid standards. The total concentration of identified FAME in the sample (mg/mL) was considered as 100%, and an individual FAME was calculated as a proportion of the total identified FAME.

2.4. Statistical analysis

Multivariate statistics were used to analyze differences in total lipid amount and total fatty acid composition for PRIMER-e 2017. Total lipid amount and all fatty acids were used in the multivariate analyses of all samples at the stations. The Bray Curtis similarity coefficient was used for PERMANOVA, principal coordinates (PCO), and CLUSTER analysis for similarity ranges. In the analyses, the fatty acid data from *C. macrostomus* were factored by weight and length groups, total lipid groups, season, gender and stations. The average total lipid, weight and length were calculated during the sampling period. Data were divided into groups according to above and below the almost-average values. The averages for the factor groups were 3.65% for total lipid, 41.36 g for weight and 14.68 cm for length in all the sampling seasons and the stations. Group 1 (TL1, n=30): ≤ 3.8; Group 2 (TL2, n=17): ≥ 3.9 for the total lipid factor; Group 1 (L1, n=24): ≤ 14.5; Group 2 (L2, n=23): ≥ 15 for the length factor; Group 1 (W1, n=12): ≤ 40; Group 2 (W2, n=21): > 40-58 for the weight factor; Group 3 (W3, n=14): > 58. The fatty acids that showed the greatest differences in all samples were investigated in the factor groups. SIMPER (Cut off for low contributions: 70%) was used to identify the fatty acids which contributed the most to the similarities between or within factor groups. Analysis of similarity (ANOSIM) was performed on the distance matrix using multiple permutations within a significant fixed effect ($p < 0.05$). The ANOSIM-R value indicated the extent to which the groups differed (R > 0.75: highly different; R= 0.50–0.75: different; 0.25–0.50: slightly different; R< 0.25: similar with some differences) (Pethybridge *et al.,* 2010). ANOVA tested for significant ($P < 0.05$) main effects of the factors (station, season, month) and their interactions on FA composition and total lipid amount. Variations and significant differences between the groups were investigated with the TUKEY HSD test using STATISTICA software.

3. RESULTS AND DISCUSSION

3.1. Effect of factor groups on the nutritional quality of *C. macrostomus*

In the present study, total lipid fatty acid composition was determined under the influence of the different factors as fatty acid composition of the edible muscle of *C. macrostomus* changed depending on various factors.

Table 1 shows the average seasonally total lipid amount, weight and length of *C. macrostomus* during the sampling season from independent stations. The most significant differences were between autumn and winter for total lipid (p=0.0005) and summer and winter for length (p=0.005), and weight (p=0.03) (Table 2). There was no difference between the Garip and Ilıcalar Stations for total lipid (3.98%, 3.06%, respectively) weight (42.30 g, 39.71 g, respectively) or length (14.86, 14.36, respectively) of *C. macrostomus* ($p < 0.05$, Tukey HSD), although the water temperature of the Ilıcalar Station was, on average, annually 5 ºC higher than the Garip Station, (Table 2). Henderson and Tocher (1987)

Table 1. Seasonal averages of the factor groups (total lipid, weight, length)

Factors	Spring (n=11)	Summer (n=5)	Autumn (n=16)	Winter (n=15)
Total lipid (%)	2.90±1.23^{a}	3.67±2.62^{ab}	5.48±2.93^{b}	2.24±0.83^{a}
Weight (g)	40.77±20.12^{ab}	66.10±6.04^{a}	46.47±24.88^{ab}	28.1±14.10^{b}
Length (cm)	15.26±2.65^{ab}	17.76±0.64^{a}	14.68±3.03^{ab}	13.23±1.94^{b}

Table 2. Seasonal water temperatures (ºC) at the Stations (Garip and Ilıcalar) during the sampling period

SPRING	SUMMER	AUTUMN	WINTER	ANNUAL
GARIP				
10.93	21.91	12.67	8.03	13.39
ILICALAR				
12.63	23.57	21.73	16.67	18.65

reported that temperature had no direct effect on body lipid content. It was emphasized by Kheriji *et al.* (2003) that when the temperature rises, an indirect effect can be seen related to the increased appetite of the fish.

All fatty acids were used in the multivariate analysis of the 47 samples. In PERMANOVA analyses of the fatty acid, data were factored by season, station, gender, total lipid, length and weight groups.

The total lipid groups gave the highest Pseudo-F (5.53) for all the factors and the lowest *P(perm)* value (0.001). Thus, changes in fatty acids in total lipid groups for all the factor groups were significantly different from each other. Season gave the second lowest *P(perm)* value (0.001, with the highest Pseudo-F (4.76). Autumn-winter and spring-autumn were significantly different from the other seasons *P(perm)*=0.001. However, autumn-winter presented the most significant difference (t=3.83) among seasons. The length groups were statistically the third most important factor group. The Pseudo-F value was 3.37, *P(perm)*=0.02. Station difference was in fourth place, with (*P(perm)*:0.02, Pseudo-F:2.74). The station factor was followed by gender with (*P(perm):*0.05, Pseudo-F:2.25) and weight with (*P(perm):*0.13, Pseudo-F:1.55). Therefore, the weight and gender were statistically the least important factor group for the fatty acid composition of *C. macrostomus;* whereas total lipid, season and station were statistically important factor groups for *C. macrostomus.* For this reason, we only used the statistically most effective factor groups (season, total lipid) in the study.

3.2. The most effective factors on nutritional quality of *C. macrostomus*

Evaluation of the stations revealed that the seasonal difference in fatty acids in *C. macrostomus* was very different at both the Garip (ANOSIM-R: 0.64, Pseudo-F: 4.68, *P(perm)*:0.001) and the Ilıcalar Stations (ANOSIM-R: 0.47, Pseudo-F: 4.13, *P(perm)*=0.001). However, locational differences in fatty acid composition within the station were higher in the Garip than the Ilıcalar Station. Although *P(perm)* values for the stations were the same (0.001), they had different ANOSIM-R values and the ANOSIM-R value was significantly different for the Garip Station.

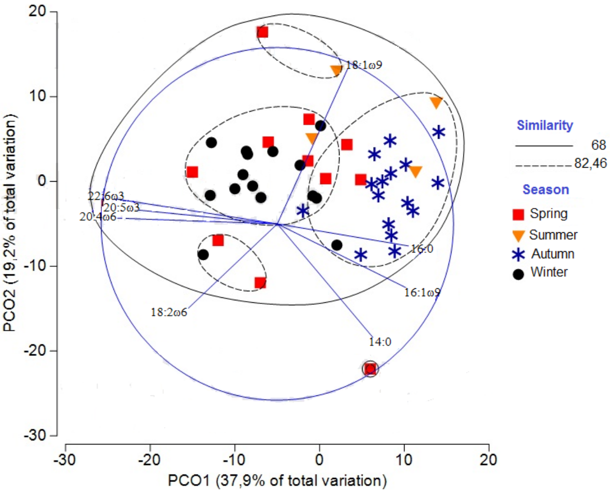

Figure 1. Two-dimensional configuration plot of a PCO analysis of a resemblance matrix of fatty acids in seasons. The lower tringular matrix was created using Bray-Curtis similarity coeffients. Pearson correlation > 0.65.

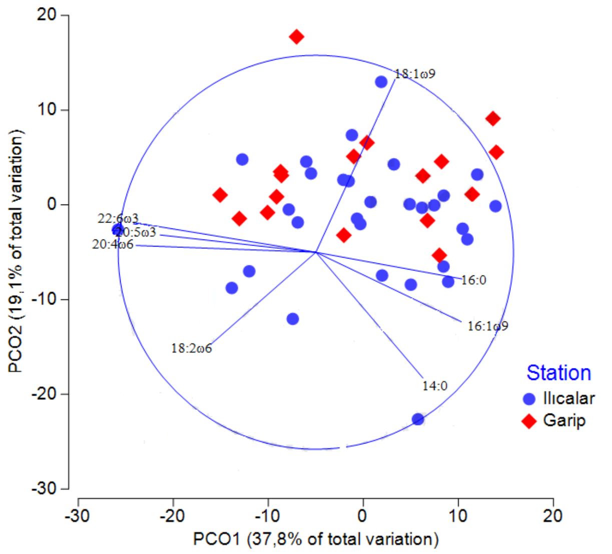

Figure 2. Two-dimensional configuration plot of a PCO analysis of a resemblance matrix of fatty acids in the stations. The lower tringular matrix was created using Bray-Curtis similarity coeffients. Pearson correlation > 0.65.

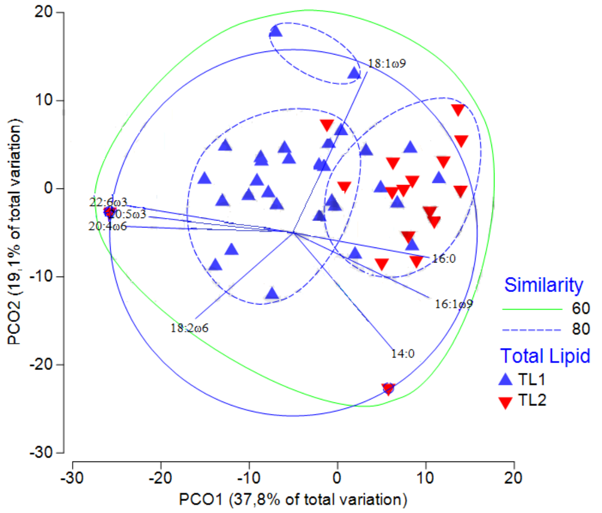

Figure 3. Two-dimensional configuration plot of a PCO analysis of a resemblance matrix of fatty acids in the total lipid groups (TL1, TL2). The lower tringular matrix was created using Bray-Curtis similarity coeffients. Pearson correlation > 0.65.

The figures show a two-dimensional configuration plot of a PCO analysis of resemblance matrix for total fatty acid data. 18:1ω9 was the major fatty acid in all the factor groups. Figure 1 shows that autumn was characterized mostly by 16:0; whereas spring, and especially winter, were characterized mostly by 18:1ω9 with 82.46% similarity. 14:0, 18:2ω6, 18:1ω9, EPA, DHA, ARA, 16:0 and 16:1ω9 were the main fatty acids for all seasons with 68% similarity. 18:1ω9 and 16:0 was the main fatty acid at both stations. However, 16:0 was more in the foreground at the Ilıcalar Station than at the Garip Station (Figure 2).

Figure 3 shows that TL1 was characterized mostly by 18:1ω9 and EPA, DHA and ARA. The fatty acids presented in TL1 are higher than TL2 with 80%. 16:0 was the most characteristic fatty acid for TL2 with 80% similarity. Also, seasonal differences for fatty acid composition were significantly important within the total lipid groups. TL1 factor group gave more seasonal differences (Pseudo-F: 2.45, *P(perm)*:0.002) than TL2 (Pseudo-F: 1.73, *P(perm)*:0.11). However, the most important difference was between summer and winter *(P(perm)*:0.002, t:2.15) in the TL2 factor group. Additionally, all the factor groups were characterized by ARA, 18:2ω6, EPA and DHA at between 60-65% similarity. Based on these results, this study showed that the most influential factors were firstly lipid amount and secondly season factors on the fatty acid composition of *C. macrostomus*. Therefore, it was decided that it would be most appropriate to examine the fatty acids that reveal the nutritional quality of *C. macrostomus* depending on these factors. The weight, especially the length and gender were not important factors in determining the fatty acid composition of *C. macrostomus*.

It was found that total MUFA was higher than total PUFA and total SFA in both stations. Only ΣPUFAs were higher than ΣMUFAs in winter at the Garip Station (Table 2). Güler *et al.* (2008) indicated that 16:0 was the primary SFA, with 14.6–16.6% for carp in all seasons. Similar results were reported by Kolakowska *et al.* (2000) for carp. Generally, fish species are relatively low in SFA (< 30%) except for some species (Güler *et al.,* 2008). Similar results were identified in this study for all seasons for *C. macrostomus* and ΣSFA varied from 24.62-32.38% (spring-autumn) at the Garip Station, with 28.85-31.76% (winter-autumn) at the Ilıcalar Station. 16:0 was the main SFA and higher in summer (20% and 25%, respectively) and autumn (22% and 23%, respectively) than the other seasons at both the Ilıcalar (Table 1) and Garip Stations (Table 1).

The ΣPUFA/ΣSFA ratio (P/S) is an index used to express the nutritional quality of dietary lipids. Generally, foods with a P/S ratio of less than 0.45 are considered undesirable for the human diet because of their potential to induce hypercholesterolemia (Fernandes *et al.,* 2014). Matos *et al.* (2019) found that the P/S ratios were as > 0.45 for the Grass, Common and Bighead carps (0.50-0.60); while the P/S was < 0.45 for Nile tilapia (cage and pond) and Silver carp (0.10 - 0.44). Ramos-Filho *et al.* (2008) reported P/S < 0.45 for freshwater fish fillets of cachara (0.44) and pacu (0.13). The P/S ratio was < 0.45 in all seasons and at both stations for *C. macrostomus* in the study. The lowest P/S ratios were 0.88 (summer), 0.73 (autumn) at the Garip Station and 0.71 (autumn) at the Ilıcalar Station. Furthermore, P/S was >1 for *C. macrostomus* in the other season at the stations. The highest ratio was 1.47 (winter) at the Garip Station and 1.22 (summer) at the Ilıcalar Station. However, the P/S ratio alone may not be sufficient to determine the nutritional quality of lipids, as it does not consider the metabolic effect of MUFAs (e.g 18:1ω9). 18:1ω9 was generally the main fatty acid in *C. macrostomus* in all the factor groups. 18:1ω9 was the highest value in summer and autumn (23%) at the Ilıcalar Station (Table 2); whereas it was the highest value in spring and summer (27%) at the Garip Station (Table 3). 18:1ω9 is the predominant MUFA in *Cyrinus carpio* from Cyprinidae (Güler *et al*., 2008).

Cengiz *et al.* (2010) researched the fatty acid compositions of total lipids in the muscle tissues of nine freshwater fish from the River Tigris, Turkey. They found that *C. macrostomus* had the low ΣMUFA (15%) and 18:1ω9 was the main MUFA. In addition, 18:1ω9 was significantly higher in summer than in winter for *Carassius gibelio* and *Sander lucioperca,* but it was higher in winter than in summer for *Cyrinus carpio* and *Leuciscus lepidus*. Also, they explained that ARA, DHA and EPA were the major fatty acids. The fact that *C. macrostomus* had both high 18:1ω9 and long-chain ω3 in all the factor groups suggested that consuming this fish fillet would be beneficial to human health. 18:1ω9 was not only the main fatty acid in ΣMUFA, but also the main fatty acid among all the fatty acids of *C. macrostomus*. 18:1ω9 has several health benefits, such as increasing HDL (high-density lipoprotein) content and lowering blood pressure (Hlais *et al.,* 2013).

18:1ω9 is used to estimate the nutritional quality of the lipids along with other hypocholesterolemic fatty acids (18:3ϖ6, C18:3ϖ3, EPA, DHA). 12:0, hypercholesterolemic fatty acids (14:0, 16:0), and 18:0 are also used to estimate the nutritional quality of lipids. The Σhypocholesterolemic/Σhypercholesterolemic fatty acid index (h/H) is an important additional index for determining the effect of individual fatty acids on cholesterol metabolism (Santos-Silva *et al.,* 2002). In terms of nutritional value, a higher h/H ratio is directly proportional to the higher PUFA content, which is considered more beneficial for human health. Carp, Nile tilapia (cage) and Grass carp have high h/H values which range between 2.15 and 2.94. These freshwater fish have a nutritional quality which is comparable to the h/H values of marine fish such as mackerel or sardines with an average of 2.46 h/H. (Fernandes *et al.*, 2014). Conversely, h/H values were reported at 1.84 for Pintado and 1.49 for Dourado from Brazilian freshwater fish fillets by Ramos-Filho *et al.* (2008). In this study, it was between 1.15-2.39 (summer-spring) at the Garip Station (Table 2) and 1.34-2.20 (autumn-summer) at the Ilıcalar Station (Table 2) for *C. macrostomus*. Therefore, summer at the Ilıcalar Station and spring at the Garip Station were the best the periods for the nutritional quality of *C. macrostomus* in terms of h/H. Also, the atherogenicity (AI) and thrombogenicity (TI) indexes are two other frequently used indexes to show the potential to stimulate platelet aggregation. [(12:0 + (4 x 14:0) + 16:0)] / (ΣMUFA + Σϖ6 + Σϖ3) is given for AI. (14:0 + 16:0 + 18:0) / [(0.5 x ΣMUFA) + (0.5 x Σϖ-6 + (3 x Σϖ3) + (Σϖ3/ Σϖ6)] is given for TI (Santos-Silva *et al.,* 2002). Foods with low AI and TI values have a greater potential to protect against coronary disease. Matos *et al.* (2019) indicated that AI values ranged between 0.34 and 0.88 in the Common carp with 0.34 and Nile tilapia (cage) with 0.42, showing the lowest AI values. The Pintado and Pacu fish species were reported to have comparable AI values (0.49, 0.86, respectively) by Ramos-Filho *et al.* (2008). The TI value was lower in the Bighead carp fillet (0.47) than the freshwater fish Cachara (0.59). However, the TI value was the lowest in the marine fish White needle (0.44) (Fernandes *et al*., 2014). AI was found between 0.38-0.49 (summer-winter) at Ilıcalar (Table 2) and 0.31-0.43 (spring-winter) at the Garip Station (Table 3) for *C. macrostomus*. TI was between 0.26-0.39 (summer-autumn) at the Ilıcalar Station and 0.22-0.40 (spring-autumn) at the Garip Station. Therefore, summer at the Ilıcalar Station and spring at the Garip Station were the best the periods for the nutritional quality of *C. macrostomus* in terms of AI and TI, similar to h/H.

Table 3. Fatty acid composition of *C. macrostomus* at Ilıcalar Station during the sampling period (% Total FAME).

Fatty Acids	SPRING (n=9)	SUMMER (n=2)	AUTUMN (n=10)	WINTER (n=9)
14:0	3.78±0.90	1.38±0.15	5.36±0.45	3.59±1.19
15:0	-	-	0.68±0.55	-
16:0	19.14±2.87	20.03±3.21	21.82±1.30	19.14±1.46
16:1ω11	1.32±1.04	0.82±0.91	0.50±0.70	1.24±0.75
16:1ω9	11.10±2.50	3.74±1.83	15.78±2.66	10.17±2.71
16:1 ω7	0.91±0.56	0.76±0.62	1.81±1.01	1.29±0.63
ι17:0	0.71±0.25	1.10±0.44	0.61±0.24	0.68±0.14
16:2ω4	0.72±0.24	-	1.07±0.54	0.52±0.31
17:0	0.94±0.23	1.13±0.44	0.94±0.23	0.67±0.51
17:1	1.13±0.56	-	0.62±0.50	0.85±0.54
16:3ω3	0.73±0.68	-	-	-
18:0	4.29±2.55	6.46±3.96	2.77±1.53	4.79±1.97
18: ω11	0.62±0.46	-	-	-
18:1ω9	19.42±9.33	22.91±13.79	22.50±2.39	19.96±3.14
18:1ω7	1.43±1.58	1.14±1.28	1.35±1.20	2.14±1.52
18:1ω6	-	0.74±1.05	-	-
18:2ω 6	2.93±0.54	2.36±0.77	2.61±0.54	2.93±0.80
18:3ω4	0.52±0.44	0.68±0.03	0.55±0.36	0.57±0.27
ALA	8.29±1.76	9.06±0.81	6.88±1.82	7.36±2.07
20:1ω9	1.22±0.73	0.75±1.06	0.83±0.82	1.05±0.83
20:2α	-	0.56±0.79	-	-
20:3ω6	0.86±0.45	1.66±0.91	0.34±0.20	0.46±0.36
ARA	1.64±0.33	2.64±1.53	0.74±0.22	1.62±0.41
20:3ω3	0.99±0.44	0.60±0.85	0.66±0.33	0.98±0.53
20:4ω3	0.78±0.24	-	-	0.65±0.47
EPA	4.27±1.32	5.14±0.95	3.31±0.95	5.54±1.09
21:5ω3	0.64±0.38	0.88±0.88	0.28±0.16	0.53±0.24
22:5ω3	1.54±1/74	1.44±0.60	0.50±0.24	0.95±0.22
DHA	6.03±2.56	10.62±3.98	3.83±0.97	8.39±1.82
24:1	-	0.54±0.15	-	-
ΣMFA*	3.69±0.98	2.72±0.78	4.80±1.76	4.42±1.45
ΣSFA	29.13±4.76	29.34±1.38	31.76±2.29	28.85±2.33
ΣMUFA	38.11±7.22	32.73±12.47	44.47±2.46	38.13±4.66
ΣPUFA	31.36±4.48	36.04±10.33	22.31±1.92	31.50±5.07
P/S	1.10±0.22	1.22±0.29	0.71±0.09	1.10±0.25
ω3	23.31±3.36	27.81±7.79	16.17±1.33	24.53±4.00
DHA/EPA	1.46±0.58	1.97±1.01	1.17±0.11	1.53±0.27
Bacterial	4.33±1.12	4.62±1.47	3.66±0.68	3.75±0.97
Zooplankton	1.26±0.74	1.17±1.12	0.83±0.82	1.05±0.83
Terrestial	11.22±2.16	11.42±0.03	9.48±1.97	10.29±2.73
ω6	6.53±1.78	7.63±4.58	4.35±0.80	5.86±0.94
ω6/ω3	0.28±0.80	0.27±0.23	0.27±0.21	0.34±0.26
16:1ω7/16:0	0.05±0.03	0.08±0.03	0.09±0.05	0.05±0.03
h/H	1.67±0.45	2.20±0.98	1.34±0.85	1.81±0.87
AI	0.50±0.12	0.38±0.09	0.67±0.12	0.49±0.05
TI	0.28±0.08	0.26±0.05	0.39±0.09	0.28±0.07

*: Minor FAs (MFAs) with mean proportion < 0.5 in all the sampling periods. Values are mean 95% confidence interval. ΣSFAs, total saturated fatty acids; ΣMUFAs, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; ALA, alpha linoleic acid; AI, atherogenicity index; TI, thrombogenicity index; h/H, The Σhypocholesterolemic/Σhypercholesterolemic fatty acid index; ι, iso-branched FAs; ω, omega fatty acids; P/S, Polyunsaturated fatty acids/Saturated fatty acids

18:3ω3 (Alpha Linolenic Acid, ALA) is the metabolic precursor to ω3 long-chain polyunsaturated fatty acids (LC-PUFA) such as EPA and DHA (Brenna, 2002). 18:2ω6 (Linoleic acid, LA) is the precursor to ARA, one of the precursors to the biosynthesis of eicosanoids, which perform important functions in the human body (Aguiar *et al.*, 2007). EPA and DHA provide human health, early development and prevention of some diseases. Therefore, dietitians have been increasingly recommending the consumption of foods containing these fatty acids (Jobling and Leknes, 2010) as ω3 LC-PUFA cannot be adequately biosynthesized by humans. Thus, the fatty acids must be obtained from the diet (Williams and Burdge, 2006). Also, fish cannot synthesize these fatty acids and obtain them from the food they consume such as algae and plankton (Falk-Petersen *et al.,* 1998). Freshwater fish lack the ability to produce certain fatty acids, especially C18 acids such as 18:2ω6 and 18:3ω6, and can directly take many long-chain polyunsaturated fatty acids such as ARA, DHA, EPA from their prey (Tocher, 2010). They are essential to the overall health of organisms and most consumers synthesize them inefficiently from their precursors (eg, 18:3ω3 and 18:2ω6). Many studies highlighted that terrestrial plants synthesize 18:3ω3+18:2ω6 fatty acids in abundance and they are used as a dietary marker in the fatty acid composition of aquatic organisms. Additionally, 18:1ω9 and 18:2ω6 are available from primary producers only. Two essential fatty acids are obtained from the diets of animals such as EPA and DHA (Parrish, 2009). Brown and red algae, vascular plants and dinoflagellates are their main sources (Kelly and Scheibling, 2012). Also, 18:1ω9 is used as a characteristic fatty acid marker for cryptophyceae along with dinophyceae and chlorophyta (Napolitano, 1999). EPAs are fatty acid markers of diatoms from Bacillariophyceae; while DHAs are fatty acid markers of dinoflagellates from Dinophyceae (Viso, and Marty, 1993). This study showed that *C. macrostomus* contain a substantial amount of PUFAs ω3 with carbon chains with C20 and C22 in all the factor groups.

Increasing water temperature increases food intake, and reduces food efficiency. The growth of fish growth can also be adversely affected by this (Norambuena *et al.*, 2016). When aquatic organisms are exposed to high water temperatures, PUFAs increase and SFAs decrease (Şen Özdemir *et al.,* 2017; Wijekoon *et al.,* 2021). 18:3ω3, ALA is lost more with the increase in temperature (Turchini and Francis, 2009). However, this higher disappearance was not associated with the higher appearance of ω3 fatty acid bioconversion products. Regarding the apparent in vivo enzymatic activities, the apparent elongations in in vivo activity were not affected by temperature considering both the ω6 and ω3 pathways (Mellery *et al.*, 2016). In the study, ALA was generally lower at the Ilıcalar Station, where the temperature was higher than at the Garip Station. It was only slightly higher at the Ilıcalar Station (8.23%) than at the Garip Station (7.92%) in spring. Here, the temperature difference between the stations was very low in spring compared to the other seasons (Table 2). PUFAs were higher at the Garip Station except for summer than at the Ilıcalar Station throughout the year (Table 1, Table 3). Omega 3 LC-PUFA comprised at least 40% of the ΣPUFA in *C. macrostomu*s in the present study. The ω3 LC-PUFA content of the plant lipids which the fish are fed was affected because of the change in the lipid bioconversion capacity (Mellery *et al.*, 2016). *C. macrostomus* can use the food taken at a high temperature (35 ºC in Sivas) to a minimal extent and the high temperature significantly affects metabolic activity. Water temperature varied between 12.63 ºC (spring) and 23.57 ºC (summer) at the Ilıcalar Station, 8.03 ºC (winter) and 21.91 ºC (summer) at the Garip Station (Table 2). Similarly, the highest ΣPUFAs (36%) of *C. macrostomus* were in low temperatures in winter (8 °C) at the Garip Station. The temperature at Ilıcalar Station was higher than at Garip Station throughout the year (Table 1). It is thought that the water temperature was especially effective in this difference.

Several researchers have suggested ω6/ω3 as a useful indicator of fish lipids' nutritional value, and a lower ratio is more effective in preventing cardiovascular diseases associated with plasma lipid levels (Rhee *et al.,* 2017). According to nutritional recommendations (FAO, 2014), the ω6/ω3 ratio should not exceed 5.0 in the human diet. Matos *et al.* (2019) reported that the ω6/ω3 ratio was 8.16 for Nile tilapia (cage), 5.40 for Carp (5.40) and 5.27 for Grass carp. High ω6/ω3 in the edible muscle of fish may be due to the high 18:2ω6 levels in terrestrial plant-based feed products used in modern aquaculture (Simat *et al.,* 2015). Also, consumption of low ω3 PUFAs and excess ω6 PUFAs is highly associated with the

Table 4. Fatty acid composition of *C. macrostomus* at Garip Station during the sampling period (% Total FAME).

Fatty Acids	SPRING (n=2)	SUMMER (n=3)	AUTUMN (n=6)	WINTER (n=6)
14:0	1.36±1.69	2.82±0.69	3.36±1.05	2.70±0.68
16:0	16.91±12.50	25.12±1.25	23.28±1.77	19.03±1.75
16:1ω11	1.41±0.35	0.53±0.57	1.28±1.07	0.62±0.43
16:1ω9	5.83±5.28	10.97±2.96	11.60±1.30	9.94±1.31
16:1ω7	-	-	-	0.66±0.43
ι17:0	0.60±0.38	0.61±0.04	0.58±0.14	-
16:2ω4	0.92±0.15	1.69±0.34	0.52±0.47	0.59±0.22
17:0	0.74±0.11	0.70±0.05	0.84±0.20	0.63±0.38
16:3ω4	-	-	0.81±1.26	-
17:1	-	0.75±0.04	0.95±0.65	0.85±0.16
18:0	5.39±1.99	2.25±2.01	4.21±0.85	4.91±0.44
18:1ω11	0.85±0.11	0.54±0.20	-	0.51±0.46
18:1ω9	26.88±11.90	26.45±4.78	24.39±4.36	19.64±3.53
18:1ω7	0.87±0.44	0.83±0.80	1.31±1.37	0.54±0.53
18:2α	0.57±0.02	-	-	-
18:2ω6	2.70±1.51	2.18±0.38	2.33±1.06	3.05±0.49
18:3ω6	0.53±0.11	-	-	-
ALA	-	-	0.65±0.49	0.53±0.29
20:1ω9	7.92±3.74	10.09±0.17	8.39±1.68	8.89±1.22
20:2ω6	-	-	1.87±1.00	-
20:3ω6	0.55±0.07	-	0.68±0.36	0.96±0.40
ARA	2.35±0.85	1.15±0.97	0.93±0.46	2.12±0.46
20:3ω3	0.85±0.32	0.94±0.24	0.86±0.30	0.87±0.12
20:4ω3	0.76±0.13	0.70±0.14	0.59±0.26	0.59±0.36
EPA	6.29±3.15	2.52±1.93	2.43±1.41	7.47±1.84
21:5ω3	0.64±0.20	-	-	-
22:5ω3	1.21±0.51	0.88±0.36	0.56±0.25	0.62±0.49
DHA	9.88±4.64	3.93±3.47	3.16±1.57	10.57±1.48
ΣMFA*	4.67±0.97	4.33±1.02	5.17±1.65	2.44±0.23
ΣSFA	24.62±18.75	31.37±4.03	32.38±1.91	27.75±0.96
ΣMUFA	37.74±23.67	40.87±4.41	42.94±3.26	33.35±2.79
ΣPUFA	36.35±18.69	26.69±7.99	23.56±3.13	38.09±3.68
P/S	1.47±0.61	0.88±0.38	0.73±0.12	1.38±0.17
ω3	27.69±14.51	19.71±6.51	16.60±2.62	29.67±2.93
DHA/EPA	1.57±0.89	1.50±0.22	1.38±0.38	1.45±0.18
Bacterial	2.77±1.97	2.94±0.16	3.48±0.74	2.75±0.49
Zooplankton	-	-	1.93±0.96	-
Terrestial	10.62±5.35	12.27±0.42	10.72±2.15	11.94±1.45
ω6	6.46±3.48	4.29±1.64	4.74±0.71	6.73±0.95
ω6/ω3	0.23±2.65	0.22±0.52	0.29±0.48	0.23±0.51
16:1ω7/16:0	0.02±0.02	0.02±0.02	0.03±0.02	0.02±0.02
h/H	2.39±1.09	1.18±0.98	1.15±0.34	1.76±0.97
AI	0.31±0.09	0.56±0.12	0.57±0.10	0.43±0.12
TI	0.22±0.05	0.35±0.07	0.40±0.04	0.24±0.03

*: Minor FAs (MFAs) with mean proportion < 0.5 in all the sampling periods. Values are mean 95% confidence interval. ΣSFAs, total saturated fatty acids; ΣMUFAs, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; ALA, alpha linoleic acid; AI, atherogenicity index; TI, thrombogenicity index; h/H, The Σhypocholesterolemic/Σhypercholesterolemic fatty acid index; ι, iso-branched FAs; ω, omega fatty acids; P/S, Polyunsaturated fatty acids/Saturated fatty acids

pathogenesis of many modern diet chronic diseases (Simopoulos, 2008). Therefore, the ω6/ω3 ratio is an important factor for food quality. The highest ω6/ω3 was 0.34 in winter (Ilıcalar Station) (Table 2) and 0.29 in autumn (Garip Station) (Table 3) for *C. macrostomus* in the study. The values did not exceed 5.0 and Σω3 fatty acids were higher than Σω6 fatty acids. *C. macrostomus* had good fatty acid nutritional quality in its natural environment because high ω3, low ω6 improves the nutritional quality of the diet.

CONCLUSIONS

Season, total lipid amount, length, station, weight and gender were used as factor groups affecting the nutritional quality of *C. macrostomus.* It was observed that the effect of fatty acids on the nutritional quality of *C. macrostomus* varied depending on the factors. While total lipid amount, season and length were found to be the most effective factors on these changes, station, gender and weight from the factor groups did not have any effect on the nutritional quality of *C. macrostomus*. Unsaturated fatty acids such as 18:1ω9, EPA, DHA were the most important fatty acids which had an effect on nutritional quality for all the factor groups. The study indicated that *C. macrostomus* had high nutritional value for humans. It would be beneficial to introduce *C. macrostomus*, which was seen to have good nutritional value, like the other freshwater fish that are widely grown and consumed. However, continued studies are needed in order to collect more information and increase our understanding of the nutritional value and health benefits of *C. macrostomus* for human consumption in different regions and aquaculture experiments. It should be kept in mind that the fish diets have a significant effect on the change in fatty acid composition, especially in aquacultural studies.

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DECLARATION OF COMPETING INTEREST

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

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Kinetic and thermodynamic studies of the oil extracted from Phoenix seeds

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SUMMARY: In this work, phoenix (*Firmiana simplex* L.) seed oil from Sterculiaceae was extracted using ultrasound-assisted and Soxhlet methods, and physicochemical properties and fatty acid composition were compared and analyzed. The kinetic and thermodynamic properties of the extraction process of Phoenix seed oil were also evaluated. The results showed that the common physicochemical properties of the oil samples extracted by the ultrasound-assisted method were lower than those of the Soxhlet extraction method. In the range of 293 K to 323 K, the effective diffusion coefficient of Phoenix seed oil was significantly different, and varied from $5.18\times10^{-13}m^2\cdot s^{-1}$ to $1.29\times10^{-12}m^2\cdot s^{-1}$. The entropy and enthalpy changes in the extraction were positive with values of 33.17 J/(mol·K) and 7.15 kJ/mol, respectively. This work provides the theoretical basis for the development of extraction process parameters and the design of an extraction process for Phoenix seed oil.

Keywords: *Fatty acid composition; Physicochemical properties; Soxhlet extraction method; Ultrasound extraction method.*

RESUMEN: ***Estudios cinéticos y termodinámicos del aceite extraído de las semillas de Phoenix***. En este trabajo se extrajo aceite de semilla de fénix (*Firmiana simplex* L.) de Sterculiaceae mediante métodos de ultrasonido y soxhlet, respectivamente, y se analizaron y compararon las propiedades fisicoquímicas y la composición de ácidos grasos. También se evaluaron las propiedades cinéticas y termodinámicas del proceso de extracción del aceite de semilla de Phoenix. Los resultados mostraron que las propiedades físico-químicas comunes de las muestras de aceite extraídas por el método asistido por ultrasonido eran más bajas que las del método de extracción soxhlet. En el rango de 293 K a 323 K, y el coeficiente de difusión efectivo del aceite de semilla de Phoenix fue significativamente diferente y varió de $5{,}18 \times 10^{-13}\ m^2\cdot s^{-1}$ a $1{,}29 \times 10^{-12}\ m^2\cdot s^{-1}$. Los cambios de entropía y entalpía de la extracción fueron positivos con valores de 33.17 J/(mol·K) y 7.15 kJ/mol, respectivamente. Este trabajo proporciona la base teórica para el desarrollo de los parámetros del proceso de extracción y el diseño del mismo del aceite de semilla de Phoenix.

Palabras Clave: *Composición en ácidos grasos; Método de extracción por ultrasonido; Método de extracción Soxhlet; Propiedades físico-químicas*

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1. INTRODUCTION

The Phoenix (*Firmiana simplex* L., a family of Sterculiaceae) is an ornamental tree found in many provinces of China (Woo *et al*., 2016). Phoenix tree leaves can be used as feedstock for biomass carbon (Tian *et al*., 2018). Phoenix tree seeds, an undeveloped woody plant resource, are also known as tung ma pea and lady's finger fruit, and have the functions of moving Qi, strengthening the spleen, stopping bleeding, and lowering blood pressure. They are effective for dietary discomfort, spleen deficiency, and stomach diarrhea. The primary fatty acids in Phoenix seed oil have been determined as sterculic acid, palmitic acid, oleic acid, and linoleic acid. In a previous study (Sun and Li, 2016), 23% sterculic acid was found in Phoenix seed oil. Sterculic acid, a cyclic propylene fatty acid, inhibits the formation of mono olefin fatty acids very effectively (James *et al*., 1968). It can be used to make isostearic acid in advanced lubricants and cosmetics. At the same time, sterculic acid can be used as a highly effective pesticide.

The oil contained in of Phoenix seed was determined to be 27.8%, which was higher than that of date palm seed (5-11%), corn (2.7-5.4%), and soybean (17.37%) (Sun and Li, 2016; Mrabet *et al*., 2015; Bauman *et al*., 1963; Politiek *et al*., 2002). However, Phoenix seeds, which produce a woody oil with a high yield, have not been developed and used in China. With the depletion of non-renewable energy sources in recent years, the development of economical and environmentally-friendly alternatives to traditional energy sources has become the focus of research (Demirbas, 2016). Due to low tailpipe emissions, and the fact that it is renewable and environmentally friendly, biodiesel has been a promising alternative (Gusniah *et al*., 2019). At present, some edible oils have been used as the feedstocks to produce biodiesel (Atapour and Kariminia, 2011), which can aggravate the imbalance between energy consumption and food consumption. Therefore, some non-edible oils, such as Phoenix seed oil, have shown great potential for biodiesel production (Liu *et al*., 2021; Sun and Li, 2020; Li *et al*., 2021; Sun *et al*., 2019; Zhou *et al*., 2021).

Vegetable oils are rich in various minerals and essential fatty acids (Zhao *et al*., 2021). Pressing, the water enzyme method, ultrasound-assisted (Komartin *et al*., 2021) and solvent extraction are often used to extract vegetable oils. In the cold pressing method, the minor active substances and protein are not destroyed. However, the residual oil rate in the meal is very high and the oil extraction rate is low. The water enzyme method is a mild method, in which no solvent is used and the process is environmentally- friendly. However, emulsification, high enzyme requirement and high production costs are the main issues. Ultrasound-assisted extraction is a green method which promotes mass diffusion and solvent penetration, and makes oil extraction easier (Barba *et al*., 2016). Therefore, ultrasound-assisted extraction has been considered an alternative to conventional solid-liquid extraction, which results in high extraction yield and short extraction time (Mrabet *et al*., 2022). Solvent extraction is a method with a high extraction rate of oils and fats, which has been widely used in industry because of its high economic efficiency.

The kinetic and thermodynamic characteristics of the Phoenix seed oil extraction process are currently not available. The diffusion coefficient and extraction temperature are the important factors for the extraction characteristics of oil. As a new type of woody oil, it is of practical significance to determine the oil diffusion coefficient and reaction activation energy, which can provide a theoretical basis for the formulation and design of the extraction process of Phoenix seed oil.

In this work, two methods (ultrasound-assisted and Soxhlet extraction) were used and compared to extract Phoenix seed oil from Phoenix seeds. The physicochemical characteristics and fatty acid composition of Phoenix seed oil extracted by the two methods were compared and analyzed. The extraction model was established and the oil diffusion coefficient and reaction activation energy of the extraction process of Phoenix seed oil were also evaluated.

2. MATERIALS AND METHODS

2.1. Materials

Phoenix tree (*Firmiana simplex* L.) seeds were provided by Anhui Bozhou Seed Industry Co. Ltd. (Anhui, China). N-hexane and anhydrous ethyl ether were purchased from Tianjin Guangfu Technology Development Co. Ltd (Tianjin, China).

2.2. Extraction of Phoenix seed oil

The Soxhlet extraction and ultrasound-assisted extraction methods were used to extract the oil samples.

Through preliminary experimental exploration, the optimal processes for oil extraction by the two methods were determined. Before extraction, the impurities in Phoenix seeds were removed. Then the Phoenix seeds were milled with a grinder and passed through a 40-mesh sieve to obtain a seed powder with an average particle size of 0.45 mm. The Soxhlet extraction method was carried out as follows: 10 g of dried and constant weight Phoenix seed powder were weighed into a round bottom flask. Next, the oil was extracted from the seed powder using n-hexane as solvent for 12 hours. N-hexane was then removed using a rotary evaporator, until no solvent was evaporated. With n-hexane being the optimal solvent, a solid-liquid ratio of 1:10 (m/v), a 15-minute extraction period, and a microwave power of 200 W were the settings for the ultrasound assisted method. A vacuum pump was used to filter the extract, and the filtrate was evaporated using a rotary evaporator.

2.3. Physical and chemical property analysis

The acid value (AV), refractive index (20 °C), peroxide value (PV), color, saponification value (SV), and phospholipid content (P) of Phoenix seed oil were determined according to the AOCS's methods Cd 3a-63, Cc 7-25, Cd 8-53, Cc 7-25, Cd 3-25, and Ca 12-55, respectively (Firestone, 2009).

For AV, 2 g oil and 20 mL mixed solvents (ethanol/ether, 1:2, v/v) were added to a 100-mL conical flask. Then, 0.2 mL 1% phenolphthalein was used as the indicator. After that, the oil solution was titrated with 0.1 N of KOH until a pink color was observed. The AV was calculated as follows:

$$AV = 56.1 \times V \times N / M \quad \text{(Eq. 1)}$$

where 56.1 represents the molecular weight of KOH; V and N represent the volume and concentration of KOH used for titration, respectively; and M represents the mass of the oil sample.

Refractive index is defined as the ratio of the speed of light in a sample to that in the vacuum (or air) at a particular wavelength. The refractive index of the oil sample was determined by an ABBE refractometer at 20 °C. Color was determined by the Rovipon colorimetric method. The color of the light passing through an oil sample with a known light range is matched to the color of the light passing through a standard glass chromatograph under the same light source.

For PV, 1 g of oil sample was weighed and added to a 100-mL conical flask. Then 20 mL mixed solvents of chloroform and glacial acetic acid (1:2, v/v), and 1 mL of saturated KI were added to the oil sample and completely mixed. After that, 20 mL of distilled water were added, and 1 ml of 1% starch solution was added and used as the indicator. Afterwards, the oil sample mixture was titrated using 0.01 N $Na_2S_2O_3$ solution. The PV was calculated as follows:

$$PV = V_{Na2S2O3} \times N_{Na2S2O3} \times 1000 / M \quad \text{(Eq. 2)}$$

where V and N represent the volume and concentration of $Na_2S_2O_3$ used for titration, respectively; and M represents for the weight of the oil sample.

For SV, 2.0 g of oil sample were weighed into a conical flask. Next, 25 mL of a 0.5 N ethanol-KOH solution were added, and then the oil sample was completely mixed. The oil solution was refluxed in a water bath for one hour. A Phenolphthalein indicator was added into the hot solution, which was titrated with 0.5 N HCl until a colorless solution appeared. The blank experiment was also used for the determination. The SV was obtained as follows:

$$SV= (A-B) \times N \times 56.1/ M \quad \text{(Eq. 3)}$$

where A and B represent the volumes of HCl consumed by the blank sample and the oil sample, respectively; N represents the concentration of HCl; and M represents the weight of the oil sample.

For P, 3.0 g of oil sample were weighed into a crucible. Then 0.5 g of zinc oxide was added, and heated on an electric furnace until the oil was completely carbonized. The crucible was put into a muffle furnace at 550-600 °C to ash for 2 hours. After the sample had been completely converted to ash, 5 mL of distilled water and 5 mL of hydrochloric acid were added to the crucible and heated gradually over low heat to a slight boil. After boiling for 5 minutes, the furnace was immediately turned off. After the solution in the crucible was cooled down to room temperature, the solution was filtered into a 100 mL flask. 50% KOH solution was then added. After a white precipitate had appeared, hydrochloric acid solution was added to make the precipitate disappear gradually, and 2 additional drops were added. At the same time, a blank control was prepared. Next, 10 mL of the solution, 8 mL of hydrazine sulfate solution and 2 mL of sodium molybdate solution were accurately pipetted, and placed in a water bath for 10 minutes. After the mixture was cooled to room temperature, the distilled water was added to adjust the volume, which was stood for 10 minutes. The prepared sample was moved into the cuvette. The UV spectrophotometer was zeroed with a blank solution, and the absorbance was measured at 650 nm. The P was calculated as follows:

$$P\ (\%) = 10\ (A-B) / (M \times V) \quad \text{(Eq. 4)}$$

where A and B represent the phosphorus content of the oil sample and blank control; M represents the weight of the oil sample; V represents the volume of the measured sample.

2.4. Total fatty acid and sn-2 fatty acid composition

FA methyl esters were prepared using potassium hydroxide-methanol in a hexane system in accordance with AOCS Ce-1b 89 (2007). The fatty acid methyl esters were analyzed using GC-MS, and the analytical procedure was the same as in the previous research (Sun and Li, 2016). The fatty acid at the sn-2 position was analyzed according to the Luddy *et al.* (1964) method with some modifications. 0.6 mL of sodium cholate solution, 2 mL of Tris-HCl buffer (1 mol/L, pH 8.0), and 0.4 mL of $CaCl_2$ were added to 0.1 g oil sample. The mixture was then combined for 1 minute in a water bath at 40 °C with 25 mg of pancreatic lipase. Finally, 1 mL of 6 mol/L HCl was added to terminate the reaction, after which 1 mL of n-hexane was added and centrifuged for separation. The supernatant was aspirated with a syringe and spotted at 1.5 cm from the bottom of the laminate. The unfolding agent was poured into the unfolding tank, and the plate was placed in the unfolding tank, which was unfolded until the solvent was 1 cm from the top of the plate. The band of the thin layer plate having glycerol monoester was scraped off for subsequent methylation operation, and the gas chromatography analysis was performed after methylation as previously described.

2.5. Kinetic study of the oil extraction

The initial stage of oil extraction is the stage where the diffusion phenomenon did not start. The kinetic model of the non-equilibrium reaction system was applied to a modified model of Fick's diffusion law with the following equation:

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=1}^{\infty} A_n \exp(-B_n t) \quad \text{(Eq. 5)}$$

where t is diffusion time; M_t is the oil extraction ratio (Phoenix seed oil/ Phoenix seeds, kg/kg) when the diffusion time is t; M_∞ is the oil extraction ratio (Phoenix seed oil/ Phoenix seeds, kg/kg) after equilibrium.

2.6. Thermodynamic study of the oil extraction

The oil extraction time should be longer than 12h, and the absolute temperatures were 293K, 303K, 313K and 323K, respectively. The extraction equation of the equilibrium constant was as follows:

$$K = \frac{(R_e)_{miscella}}{(R_e)_{soild}} \quad \text{(Eq. 6)}$$

where K is the extraction equilibrium constant; $(Re)_{miscella}$ is the extraction rate at extraction equilibrium; $(Re)_{soild}$ is the residual oil rate at extraction equilibrium.

Van't Hoff's equation was as follows:

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad \text{(Eq. 7)}$$

where K is the extraction equilibrium constant for each temperature condition; ΔS^0 is the entropy change; ΔH^0 is the enthalpy change; R is the general gas constant.

Gibbs free energy equation for the extraction was as follows:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad \text{(Eq. 8)}$$

2.7. Statistical analysis

All of the experiments were carried out three times, and the results were analyzed and processed using SPSS 20.0 and Origin 9.0 software. The results were expressed as mean ± SD. ANOVA analysis was used to analyze the differences in means. And $p < 0.05$ indicated that there was a significance difference.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties analysis

As shown in Table 1, the PV, P, SV, and AV of the oil extracted by the ultrasound-assisted method were 0.37±0.04 mmol/kg, 0.12±0.03%, 188.73±1.76 mg KOH/g, and 47.60±1.35 mg KOH/g, respectively, which were lower than the oil using Soxhlet extraction method (1.42±0.30 mmol/kg, 0.48±0.05%, 195.11±2.46 mg KOH/g, and 51.43±1.75 mg KOH/g). High AV was found for Phoenix seed oil, which was significantly higher than that of corn oil (5.13±0.51 mg KOH/g), camellia seed oil (3.7 mg KOH/g), or soybean oil (< 2.0 mg KOH/g) (Wang *et al.*, 2021; Chen *et al.*, 2017). The high AV of Phoenix seed oil was due to the hydrolysis of endogenous lipases in the seeds. These were also confirmed by the experiment which, after the seeds were stored for one year, a higher AV (60.3 mg KOH/g) of the oil extracted from the seeds was found (Li *et al.*, 2021). Compared with Soxhlet extraction method, the lower peroxide value, phospholipid content, saponification value, and acid value of Phoenix seed oil obtained by the ultrasound-assisted method may be due to the fact that the high temperature and long oil extraction time were used in the Soxhlet extraction method, which resulted in some oxidation and hydrolysis of the oil. Similar oxidation and hydrolysis were found during the extraction of papaya seed oil by Samaram *et al.* (2014). Phospholipids have antioxidant properties, and Phoenix seed oil has a low phospholipid level when compared to other oils (such as soybean oil, which has a phospholipid content of 3.2%). This makes Phoenix seed oil difficult to store (Gustone, 2002).

3.2. Fatty acid composition analysis

Table 2 shows that the different extraction methods had a minor effect on the composition and content of total fatty acids. However, some small differences in sn-2 fatty acid composition and content were found. Phoenix seed oil has the saturated fatty acid contents

Table 1. Physicochemical properties of Phoenix tree seed oil.

Properties	Ultrasound-assisted	Soxhlet extraction
Refractive index (20 °C)	1.4650 ± 0.0001	1.4662 ± 0.0003
Peroxide value (mmol/kg)	0.37 ± 0.04	1.42 ± 0.30
Phospholipid content (%)	0.12 ± 0.03	0.48 ± 0.05
Saponification value (mg KOH/g)	188.73 ± 1.76	195.11 ± 2.46
Color (Lovibond, 1 inch)	Y 5.8, R 2.5	Y 5.8, R 2.9
Acid value (mg KOH/g)	47.60 ± 1.35	51.43 ± 1.75

The values are the mean ± SD of the two different oil extraction methods used and the results are the average values of three replicates.

Table 2. Fatty acid composition of Phoenix tree seed oil (%, w/w).

Fatty acid	Total fatty acid		Sn-2 fatty acid	
	Ultrasound-assisted	Soxhlet extraction	Ultrasound-assisted	Soxhlet extraction
Palmitoleic acid	1.33 ± 0.09^{a}	1.33 ± 0.07^{a}	1.64 ± 0.06^{b}	1.18 ± 0.07^{c}
Palmitic acid	18.20 ± 0.56^{a}	18.53 ± 0.74^{a}	3.84 ± 0.07^{b}	1.83 ± 0.07^{c}
Malvalic acid	1.00 ± 0.34^{a}	1.00 ± 0.04^{a}	0.29 ± 0.03^{b}	0.22 ± 0.02^{b}
Linoleic acid	33.36 ± 0.21^{a}	32.83 ± 0.43^{a}	50.28 ± 0.03^{c}	48.26 ± 0.28^{b}
Oleic acid	21.52 ± 0.38^{a}	21.63 ± 0.96^{a}	29.20 ± 0.89^{b}	30.62 ± 0.34^{c}
Stearic acid	2.60 ± 0.21^{a}	2.67 ± 0.14^{a}	1.07 ± 0.16^{b}	0.86 ± 0.03^{b}
Sterculic acid	19.30 ± 1.79^{a}	19.43 ± 0.17^{a}	11.55 ± 0.73^{b}	13.80 ± 0.25^{c}
Nonadecenoic acid	0.77 ± 0.25^{a}	0.81 ± 0.04^{a}	0.44 ± 0.03^{b}	0.31 ± 0.02^{b}
Eicosadienoic acid	0.36 ± 0.03^{b}	0.18 ± 0.06^{a}	0.50 ± 0.07^{c}	1.75 ± 0.04^{d}
Others	1.55 ± 0.16^{a}	1.58 ± 0.06^{a}	1.17 ± 0.03^{b}	1.15 ± 0.09^{b}
Saturated fatty acids	20.8 ± 0.21^{a}	21.2 ± 0.08^{a}	4.91 ± 0.04^{b}	2.69 ± 0.05^{c}
Unsaturated fatty acids	77.65 ± 0.45^{a}	77.22 ± 0.09^{a}	93.92 ± 0.13^{b}	96.16 ± 0.04^{c}

The values are the mean ± SD of the two different oil extraction methods used and the results are the average values of three replicates. According to Duncan's multiple range test, different superscript letters (a, b, and c) in the same row show significant differences ($p < 0.05$) in the fatty acid composition of Phoenix tree seed oil between the two extraction methods.

of 20.8-21.2% for total fatty acids and 2.69-4.91% for sn-2 fatty acids. Among the saturated fatty acids, the content of palmitic acid was higher than stearic acid. Further, the content of unsaturated fatty acids was up to more than 75%. And the contents of oleic and linoleic acid in the total fatty acids were 21.52-21.63 and 32.83-33.36%, respectively, and the contents of oleic and linoleic acid in sn-2 fatty acids were classified as 29.20-30.62 and 48.26-50.28%. The high contents of oleic and linoleic acid in Phoenix seed oil means it may have the function of lowering LDL (low density cholesterol), which has a significant effect on the prevention and treatment of myocardial infarction, atherosclerosis and other diseases. Table 2 also shows that sn-2 fatty acids were mainly unsaturated (oleic acid and linoleic acid); whereas saturated fatty acids were mainly concentrated on the sn-1 and sn-3 positions, which was compatible with the fatty acid distribution pattern on the glycerol ester of vegetable oils. In addition, compared to other vegetable oils, Phoenix seed oil has two special fatty acids, sterculic acid and malvalic acid, both of which have special physiological effects. Sterculic acid and malvalic acid were mainly distributed on the sn-1 and sn-3 positions in the glycerol ester of Phoenix seed oil.

3.3. Kinetic study

In this study, different extraction temperatures (20, 30, 40 and 50 °C) were used (Figure 1). The initial stage of Phoenix seed oil extraction is the stage in which the oil on the surface of Phoenix seed powder was washed using n-hexane. At this stage, the extraction of oil from the surface of the powder was very fast. The extraction rate increased substantially at 50 °C when the extraction duration was less than 15 minutes. When the extraction time was 15 min, the extraction rate of Phoenix seed oil was 18.84% (50 °C), which was 73% of the extraction rate of Phoenix seed oil when the equilibrium was reached. However, when the extraction time was longer than 30 min, the diffusion phase of the extraction process was reached, and the increasing trend of the extraction rate of Phoenix seed oil was to slow down. When the extraction time was longer than 10 h, the equilibrium stage of the oil extraction was reached, and the maximum extraction rate of Phoenix seed oil (25.81%) was obtained.

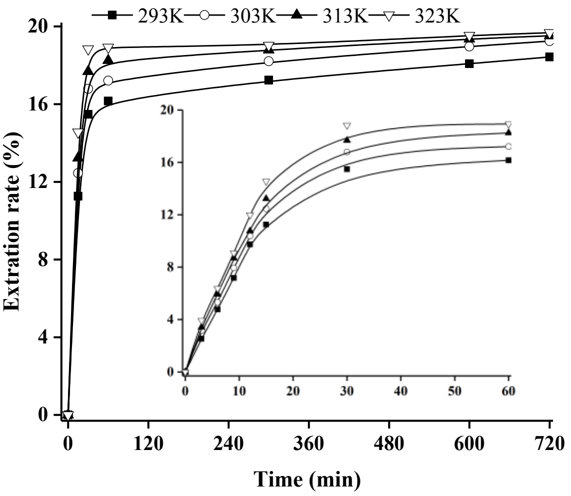

Figure 1. Effect of extraction time on the extraction rate of Phoenix seed oil with a reaction system substrate ratio (Phoenix seeds: hexane, w/v) of 1:10. The results reported are mean values, and the experiments were repeated three times.

When the extraction temperature was 20-50 °C (or 293 K to 323 K), the mass transfer between phoenix seed powder and extraction solvent was accelerated (Figure 1), and the extraction rate of Phoenix seed oil increased as the extraction temperature was raised, which was due to the acceleration of mass transfer between Phoenix seed power and the extraction solvent at a high temperature. However, in the washing stage, the increase trend was pronounced. When the extraction entered the diffusion stage, the temperature had a minor influence on the extraction rate of Phoenix seed oil.

According to the kinetic model of Fick's diffusion law by Eq. 5, the kinetic data obtained from the experiments were curve-fitted and the regression coefficients are shown in Table 3. The experimental data showed high correlation ($R^2 > 0.97$), which indi-

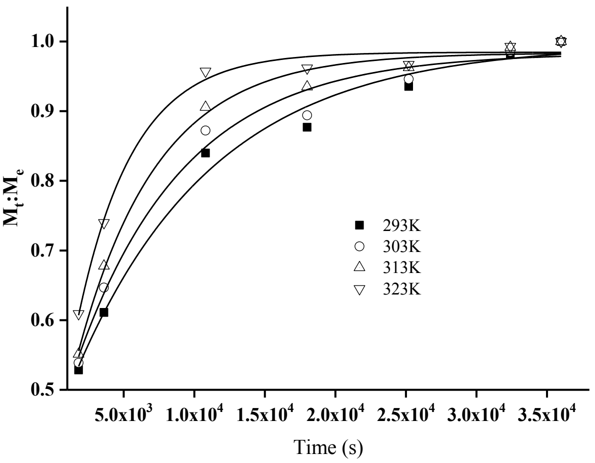

Figure 2. Extraction kinetic curve of Phoenix seed oil. The results reported are mean values, and the experiments were repeated three times.

Table 3. Parameters of Fick's diffusion law kinetic model.

Parameters	Absolute temperature (K)			
	293	303	313	323
A	0.5553^a	0.5514^a	0.5878^b	0.5944^b
$B_1(s^{-1})$	$1.01 x 10^{-4}$	$1.30 x 10^{-4}$	$1.76 x 10^{-4}$	$2.52 x 10^{-4}$
R_e	18.83^a	19.25^{ab}	19.52^b	19.68^b
R^2	0.982	0.978	0.989	0.989

The results reported are mean values, and the experiments were repeated three times. Values with different letters in the same row are significantly different at $p < 0.05$ according to one-way ANOVA followed by Duncan's multiple range test.

cated that the kinetic model could be used to characterize the extraction kinetics of Phoenix seed oil. The curve fit of Phoenix seed oil kinetic data is shown in Figure 2.

As shown in Table 3, Fick's diffusion law kinetic model parameter B_1 was the crucial factor for the effective diffusion coefficient of Phoenix seed oil extraction. The diffusion coefficient increased as the extraction temperature was raised. This indicated that the diffusion coefficient of Phoenix seed oil increased as well at the higher extraction temperature. The effective diffusion coefficient of Phoenix seed oil extraction at 20 °C was $5.18 \times 10^{-13}\ m^2 \cdot s^{-1}$. The effective diffusion coefficient of Phoenix seed oil extraction at 50 °C was $1.29 \times 10^{-12}\ m^2 \cdot s^{-1}$. The effective diffusion coefficient of rapeseed oil extracted with hexane was $1.3 \times 10^{-12}\ m^2 \cdot s^{-1}$ - $3.0 \times 10^{-12}\ m^2 \cdot s^{-1}$ (Fernandez *et al.*, 2012). The effective diffusion coefficient of sunflower seed oil extracted with hexane was $2.06 \times 10^{-12}\ m^2 \cdot s^{-1}$ - $5.03 \times 10^{-12}\ m^2 \cdot s^{-1}$ (Perez *et al.*, 2011). The effective diffusion coefficient of Phoenix seed oil extraction was lower than that of canola and sunflower seed oils, which may be due to the presence of cyclic acrylic acid in Phoenix seed oil.

3.4. Thermodynamic study

From the Van't Hoff equation (Eq. 7), the extraction equilibrium coefficient of Phoenix seed oil was used to characterize its extraction thermodynamics. The thermodynamic constants of Phoenix seed oil extraction were calculated from Eq. 6, as shown in Table 4. The equilibrium coefficient of Phoenix seed oil extraction increased with the increase in temperature, which indicated that the extraction was an endothermic process. Meanwhile, the increase in extraction temperature could accelerate the mass transfer between the oil in the Phoenix seed powder and the extraction solvent, which was beneficial to the increase in the extraction rate of Phoenix seed oil.

Table 4. Thermodynamic constants for the extraction of Phoenix seed oil at different extraction temperatures.

Temperature (K)	$(R_e)_{miscell}$	$(R_e)_{solid}$	K	lnK
293	18.43^a	6.54^a	5.45^b	1.04^a
303	19.25^b	2.82^a	3.58^b	1.21^b
313	19.52^b	5.72^b	5.29^b	1.28^b
323	19.68^b	3.37^b	3.72^b	1.31^b

The results are mean values, and the experiments were repeated three times. Values with different letters in the same column are significantly different at $p < 0.05$ according to one-way ANOVA followed by Duncan's multiple range test.

Table 5. Thermodynamic parameters of Phoenix seed oil extraction.

Temperature (K)	ΔH^0 (KJ/mol)	ΔS^0 ($Jmol^{-1}K^{-1}$)	ΔG^0 (KJ/mol)
293	7.15	33.17	-2.57^a
303	7.15	33.17	-2.90^a
313	7.15	33.17	-3.23^b
323	7.15	33.17	-3.56^b

The results reported are mean values, and the experiments were repeated three times. Values with different letters in the same column are significantly different at $p < 0.05$ according to one-way ANOVA followed by Duncan's multiple range test.

From Equations 7 and 8, it can be determined that the changes in Gibbs free energy, enthalpy and entropy during the extraction of Phoenix seed oil at different temperatures were obtained, as shown in Table 5. During the extraction of Phoenix seed oil, energy absorption was required to break through the hindrance of oil transfer from the solid to liquid phases. The entropy and enthalpy changes in the extraction were 33.17 J/(mol·K) and 7.15 kJ/mol, respectively, which also showed that the process was an endothermic process. This was mainly because the transfer of oil from the solid phase to the liquid phase was hindered during the extraction process of Phoenix seed oil, which required energy absorption. These were slightly lower than the extraction of other vegetable oils. The entropy and enthalpy changes in coconut oil extraction were 36.73 J/(mol·K) and 12.16 kJ/mol, respectively (Sulaiman *et al.*, 2013). The entropy and enthalpy changes in olive oil extraction were 59.33 J/(mol·K) and 12.91 kJ/mol, respectively (Meziane and Kadi, 2008). Compared to the extractions of coconut oil and olive oil, the enthalpy and entropy changes in Phoenix seed oil extraction were lower, which indicated that less heat was absorbed during the extraction of Phoenix seed oil and that the extraction could be performed at a low temperature. The enthalpy and entropy changes in the n-hexane extraction of Phoenix seed oil were positive and the Gibbs free energy was negative, which indicated that the extraction of Phoenix seed oil is a heat-absorbing, irreversible, and spontaneous process.

4. CONCLUSIONS

In this paper, the ultrasound-assisted and Soxhlet extraction methods were used to extract Phoenix seed oil, and the physicochemical properties and fatty acid composition of the extracted Phoenix seed oils were compared. The peroxide value, phospholipid content, saponification value, and acid value of the oil extracted by the Soxhlet extraction were higher than those of the oil using the ultrasound-assisted method. The different extraction methods had a minor impact on the composition and content of total fatty acids. However, there are some small differences in sn-2 fatty acid composition and content. In the extraction temperature ranging from 20 (293 K) to 50 °C (323 K), the effective diffusion coefficient of Phoenix seed oil was significantly different and increased from 5.18×10^{-13} $m^2{\cdot}s^{-1}$ to 1.29×10^{-12} $m^2{\cdot}s^{-1}$. The entropy and enthalpy changes of the extraction using n-hexane were positive with the values of 33.17 J/(mol·K) and 7.15 kJ/mol, respectively. However, the Gibbs free energy of the extraction using n-hexane was negative, with a value of -3.56 KJ/mol at an extraction temperature of 50 °C. This indicated that solvent extraction of Phoenix seed oil using n-hexane is a spontaneous and heat-absorbing process. This research could provide the theoretical information for the oil extraction and the design of the extraction process for Phoenix seed oil.

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