Grasas y aceites

International Journal of Fats and Oils

Volumen 74 | Nº 3 | July-September 2023 | Sevilla (España) | ISSN-L: 0017-3495

CSIC
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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

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Volume 74 • N.º 3 July-September 2023 Sevilla (España) ISSN-L: 0017-3495

MINISTERIO
DE CIENCIA
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CSIC

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Volume 74 • N.º 3 July-September 2023 Sevilla (España) ISSN-L: 0017-3495

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REVISTA PUBLICADA POR EL INSTITUTO DE LA GRASA, CSIC

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REDACCIÓN E INFORMACIÓN
Revista Grasas y Aceites
INSTITUTO DE LA GRASA, CSIC
Universidad Pablo de Olavide - Edificio 46
Ctra. de Utrera, km. 1 - 41013, Sevilla (Spain)
Tel.: +34 954 611 550
e-mail: grasasyaceites@ig.csic.es
http://grasasyaceites.revistas.csic.es

DISTRIBUTION, SALES and SUBSCRIPTIONS
Editorial CSIC / CSIC Press
c/ Vitruvio 8
28006 Madrid
Tel: +34 915 681 402
Email: publ@csic.es
editorial.csic.es

Librería Científica del CSIC
Calle de Serrano, 123 28006 Madrid
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Sale of Printed edition available until 2013 at:
http://editorial.csic.es/publicaciones/revista/17/1/10/grasas-y-aceites.html:
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ISSN-L: 0017-3495
NIPO (en línea): 833-20-022-X
Depósito legal: M 862-1958
Impreso en España - Printed in Spain.
En esta edición se ha utilizado papel ecológico sometido a un proceso de blanqueado TCF, cuya fibra procede de bosques gestionados de forma sostenible.
Maquetación: Editorial MIC

2023 CALIDAD REVISTAS CIENTÍFICAS ESPAÑOLAS
GOBIERNO DE ESPAÑA | MINISTERIO DE CIENCIA E INNOVACIÓN
FECYT INNOVACIÓN
FECYT-357/2023
Fecha de certificación: 12 de julio de 2019 (6ª convocatoria)
Válido hasta: 28 de julio de 2024

GRASAS Y ACEITES

Volume 74 • N.º 3 July-September 2023 Sevilla (España) ISSN-L: 0017-3495

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GRASAS Y ACEITES 74 (3)
July-September 2023, e510
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0909212

Antioxidant capacity of value-added sandwich cookie creams based on red palm olein during storage

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Submitted: 30 September 2021; Accepted: 17 October 2022; Published online:10 October 2023

ABSTRACT: Red palm olein (RPOL) is naturally rich in antioxidants, yet changes in its antioxidant capacity during storage were uncertain, which limited the application of RPOL in fat-based food products. Therefore, this study incorporated shortenings composed of 0, 5, and 10% (w/w) RPOL into sandwich cookie creams of SCC-0, SCC-5, and SCC-10, respectively, and determined their antioxidant capacity during storage for six months at 20, 30, and 35 ºC. Both SCC-5 and SCC-10 exhibited higher carotene, tocopherol, tocotrienol, and total phenolic content (TPC) than that of SCC-0, yet all SCCs were comparable in their free fatty acid (FFA) and UV-total oxidation values. After six months, the SCCs exhibited low TPC and FFA, yet were high in DPPH scavenging activity. At 20 and 30 ºC, both SCC-5 and SCC-10 oxidized more slowly than that of SCC-0. These findings proved the antioxidant capacity of RPOL, which delayed oxidation reactions in the SCCs during storage.

KEYWORDS: *Functional food; Nutrition; Oxidation; Palm oil; Phytonutrient; Vitamin*

RESUMEN: ***Capacidad antioxidante de cremas para galletas tipo sándwich con valor agregado a base de oleína roja de palma durante el almacenamiento.*** La oleína roja de palma (RPOL) es naturalmente rica en antioxidantes, pero los cambios en la capacidad antioxidante durante el almacenamiento no estaban claros y limitaron la aplicación de RPOL en productos alimenticios a base de grasas. En este estudio se incorporó mantecas compuestas por 0, 5 y 10 % (p/p) de RPOL en las cremas de galletas tipo sándwich (SCC): SCC-0, SCC-5 y SCC-10, respectivamente, y se determinó su capacidad antioxidante durante el almacenamiento durante seis meses a 20, 30 y 35 ºC. Tanto SCC-5 como SCC-10 exhibieron un mayor contenido de caroteno, tocoferol, tocotrienol y fenoles totales (TPC) que SCC-0, sin embargo, todas las SCC fueron comparables en sus valores de oxidación total de ácidos grasos libres (FFA) y UV. Después de seis meses, las SCC exhibieron TPC y FFA más bajos, pero fueron más altos en la actividad de eliminación de DPPH. A 20 y 30 ºC, tanto SCC-5 como SCC-10 se oxidaron más lentamente que SCC-0. Estos hallazgos demostraron la capacidad antioxidante de RPOL que retrasó las reacciones de oxidación en las SCC durante el almacenamiento.

PALABRAS CLAVE: *Aceite de palma; Alimentos funcionales; Fitonutriente; Nutrición; Oxidación; Vitamina*

Citation/Cómo citar este artículo: Mohamad Shah NK, Sanny M, Ab Karim NA, Kuppan K, Yahaya NA, Mat Yusoff M. 2023. Antioxidant Capacity of Value-Added Sandwich Cookie Creams based on Red Palm Olein During Storage. *Grasas Aceites* **74** (2), e510. https://doi.org/10.3989/gya.0909212

1. INTRODUCTION

Red palm olein (RPOL) is a novel palm-based product which, upon refining, retained approximately 80% phytonutrient compounds from crude palm oil. The phytonutrients are mainly composed of carotenoids, tocopherols, and tocotrienols which are natural vitamin A and vitamin E in the human diet (Kannan and Gundappa, 2014; Teh and Lau, 2021). The carotenoids possess antioxidative properties which contribute to longer shelf life of the oil. Both α- and β-carotenes have been reported as phytochemicals of high value and reviewed in terms of their functionality in inhibiting lipid oxidation, besides being widely associated with improving human health (Teh and Lau, 2021). Specifically, β-carotene can effectively be converted into vitamin A and provitamin A, which contribute to resolving vitamin A deficiency among malnourished and poor populations suffering from severe complications such as blindness and stunted growth followed by death (Gurmu *et al.*, 2014). A number of studies have reported the potential of palm carotenoids to function synergistically with vitamin E as a super natural antioxidant in the oil (Loganathan *et al.*, 2020). Vitamin E is present naturally as tocotrienols and tocopherols in the form of several isoforms of α-, β-, γ-, and δ- (May and Nesaretnam, 2014). Vitamin E works together with carotenoids as antioxidants by neutralizing free radicals and reactive oxygen species directly attributed to the donation of hydrogen molecules (Peh *et al.*, 2016). Several human clinical studies proposed tocotrienols, especially α-tocotrienols, to exhibit outstanding anticancer activity (Meganathan and Fu, 2016; Peh *et al.*, 2016), and upon long-term supplementation, have lowered cardiovascular health risk, depending on the dose and subject matter (Meganathan and Fu, 2016).

The presence of beneficial compounds in RPOL is undisputable. Besides their nutritional and medicinal values, these compounds may also delay the oxidation reaction in a food's fat portion during storage and simultaneously enhance the product's shelf life (Ayu *et al.*, 2016; Kumar *et al.*, 2016). Fortification of RPOL in bakery and confectionery products was able to provide 15-200% of the recommended dietary allowances (RDA) for β-carotene per portion of product consumed (Benede, 2001). El-Hadad *et al.* (2010) and El-Hadad *et al.* (2011) found enhanced oxidative stability of biscuits and chocolate spread incorporated with RPOL during storage, respectively. Manorama (2014) proved the contribution of RPOL as a food supplement in combating against vitamin A-deficiency among children in developing countries. Nurkhuzaiah *et al.* (2015) have used RPOL for frying, which resulted in higher antioxidant content in fried chicken nuggets. Loganathan *et al.* (2020) reported enhanced heat stability and low oxidative degradation of RPOL as cooking oil upon heating using different cooking techniques. These studies highlighted the growing interest in the use of RPOL due to its phytonutrients which can act as natural antioxidants in food products comparable to the synthetic ones.

Among the fat-based food products of interest is sandwich cookie cream (SCC), also termed as biscuit cream or cream filling, a confectionery product applied in between two pieces of cookies or wafers to enhance palatability. Generally, SCC should possess good shelf-stability, high resistance to oxidation and a less greasy sensation in the mouth. These properties are mainly due to its fat portion as the major ingredient in an SCC formulation (Mat Yusoff *et al.*, 2013; Cruz Serna and Gonzalez, 2014). An SCC is composed of 20-40% fat and 20-22% moisture, which contribute to its desirable stability and palatability, besides having low water activity of 0.2-0.3 in ensuring its long shelf life. The fat portion is also important in providing a smooth and creamy texture at body temperature, and in developing good creaming, quick-setting, and firm structural properties of an SCC at room temperature (Mat Yusoff *et al.*, 2013; Biswas *et al.*, 2017). Despite these functional properties, the fat portions were always composed of high saturated fat which are undesirable in terms of consumer health, yet are important in imparting the desirable physicochemical properties of the SCC. Therefore, recent studies attempted to improve an SCC formulation by reducing the fat content, besides replacing the fat portion especially the saturated fat with those of plant-based fat replacers or fats with a lower degree of saturation. The focus of these studies was mainly to obtain newly-formulated SCCs which were similar to the original SCCs in terms of physicochemical properties (Cruz Serna and Gonzalez, 2014; Biswas *et al.*, 2017).

In addition to these previous attempts, it is noteworthy that the incorporation of RPOL can further contribute to the development of value-added SCCs with enhanced antioxidant capacity. However, the antioxidant compounds undergo oxidation themselves to preserve the RPOL quality. Due to this undesirable reaction, the stability of these antioxidants in different storage conditions is uncertain, which limited the incorporation of RPOL into fat-based food products. In addition, there are very few reports on the enhancement of the nutritional properties of SCC products. Therefore, in order to fill these research gaps, this study aimed to develop and determine the storage stability of SCCs formulated with shortenings composed of 0 (SCC-0), 5 (SCC-5), and 10% (SCC-10) RPOL (w/w). The storage stability was determined based on the antioxidant capacity of the extracted fat portions of SCCs during six months of storage at 20, 30, and 35 °C, which represented variation in storage conditions upon display in the hypermarket, keeping at room temperature at home, and during transportation.

2. MATERIALS AND METHODS

2.1. Materials

Blends of RPOL:PO:PS were prepared in shortenings at different ratios of SH-0 (0:50:50), SH-5 (5:45:50), and SH-10 (10:40:50) (Mohamad Shah *et al.*, 2021). These fat portions and sunflower lecithin were provided by Sime Darby Plantation Research Sdn Bhd, Selangor, Malaysia. Other ingredients (Table 1) and plain black cookies were purchased from local bakery shops.

Table 1. Sandwich cookie cream formulation

Ingredients		Amount (% w/w)
Fat-based	Shortening	25.00
	Sunflower lecithin	1.50
Dry phase	Icing sugar	40.00
	Skim milk powder	18.00
	Wheat flour	10.00
	Salt	0.90
	Potassium sorbate	0.10
Wet phase	Vanilla extract	2.00
	Water	3.00

For the analysis methods, the chemicals were acetic acid, chloroform, Follin-Ciocalteu reagent, iso-octane, iso-propanol, methanol, n-heptane, n-hexane, petroleum ether (Merck, Darmstadt, Germany), phenolphthalein, potassium hydroxide, potassium iodide, starch, sodium carbonate, sodium thiosulphate (Fisher Scientific, Pittsburgh, PA), α-tocopherol, α-, β-, γ-, and δ- tocotrienol reagents (Davos Chemical Corp, New Jersey, USA), gallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent (Sigma-Aldrich Chemical Co., St. Louis, MO).

2.2. Preparation and storage of sandwich cookie cream

The shortenings were further used to develop SCCs (SCC-0, SCC-5, and SCC-10) based on the formulation in Table 1 and the preparation method described by Mat Yusoff *et al.* (2013) with some modifications. Fat-based ingredients were mixed and beaten in a Hobart food mixer (N50 5-Quart Mixer, Ohio, USA) at low speed (1 min), while the dry and wet ingredients were mixed separately in different bowls. The dry ingredients were further mixed into the fat-based ingredients in the mixing bowl, followed by the wet ingredients. The mixture was continuously beaten at medium (5 min) and high (5 min) speeds until it became intact. During the mixing, the mixture was scrapped and folded for every 2 min interval. Lastly, the mixture was beaten at slow speed (1 min), forming SCC. Each SCC was layered between two cookie pieces of circle-shaped cookies to mimic the commercial SCC samples and packed into a single packaging. The single packages were packed into bulk-sealed packages before being placed into containers. A similar type of packaging material was used for all samples to minimize variations due to oxygen permeability. These containers were stored in FRIOCELL incubators to minimize light exposure (MMM Medcenter Einrichtungen, GmBH, Germany) for six months at 20, 30, and 35 °C (Figure 1). The 2-, 4-, and 6-month periods were used for sample collection and analysis based on our preliminary studies which showed that changes in fat properties took place slowly in food products. Therefore, a shorter storage period would not show any significant changes in fat properties. The shelf life of a commercial sandwich cookie cream product is up to one year. Therefore, the samples developed

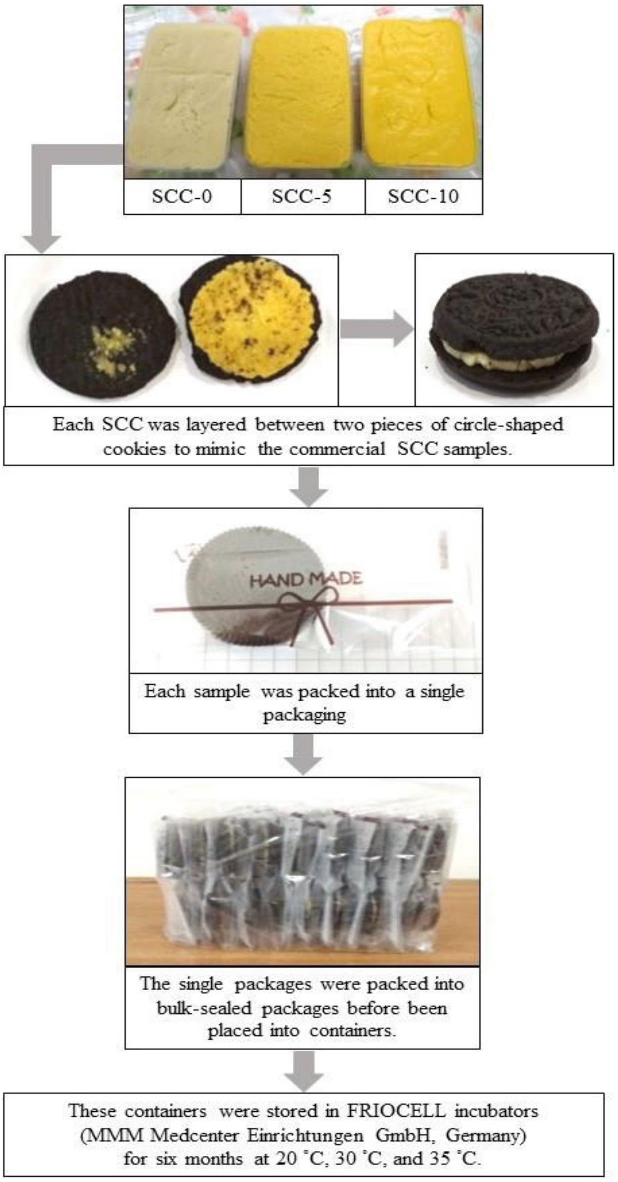

Figure 1. Preparation of sandwich cookie creams (SCC) for storage study

in this study were stored for six months only, since they were produced in the laboratory and not treated with any preservation method.

2.3. Fat extraction from sandwich cookie cream

After two, four, and six months of storage, the fat portions of the SCCs stored at all temperatures were extracted according to the MPOB Test Method (2005) with minor modifications. For each SCC, approximately 25 g were mixed with 100 ml petroleum ether and left at room temperature for 24 h. The mixture was further filtered into a round-bottom flask using Whatsman filter paper No. 1 (90 mm Ø), and the petroleum ether was removed from the mixture with a rotary evaporator (2422A0 RII Diagonal Rotary Evaporator System, Buchi, Flawil, Switzerland) at 50 °C. The flask containing the extracted fat was further heated in an oven (60 °C, 5 min) to remove the remaining petroleum ether. The extracted fat was transferred to a consumable tube and kept at 4 °C prior to the determination of antioxidant capacity.

2.4. Determination of carotene content and degree of bleachability index

The carotene content and degree of bleachability index (DOBI) (MPOB Test Method, 2005) were determined with a UV-VIS spectrophotometer (Varian Cary®, Agilent, USA). The oil sample (0.1 g) was weighed to the nearest of 0.001g into a 25-mL volumetric flask and dissolved with n-hexane. The diluted mixture was filled into a 10-mm cell and read at 446 nm for determining the carotene [Eq.1], and at 446 nm and 269 nm for the DOBI [Eq.2].

$$\text{Carotene content (ppm)} = \frac{25\ X\ a466\ X\ 10000}{W\ X\ 2610} \qquad \text{[Eq.1]}$$

a466 = absorbance reading of the oil sample
W = weight of the oil sample (g)

$$\text{DOBI} = \frac{\text{Absorbance at 446 nm}}{\text{Absorbance at 269 nm}} \qquad \text{[Eq.2]}$$

2.5. Determination of tocopherol and tocotrienol contents

Tocopherol and tocotrienol contents were determined using HPLC (MPOB Test Method, 2005). A melted sample of 0.02 g was dissolved into 5 mL n-heptane, followed by the injection of 20 µl of the mixture into the Agilent 1100 series HPLC equipped with a fluorescence detector (Agilent, USA). Emission and excitation wavelengths were set at 330 nm and 290 nm, respectively. The sample was eluted with a mobile phase consisting of heptane and isopropanol (99.5:0.5 v/v) at a flow rate of 1.4 mL/min and running time of 30 min. The column used was Luna® 5 µm silica of 250 mm length x 4.6 mm diameter (Phenomenex, California, USA) set at 35 °C, and a 95.5% tocols standard (Darvo Life Science, KLK OLEO) was used for the identification of compounds.

2.6. Extraction and determination of total phenolic content

The extraction of phenolic compounds was carried according to Abdullah *et al.* (2018) with minor alterations. The fat sample (5 g) was dissolved with n-hexane (5 ml) in a separating funnel and 10 ml methanol were added three times. For every methanol wash, the methanolic mixture was shaken for 5 min and rewashed with 10 ml n-hexane for another 1 min before being transferred to a round-bottom flask. The mixture was subjected to rotary evaporation (2422A0 RII Diagonal Rotary Evaporator System, Buchi, Flawil, Switzerland) at 50 °C for 5 min to remove the methanol and hexane. Then, the round-bottom flask was left in an oven at 40 °C for 3 hours to remove the remaining solvents. The phenolic extract was collected and kept in consumable tubes at -4 ºC prior to analysis.

The total phenolic content (TPC) was further determined using the Follin-Ciocalteu method according to Abdullah *et al.* (2018) with minor modifications. The calibration curves were prepared by using the gallic acid concentration at serial dilutions of 1000, 100, 50, 25, 12.5, and 6.25 ppm. The equation of plotted calibration curves was obtained as y = 0.007x + 0.0115, with $R^2 = 0.9985$ ($n = 6$). For sample preparation, the phenolic extracted previously was mixed with diluted Folin–Ciocalteu's reagent and sodium carbonate (Na_2CO_3). Methanol was used as blank solvent, while a mixture of methanol, Folin–Ciocalteu's reagent and Na_2CO_3 was used as a negative control. The prepared samples, standard, blank and negative control were loaded into a 96-well microplate and incubated for 30 min in a dark place, and then read at 765 nm using microplate readers (Synergy™ H1 microplate reader, BioTek Instruments, Inc, USA). The TPC was calculated according to Eq.3.

$$\text{TPC (mg GAE/g)} = \frac{(\text{absorbance}-\text{blank})-\text{c}}{\text{m}} \qquad \text{[Eq.3]}$$

c = 0.0115 (y-intercept obtained from the gallic acid standard equation)
m = 0.007 (gradient obtained from the gallic acid standard equation)

2.7. Determination of DPPH scavenging assay

A scavenging assay of DPPH was used to determine the free radical-scavenging activity of the phenolic extract according to Antoniewska *et al.* (2018) with minor alterations. For sample preparation, 50 µL of the phenolic extract were mixed with a 100 µL diluted DPPH solution. For the blank sample, 50 µL of the phenolic extract were diluted with 100 µL methanol. A mixture of methanol and diluted DPPH solution (1:2) was used as the blank solvent, while a mixture of gallic acid and diluted DPPH solution (1:2) was used as the negative control. Each mixture was loaded into a 96-well microplate which was further incubated in a dark place at room temperature for 30 min. The readings were taken at 517 nm with a microplate reader (Synergy™ H1 microplate reader, BioTek Instruments, Inc, USA) and were further used to calculate the scavenging activity according to Eq.4, expressed as mg GAE/100 g phenolic extract.

$$\text{\% DPPH Scavenging Activity} = \frac{(\text{BSO})-(\text{SA}-\text{BSA})}{(\text{BSO})} X\ 100 \qquad \text{[Eq.4]}$$

SA = absorbance of sample dilution (nm)
BSA = absorbance of blank sample dilution (nm)
BSO = blank solvent dilution of phenolic extract of the SCCs

2.8. Determination of free fatty acid

Free fatty acid (FFA) was determined according to the AOCS Official Method Ca 5a-40 (2003) using the titration method with potassium hydroxide until the solution turned pink in color. The % of FFA was calculated using the following equation:

$$\text{\% of FFA} = \frac{v\ X\ N\ X\ 256}{10\ X\ W} \qquad \text{[Eq.5]}$$

V = Volume of potassium hydroxide (ml);
N = Normality of potassium hydroxide (eq./ml);
W = Weight of the fat sample (g);
256 = Molecular weight of palmitic acid (g/mol)

2.9. Determination of peroxide value

Peroxide value (PV) was determined according to the AOCS Official Method Ca 8-53 (2003) using the titration method with 0.01 N sodium thiosulphate until the solution turned clear. The PV was calculated by using the following equation:

$$\text{PV (meq } O_2/g) = \frac{1000\,(v1-v2)N}{W} \quad \text{[Eq.6]}$$

v1 = Volume of sodium thiosulphate without fat sample (ml);
v2 = Volume of sodium thiosulphate used to titrate the blank sample (ml);
N = Normality of sodium thiosulphate (0.01 N) (eq./ml);
W = Weight of fat sample used (g)

2.10. Determination of UV-total oxidation (UV-TOTOX) value

UV-TOTOX was determined with a UV-VIS spectrophotometer (Varian Cary®, Agilent, USA) at 233 and 269 nm and was calculated by using Eq.7-10 (MPOB Test Method, 2005).

$$\text{Volume of sample mixtures} = \frac{\text{Volume of iso-octane (ml)}}{100 \text{ X weight of extracted fats (g)}} \quad \text{[Eq.7]}$$

$$\text{EC269} = \frac{[\text{volume of sample mixtures (ml) X Absorbance 269}]-(0.18\,)}{383 \text{ X carotene content (ppm)}}$$

[Eq.8]

$$\text{EC233} = \frac{[\text{volume of sample mixtures (ml) X Absorbance 233}]-(0.06)}{383 \text{ X carotene content (ppm)}}$$

[Eq.9]

$$\text{UV TOTOX} = \text{EC269} + \text{EC233} \quad \text{[Eq.10]}$$

2.11. Statistical analysis

All results were expressed as mean ± standard deviation (*n*=6) using MINITAB$^{\text{TM}}$ Statistical Software (MINITAB$^{®}$ 14.12.0, New York, USA). One-way analysis of variance (ANOVA) and Tukey's multiple comparison test at 95% confidence level was used to determine significant differences among three or more sets of data, while a two-sample t-test was used for two sets of data.

3. RESULTS AND DISCUSSION

3.1. Effect of storage conditions on the antioxidative properties of the fat portions in sandwich cookie creams.

On day 0, the carotene content in SCC-10 (84.75 ± 1.22 ppm) was significantly higher than those of SCC-5 (50.32 ± 7.94 ppm) and SCC-0 (18.47 ± 0.42 ppm) (Table 2(a)). This trend took place throughout the storage period and was in line with El-Hadad *et al.* (2011), who reported an increase in carotene from 10.0 to 148.0 ppm as RPOL increased from 0 to 20% in chocolate spread. Changes in the carotene were clearly observed after 4 and 6 months. At 20 °C, a significant decrease ($p < 0.05$) in the carotene took place only after 6 months in all SCCs. At 30 and 35 °C, the carotene started to decrease faster in SCC-0 after 4 months, and in SCC-10 after 2 months. At 35 °C, the carotene in SCC-5 started to decrease significantly ($p < 0.05$) after 4 months. These findings concluded faster carotene degradation at higher temperatures, which also occurred due to lipid oxidation. Therefore, a simultaneous decrease and increase occurred in the DOBI (Table 2(b)) and UV-TOTOX (Table 3(c)), respectively.

DOBI indicates oil stability and quality through the measurement of bleaching earth required to refine the oil, which further depends on the oil's carotene content. Greater DOBI values indicate better oil quality (Tan *et al.*, 2017). However, for RPOL, its DOBI ranged below one (< 1) due to the fact that RPOL did not undergo an intense bleaching step during the refining process (Kannan and Gundappa, 2014). Based on Table 2(b), DOBI of the developed SCCs on day 0 ranged between 0.01-0.44. Throughout the storage period, SCC-10 was significantly higher ($p < 0.05$) in DOBI, which proved its greater quality due to its higher RPOL, followed by SCC-5 and SCC-0. Despite this finding, the DOBI of both SCC-5 and SCC-10 significantly decreased ($p < 0.05$) after 2 months and remained unchanged for up to 6 months at all temperatures. The sudden decrease indicated rapid oxidation in food incorporated with RPOL, as DOBI is more affected by the oxidation level of oil and the presence of antioxidants and contaminants besides the carotene content (Corley and Tinker, 2003). After 2 and 4 months, the different temperatures insignificantly affected ($p > 0.05$) the DOBI in all SCCs, yet the

Table 2. Antioxidant properties of red palm olein-based sandwich cookie creams (SCC) during storage for six months at different temperatures as described by their (a) carotene, (b) degree of bleachability index, (c) tocopherol, (d) tocotrienol, (e) total phenolic content, and (f) DPPH scavenging activity.

(a) Carotene content

Storage temperature (ºC)	Storage month	SCC-0	SCC-5	SCC-10
-	0	18.47 ± 0.42^{cA}	50.32 ± 7.94^{bA}	84.75 ± 1.22^{aA}
20	2	18.31 ± 6.02^{cA}	48.09 ± 1.31^{bAB}	72.35 ± 0.12^{aABC}
	4	10.58 ± 0.82^{cABC}	47.06 ± 7.73^{bAB}	73.36 ± 6.31^{aAB}
	6	5.10 ± 0.60^{cC}	21.72 ± 0.65^{bCD}	42.62 ± 0.26^{aDE}
30	2	11.01 ± 1.15^{cABC}	40.97 ± 2.71^{bAB}	67.05 ± 3.12^{aBC}
	4	8.43 ± 1.18^{cBC}	42.20 ± 3.90^{bAB}	64.07 ± 2.72^{aBC}
	6	6.07 ± 1.23^{cBC}	35.24 ± 2.19^{bBCD}	56.55 ± 3.54^{aCD}
35	2	14.89 ± 7.48^{bAB}	44.62 ± 9.55^{aAB}	65.35 ± 8.22^{aBC}
	4	7.26 ± 0.20^{cBC}	36.36 ± 1.74^{bABC}	64.98 ± 2.33^{aBC}
	6	5.80 ± 0.54^{cC}	20.66 ± 3.30^{bD}	40.73 ± 5.64^{aE}

(b) Degree of bleachability index (DOBI)

Storage temperature (ºC)	Storage month	SCC-0	SCC-5	SCC-10
-	0	0.01 ± 0.01^{cA}	0.25 ± 0.02^{bA}	0.44 ± 0.00^{aA}
20	2	0.01 ± 0.00^{cA}	0.06 ± 0.00^{bB}	0.08 ± 0.00^{aBC}
	4	0.01 ± 0.00^{cA}	0.06 ± 0.00^{bB}	0.09 ± 0.01^{aB}
	6	0.03 ± 0.02^{bA}	0.06 ± 0.01^{aB}	0.08 ± 0.00^{aBC}
30	2	0.01 ± 0.00^{cA}	0.04 ± 0.00^{bB}	0.07 ± 0.00^{aC}
	4	0.02 ± 0.01^{cA}	0.04 ± 0.00^{bB}	0.07 ± 0.00^{aC}
	6	0.02 ± 0.01^{bA}	0.06 ± 0.01^{aB}	0.07 ± 0.00^{aC}
35	2	0.02 ± 0.01^{cA}	0.06 ± 0.00^{bB}	0.09 ± 0.01^{aB}
	4	0.01 ± 0.01^{cA}	0.04 ± 0.01^{bB}	0.07 ± 0.01^{aBC}
	6	0.01 ± 0.00^{bA}	0.05 ± 0.01^{aB}	0.07 ± 0.00^{aC}

(c) Tocopherol content (ppm)

Storage temperature (ºC)	Storage month	SCC-0	SCC-5	SCC-10
-	0	142.67 ± 2.08^{bA}	140.33 ± 1.53^{bA}	150.33 ± 3.06^{aA}
20	2	144.00 ± 7.00^{aA}	143.00 ± 1.73^{aA}	145.33 ± 4.04^{aA}
	4	117.67 ± 1.16^{cCD}	125.33 ± 1.15^{bBC}	128.33 ± 0.58^{aA}
	6	106.33 ± 1.53^{cE}	114.00 ± 2.65^{bF}	120.33 ± 1.53^{aA}
30	2	123.33 ± 2.08^{aBC}	129.00 ± 1.73^{aB}	168.67 ± 54.88^{aA}
	4	112.67 ± 4.73^{bDE}	121.67 ± 1.53^{aCD}	121.33 ± 2.08^{aA}
	6	109.00 ± 0.00^{cDE}	114.67 ± 0.58^{bEF}	123.67 ± 2.08^{aA}
35	2	131.33 ± 0.58^{bB}	125.67 ± 1.53^{cBC}	136.00 ± 0.00^{aA}
	4	113.00 ± 1.00^{bDE}	119.3 ± 1.53^{abDE}	121.00 ± 4.36^{aA}
	6	114.00 ± 3.61^{aDE}	120.67 ± 2.89^{aCD}	124.67 ± 5.77^{aA}

(d) Tocotrienol content (ppm)

Storage temperature (ºC)	Storage month	SCC-0	SCC-5	SCC-10
-	0	425.00 ± 4.36^{aA}	431.00 ± 1.00^{aA}	399.67 ± 54.50^{aABC}
20	2	439.00 ± 24.76^{aA}	428.00 ± 2.65^{aA}	437.33 ± 16.50^{aA}
	4	348.00 ± 3.46^{cC}	363.67 ± 1.53^{bC}	370.33 ± 0.58^{aBCD}
	6	311.33 ± 4.04^{cD}	326.33 ± 6.66^{bE}	344.00 ± 2.00^{aD}
30	2	386.00 ± 7.00^{aB}	393.00 ± 4.36^{aB}	399.67 ± 10.07^{aABC}
	4	334.67 ± 13.32^{bCD}	362.33 ± 10.21^{aCD}	349.33 ± 5.69^{abCD}
	6	320.33 ± 1.53^{cCD}	328.67 ± 1.15^{bE}	355.67 ± 4.62^{aBCD}
35	2	387.67 ± 2.89^{bB}	380.67 ± 6.11^{bB}	406.67 ± 1.53^{aAB}
	4	332.33 ± 1.53^{aCD}	348.33 ± 4.16^{aCD}	346.33 ± 12.74^{aCD}
	6	335.00 ± 9.85^{aCD}	347.33 ± 7.77^{aD}	356.00 ± 16.17^{aBCD}

(e) Total phenolic content (mg GAE/g)

Storage temperature (ºC)	Storage month	SCC-0	SCC-5	SCC-10
-	0	9.99 ± 8.08^{aA}	14.13 ± 2.65^{aA}	15.08 ± 0.40^{aA}
20	2	6.71 ± 0.12^{aA}	7.14 ± 0.80^{aAB}	7.67 ± 0.08^{aCD}
	4	4.07 ± 0.50^{aA}	4.55 ± 0.46^{aAB}	5.27 ± 1.29^{aCDE}
	6	3.08 ± 0.33^{bA}	4.08 ± 0.46^{abB}	4.79 ± 0.48^{aF}
30	2	5.21 ± 0.74^{aA}	7.05 ± 0.66^{aAB}	7.86 ± 4.27^{aB}
	4	3.83 ± 0.22^{aA}	4.37 ± 1.07^{aAB}	5.06 ± 0.11^{aC}
	6	2.13 ± 0.54^{aA}	2.94 ± 0.36^{aAB}	4.04 ± 11.29^{aEF}
35	2	4.21 ± 0.48^{bA}	6.14 ± 0.47^{aAB}	6.64 ± 0.30^{aB}
	4	3.63 ± 0.37^{bA}	4.50 ± 0.46^{abAB}	4.79 ± 0.16^{aC}
	6	1.94 ± 0.17^{aA}	2.56 ± 0.08^{aAB}	3.04 ± 0.76^{aDEF}

(f) DPPH scavenging activity (mg GAE/100g)

Storage temperature (˚C)	Storage month	SCC-0	SCC-5	SCC-10
-	0	25.88 ± 13.07^{aAB}	30.68 ± 6.56^{aA}	30.90 ± 3.88^{aA}
20	2	25.64 ± 2.00^{aAB}	23.23 ± 0.14^{aABCD}	24.89 ± 0.29^{aA}
	4	17.10 ± 1.65^{aB}	16.82 ± 0.39^{aE}	17.10 ± 0.46^{aB}
	6	30.90 ± 0.68^{bA}	27.41 ± 0.11^{cBCD}	33.48 ± 0.97^{aAB}
30	2	24.81 ± 0.90^{abAB}	24.64 ± 0.52^{bABCD}	26.31 ± 0.00^{aA}
	4	$14. 77 \pm 0.23^{bB}$	18.56 ± 1.72^{aDE}	19.47 ± 0.57^{aB}
	6	23.46 ± 1.46^{aAB}	22.95 ± 1.32^{aBCD}	25.14 ± 0.34^{aAB}
35	2	26.48 ± 1.53^{aAB}	27.48 ± 0.80^{aAB}	28.14 ± 0.76^{aA}
	4	15.46 ± 0.60^{aB}	16.93 ± 1.95^{aCDE}	17.35 ± 0.26^{aB}
	6	22.82 ± 2.74^{aA}	24.24 ± 2.63^{aABC}	26.70 ± 7.18^{aA}

MINITAB™ Statistical Software (MINITAB® 14.12.0, New York, USA) was used for the one-way analysis of variance (ANOVA) and Tukey's multiple comparison test at 95% confidence level to determine significant differences between three or more sets of data. Means ± standard deviation ($n = 6$) with different small letters in each row, and means ± standard deviation ($n = 6$) with different capital letters in each column, are significantly different ($P < 0.05$). SCC-0 (0:50:50), SCC-5 (5:45:50), and SCC-10 (10:40:50) are SCCs composed of different ratios of red palm olein: palm oil: palm stearin

DOBI of SCC-10 significantly decreased ($p < 0.05$) at 30 °C and 35 °C after 6 months, which further proved the greater oxidation rate at higher storage temperature (Leonardis *et al.*, 2016).

With reference to Table 2(c), on day 0, SCC-10 (150.33 ± 3.06 ppm) was significantly higher ($p < 0.05$) in tocopherol content followed by SCC-0 (142.67 ± 2.08 ppm) and SCC-5 (140.33 ± 1.53 ppm). At 20 °C, the tocopherol in both SCC-0 and SCC-5 decreased significantly ($p < 0.05$) after 4 months, while no significant changes ($p > 0.05$) took place in SCC-10. At 30 °C, both SCC-0 and SCC-5 started to decrease ($p < 0.05$) in tocopherol sooner, after 2 months, and further decreased ($p < 0.05$) after 4 months, while in SCC-10, it started to decrease ($p < 0.05$) later, after 4 months. These findings highlighted the decreasing tocopherol in the SCCs due to prolonged storage time, which took place faster at higher temperatures. Similar trends were observed in the case of tocotrienol (Table 2(d)). The results also revealed 15-20% loss in total tocopherol and tocotrienol contents in all SCCs, which was most likely due to oxidative activity in the fats throughout the storage period. The antioxidant compounds, including tocopherol and tocotrienol had to self-immolate in order to scavenge free radicals prior to lipid oxidation (Ayu *et al.*, 2016), thus their amount decreased with storage.

The DPPH scavenging activity (Table 2(f)) was determined based on reactivity of TPC (Table 2(e)) as an antioxidant to resist rapid lipid oxidation. SCC-10 (15.08 ± 0.40 mg GAE/g) was significantly higher ($p < 0.05$) in TPC than those of SCC-5 (14.13 ± 2.65 mg GAE/g) and SCC-0 (9.99 ± 8.08 mg GAE/g), and this trend continued throughout the storage period. On the other hand, the scavenging activities were insignificantly different ($p > 0.05$) from each other (25.88-30.90 mg GAE/100g) on day 0, and were insignificantly affected ($p > 0.05$) by temperature after 2 and 6 months. At all temperatures, the scavenging activity of the SCCs at month 4 were significantly lower ($p < 0.05$), while those of month 6 were insignificantly different ($p > 0.05$) from those of day 0. These findings concluded that the scavenging activity of the SCCs were significantly affected by storage time, but not the temperatures. In relation to the TPC after 6 months, the TPC was significantly lower ($p < 0.05$), whilst the scavenging activity was higher ($p < 0.05$). These trends indicated that the phenolic compounds underwent scavenging activity, and thus their values decreased as the scavenging activity increased (Kumar *et al.*, 2016), and the changes were more obvious after 6 months. Moreover, both TPC and scavenging activity were insignificantly affected ($p > 0.05$) by temperature. Fluctuating scavenging activity and antioxidant content during storage were also reported in blends of groundnut and sunflower oils (Sunil *et al.*, 2015), which in the latter case was most likely dependent on the rate of lipid oxidation and reactivity of antioxidant compounds.

Table 3 summarizes the antioxidants present in the SCCs in comparison with other RPOL-based food products. The presence of 5% (w/w) and 10% (w/w) RPOL in the shortenings contributed to 1.25% (w/w) and 2.50% (w/w) RPOL in the SCC-5 and SCC-10, respectively. These small amounts of RPOL contributed to higher tocopherol contents as compared to biscuits (El-Hadad *et al.,* 2010) and chocolate spread (El-Hadad *et al.*, 2011), and approximately similar tocotrienol content to that of biscuits. Moreover, the carotene content in the SCCs was comparable to the lower amount of carotene present in chicken nuggets, comprising 50-100% (w/w) RPOL (Nurkhuzaiah *et al.*, 2015). These findings highlighted the potential of SCC as another value-added food product to be developed based on RPOL.

3.2. Effect of storage conditions on the oxidative properties of the fat portions in sandwich cookie cream

The determination of FFA (Table 4(a)) is important for developing fat-based food products because it indicates the level of fat deterioration in the food (Leonardis *et al.*, 2016). On day 0, there was no significant difference ($p > 0.05$) between the FFA (0.05-0.06%) of SCC-0, SCC-5, and SCC-10, which were lower than the acceptable FFA for fresh palm-based products (< 5.0%) (Codex Alimentarius, 2017), thus indicating their low oxidation state. However, the values significantly increased ($p < 0.05$) after 2 and 4 months at all temperatures due to lipid oxidation as most likely affected by the storage conditions. Oxidative degradation still took place after 6 months, yet in unsaturated fatty acids such as linoleic and linolenic acids, which increased levels of hexanal

Table 3. Antioxidant compounds in sandwich cookie creams (SCC) composed of red palm olein-based shortenings (SH), and other red palm olein-based food products

	Sandwich cookies creams			**Red palm olein-based food products**		
	SCC-0	**SCC-5**	**SCC-10**	**Chicken nuggets (Nurkhuzaiah *et al.*, 2015)**	**Chocolate spread (El-Hadad *et al.*, 2011)**	**Biscuits (El-Hadad *et al.*, 2010)**
Red palm olein (% w/w)	0 (0% in SH-0)	1.25 (5% in SH-5)	2.50 (10% in SH-10)	50-100	0-20	40-60
Antioxidant compounds						
Carotene (ppm)	18.47 ± 0.42^{c}	50.32 ± 7.94^{b}	84.75 ± 1.22^{a}	53-505	148.0	173.0-188.0
Tocopherol (ppm)	142.67 ± 2.08^{b}	140.33 ± 1.53^{b}	150.33 ± 3.06^{a}	> 200	60.8	93.0-116.0
Tocotrienol (ppm)	425.00 ± 4.36^{a}	431.00 ± 1.00^{a}	399.67 ± 54.50^{a}	> 600	221.2	387.3-462.2
Total phenolic content (mg GAE/g)	9.99 ± 8.08^{a}	14.13 ± 2.65^{a}	15.08 ± 0.40^{a}	nd	nd	nd

nd: not determined. MINITAB^{TM} Statistical Software (MINITAB® 14.12.0, New York, USA) was used for the one-way analysis of variance (ANOVA) and Tukey's multiple comparison test at 95% confidence level to determine significant differences between three or more sets of data. For the SCC, means ± standard deviation (n = 6) with different small letters in each row are significantly different ($P < 0.05$).

but not the FFA (Heydanek and McGorrin, 1988), thus the FFA significantly decreased ($p < 0.05$). After 4 and 6 months, the FFA in all SCCs increased with increasing temperature, which highlighted the significant effect of high temperature in promoting oxidation in the SCCs.

In addition to TPC, the trend in changes in DPPH scavenging activity (Table 3(d)) from day 0 to month 6 was also in line with the trend in changes in FFA at most temperatures. On day 0, the scavenging activity was the highest while FFA was the lowest, which indicated a low oxidative state of the fat portions. After 4 months, the scavenging activity decreased significantly ($p < 0.05$) and was the lowest, while the FFA significantly increased ($p < 0.05$) and was the highest, signifying increased oxidation as storage progressed. However, after 6 months, both TPC and FFA significantly decreased ($p < 0.05$) while the scavenging activity significantly increased ($p < 0.05$) which indicated the effectiveness of TPC in decreasing the oxidation reaction.

Peroxide value (PV) indicates the initial stage of oil deterioration in terms of peroxides and hydroperoxides as primary oxidation products (Tan *et al.*, 2017). The standard limit for the PV of fresh fats and oils is less than 10.0 meq O$_2$/g (Codex Alimentarius, 2017). Low PV may also indicate the degradation of primary oxidation products into secondary oxidation products with greater oxidation reaction (Tan *et al.*, 2017). As shown in Table 4(b), on day 0, the PV of SCC-0 was 0.88 ± 0.20 meq O$_2$/g. The presence of RPOL most likely lowered the oxidation rate of both SCC-5 and SCC-10, thus their PVs were too low and could not be determined. At all temperatures, the PV of all SCCs significantly increased ($p < 0.05$), indicating that all SCCs continuously underwent oxidation throughout storage, with the highest PVs ($p < 0.05$) recorded after 6 months. Due to absence of RPOL, SCC-0 showed the highest values compared to both SCC-5 and SCC-10. In particular, after 4 months, SCC-10 exhibited insignificantly different ($p > 0.05$) PV at all temperatures. However, after 6 months, SCC-10 exhibited higher PV at 20 °C compared to 30 °C ($p > 0.05$) and 35 °C ($p < 0.05$). This finding was most likely due to the higher oxidation rate at higher temperatures (30-35 °C), which led to the degradation of primary oxidation products into secondary oxidation products and thus lowered the PV (Tan *et al.*, 2017). Furthermore, after 6 months, except for SCC-10, the PV of the SCCs (11.71-17.21 meq O$_2$/g) had exceeded the limit of PV for fresh-produced fats and oils (<10.0 meq O$_2$/g) (Codex Alimentarius, 2017). Yet, in most cases, commercial SCCs have a shelf life of at least 1 year (Daglioglu *et al.*, 2004). According to Gotoh and Wada (2006), the threshold value for PV in fat-based food should be not more than 30 meq O$_2$/g. Thus, it can be concluded that the SCCs produced in

Table 4. Oxidative properties of red palm olein-based sandwich cookie creams (SCC) during storage for six months at different temperatures as described by their (a) free fatty acid, (b) peroxide value, and (c) UV-total oxidation (TOTOX) value.

(a) Free fatty acid (%)

Storage temperature (˚C)	Storage month	SCC-0	SCC-5	SCC-10
-	0	0.05 ± 0.01^{aE}	0.06 ± 0.01^{aE}	0.06 ± 0.01^{aE}
20	2	0.96 ± 0.04^{bD}	1.10 ± 0.04^{aC}	1.08 ± 0.04^{aCD}
	4	1.32 ± 0.02^{aB}	1.31 ± 0.03^{aB}	1.30 ± 0.02^{aBC}
	6	0.94 ± 0.07^{aD}	0.88 ± 0.01^{aD}	0.85 ± 0.03^{aD}
30	2	1.14 ± 0.04^{aC}	1.14 ± 0.10^{aC}	1.07 ± 0.07^{aCD}
	4	1.50 ± 0.02^{cA}	1.64 ± 0.03^{aA}	1.56 ± 0.03^{bAB}
	6	1.16 ± 0.03^{aC}	1.23 ± 0.06^{aBC}	1.05 ± 0.17^{aCD}
35	2	1.12 ± 0.07^{aC}	1.12 ± 0.06^{aC}	1.12 ± 0.00^{aCD}
	4	1.59 ± 0.04^{bA}	1.69 ± 0.01^{aA}	1.61 ± 0.02^{bA}
	6	1.06 ± 0.04^{aCD}	1.32 ± 0.09^{aB}	1.18 ± 0.19^{aCD}

(b) Peroxide value (meq O_2/g)

Storage temperature (˚C)	Storage month	SCC-0	SCC-5	SCC-10
-	0	0.88 ± 0.20^{aB}	Null^{bC}	Null^{bE}
20	2	4.48 ± 1.93^{aB}	1.41 ± 0.14^{bBC}	1.24 ± 0.00^{bDE}
	4	4.66 ± 0.11^{aB}	4.70 ± 0.20^{aB}	3.88 ± 0.23^{bCDE}
	6	15.86 ± 0.09^{aA}	11.71 ± 0.56^{bA}	15.22 ± 0.90^{aA}
30	2	3.81 ± 0.15^{aB}	3.07 ± 0.14^{bBC}	2.82 ± 0.14^{bDE}
	4	4.86 ± 0.17^{aB}	4.52 ± 0.37^{aB}	4.71 ± 0.54^{aBCD}
	6	16.89 ± 0.45^{aA}	13.46 ± 2.76^{aA}	8.83 ± 0.83^{bABC}
35	2	4.43 ± 3.59^{aB}	3.79 ± 2.21^{aB}	3.57 ± 1.81^{aDE}
	4	4.66 ± 0.16^{aB}	4.83 ± 0.55^{aB}	4.61 ± 0.82^{aBCD}
	6	17.21 ± 4.90^{aA}	12.14 ± 1.33^{abA}	8.45 ± 1.87^{bB}

(c) UV-Total oxidation value (UV-TOTOX)

Storage temperature (˚C)	Storage month	SCC-0	SCC-5	SCC-10
-	0	1.86 ± 0.03^{bC}	2.03 ± 0.10^{aC}	1.79 ± 0.03^{bC}
20	2	4.81 ± 0.09^{aB}	4.85 ± 0.04^{aB}	4.85 ± 0.03^{aB}
	4	5.14 ± 0.19^{aAB}	5.05 ± 0.12^{aAB}	5.03 ± 0.15^{aAB}
	6	5.33 ± 0.09^{aAB}	4.89 ± 0.10^{bB}	5.32 ± 0.14^{aA}
30	2	5.02 ± 0.01^{aAB}	4.91 ± 0.14^{aB}	4.87 ± 0.10^{aB}
	4	5.38 ± 0.49^{aA}	5.22 ± 0.10^{aAB}	5.19 ± 0.15^{aAB}
	6	5.36 ± 0.00^{aA}	5.13 ± 0.12^{bAB}	5.29 ± 0.10^{abA}
35	2	5.07 ± 0.09^{aAB}	4.93 ± 0.14^{aB}	4.93 ± 0.15^{aB}
	4	5.50 ± 0.14^{aA}	5.42 ± 0.07^{aA}	5.38 ± 0.13^{aA}
	6	5.48 ± 0.06^{aA}	5.24 ± 0.31^{aAB}	5.13 ± 0.14^{aAB}

MINITAB™ Statistical Software (MINITAB® 14.12.0, New York, USA) was used for the one-way analysis of variance (ANOVA) and Tukey's multiple comparison test at 95% confidence level to determine significant differences between three or more sets of data. Means ± standard deviation ($n = 6$) with different small letters in each row, and means ± standard deviation ($n = 6$) with different capital letters in each column, are significantly different ($P < 0.05$). SCC-0 (0:50:50), SCC-5 (5:45:50), and SCC-10 (10:40:50) are SCCs composed of different ratios of red palm olein: palm oil: palm stearin this study were still acceptable in the market after 6 months at all temperatures tested.

Table 4(c) shows the UV-TOTOX values of the SCCs in different storage conditions which indicate the level of lipid oxidation, similar to DOBI (Tan *et al.*, 2017). On day 0, SCC-5 (2.03 ± 0.10) was significantly higher ($p < 0.05$) in UV-TOTOX, followed by SCC-0 (1.86 ± 0.03) and SCC-10 (1.79 ± 0.03). After 2 months, all SCCs significantly increased ($p < 0.05$) in UV-TOTOX, yet no critical changes were further observed afterwards for up to 6 months at all temperatures. These findings were inversely correlated with the trends observed in their DOBI. On day 0, both SCC-5 and SCC-10 were significantly higher ($p < 0.05$) in DOBI and were significantly lower in UV-TOTOX. After 2 months, the DOBI significantly decreased ($p < 0.05$), while the UV-TOTOX significantly increased, which indicated rapid oxidation at the early stage of storage as described earlier. However, all values were insignificantly affected ($p > 0.05$) by the different storage time and temperatures afterwards. Different observations were made in SCC-0, where the DOBI values remained unchanged, yet its UV-TOTOX significantly increased ($p < 0.05$) after 2 months and onwards. Such findings are common to the oxidation of vegetable oils with lower or minute amounts of carotene (Table 2(a)), such as the shortening used in the SCC-0, which was not incorporated with RPOL. In this case, oxidation produced oxidative products which led to higher UV-TOTOX after 2 months, yet the DOBI were not critically affected due to the lower amount of carotene compared to those of SCC-5 and SCC-10.

4. CONCLUSIONS

The development of value-added SCCs incorporated with RPOL was carried out. The carotene, tocopherol, tocotrienol, and TPC were higher in SCC-5 and SCC-10 compared to SCC-0, yet both FFA and UV-TOTOX were comparable in all SCCs. Due to storage, the beneficial compounds underwent degradation which in most cases was reflected by increased DOBI and decreased PV and FFA. The lowest TPC after 6 months was reflected by the highest DPPH scavenging activity and the lowest FFA in the SCCs. In addition to these findings, the degradation rate in SCC-5 and SCC-10 was lower than that of SCC-0 at 20 and 30 °C. Overall, higher RPOL enhanced the antioxidant capacity and assisted in delaying oxidation reactions in the SCCs in all storage conditions tested, with SCC-10 showing the best results.

ACKNOWLEDGMENTS

The authors specially thank Sime Darby Plantation Research Sdn. Bhd. in Klang, Selangor, for providing the raw ingredients, chemicals, and facilities for most of the analysis methods.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e511
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0220221

Palm olein and perilla seed oil blends for the improvement of nutritional and thermal stability

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Submitted: 11 February 2022; Accepted: 25 October 2022; Published online: 10 October 2023

SUMMARY: This study aimed to develop a healthy blended oil with a balanced fatty acid ratio, as well as high thermal and oxidative stability. The blending of highly saturated Palm olein (PO) with polyunsaturated fatty acid-rich (PUFA) Perilla seed oil (PeO) in two different proportions, 70:30 (B1) and 80:20 (B2) v/v was studied. The physicochemical parameters, fatty acid composition, and oxidative stability of cold-pressed perilla seed oil (PeO), palm olein, and their blends were analyzed. The blends presented higher oxidative stability (6.5 h) with enhanced α-linolenic acid content (18%) than pure oils. The fatty acid ratio in both blend (B1- 1:1.4:1 and B2- 1.5:1.5:1) was found close to the WHO recommended ratio i.e., 1:1-5:1. The evaluation of the thermal stability of the blended oils revealed that PeO oxidized quickly during heating (Peroxide value-15.16 meq O_2/kg); whereas thermal stability improved with blending (Peroxide value: B1-7.92 and B2- 7.69 meq O_2/kg).

KEYWORDS: *α-Linolenic acid; Blending; Perilla seed oil; Thermal stability.*

RESUMEN: ***Mezclas de aceites de semilla de perilla y oleína de palma para mejorar la estabilidad nutricional y térmica.*** Este estudio tuvo como objetivo desarrollar una mezcla de aceites saludables con una proporción equilibrada de ácidos grasos, alta estabilidad térmica y oxidativa. Se ha estudiado la mezcla de oleína de palma (PO) altamente saturada con aceite de semilla de perilla (PeO) rico en ácidos grasos poliinsaturados (PUFA) en dos proporciones diferentes, 70:30 (B1) y 80:20 (B2) v/v. Se analizaron los parámetros fisicoquímicos, la composición de ácidos grasos y la estabilidad oxidativa del aceite de semilla de perilla prensado en frío (PeO), la oleína de palma y sus mezclas. Las mezclas presentaron mayor estabilidad oxidativa (6,5 h) con mayor contenido de ácido α-linolénico (18%) que los aceites puros. La proporción de ácidos grasos de ambas mezclas (B1- 1:1,4:1 y B2- 1,5:1,5:1) se encontró cerca de la proporción recomendada por la OMS, es decir, 1:1-5:1. La evaluación de la estabilidad térmica de los aceites mezclados reveló que el PeO se oxidaba rápidamente durante el calentamiento (Valor de peróxido: 15,16 meq O_2/kg), mientras que la estabilidad térmica mejoraba con la mezcla (Valor de peróxido: B1-7,92 y B2- 7,69 meq O_2/kg).

PALABRAS CLAVE: *Aceite de semilla de perilla; Ácido α-linolénico; Estabilidad térmica; Mezcla.*

Citation/Cómo citar este artículo: Dhyani A, Chopra R, Singh PK, Garg M. 2023. Palm olein and perilla seed oil blends for the improvement of nutritional and thermal stability. *Grasas Aceites* **74** (2), e511. https://doi.org/10.3989/gya.0220221

1. INTRODUCTION

Fats and oils are essential constituents of the daily diet and play a crucial role as a cooking medium. Due to the significant prevalence of Non-communicable diseases, people's focus is now shifted toward consuming healthy cooking oil. The nutritional quality of fats and oils is dependent on the type of fatty acid, degree of unsaturation, and arrangement of fatty acid in the triacylglycerol structure (Dorni *et al.*, 2018). The WHO has given a particular recommendation for healthy oil, i.e., the ratio of saturated, mono, and polyunsaturated fats should be 1:1.5:1; the balance of essential fatty acid, linoleic acid (n-6), and α-linolenic acid (n-3) should be 5-10:1 or less, with the presence of antioxidants (WHO, 2008). Most commonly-consumed oils are rich in saturated and monounsaturated fatty acids and deficient in polyunsaturated fatty acids, mainly omega-3 fatty acids (α-linolenic fatty acid). Omega-3 fatty acids are essential fatty acids, and their consumption prevents various non-communicable diseases such as coronary artery disease, diabetes, and cancer. As per the WHO reports, almost 61% of deaths globally are solely due to NCDs. But the major limitation of omega-3-rich oils is that they cannot be used for culinary purposes because they are highly susceptible to oxidation due to the presence of double bonds.

The vegetarian source of essential fatty acid (omega-3) is very limited. In recent years, the need for unconventional oils has increased due to their bioactive components and essential fatty acid composition (Al- Farga *et al.*, 2020). Perilla frutescens is an underutilized herb of the mint family (Lamiaceae), mainly found in Asian countries like China, Japan, Thailand, and India. It has been listed as one of the edible medicinal plants by the Ministry of Health of the People's Republic of China (Yu *et al.*, 2017; Dhyani *et al.*, 2019). This plant is receiving attention in western countries due to its medicinal importance. Perilla seeds contain 35-51% oil and play a significant role in the human diet and health due to their fatty acid composition. The foremost vital characteristic of PeO is that is one of the rich plant sources of omega-3 fatty acid. However, PeO, a rich source of n-3 PUFA, cannot be utilized for culinary purposes due to its high susceptibility to oxidation. Our previous study showed the poor oxidative stability of PeO (<1 h), measured by the Rancimat method (Dhyani *et al.*, 2021).

Nowadays, the oil industry's primary challenge is finding the most economically feasible nutritional oil with desirable functionality and improved oxidative and thermal stability. Unfortunately, a single oil could not provide all these quality characteristics. The blending of oil is the most feasible and economical way to modify oil's physicochemical and nutritional properties and improve its oxidative stability or make use of the properties of different oils in a single oil; blending increases bioactive and antioxidant composition. Nowadays, oil blending has become common in various countries (Hashempour-Baltork, *et al.*, 2016). Various studies have proven the nutritional and physicochemical benefits of oil blending. The cardiologist society of India defined "blending as a positive approach toward the enhancement of oxidative and thermal stability of oils" Also, it is recommended that replacing commonly-consumed cooking oil with blended oil is a feasible alternative to fulfill dietary recommendations and to reduce the risk of coronary heart diseases (CHD) (Manchanda and Passi, 2016).

Palm olein is mainly utilized as a frying oil due to its high smoke point and resistance to thermal degradation. It is mainly used to prepare fried food due to its high oxidative stability. Palm olein is a saturated fat but deficient in essential fatty acids (α-linolenic acid). Perilla seed oil is a major source of essential fatty acids (linoleic acid and α-linolenic acid). By blending PO and PeO, we could improve fatty acids and the n-6:n-3 ratio in the blend without compromising oxidative stability, thermal stability and long term storage stability. Various studies have proven that blending PUFA-rich oils with palm olein improved its nutritional as well as functional properties (Dhyani *et al.*, 2022; Joshi *et al.*, 2021; Siddique *et al.*, 2010). To the best of our knowledge, a detailed study on the effect of temperature (thermal stability) and time (storage stability) on the stability of perilla oil, palm olein and their blends has not been reported to date. Thus, the present study investigates the effects of blending n-3 PUFA-rich PeO with PO on the Physico-chemical properties, fatty acid composition, oxidative stability, thermal stability, and storage stability of the blend. This study may provide a new oil alternative with balanced fatty acid composition and improved oxidative and thermal stability at an affordable price.

2. MATERIALS AND METHODS

2.1. Materials

Perilla seeds were procured from the Forest Research Institute (FRI), Dehradun, India. Refined cooking oil, such as Palm olein was procured from the local market (Delhi, India). All reagents and chemicals were acquired from E. Merck or Sigma Aldrich.

2.2. Methods

2.2.1. Oil extraction and blends formulation

Perilla seed oil was extracted according to the cold-pressed method (Screw-press Model, 40kg, capacity 8-10 kg/hour, Power 1.2 KW). The extracted oil was kept overnight at room temperature for sedimentation. The decanted oil was first filtered through a muslin cloth and then Whatman filter paper no. 2 to remove impurities. The filtered oil was stored in a sealed, dark amber bottle until further use.

PO oil blends with PeO were prepared in two ratios: B1 (PO:PeO 70:30 v/v) and B2 (PO:PeO 80:20 v/v) as recommended by FSSAI. The uniform blends were prepared using a magnetic stirrer at 180 rpm for 15 minutes. Then, all the oils and blends were stored in a dark amber glass bottle at 4 °C until further analysis.

2.2.2. Initial physicochemical characterization of the blends

Fatty acid composition. Blended oils were analyzed for their fatty acid composition by gas chromatography as per the method provided by Choudhary, Grover, and Kaur, 2015. The fatty acids in the oil samples were converted to fatty acid methyl esters (FAMEs) using the IUPAC standard method. FAMEs were analyzed with a gas chromatograph (Agilent 7890 B), equipped with a flame ionization detector (FID) and FP 2560 cephalic column (100 mm x 0.25 µm x 0.2 µm) coated with CP-SIL 88 as the stationary phase. The temperature of the oven was at 200 °C. The injector and FID temperature was 250 °C. The FAMEs were expressed as relative area percentages.

Determination of other physicochemical parameters. The color was measured using the Lovibond tintometer (Model F) method. The color intensity was measured in 1” cells in the transmittance mode of a white glass filter, yellow glass filter, and red glass color and expressed as 5R+Y Lovibond units, according to the method described in AOCS (2017). The viscosity of the pure and blended oil was determined using a Brookfield viscometer at constant temperature (30 °C). The smoke point was determined as per the method described by Das *et al*. (2013).

The determinations of peroxide value (PV), acid value (AV), iodine value (IV), saponification value (SV) were performed according to the method provided by AOCS (2017).

2.2.3. Determination of oxidative stability by Rancimat method

The oxidative stability of oils can be determined by conducting an accelerated oxidation test and measuring the induction period (IP hour). Oxidation induction times were measured by a Rancimat model 743 using 3 g of oil, heated at 120 °C with a 20 L/h airflow. At the end of the process, volatile and secondary products were formed, absorbed by measuring a vessel containing deionized water, and then the electrical conductivity was measured (Ben Hammouda *et al.,* 2018).

2.2.4. Determination of thermal stability by heating cycle method

The heating procedure was conducted as per the method reported by Anwar and colleagues with slight modifications in time duration (Anwar *et al.*, 2007). In this method, freshly blended oils were heated at 180 °C in an electric fryer with temperature control (Inalsa Professional 2 fryer, 18/8 steel, 2 L, digital timer). Successive heating was conducted over 4 days for 6 hours each day, giving a total heating time of 24 hours. After completing the heating cycle, 100 mL of oil sample were drawn and stored in amber glass bottles at 4 °C for further analysis. The thermo-oxidative degradation level in the oils was assessed by measuring changes in color, peroxide value, free fatty acid, *p*-anisidine value, totox value, oxidative stability (Rancimat), and total polar components (TPC) after each cycle. The methodology for color, peroxide value, and free fatty acid is discussed in section 2.4.

Total polar compounds (TPC). The TPC of the frying oils was determined by the Column Chromatography technique, as per the Official International Union of Pure and Applied Chemistry (IUPAC) method. It is based on separating polar compounds from non-polar components as provided by Arslan *et al*. (2017). The percentage of TPCs was calculated using the equation:

$$\text{Total Polar compounds (\%)} = \frac{\text{weight of oil sample} - \text{weight of non-polar fraction}}{\text{weight of oil samples}} \times 100$$

p-*Anisidine value (*p*-AV).* The *p*-AV is a measurement of secondary oxidation products (aldehyde content) in the oil; it was determined according to the method provided by the AOCS (2017).

Totox value. Totox value was calculated using *p*-AV and PV, and the formula used was TV = 2PV + *p*-AV. However, since PV is an important parameter affecting refined oil's stability, the *p*-AV, 2 was used as a factor for multiples of PV (Nayak, 2017).

2.2.5. Determination of storage stability of PeO, PO and their blends

The PeO, PO, and blend were subjected to long-term storage to study their storage stability. Storage was done in amber glass bottles at room temperature (25 °C±2) for 180 days. The stability was assessed periodically after every 45 days in order to measure PV, *p*-Anisidine, TOTOX, color, and fatty acid composition.

2.3. Statistical analysis

All the data are reported as means ± standard deviation (*SD*). The data were analyzed using one-way analysis of variance (ANOVA) with IBM SPSS statistical software 20. In addition, for all thermo-degradative parameters, a two-way analysis of variance (ANOVA) followed by the Duncan's post-hoc test was employed to express the significant differences among the mean values at the 0.05 level ($p < 0.05$).

3. RESULTS

3.1. Initial characterization of oil and their blends

3.1.1. Fatty acid composition

Table 1 shows the fatty acid composition of PeO, PO, and their blends. Fatty acid composition plays a crucial role in deciding the nutritional value, functional value, oxidative stability, and industrial application of edible oils. PO's major primary fatty acids were palmitic, oleic, and stearic, accounting for 40.79%, 42.45%, and 4%, respectively. PO was found to be deficient in α-linolenic fatty acid (0.18%). PeO indicated the highest level of α- linolenic acid (55.92%), followed by oleic acid (20.58%) and linoleic acid (13.58%). The fatty acid profile of PeO was in agreement with results reported by other authors (Scapin *et al*., 2017; Yu *et al*., 2017).

The blended oil samples B1 and B2 showed different nutritional properties in terms of fatty acid

TABLE 1. Fatty acid composition (%) of perilla seed oil (PeO), palm olein (PO), and their blends.

Fatty acid composition (%)	**Oil samples**			
	PO	**PeO**	**B1**	**B2**
Palmitic acid (C16:0)	40.79 ± 0.50^{A}	7.44 ± 0.06^{D}	27.71 ± 0.08^{C}	32.72 ± 0.14^{B}
Palmitoleic acid (C16:1)	0.19 ± 0.00^{B}	0.23 ± 0.02^{A}	0.18 ± 0.00^{B}	0.19 ± 0.00^{B}
Stearic acid (C18:0)	3.99 ± 0.21^{A}	2.24 ± 0.19^{C}	3.49 ± 0.23^{B}	3.35 ± 0.03^{B}
Oleic acid (C18:1)	42.45 ± 0.50^{A}	20.54 ± 0.20^{D}	38 ± 0.38^{B}	37.75 ± 0.15^{B}
Linoleic acid (C18:2)	11 ± 0.23^{C}	13.75 ± 0.15^{A}	11.79 ± 0.20^{B}	11.86 ± 0.11^{B}
α-Linolenic acid (C18:3)	0.18 ± 0.00^{D}	55.80 ± 0.53^{A}	17.86 ± 0.18^{B}	12.96 ± 0.16^{C}
SFA	46.18 ± 0.19^{A}	9.68 ± 0.25^{D}	32.06 ± 0.28^{C}	37.24 ± 0.11^{B}
MUFA	42.64 ± 0.41^{A}	20.77 ± 0.19^{D}	38.18 ± 0.38^{C}	37.94 ± 0.15^{B}
PUFA	11.17 ± 0.24^{D}	69.55 ± 0.42^{A}	29.68 ± 0.41^{B}	24.82 ± 0.26^{C}
SFA: MUFA: PUFA	4.1:3.8:1	1:2.1:7.1	**1:1.3:1**	**1.5:1.5:1**

Values are taken in triplicate and expressed as Mean ±SD; $^{A-D}$ Means within each row with different superscripts are significantly different ($P \leq 0.05$) according to one-way analysis of variance (Duncan's post-hoc test); SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, ND not detected, PeO Perilla seed oil; PO Palm olein; B1- PO: PeO (70:30), B2 - PO: PeO (80:20) compared to pure PeO and PO. A peculiarity of PeO was its high level of omega -3 fatty acids (α-linolenic fatty acid) at a concentration of about 55.92%. On the other hand, PO had a minimal percentage of omega 3 acids (0.18%) and higher saturated fatty acid levels (46%). This study indicates that blending leads to balancing the fatty acid composition (Table 1). Thus, the addition of 30% PeO into PO (B1) showed a significant (< 0.05) increase in α-linolenic fatty acid content by 98.9% from 0.18 to 17.86 in B1 as compared to PO. Various studies have proven that blending omega -3 rich oil with other vegetable oil increases the α - linolenic acid content of the resultant mixture (Wang *et al.*, 2016; Hashempour-Baltork *et al.*, 2016).

It has been proven that a low level of PUFA:SFA in the diet is the leading cause of increased blood cholesterol levels (below 0.45). According to the results, the PUFA:SFA of PeO was very high (7.16), and with the blending process, the PUFA:SFA ratio of the final blends (B1 – 0.88 and B2- 0.67) of PeO and PO improved. Although, according to WHO, the recommended ratio of SFA:MUFA:PUFA is 1:1-5:1, both blends of PO and PeO were near the recommended value (B1- 1:1.4:1 and B2- 1.5:1.5:1) (WHO, 2008). Thus, blended oils presented an increase in omega-3 fatty acid (α-linolenic acid) content and decreased MUFA and SFA levels compared to pure oils.

3.2. Determination of other physico-chemical parameters of the blends

The quality of native oil and its blend before heating was analyzed by evaluating Physico-chemical properties (Table 2). Table 2 summarizes the physical properties of PO, PeO, and their blends. The oil color is one of the superior physical properties that influences consumer acceptance. Fresh PO had a lighter color at 11.43 ± 0.40. However, PeO had significantly (P ≤ 0.05) the highest color value (34.83 ± 0.76). The dark color of PeO was attributed to its high level of pigments and polyphenolic compounds (Yu *et al.*, 2017). When PO was blended with PeO, a significant decrease in the golden color of the blended oil was observed due to dilution with a lighter color, thus improving the blended oil's color and acceptability. No significant difference (P ≤ 0.05) was observed between the color value of blends (B1 and B2).

Viscosity is another important indicator of the quality and stability of edible oil. PO showed the highest viscosity (45mPa.s); whereas PeO displayed the lowest (28mPa.s). After blending, there was a slight decrease observed in the viscosity of blended oil compared to the Palm olein; whereas no significant difference (P ≤ 0.05) was found in the viscosity blends B1 and B2. The result was in agreement with the findings of previous studies, where authors reported that the blending of oil with PUFA-rich oil leads to a decrease in oil viscosity as viscosity decreases with the increase in PUFA content (Debnath et al., 2012; Flores et al.,2021).

The temperature at which fats and oils produce continuous smoke during heating is known as the smoke point (Hashempour-Baltork *et al.*, 2016). The highest smoke point was reported for PO (241.5 °C ± 2.8) and lowest for PeO (201 °C ±3.2) (Table 2). However, the blends showed better smoke point than perilla seed oil and no significant differences (P ≤ 0.05) between B1and B2 were observed. Therefore, the blended oil sample reported a smoke point to fulfill the recommended value for frying oils, i.e.,

Table 2. Physicochemical parameters of Palm olein (PO), Perilla oil (PeO), and their blends

Samples	**Physicochemical Parameters**						
	Color (5R+Y Lovibond units)	**Viscosity (mPa.s)**	**Smoke point (°C)**	**Peroxide Value (meq O_2/kg)**	**Acid Value (mg KOH/g)**	**Iodine Value (g/100g)**	**Saponification value (mg KOH/g)**
PeO	34.83±0.76^{a}	28±0.16^{c}	201±3.^{2}b	4.81 ± 0.40^{a}	1.61±0.02^{a}	192.33±1.25^{a}	180.66 ±1.25^{d}
PO	11.43±0.40^{c}	45±0.23^{a}	241.5±2.8^{a}	0.95±0.15^{d}	0.33±0.01^{b}	67.00±1.00^{d}	190.87±1.02^{a}
B1	25.83±0.76^{b}	43±0.30^{b}	232±2.51^{c}	2.49±0.13^{bc}	0.28±0.01^{c}	86.50±1.80^{b}	186.83±1.75^{b}
B2	25.16±0.28^{b}	43±0.40^{b}	232±2.35^{c}	2.20±0.17^{c}	0.26±0.02^{c}	77.00±0.50^{c}	184.44±0.68^{c}

Values are taken in triplicate and expressed as Mean ± SD**;** $^{a-d}$ Means within each column with different superscripts are significantly different (P ≤ 0.05) according to one-way analysis of variance (Duncan's post-hoc test)**;** PeO Perilla seed oil; PO Palm olein; B1- PO: PeO (70:30), B2 - PO: PeO (80:20) above 200 °C (AOCS, 2003). Blends of flaxseed oil with palm olein showed a higher smoke point than flaxseed oil. Therefore, the blending of oils helps to achieve a higher smoke point (Joshi et al., 2021).

The results of the chemical parameters such as peroxide, acid, iodine, and saponification value of the blended oils are presented in Table 2. Peroxide value is a measure of primary oxidation products (hydroperoxides) in oils, and it is correlated with the quality of the oil. The peroxide value (PV) of PeO was the highest (4.81 ± 0.40 meq O_2/kg) among the oils and the blends (Table 3). This may be due to the high PUFA content in PeO. A similar result was reported by Pan *et al.*, (2019). The addition of PO to cold-pressed PeO resulted in a significant decline in their PV, therefore enhancing the oxidative stability of blends. On the other hand, PO showed the lowest PV (0.95 meq O_2/kg), and the peroxide value of all the blended oil samples was within the permissible limit (10 meq O_2/kg) for edible oils.

The acid value measures triacylglycerol hydrolysis, and it is also a crucial quality parameter for edible oil. The acid value of PeO was found to be highest among all the blended oil samples, and it decreased with the increase in the volume of PO. A higher AV of any vegetable oil is due to lipase activity, and it can be reduced by employing certain pre-processing treatments such as roasting and microwaving. Furthermore, lipase enzyme activity is responsible for the hydrolysis of triacylglycerol molecules, leading

Table 3. Effect of heating cycle on chemical parameters of perilla seed oil (PeO), palm olein (PO), and their blends (180 ± 5 °C)

Oil sample/blends	**Heating Periods (days) (180 ± 5 °C)**				
			Peroxide value (meq O_2/kg)		
	day 0	**1st day**	**2nd day**	**3rd day**	**4th day**
PeO	4.81±0.40^{aE}	8.05±0.35^{aD}	10.98±0.35^{dC}	14.04±0.93^{aB}	15.16±0.23^{aA}
PO	0.95±0.15^{cE}	1.72±0.25^{cD}	2.47±0.06^{cC}	4.32±0.20^{cB}	4.92±0.27^{cA}
B1	2.6±0.17^{bD}	4.24±0.23^{bC}	5.75±0.13^{bB}	7.69±0.16^{bA}	7.92±0.09^{bA}
B2	2.2±0.14^{bE}	3.8±0.34^{bD}	5.18±0.20^{aC}	6.98±0.37^{bB}	7.69±0.16^{bA}
			Free Fatty acid (% oleic acid)		
PeO	0.93±0.05^{aE}	2.12±0.02^{aD}	2.34±0.05^{aC}	2..51±0.02^{aB}	2.78±0.07^{aA}
PO	0.15±0.0^{bC}	0.15±0.0^{cC}	0.19±0.02^{dB}	0.51±0.01^{cA}	0.56±0.05^{dA}
B1	0.17±.01^{bD}	0.18±0.05^{bC}	0.28±0.00^{cB}	0.76±0.05^{bB}	1.01±0.15^{bA}
B2	0.14±0.01^{bE}	0.18±0.03^{bD}	0.36±0.05^{cC}	0.71±0.02^{bB}	0.83±0.05^{cA}
			***p*-Anisidine value**		
PeO	2.56±0.39^{aE}	7.12±0.07^{aD}	15.89±0.80^{aC}	20.54±0.72^{aB}	25.64±0.41^{aA}
PO	0.41±0.15^{cE}	3.67±0.24^{bD}	7.4±0.42^{bC}	12.09±0.14^{bB}	15.05±0.60^{cA}
B1	1.22±0.19^{bE}	2.27±0.20^{cD}	7.7±0.25^{bC}	12.06±0.17^{bB}	20.10±0.06^{bA}
B2	0.76±0.17^{bcE}	1.62±0.45^{dD}	5.16±0.31^{cC}	8.65±0.48^{cB}	11.22±0.21^{dA}
			Totox value (2PV+ *p*-AV)		
PeO	12.18±1.01^{aE}	23.23±0.64^{aD}	37.86±1.42^{aC}	48.62±0.92^{aB}	55.96±0.88^{aA}
PO	2.31±0.39^{dE}	4.71±0.30^{dD}	10.35±0.32^{cC}	20.73±0.49^{dB}	24.86±1.15^{dA}
B1	6.42±0.15^{bE}	10.73±0.64^{bD}	18.07±0.42^{bC}	27.47±0.12^{bB}	35.95±0.16^{bA}
B2	5.16±0.52^{cE}	9.24±0.94^{cD}	16.66±0.12^{bC}	22.61±0.77^{cB}	26.60±0.54^{cA}
			Total Polar compounds		
PeO	5.25±0.25^{aE}	17.37±0.80^{aD}	20.71±0.84^{aC}	25.87±1.36^{aB}	34.63±0.78^{aA}
PO	2.44±0.20^{dD}	6.50±0.05^{dC}	7.79±0.69^{dB}	8.79±0.67^{dB}	10.87±0.79^{dA}
B1	4.65±0.30^{bE}	11.77±0.11^{bD}	14.21±0.39^{bC}	16.57±0.16^{bB}	19.80±0.62^{bA}
B2	4.00±0.35^{cE}	10.57±0.51^{cD}	11.65±0.30^{cC}	14.64±0.20^{cB}	16.83±0.72^{cA}

Values are taken in triplicate and expressed as Mean ± SE**;** $^{a\text{-}d}$ Means within each column with different superscripts are significantly different ($P \leq 0.05$); $^{A\text{-}E}$ Means within each row with different superscripts are significantly different ($P \leq 0.05$) according to two-way analysis of variance (Duncan's post-hoc test); PeO Perilla seed oil; PO Palm olein; B1- PO: PeO (70:30), B2 - PO: PeO (80:20) to free fatty acid formation in edible oils (Mazaheri *et al.*, 2019).

Iodine value is the indicator for the degree of unsaturation in fats and oils and is one of the important quality parameters of edible oils. The iodine value of PO and PeO was 67 and 192 g/ 100 g, respectively. The higher iodine value of PeO confirms the high degree of unsaturation. There was a significant ($P \leq 0.05$) reduction observed in the iodine value of blended oils compared to PeO. This reduction was observed due to the decrease in the predominance of polyunsaturated fatty acids in the blended oils. A study reported that the proportion of Palm olein caused a decrease in IV in Palm olein and olive oil blends, thus improving their stability against oxidative rancidity (Naghshineh *et al.*, 2010).

The saponification value represents the length of the carbon chain of the acid moiety of the lipid molecule. A greater amount of short-chain acids in fats and oil leads to a higher saponification value. The results for the SV of the blended oil were within the range, as reported in another study by Pan *et al.*, (2019).

3.3. Oxidative stability of oil and their blends

Oxidative stability is a vital quality parameter of edible vegetable oils. The stability of oils depends on various factors such as their fatty acid composition, free fatty acid, storage conditions, refining method, and processing methods. The oxidative stability index (OSI) of PUFA-rich fats and oils is low and could be improved by blending it with saturated fats and MUFA-rich oils. It is determined by the rancimat method, in which the induction time is measured, which is directly proportional to the stability of oils.

The results for the oxidative stability of PO, PeO, and their blends are presented in figure 3. The PO showed a remarkably high OSI value (12.29 h > 0.50 h) for induction time. As expected, the higher concentration of PUFA content in PeO is the reason for the low OSI value, i.e., 0.50 h (Torri *et al.*, 2019). The blends B1 and B2 showed intermediate values, i.e., 5.76 and 6.67. Hence, the increased PO levels in blended oil markedly correlated with increased oxidative stability. Therefore, the chemical composition and oxidative stability of the developed blends showed improved oxidative and nutritional stability.

3.4. Effect of heating cycle method on physicochemical parameters of blends

Color is the characteristic parameter used by the food industry for the rapid screening of frying oil quality. The color value of oil increases with increasing heating temperature and time. The color value increases due to the oxidation and polymerization of unsaturated fatty acids in the oil and the solubilization of other non-polar compounds in the oil (Wang *et al.*, 2016). The results in Figures 1 and 2 show that the color value was changed from the beginning to subsequent heating days. A significant ($P \leq 0.05$) increase in the color value of all the blended oils was observed during 24 hours of the heating process. The PeO showed the highest initial color value (34.83), which increased (134.20) by 74.04% after the 4th day of heating; while PO showed the lowest initial color value (11.43). On the other hand, the blending of PeO with PO showed a significant decrease ($P \leq 0.05$) in the color value of blended oils, due to the lower level of degradation in blended oil. The color values for both the blends PO:PeO (70:30) and PO:PeO (80:20) significantly increased by ($P \leq 0.05$) 33.04 and 29.87%, respectively, during the 4th day of heating. These results indicate that blended oil was more stable against color change during frying than pure PeO. The darkening of color is related to the formation of hydroperoxides, aldehydes, ketones, and hydroxides during heating. However, various studies reported that blending slows down the rate of increased color value or darkening of oil due to

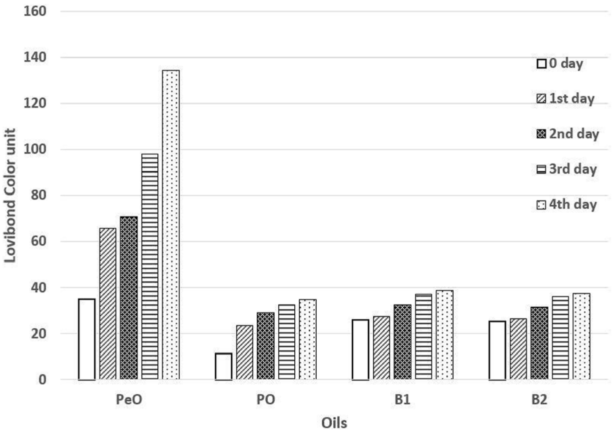

Figure 1. Change in Lovibond color unit of oil during the heating cycle (180 ± 5 °C). Values are taken in triplicate and expressed as Mean ± SE; Perilla seed oil (PeO), Palm olein (PO), B1- PO: PeO (70:30), B2 - PO: PeO (80:20)

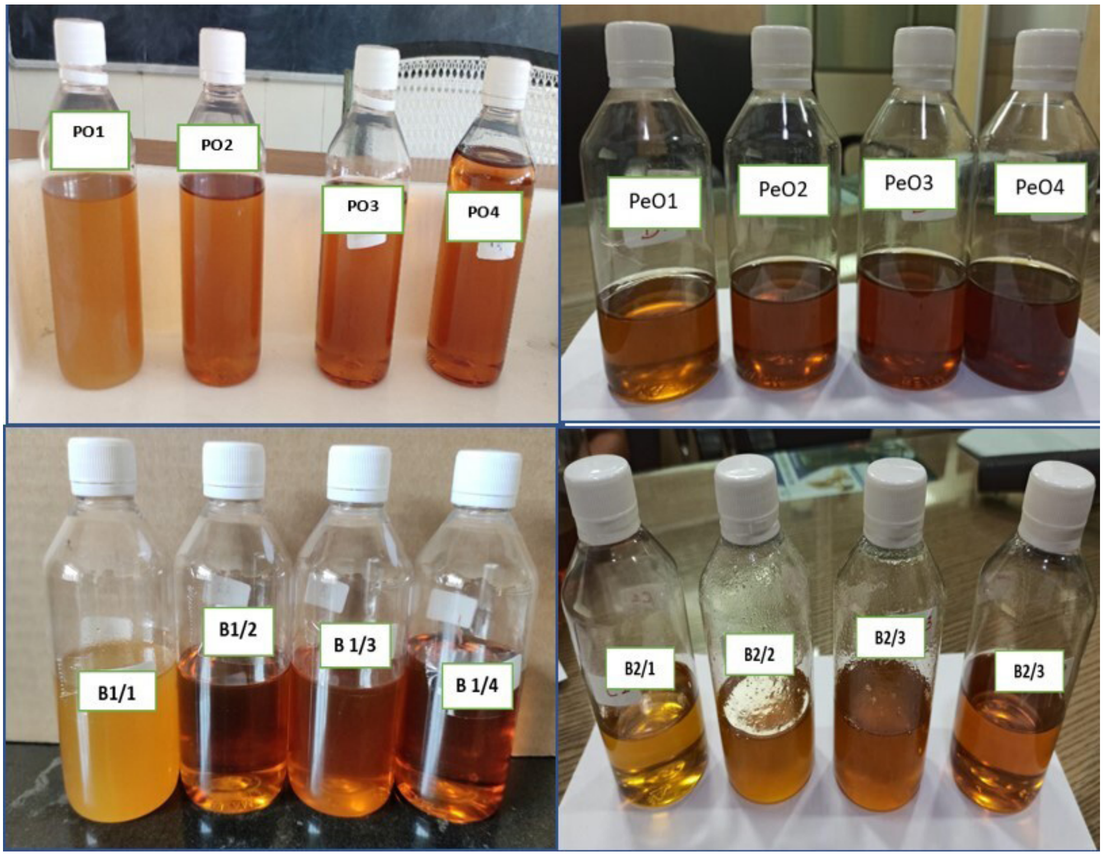

Figure 2. Changes in color of oil during the heating cycle (180 ± 5 °C). Palm olein (PO), Perilla seed oil (PeO), B1- PO: PeO (70:30), B2 - PO: PeO (80:20). 1,2,3, and 4 represent the subsequent days of heating.

heating or frying (Wang *et al.*, 2016; Ben Hammouda *et al.*, 2018).

The changes in peroxide value (PV) of the pure and blended oil during the heating process at 180 °C are shown in Table 3. The peroxide value of all the oil samples increased significantly at the end of the 4-day (24 hours) heating process. The peroxide value of PeO was the highest due to the high content of α-linolenic acid, which is more prone to oxidation. In contrast, the slower increment in the rate of PV was analyzed in PO and blended oil (B1 and B2). However, there was a significant ($P \leq 0.05$) increase in the PV in PO, PeO, and their blends at the end of each heating cycle, but PV was less than 10 meq O_2/kg. As expected, the blending of PO with PeO resulted in a stable blend with respect to PV. Thus, according to the codex, these blended oil samples were in the acceptable range of fresh oil; whereas the PV of PeO was higher than 10 meq O_2/kg at the end of the heating cycle and thus cannot be used in domestic cooking. In a study, Arslan *et al.*, (2017) compared cottonseed and Palm olein's frying and oxidative stability with their blends. It was reported that the PV of cottonseed and Palm olein blends lies within the acceptable range for fresh oil even after 10-hours of frying. However, peroxide value alone cannot be

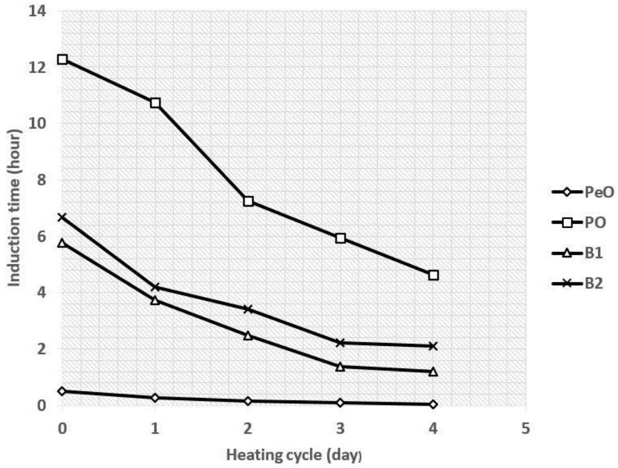

Figure 3. Oxidative stability index (OSI) of Perilla, Palm, and their blends during the heating cycle (180 ± 5 °C). Values are taken in triplicate and expressed as Mean ± SE; PeO Perilla seed oil; PO Palm olein; B1- PO: PeO (70:30), B2 - PO: PeO (80:20)

used as a measure of stability because peroxides and hydroperoxides are volatile compounds and further decompose into aldehydes and ketones, thus reducing the peroxide value. From the data for PV in Table 3, it was found that the rate of increment in PV is significantly ($P \leq 0.05$) decreased in the third and fourth heating cycles compared to initial heating cycles. This finding could be explained by the fact that peroxides are volatile, and upon heating, they further decompose into secondary oxidation products.

The free fatty acid (FFA) content in the blended oil samples during the heating process is presented in Table 3. Free fatty acid is an essential parameter in the degradation of frying or heating oil, and FFA increases with heating due to hydrolytic degradation. However, free fatty acid cannot be taken alone as a parameter of oil deterioration during frying or heating. It should be considered with other methods such as peroxide value and *p*-Anisidine (Abdulkarim *et al.*, 2007). The free fatty acid content in PeO was the highest and the lowest was in PO; whereas no significant difference ($P \leq 0.05$) was observed in their blends. There was a significant($P \leq 0.05$) increase in the FFA of all the oil samples after each heating cycle, and similar increments were observed upon frying in various studies (Wang *et al.*, 2016). The FFA content in Palm olein during the heating cycle increases by 2.7 times; whereas in the case of PeO, it increased approximately by 3 times. The higher free fatty acid content in oil indicates volatile and non-volatiles degradation products which deteriorate the quality of the oil.

The *p*-Anisidine value (*p*-AV) is always used with the peroxide value to measure the oxidative rancidity of oil at high frying temperatures. However, *p*-AV is a more accurate test than PV as it reflects the presence of secondary oxidation products such as 2-alkenals and 2, 4-alkadienals, which are more stable during frying than peroxides. The results for the *p*-AV of PeO, PO, and their blends are shown in Table 3. There was a rapid increase in the value of *p*-AV after each heating cycle regardless of the type of oil sample. This was observed due to the further decomposition of primary oxidation products (hydroperoxides) into secondary oxidation products (aldehydes), which leads to the development of off-flavor and odor. At the end of the 4-day heating period (24 hours), the *p*-AV of PeO was the highest; whereas it was recorded lowest for PO. Therefore, the significantly higher *p*-AV of PeO compared to PO and their blends indicated extensive degradation of oxidized PUFAs.

The addition of PO into the PeO significantly lowered the *p*-AV of blended oils compared to PeO, which is due to a decrease in the percent of polyunsaturated acids. They are the chief targets of thermal-oxidative reactions. These results indicated that blended oils were more resistant to oxidative stability during frying than pure PeO. Similar results were obtained in various studies (Mishra and Sharma, 2014; Wang *et al.*, 2016).

The results of the totox value are reported in Table 4. The totox value for PO is significantly ($P \leq 0.05$) lower than PeO and their blends because of its higher saturated fatty acid content. The higher totox value of PeO indicates a higher susceptibility of PeO to oxidative rancidity than other oils due to the presence of high PUFA content. The results indicated that with the addition of PO in PeO, the PUFA content decreases, thus improving the oxidative stability of blended oils.

The results of the heating cycle of the oxidative stability index (OSI) for blended oil are reported in Figure 4. A higher degree of unsaturation is directly proportional to lower OSI. Therefore, the OSI of the oil sample decreases with heating time due to an increase in the deterioration of oil resulting from the heating process. To compare the rates of OSI increment between the native and blended oils during frying, the raised percentages were calculated for the initial and the end of frying sessions (day 4). Therefore, the fastest increment was found for pure PeO (about 92%) from 0.5h to 6.67h; whereas the lowest increment was found for B1 (about 38%), from 0.02h to 2.21h.

Total polar compound (TPC) is the most accurate measurement of oil deterioration due to its higher accuracy and reproducibility. It includes all the degraded products formed in oils due to thermal oxidation reactions or hydrolytic breakdown of triglyceride molecules during heating or frying. All vegetable oils consist of some parts of polar compounds, but the rate of formation of TPC increases with the application of frying or heating. The quality and shelf life of fried food depend upon frying oil quality. Henceforth, various countries have formulated and reported many quality standards for frying oils. The recommended minimum standard limit for TPC varies from country to country and mainly ranges from 23-29% (Stier, 2013). Table 3 shows the TPC of PO, PeO, and their blends at different heating cycles. The initial TPC of fresh oils was very low, reflecting their sound quality. As we can see, the heating caused a significant and rapid increase in the TPC value of pure and blended oils after every heating cycle. This change in the TPC value was linearly associated with heating temperature and heating time. At the end of the frying cycle (day 4 day), the TPC level of PeO was higher (34.63%) for PeO as compared to PO (10.87%). When PO was added to PeO the TPC level was significantly ($P \leq 0.05$) reduced in the blended oils. The low value of TPC in blended oil compared to PeO was due to an increase in the amount of SFA in blends. Similar findings were also reported by Ben Hammouda *et al* (2018). They suggested that blending PUFA-rich oil with SFA-rich oil results in low TPC during the frying cycle and is less prone to thermo-oxidation.

In the case of PeO, the TPC content reached the rejection range during the frying process, while its blends did not. Assuming a TPC content limit of 23-29%, the number of frying sessions necessary to reach this limit was used to measure frying stability. The frying stability of pure PeO was much lower than that of its blends. As a result, when compared to pure PeO, the blended oil performed well and was of good quality.

3.5. Storage stability of PeO, PO and their blends

The shelf life data (PV, AV, *p*-AV, and Totox value) for the PeO, PO, and their blends is shown in Table 4. The PV of the PeO, PO, B1, and B2 increased significantly throughout the study period. After 180 days of storage at 25 ±2 °C, only PeO exceeded the upper limit of PV (10.0 meqO_2/kg) as given by Codex Alimentarius. The legal upper limit of PeO was reached at between 45 and 90 days of storage. The PV of B1 and B2 after 180 days of storage was 10.32 ± 0.14 meqO_2/kg and 10.05 ± 0.07 meqO_2/kg, respectively. The change in the PV value during storage suggests the formation of primary oxidation compounds such as hydroperoxide. It was also found that there is less formation of hydroperoxide in B1 and B2 than in pure PeO as the PeO had higher PUFA, which results in higher oxidation.

The result showed that the acid value increased with storage. The PeO showed significant ($P \leq 0.05$) changes in acid value. This could be due to the higher

Table 4. Effect on storage stability on chemical parameters in Perilla seed oil (PeO), Palm olein (PO), and their blends.

Parameters	Oil/blends	Storage Days				
		0 day	**45 days**	**90 days**	**135 days**	**180 days**
Peroxide value (meq O_2/kg)	**PeO**	4.81 ± 0.40^{aE}	8.40 ± 0.22^{aD}	14.11 ± 0.16^{aC}	16.16 ± 0.22^{aB}	20.12 ± 0.17^{aA}
	PO	0.95 ± 0.15^{cC}	1.48 ± 0.12^{dC}	2.38 ± 0.09^{dB}	4.36 ± 0.43^{dA}	4.93 ± 0.09^{cA}
	B1	2.6 ± 0.17^{bE}	4.82 ± 0.11^{bD}	7.88 ± 0.16^{bC}	9.23 ± 0.30^{bB}	10.32 ± 0.14^{bA}
	B2	2.2 ± 0.14^{bE}	3.93 ± 0.09^{cD}	6.78 ± 0.33^{cC}	8.32 ± 0.17^{cB}	10.05 ± 0.07^{bA}
Acid value	**PeO**	1.61 ± 0.02^{aD}	1.72 ± 0.39^{aD}	2.11 ± 0.16^{aC}	4.64 ± 0.19^{aB}	6.11 ± 0.16^{aA}
	PO	0.33 ± 0.01^{cD}	0.98 ± 0.02^{cC}	1.05 ± 0.15^{dBC}	1.15 ± 0.14^{dB}	1.40 ± 0.11^{dA}
	B1	0.38 ± 0.01^{bE}	1.08 ± 0.02^{bD}	1.72 ± 0.14^{bC}	2.92 ± 0.14^{bB}	3.32 ± 0.06^{bA}
	B2	0.36 ± 0.01^{bE}	1.06 ± 0.01^{bD}	1.64 ± 0.16^{bC}	2.76 ± 0.10^{bB}	3.27 ± 0.08^{bA}
***p*-Anisidine value**	**PeO**	2.56 ± 0.39^{aE}	5.48 ± 0.11^{aD}	6.60 ± 0.14^{aC}	8.72 ± 0.31^{aB}	11.04 ± 0.05^{aA}
	PO	0.41 ± 0.15^{cD}	0.54 ± 0.01^{cD}	0.98 ± 0.02^{dC}	1.47 ± 0.08^{dB}	2.33 ± 0.31^{dA}
	B1	1.02 ± 0.19^{bD}	1.19 ± 0.08^{bD}	2.27 ± 0.07^{bC}	5.72 ± 0.08^{bB}	7.06 ± 0.08^{bA}
	B2	0.76 ± 0.17^{bcE}	1.03 ± 0.07^{bD}	2.08 ± 0.11^{cC}	3.55 ± 0.07^{cB}	5.47 ± 0.22^{cA}
Totox value	**PeO**	12.18 ± 1.01^{aE}	22.28 ± 0.56^{aD}	34.83 ± 0.46^{aC}	40.82 ± 1.08^{aB}	50.79 ± 1.11^{aA}
	PO	2.31 ± 0.39^{dE}	3.51 ± 0.22^{dD}	5.74 ± 0.21^{dC}	10.19 ± 0.96^{dB}	12.20 ± 1.13^{dA}
	B1	6.42 ± 0.15^{bE}	10.83 ± 0.31^{bD}	18.03 ± 0.26^{bC}	24.19 ± 0.52^{bB}	27.70 ± 0.24^{bA}
	B2	5.16 ± 0.52^{cE}	8.89 ± 0.26^{cD}	15.64 ± 0.77^{cC}	20.19 ± 0.98^{cB}	25.57 ± 0.07^{cA}

Values are taken in triplicate and expressed as Mean ± SE**;** $^{a\text{-}d}$Means within each column with different superscripts are significantly different ($P \leq 0.05$); $^{A\text{-}E}$Means within each row with different superscripts are significantly different ($P \leq 0.05$) according to two-way analysis of variance (Duncan's post-hoc test); PeO Perilla seed oil; PO Palm olein; B1- PO:PeO (70:30), B2 - PO:PeO (80:20)

PUFA content in PeO. It was observed that there is no significant difference ($P \leq 0.05$) in the AV of either of the blended oils during storage. Perilla seed oil showed a higher *p*-AV at the initial stage of storage (t = 0) when compared to PO. On the contrary, in the case of blended oil, *p*-AV was reduced due to the blending of PO with PeO. The results indicated that the high content of PUFA in PeO leads to the formation of a high number of secondary oxidation products during storage. However, blending PeO with PO results in low oxidation product formation, leading to higher oxidative stability.

4. CONCLUSIONS

The result demonstrated that the objective of balancing the fatty acid ratio in oil blends was accomplished by selecting oil with low PUFA content such as PO and blending with PeO, which is rich in PUFA content, mainly ALA, so as to get a balanced fatty acid and LA/ALA ratio below 5:1.

The physico-chemical charaterization of the newly developed blends showed its suitability as good cooking oil. The determination of chemical parameters (peroxide value, free fatty acid, *p*-anisidine value, totox value, OSI, total polar compounds) confirmed that these blends are thermally stable up to a certain time during heating. Furthermore, the study showed that the blends exhibited higher thermal stability after a 4-day heating cycle than PeO. As a result, blended oil has a better SMP ratio than pure PeO or PO, with good oxidative stability which fluctuates between 6 and 7 hours at 120 °C and thermal stability of 24 hours at 180 °C.

Finally, the six-month storage stability study indicated that the addition of PO could provide oxidative stability to the blend containing oxidatively vulnerable PeO. Based on our findings, the formulation of perilla seed oil blends is crucial to the food industry, which is presently interested in nonconventional oils and functional foods to improve health and human nutrition.

ACKNOWLEDGMENTS

This study is a part of a Ph.D. dissertation. The authors gratefully acknowledge the financial support provided for this work by the University Grant Commission (UGC), India

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GRASAS Y ACEITES 74 (3)
July-September 2023, e512
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0980221

Untargeted lipidomics approach using LC-Orbitrap HRMS to discriminate lard from beef tallow and chicken fat for the authentification of halal

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Submitted: 04 September 2022; Accepted: 03 November 2022; Published online: 10 October 2023

SUMMARY: This research aimed to perform a lipidomics study using liquid chromatography-high resolution mass spectrometry (LC-HRMS) to identify lard, beef tallow and chicken fat. A total of 292, 345, and 403 lipid compounds were observed in lard, beef tallow, and chicken fat, respectively. The lipid groups of AcHexStE (acyl hexosyl stigmasterol ester), biotinylPE (biotinylphosphoetanolamine), LPC (lysophosphatidylcholine), MePC (monoetherphosphatidylcholine), PC (phosphatidylcholine) and PI (phosphoinocitol) were found to be specific for lard. The principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) successfully differentiated lard from beef tallow and chicken fat. This research suggested that the untargeted lipidomics technique using LC-HRMS combined with chemometrics could be used to discriminate lard from beef tallow and chicken fat. This method is a promising technique for the detection of lard adulteration in beef tallow and chicken fat for halal authentication purposes.

KEYWORDS: *Chemometrics; Halal Authentication; Lard; LC-HRMS; Metabolomics; Untargeted*

RESUMEN: ***Enfoque de lipidómica no dirigida utilizando LC-Orbitrap HRMS para discriminar manteca de cerdo, sebo de res y grasa de pollo para la autenticación halal.*** Esta investigación tuvo como objetivo realizar un estudio de lipidómica utilizando cromatografía líquida-espectrometría de masas de alta resolución (LC-HRMS) para discriminar manteca de cerdo, sebo de res y grasa de pollo. Se pudo observar un total de 292, 345 y 403 compuestos lipídicos en manteca de cerdo, sebo de res y grasa de pollo, respectivamente. Se encontró que los grupos lipídicos de AcHexStE (éster de acil hexosil estigmasterol), biotinilPE (biotinilfosfoetanolamina), LPC (lisofosfatidilcolina), MePC (monoéterfosfatidilcolina), PC (fosfatidilcolina) y PI (fosfoinocitol) son específicos para la manteca de cerdo. El análisis de componentes principales (PCA) y el análisis discriminante de mínimos cuadrados parciales (PLS-DA) diferenciaron con éxito la manteca de cerdo del sebo de res y la grasa de pollo. Esta investigación sugirió que la técnica de lipidómica no dirigida que usa LC-HRMS combinada con quimiometría podría usarse para discriminar la manteca de cerdo del sebo de res y la grasa de pollo. Este método es una técnica prometedora para la detección de la adulteración de manteca de cerdo en sebo de res y grasa de pollo con fines de autenticación halal.

PALABRAS CLAVE: *Autenticación Halal; LC-HRMS; Manteca de cerdo; Metabolómica no dirigida; Quimiometría.*

Citation/Cómo citar este artículo: Windarsih A, Bakar NKA, Rohman A, Riswanto FDO, Erwanto Y. 2023. Untargeted lipidomics approach using LC-Orbitrap HRMS to discriminate lard from beef tallow and chicken fat for halal authentication. *Grasas Aceites* **74** (2), e512. https://doi.org/10.3989/gya.0980221

1. INTRODUCTION

Food authentication has become the main important issue in the world recently because it is associated with many aspects of food, such as quality, safety, and the halal status of food products (Balkir *et al.*, 2021). The adulteration and mislabelling of food products are often carried out in high quality food products by unethical players. The main reason for such adulteration is related to the aim of obtaining higher profits (Danezis *et al.*, 2016). Edible fat is one of the food products which is prone to adulteration and mislabelling because it is easy to mix a fat with other types of fats. High quality fats such as beef tallow and chicken fats have many functions in various food products, for instance to obtain a desired flavour, typically in breads, baked goods, meat products, and many more. Thus, it is susceptible to adulteration with lower quality fats such as lard (Lee *et al.*, 2018). Lard, a type of fat obtained from pork, is known as the cheapest fats. It spreads widely in the markets and has been utilized in numerous food products (Taylan *et al.*, 2020). However, the consumption of lard is prohibited by certain religions such as Muslim and Jewish (Hossain *et al.*, 2020). Lard is categorized as containing non-halal lipids which are not allowed to be consumed according to Shariah law. Differentiating lard from beef tallow and chicken fat is obviously difficult due to their similar appearance and characteristics (Rohman and Windarsih, 2020). Thus, analytical methods capable of discriminating lard from other fats such as beef tallow and chicken fat are required.

Various analytical techniques have been developed and validated for the analysis of fats including spectroscopy and chromatography, especially in combination with multivariate data analysis or chemometrics (Valdés *et al.*, 2018). Gas chromatography using a flame ionization detector (GC-FID) and mass spectrometer (GC-MS) have evolved as the most common methods used for fat analysis (Guntarti *et al.*, 2020). Both GC-FID and GC-MS analyse fats through the fatty acid compositions. GC-FID has been used for the analysis of lard, chicken fat, and beef tallow based on their fatty acid compositions. The results showed that the fatty acid of *cis* C18: 2 become the major fatty acid found in lard (Dahimi *et al.*, 2014). Apart from GC-FID, GC-MS has been more widely utilized in the analysis of fats due to its high specificity and sensitivity. GC-MS combined with PCA has been used for the analysis of dog fats in beef meatballs (Guntarti, 2018). However, GC-based methods require complex preparation steps including the derivatization of fatty acids, which becomes time consuming. Vibrational spectroscopy such as Fourier transform infrared (FTIR) spectroscopy has been widely used for the análisis of fats and oils. FTIR spectroscopy is known as the most rapid screening method for the analysis of fats and oils (Li *et al.*, 2019). Combined with chemometrics, FTIR spectroscopy has been successfully used to identify, differentiate, and classify fat samples (Jiménez-Sotelo *et al.*, 2016; Jamwal *et al.*, 2021). However, FTIR spectroscopy is not a confirmatory method, and could not be used to identify unknown samples.

The emerging of omics-based techniques such as metabolomics, proteomics, genomics, and transcriptomics have boosted research in food authentication (Böhme *et al.*, 2019). Metabolomics is the comprehensive study of metabolites, including amino acids, lipids, organic acids, nucleosides, phenolic compounds, alkaloids, flavonoids, sugars and many more in biological samples under particular conditions (Castro-Puyana *et al.*, 2017). Lipidomics, a subsection of metabolomics, focuses on the study of lipid metabolites. Lipidomics provides a comprehensive lipid analysis to identify as many lipid compounds as possible in food samples (Sun *et al.*, 2020). Untargeted lipidomics has advantages in the global screening of lipids in samples. Therefore, we can identify a global lipid overview in samples. It does not only analyzing one or few lipids as in a targeted approach. Moreover, the identification of discriminating lipids can be further used as potential biomarkers to differentiate samples through chemometrics análisis. Recently, the use of untargeted lipidomics in food analysis has become more widespread due to its ability to identify lipid compositions from different types of food samples (Wu *et al.*, 2021). It could be used to analyze not only fatty acids but also other types of lipids such as phospholipids, glycolipids, ceramides, sphingolipids and many more (Lee and Yokomizo, 2018; Song *et al.*, 2022). Thus, it offers potential advantages for the comprehensive identification of lard, chicken fat, and beef tallow to identify the potential biomarkers of each fat.

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) techniques are the most common methods used for lipidomic analysis due to their applications in throughput analysis (Balkir *et al*., 2021). NMR offers minimum sample preparation, rapid analysis, and minimum use of solvent. However, it has lower sensitivity and lower resolution compared to MS-based techniques (Li *et al*., 2017). MS-based techniques coupled with a chromatography technique such as liquid chromatography offer potential advantages for lipid separation, thus enhancing the resolution, and obtaining greater lipid metabolites. (Sun *et al*., 2020). Liquid chromatography-high resolution mass spectrometry (LC-HRMS) could be used for throughput screening of metabolites including lipids with high sensitivity and high specificity. The utilization of an Orbitrap mass analyzer has advantages in resolving complex samples with high resolution due to its high resolving power (Zeki *et al*., 2020). Combinations with advanced statistical tools such as chemometrics are required to process the huge amount of data on lipids which is obtained from the measurement of LC-HRMS. Pattern recognition chemometrics such as principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA), orthogonal projections to latent structures-discriminant analysis (OPLS-DA) and soft independent modelling class analogy (SIMCA) have been widely used in metabolomic and lipidomic analyses (Jia *et al*., 2022; Mi *et al*., 2018).

The lipidomic approach has been successfully used to differentiate beef and pork as well as to detect pork adulteration in beef meat. Analysis was carried out using LC-MS LTQ-Orbitrap combined with PLS-DA (Trivedi *et al*., 2016). A lipidomic study using liquid chromatography-Quadrupole time of flight mass spectrometry (LC-QTOF-MS) has also been used for the characterization and discrimination of China´s selected domestic pork. PCA and PLS-DA were successfully used to differentiate and classify different samples of China’s domestic pork. One hundred variables consisted of glycerolipids, glycerophospolipids, sterol lipids, phospholipids, polyketides, fatty acids and prenol lipids were found as potential biomarkers to differentiate among samples (Mi *et al*., 2019). In addition, lipidomic analysis has been applied for the analysis of phospholipids in Tan sheep meat subjected to thermal processing. The quantification of ninety lipids from six subclasses, namely ceramide, triacylglycerol, phosphatidylcholine, lysophosphatidylcholine, phosphatidyletanolamine and sphingomyelin, was performed with Tan sheep meat with and without thermal processing (Jia *et al*., 2021).

To the best of our knowledge, studies on the discrimination of non-halal fats such as lard from beef tallow and chicken fat using a lipidomic approach employing LC-Orbitrap HRMS are still limited. Therefore, the aim of this research was to develop an untargeted lipidomic approach using LC-Orbitrap HRMS and chemometrics to identify lipid compositions for the discrimination of lard, beef tallow, and chicken meat as well as to detect lard adulteration in beef tallow and chicken fat based on their lipid compositions.

2. MATERIALS AND METHODS

2.1. Materials

Methanol, acetonitrile, water and isopropanol were all LC-MS grade and obtained from Thermo Fisher Scientific (Fairlawn, NJ, USA). Ammonium format, formic acid and HPLC-grade methanol were purchased from E. Merck (Darmstadt, Germany). A calibrant solution of Pierce LTQ Velos positive and Pierce negative was obtained from Thermo Fisher Scientific (Rockford, IL, USA).

2.2. Sample preparation

Lard, chicken fat, and beef tallow were obtained from the rendering of corresponding animals’ adipose tissues according to Rohman & Che Man (2010). An amount of 20 mg fat sample was weighed and placed in a 2-mL microcentrifuge tube. Samples of pure lard, pure chicken fat, and pure beef tallow were prepared. The adulterated beef tallow and chicken fat with lard were prepared by mixing beef tallow and chicken fat with lard using a ratio of 50:50 (% w/w). It was aimed to observe the profile of beef tallow and chicken fat when the adulteration was present. The ratio (50:50) was chosen because adulteration is usually performed in high concentrations. Each sample was dissolved in 1 mL isopropanol, then vortexed for 1 min at room temperature. Subsequently, the sample was ultrasonicated at room temperature for 30 min. After sonication finished, the sample was then centrifuged at 12,000 x *g* for 10 min at 4 °C. The supernatant was collected and filtered using PTFE filter 0.22 μm and placed into a clear HPLC vial for lipidomic analysis using LC-HRMS. Each sample was prepared in three replicates.

2.3. Lipidomics analysis using LC-HRMS

The lipidomic analysis was performed using an ultra-high-performance liquid chromatography (Thermo Scientific™ Vanquish™ UHPLC binary pump) and high-resolution mass spectrometry-Orbitrap (Thermo Scientific™ Q-Exactive™ Hybrid Quadrupole-Orbitrap™ High Resolution Mass Spectrometer). The separation of analyte was carried out using an analytical column of Thermo Scientific™ Accucore™ C-18 (100 mm x 2.1 mm ID x 2.6 µm). Analysis was performed according to Jia *et al*. (2022) with modifications. Lipidomic analysis was performed using a mobile phase of water:acetonitrile (40:60 v/v) containing 40 mM ammonium format and 0.1% formic acid as the mobile phase A and isopropanol:acetonitrile (90:10 v/v) containing 40 mM ammonium format and 0.1% formic acid as the mobile phase B. The gradient mode was applied as follows: initially, the mobile phase B was set at 32% B for 1.5 min, then increased to 45% B until reaching a minimum of 4.0. After that, it was increased to 54% B (4.01-5.0 min), 58% B (5.01-8.0 min), 66% B (8.01-11 min), 70% B (11.01-14.00 min), 75% B (14.01-18.00 min), 97% B (18.01-21.00 min), then held at 97% B for 25 min. At the end, the process was returned to its initial condition (32% B) for 25.01–30.00 min. The flow rate of the mobile phase was 0.260 mL/min with a sample injection volume of 5 µL. The temperature of the sampler was set at 25 °C, while the column temperature was maintained at 40 °C. The mass spectrometry condition for untargeted lipidomic screening was carried out using full MS/dd-MS2 acquisition mode. Lipid analysis was performed both in positive and negative ionization modes. The sheath gas flow rate, auxiliary gas flow rate, and sweep gas flow rate applied in this research were set at 32, 8, and 4 arbitrary unit (AU), respectively. The electrospray ionization used spray voltage of 3.30 kV with capillary temperature set at 320 °C. The auxiliary gas heater temperature was set at 30 °C. The analysis was performed using a scan range of 100-1500 m/z and a resolution of 70,000 for full MS and 17,500 for dd-MS2. The mass spectrometer instrument was weekly calibrated using Thermo Scientific Pierce ESI calibration solutions both in positive and negative modes to warrant the mass accuracy.

2.4. Data processing and identification of lipids

The raw data of the total ion chromatogram (TIC) obtained from the LC-HRMS measurement both in positive and negative ionization modes were analyzed using Lipid Search 4.2 software (Thermo Scientific, USA) for peak alignment, baseline correction, background correction, retention time alignment (0.2 min tolerance) and mass tolerance (5 ppm). The identification of the lipid compositions was compared to the predicted in silico spectra from various lipid compounds. The results of lipid metabolomes were classified according to their lipid groups and lipid ions. Data were filtered using RSD (relative standard deviation) < 20 and S/N ratio > 10. The molecules with RSD at more than 30% and missing values exceding 50% were deleted.

2.5. Chemometrics analysis

Chemometrics was carried out using variables of lipid ions and the relative areas. Analysis was performed using SIMCA 14.0 software (Umetrics, Sweden). Principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were used in this study. The PCA model was evaluated using PCA score plot, R^2 value and Q^2 value. In addition, the PLS-DA model was evaluated using PLS-DA score plot, R^2X, R^2Y, and Q^2 values. The permutation test using 999 permutations and receiver operating characteristics (ROC) value were used to validate the PLS-DA model. The identification of potential biomarkers which are important for sample discrimination was performed using the variable importance for projections (VIP) value in the PLS-DA analysis. Variables with a VIP value higher than 1 were considered as discriminating metabolites which are potential for biomarkers.

3. RESULTS AND DISCUSSION

3.1. Lipid compositions of pure lard, beef tallow, and chicken fat

The physical appearance of lard, beef tallow (BT), and chicken fat (CF) are similar, thus making them vulnerable for adulteration and mislabelling. Figure 1 shows the total ion chromatogram (TIC) of lard, BT, and CF obtained from the LC-Orbitrap HRMS measurement. The TIC of those three samples were very similar, thus it is very difficult to dif-

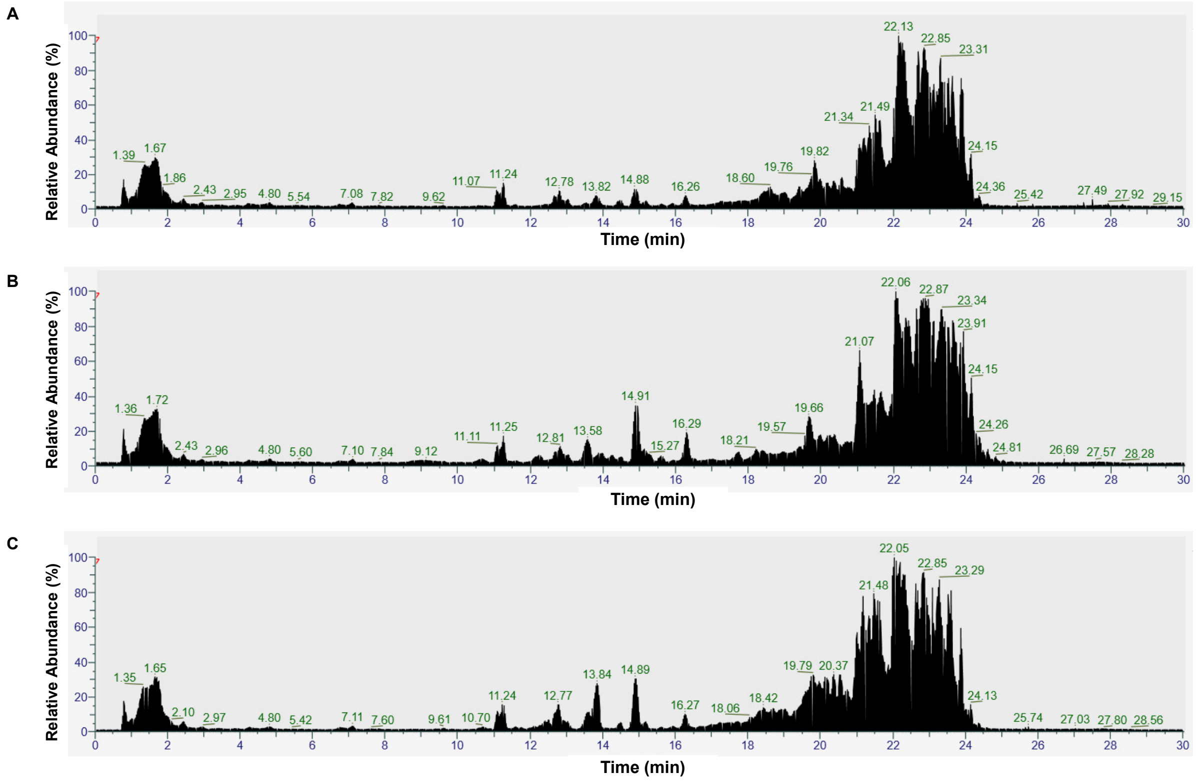

Figure 1. Total ion chromatogram (TIC) of lard (A), beef tallow (B), and chicken fat (C).

ferentiate lard, BT, and CF only by using visual observation on the TIC. The lipid compositions of lard, BT, and CF were successfully identified using Lipid Search software by extracting the raw TIC data. A total of 281 lipid ions from 18 lipid groups was obtained in lard using the positive ionization mode and 11 lipid ions from two lipid groups were observed using the negative ionization mode. The main lipid composition of lard was triglycerides (TG = 51.03%) followed by diglycerides (DG = 19.52%) and ceramides (Cer = 8.90%). BT contained 339 lipid compounds from 12 lipid groups observed in the positive ionization mode as well as 6 lipid compounds from the negative ionization mode. The mos abundant lipid compositions in BT were TG (53.33%), DG (25.80%), and cer (8.99%), respectively. In addition, the main compositions of lipids in CF were also the same as lard and BT, which were TG (58.31%), DG (25.31%), and cer (5.21%), respectively. The total lipid compounds observed in CF were 395 compounds from the positive ionization mode and 8 compounds from the negative ionization mode.

Many lipid compounds in lard, BT, and CF could be found from various lipid groups. Figure 2 illustrates the Venn diagram of the lipid metabolites contained in the three types of fats. According to the diagram, it could be observed that 117 lipids were found only in lard while 172 and 210 lipids were only found in BT, and CF, respectively. On the other hand, 82 lipid compounds were identified in all three types of fats (lard, CF and BT). Further investiga-

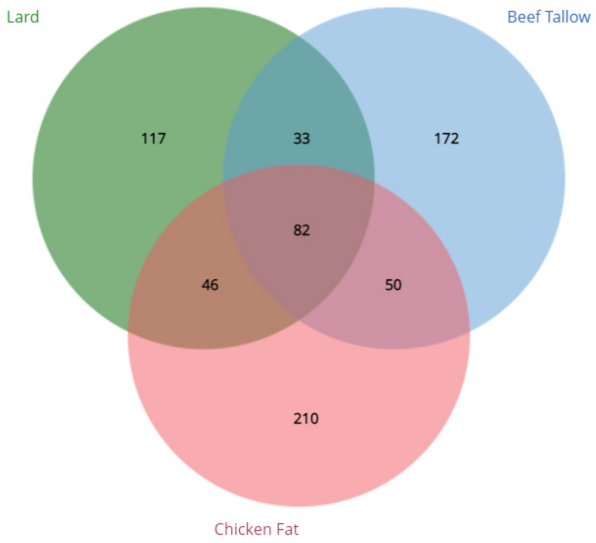

Figure 2. Venn diagram of lipid compositions in lard, beef tallow, and chicken fat

tion detected specific lipid groups only found in lard such as AcHexStE (acyl hexosyl stigmasterol ester), BiotinylPE (biotinyl phosphoetanolamine), LPC (lysophosphatidylcholine), MePC (monoether phosphatidylcholine), PC (phosphatidylcholine) and PI (phosphoinocitides). These lipid groups were absent from CF and BT. Lipid groups of SPH (sphingomyelin) and WE (wax esters) were found to be specific to CF, whereas lipid groups of MG (monoglyceride) and SiE (silyl ether) were observed only in BT. This information is very useful for the differentiation of lard, BT, and CF in order to avoid adulteration and mislabelling. The details of the specific lipid groups found in lard, CF, and BT with their lipid compounds for each group are presented in Table 1. Previous research on the discrimination of lard from other fats such as chicken fat, goat fat, and cattle fat has been performed based on fatty acid profiles using GC-TOF-MS. It was found that three fatty acid methyl esters of methyl trans-9,12,15-octadecatrienoate (C18:3 n3t), methyl 11,14,17-eicosatrienoate (C20:3 n3t) and methyl 11,14-eicosadienoate (C20:2 n6) could be used as potential discriminating lipids of lard from other animal fat samples (Indrasti *et al*., 2010). However, it is only capable of identifying fatty acids, not the comprehensive types of lipids. Another study aimed to apply different analytical approaches such as gas liquid chromatography

Table 1. Specific lipid compounds in lard, beef tallow, and chicken fat observed in untargeted lipidomics using LC-Orbitrap HRMS

Types	Lipid Groups	Compounds	Ionization mode
Lard	BiotinylPE	BiotinylPE(31:0)	Positive
	LPC	LPC(18:0)	Positive
	MePC	MePC(33:0)	Positive
		MePC(33:0e)	Positive
		MePC(33:1)	Positive
		MePC(35:0)	Positive
		MePC(35:1)	Positive
		MePC(35:2)	Positive
	PC	PC(16:0_18:1)	Negative
		PC(16:0_18:2)	Negative
		PC(18:0_18:1)	Negative
		PC(18:0_18:2)	Negative
		PC(34:2)	Positive
		PC(36:1)	Positive
		PC(36:2)	Positive
		PC(36:3)	Positive
		PC(36:4)	Positive
		PC(37:3e)	Positive
		PC(39:4e)	Positive
	PI	PI(18:0_20:4)	Positive
Beef Tallow	MG	MG(34:0)	Positive
		MG(34:1)	Positive
	SiE	SiE(28:0)	Positive
Chicken Fat	SPH	SPH(d22:1)	Positive
	WE	WE(6:0_16:3)	Positive
	LPA	LPA(15:0)	Negative

BiotinylPE = biotinyl phosphoetanolamine, LPC = lysophosphatidylcholine, MePC = monoetherglycerophosphocoline, PC = phosphatidylcholine, PI = phosphatidylinositol, MG = monoglycerides, SiE = SPH = sphingomyelin, WE = wax esters, LPA = lysophospatidic acid

(GLC), HPLC, and differential scanning calorimetry (DSC) to discriminate lard from beef tallow, mutton tallow, and chicken fat. The GLC method was not suitable for discriminating lard from the others by using overall fatty acid compositions. Triacylglycerol (TAG) análisis using HPLC showed a TAG profile for lard that differs from beef tallow and mutton tallow, but similar to chicken fat. The analysis of lard using DSC showed a different melting temperature for lard compared to other animal fats, although further analysis is still required in order to be more specific (Marikkar *et al.*, 2021).

Overall, liquid chromatography-high resolution mass spectrometry using the Orbitrap mass analyzer could be used for the comprehensive identification of lipid compositions in lard, BT, and CF. Some differences in the lipid groups were detected, which is important for the differentiation of lard from BT and CF. The chemometric analysis could be used to identify the metabolite pattern, in this case lipids, to differentiate and classify lard, beef tallow, and chicken fat.

3.2. Lipidomics using LC-HRMS and chemometrics to detect lard adulteration in BT and CF

Lipidomic analysis using LC-HRMS could be used to detect the presence of lard adulteration both in BT and CF at a ratio of 50% adulteration. The TIC of adulterated CF and BT with 50% lard was still similar to samples of pure BT and pure CF (data not shown). The main composition of lipid groups in adulterated BT and CF with lard, such as triglycerides, followed by diglycerides and ceramides, was similar to pure samples. Investigations using lipid compositions showed that BT and CF adulterated with lard could be differentiated from pure BT and CF samples. The specific lipid groups in lard could be detected in adulterated imples of BT, namely LPC (lysophosphatidylcholine), MePC (monoetherglycerophosphocoline), PC (phosphatidylcholine), and PI (phosphatidylinositol). These lipid groups were absent from pure BT. Therefore, it can be used to indicate the presence of lard in BT. At the same time, in adulterated CF with 50% lard, the specific lipids of lard which were absent from CF such as biotinylPE (biotinylphosphoetanolamine), PC, LPC, MePC and DG were detected.

Figure 3A shows the Venn diagram of lipid metabolites between pure lard, pure BT and adulterated BT with 50% lard. The results showed that 99 lipid compounds were present in lard, BT and adulterated BT. 161, 119, and 85 lipid compounds were found specific to BT, lard, and adulterated BT, respectively.

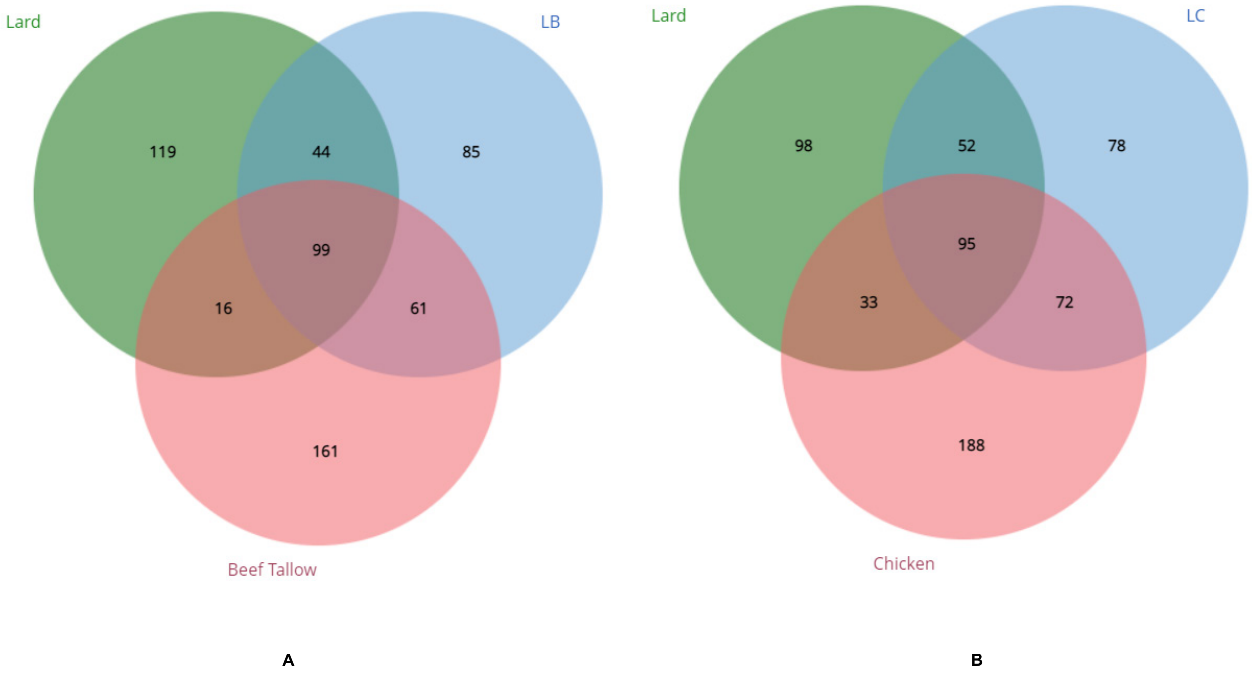

FIGURE 3. Venn diagrams of lipid compositions in lard, beef tallow, and beef tallow adulterated with 50% lard (LB) (A) and lard, chicken fat and chicken fat adulterated with 50% lard (LC) (B)

On the other hand, the results of the Venn diagram from pure lard, pure CF and adulterated CF with 50% lard as depicted in Figure 3B show that 98 lipid compounds were found only in lard, 188 lipids were specific to CF, and 78 lipids were observed only in adulterated CF. These lipids could be used to identify the authentication purposes of CF from lard. Meanwhile a number of 95 lipid compounds were found in lard, CF, and CF adulterated with lard.

The chemometric analysis using PCA successfully differentiated between pure samples of BT and CF and the adulterated ones using lard as shown in the PCA score plot in Figure 4A. The PCA performed with six principal components successfully differentiated adulterated samples from pure samples with $R^2 = 0.999$ and $Q^2 = 0.996$. A high R^2 value indicated high model accuracy, whereas a high value for Q^2 (> 0.500) showed good model predictability. (Bevilacqua *et al.*, 2017). All adulterated samples of BT and CF with 50% lard appeared around the score plot for lard. PLS-DA using three components was successfully used for discrimination and classification between pure and adulterated samples of BT and CF with lard as depicted in the PLS-DA score plot in Figure 3B. The goodness of fit of the PLS-DA model was shown by R^2X (0.706) and R^2Y (0.988) values. Meanwhile, the Q^2 value (0.976) demonstrated the good predictability of the model. In addition, all the adulterated samples of BT and CF could be correctly classified as adulterated samples with 100% accuracy. The PLS-

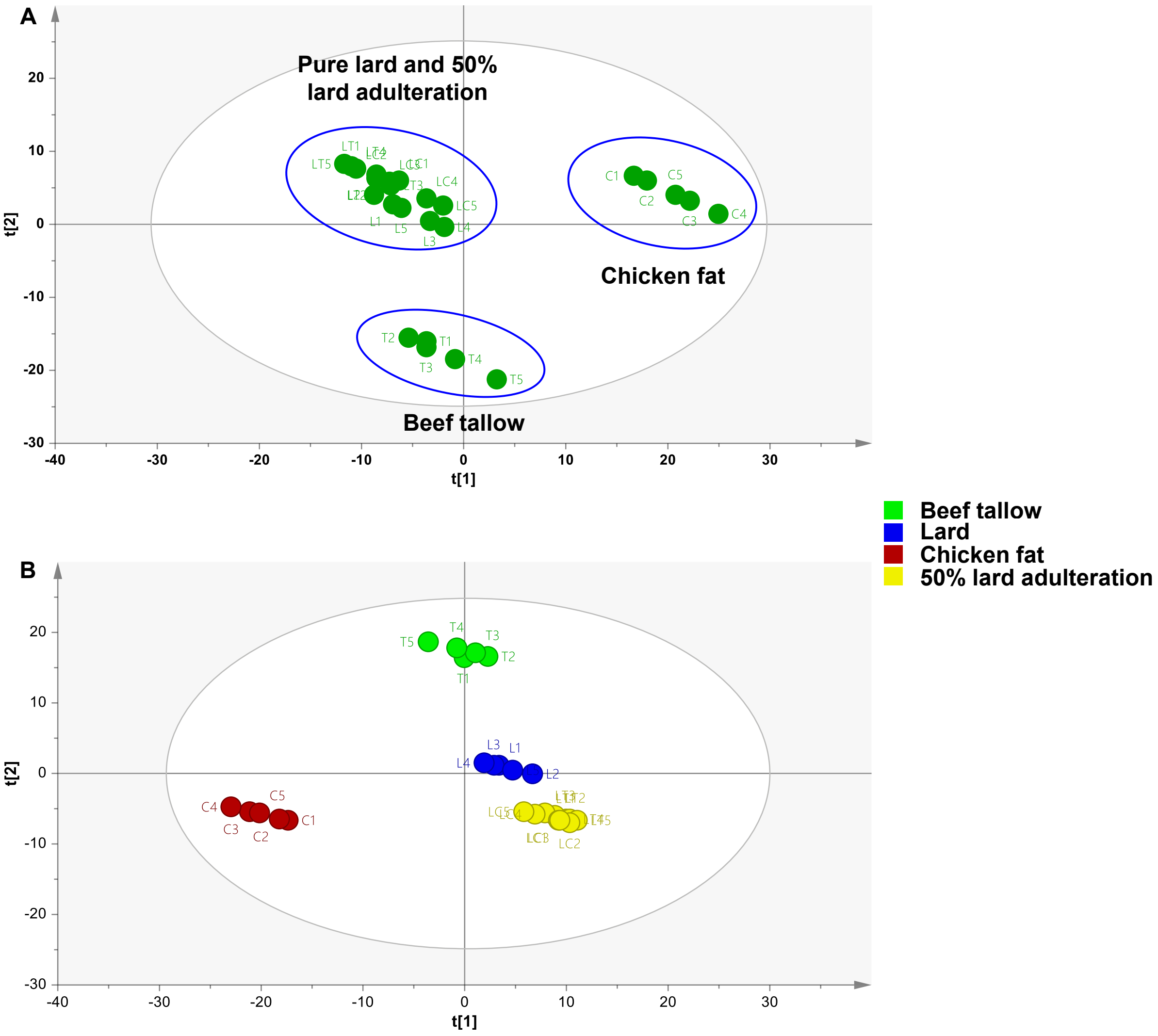

FIGURE 4. PCA score plot (A) and PLS-DA score plot (B) for differentiation of lard, beef tallow, chicken fat, and adulterated beef tallow and chicken fat with 50% lard [L1-L5= lard (n=5), T1-T5 = beef tallow (n=5), C1-C5 = chicken fat (n=5), LT1-LT5 = mixture of 50% lard and 50% beef tallow (n=5), LC1-LC5 = mixtures of 50% lard and 50% chicken fat (n=5)]

DA model was evaluated by means of a permutation test and ROC value to validate the PLS-DA model as shown in Figure 5. The permutation test used 999 permutations to confirm the validity of the PLS-DA model. All permutated models on the left side were lower than the original models on the right side (Figure 5A). In addition, the intercept of Q2 was zero and lower than zero (0.0, -0.43), thus indicating good model validity. The analysis of ROC was evaluated using the area under the curve (AUC) value. The resulting AUC value was 1 for each class (Figure 5B), which confirmed the validity of the model (Rivera-Pérez *et al*., 2021). In addition, the analysis of variable importance projections (VIP) value in PLS was used to identify potential lipids which play important roles in discriminating between pure fat samples (BT and CF) and those adulterated with lard. Table 2 shows the potential lipid biomarkers obtained from the VIP analysis. Variables with a VIP value greater than 1 are considered important variables as potential biomarkers for sample discrimination. Most of them were glycerolipids (DG and TG).

Previous research on lipidomic analysis using DART-TOF-MS (direct analysis in real time-time of flight-mass spectrometry) has been successfully used for the authentication of beef tallow. This research focused on triacylglycerol (TAG) compositions. A chemometric linear discriminant analysis (LDA) using TAG compositions was performed to successfully discriminate between pure and adulterated beef tallow

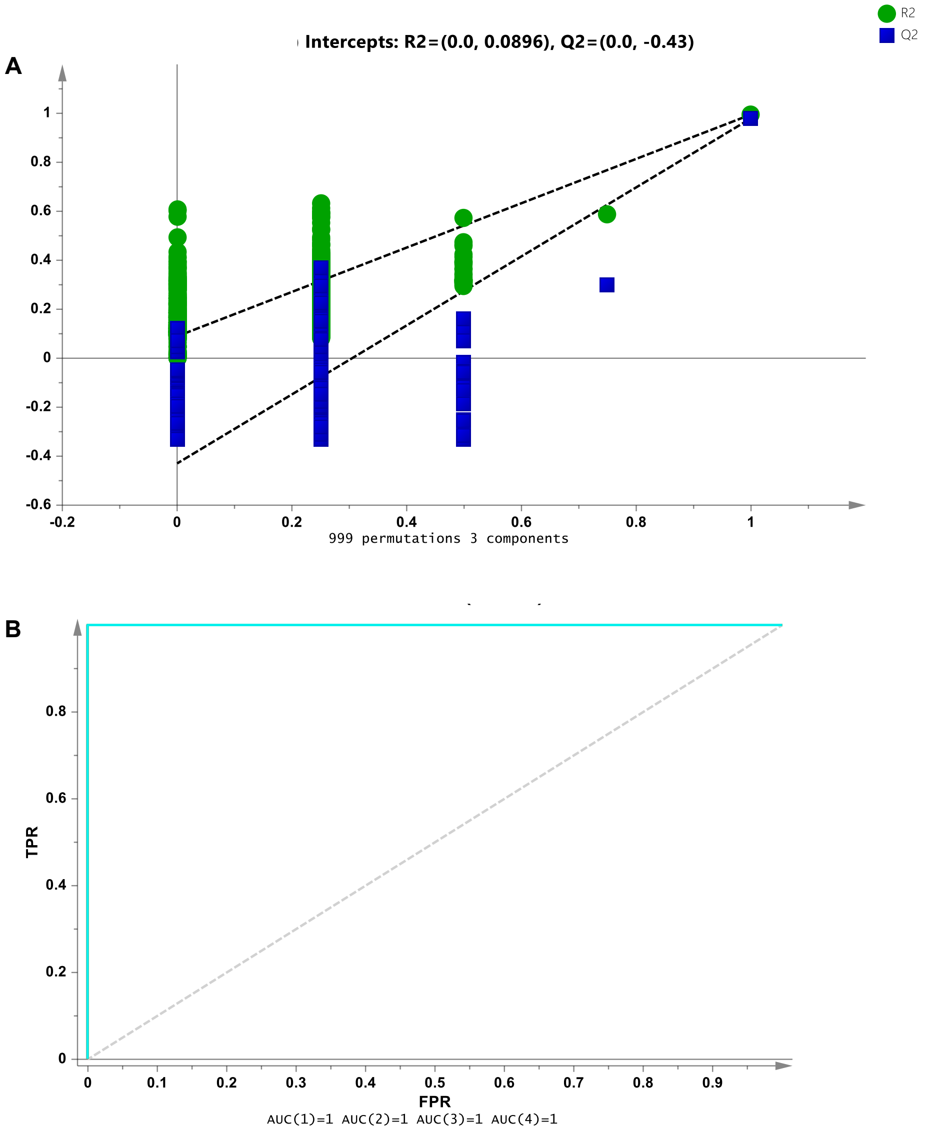

FIGURE 5. Permutation test (A) and receiver operating characteristic value (B) of PLS-DA model.

Table 2. Potential lipid biomarkers for discrimination between pure beef and adulterated beef tallow and chicken fat with 50% lard obtained from PLS-DA

No.	Lipids	VIP Value
1	TG(16:0_14:0_18:3)	1.31
2	TG(16:1_18:1_18:3)	1.31
3	TG(16:0_10:0_18:2)	1.31
4	TG(18:1e_16:0_18:2)	1.31
5	TG(16:0_18:1_18:2)	1.31
6	TG(16:0_14:4_16:0)	1.30
7	TG(18:0_16:0_18:3)	1.30
8	TG(8:0_14:1_18:2)	1.30
9	TG(16:0_17:1_18:3)	1.30
10	TG(15:0_18:1_18:2)	1.30
11	DG(32:3e)	1.30
12	DG(18:0_18:1)	1.30
13	DG(18:0_16:0)	1.30
14	DG(34:3e)	1.30
15	DG(34:4e)	1.30
16	TG(16:1_16:1_18:2)	1.30
17	TG(18:2_18:2_18:2)	1.30
18	TG(18:1_10:1_18:2)	1.30
19	TG(18:0e_18:1_18:2)	1.30
20	TG(17:0_18:1_18:2)	1.30
21	DG(18:0_17:0)	1.29
22	DG(54:3)	1.29
23	DG(17:0_18:2)	1.29
24	DG(19:1_18:1)	1.29
25	DG(20:3_18:2)	1.29
26	TG(18:1_14:4_18:1)	1.29
27	TG(18:3_18:2_18:2)	1.29
28	TG(16:0_12:3_18:1)	1.29
29	TG(18:1_18:1_18:2)	1.29
30	TG(18:0_18:0_18:0)	1.29
31	TG(18:0_18:0_18:1)	1.29
32	ZyE(35:6)	1.29
33	TG(16:0_16:0_18:1)	1.29
34	TG(70:2)	1.29
35	TG(18:1_18:2_22:3)	1.29
36	TG(16:1_14:1_14:2)	1.29
37	TG(16:0_17:0_18:1)	1.29
38	TG(4:0_16:0_18:1)	1.29
39	TG(16:0_16:0_18:2)	1.29
40	TG(16:0_16:1_18:2)	1.29

VIP = variable importance for projections, TG = triglycerides, DG = diglycerides, ZyE = zymosteryl ester

samples with lard (Vaclavik *et al.*, 2011). Our study provided more comprehensive lipid compounds because it is focused not only on the TAG compositions but a wider range of lipid compounds as well.

4. CONCLUSIONS

In the current study, liquid chromatography-Orbitrap high resolution mass spectrometry provided high throughput screening for the lipidomic analysis of lard, beef tallow, and chicken fat. The identification of lipid composition could be used to differentiate lard, beef tallow, and chicken fat. A combination with chemometrics such as PCA and PLS-DA could be used to detect the adulteration of chicken fat and beef tallow with 50% lard. Some potential lipid markers could be identified to detect and discriminate lard in beef tallow and chicken fat. This method is promising as a feasible strategy to discriminate lard from beef tallow and chicken fat for food authentication purposes. This research also supports the authorities responsible for halal authentication testing by providing effective and powerful analytical techniques for halal authentication of fat products. Future research using larger samples is required to validate the lipid markers of lard and to ensure the consistency of the results.

ACKNOWLEDGMENTS

This research was funded by The Ministry of Education, Culture, Research, and Technology through Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2022 with contract number 1817/UN1/DITLIT/Dit-Lit/PT.01.03/2022. The authors are grateful to LPDP (Lembaga Pengelola Dana Pendidikan) Scholarship (Indonesian Endowment Fund for Education), Ministry of Finance, Republic of Indonesia for supporting this research. The authors acknowledge The Research Center for Food Technology and Processing (PRTPP), National Research and Innovation Agency (BRIN), Playen area, Gunungkidul, Yogyakarta for providing the LC-Orbitrap HRMS used for the lipidomic analysis. The authors thank Hendy Dwi Warmiko for providing the Lipid Search software used in this research.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e514
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0992221

The degradation of some volatile compounds in evening primrose oil under UV/TiO$_2$ process

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Submitted: 25 September 2022; Accepted: 16 November 2022; Published online: 10 October 2023

SUMMARY: Off-flavor is one of the limiting factors in the quality and commercial acceptability of evening primrose oil (EPO). The results of this study demonstrated that ultraviolet light irradiated with titanium dioxide (UV/TiO$_2$) was able to effectively reduce odorous aldehyde concentrations, which would produce undesired flavors. Specifically, reductions in the E-2-Decenal, 1-octen-3-ol and hexanoic acid in EPO reached 50, 75.2 and 61.4% after a UV/TiO$_2$ process of 5 min, respectively. The odor active values (OAV) and hierarchical cluster analysis (HCA) showed that the result of the 5 min group was similar to that of the original oil. In addition, the physicochemical characteristics of EPO after processing did not change significantly. The result of the aroma profile analysis was consistent with the OAV and HCA results. Therefore, it has been concluded that 5 min UV/TiO$_2$ treatment could degrade some volatile compounds and provide a potential deodorization method for industry.

KEYWORDS: *Evening primrose oil; Fatty acid composition; Volatile components; UV/TiO$_2$.*

RESUMEN: ***Degradación de algunos compuestos volátiles de aceites de onagra bajo un proceso UV/TiO$_2$.*** El mal sabor es uno de los factores limitantes de la calidad y aceptabilidad comercial del aceite de onagra (EPO). Los resultados de este estudio demostraron que la luz ultravioleta irradiada con dióxido de titanio (UV/TiO$_2$) fue capaz de reducir eficazmente las concentraciones de aldehídos volátiles, que darían olores no deseados. En concreto, la reducción del E-2-Decenal, 1-octen-3-ol y el ácido hexanoico en EPO alcanzó el 50%, 75,2% y 61,4% respectivamente tras un proceso UV/TiO$_2$ de 5 min. Los valores de olor activo (OAV) y el análisis de conglomerados jerárquicos (HCA) mostraron que el resultado del grupo de 5 min fue similar al del aceite original. Además, las características fisicoquímicas del EPO después del procesamiento no cambiaron significativamente. El resultado del análisis del perfil de aroma fue consistente con nuestro resultado de OAV y HCA. Por lo tanto, se ha llegado a la conclusión de que el tratamiento con UV/TiO$_2$ durante 5 min podría degradar algunos compuestos volátiles y proporcionar un método de desodorización potencial para la industria.

PALABRAS CLAVE: *Aceite de onagra; Componentes volátiles; Composición de ácidos grasos; Ultravioleta/TiO$_2$.*

Citation/Cómo citar este artículo: Pan FG, Chen XM, Yang EQ, Liu BQ. 2023. The degradation of some volatile compounds in evening primrose oil under UV/TiO2 process. *Grasas Aceites* **74** (3), e514. https://doi.org/10.3989/gya.0992221

1. INTRODUCTION

Evening Primrose Oil (EPO) is obtained from the seeds of evening primrose by the cold-pressed process. Due to its high content in unsaturated fatty acids, especially γ-linolenic acid, it is widely consumed and used to relieve the effects of dermatitis, psoriasis, premenstrual and menopausal syndromes (de Santana *et al.,* 2019). However, these unsaturated fatty acids are also easily oxidated and some oxidation products with low molecular weight are formed. Among the complex production, some compounds could directly influence the overall flavor of EPO and contribute to off-flavor. As a consequence, there is a decline in commercial acceptability.

TiO_2 photocatalysis is considered a very promising technology due to its environmental protection, high antibacterial activity, low toxicity, and low cost. Hence, its application has been steadily growing in the food industry (Rashid *et al.,* 2021). One of these technologies is TiO_2 photocatalysis under UV, which is always used for the decomposition of organic compounds (Fonseca *et al.,* 2021). Zhang *et al.* (2016) have established a nanocomposite based on TiO_2, which showed excellent ability to degrade acetone in air. Similarly, the combination of UV and H_2O_2 successfully removed organic chemicals from waste water (Miklos *et al.,* 2018). However, the oxidation of fatty acids should be taken into consideration when applying UV to oil compared to the aqueous system (Sun *et al.,* 2019). As early as 2006, Luna *et al.* (2006) discovered that UV irradiation would cause virgin olive oil oxidation, and the concentration of volatile compounds responsible for the rancid sensory increased. However, a similar result was not found in essential oil. Li *et al.* (2016) demonstrated some aldehydes in citrus essential oil were degraded after UV irradiation. In addition, UV/TiO_2 was used to remove aflatoxins which were harmful to humans from oil. Researchers used UV radiation combined with TiO_2 to remove aflatoxins from peanut oil and corn oil. They found that the effect was satisfactory, and the physicochemical characteristics of the oil were not significantly affected (Magzoub *et al.,* 2019; Sun *et al.,* 2019). Therefore, it is necessary to explore the changes in the volatile compounds in EPO due to the UV/TiO_2 process and to evaluate the impact on oil quality.

In recent years, headspace solid-phase microextraction-mass spectrometry (HS-SPME-GC-MS) was widely developed in the analysis of volatile compounds, It was useful to detect the composition and concentration of volatile compounds in the sample (Song *et al.,* 2022). However, the intensity of the fragrance is not only related to the concentration, but also the threshold. Thus, the odor activity value (OAV) was calculated to indicate the importance of the odor in the overall flavor. Generally speaking, the larger the OAV, the greater the effect it has. Fang *et al.* (2022) evaluated the effect of the deodorization of oil after the nanofiltration membrane process by OAV analysis. In addition to the methods described above, a cluster heat map was also used as an important tool for the analysis of large amounts of data.

A study from our previous investigation has established the volatile compounds of original evening promise oil (Pan *et al.,* 2022). As shown in Figure 1,

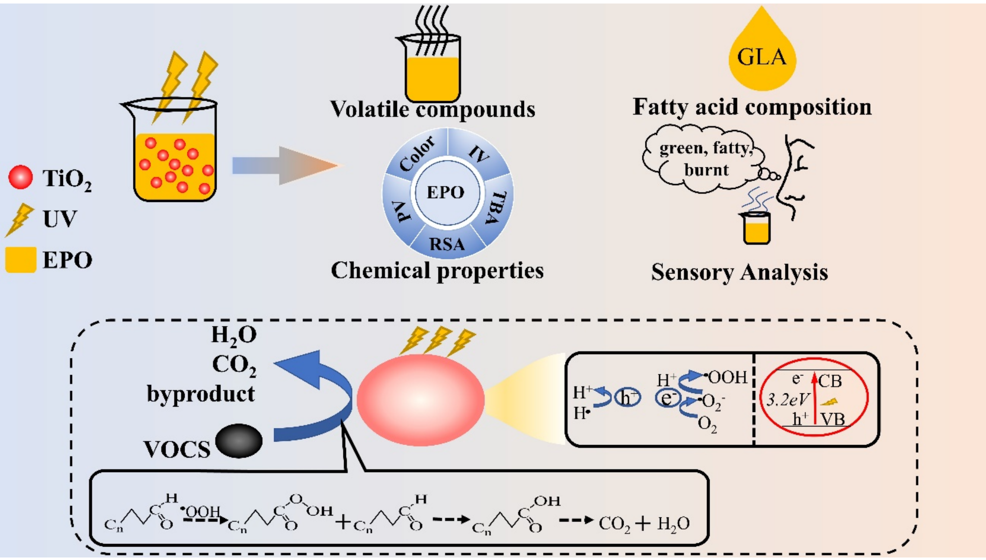

FIGURE 1. Schematic diagram of the study on the influence of UV/TiO2 process in EPO.

this study aimed to explore the volatile compounds of EPO subjected to UV/TiO_2 irradiated at different times (0, 5, 15, 30, 45, 60 min), separately. A sensory evaluation was conducted to compare the aroma characteristics of EPO before and after treatment. The relationship between the volatile compounds and the irradiation time was also revealed. In addition, the chemical properties of EPO were discussed.

2. MATERIALS AND METHODS

2.1. Oil sample preparation

Evening primrose oil came from the Changchun Baili biotech company (Changchun, Jilin province, China). TiO_2 nanopowder (P25) was commercially obtained from the Benze Reagent company, manufactured in Germany. All chemical reagents, including solvents, were of analytical grade and purchased from local suppliers.

Before irradiation, EPO (10 g) and TiO_2 (10 mg) were stirred in the dark for 30 min to achieve equilibrium. The samples (10 g EPO stirred with 10 mg TiO_2) were placed under ultraviolet light (254 nm) for 5, 15, 30, 45, and 60 minutes, and stored at 4 °C.

2.2. The identification of volatile compounds

The extraction of volatile compounds in EPO was carried out as described by Wang *et al.* (2022) with slight modifications. The volatile compounds of the irradiated and control samples were extracted with 1 cm of Divinylbenzene / Carboxen / Polydimethylsiloxane (DVB / CAR / PDMS) SPME fiber (50/30μm), (grey, Supelco, USA), and then analyzed by GC-MS (GC-MS-QP2020, Shimadzu, Kyoto, Japan). According to the manufacturer's suggestion, the fiber was heated before use at 250 ºC in the gas chromatographic injection port for 30 minutes. The sample (2.5 g) was evenly distributed in a 15-mL headspace vial, and then 1 μL of 2-methyl-3-Heptanone (14 μg/μL in n-hexane) was added as internal standard. The sample was equilibrated at 50 °C for 10 minutes, then adsorbed by fiber at 50 °C for 30 minutes, and finally thermally adsorbed into the GC-MS at 250 °C for 5 minutes.

2.3. Qualitative and quantitative analysis of volatile compounds

The relative concentration of volatile compounds in the evening primrose oil samples was quantitatively calculated based on internal standards. The detected compounds were identified by comparing the mass spectra obtained from the NIST17 library.

OAV is equal to the ratio of compound concentration (ci) to the odor threshold (oti). Threshold intensity in the oil was found in the book and the compounds with OAV ≥ 1 were generally considered to contribute greatly to aromatic volatile compounds (Fang *et al.,* 2022).

2.4. Determination of the fatty acid composition in oil

The fatty acid composition of the samples was determined by GC-MS, and area normalization was used to quantify the fatty acid. The results were expressed as a percentage of the relative peak area. The method was based on our previous study with slight modifications (Pan *et al.,* 2020).

2.5. Radical scavenging activity

The radical scavenging capacity (RSA) of EPO was measured according to the methods applied in the previous study with slight modifications (Szydłowska-Czerniak and Łaszewska, 2015). Briefly, the sample was extracted with methanol, and the absorbance of the obtained solutions was measured using a UV−Vis spectrophotometer and automated microplate reader (Bio TEK, USA). The radical scavenging activity was estimated by the following equations:

$$\text{Radical scavenging capacity (\%)} = \frac{A_C - A_S}{A_C} \times 100\%$$

where A_S and A_C represent the absorbance of the oil samples and control group, respectively.

2.6. Chemical properties

The peroxide value (PV) and iodine value (IV) of EPO were determined by the procedure of the AOCS (2003) Official Method. Thiobarbituric acid (TBA) value was evaluated according to Baghdadi *et al.* (2019). The color parameters (L, a* and b* values) of EPO were determined using a Chroma Meter (CR-400, Konica Minolta, Japan) at 25 °C.

2.7. Sensory analysis

Trained panelists (5 females and 5 males between the ages of 20−35 years) with experience in sensory analysis of oil were selected from Jilin University to evaluate the intensity of odor attributes in the samples. Prior to analysis, several aroma qualities were selected by a descriptive test. The intensities of selected odor attributes (green, fruity, fatty, vinegar-like, burnt) were rated using a linear scale from 0 (not perceivable) to 5 (strong, high intensity) on a five-point scale in steps of 0.5. Each sample was analyzed three times by the panelist, and the data was expressed as the mean. Adequate time was provided between the different samples to avoid tiredness on the part of the panelists. The test was carried out at a comfortable temperature (25 °C) and the samples were poured into the glasses for further analysis.

2.8. Data analysis

All experiments were carried out in triplicate and the results are given as mean ± standard deviation. Variance analysis was executed using SPSS Statistics software v.21.0 (SPSS Inc., Chicago, IL, USA) with a p value < 0.05.

3. RESULTS AND DISCUSSION

3.1. Analysis of volatile compounds in evening primrose oil

The previous report confirmed that the increase in temperature has a negative effect on oil quality, which will lead to the autoxidation and the decomposition of hydroperoxides, and generate undesired flavors (Bao *et al.,* 2022). To avoid raising the temperature during irradiation, an ice bath was used to make the experiment work at a lower temperature. As the results in Table 1 show, a total of 56 dominant odorants (identified by adding 2-methyl-3-heptanone as internal standard) were extracted from our samples by fiber and identified by the machine. They can be classified into seven chemical categories according to different properties, including 7 alcohols, 18 aldehydes, 4 ketones, 8 acids, 4 esters, 14 hydrocarbons and 1 other compound. Among them, hydrocarbons were the most abundant volatile compound in all samples, followed by aldehydes and alcohols. However, due to the high odor thresholds (Zhang *et al.,* 2021), hydrocarbons were considered inadequate aroma contributors for our samples despite having the highest content determined in the EPOs. The alcohols and aldehydes were formed by the decomposition of fatty acids. The analysis of our study confirms again that the aldehydes, which are considered to have contributed to the overall odor in a large quantity of edible oil, such as Camellia oleifera Seed Oil, virgin olive oil, cold-pressed peanut oil (Angerosa *et al.,* 2004), are also major compounds in EPO. It is worth noting that the aldehydes, which provide a typical grassy and fatty odor in oil, may be harmful to the sensory quality of the product. Esters were produced by the esterification of alcohols with free small molecular fatty acids, generally described as fruit and flower aromas (Zhang *et al.,* 2021). They were also detected in our sample and contributed to a positive odor in EPO. Besides the above four kinds of components, the remaining substances also play a role in the overall flavor. They interact with and influence each other and constitute the complicated aroma profile of EPO.

The evolution of key VOCs mainly affected by the irradiation of the EPO is shown in Figure 2. E-2-Decenal, 1-octen-3-ol and hexanoic acid showed very similar trends with diverse amounts. They rapidly decreased under 5 min irradiation and then reached their maximum concentration after 60 min irradiation. (E, E)-2,4-decadienal and 3-methyl-1-pentanol showed similar behavior in that they initially decreased and then increased with the increase in UV/TiO_2 pretreatment time, and reached the lowest concentration at 30 min, which means that the decomposition capacity of UV/TiO_2 was different for each molecule. In addition, the concentration of E-2-octenal, (E, E)-2,4-Nonadienal and nonanal decreased after the UV/TiO_2 irradiation. Therefore, UV/TiO_2 treatment for a short time, less than 30 min, was considered beneficial for EPO flavor. In fact, nonanal was an important odorant in oil, including olive oil and soybean oil, derived from the autoxidation of oleic acid, and was previously considered to be a suitable index of the degree of oxidation (Vichi *et al.,* 2003). It showed very low amounts, approximately 0.05 mg/kg in EPO with 60 min UV irradiation. As already pointed out in the literature, (E)-2-Octenal was detected in the deep-frying process and suggested to be responsible for the rancid defect (Multari *et al.,* 2019). In our study, the concentration of (E)-2-Octenal in the sample of 60 min decreased by approximately 5 times compared to the original sample. Hexanal showed a similar amount in EPO at 0, 5, and 15 min and a significant decrease over time. Heptanal exhibited a different behavior from hexanal. It increased significantly after 30, 45,

Table 1. Identification of volatile compounds extracted by HS-SPME

Nr	**Compounds**	**Identification method**	**Concentration (mg/kg)**					
			0 min	5 min	15 min	30 min	45 min	60 min
	Alcohol							
1	(E)-2-Octenol	MS	0.13 ± 0.04^{a}	0.02 ± 0.00^{a}	0.06 ± 0.03^{a}	0.04 ± 0.01^{a}	0.03 ± 0.00^{a}	0.06 ± 0.04^{a}
2	(Z, Z)-9,12-Octadecadien-1-ol	MS	ND	0.04 ± 0.00^{a}	0.02 ± 0.01^{c}	0.01 ± 0.01^{c}	0.02 ± 0.01^{c}	0.03 ± 0.01^{b}
3	2-ethyl-1-Hexanol	MS	0.31 ± 0.06^{b}	0.28 ± 0.10^{b}	0.32 ± 0.07^{b}	0.24 ± 0.05^{b}	0.25 ± 0.07^{b}	0.47 ± 0.08^{a}
4	2-Propanol	MS	0.03 ± 0.01^{a}	0.02 ± 0.01^{ab}	0.01 ± 0.01^{ab}	ND	0.00 ± 0.01^{b}	0.03 ± 0.01^{a}
5	1-Octen-3-ol	MS	1.25 ± 0.03^{a}	0.31 ± 0.04^{e}	0.48 ± 0.06^{d}	0.77 ± 0.04^{c}	0.82 ± 0.08^{c}	1.00 ± 0.08^{b}
6	3-methyl-1-Pentanol	MS	0.26 ± 0.04^{a}	0.22 ± 0.05^{ab}	0.16 ± 0.10^{ab}	0.14 ± 0.04^{b}	0.22 ± 0.05^{ab}	0.21 ± 0.03^{ab}
7	1-butanol	MS	2.98 ± 0.61^{a}	2.73 ± 0.37^{a}	2.56 ± 0.24^{a}	2.67 ± 0.13^{a}	2.81 ± 0.18^{a}	2.92 ± 0.14^{a}
	Aldehydes							
8	(E)-2-Heptenal	MS	0.20 ± 0.07^{a}	0.25 ± 0.12^{a}	0.16 ± 0.05^{a}	0.17 ± 0.03^{a}	0.19 ± 0.03^{a}	0.29 ± 0.04^{a}
9	Nonanal	MS	0.81 ± 0.06^{a}	0.35 ± 0.11^{b}	0.21 ± 0.05^{c}	0.26 ± 0.05^{bc}	0.09 ± 0.06^{d}	0.05 ± 0.01^{d}
10	Hexanal	MS	0.82 ± 0.26^{ab}	0.75 ± 0.03^{ab}	0.89 ± 0.14^{a}	0.76 ± 0.04^{ab}	0.60 ± 0.08^{b}	0.59 ± 0.10^{b}
11	(E)-2-Nonenal	MS	0.11 ± 0.02^{ab}	0.05 ± 0.01^{de}	0.15 ± 0.05^{a}	0.05 ± 0.04^{bc}	0.04 ± 0.01^{e}	0.10 ± 0.04^{ab}
12	Decanal	MS	ND	ND	0.01 ± 0.01^{a}	ND	ND	0.01 ± 0.01^{a}
13	(E, E)-2,4-Dodecadienal	MS	0.11 ± 0.04^{b}	0.19 ± 0.04^{a}	0.11 ± 0.02^{b}	0.15 ± 0.03^{ab}	0.13 ± 0.03^{ab}	0.13 ± 0.03^{ab}
14	(E, E)-2,4-Decadienal	MS	0.65 ± 0.02^{a}	0.33 ± 0.09^{bc}	0.26 ± 0.05^{bc}	0.21 ± 0.02^{c}	0.24 ± 0.04^{bc}	0.42 ± 0.01^{b}
15	(E)-2-Octenal	MS	1.24 ± 0.10^{a}	0.41 ± 0.18^{b}	0.22 ± 0.05^{bc}	0.28 ± 0.07^{bc}	0.17 ± 0.02^{c}	0.24 ± 0.11^{bc}
16	2,5-Dihydroxybenzaldehyde	MS	ND	0.42 ± 0.09^{a}	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}	0.01 ± 0.00^{b}	0.02 ± 0.01^{b}
17	Octanal	MS	1.93 ± 0.15^{a}	1.61 ± 0.33^{a}	1.65 ± 0.26^{a}	1.72 ± 0.31^{a}	1.63 ± 0.11^{a}	1.78 ± 0.28^{a}
18	(Z)-9,17-Octadecadienal	MS	1.35 ± 0.37^{ab}	1.76 ± 0.23^{a}	0.98 ± 0.13^{b}	1.23 ± 0.65^{ab}	1.59 ± 0.39^{ab}	1.22 ± 0.22^{ab}
19	Dodecanal	MS	0.70 ± 0.05^{b}	0.81 ± 0.18^{b}	1.14 ± 0.36^{b}	1.71 ± 0.37^{a}	1.61 ± 0.26^{a}	1.64 ± 0.28^{a}
20	(E)-4-Nonenal	MS	ND	ND	0.01 ± 0.00^{b}	0.01 ± 0.01^{a}	ND	ND
21	Pentanal	MS	0.6 ± 0.15^{a}	0.89 ± 0.16^{a}	0.65 ± 0.13^{a}	0.63 ± 0.12^{a}	0.60 ± 0.29^{a}	0.76 ± 0.16^{a}
22	Heptanal	MS	0.03 ± 0.03^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.07 ± 0.02^{a}	0.05 ± 0.00^{ab}	0.05 ± 0.01^{ab}
23	(E, E)-2,4-Nonadienal	MS	0.07 ± 0.05^{a}	0.03 ± 0.02^{ab}	0.01 ± 0.01^{b}	0.03 ± 0.01^{ab}	0.02 ± 0.01^{ab}	0.04 ± 0.01^{ab}
24	(E)-2-Decenal	MS	0.06 ± 0.03^{bc}	0.03 ± 0.01^{c}	0.11 ± 0.04^{bc}	0.15 ± 0.06^{ab}	0.12 ± 0.02^{ab}	0.20 ± 0.06^{a}
25	(E, Z)-2,4-Decadienal	MS	0.31 ± 0.07^{d}	0.35 ± 0.10^{d}	0.52 ± 0.11^{cd}	0.63 ± 0.12^{bc}	0.77 ± 0.12^{b}	1.02 ± 0.23^{a}
	Ketone							
26	5-Tridecanone	MS	0.16 ± 0.03^{a}	0.15 ± 0.04^{a}	0.14 ± 0.06^{a}	0.15 ± 0.03^{a}	0.18 ± 0.03^{a}	0.21 ± 0.03^{a}
27	Elsholtzia ketone	MS	ND	0.04 ± 0.03^{b}	0.02 ± 0.02^{a}	0.03 ± 0.01^{b}	0.04 ± 0.01^{b}	0.06 ± 0.01^{a}
28	3-Octen-2-one	MS	0.05 ± 0.02^{c}	0.27 ± 0.11^{ab}	0.34 ± 0.10^{a}	0.28 ± 0.05^{ab}	0.14 ± 0.14^{bc}	0.29 ± 0.09^{ab}
29	1-Nonen-3-one	MS	0.12 ± 0.07^{ab}	0.22 ± 0.09^{a}	0.08 ± 0.05^{b}	0.15 ± 0.02^{a}	0.16 ± 0.03^{ab}	0.20 ± 0.01^{a}
	Acid							
30	2-Hexenoic acid	MS	0.06 ± 0.06^{a}	0.03 ± 0.05^{a}	0.03 ± 0.04^{a}	0.04 ± 0.01^{a}	0.19 ± 0.25^{a}	0.03 ± 0.01^{a}
31	2-Octynoic acid	MS	0.34 ± 0.28^{a}	0.40 ± 0.05^{a}	0.30 ± 0.06^{a}	0.32 ± 0.09^{a}	0.38 ± 0.08^{a}	0.36 ± 0.05^{a}
32	Acetic acid	MS	0.03 ± 0.03^{a}	ND	ND	ND	ND	ND
33	Heptanoic acid	MS	0.13 ± 0.07^{a}	0.17 ± 0.09^{a}	0.22 ± 0.04^{a}	0.13 ± 0.01^{a}	0.12 ± 0.06^{a}	0.16 ± 0.02^{a}
34	Hexanoic acid	MS	1.27 ± 0.48^{a}	0.49 ± 0.20^{b}	0.59 ± 0.15^{ab}	0.62 ± 0.09^{ab}	0.78 ± 0.13^{ab}	0.95 ± 0.11^{ab}
35	(Z, Z)-9,12-Octadecadienoic acid	MS	ND	0.07 ± 0.02^{b}	0.02 ± 0.02^{c}	0.06 ± 0.01^{b}	0.08 ± 0.03^{b}	0.12 ± 0.01^{a}
36	Octadecanoic acid	MS	0.03 ± 0.04^{a}	ND	ND	ND	ND	ND
37	(E)-2-Hexenoic acid	MS	ND	0.05 ± 0.03^{a}	0.03 ± 0.01^{a}	0.05 ± 0.01^{b}	0.09 ± 0.06^{a}	0.05 ± 0.02^{a}
	Ester							
38	Docosanoic acid, docosyl ester	MS	0.40 ± 0.06^{ab}	0.43 ± 0.13^{a}	0.28 ± 0.10^{ab}	0.31 ± 0.04^{ab}	0.17 ± 0.04^{b}	0.18 ± 0.08^{b}
39	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	MS	1.23 ± 0.24^{ab}	1.49 ± 0.49^{a}	0.46 ± 0.12^{c}	0.82 ± 0.30^{bc}	1.30 ± 0.27^{ab}	1.45 ± 0.42^{ab}
40	Hexadecanoic acid, methyl ester	MS	0.60 ± 0.27^{a}	0.25 ± 0.11^{bc}	0.58 ± 0.17^{ab}	0.29 ± 0.08^{bc}	0.18 ± 0.15^{c}	0.28 ± 0.10^{bc}
41	Octadecanoic acid, 9-octadecenyl ester, (Z)-	MS	0.04 ± 0.02^{a}	0.01 ± 0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}	0.02 ± 0.03^{a}	ND
	hydrocarbon							
42	4,6-dimethyl-Dodecane	MS	3.85 ± 0.53^{a}	3.41 ± 1.62^{a}	3.64 ± 0.69^{a}	3.94 ± 0.28^{a}	3.34 ± 1.18^{a}	4.65 ± 0.47^{a}
43	2,6,10-Trimethyltridecane	MS	0.15 ± 0.05^{a}	0.15 ± 0.08^{a}	0.15 ± 0.08^{a}	0.13 ± 0.03^{a}	0.16 ± 0.02^{a}	0.22 ± 0.04^{a}
44	3-Octadecene, (E)-	MS	0.93 ± 0.07^{b}	1.24 ± 0.39^{ab}	1.59 ± 0.37^{a}	0.99 ± 0.27^{b}	1.01 ± 0.08^{b}	1.24 ± 0.30^{ab}
45	Cyclononasiloxane, octadecamethyl-	MS	0.86 ± 0.18^{a}	0.40 ± 0.09^{b}	0.39 ± 0.14^{b}	0.40 ± 0.09^{b}	0.38 ± 0.09^{b}	0.43 ± 0.08^{b}
46	Decane, 3,7-dimethyl-	MS	0.11 ± 0.06^{ab}	0.15 ± 0.05^{a}	0.09 ± 0.01^{ab}	0.06 ± 0.02^{b}	0.09 ± 0.00^{ab}	0.14 ± 0.03^{a}
47	Dodecane	MS	0.10 ± 0.06^{a}	0.13 ± 0.03^{a}	0.11 ± 0.04^{a}	0.10 ± 0.04^{a}	0.08 ± 0.07^{a}	0.09 ± 0.02^{a}
48	Heptadecane	MS	0.40 ± 0.05^{a}	0.33 ± 0.06^{a}	0.12 ± 0.04^{a}	0.07 ± 0.05^{a}	0.36 ± 0.45^{a}	0.41 ± 0.08^{a}
49	Hexadecane	MS	ND	0.14 ± 0.09^{a}	0.14 ± 0.04^{a}	0.08 ± 0.02^{ab}	0.05 ± 0.05^{bc}	0.07 ± 0.03^{ab}
50	Octadecane	MS	0.07 ± 0.05^{ab}	0.01 ± 0.01^{b}	0.01 ± 0.01^{b}	0.01 ± 0.00^{b}	0.05 ± 0.05^{b}	0.12 ± 0.03^{a}
51	Pentadecane	MS	ND	0.03 ± 0.04^{a}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.03^{a}	0.04 ± 0.04^{a}
52	Pentane	MS	1.13 ± 0.09^{a}	0.85 ± 0.10^{b}	0.56 ± 0.07^{c}	0.52 ± 0.09^{c}	0.49 ± 0.02^{c}	1.24 ± 0.20^{a}
53	Tetradecane, 5-methyl-	MS	1.92 ± 0.60^{a}	1.84 ± 0.54^{a}	1.72 ± 0.23^{a}	1.67 ± 0.20^{ab}	1.63 ± 0.15^{ab}	0.75 ± 0.93^{b}
54	Tridecane	ND	1.26 ± 0.32^{a}	0.72 ± 0.14^{bc}	0.66 ± 0.07^{c}	0.80 ± 0.14^{bc}	0.77 ± 0.05^{bc}	1.17 ± 0.41^{ab}
55	Undecane	ND	0.45 ± 0.16^{a}	0.56 ± 0.12^{a}	0.54 ± 0.09^{a}	0.62 ± 0.11^{a}	0.55 ± 0.04^{a}	0.44 ± 0.10^{a}
	Other							
56	Ethylene glycol - Adipate - Diethylene glycol	MS	ND	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}
	TOTAL		$\mathbf{34.92\pm5.04^{a}}$	$\mathbf{28.03\pm2.75^{bc}}$	$\mathbf{26.71\pm1.68^{c}}$	$\mathbf{28.02\pm2.00^{bc}}$	$\mathbf{28.74\pm1.53^{bc}}$	$\mathbf{33.38\pm2.65^{ab}}$

Each value in the table represents the mean ± SD (n=3). Valued labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test. ND: not detected in the sample, HS-SPME: headspace solid-phase microextraction, MS: mass spectrometry.

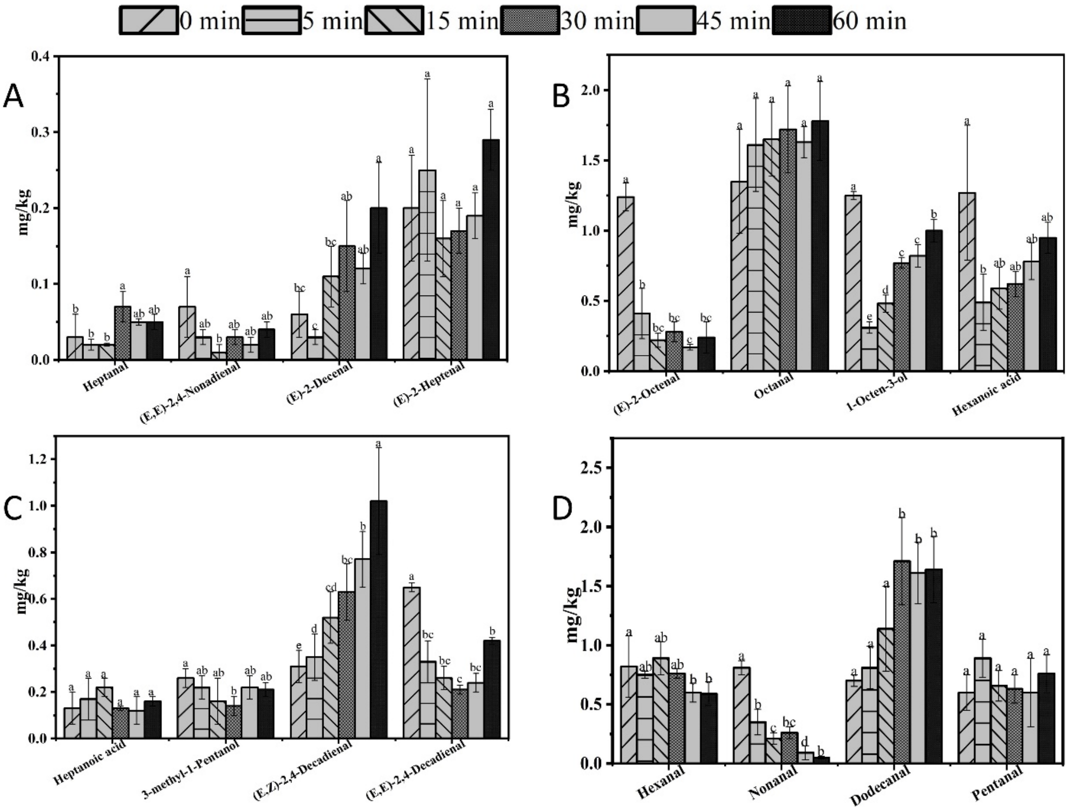

Figure 2. Evolution of the content of some volatile compounds subjected to different irradiation times. Each value represents the mean ± SD (n=3). Values labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test.

60 min irradiation because of the oxidation of free fatty acids. Heptanoic acid, (E)-2-Heptenal and pentanal displayed an insignificant change in all samples, which suggested that they were stable after a short time of irradiation. Apart from the compounds mentioned above, octanal, (E, Z)-2,4-decadienal and dodecanal showed an increasing trend. More specifically, (E, Z)-2,4-decadienal showed significant (and in some cases very strong) growth with the increase in processing time. The concentration of octanal was higher than original EPO, although there was no significant difference.

In our experiment, the concentration of some aldehydes was reduced. The change in volatile substances appears to be different between a short time and continuous irradiation for a long time of more than 1 day. According to the previous study, the decomposition of the organic compound was related to the oxidation reaction (Jamil *et al*., 2017; Jo and Dietrich, 2009; Tsang *et al.,* 2019). Therefore, the possible photocatalytic mechanism of TiO_2 on reduction of volatile compounds was discussed. Comprehensively, the electron (e^-) would be removed and a positive hole (h^+) would be generated, when the TiO_2 was exposed to light with energy greater than the band gap energy (higher than 3.2 ev). They would migrate to the surface of TiO_2. Then the combination of oxygen and e^- would produce active superoxide radical ion ($\bullet O_2^-$) and hydroperoxyl radical (•OOH), respectively. The volatile substances such as E-2-Decenal, 1-octen-3-ol and hexanoic acid would be oxidized, and degraded, leading to a decrease in their contents.

3.2. The analysis of the OAVs of the aroma compounds in EPO

The composition and content of the volatile substances are discussed above. However, the analysis does not fully represent the aroma characteristics of EPO. The threshold of the volatile compounds also influences their contribution to the aroma of EPO. The OAV, calculated by the ratio of its concentration to its odor threshold, was a key indicator in flavor analysis. As shown in table 2, 16 key aroma compounds were selected, including 12 aldehydes, 2 alcohol, and 2 acids. Among them, (E)-2-Hepte-

Table 2. Identification of volatile flavor compounds by OAV analysis

Compounds	Odor description [a]	OAV						
		Thresholds (mg/kg)[a]	0 min	5 min	15 min	30 min	45 min	60 min
(E)-2-Heptenal	Intense green, fatty, oily	0.01	15.38	19.23	12.31	13.08	14.62	22.31
Hexanal	Green, fatty, leafy	0.08	10.93	10.00	11.87	10.13	8.00	7.87
Nonanal	Strong, floral	0.15	5.40	2.33	1.40	1.73	0.60	0.33
(E, E)-2,4-Decadienal	Fatty, chicken, fried,	0.14	4.81	2.44	1.93	1.56	1.78	3.11
(E)-2-Octenal	green, pungent, spicy,	0.04	31.00	10.25	5.50	7.00	4.25	6.00
(E, Z)-2,4-Decadienal	fried fatty, green, waxy	0.18	1.72	1.94	2.89	3.50	4.28	5.67
Octanal	fat, soap, lemon, green	0.90	2.14	1.79	1.83	1.91	1.81	1.98
Dodecanal	Soapy, waxy, citrus	0.90	0.78	0.90	1.27	1.90	1.79	1.82
Pentanal	almond, malt, pungent	0.15	4.00	5.93	4.33	4.20	4.00	5.07
Heptanal	fat, citrus, rancid	0.05	0.60	0.40	0.40	1.40	1.00	1.00
(E, E)-2,4-Nonadienal	geranium, pungent	0.46	0.15	0.07	0.02	0.07	0.04	0.09
(E)-2-Decenal	green, must	0.15	0.40	0.20	0.73	1.00	0.80	1.33
3-methyl-1-Pentanol	pungent	0.47	0.55	0.47	0.34	0.30	0.47	0.45
1-Octen-3-ol	green, oily	0.04	34.72	8.61	13.33	21.39	22.78	27.78
Heptanoic acid	Cheesy, waxy, sweaty	0.10	1.30	1.70	2.20	1.30	1.20	1.60
Hexanoic acid	mild, fatty	2.50	0.51	0.20	0.24	0.25	0.31	0.38

[a] The Odor description and Thresholds of volatile compounds were referenced from www.thegoodscentscompany.com, http://www.flavornet.org/flavornet.html. OAV: odor active values

nal, 1-octen-3-ol, (E, E)-2,4-Decadienal, Nonanal, (E)-2-Octenal, (E, Z)-2,4-Decadienal, octanal, Pentanal and Heptanoic acid have a major impact on the overall flavor. By comprehensively considering the OAV, it seems that the EPO is more acceptable after 5 min of treatment.

The volatile compound with the maximum OAV in the original oil was 1-Octen-3-ol, followed by E-2-Heptenal, Hexanal and Nonanal. E-2-Heptenal was reported as the production generated by the linoleic acid, known to have a low odor threshold value, and it is usually described as oxidized, green, grassy, and pungent (Cecchi *et al.,* 2019; Tsitlakidou *et al.,* 2019). Although the concentration of E-2-Heptenal was only 0.2 mg/kg, the OAV was 15.13. 1-Octen-3-ol is generally considered to be mushroom-like and described as a secondary oxidation product of linoleic acid in the previous study (Choe and Min, 2006). It has an important contribution to the overall flavor profile because the OAV is 34.72, larger than 1. In the processed sample, the OAV was changed due to the change in concentration. The lowest OAV of E-2-Heptenal was 12.30 after 15 min treatment, followed by the OAV of 13.08 after 30 min. The OAV of 1-octen-3-ol decreased to 8.61, 13.33, 21.39, 22.78, 27.78, respectively. (E, Z)-2,4-Decadienal was described as deep-fried, and green, which is unexpected because it would give a negative flavor to the EPO.

To make a clearer comparison among the samples, the volatile compounds were investigated using a clustering heatmap based on the OAV in table 2. A total of 16 compounds were input and the plot is shown in Figure 3. The heatmap provided the profile of the input value by the colored box, and HCA was used for classifying the group of samples displayed on the left of the plot. The intensity of the color represents the relative contents of the value. The darker the yellow, the larger the contribution to the overall odor. As Figure 3 shows, the samples irradiated for 5 minutes and other periods are grouped into a cluster and distinguished from other EPOs. Considering a comparatively comprehensive and systematic study of their sensory description, we can state again that the EPO with 5 min irradiation was more acceptable. The figure also shows that 1-octen-3-ol, (E)-2-octenal and (E)-heptenal have a significant effect on all samples. On the top of the graph, the compounds were classified by HAC as well.

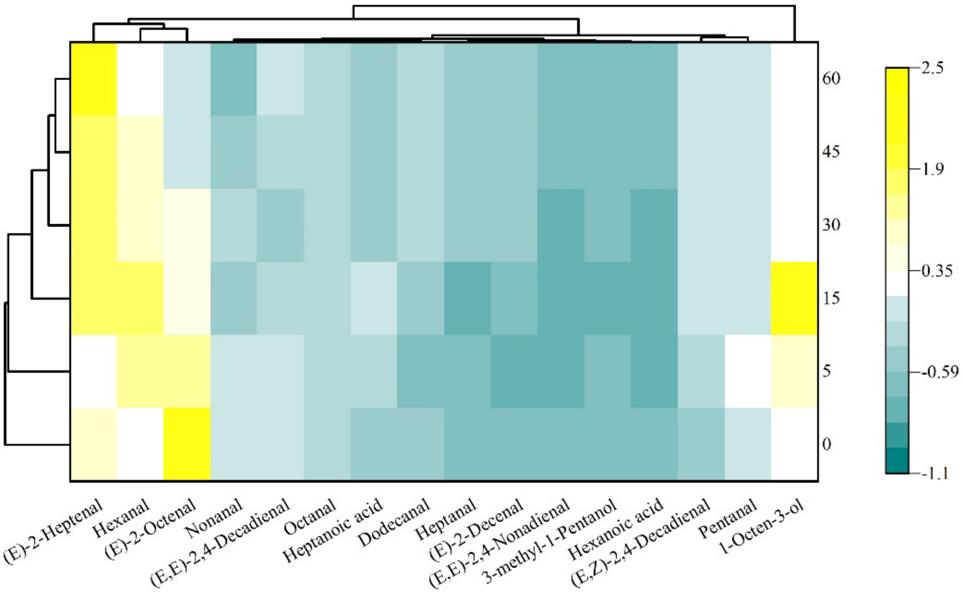

Figure 3. HCA of volatile compounds in EPO after different irradiation times.

3.3. Fatty acid composition of the EPO

We hope that UV irradiation has no remarkable negative effect on the quality of the oil, especially on the fatty acids. Previous reports have determined that fatty acids play an essential role in human organisms. They provide energy for our body, are beneficial against some diseases and have a distinctive sensory performance. The composition and percentage of fatty acids have an important impact on the quality of the oil. So, it is necessary to determine the fatty acids in EPO. The fatty acid composition was determined by converting the oil into fatty acid methyl esters (FAME) and detecting it using GC-MS. The results are presented in table 3. From the table, we can see that linoleic acid (C18:2) showed the highest percentage in EPO, followed by γ-linolenic (C18:3n6), oleic (C18:1), palmitic (C16:0), and stearic (C18:0) acids. The result showed that after irradiation the unsaturated acids remained relatively stable. Although a slight reduction existed in some fatty acids, there were no statistically significant differences. γ-linolenic, which is believed to have benefits for human health, is one of the most prominent fatty acids in evening primrose oil. The content of γ-linolenic was 12.15 in the original EPO sample, which is consistent with our previous study (Pan *et al.,* 2020). As the time increased, the content decreased to 11.99, 11.91, 12.01, 11.93 and 11.86, respectively. Luna *et al.* (2006) monitored the content of UFA in virgin olive oil. They found there is no significant reduction during the first 5 days of irradiation (Luna *et al.,* 2006). Our result showed the same trend. The composition and content of fatty acids in EPO were steady. It seemed 30 min irradiation had a minimal impact on EPO. Sun *et al.* (2021) also report that magnetic graphene oxide/TiO_2 nanocomposite can reduce aflatoxin B1, but the treatment does not affect the quality of corn oil. During storage, the main fatty acid contents in corn oil showed no significant change ($P \geq 0.05$). Magzoub *et al.* (2019) reported a similar result for Sudanese peanut oil. They found after irradiation, the fatty acid profiles were similar in the untreated and treated peanut oil samples, but the aflatoxins were reduced by immobilized titanium dioxide. This demonstrates the use of UV/TiO_2 an application which does not cause any unexcepted alterations to the nutritional qualities of EPO, especially when it irradiates under moderate conditions.

3.4. Free radical scavenging capacity

The free radical scavenging capacities of EPO were determined by DPPH and ABTS. The results are shown in Figure 4. The results showed that the antioxidant capacity of EPO with UV irradiation was weaker than the original sample, especially the sample with longer UV pretreatment time (45 and 60 min), which was attributed to the UV irradiation. The DPPH scavenging abilities of EPO with 0, 5,

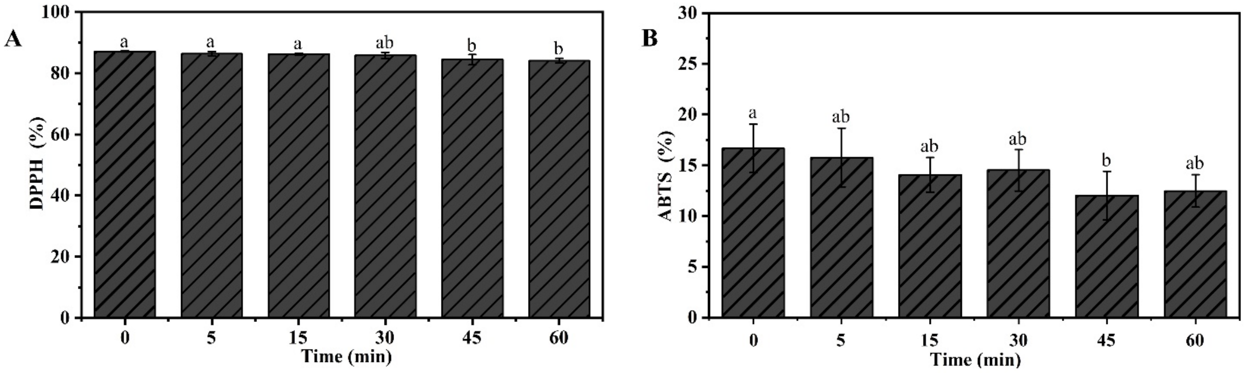

Figure 4. DPPH (A) and ABTS scavenging capacity (B) of different samples. Each value in the table represents the mean ± SD (n=3). Values labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test.

Table 3. Fatty acid composition and content of EPO subjected to UV/TiO_2 irradiation

Fatty acid	0 min	5 min	15 min	30 min	45 min	60 min
C14:0	0.05±0.01^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.05±0.00^{a}	0.05±0.01^{a}
C16:0	8.19±0.10^{a}	8.06±0.23^{a}	8.00±0.41^{a}	8.36±0.52^{a}	8.05±0.20^{a}	8.00±0.30^{a}
C16:1	0.04±0.00^{b}	0.04±0.01^{b}	0.03±0.00^{b}	0.04±0.01^{b}	0.03±0.01^{b}	0.05±0.01^{a}
C17:0	0.09±0.01^{a}	0.09±0.01^{a}	0.09±0.01^{a}	0.09±0.00^{a}	0.10±0.01^{a}	0.09±0.02^{a}
C18:0	2.85±0.09^{a}	2.93±0.23^{a}	2.90±0.07^{a}	2.91±0.16^{a}	2.91±0.05^{a}	2.88±0.11^{a}
C18:1	5.47±0.11^{a}	5.65±0.04^{a}	5.65±0.16^{a}	5.62±0.34^{a}	5.67±0.28^{a}	5.67±0.14^{a}
C18:2	62.91±0.48^{a}	62.88±1.90^{a}	63.07±2.17^{a}	62.87±0.40^{a}	62.94±1.27^{a}	63.12±2.57^{a}
C18:3n3	0.79±0.12^{a}	0.80±0.01^{a}	0.80±0.01^{a}	0.80±0.10^{a}	0.80±0.06^{a}	0.79±0.14^{a}
C18:3n6	12.15±0.44^{a}	11.99±0.18^{a}	11.91±0.96^{a}	12.01±0.13^{a}	11.93±0.30^{a}	11.86±0.65^{a}
C20:0	0.50±0.04^{a}	0.50±0.01^{a}	0.51±0.07^{a}	0.51±0.01^{a}	0.52±0.08^{a}	0.51±0.08^{a}
C20:1	0.08±0.01^{a}	0.08±0.01^{a}	0.09±0.02^{a}	0.08±0.01^{a}	0.08±0.00^{a}	0.08±0.00^{a}
C20:2	0.29±0.02^{a}	0.25±0.05^{a}	0.30±0.01^{a}	0.30±0.02^{a}	0.25±0.03^{a}	0.25±0.07^{a}
SFA	11.68±0.19^{a}	11.62±0.45^{a}	11.54±0.48^{a}	11.92±0.61^{a}	11.63±0.08^{a}	11.53±0.26^{a}
UFA	81.73±0.56^{a}	81.69±2.00^{a}	81.85±2.12^{a}	81.72±0.41^{a}	81.70±1.36^{a}	81.82±1.32^{a}
MUFA	5.59±0.11^{a}	5.76±0.40^{a}	5.77±0.16^{a}	5.73±0.33^{a}	5.79±0.28^{a}	5.80±0.14^{a}
PUFA	76.14±0.58^{a}	75.93±1.98^{a}	76.08±2.17^{a}	75.98±0.58^{a}	75.91±1.22^{a}	76.01±1.19^{a}

Each value in the table represents the mean ± SD (n=3). Values labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test. EPO: evening primrose oil, UV/TiO_2: ultraviolet light irradiation with titanium dioxide. C14:0: Myristic acid, C15:0: Pentadecanoic acid, C16:0: Palmitic acid, C16:1: Palmitoleic acid, C17:0: Margaric acid, C18:0: Stearic acid, C18:1: Oleic acid, C18:2: Linoleic acid, C18:3n3: α-Linolenic acid, C18:3n6: γ-Linolenic acid, C20:0: Arachidic acid, C20:1: Eicosenoic acid, C20:2: Eicosadienoic Acid, SFA: saturated fatty acids, UFA: unsaturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

15, 30, 45, 60 min were 87.12, 86.38, 86.25, 85.79, 84.40 and 84.12%, respectively, which were higher than the value obtained with ABTS methods. This might be related to the machine used in these methods. In general, the antioxidant capacity of the original EPO is better than that of the irradiated sample, which suggests again that a period of less than 5 min is more suitable for EPO.

3.5. Quality parameters of PSO

In our experiment, we explored the effect of UV/TiO_2 processing on the quality of EPO. The results are shown in table 4. These results indicate that a short irradiation time is appropriate, and the EPO remained relatively stable under such treatment. Overall, these findings are in accordance with the findings reported

Table 4. Quality characteristics of EPO subjected to UV/TiO$_2$ irradiation

	0 min	5 min	15 min	30 min	45 min	60 min
TBA (mg/kg)	0.03±0.00^{a}	0.03±0.00^{a}	0.04±0.00^{b}	0.04±0.00^{b}	0.04±0.00^{b}	0.04±0.00^{b}
POV (meq/kg)	0.86±0.05^{a}	0.91±0.09^{a}	0.91±0.12^{a}	0.95±0.05^{a}	0.99±0.06^{a}	0.97±0.07^{a}
IV (gI$_2$/100g)	184.30±1.48^{a}	176.48±3.60^{ab}	183.16±2.27^{ab}	174.95±3.10^{ab}	173.10±5.03^{b}	174.83±6.33^{ab}
L	27.82±0.52^{a}	27.44±0.42^{a}	27.56±0.59^{a}	27.55±0.69^{a}	26.77±0.79^{a}	27.42±0.60^{a}
a*	0.55±0.08^{a}	0.57±0.03^{a}	0.54±0.03^{a}	0.53±0.02^{a}	0.53±0.03^{a}	0.54±0.02^{a}
b*	5.44±0.25^{a}	5.53±0.32^{a}	5.29±0.06^{a}	5.33±0.10^{a}	5.31±0.11^{a}	5.31±0.20^{a}

Each value in the table represents the mean ± SD (n=3). Values labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test. EPO: evening primrose oil, UV/TiO$_2$: ultraviolet light irradiation with titanium dioxide. TBA: thiobarbituric acid values, POV: Peroxide value, IV: Iodine value.

by (Sun *et al.,* 2021), who also found that the quality of corn oil subjected to less than 30 min irradiation was acceptable. Table 4 demonstrates how POV undergoes a significant change due to a short irradiation time, but remained lower than the national limits (POV ≤ 19.7 mmol/kg). TBA and IV also showed a statistically significant change in EPO. The values for TBA and IV varied from 0.03 to 0.04 and 184.30 to 174.83. The deterioration was due to the treatment of UV/TiO$_2$. UV irradiation has an impact on the oxidation of EPO , although the deterioration is negligible. Color is one of the essential factors which determines the selection during a consumer's purchase. As shown in table 4, the changes in L, a* and b* values in EPO were not significant, which means UV/TiO$_2$ treatment does little damage to consumer perception.

3.6. Sensory analysis

To further obtain the differences in the overall flavor between the control group and treatment group, a flavor profile analysis was performed. The flavor attributes, including green, fruity, fatty, vinegar-like, and burnt were used to describe the EPO. The results are shown in Figure 5. Compared to the EPO with 5 min irradiation, the control group showed high scores green and fatty flavors, and lower intensities in fruity, vinegar-like and burnt. This suggests that undesired flavors are strongly perceived in EPO after 5 min of UV exposure as the intensities of green flavor decreased. This is consistent with what found in the previous experiment, a distinct slip in some volatile compounds (E-2-Decenal, 1-octen-3-ol) existed, which led to a reduction in the scores for green and fatty flavors. The sample treated for 5 min showed a better evaluation and higher sensory quality than other treatment-time groups.

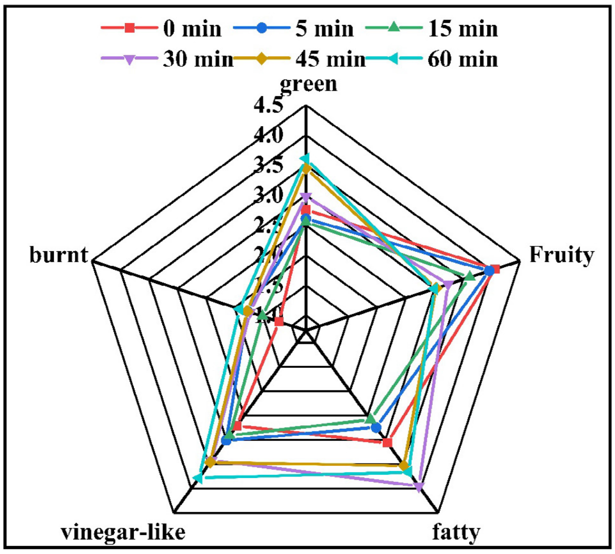

Figure 5. Aroma profile of EPO under different treatment conditions

4. CONCLUSIONS

In conclusion, the UV/TiO$_2$ process was able to effectively reduce odorous compound concentrations, such as E-2-Decenal, 1-octen-3-ol and hexanoic acid, which reached 50, 75.2 and 61.4% reduction after the UV/TiO$_2$ process of 5 min, respectively. It may be due to the positive hole (h$^+$) active superoxide radical ion (•O$_2^-$) generated by the UV radiation, which exerts a strong oxidative capacity and promotes the decomposition of these volatile substances. The HCA result and sensory analysis show that the sample with a 5 min process was similar to the control group. The results also indicate a reduction in some volatile compounds due to the UV/TiO$_2$ treatment, which does not have a significant impact on the quality of corn oil. These results have demonstrated that 5 min UV/TiO$_2$ processing is appropriate and for a large-scale application for deodorization in the EPO industry based on ease of operation and low cost,

ACKNOWLEDGMENTS

The authors acknowledge the financial support provided by the Jilin Province Science and Technology Development Project (NO.20220202074NC).

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GRASAS Y ACEITES 74 (3)
July-September 2023, e515
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1128212

Optimization and evaluation of a hydration method for producing high quality oil and defatted meal from hemp seed kernels

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Submitted: 19 November 2021; Accepted: 29 November 2022; Published online 10 October 2023

SUMMARY: A green and efficient method for producing high quality oil and defatted meal is in high demand, which has promoted the development of a hydration method for extracting oils from hem seed kernels. The hydration method optimized in this study recovered > 96% oil, which was further proved by infrared spectra, and extracted > 91% tocopherols, vitamin A, carotenoids, coenzyme Q10, phytosterols and squalene into the oil phase; while only small portions of flavonoids, other phenolic compounds and free fatty acids were extracted. The defatted meal was rich in water-soluble vitamins (including thiamin, riboflavin, niacin, pyridoxine, folate and vitamin C), proteins, dietary carbohydrates and phospholipids. The hydration method produced oils with lower AV or PV compared to solvent extraction, supercritical CO_2 and cold-pressing methods and a defatted meal with a protein content (52.69%) which was significantly higher than that obtained by supercritical CO_2 and cold-pressing methods. The hydration method is a type of green technology for sustainablly processing hempseeds.

KEYWORDS: *Green technology; High oil recovery rate; Hydration of non-oil solids; Sustainability.*

RESUMEN: ***Optimización y evaluación de un método de hidratación para producir aceite de alta calidad y harina desgrasada a partir de semillas de cáñamo.*** Se demanda métodos verdes y eficientes para producir aceite y harina desgrasada de alta calidad, lo que promovió el desarrollo de un método de hidratación para extraer aceites de semillas de cáñamo. El método de hidratación optimizado en este estudio recuperó >96 % de los aceites que, además, se demostró mediante espectros infrarrojos, extrajo >91 % de tocoferoles, vitamina A, carotenoides, coenzima Q10, fitoesteroles y escualeno en la fase oleosa, mientras que solo se extrajeron pequeñas porciones de flavonoides, otros compuestos fenólicos y ácidos grasos libres. La harina desgrasada era rica en vitaminas hidrosolubles (incluyendo tiamina, riboflavina, niacina, piridoxina, folato y vitamina C), proteínas, carbohidratos dietéticos y fosfolípidos. El método de hidratación produjo aceites con AV o PV más bajos en comparación con los métodos de extracción por solventes, CO_2 supercrítico y prensado en frío y harina desgrasada con un contenido de proteína (52,69%) significativamente mayor que el obtenido por los métodos de CO_2 supercrítico y prensado en frío. El método de hidratación es un tipo de tecnología ecológica para el procesamiento sostenible de semillas de cáñamo.

PALABRAS CLAVE: *Alta tasa de recuperación de aceite; Hidratación de sólidos no oleosos; Sostenibilidad; Tecnología verde.*

Citation/Cómo citar este artículo: Wang S, Wu W. 2023. Optimization and evaluation of a hydration method for producing high quality oil and defatted meal from hemp seed kernels. *Grasas Aceites* **74** (3), e515. https://doi.org/10.3989/gya.1128212

1. INTRODUCTION

The whole seeds of Hemp (*Cannabis sativa* L.) with < 0.2% δ-9-tetrahydrocannabinol, widely cultivated as an industrial crop (Petrović *et al.*, 2015) usually contain ca. 30% oil and ca. 24% protein, as well as a significant amount of dietary fiber, vitamins and minerals (House *et al.*, 2010). The Organization for Economic Cooperation and Development - Food and Agriculture Organization of the United Nations (OECD-FAO) projected that the annual per capita consumption of vegetable oils as foods may reach 27 kg in developed countries with an annual growth of 0.9% from 2020 to 2029 (OECD/FAO, 2020). Oils should also be good material for making such products as surfactants, lubricants and biodiesel (Pal *et al.*, 2019; Farooq *et al.*, 2019) and extracting fat-soluble bioactive compounds (Yara-Varón *et al.*, 2017). Furthermore, the annual protein demand for food security in the world was projected to be 31 kg per capita, while annual protein intake will reach 41 kg per capita by 2029 (OECD/FAO, 2020). Proteins are also applicable to other industries, such as protein fibers in the textile industry (Stenton *et al.*, 2021) or in medicine (DeFrates *et al.*, 2018). If animal meats are used for the protein source needed, greenhouse gas emissions (GHGE) will be huge. For example, agricultural GHGE may increase from 11 to 24% by 2029, 2/3 of it should be contributed by livestock (for obtaining animal meat proteins) (OECD/FAO, 2020). Nonmeat protein alternatives such as proteins from oilseeds are able to greatly decrease GHGE, which is valuable for environmental protection. Therefore, a method which can simultaneously produce high quality oil and defatted meal from hemp seeds is very valuable.

Solvent extraction is currently the most common method used for the commercial separation of seed oils, but the disadvantages of time-consuming, solvent consumption, high fire or explosion risk, residual toxicity, adverse effect on environment and high equipment investment costs have also been reported (Escorsim *et al.*, 2018; Crimaldi *et al.*, 2017). The application of supercritical fluids (such as supercritical CO_2) is presently restricted because this method can only be operated in batches and production on a large scale is difficult (Aladic *et al.*, 2014). Cold pressing has a low recovery rate (< 80%) of hemp seed oils (Crimaldi *et al.*, 2017). Hot pressing produces defatted meal with very poor quality.

Therefore, the purpose of this study is to develop a new method, i.e. hydration, for the production of oils which are rich in fat-soluble bioactive compounds and defatted meal which is rich in proteins and water-soluble bioactive compounds from hemp seeds. Experimental works include the optimization of operating conditions of the method and the evaluation of its efficiency compared to other methods.

2. MATERIALS AND METHODS

2.1. Materials

Hempseeds (grown in Bama, Guangxi, China) were bought from Fangyitan Economy and Trade Co. Ltd, Changchun, Jilin and stored in a cool, dry, sealed bag. Hulled hempseeds contained 6.12% water and 48.10% fat (on wet basis), and were used in all experiments. NaCl (salt) was food and analytical grade.

2.2. General procedure used in the experimentation

The hulled hemp seeds were heated in a microwave oven (TOSHIBA, ER-SS20CNW) at certain output power (variable) and time (variable), cooled, pre-crushed using a blade crusher for 30 s and finally ground by hand to pass through a sieve with certain meshes (variable). The ground hemp seed kernel (GHSK; 10.00 g) and a certain amount (variable) of pure water with a certain amount (variable) of sodium chloride were placed in a 50 mL centrifuge tube. The mixture in the centrifuge tube was agitated for a certain time (variable) and at a certain speed (variable) at room temperature (20 °C) by using a digital display electric mixer (JJ-1A 100W) with a self-made screw-type mixing head. Free oil was collected by centrifugation at 4000 rpm and weighed. The centrifugation residue was quantitatively collected and cold-pressed three times. The cold-pressed residue was quantitatively collected, dried at 50 °C and weighed. All measurements were repeated three times.

2.3. Condition optimization by single factor experimentation

According to the steps (Figure 1) and general procedure ("2.2.") described above, the single fac-

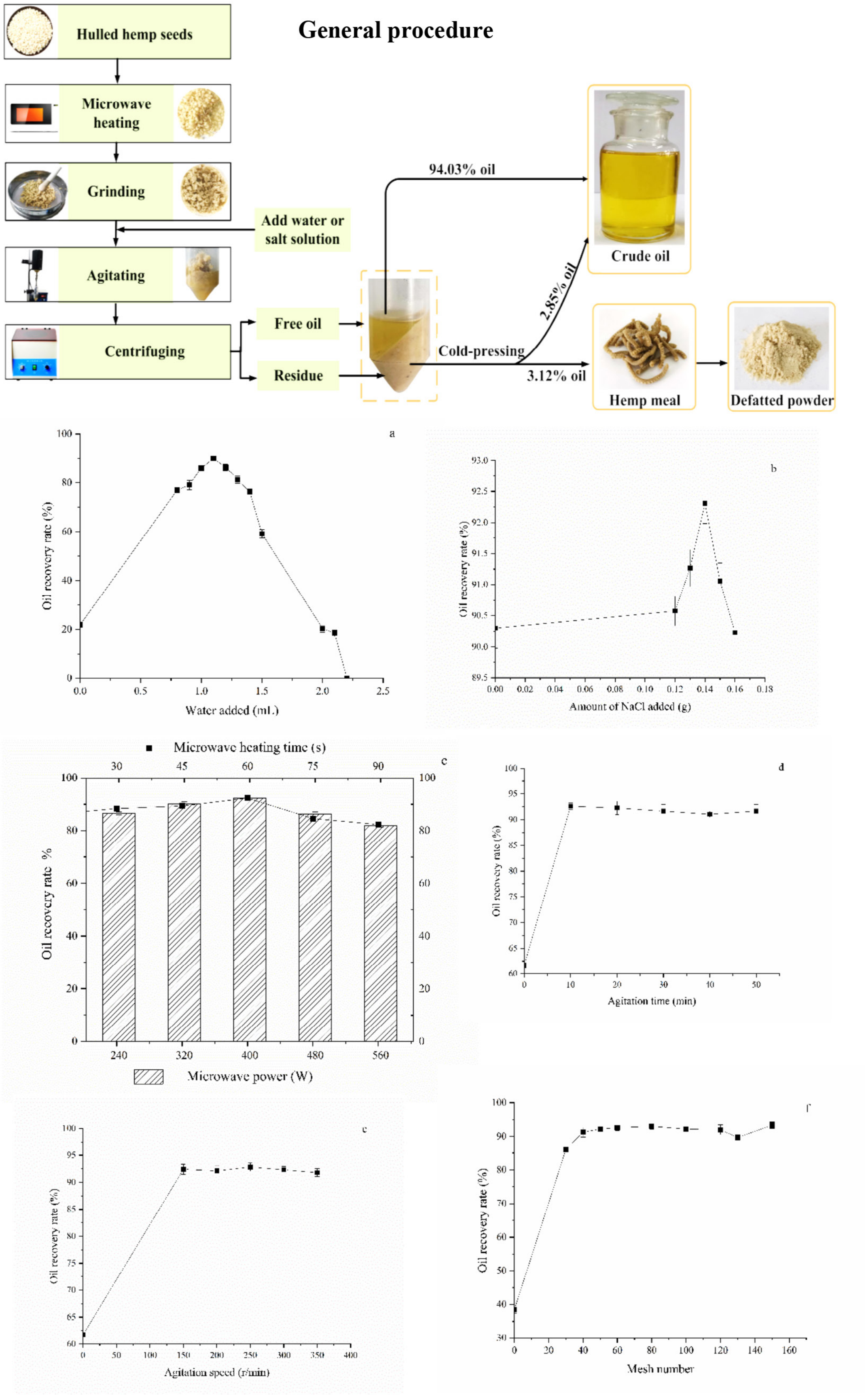

Figure 1. General procedure of hydration method and the effect of extent of hydration, salt, microwave power and microwave heating time, agitation time and speed, and mesh number of sieve on the oil recovery rate (mean±SD: n=3). Note: The average of 3 replicates was calculated.

tor experiment was performed to optimize the extraction conditions. The effect of different amounts (0, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.1 and 2.2 mL/10 g) of water added on oil recovery rate (ORR) was investigated, while other parameters were fixed to be heated at 400 W for 60 s, the GHSK was passed through a 60-mesh sieve and agitated at room temperature (20 ºC) at 150 r/min for 10 min. The effect of different amounts (0, 0.12, 0.13, 0.14, 0.15 and 0.16 g/10 g) of NaCl added on ORR was investigated, while other parameters were fixed to be heated at 400 W for 60 s, the GHSK was passed through a 60-mesh sieve, 1.10 mL water were added and the mixture was agitated at room temperature (20 ºC) at 150 r/min for 10 min. The effect of different microwave heating times (0, 30, 45, 60, 75 and 90 s) on ORR was investigated, while other parameters were fixed to be heated at 400 W, the GHSK was passed through a 60-mesh sieve, 1.10 mL water and 0.14 g NaCl were added and the mixture was agitated at room temperature (20 ºC) at 150 r/min for 10 min. The effect of different microwave power settings (240, 320, 400, 480 and 560 W) on ORR was investigated, while other parameters were fixed to be heated for 60 s, the GHSK was passed through a 60-mesh sieve, 1.10 mL water and 0.14 g NaCl were added and the mixture was agitated at room temperature (20 °C) at 150 r/min for 10 min. The effect of different agitation speeds (0, 150, 200, 250, 300 and 350 r/min) on ORR was investigated, while other parameters were fixed to be heated at 400 W for 60 s, the GHSK was passed through a 60-mesh sieve, 1.10 mL water and 0.14 g NaCl were added and the mixture was agitated at room temperature (20 ºC) for 10 min. The effect of different agitation times (0, 10, 20, 30, 40 and 50 min) on ORR was investigated, while other parameters were fixed to be heated at 400 W for 60 s, the GHSK was passed through a 60-mesh sieve, 1.10 mL water, and 0.14 g NaCl were added and the mixture was agitated at room temperature (20 ºC) at 150 r/min. The effect of different sieve meshes (30, 40, 50, 60, 80, 100, 120, 130 and 150) on ORR was investigated, while other parameters were fixed to be heated at 400 W for 60 s, 1.10 mL water and 0.14 g NaCl were added and the mixture was agitated at room temperature (20 ºC) at 150 r/min for 10 min.

The residual oil content in the cold-pressed residue was determined by the Soxhlet method (using petroleum ether as a solvent). The oil recovery rate (ORR) was calculated using the following formula:

$$\text{ORRC (\%)} = \frac{\text{Oil}_c}{\text{Oil}_t} \text{x100 or}$$

$$\text{ORRCP (\%)} = \frac{\text{Oil}_t - \text{Oil}_r}{\text{Oil}_t} \text{x100}$$

In the formula, Oil$_c$ represents the weight of free oil obtained by centrifugation (g); Oil$_t$ represents total oil in 10 g GHSK (g; 10 g x oil fraction in GHSK); Oil$_r$ represents oil in the centrifuged and then cold-pressed residue (g; the weight of quantitatively collected dry cold-pressed residue x its oil fraction); ORRC represents the oil recovery rate obtained by centrifugation; ORRCP represents the oil recovery rate obtained by centrifugation followed by cold-pressing.

2.4. Extraction of oils by solvent

The hulled hemp seeds were heated at 330 W for 60 s, cooled and crushed for 30 s using a blade crusher. The crushed hemp seed was extracted with Petroleum ether at 50 ºC for 7 h in a Soxhlet extractor. The residual solvent in the extract was completely removed by vacuum evaporation. The extracted oil was then put into a blast drying oven at 50 ºC and dried to constant weight with the quantity difference of less than 2 mg. The refining of crude oil was carried out according to the method reported by Ma *et al.* (2017).

2.5. Analytical methods

The Chinese National Standard Methods (GB/T 14488.1-2008, GB 5009.229-2016, GB 5009.227-2016, GB 5009.236-2016, GB 5009.262-2016, GB 5009.5-2016, GB/T25223-2010, GB5009.83-2016, GB 5009.82-2016, GB 5009.84-2016, GB 5009.85-2016, GB 5009.89-2016, GB 5009.211-2014, GB 5009.82-2016, GB 5009.86-2016, GB 5009.272-2016, GB 5009.4-2016, GB/T 5510-2011, GB 5009.88-2014, GB 5009.8-2016 and GB 5009.153-2016) were followed to determine the content of crude oil, acid value (AV), peroxide value (PV), moisture and volatile, residual solvent, crude protein, phytosterols, carotenoids, vitamin A, thiamine, riboflavin, niacin, pyridoxine, folate, vitamin C, phospholipids, ash, free fatty acids, fibers, sucrose/glucose and phytates, respectively. The content of total phenolic compounds was determined according to the methodology reported by Wu and Sun (2011) while that of total flavonoids was measured by the method defined by Han *et al.* (2019). Cannabinoid content was determined following the method developed by Pellegrini *et al.* (2005). After completely extracting the lipids of GHSK and defatted GHSK samples three times (each time 4 h) with petroleum ether by the Soxhlet method and estimating the crude oil contents of the samples, the wax content in the extracted crude oil was then measured according to the method published by The International Olive Council (2017), while squalene and coenzyme Q10 (Co-Q10) were determined according to LS/T6120-2017 (2017) and the method developed by Karpinska and Mularczyk (2004), respectively. The color was analyzed with a colorimeter (HanterLab UltraScan Pro., Hunter Associates Laboratory, Inc., Reston, VA).

2.6. Infrared spectra

A Fourier Transform Infrared Spectrometer with ATR accessory (Spectrum 100, PerkinElmer, Inc.) was used to reveal the functional groups in oils extracted (HMEO) and defatted meal (HMDM) obtained by the hydration method or that (SEO and SEDM) obtained by solvent extraction, respectively. The proper amount of sample ground to pass a 60-mesh sieve was put on an ATR plate which was washed with ethanol before and after the test to remove any oil residue. The sample spectra were recorded in the wave number range of 4000-600 cm^{-1} by cumulating 16 scans at a resolution of 4 cm^{-1}.

2.7. Statistical analysis

Each sample was analyzed in triplicate. Data were obtained as means ± standard deviations (SD) and analyzed by a one-way analysis of variance (ANOVA) and least-significant difference. Differences in paired data and those of more than 3 were estimated by t-test and F-test, respectively.

3. RESULTS AND DISCUSSION

3.1. Optimization of hydration method

3.1.1. Division 1. Optimization by single factor experiments

Hydration. The effect of the extent of hydration on ORR is shown in Figure 1a. Only 21.9% of the total oil from GHSK was recovered by centrifugation without the addition of water. Increases in water addition from 0.00 to 1.10 mL/10 g GHSK significantly increased ORR. However, when water addition increased to be > 1.10 mL/10 g GHSK, ORR gradually decreased. The addition of 1.10 mL water/10 g GHSK resulted in the maximum ORR (90.30%); whereas that of 2.20 mL water/10 g GHSK decreased ORR to zero.

The ORR (90.30%) obtained by the absorption of 1.10 mL water for 10 g GHSK should be considered meaningful since it is much higher than that obtained by cold-pressing (Crimaldi *et al.*, 2017) and enzymatic aqueous extraction (Hou and Wu, 2014). It should be noted that the ORR at 1.10 mL water/10 g GHSK was obtained before the optimization of other conditions. This means that higher ORR is possible when other conditions are optimized.

Another important characteristic of obtaining the maximum ORR at 1.10 mL water/10 g GHSK was that the moisture content of defatted meal was only ca.25%. This moisture content means that drying the defatted meal is quite easy. Therefore, oil separation by hydration is superior to traditional aqueous (including enzyme-assisted) methods in that the discharge of waste water is huge.

Salt addition. The effect of NaCl addition on ORR is shown in Figure 1b. As the amount of NaCl increased from 0.00 g to 0.14 g, ORR continued to increase. However, the addition of more than 0.14 g continued to decrease ORR. The maximum ORR (92.31%) was obtained with NaCl addition at the level of 0.14 g/10 g GHSK, which was significantly higher than that (90.30%) obtained without NaCl addition ($p < 0.05$), but the absolute difference between the ORR obtained with and without the addition of NaCl was only 2.01%, which was not significant ($p > 0.05$).

The amount of salt needed to obtain the maximum ORR only resulted in a salt content of ca. 2% on wet weight basis and ca. 2.7% on dry weight basis in defatted meal. The defatted meal with this range of salt content should be consumable directly. Furthermore, the defatted meal with this range of salt should be applicable for making products such as meat substitute.

Microwave heating time and power. The effect of microwave heating time and power on ORR is shown in Figure 1c. It can be seen that only 86.14%

ORR was obtained without microwave heating. When microwave power was fixed at 400 W, ORR gradually increased as heating time increased from 0 to 60 s, but further extension of heating time gradually reduced ORR. The maximum ORR (92.31%) was obtained with heating for 60 s, which was higher than that obtained by using any other heating time ($p < 0.05$). When heating time was constant at 60 s, increases in microwave power from 240 to 400 w significantly increased ORR, but higher than 400 w gradually reduced ORR. The maximum ORR obtained was not significantly different from that obtained in the experiment on any other microwave power ($p > 0.05$).

The microwave oven used was able to treat 800 g samples. The electricity consumption of microwave treatment for obtaining the maximum ORR was only ca. 0.0083 kwh/kg (i.e. 0.4 (kw) x 1/60 (h) ÷ 0.8 (kg)). This means that microwave treatment not only has a high efficiency for recovering oils, but also saves energy and time, which is beneficial to environmental protection and economics.

Agitating time and speed. The effects of agitating time and speed on ORR are indicated in Figures 1d and 1e, respectively. Figure 1d shows that ORR was only 61.74% when agitation was not carried out, but ORR increased rapidly to 92.50% when the mixture of water and GHSK was agitated for 10 min. As the agitation time extended from 10 min to 50 min, ORR remained relatively stable (not significantly different; $p > 0.05$). Figure 1e shows that increases in the rotating speed of the screw head of the agitator from 150 to 350 r/min had little effect on ORR.

The agitation time of 10 min for obtaining the maximum ORR should facilitate the shortening of the total process circle. This should significantly reduce the risk of microbial growth, save time and energy, and decrease production costs. The range of 0 – 150 r/min was not investigated for the rotation speed of the screw head of the agitator, which does not mean that this range is not worth investigating in future.

Mesh number of sieve. The effect of the sieve mesh number for passing GHSK is indicated in Figure 1f. Crushing the baked HSK for 30 s by using a blade crusher resulted in only 38.46% ORR. When the crushed HSK was further ground to pass through a sieve with 30 – 40 mesh numbers, ORR increased from 86.07% to 92.90%. ORR remained almost the same (ca. 93.20%) when crushed HSK was further ground to pass through a sieve with 60 – 150 mesh numbers, but was not significantly different from that obtained by passing through a number 40 mesh ($p > 0.05$).

A sieve with 40 or 60 mesh number has a pore size of 0.38 mm or 0.25 mm, respectively. HSK ground to pass through a sieve in these ranges of pore size is quite easy to achieve. The ORR obtained by grinding HSK to pass through a sieve with mesh number 40 should be meaningful since it is higher than 92.90%. As viewed from the achievement of the lowest processing cost, the sieve with smaller mesh numbers may be preferred. However, grinding HSK to pass through a sieve with mesh number 60 should also be applicable since this operation is quite easy to achieve and not costly.

Optimal conditions. The optimal operating conditions obtained by single factor experimentation was as the following: heating HSK for 60 s by 400 w microwave, preparing GHSK by crushing the baked HSK for 30 s followed by grinding to pass through a sieve with 40 or 60 mesh number, adding 1.10 mL water and 0.14 g salt (optional), agitating the mixture of GHSK, water and salt for 10 min at 150 r/min and room temperature. Under the conditions established in the general procedure described in section 2.2., and employing the optimal conditions mentioned above, the maximum ORR obtained reached 93.20%.

3.1.2. Division 2. Further optimization by response surface method

The experimental results obtained by response surface method are shown in Table 1. The highest ORR obtained was 92.52% (run 17); whereas the lowest ORR was only 77.23% (run10). The quadratic multiple regression model based on coding factors was as follows:

$ORR = 90.73 + 4.08A + 0.7710B - 1.13C + 0.0606D - 2.13AB + 2.08AC - 1.22AD + 1.33BC - 2.65BD + 0.7277CD - 2.06A^2 - 1.80B^2 - 4.32C^2 - 2.71D^2$

(actual equation: $ORR = -727.649000 + 769.282740A + 5593.728340B - 0.777662C + 0.006605D -2130.977130AB + 1.386000AC - 0.152677AD + 8.835760BC + 3.313410BD + 0.000606CD - 205.561330A^2 - 17957.380460B^2 - 0.019185C^2 - 0.000423D^2$)

The statistical significance of the response surface model is shown in Table 2. The model was extremely

Table 1. Original and coded values of process parameters, experimental design matrix and results of response surface experimentation

Run	A: Water added (mL)	B: NaCl added (g)	C: Microwave heating time (s)	D: Microwave power (W)	Oil recovery rate (%)
1	-1	0	0	-1	80.87
2	0	0	1	1	82.95
3	1	0	0	1	89.40
4	0	0	0	0	90.44
5	1	0	1	0	90.23
6	0	1	0	1	88.77
7	-1	-1	0	0	79.31
8	0	1	-1	0	85.45
9	0	0	0	0	91.06
10	-1	0	1	0	77.23
11	-1	0	0	1	85.14
12	0	1	0	-1	85.86
13	1	1	0	0	89.50
14	0	0	0	0	89.81
15	-1	1	0	0	84.82
16	0	0	0	0	90.64
17	1	-1	0	0	92.52
18	0	0	-1	1	84.72
19	0	-1	-1	0	87.01
20	0	-1	0	-1	88.88
21	0	0	0	0	91.68
22	0	-1	1	0	81.91
23	-1	0	-1	0	82.54
24	0	0	1	-1	80.56
25	1	0	-1	0	87.21
26	1	0	0	-1	90.02
27	0	0	-1	-1	85.24
28	0	-1	0	1	81.19
29	0	1	1	0	85.65

Variable	Symbol	Coded variables levels		
		-1	0	1
Water added/mL	A	1.0	1.1	1.2
NaCl added/g	B	0.13	0.14	0.15
Microwave heating time/s	C	45	60	75
Microwave power/W	D	320	400	480

significant ($p < 0.01$), whereas the lack of fit was not statistically significant ($P > 0.05$), indicating that the model was effective. Furthermore, the R^2 (= 0.9574) of the quadratic model and the low variance (1.39%) indicate that the multiple quadratic regression model (established on the basis of experimental values) simulated the relationship well among all the conditions examined and ORR with high accuracy, high reliability and repeatability. The quadratic multiple regression equation model predicted that the optimal process conditions for extracting hemp seed oil according to the hydration method were as follows: A-1.28 mL, B-0.13 g, C-61 s, D-330 W. The ORR was theoretically predicted to be 94.78%. Under the conditions of the general procedure described in section 2.2. and employing the optimal conditions mentioned above, a verification study was carried out. The study found that the actual ORR was 94.03% while the residual standard error (RSD) was 0.83% and therefore the measured value was basically in line with the predicted value (Less than ± 5% of the RSD should indicate that the model was reliable and the optimization result was credible).

In addition, the effect of the amount of water (A) and salt (B) added, microwave treating time (C) A^2, B^2, C^2, D^2 (microwave power) as well as the interaction of water and salt (A×B), water and microwave treating time (A×C), salt and microwave treating time (B×C) or salt and microwave power (B×D) on ORR was statistically significant ($p < 0.05$). The contours and response surfaces for the effect of all parameters studied are shown in Figure 2.

The surface response method further optimized operating conditions. The optimal condition combination was different from that obtained by single factor experimentation, but the difference (94.03% – 93.20% = 0.83%) in the ORRs obtained by using the two optimal condition combinations was not significant ($p > 0.05$).

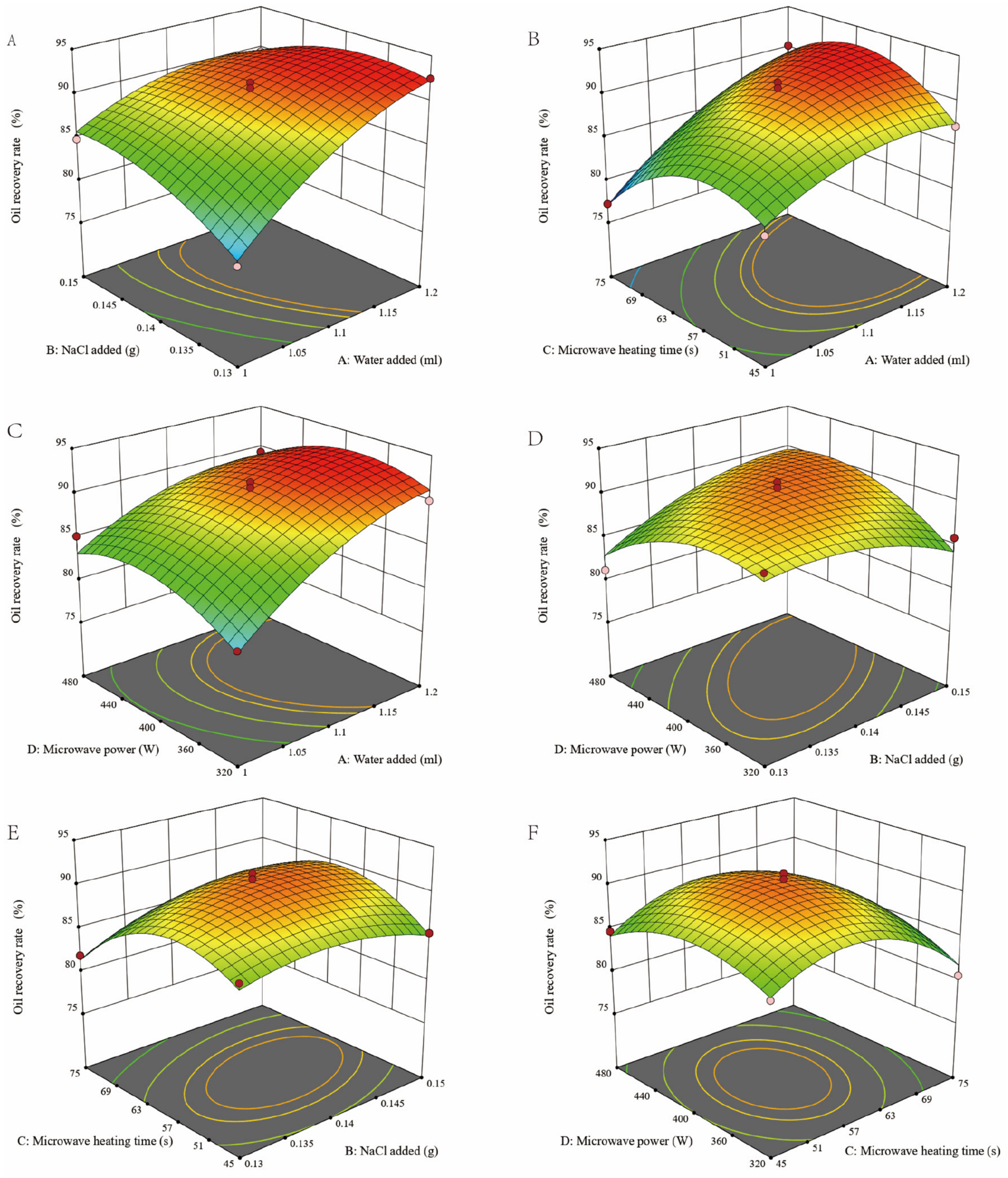

Figure 2. Response surfaces for the cross-effect of the addition of water and NaCl, water and microwave time, microwave power and the addition of water, microwave power and NaCl added, microwave heating time and NaCl added as well as microwave heating time and microwave power on the oil recovery rate.

Table 2. ANOVA for response surface quadratic model and the analysis of variance table (partial sum of squares—Type III); response: oil recovery rate

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	454.12	14	32.44	22.49	< 0.0001**
A-Water added	199.76	1	199.76	138.49	< 0.0001**
B- NaCl added	7.13	1	7.13	4.94	0.0431*
C- Microwave time	15.45	1	15.45	10.71	0.0056**
D- Microwave power	0.0441	1	0.0441	0.0306	0.8637
AB	18.16	1	18.16	12.59	0.0032**
AC	17.29	1	17.29	11.99	0.0038**
AD	5.97	1	5.97	4.14	0.0614
BC	7.03	1	7.03	4.87	0.0445*
BD	28.11	1	28.11	19.49	0.0006**
CD	2.12	1	2.12	1.47	0.2457
A^2	27.41	1	27.41	19.00	0.0007**
B^2	20.92	1	20.92	14.50	0.0019**
C^2	120.86	1	120.86	83.79	< 0.0001**
D^2	47.47	1	47.47	32.91	< 0.0001**
Residual	20.19	14	1.44		
Lack of Fit	18.24	10	1.82	3.73	0.1079
Pure Error	1.95	4	0.4884		
Cor Total	474.32	28			
R^2	0.9574				
Adj R^2	0.9149				
C.V.	1.39				

Note: “*” indicates significant, $p < 0.05$; “**” indicates extremely significant, $p < 0.01$; Values greater than 0.1000 indicate the model terms are not significant.

3.2. Efficiency of methods for free oil collection

The ORR described in section 3.1., *Divisions 1* and *2* was calculated on the basis of oils collected by centrifugation at 4000 rpm. The maximum ORR (94.03%) corresponded to 5.24% residual oil content in the defatted meal obtained by only centrifugation. Part of the small amount of residual oil in the centrifugation residue can be recovered by cold-pressing. The residual oil in the defatted residue obtained by centrifugation combined with cold-pressing was as low as 2.81%, which corresponded to 96.88% ORR. However, it should be noted that cold-pressing alone without centrifugation to obtain defatted meal with residual oil content as low as 2.81% was found to be difficult.

3.3. Evaluation of efficiency of hydration method by comparison with other methods

A comparison of the hydration method with other methods in terms of extraction conditions, oil extraction or recovery rate as well as oil and defatted meal qualities is shown in Table 3. Although the hydration method had a lower oil extraction rate, it had an identical ORR compared to solvent extraction, which produced crude oil which requires refining responsible for leading to significant losses in neutral oils and bioactive compounds. The hydration method had significantly higher ORR compared to supercritical CO_2 and cold-pressing methods. Figure 4 indicates that absorption peaks at 3008, 2924, 2854 and 1744 cm^{-1} corresponding to oils disappeared in the spectrum of defatted meal (containing 1.37% residual lipids) obtained by solvent extraction. This may mean that the 1.37% residual lipids were not oils. Therefore, the maximum residual oil in the defatted meal obtained by the hydration method might be only 1.44% (i.e. 2.81% – 1.37%), so that the real ORR of this method should be higher than 96.88%.

Table 3 also indicates that the AV and PV of oils extracted by the hydration method were lower than those obtained by solvent extraction or cold-pressing. Only a small amount of water and volatiles (0.06%) were found in the oil sample. Compared to solvent extraction, the hydration method had the advantage of no residual solvent in the extracted oil, which means it can be directly consumed.

Table 3. Comparison of hydration method with other methods in terms of extraction conditions, oil extraction or recovery rate as well as partial oil and defatted meal quality.

Items	Hydration method[a]	Solvent extraction[b]	Supercritical CO_2 extraction[c]	Cold-pressed
Extraction conditions	Material-to-liquid ratio:1:0.13, microwave heating at 330 W for 61 s, GHSK passed a sieve with 60 meshes (0.42 mm)	Microwave heating at 330 W for 61 s, extracting at 50 °C for 7 h	40 °C, 300 bar and, 195 min, <1 mm particle size, 10 mL/min CO_2	Temperature of output press head at 60 °C, frequency of 20 Hz, and nozzle of ID 6 mm (Aladić *et al.*, 2014)
Oil extraction (%)	96.88±1.86	98.63±1.21	93.19	70.00 (Aladić *et al.*, 2014)
ORR (%)	96.88±1.56	94.21±1.32	93.19	-
AV (mg KOH/g)	1.09±0.15	2.19±0.21	-	1.76 (Teh and Birch, 2013)
PV (mmol/kg)	0.93±0.06	1.89±0.18	2.75	1.94 (Teh and Birch, 2013)
Moisture and volatile content (%)	0.06±0.01	-	-	0.08 (Aladić *et al.*, 2014)
Residual solvent (g/kg)	-	1.59±0.17	-	-
Lipid content in defatted meal (%)	2.81±0.19	1.37±0.21	3.27	10.33 (Aladić *et al.*, 2014)
Protein content in defatted meal (%)	52.69±1.25	54.30±1.16	51.20	45.63

[a,b]The average of 3 replicates was calculated; [c]Aladić *et al.* (2014).

Figure 3 also shows that the spectrum of oils extracted by the hydration method was identical to that of the oils extracted by solvent extraction. This may mean that the major compounds in the oils extracted by both methods were identical.

Table 4 indicates that more than 91% of some fat-soluble bioactive compounds were extracted into the oil phase by the hydration method. Although solvent extracted more fat-soluble bioactive compounds into the oil phase, the majority of them should be lost during refining. Compared to solvent extraction, the hydration method had another advantage in that the extraction rate of compounds containing hydrophilic groups into the oil phase was much lower, especially those adversely affecting the stability of the oils such as waxes and free fatty acids.

Table 3 also shows that the hydration method produced the defatted meal with higher or slightly lower protein contents compared to supercritical CO_2 and cold-pressing methods or solvent extraction, respectively. The defatted meal produced by the hydration method should be a good material for making meat analogue or texturized protein in the food industry since it had a low residual oil content (< 5%) and high protein content (> 45%). It also shows potential for many applications in other products in the food industry. It can be a good material for making many products in other industries, such as solar energy conversion or absorption (strong alkali treatment to form black protein) products and graphene oxide-protein conjugates (Simsikova and Sikola, 2017).

In addition, defatted meal obtained by the hydration method was rich in some major water-soluble bioactive compounds: thiamin (2.75 ± 0.11 mg/100 g), riboflavin (0.59 ± 0.05 mg/100 g), niacin (20.07 ± 1.93 mg/100 g), pyridoxine (1.33 ± 0.09 mg/100 g), Folate (0.24 ± 0.02 mg/100 g) and vitamin C (1.07 ± 0.08 mg/100 g). It contained 10.33% total carbohydrates (including insoluble and soluble fibers), 14.39% phytates and 11.95% ash in dry matter.

Table 5 shows that the optimal conditions for the extraction of hemp seed kernel oils by the hydration

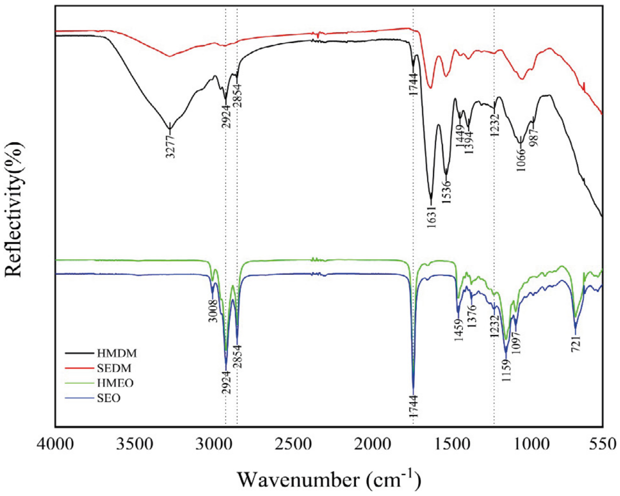

Figure 3. FTIR spectra of hemp oil obtained by different methods and corresponding defatted samples. HM-DM and SE-DM: defatted meal of the hydration method and solvent extraction, respectively; HMEO and SEO: oil extracted by hydration and solvent, respectively. Main band contribution of functional groups (ν: Telescopic or frame vibration; δ: Bending vibration; vf: skeleton; Amide II: δ(N-H)+ν(C-H)): 3277-ν(O–H) of hydrogen bonds from water and polysaccharides; 3008-ν(=CH) from lipids; 2924 and 2854-ν(C–H) of methyl (CH$_3$) and methylene (CH$_2$) groups from lipids; 1744-ν(C=O) of carbonyl and ester groups from pectin, xylan and lipids; 1631-δ(N-H) from water and protein; 1536- amide II from protein; 1459 and 1449-δ(CH$_2$) from polysaccharides; 1394 and 1376-δ(CH$_2$), n(CC), ns(COOH) from polysaccharides (pectin); 1232-ν(C-O) from polysaccharides (disaccharides sucrose); 1159-n(C–O–C), ring from polysaccharides (pectin); 1097-n(CO), n(CC), ring from polysaccharides (pectin); 1066-ν(CO), ν(CC), δ(OCH) from polysaccharides (cellulose); 987-OCH$_3$ from polysaccharides (cellulose); 721-vf(C-C).

method established in this study were significantly different from those developed for extracting oils from other oilseeds. Therefore, this study obtained new findings.

The results of these evaluation studies proved that the hydration method developed was efficient for the simultaneous production of high-quality oil and defatted meal as compared to other methods. This should mean that the hydration method has potential for replacing other methods which are currently used for the commercial separation of oil and defatted meal.

3.4. Analysis of the environmental impact of the hydration method

Oil extraction by the hydration method uses quite a small amount of water (a green reagent) which is completely absorbed by the solids of GHSK so that no wastewater is discharged into the environment during the extraction process. Another characteristic of the hydration method is that it avoids harmful chemical input and output. Furthermore, the hydration method utilizes whole hemp seed kernels so that no solid waste is discarded into the environment. The utilization of whole raw material saves valuable resources such as proteins and other bioactive com-

Table 4. Comparison of percentage of bioactive substances with different polarity extracted into the oil phase from hemp seed kernels by hydration method or solvent extraction

Bioactive substances	Hempseed kernel (mg/100 g)	Defatted meal (mg/100 g)		Percentage extracted into oil phase (%)	
		Hydration method	Solvent extraction	Hydration method	Solvent extraction
Total tocopherols	40.01±0.68	7.34±0.18	3.31±0.16	91.60±1.16	96.21±1.54
Vitamin A	1.60±0.17	0.31±0.06	0.15±0.02	91.06±1.11	95.65±1.44
Carotenoids	1.76±0.12	0.27±0.02	0.14±0.03	92.90±1.22	96.28±2.01
Co-Q10[a]	2.22±0.11	0.41±0.03	0.08±0.01	91.48±1.49	98.37±2.09
Phytosterols	432.44±2.68	78.02±1.19	51.20±1.02	91.74±1.35	94.58±1.26
Squalene	27.81±0.61	5.05±0.22	2.20±0.12	91.68±1.25	96.38±1.51
Cannabinoids	198.16±1.46	427.62±1.22	380.30±1.82	1.21±0.15	12.14±1.21
TF[b] (as QE equivalent)	291.00±5.01	620.00±3.01	614.00±2.02	2.43±0.37	3.40±2.24
TPC[c] (as GAE equivalent)	276.00±3.03	572.00±8.18	557.00±2.03	5.17±5.15	7.67±0.16
Phospholipid	2397.69±12.30	2890.00±18.11	1320.35±7.01	1.78±0.19	25.21±0.24
Free fatty acids (as oleic acid equivalent)	0.85±0.29	1.28±0.11	0.70±0.06	31.03±0.29	62.35±0.20
Waxes	4.73±0.39	10.32±0.98	0.86±0.07	undetectable	91.68±1.26

[a]Coenzyme Q10; [b]Total flavonoids while QE represents quercitin; [c]Total phenolic compounds while GAE represents gallic acid; [d]the average of 3 replicates was calculated.

Table 5. Comparison of process conditions optimized for processing different oilseeds by the hydration method

Oilseeds	Process conditions							
	H or P[a]	BT[b]	Bt[c]	PSSP[d]	W or S[e]	AT[f]	At[g]	ORR[h]
Peanut[1]	Hulled, peeled	110	90	150	1.5 mL H_2O+0.1 g NaCl per 10 g kernel slurry	20	Till free oil observed	96
Walnut[1]	Hulled	115	90	150	1.5 mL H_2O+0.1 g NaCl per 10 g kernel slurry	20	Till free oil observed	97
Sunflower[1] seed	Hulled	115	110	48	1.8 mL H_2O+0.1 g NaCl or 0.03 g Na_2CO_3 per 10 g kernel slurry	70	Till free oil observed	95
White sesame[2]	-	115	1	154	1.95 mL 6.00% (w/w) salt solution per 10 g ground seeds	65	25	96.06
Black[3] sesame	-	115	1	154	1.95 mL 6.79% (w/w) salt solution per 10 g ground seeds	65	30	96.54
Almond[4]	Hulled	110	3	58	1.37 or 1.40 mL H_2O per 10 g kernel slurry	RT[i]	40 or 45	96.38
Tea seed[5]	Hulled	115	90	74	1.2 mL H_2O+0.1 g NaCl or 0.08 g Na_2CO_3 per 10 g kernel slurry	65	40	94.47
Rape seed[6]	Hulled	115	2	61	1.5 mL H_2O per 10 g kernel slurry	50	30	94.64
Soybean[7]	Peeled	120	5	150	1.3 mL H_2O+0.08 g NaCl per 10 g kernel slurry(3 parts oil+5 parts powder)	75	30	81
Pumpkin seed[8]	Hulled	110	1	150	1.6 mL H_2O+0.08 g NaCl per 10 g kernel slurry	30	30	94.08
Hemp seed	Hulled	330 W microwave	1.02	380 or 250	1.28 mL H_2O+0.13 g NaCl per 10 g kernel slurry	RT[i]	10	96.88

[a]Hulling or peeling; [b]Baking temperature (°C) in oven; [c]Baking time (min) in oven; [d]Pore size (μm) of sieve passed through by ground oilseeds or kernels; [e]Water or salt added; [f]Agitation temperature (°C); [g]Agitation time (min); [h]Oil recovery rate (%); [i]Room temperature; [1]Tu *et al.* (2017); [2]Lv and Wu (2020); [3]Lv and Wu (2021); [4]Fu and Wu (2019); [5]Lv and Wu (2019a); [6]Lv and Wu (2019b); [7]Tu and Wu (2019); [8]Fu and Wu (2022).

pounds so that energy input or land for agricultural production can be significantly reduced and greenhouse gas emission is decreased. In particular, the high-quality protein in the defatted meal obtained by the hydration method may be a good material for making alternative meat products, which can significantly reduce the production of animal meats and therefore decrease greenhouse gas or methane emissions. Therefore, the hydration method is undoubtedly a kind of green technology for simultaneously producing high-quality oils and defatted meal from hempseeds or other oilseeds.

4. CONCLUSIONS

It was concluded that the hydration method optimized in this study was able to efficiently produce high-quality oils and defatted meal with an identical ORR compared to solvent extraction and a significantly higher ORR compared to supercritical CO_2 and cold-pressing methods. This method extracted more than 91% tocopherols, vitamin A, carotenoids, coenzyme Q10, phytosterols and squalene into the oil phase while only small portions of flavonoids, other phenolic compounds and free fatty acids were extracted. Defatted meal was rich in water-soluble vitamins including thiamin, riboflavin, niacin, pyridoxine, folate and vitamin C as well as proteins, dietary carbohydrates and phospholipids. The hydration method produced oils with lower AV or PV compared to solvent extraction, supercritical CO_2 and cold-pressing methods and defatted meal with protein content significantly higher than that obtained by supercritical CO_2 and cold-pressing methods. The hydration method is a kind of green technology for environmentally-friendly and simultaneous production of high-quality oils and defatted meal from hempseeds or other oilseeds.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e516
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0984221

Stabilization of organic extra virgin olive oil using maceration and ultrasound-assisted extraction of natural antioxidants from *Artemisia absinthium* leaves

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Submitted: 11 September 2022; Accepted: 12 December 2022; Published online: 10 October 2023

SUMMARY: Olive oil is the most popular product derived from *Olea europaea* L. due to its organoleptic characteristics and its beneficial effects on human health. Nowadays, there is a growing interest in olive oil enrichment with medicinal plants. In this regard, *Artemisia absinthium* L. is an edible source of antioxidants. The aim of the present study was to improve the oxidative stability and the nutritional value of Organic Tunisian *Chetoui* Olive oil by its enrichment with the phenolic compounds in *Artemisia absinthium*. The enrichment carried out by maceration and ultrasound-assisted extraction did not affect the organic criteria of olive oil. The comparative study, performed during one year of storage, showed that ultrasound-assisted extraction led to the lowest final peroxide and extinction coefficient values. At the end of storage, this method increased oleic acid content and biophenol contents. Furthermore, the anti-radical activities of enriched samples were higher than the control.

KEYWORDS: Artemisia absinthium *leaves; Enrichment; Maceration; Organic extra virgin olive oil; Ultrasound-assisted extraction.*

RESUMEN: ***Estabilización de aceite de oliva virgen extra ecológico con antioxidantes naturales de hojas de* Artemisia absinthium *obtenidos por maceración y extracción asistida por ultrasonido***. El aceite de oliva es el producto derivado de *Olea europaea* L. muy popular debido a sus características organolépticas y sus efectos beneficiosos para la salud humana. En la actualidad existe un interés creciente por enriquecer el aceite de oliva con plantas medicinales. En este sentido, *Artemisia absinthium* L. es una fuente comestible de antioxidantes. El objetivo del presente estudio fue la mejora de la estabilidad oxidativa y el valor nutricional del aceite de oliva Chetoui tunecino orgánico mediante su enriquecimiento con compuestos fenólicos de *Artemisia absinthium*. El enriquecimiento realizado por maceración y extracción asistida por ultrasonidos no afectó a los criterios ecológicos del aceite de oliva. El estudio comparativo, realizado durante un año de almacenamiento, mostró que la extracción asistida por ultrasonidos condujo a los valores finales de peróxido y coeficientes de extinción más bajos. Al final del almacenamiento, este método aumentó el contenido de ácido oleico y de biofenoles. Además, las actividades antirradicales de las muestras enriquecidas fueron mayores que las del control.

PALABRAS CLAVE: *Aceite de oliva virgen extra ecológico; Enriquecimiento; Extracción asistida por ultrasonido; Hojas de* Artemisia absinthium; *Maceración.*

Citation/Cómo citar este artículo: Bezzezi A, Boulares M, Arfaoui M, Ben Moussa O, Hassouna M. 2023. Stabilization of organic extra virgin olive oil using maceration and ultrasound-assisted extraction of natural antioxidants from *Artemisia absinthium* leaves. *Grasas Aceites* **74** (3), e516. https://doi.org/10.3989/gya.0984221

1. INTRODUCTION

In recent years, the interest in natural extracts from plants and by-products which are rich in bioactive compounds has increased in the agronomic, pharmaceutical, and cosmetic industries. In fact, there is a growing demand to produce novel functional foods with significant health benefits. The use of plant extracts as natural preservatives for various vegetable oils has been extensively researched (Allane and Benamara, 2019). In this respect, wormwood is a promising medicinal plant due to its richness in a large variety of secondary metabolites such as saponins, tannins, alkaloids, sterols, triterpenoids, phenolic, flavonoids and glycosides, exhibits biological activities (Dahal *et al.,* 2021). In fact, the powerful antioxidant, antimicrobial, anti-inflammatory and anti-diabetic properties of wormwood are due to its high content in lipophilic antioxidant molecules (Allane and Benamara, 2019). Nowadays, literature reports that *Artemisia* extracts and Covid-Organics exhibit some anti-viral activity against SARS-CoV-2 in vitro (Nie *et al.,* 2021). Furthermore, *Artemisia absinthium* leaves are used in the food industry as the main ingredient in the famous absinthe drink, in many herbal tea blends (Judžentiene, 2016) and for the improvement in the oxidative stability of butter. In addition, the essential oils and phenolic extracts of *Artemisia* areal parts presented strong antimicrobial and antioxidant capacities (Dahal *et al.,* 2021). These biological activities have sparked the interest in extracting the antioxidant compounds of these plants to take advantages of their properties and incorporate them into food products. Indeed, new extraction techniques were developed including ultrasound-assisted extraction. This method is an inexpensive, simple and efficient alternative to conventional extraction techniques such as heating, boiling, and maceration, which present disadvantages such as the loss of phenolic compounds due to oxidation, ionization and hydrolysis during processing with long extractions times (Chemat *et al.,* 2017). Ultrasound-assisted extraction contributes to environmental preservation by reducing the use of water and solvents and leads to high yield extraction in a short time. It maintains the good quality of extracts and improves the oxidative stability and organoleptic properties of newly-developed products (Chemat *et al.,* 2017).

In Tunisia, olive oil with its exceptional nutritional quality is a strategic branch in the national economy and is the number one exported Tunisian agricultural product. Thanks to its richness in antioxidants, olive oil is partially protected against oxidation but remains sensitive to photo-oxidation which causes the degradation of its organoleptic and nutritional quality during storage (Sanmartin *et al.,* 2018). In this context, several works studied the enrichment of olive oil using the maceration of vegetable matrices (oregano, Rosemary, Laurel, Basil, olive leaves) (Sousa *et al.,* 2015; Boulares *et al.,* 2022), and their essential oils (Boulares *et al.,* 2022) in order to delay oxidation or to improve their nutritional and organoleptic qualities. As far as we knew, no studies have been performed on the enrichment of olive oil with the natural extracts of *Artemisia absinthium*.

In this study, the oxidative stability of Organic Tunisian *Chetoui* Olive oil (OTCOO) was determined following its enrichment with natural antioxidants from *Artemisia absinthium* L., which were extracted by maceration or ultrasound-assisted extraction. The quality parameters, antioxidant activity and sensorial characteristics of enriched olive oil were assessed over one year of storage.

2. MATERIALS AND METHODS

The *Artemisia absinthium* L. plant originating from north Tunisia (Bizerte) was collected during January 2021. This plant was identified by a specialist in botany and certified specimens (VS1-QS2020/01) were deposited at the Herbarium run. The leaves were dried at room temperature for three weeks.

2.1. Chemical characterization

The moisture, ash, protein and fat of *Artemisia absinthium* leaf powder were determined according to the AOAC (2000) procedures.

2.2. Preparation of raw materials

In the current study, the "*Chetoui*" variety was used. All samples were purchased during the 2019/2020 crop season from the Aljazzira-Morneg oil mill in Tunis. *Artemisia absinthium* leaves were collected from the region of Bizerte in the North of Tunisia. They were washed, dried at room temperature and stored until use. The enrichment of OTCOO with the natural antioxidants from *Artemisia* leaves was carried out using two methods, maceration and ultrasound-assisted extraction. For this reason, the sample of organic extra virgin olive oil was subdivided into three batches. The first batch served as the untreated control. In the second batch, the natural antioxidants were extracted using the maceration method by incorporating the *Artemisia absinthium* leaves into the OTCOO at a concentration of 2% (w/v) (Arfaoui *et al.,* 2021). The remained batch was enriched by natural polyphenols using ultrasound-assisted extraction. For this reason, twenty grams of dried and crushed *Artemisia absinthium* leaves were added to 1 liter of OTCOO (2%). The extraction was performed for 45 min using an ultrasonicator PEX3 (R.E.U.S., Contes, France) under optimal conditions of 60 W and 16 °C (Achat *et al.,* 2012). Finally, the control and enriched OTCOO were stored in black glass bottles in the dark at room temperature for 12 months. The sampling was done each three months during storage with three replicates.

2.3. Pesticides contents measurement

The OTCOO was analyzed to detect the presence of pesticides using the high-performance liquid chromatography (shimadzu RP-HPLC-PDA system), at the beginning and the end of storage. The described analytical procedure was validated according to the Arfaoui *et al.* (2021) protocol for analytical techniques for pesticide residue analysis in food and feed. This procedure fulfils the European Decision 2002/657/EC requirements to verify if the flavoring affects the organic criterion of the studied oil.

2.4. Fatty acids composition

In the beginning of storage, the total fatty acid compositions of all the studied olive oil samples were determined according to the international standard ISO 5509 (2000). The fatty acid composition was determined by preparing the methyl esters, and analyzing them by gas chromatography (GC) (HP. Hewlett. Packard model 6890) according to the ISO 5509 (2000).

The separation and determination of the fatty acid methyl esters were carried out by GC using a Hewlett HP chromatograph. Hewlett. Packard model 6890 equipped with a capillary column of 30 m length and 0.52 mm internal diameter with a film thickness of 1µm. This technique for separating the components of a mixture is based on the difference in affinity of the substances to be analyzed with respect to a common mobile phase and a stationary phase. In GC, the mobile phase is called carrier gas (nitrogen), which had a flow rate of 13 ml/min. The split-splitless injector (division 1/10) was heated to 240 °C. At the exit of the column, which was held at an isothermal temperature of 230 °C throughout the analyses, the compounds were detected by a FID (Flame Ionization Detector) heated to 260 °C. Elution was carried out in ascending order of molecular weight and number of unsaturation.

2.5. Quality parameters measurements

The fatty acidity (FA), peroxide value (PV) and extinction coefficients (K_{232}, K_{270}) were carried out every three months of storage, according to Boulares *et al.* (2022). The chlorophyll and carotenoid levels were determined according to the method described by Boulares *et al.* (2022). A total of 7.5 g of oil sample were placed in a 25 ml volumetric flask, filled to the mark with cyclohexane. The absorbance of the fat solution obtained was measured against that of the solvent at 670 nm for chlorophylls and 470 nm for carotenoids, using a spectrophotometer Jenway Model 6315. The pigment content was calculated using the following formula:

Chlorophylls (mg/kg) = (A670 x 10^6) / (E1x 100 x d)

Carotenoïds (mg/kg) = (A470 x 10^6) + (E2 x 100 x d)

With: d: optical path = 1 cm; A 670: absorbance at 670 nm; A 470: absorbance at 470 nm; E1: coefficient linked to the spectrophotometer = 613; E2: coefficient linked to the spectrophotometer = 2000.

2.6. Biophenol content

The determination of biophenol content was made in accordance with IOC (2009). The minor polar compounds were directly extracted from olive oil samples with a methanolic solution. Then, high performance liquid chromatography (Agilent 1260 Infinity HPLC chromatograph), using an UV developer at 280 nm was carried out. The biophenol contents were expressed in mg / kg of tyrosol. Syringic acid was used as internal standard.

2.7. Antioxidant activity measurements

The anti-radical activity of OTCOO samples was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. It was expressed as a percentage inhibition of the DPPH. 1g of the sample was mixed with 10 ml of ethanol/water (4V/1V). The mixture was left to stand for 1.5 hours in the dark. Finally, this mixture was filtered. 0.1 ml aliquot of the extract was added to 1000 µL of a DPPH solution (0.2 mM) and 2.9 mL of ethanol. After vigorous shaking of the mixture, it was kept in the dark for 30 min. The absorbance was measured at 517 nm using a UV/visible spectrophotometer, referring to a control without extract (Antoniewska *et al.,* 2018). The calculation of antioxidant activity was determined according to the following formula:

% Inhibition = 100 x [(control OD - extract OD) / (control OD)]

Inhibition rate in % OD control: absorbance at 517 nm of the control, OD extract: absorbance at 517 nm of the extract solution.

2.8. Sensory evaluation

The sensorial analysis of all olive oils samples was performed at the beginning and the end of the storage period. A descriptive sensory evaluation was carried out by eight experts from the Tunisian National Oil Office. Olive oil samples were served at room temperature in tasting glasses. The expert panelists noted on a profile sheet the intensity of each negative (fusty/muddy, musty, winey, metallic, rancid, wet wood) and positive (fruity, bitter, pungent) descriptor IOC (2018).

2.9. Statistical analysis

Statistical analysis of all data was performed using the ANOVA test according to Duncan's multiple range in SPSS 23.0 (SPSS IBM 2017). All the tests were processed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Chemical characterization

In order to valorize the wormwood, improve its value and its potential application in food industries to produce functional foods, the biochemical properties of its leaf powder were determined. The moisture content in the aerial parts of *Artemisia absinthium* was 4.18 ± 0.04%. The ash content representing the total amount of minerals was 3.34 ± 0.03%. This finding was in accordance with the findings of Iqbal *et al.* (2012), who observed 7% ash content in *Artemisia annua*. The average protein and carbohydrate contents were 11.97 ± 0.03% and 6.93 ± 0.06%, respectively. In this study, a higher fat content (8.8 ± 0.3%) was obtained in wormwood when compared to that (6.07%) reported in the study of Iqbal *et al.* (2012) on *Artemisia annua*. This could be due to variations in climate, soil and species (Riahi *et al.,* 2015). Thus, the promising nutritional value of wormwood leaves which contributes to their health promoting efficiency was shown.

3.2. Organic criteria and initial composition

The obtained results on the pesticide level in olive oil samples, at the beginning and the end of the storage period, showed that their enrichment with *Artemisia* leaves maintained the organic criteria of the olive oil. In fact, the results proved that the phthalate levels remained below the quantification limit recommended by the norm throughout storage and for the three analyzed olive oils (data not shown). In addition, the identification and quantification of 11 analyzed pesticides showed that they remained absent throughout the storage period (data not shown), which confirms the organic criteria of the analyzed olives.

Moreover, the initial values for moisture (0.066%) and impurities (0.0062%) of the analyzed samples demonstrated the high quality of extracted olive oils. These values were lower than those required by the IOC (2019), which are 0.2 and 0.1%, respectively. Furthermore, the initial obtained peroxide value (PV), free fatty acid (FFA) content and extinction coefficients (K_{232}, K_{270}) were 7.38 meq O_2/Kg; 0.23%; 1.87 and 0.17, respectively. These findings were in agreement with the recommended standards IOC (2019), which confirmed that the studied OTCOO had good quality and was under the nomination "extra virgin".

3.3. Effect of enrichment using *Artemisia absinthium* on the quality of OTCOO

3.3.1. Effect on the fatty acid composition

The results on the variations in fatty acid compositions in all samples are shown in table 1. In fact, the control showed the predominance of oleic acid (C18:1) and a richness in linoleic and palmitic acids with respective values of 64.02 ± 0.28%;

Table 1: Initial fatty acids composition (%) of control and enriched organic extra-virgin olive oils

	OC	OM	OU	Limit (IOC, 2019)
Palmitic acid $C_{16:0}$	10.78 ±0.38^{a}	10.78 ±0.12^{a}	11.38 ±0.22^{b}	7.20-20.00%
Palmitoleic acid $C_{16:1}$	0.48 ±0.04^{a}	0.44 ±0.02^{a}	0.49 ±0.04^{a}	0.30-3.50%
Margaric acid $C_{17:0}$	0.05 ±0.00^{a}	0.05 ±0.01^{a}	0.05 ±0.00^{a}	≤0.40
Ginkgolic $C_{17:1}$	0.05 ±0.01^{a}	0.05 ±0.00^{a}	0.06 ±0.01^{a}	≤0.60
Stearic acid $C_{18:0}$	3.06 ±0.0^{a}	3 ±0.01^{a}	3.11 ±0.00^{b}	0.5-5.00%
Oleic acid $C_{18:1}$	64.02 ±0.28^{b}	63.46 ±0.52^{a}	65.38 ±0.23^{c}	55.00-83.00%
Linoleic acid $C_{18:2}$	20.65 ±0.53^{b}	20.37 ±0.47^{b}	19.15 ±0.18^{a}	2.50-21.00%
Linolenic acid $C_{18:3}$	0.71 ±0.02^{a}	0.7 ±0.00^{a}	0.71 ±0.03^{a}	≤1.00
Arachidic acid $C_{20:0}$	0.43 ±0.02^{a}	0.45 ±0.00^{a}	0.48 ±0.01^{b}	≤ 0.60%
Gadoleic acid $C_{20:1}$	0.36 ±0.06^{a}	0.39 ±0.01^{b}	0.38 ±0.04^{ab}	≤0.50

OC: control olive oil; **OM:** enriched olive oil using maceration; **OU:** enriched olive oil using ultrasound-assisted extraction.The values are expressed as means ± standard deviation, n=3. Different letters in each column indicate significant differences ($P < 0.05$) using Duncan's multiple range test.

20.65 ± 0.53% and 10.78 ± 0.38%. These findings are higher than those reported by Boudiche *et al.* (2003) on Spanish, Greek and Italian olive oils. The enrichment of olive oil using the maceration of olive leaves resulted in no significant difference ($p > 0.05$) in linoleic acid content compared to the control sample, although the oleic acid content was significantly ($p< 0.05$) lower than that of the control sample. This result was attributed to the greater richness of olive leaves from the variety "*Chetoui*" in oleic acid than in linoleic acid as described before. The richness in C18:1, known as the major monounsaturated fatty acid in olive oil is associated with beneficial effects on prevention of cancer, inflammatory and autoimmune diseases (Boulares *et al.,* 2022).

On the other hand, olive oil enriched with natural antioxidants obtained using ultrasound extraction had a significantly high amount ($p < 0.05$) of oleic acid 65.38 ± 0.23% and low amount of linoleic acid (19.15 ± 0.18%) compared to the control. Similar results were found by Ammar *et al.* (2017) where the oleic acid content increased significantly with the addition of *O. ficus-indica* flowers in olive oil. In addition, the results obtained by Sousa *et al.* (2015) revealed that the amounts of mono-unsatured fatty acids increased significantly with the addition of bay leaf and oregano to olive oil. These findings are in perfect agreement with those highlighted by Jaber *et al.* (2012), who showed that the enrichment of a refined olive oil with a chlorophyll extract of olive leaves caused an increase in the content of oleic acid and a decrease in that of linoleic acid.

3.3.2. Effect on free acidity

Initially, no significant differences ($p < 0.05$) were detected between the free acidity values of the control and enriched samples. A significant increase in initial free acidity was noted for all olive oil samples during storage. Then, acidity increased especially in macerated olive oil to reach the values of 0.61 ± 0.01%; 0.53 ± 0.03% and 0.72 ± 0.01%, respectively, for the control (OC), olive oil enriched by ultrasonic extracts (OU) and using maceration (OM). These findings can be explained by the enzymatic activity caused by lipolytic reactions in olive oil (Sousa *et al.,* 2015). In general, adding medicinal and aromatic plants or their extracts to olive oil leads to a slight increase in acidity, which has been shown by several authors. In fact, the addition of the phenol extract of olive leaves during the mixing step resulted in an increase in acidity (Arfaoui *et al.,* 2021). Similar results were found by Arfaoui *et al.* (2021), who reported that adding olive leaves or their ultrasonic extracts significantly increased acidity values compared to the control.

3.3.3. Effect on peroxide value

In this study, the peroxide value (PV) increased significantly ($p > 0.05$) during the storage period for all the analyzed samples (Table 2). At the beginning of storage, no significant differences ($p < 0.05$) were observed between any of the studied olive samples. Besides, after 6 months of storage, only the PV of the OU sample remained below the recommended limit. Furthermore, the enriched oils were more stable than the control sample during storage especially the olive

Table 2: Evaluation of quality parameters, pigment, biophenol contents and inhibition percentage (IP) of control and enriched organic extra-virgin olive oil during 12 months of storage

Analyses	**Storage time (Months)**	**OC**	**OM**	**OU**
Acidity (%)	M_0	0.23 ± 0.01^{aA}	0.25 ± 0.02^{aA}	0.25 ± 0.01^{aA}
	M_3	0.30 ± 0.01^{aB}	0.32 ± 0.04^{aBC}	0.32 ± 0.01^{aBC}
	M_6	0.42 ± 0.01^{aC}	0.35 ± 0.01^{aC}	0.36 ± 0.01^{aC}
	M_9	0.54 ± 0.04^{bD}	0.49 ± 0.02^{bD}	0.42 ± 0.02^{aCD}
	M_{12}	0.61 ± 0.01^{bE}	0.72 ± 0.01^{cE}	0.53 ± 0.03^{aD}
PV (meq O_2/Kg)	M_0	$7.38 \pm 0,45^{aA}$	6.85 ± 0.29^{aA}	7.6 ± 0.33^{aA}
	M_3	10.77 ± 0.48^{bB}	10.47 ± 0.21^{bB}	9.09 ± 0.58^{aB}
	M_6	13.38 ± 0.52^{bC}	13.12 ± 0.52^{bC}	12.23 ± 0.39^{aC}
	M_9	16.84 ± 0.52^{cD}	16.17 ± 0.49^{bD}	15.65 ± 0.55^{aD}
	M_{12}	21.23 ± 0.15^{cE}	18.64 ± 0.22^{bE}	17.07 ± 0.42^{aE}
K_{232}	M_0	1.87 ± 0.04^{aA}	1.8 ± 0.03^{aA}	1.86 ± 0.03^{aA}
	M_3	2.05 ± 0.04^{bB}	2.02 ± 0.04^{aB}	2.01 ± 0.02^{aB}
	M_6	2.33 ± 0.02^{bC}	2.27 ± 0.05^{aC}	2.28 ± 0.05^{aC}
	M_9	2.47 ± 0.11^{bC}	2.37 ± 0.11^{bC}	2.32 ± 0.05^{aC}
	M_{12}	2.87 ± 0.02^{bD}	2.53 ± 0.02^{bD}	$2.42 \pm 0,05^{aD}$
K_{270}	M_0	0.17 ± 0.01^{aA}	0.17 ± 0.02^{aA}	0.17 ± 0.02^{aA}
	M_3	0.19 ± 0.01^{aAB}	0.18 ± 0.01^{aAB}	0.18 ± 0.01^{aAB}
	M_6	0.22 ± 0.01^{bB}	0.19 ± 0.01^{aB}	0.19 ± 0.01^{aB}
	M_9	0.22 ± 0.02^{aB}	0.20 ± 0.03^{aB}	0.19 ± 0.02^{aB}
	M_{12}	0.23 ± 0.03^{bB}	0.21 ± 0.02^{abB}	0.20 ± 0.02^{aB}
Chlorophylls	M_0	4.51 ± 0.05^{bE}	4.61 ± 0.04^{aE}	4.78 ± 0.03^{cE}
	M_3	4.07 ± 0.03^{aD}	3.98 ± 0.04^{bD}	4.32 ± 0.07^{cD}
	M_6	3.13 ± 0.06^{aC}	3.41 ± 0.03^{bC}	3.86 ± 0.08^{cC}
	M_9	2.89 ± 0.05^{aB}	3.26 ± 0.04^{aB}	3.67 ± 0.03^{aB}
	M_{12}	2.42 ± 0.06^{aA}	2.97 ± 0.03^{aA}	3.48 ± 0.04^{aA}
Carotenoids	M_0	1.52 ± 0.04^{aD}	1.56 ± 0.03^{aC}	1.69 ± 0.06^{bE}
	M_3	1.14 ± 0.04^{aC}	1.38 ± 0.05^{bB}	1.42 ± 0.07^{cD}
	M_6	0.54 ± 0.04^{aB}	1.06 ± 0.04^{bA}	1.21 ± 0.02^{cC}
	M_9	0.21 ± 0.01^{aA}	0.96 ± 0.04^{bA}	1.05 ± 0.05^{cB}
	M_{12}	n.d	0.52 ± 0.01^{aA}	0.94 ± 0.02^{bA}
Biophenols	M_0	231 ± 0.05^{aC}	269 ± 0.02^{bD}	338 ± 0.01^{cD}
	M_3	128 ± 0.06^{aB}	162 ± 0.03^{bC}	233 ± 0.05^{cC}
	M_6	61 ± 0.05^{aA}	132 ± 0.05^{bB}	197 ± 0.05^{cB}
	M_9	n.d	64 ± 0.05^{aA}	102 ± 0.05^{bA}
	M_{12}	n.d	0. n.d	n.d
IP (%)	M_0	37.82 ± 0.14^{aE}	44.74 ± 0.12^{bE}	49.39 ± 0.18^{cE}
	M_3	25.9 ± 0.11^{aD}	38.73 ± 0.12^{bD}	42.73 ± 0.12^{cD}
	M_6	13.16 ± 0.16^{aC}	29.89 ± 0.12^{bC}	35.72 ± 0.12^{cC}
	M_9	9.17 ± 0.09^{aB}	22.82 ± 0.12^{bB}	26.39 ± 0.12^{cB}
	M_{12}	1.32 ± 0.03^{aA}	8.09 ± 0.01^{bA}	16.48 ± 0.04^{cA}

OC: control olive oil; **OM:** enriched olive oil using maceration; **OU:** enriched olive oil using ultrasound-assisted extraction. The values are expressed as means ± standard deviation, n=3 using Duncan's multiple range test. Mean values with different lowercase letters indicate significant differences ($p < 0.05$) between samples. Mean values with different uppercase letters indicate significant differences ($p < 0.05$) during storage period.

oil sample enriched with ultrasonic extracts. In fact, enriched samples had the lowest PV values of 18.64 ± 0.22 and 17.07 ± 0.42 for OM and OU, respectively, at the end of the storage period. The obtained results showed that enrichment using maceration or ultrasonic phenolic extracts from *Artemisia absinthium* leaves reduced the formation of lipid hypdroperoxides and consequently reduced the oxidation of the oil compared to the control. This result was attributed to the antioxidant activity of the alkaloid polyphenols, flavonoids, saponin, tannin, glycosides, phenols, and anthroquinonones present in this plant (Dahal *et al.,* 2021).

3.3.4. Effect on specific extinction coefficients

With regards to the specific extinction coefficients K_{232} and k_{270}, which give an indication of primary and secondary oxidation, a significant ($p > 0.05$) increase in these parameters was observed during the 12 months of storage for all analyzed samples. The increase rate of the enriched samples was slightly slower that of the control sample. The obtained results proved that enrichment using ultrasound-assisted extraction is more efficient in preventing oxidation than the maceration method. These findings were in agreement with those of Sousa *et al.* (2015), who showed that enriched olive oils are more stable against primary oxidation. Indeed, from the third month of storage, the specific extinction coefficient K_{232} of the control sample became significantly higher ($p > 0.05$) than those of enriched samples.

Based on all findings related to quality parameters, it was concluded that olive oil enriched with the ultrasonic extracts of *Artemisia absinthium* remained the only sample classified as “extra virgin olive oil” after 12 months of storage according to the IOC (2019). Therefore, this treatment was the most efficient for oxidative stability due to the richness of *Artemisia* in natural antioxidants (Dahal *et al.,* 2021).

3.3.5. Effect on pigment contents

As shown in Table 2, chlorophyll contents decreased in all samples to reach values of 2.42 ± 0.06 ppm, 3.48 ± 0.04 ppm and 2.97 ± 0.03 ppm, respectively, for the control, OU and OM at the end of storage. In addition, at the end of storage, the carotenoids were not detected in the control sample. In fact, the obtained chlorophyll contents showed a significant decrease ($p < 0.05$) during storage. These findings were in accordance with those of Jaber *et al.* (2012), who reported a decrease in pigment contents throughout the storage period. However, carotenoid contents were 0.94 ± 0.02 ppm and 0.52 ± 0.01 ppm, respectively in OU and OM samples. It was observed that the chlorophyll and carotenoid contents in enriched olive oil with ultrasonic extracts were significantly ($p < 0.05$) higher than those of the macerated sample. These findings can be explained by the mechanical effect caused by the implosion of micro-bubbles of ultrasonic extraction, which cause rapid breakdown of the tissues, allowing the release of compounds into the solvent (Toma *et al.,* 2001). This richness in pigments can contribute to a promising antioxidant activity compared to the other samples. These findings are in line with those of Malheiro *et al.* (2013), who showed that OTCOO enriched by the addition of olive leaves during the extraction process presented much higher lutein and β-carotene contents than the control.

3.3.6. Effect on biophenol content

The term «biophenol» is used to designate the bioactive phenols in order to replace the more common chemically vague term “polyphenol” (Arfaoui *et al.,* 2021).

The registered initial biophenol contents in the analyzed olive oil samples showed that the highest value (338 ± 0.01 ppm) was noted for OU followed by OM (269 ± 0.02 ppm) and then the control (231 ± 0.05 ppm). This result can be explained by the richness of *Artemisia absinthium* in polyphenols (Riahi *et al.,* 2015). During one year of storage, a significant decrease ($p < 0.05$) was registered in terms of biophenol contents for all the analyzed OTCOO samples. These findings were in agreement with those of previous researchers, who showed that total biophenol content decreased in olive oil even after its enrichment with chlorophyll pigment (Jaber *et al.,* 2012) or natural antioxidants extracted from plants using various methods. Furthermore, from the ninth month, the results showed that the phenolic compounds were absent from the control sample and that their content was significantly higher in the OU compared to the macerated sample.

The olive oil enriched with natural antioxidant extracted from *Artemisia absinthium* leaves can be considered a functional food. In fact, its consumption could reduce the risk of chronic diseases such as cardiovascular diseases, diabetes and hypertension due to its high polyphenol content (Boulares *et al.,* 2022).

3.3.7. Effect on variation in antioxidant activity

The obtained results on all analyzed samples showed a significant decrease ($p < 0.05$) in the antioxidant activity over the 12 months of storage. The initial inhibition percentage (IP %) decrease to reach values of 1.32 ± 0.03, 16.48 ± 0.04 and 8.09 ± 0.01%, respectively, for the control, OU and OM at the end of storage. These findings can be assigned to the auto-oxidation of natural antioxidants such as pigments, sterols and polyphenols, which lead to their degradation and a decrease in anti-radical activity (Antoniewska *et al.,* 2018). Furthermore, the antioxidant activities of enriched olive oil samples were higher ($p < 0.05$) than that of the control after 12 months of storage. The highest radical scavenging activity of the enriched samples with ultrasonic extracts throughout the storage period was attributed to the mechanism of applied ultrasound-assisted extraction. In fact, cavitation, mechanical forces and thermal impact lead to the disruption of cells walls, and the reduction in particle size, which enhance mass transfer across cell membranes, resulting in higher contents of phenolic compounds and pigments (Şahin *et al.,* 2017). These results are in agreement with those of Boulares *et al.* (2022), who showed that olive oils stored in black glass boxes for 90 days and incorporated with various essential oils are characterized by a better antioxidant activity compared to control samples.

3.3.8. Effect on sensorial properties

The sensory analysis was carried out at the beginning and at the end of the storage period all the studied samples (Table 3). At the beginning of storage, all the OTCOO samples were devoid of defects. Regarding the positive attributes (fruity, bitter, pungent), a significant difference ($p < 0.05$) was observed among all samples. In fact, the sample enriched with ultrasonic extracts had a stronger fruity smell (4), bitter smell (3.5) and pungent taste (3.5), which are the most important criteria for the Tunisian consumers. These findings were in agreement with the results found by Şahin *et al.* (2017), who proved that sensorial attributes were enhanced in terms of fruity taste and green color with the addition of olive leaves. In this context, wormwood was known to contain bitter substances such as absinthin (Judžentiene, 2016). Therefore, these molecules may have caused the bitter taste in the enriched olive oils, especially the OU sample. Moreover, at the end of the storage period, the fusty defect was detected in the control, which also had the lowest fruity intensity (0.8). In addition, the macerated OTCOO was musty (0.8) and winey (2), with an unacceptable taste and odor. However, the extraction of natural antioxidants using ultrasound-assisted extraction in olive oil had no negative effect on the product, which remained devoid of defects until the end of storage. These findings showed that the enrichment of olive oil using ultrasonic extracts improved the sensory characteristics of this product and inhibited the musty and fusty defects in olive oil. This finding confirmed that the ultrasound-assisted extraction method is a good alternative to conventional methods.

4. CONCLUSIONS

The present data proved that the enrichment of OTCOO with *Artemisia absinthium* leaf extracts was a preventive tool which gave better oxidative stability to the olive oil by reducing its PV, free acidity and the primary metabolite of oxidation compared to the control. Moreover, the enrichment of OTCOO with natural antioxidants using ultrasound-assisted extraction was shown to be the most efficient technique compared to conventional methods such as maceration by significantly improving the oxidative

Table 3: Variation in sensory properties of control and enriched organic extra-virgin olive oils at the beginning and end of the storage period

		Musty	Winey	Wet wood	Metallic taste	Rancid taste	Fruity taste	Pungent taste	Bitter taste
OC	M_0	n.d	n.d	n.d	n.d	n.d	3.00 ± 0.30^{aB}	3.10 ± 0.20^{aB}	3.0 ± 0.20^{aB}
	M_{12}	1.00 ± 0.10^{bA}	1.50 ± 0.13^{cA}	n.d	n.d	2.02 ± 0.25^{dA}	0.81 ± 0.10^{aA}	0.80 ± 0.11^{aA}	1.5 ± 0.13^{cA}
OM	M_0	n.d	n.d	n.d	n.d	n.d	3.25 ± 0.40^{aB}	3.04 ± 0.20^{aB}	3.2 ± 0.15^{aB}
	M_{12}	0.40 ± 0.13^{aA}	2.02 ± 0.11^{aA}	n.d	n.d	1.50 ± 0.13^{bA}	1.51 ± 0.12^{bA}	1.50 ± 0.21^{bA}	2.2 ± 0.10^{cA}
OU	M_0	n.d	n.d	n.d	n.d	n.d	4.01 ± 0.50^{bB}	3.50 ± 0.00^{aB}	3.5 ± 0.10^{aB}
	M_{12}	n.d	0.50 ± 0.10^{aA}	n.d	n.d	0.51 ± 0.10^{aA}	2.50 ± 0.10^{dA}	2.10 ± 0.10^{bA}	2.2 ± 0.10^{cA}

OC: control olive oil; **OM:** enriched olive oil using maceration; **OU:** enriched olive oil using ultrasound-assisted extraction. The values are expressed as means ± standard deviation, n=3 using Duncan's multiple range test. Mean values with different lowercase letters indicate significant differences ($p < 0.05$) between samples. Mean values with different uppercase letters indicate significant differences ($p < 0.05$) during storage period.

stability of olive oil. This treatment also showed a number of particular advantages, such as increasing oleic acid content, pigments and biophenols and improving antioxidant activity compared to control and macerated oil. Furthermore, the sensorial analysis showed an improvement in taste and odor of the olive oil enriched with ultrasonic extracts. These findings encourage the application of *Artemisia absinthium* leaves as an alternative source of natural antioxidants, especially using ultrasound-assisted extraction to accelerate mass transfer with shorter processing times. All of these advantages will contribute to decreasing operating costs and environmental issues and lead to the production of olive oil with good nutritional value.

Conflict of interest:

All the authors declare that there is no conflict of interest in this work.

Acknowledgements

This study was sponsored by the «Tunisian Ministry of Higher Education and Scientific Research» and the «Tunisian Institution for Agricultural Research and Higher Education ». This work was performed in the Research Laboratory: “Technological Innovation and Food Safety LR22-AGR 01-ESIAT”, in the Higher Institute of Food Industries of Tunisia.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e517
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0990221

Antioxidant and anticancer activities of peanut (*Arachis hypogaea* L.) skin ultrasound extract

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Submitted: 21 September 2022; Accepted: 16 January 2023; Published online: 10 October 2023

SUMMARY: This study evaluates the effect of ultrasound-assisted extraction on the extractability of polyphenols from peanut skins (PS) and their antioxidant, and anticancer activities. The extraction was performed with solid/solvent ratios of 1:20 and 1:30 (w/v) at ultrasound intensity ranging from 5.8 to 15.4 W/cm^2 for different extraction times (10, 20, 30 and 40 min). The highest polyphenol yield was 167.46 mg GAE/g dried PS. The most abundant polyphenols were catechin, syringic acid, and vanillic acid. The PS ultrasound extract (PSUE) increased the oxidative stability of sunflower oil by four times its initial level. PSUE possessed high inhibitory activity against MCF-7, HepG-2, HCT-116, and PC-3 cancer cell lines, with IC$_{50}$ ranging from 1.85 ± 0.13 to 6.1 ± 0.43 µg/ml. In addition, the cytotoxicity of PSUE was examined on HFB4 human normal melanocytes using the MTT assay. These results suggest that PSUE can be used as a natural antioxidant and anticancer agent.

KEYWORDS: *Anticancer; Antioxidant; Peanut skin; Polyphenols; Sunflower oil; Ultrasound-assisted extraction.*

RESUMEN: ***Actividades antioxidantes y anticancerígenas del extracto obtenido por ultrasonido de piel de maní (Arachis hypogaea L.).*** Este estudio evalúa el efecto de la extracción asistida por ultrasonido sobre la extractabilidad de los polifenoles de la piel de maní (PS) y sus actividades antioxidantes y anticancerígenas. La extracción se realizó con relaciones sólido/solvente de 1:20 y 1:30 (p/v) a una intensidad de ultrasonido que varió de 5,8 a 15,4 W/cm^2 para diferentes tiempos de extracción (10, 20, 30 y 40 min). El mayor rendimiento de polifenoles fue de 167,46 mg GAE/g de PS seco. Los polifenoles más abundantes fueron la catequina, el ácido siríngico y el ácido vanílico. El extracto de ultrasonido PS (PSUE) aumentó cuatro veces la estabilidad oxidativa del aceite de girasol. PSUE poseía una alta actividad inhibitoria contra las líneas celulares de cáncer MCF-7, HepG-2, HCT-116 y PC-3, con IC50 que oscilaba entre 1,85 ± 0,13 y 6,1 ± 0,43 µg/ml. Además, se examinó la citotoxicidad de PSUE en melanocitos humanos normales HFB4 utilizando el ensayo MTT. Estos resultados sugieren que el PSUE puede usarse como un antioxidante natural y un agente anticancerígeno.

PALABRAS CLAVE: *Aceite de girasol; Anticancerígeno; Antioxidante; Extracción asistida por ultrasonido; Piel de maní; Polifenoles.*

Citation/Cómo citar este artículo: Hammad KSM, El-Roby AM, Galal SM. 2023. Antioxidant and anticancer activities of peanut (*Arachis hypogaea* L.) skin ultrasound extract. *Grasas Aceites* **74** (3), e517. https://doi.org/10.3989/gya.0990221

1. INTRODUCTION

Recent studies indicate that many food wastes are rich sources of bioactive compounds which could be used as nutraceuticals and functional foods (Leichtweis *et al*., 2021). The peanut (*Arachis hypogaea* L.) is an important commercial crop used to produce oil. It is an ingredient in peanut butter, confections, and other finished products. The worldwide production of peanuts with shells in 2020 was 53 million tonnes (FAOSTAT, 2022). PS is the pink-red coat which is produced as waste after peanut kernels are roasted and blanched. It represents 3% of the fruit weight. It has limited industrial applications due to its high level of tannins, bitter flavor, low-calorie level, and poor commercial value. However, it is rich in various bioactive compounds which belong to polyphenols. Composite film containing PS polyphenol extract demonstrated DPPH and ABTS radical scavenging activity (Dai *et al*., 2022).

The oxidation of oils deteriorates the quality characteristics of food during storage. The use of synthetic antioxidants in the food sector is increasingly restricted because they have the potential to cause cancer (Bhadresha *et al*., 2022). This trend is accompanied by the expansion of the use of natural antioxidants such as polyphenols from plant sources. The cytotoxicity of polyphenols from different plant sources has been investigated in cancer cell lines. Olive pomace methanolic extract showed anticancer activity against HepG2, MCF-7, PC3 and HCT116 cell lines (Mahmoud *et al*., 2018). Meanwhile, moringa leaf aqueous extract exhibited the same effect on human lung cancer A549 cells (Bhadresha *et al*., 2022).

Ultrasound-assisted extraction (UAE) is becoming more widely used because of issues with traditional extraction processes. Compared with conventional techniques, the recent ones are more efficient, require less energy, and yield high-quality extracts (Sridhar *et al*., 2021). Khaopha *et al*. (2015) indicated that PS methanolic extract obtained by maceration had an anticancer effect against HeLa, HT29, HCT116 and Jurkat cells.

There is a lack of studies about the antioxidant and cytotoxic activities of PSUE which is rich in polyphenols on colon, breast, and prostate carcinoma cells. The objectives of the present study were maximizing the recovery of polyphenols from PS using ultra-sound assisted extraction, the characterization of the phenolic extract, and evaluating its ability to extend the shelf-life of refined sunflower oil, besides its cytotoxicity on different carcinoma cell lines.

2. MATERIALS AND METHODS

2.1. Materials

Three kg of skins from roasted peanuts were obtained from the local Roasting and Peeling Plant of peanuts, Cairo, Egypt. Completely refined sunflower oil (RBD) without added antioxidants was provided by Cairo Oil and Soap Company (Egypt). Folin–Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), Trypan blue dye, 0.25% Trypsin-EDTA solution, L-glutamine, gentamycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and all the HPLC standards used for the identification of polyphenols were purchased from Sigma (St.Louis, MO, USA). Fetal Bovine serum (FBS) and Dulbecco's Modified Eagle's medium (DMEM) were purchased from Lonza (Walkersville, USA). HepG-2, HCT-116, MCF-7, and PC-3 cells were obtained from the VACSERA Tissue Culture Unit.

2.2. Preparation and chemical composition of PS

A PS sample (500 g) was ground and sieved in a 20-30 mesh. The chemical composition of PS powder was determined according to AOAC (2012). The Kjeldahl method was used to determine the total nitrogen content. The protein content on a nitrogen basis was measured using a conversion factor of 6.25. Ether extract was obtained using diethyl ether in a Soxhlet system. The ash content was determined by incineration in a muffle furnace at 550 °C. Crude fiber was determined gravimetrically after digestion of the sample with 1.25% sulfuric acid solution and 1.25% sodium hydroxide solution.

2.3. Extraction of polyphenols

Polyphenols were extracted from a roasted peanut skin powder sample with 80% ethanol aqueous solution (v/v). Extraction was performed for 10, 20, 30, and 40 min using a Fisher Sonic Dismembrator (Model 300, USA) and solid/solvent ratios of 1:20 and 1:30 (w/v). Ultrasound power (P) and ultrasound intensity (UI) were calculated using the following equations by Vernes *et al*. (2019).

$$Power\ (\mathrm{W}) = \left(\frac{dT}{dt}\right) \times Cp \times M \qquad \text{Eq. (1)}$$

Where (dT/ dt) is the increase of °C/min of 200 ml of aqueous ethanol, *Cp* is the heat capacity of 80% aqueous ethanol (2746 J/kg·°C), and M is the mass (kg) of 80% aqueous ethanol. When the generator was set to 95, 100, 105, and 110 W, the estimated *P* was 16.5, 26, 37.8, and 43.7 W, respectively.

The UI (W/cm^2) was calculated as follows

$$UI = 4P/\pi D^2 \qquad \text{Eq. (2)}$$

where D is the diameter (cm) of the ultrasound probe. Supernatants were saved after extraction and kept at -20 °C until analysis.

2.4. Total polyphenols

The total polyphenol content of the PSUE was determined using the Folin-Ciocalteu method as described by Arnous *et al.* (2002) at 760 nm. Results are expressed as mg gallic acid equivalent (GAE)/g dried PS.

2.5. Total flavonoids

The flavonoid content in the extracts was determined as described by Formagio *et al.* (2014). The absorption of the reaction mixture against the blank was recorded at 510 nm. The analysis was performed in triplicate. Values are expressed as mg quercetin equivalents (QE)/g dried PS.

2.6. Identification and quantification of polyphenols using HPLC

The PSUE with the highest level of polyphenols was concentrated at 40 °C under vacuum using a rotary evaporator. The HPLC analysis of the concentrated extract was carried out according to Kim *et al.* (2006). An Agilent Technologies 1100 series liquid chromatograph equipped with a diode-array detector was used. The Eclipse XDB-C$_{18}$ column (150 mm x 4.6 µm x 5 µm) with a C$_{18}$ guard column (Phenomenex, Torrance, CA) was also used. Before injection, the sample was filtered through an 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI). The injection volume was 20 µl. The mobile phase contained (A) acetonitrile and (B) 2% acetic acid. Gradient flow was conducted at 0.8 ml/min. Peaks were observed simultaneously at 280, 320, and 360 nm. The peaks were identified by UV spectra and retention times and their values were compared with those of the standards.

2.7. Antioxidant assays

2.7.1. DPPH assay

The assay was carried out as described by Brand-Williams *et al.* (1995). An aliquot (0.2 ml) of the extract was mixed with 2.7 ml of DPPH solution (45 mg/L) and the mixture was kept for 30 min in the dark. The absorbance was read at 515 nm using a Vis-UV spectrophotometer. BHT was used as a reference. The following formula was used to calculate the percentage of radical inhibition:

$$Inhibition\ percentage\ = \left[\frac{A0 - A1}{A0}\right] \times 100$$

A$_0$ denotes absorbance in the absence of sample, while A$_1$ denotes absorbance in the presence of sample. The IC$_{50}$ value represented the concentration of the extract required to decrease the initial absorbance of the DPPH solution by 50%.

2.7.2. Reducing power assay

The reducing power of various concentrations of the PSUE was measured according to the method described by Chang *et al.* (2002) at 700 nm. From the linear regression analysis, the extract concentration which produced 0.5 absorbance (IC$_{0.5}$) was determined. Results were compared with BHT as a standard.

2.8. Quality characteristics of oil

The acid value and peroxide value of the RBD sunflower oil were determined according to the recommended methods of AOCS (2009). The color of the oil samples was measured with the Lovibond Tintometer (Tintometer Ltd., United Kingdom), and a 5.25-inch cell, according to ISO 15305 (1998).

2.9. Rancimat analysis

The efficiency of the investigated PSUE (concentrated extract) in protecting sunflower oil against accelerated oxidation was carried out using Rancimat 743 (Metrohm, Switzerland) according to AOCS (2009). The concentrated extract was added directly to the oil samples at the investigated levels. The oil samples enriched with 200 and 400 mg GAE from PSUE (concentrated extract)/kg oil were heated at 110 °C and 120 °C, respectively. Sunflower oil without any added antioxidants was used as a control. BHT was used at a concentration of 200 mg/kg oil. The oxidative stability was expressed by the induction period (h). The airflow rate was set at 20 L/h.

2.10. Determination of anti-cancer activity

The investigated human cancer cell lines and human normal melanocytes (HFB-4) were propagated in DMEM supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 50 µg/ml gentamycin. All cells were kept at 37 °C in a humidified atmosphere which contained 5% CO_2. The colorimetric method of Mosmann (1983) was used to assess the PSUE's cell cytotoxicity. The absorbance was read at 570 nm with a microplate reader (SunRise, TECAN, Inc., USA). IC_{50} values were calculated from a dose-response curve.

2.11. Statistical analyses

All analyses were carried out in triplicate, except for the Rancimat analysis (two repetitions) and HPLC analysis (single determination). Statistica software (StatSoft Inc., Tulsa, OK, USA) was used to analyze the variance of the results. The results were presented in terms of means and standard deviation.

3. RESULTS AND DISCUSSION

3.1. Chemical composition of the PS

The ether extract, protein, ash, and crude fiber contents of PS were 8.32 ± 0.22, 11.57 ± 0.27, 2.57 ± 0.08, and 48.04 ± 2.14% on a dry weight basis, respectively. These results supported earlier findings of Muñoz-Arrieta *et al.* (2021) and proved that PS is a relatively rich source of protein and could be used in the future for the extraction and purification of this nutritional component. PS composition varies with seed maturity and cultivar.

3.2. Polyphenol extraction yield

The results in Figure 1a illustrate that increasing extraction time to 20 min at the highest solid/solvent ratio increased the yield of polyphenols, after which the yield decreased significantly ($p < 0.05$) at each UI used.

The extracted polyphenols with the lowest solid/solvent ratio and at each UI did not significantly ($p > 0.05$) increase when the extraction duration was extended to 40 min (Figure 1b). The yield in polyphenols increased significantly ($p < 0.05$) with the increase in UI from 5.8 to 9.2 W/cm^2 during extractions lasting 30 and 40 min, regardless of the solid/solvent ratio. However, increasing UI from 9.2 to 15.4 W/cm^2 did not significantly ($p > 0.05$) enhance polyphenol extraction. The decrements of extraction yield of polyphenols at higher ultrasonic power could be due to the decomposition of the components (Wang *et al.*, 2018). The maximum polyphenol yield (167.46 ± 0.89 mg GAE/g) was obtained with a relatively high solid/solvent ratio for 20 min at UI of 5.8 W/cm^2 (Figure 1a).

The results of the current study demonstrated that the yield of polyphenols achieved with ultrasonic assistance was superior to that of other researchers (Taha *et al.*, 2012) who relied on conventional techniques (41.5 mg GAE/g dry PS). This proved that ultrasound-assisted extraction is an efficient method for extracting polyphenols as reported by Sridhar *et al.* (2021).

The results of this investigation indicated that the highest level of flavonoids reached 321.76 ± 2.26 mg of QE/g of PS. Meng *et al.* (2020) found that the flavonoid content in the PS methanolic extract was 234.33 mg rutin equivalents/g PS. These differences in total polyphenol and flavonoid contents could be attributed to cultivar variations, growing conditions, and extraction techniques.

3.3. Identified phenolic compounds in the PSUE

The phenolic composition of PSUE (concentrated extract, 41.55 mg/ml) was analyzed by HPLC. The results are presented in Figure 2 and Table 1.

The PSUE was characterized by a high level of syringic acid and vanillic acid, followed by protocatechuic and *p*-coumaric acids. Rosmarinic, caffeic, and chlorogenic acids were also identified in the examined extract. The analysis demonstrated that catechin was the most abundant flavonoid compound. This result is in line with the results found by Bodoira *et al.* (2022). Moderate amounts of kaempferol, chrysin, and apigenin were also detected in the extract. Francisco and Resurreccion (2009) examined the polyphenols

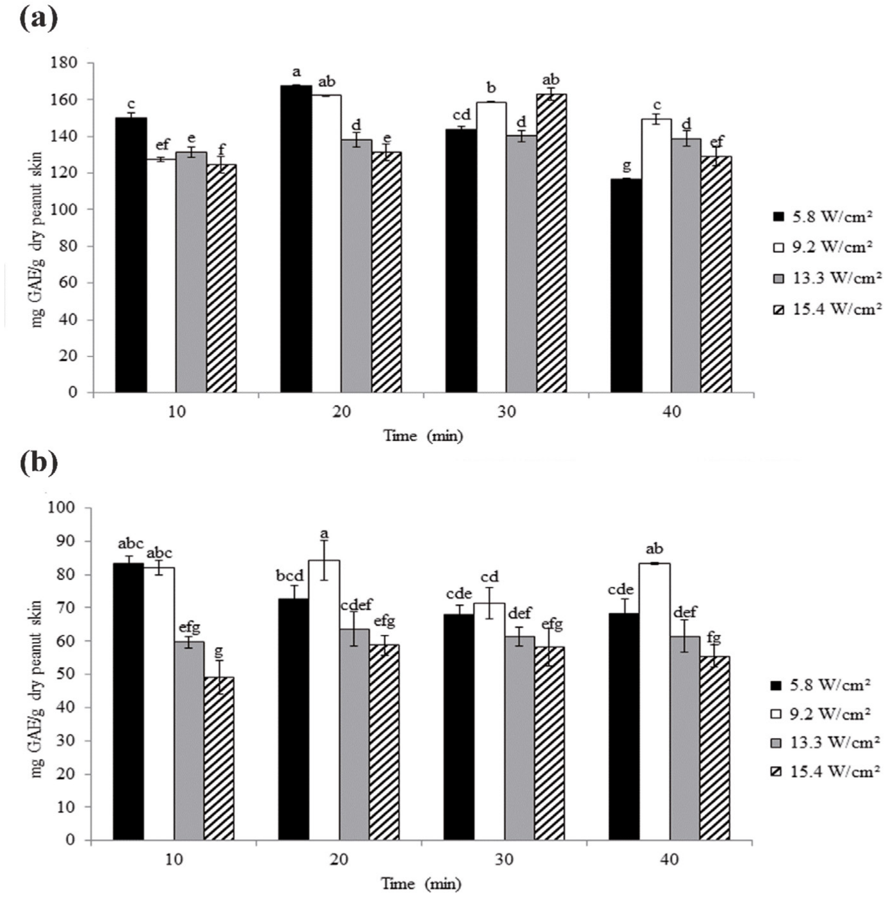

FIGURE 1. Total phenolic contents (mg GAE/g dry skin) of the ultrasound extract obtained by, (a) peanut skin/aqueous ethanol ratio of 1:20 (w/v) and (b) peanut skin /aqueous ethanol ratio of 1:30 (w/v). Values are means ± standard deviation of three replicates. Bars with different letters indicate significant difference ($p < 0.05$) by Tukey's test

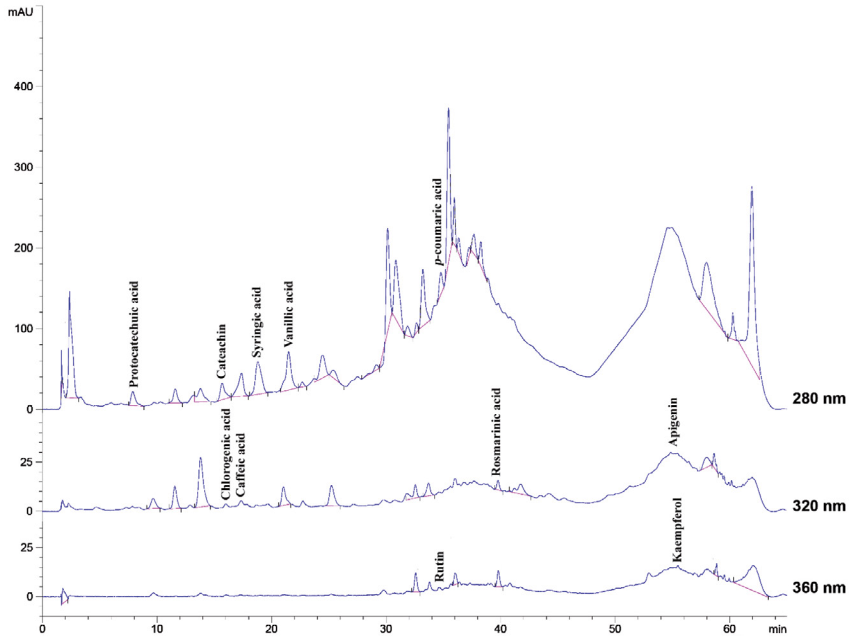

FIGURE 2. HPLC profiles of phenolic compounds of peanut skin extract simultaneously recorded at 280 nm, 320 nm, and 360 nm. The values refer to a single determination

Table 1. Phenolic and flavonoid compounds of peanut skin ultrasound extract

Identified compounds	Concentration (mg/g dry extract)
Polyphenols	
Gallic acid	ND*
Protocatechuic acid	19.70
ρ-hydroxybenzoic acid	ND*
Gentisic acid	ND*
Chlorogenic acid	1.14
Caffeic acid	1.54
Syringic acid	64.74
Vanillic acid	42.29
Ferulic acid	ND*
Sinapic acid	ND*
ρ-coumaric acid	17.74
Rosmarinic acid	2.92
Cinnamic acid	ND*
Flavonoids	
Catechin	119.98
Rutin	1.36
Apigenin-7-glucoside	ND*
Quercetin	ND*
Apigenin	5.11
Kaempferol	22.30
Chrysin	21.50

* Not detected. The values refer to a single determination

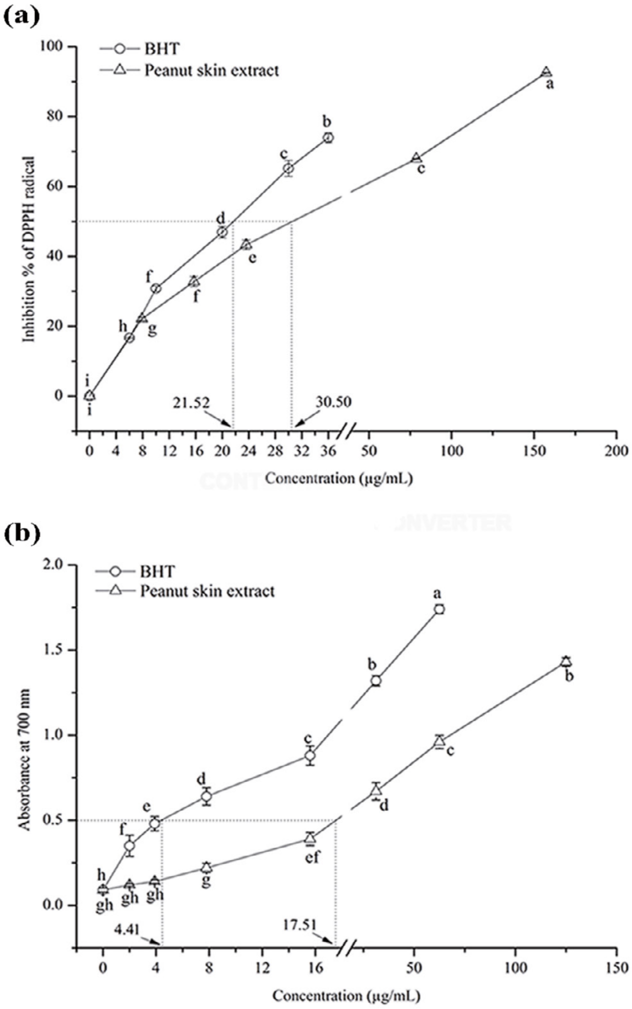

Figure 3. Antioxidant activity of the peanut skin extract compared to BHT as assessed by: (a) DPPH radicals, (b) Ferric reducing power. The results are represented as average values of three replicates ± SD. Bars with different letters indicate significant differences ($p < 0.05$) by Tukey's test

composition of skin extracts from three peanut types (Runner, Virginia, and Spanish). They reported that the discrepancy in the polyphenol levels could be due to the differences in peanut cultivar and skin type.

3.4. Antioxidant activity

The scavenging activity of PSUE against DPPH radicals is shown in Figure 3a.

The results illustrated the positive correlation between PSUE concentration and its activity against DPPH radicals. Compared to BHT, PSUE had a significantly ($p < 0.05$) lower scavenging action on DPPH radicals. PSUE inhibited more than 92% of the DPPH radicals at 157.3 µg GAE/ml. However, the IC_{50} values of PSUE and BHT were 30.5 ± 0.43 µg GAE/ml and 21.65 ± 0.3 µg/ml, respectively. A decrease in the IC_{50} value indicates better antioxidant activity (Brand-Williams *et al*., 1995).

The reduction in ferric ions (Fe^{3+}) from $K_3Fe(CN)_6$ to ferrous ions (Fe^{2+}) by the PSUE is shown in Figure 3b. Increasing extract concentration was accompanied by an increase in reducing power. The results indicated that PSUE had lower reducing power ($IC_{0.5}$=17.51 ± 0.75 mg GAE/ml) than BHT (4.48 ± 0.5 mg/ml). At a concentration of 15.6 µg/ml, PSUE and BHT had reducing power values corresponding to 0.39 ± 0.038 and 0.88 ± 0.057, respectively. These results demonstrate that the PSUE showed concentration-dependent antioxidant activity as reported by Wang *et al*. (2018).

3.5. Oxidative stability of PSUE enriched oil

The acid and peroxide values for the oil were 0.21 ± 0.01 mg KOH/g and 0.73 ± 0.004 meq/Kg, respectively, which supported Codex (2021) requirements for acceptable quality. The results in Table 2 show that the addition of PSUE (concentrated extract) at 200 mg GAE/Kg sunflower oil prolonged the induction period of the oil (tested at 110 °C) by 29%. Meanwhile, enriching oil with BHT at the same level enhanced its stability against oxidation by only 21%.

The addition of 400 mg GAE from PSUE (concentrated extract)/kg oil increased its oxidative stability (measured at 120 °C) by 354%. These results prove that PSUE inhibits lipid oxidation without pro-oxidative effects at a higher concentration.

No significant difference was found in oxidative stability (measured by peroxide value) between sunflower oil samples (control) and those mixed with 0.2% (w/w) of PS ethanol extract, obtained by maceration at room temperature, after 3 days of storage at 60 °C (Larrauri *et al*., 2016). The addition of 1.56 g GAE of PS subcritical fluid extract/kg chia oil increased its oxidative stability to that obtained with TBHQ at 0.2 mg/kg (Bodoira *et al*., 2022).

Color is an important oil quality parameter, which increased as the extract level increased. The addition of PSUE at the highest investigated level produced a more highly colored oil (0.8 ± 0.05 Red/4 Yellow) than the oil prepared with half the concentration of the same extract (0.5 ± 0.05 Red/4 Yellow) or 200 mg BHT/kg oil (0.3 ± 0.00 Red/4 Yellow). However, the color of the studied oil samples was less intense than the red and yellow hues of the bleached sunflower oil (2.5 Red/25 Yellow), according to American trading rules as reported by Guliyev *et al.* (2018).

3.6. Cytotoxic activity of PSUE

The cytotoxicity of the concentrated PSUE (concentrated extract, 41.55 mg GAE/ml) was studied on HepG2, HCT-116, MCF-7, and PC-3 carcinoma cells (Figure 4). The inhibitory activity of the investigated extract against HFB4 human normal melanocytes was also assayed.

The PSUE demonstrated a concentration-dependent reduction in cancer cell viability. The inhibitory activities (IC_{50} value) of the PSUE against HepG-2 (Figure 4a), HCT-116 (Figure 4b), MCF-7 (Figure 4c), and PC-3 (Figure 4d) carcinoma cells were 1.85 ± 0.13, 1.99 ± 0.07, 5.32 ± 0.62 and 6.1 ± 0.43 µg/ml, respectively, compared to 2.93 ± 0.18, 3.5 ± 0.46, 5.9 ± 0.71 and 42.4 ± 2.7 µg/ml for the standard drug. A low IC_{50} value indicates the high sensitivity of a cell line to PSUE or Vinblastine sulphate.

The results showed that PSUE at 31.2 and 15.6 µg/ml had significantly ($p < 0.05$) similar inhibition effects on HepG-2 (Figure 4a) and HCT-116 (Figure 4b) cells as vinblastine sulfate, respectively. It is interesting to note that PSUE at 62.5 µg/ml inhibited 80% of the viability of MCF-7 cells (Figure 4c). The standard drug at 125 µg/ml (Figure 4c) recorded this level of inhibition of the same cells. PSUE at 31.25 µg/ml had a significantly ($p < 0.05$) similar inhibitory effect (~75%) on PC-3 cells as did vinblastine sulfate at 125 µg/ml (Figure 4d).

The PSUE proved more effective than vinblastine sulphate at inhibiting the human cancer cells under investigation. The presence of syringic acid, protocatechuic acid, and catechin might have contributed to the cytotoxic effect of the investigated extract as reported in different studies against cancers of different origins (Elansary *et al*., 2019; Mihanfar *et al*., 2021).

TABLE 2. Induction time and protection factor of sunflower oil enriched with peanut extract at different concentrations

Sample	Induction time (h)	Protection factor [c]
Sunflower oil [a]	3.23 ± 0.28	1.00
Sunflower oil [b]	1.37 ± 0.11	1.00
BHT (200 mg/kg oil) [a]	3.92 ± 0.40	1.21
Extract (200 mg GAE /kg oil) [a]	4.17 ± 0.32	1.29
Extract (400 mg GAE /kg oil) [b]	6.22 ± 0.53	4.54

The values are expressed as mean ± SD of two independent experiments. [a] The induction time was determined at 110 °C. [b] The induction time was determined at 120 °C. [c] Induction time of oil containing antioxidant/Induction time of oil measured at the same temperature.

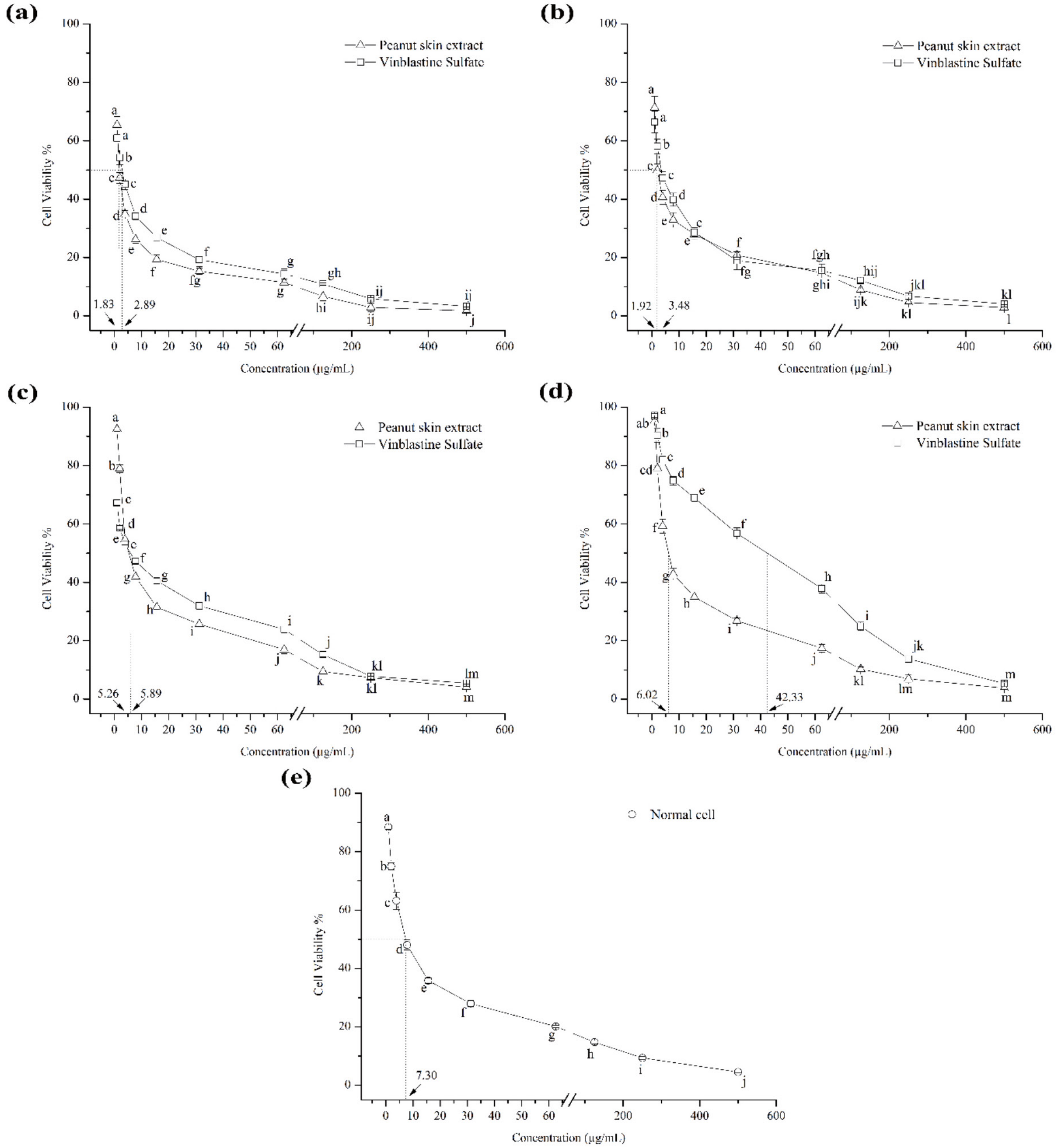

FIGURE 4. Cytotoxicity effect of peanut skin extract and Vinblastine sulfate concentrations (µg/ml) against (a) HepG-2 hepatocellular cancer cell line, (b) HCT-116 human colon cancer cell line, (c) MCF-7 human breast cancer cell line, (d) PC-3 prostate cancer cell line, and (e) HFB4 human normal melanocytes. The results are represented as average values of three replicates ± SD. Values with different letters indicate significant differences ($p < 0.05$) by Tukey's test

The investigated extract had a low toxicity (CC_{50}= 7.3 ± 0.5 µg/ml) to HFB4 human normal melanocytes (Figure 4e). The selective toxicity levels (IC_{50} values for normal fibroblast cells/IC_{50} value for cancer cells ratio) for HepG-2 and HCT-116 were 3.9 and 3.80, respectively, showing considerable selective ability (> 2) against those cancer cells according to Valderrama *et al*. (2016). Rossi *et al*. (2020) indicated that the PS ethanolic extract did not exert cytotoxicity against human peripheral blood mononuclear cells.

On the other hand, PS methanolic extract was found to have IC_{50} values of 10.9 and 19.3 µg/ml on HCT-116 and HepG-2 cancer cells, respectively, but did not affect breast carcinoma cells (Taha *et al.*, 2012). Furthermore, PS methanolic extract which was obtained by maceration induced the apoptosis of HCT-116 cancer cells (IC_{50} =50.68 µg/ml) (Khaopha *et al.*, 2015). They found that MCF-7 cells were less sensitive to the extract (IC_{50} > 90 µg/ml). The results of this study showed that the ultrasound-investigated extract was a more effective anticancer agent than those acquired using other methods from earlier investigations.

Plant-derived medications can advance into clinical trials for further therapeutic development if they are non-toxic to normal cell lines, exhibit cytotoxicity in cancer cell lines, and have high selective toxicity (Rossi *et al.*, 2020).

4. CONCLUSIONS

At room temperature, a high yield of polyphenols was successfully extracted from PS with aqueous ethanol using ultrasound. Within the examined ranges, increasing UI and duration had a favorable impact on extraction yields. The incorporation of PSUE into sunflower oil at 200 and 400 mg/kg increased its oxidative stability compared to the samples containing the synthetic antioxidant BHT. The extract proved to have *in vitro* anticancer activity on HepG2, MCF-7, HCT-116, and PC-3 with lower IC_{50} values than those of the standard drug (Vinblastine Sulfate). This suggests that PSUE can be safely used in the preparation of functional foods with antioxidant bioactive ingredients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e518
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0224221

What are the most effective biotic and abiotic factors affecting fatty acid composition of *Garra rufa* (Heckel, 1843)?

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Submitted: 22 February 2022; Accepted: 30 January 2023; Published online: 10 October 2023

SUMMARY: Specimens of *Garra rufa* were collected from a warm river and a cool stream in the Bingöl Province, Turkey, once a month over a period of one year. The effects of month, season, gender and location on the fatty acid composition in the muscle and the lipid content were investigated and dietary marker fatty acids were used to obtain dietary preferences in different locations (Ilıcalar, Garip) and periods. Total lipid change was seasonally significant (ANOSIM-R=0.49) at both locations and 18:1ω9, 20:5ω3 and 20:6ω3 were the most abundant dietary fatty acids. Although *G. rufa* are predominantly herbivores, they can also feed omnivorously on mixed diets depending on the presence and absence of their primary diet. The effect of season was significant on fatty acid composition, regardless of the location (P_{perm}=0.001). Significant seasonal changes in all the fatty acid compositions could be attributed to seasonal changes in the abundance and diversity of dietary sources in the environment due to the effect of temperature.

KEYWORDS: *Dietary fatty acids; DHA EPA; Garra rufa; 18:1ω9*

RESUMEN: ***¿Cuáles son los factores bióticos y abióticos más efectivos que afectan a la composición de ácidos grasos de la* Garra rufa *(Heckel, 1843)?.*** Se recolectaron especímenes de *Garra rufa* (pez doctor) de un río cálido y un arroyo frío en la provincia de Bingöl, Turquía, mensualmente durante un año. Se investigaron los efectos del mes, la estación, el género y la ubicación en la composición de ácidos grasos musculares y el contenido de lípidos y se utilizaron los ácidos grasos como marcadores dietéticos para obtener preferencias dietéticas en diferentes lugares (Ilıcalar, Garip) y períodos. El cambio total de lípidos fue estacionalmente significativo (ANOSIM-R=0,49) en ambos lugares y 18:1ω9, 20:5ω3 y 20:6ω3 fueron los ácidos grasos dietéticos más abundantes. Aunque *G. rufa* son predominantemente herbívoros, también pueden alimentarse de forma omnívora con dietas mixtas según la presencia o ausencia de la dieta principal. El efecto de las estaciones fue significativo en la composición de ácidos grasos independientemente de las localizaciones (P_{perm}=0.001). Los cambios estacionales significativos en todas las composiciones de ácidos grasos podrían atribuirse a los cambios en la abundancia y diversidad de las fuentes dietéticas en el medio ambiente debido al efecto de la temperatura.

PALABRAS CLAVE: *Ácidos grasos dietéticos; DHA; EPA; Garra rufa; 18:1ω9*

Citation/Cómo citar este artículo: Şen Özdemir N. 2023. What are the most effective biotic and abiotic factors affecting fatty acid composition of *Garra rufa* (Heckel, 1843)? *Grasas Aceites* **74** (3), e518. https://doi.org/10.3989/gya.0224221

1. INTRODUCTION

Garra rufa (Heckel, 1843), one of the smallest members of the Cyprinidae family belongs to the genus *Garra,* which includes about 73 species (Coad, 2010). It is used in ichthyotherapy as an alternative treatment for healing some skin diseases such as psoriasis and eczema. It is therefore called “doctor fish” (Yedier *et al.,* 2016).

Lipids are among the most important energy sources for animals and the fatty acids (FAs) in their structure form the building blocks of cell membranes (Iverson, 2009). Furthermore, they provide the organism with essential fatty acids (EFAs), a key nutrient for proper develeopment (Parrish, 2009). The natural diets of many fish species contain large amounts of long-chain polyunsaturated fatty acids (LC-PUFAs). Unlike terrestrial animals, the lipid composition of aquatic organisms contains high levels of PUFAs, predominantly omega 3 (ω3) FAs (Parrish, 2013). In addition, ω3 FAs play an essential role in the normal development of the embryos and larvae of freshwater fish and in the regular functioning of nervous systems and sensory organs. These processes occur in different ways in different species or subspecies and even in male and female individuals of the same species (Kaushik *et al*., 2006). In addition, the fatty acid composition of fish species varies according to the geographic location, diet, feeding, gender and reproductive cycles. Seasonal variations may also be effective in changing the FAs composition of fish (Kaçar and Başhan, 2015).

Aquatic organisms are dependent on the availability of nutrients, and conducting research on the essential nutrients of these organisms has become important in ecology. Traditionally, understanding the food web is derived from detailed analysis of stomach contents. However, since stomach content analysis only provides a snapshot of an animal’s diet, large numbers of samples are required to be analyzed, meaning that sampling can be logistically restrictive or unsustainable. Conversely, the biochemical composition of muscle tissue is the result of long-term feeding histories. Thus, techniques such as stable isotope and fatty acid analysis are increasingly used to reveal complex ecological information (Dalsgaard *et al*., 2003). Stable isotopes also provide a measure of trophic position but can be confounded by differences at the bottom of the food chain. However, fatty acid composition can help identify many unique synthesized structures (Revill *et al*., 2009). Therefore, this study aimed to determine and compare the FAs composition of *G. rufa* in relation to biotic and abiotic factors such as season, gender and different stations of the same region, and to reveal the dietary preferences of *G. rufa* in different periods using dietary marker fatty acids.

2. MATERIALS AND METHODS

2.1. Sampling area and samplings

Samples were taken monthly from two locations, namely Garip and Ilıcalar, on the Garip Stream of the Murat River in the Bingöl Province (Figure 1). The Ilıcalar location (36°59’01.5” N, 40°40”58.9” E) has temperatures above seasonal averages; whereas the Garip location (30°47’10.7”N, 40°32’58.7”E) has colder waters. Water samplings were taken from the same location at both stations between March 2017 and February 2018. Nets with different mesh sizes (12×12 mm, 16×16 mm, 22×22 mm, 32×32 mm) were used for catching the fish. Water samples were taken to determine the level of chlorophyll-*a* (Chl-a). Water temperatures were measured *in situ* at both stations.

2.2. Laboratory studies

The fish samples collected were brought to the laboratory, and at least 3-5 samples for each season during the sampling period were examined. A total of 25 individuals from Garip and 39 individuals from Ilıcalar locations were taken during the sampling period (March 2017-February 2018). Sexually mature fish were used in the analyses. Gender was also determined in the fish samples used for the total lipid and fatty acid analyses. The gender of the fish samples was determined macroscopically from the gonads of the fish samples. The samples to be used in the biochemical analysis were obtained from the edible muscle tissues. Each fish muscle from the non-posterior part was cut into uniform pieces (2.0 × 2 × 1 cm; ~1-2 g) using a scalpel. All the fish muscle samples were stored in a freezer at -80 °C until analysis.

2.2.1. Determination of chlorophyll-a

Chlorophyll-a (chl-a) measurements were made according to the spectrophotometric method (Parsons *et al*., 1984). For the determination of chl-a content, 1

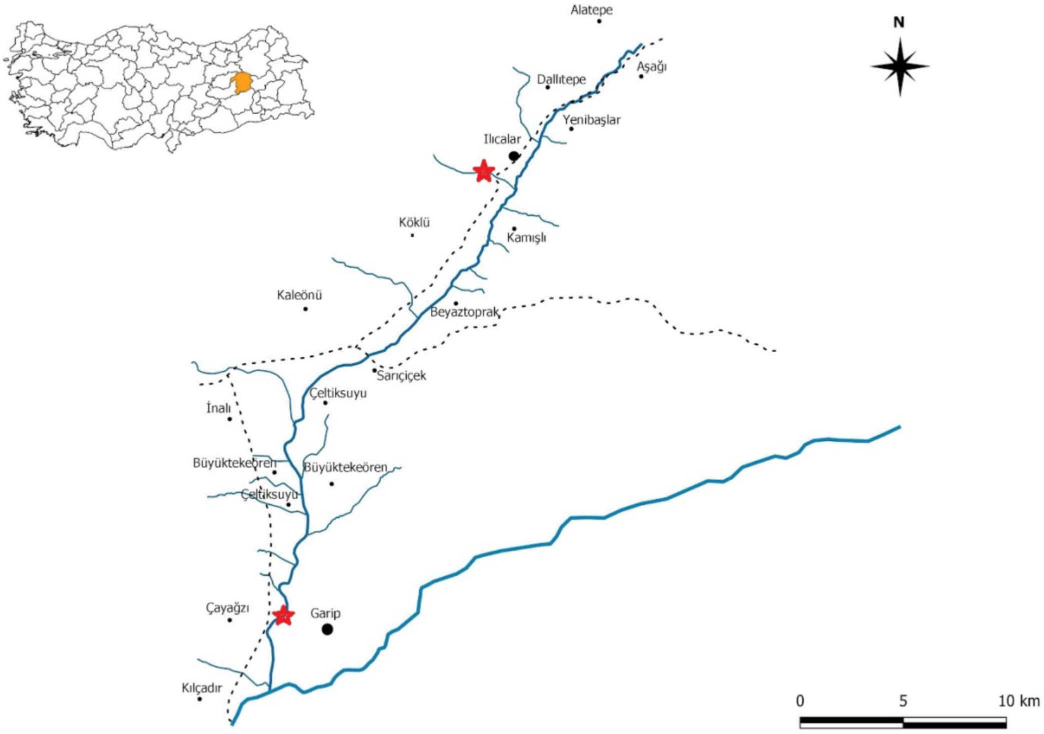


Figure 1. Sampling Area

liter of water was sampled monthly from the specified stations. The sample was then filtered through GFC filters with a pore size of 1 µm. The filter papers were folded and placed in 15 mL centrifuge tubes and 10 mL 90% acetone solution were added to the centrifuge tubes. They were kept in the refrigerator at 4 °C for 24 hours. Then the samples were brought to room temperature and their absorbances were determined at 750, 664, 647, and 630 nm wavelengths by means of a spectrophotometer (SHIMADZU UV 2100).

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 of wet weight (WW). The wet weight of each sample was about 1-2 g. Hexane/isopropanol (3/2) was used for lipid extraction as suggested by Hara and Radin (1978). For fatty acid transmethylation, 20 g methanolic sulfuric acid were mixed with 1 liter of distilled water to prepare a 2% methanolic sulfuric acid solution. Five mL of this solution were added to a test tube and completely mixed by vortex. The mixture was left to methylate in an oven at 55 °C for 15 hours. At the end of this period, 5 mL of 5% NaCl were added to it and mixed thoroughly. Afterwards, 5 mL of hexane were added to the fatty acid methyl esters formed in the tubes, and the tubes were mixed (Christie, 1992). After waiting three hours at room temperature, the hexane phase formed was taken from the top, 5 mL of 2% $KHCO_3$ solution were added to the tubes and the sample was dried in a nitrogen evaporator (Allsheng WD-12). A weight measurement was taken on a precision scale to determine the dry lipid content after the evaporation process, and the average total lipid content (%) per individual was calculated. Then, the dry lipid layer was hydrated with the addition of 1 mL hexane, and then vortexed. The samples were transferred to 2 mL capped autosampler vials and analyzed in a gas chromatograph/mass spectrometer (GC/MS) system.

2.2.3. GS/MS analysis

The fatty acids were analyzed with a GC/MS system (Agilent 5975 C). A Macherey-Nagel (Germany) capillary column (100 m x 0.25 mm, 0.25 µm) was used for the analysis. The column temperature was kept at 120-220 °C; whereas the injection temperature was 240

°C and the detector temperature was 280 °C throughout the analysis. The column temperature program was set from 120 to 220 °C. The temperature ramp was set at 5 °C / min up to 200 °C and at 4 °C / min from 200 to 220 °C. It was held at 220 °C for 8 min and the total time was 52 min. Helium (0.5 ml/min) was used as carrier gas. The fatty acid methyl esters (FAMEs) of the samples were identified initially based on the retention time of each fatty acid by using the analytical standard of FAMEs (Supelco Component FAME Mix). After analysis, wsearch32 mass spectrometry software (Wsearch 2008; version 1.6 2005, Sidney, Australia) was used to confirm the peak identities of each fatty acid.

2.3. Statistical analysis

Multivariate statistics were used to analyze the differences in total lipid contents and total fatty acid compositions in PRIMER-e 2017. The Bray Curtis similarity coefficient was employed for PERMANOVA and principal coordinates (PCO) and cluster analysis for similarity ranges. In the analyses, the fatty acid data of *G. rufa* were factored by month, season, gender and location (stations). The fatty acids which showed the greatest difference in all samples were investigated in the factor groups. A similarity percentage (SIMPER) analysis (cut-off for low contributions: 70%) was used to identify the fatty acids which contributed the most to the similarities between/within the factor groups. The analysis of similarity (ANOSIM) was performed on the distance matrix using multiple permutations within a significant fixed effect. The ANOSIM-R value indicated the extent to which the groups differed ($R > 0.75$: well-separated groups, highly different; R=0.50–0.75: separated but overlapping groups, different; R=0.25–0.50: separated but strongly overlapping groups; $R<0.25$: barely separated groups, similar with some differences) (Pethybridge *et al.*, 2010).

ANOVA test was performed to determine significant ($p < 0.05$) main effects of the factors (station, season, month) and their interactions on FA compositions and total lipid content. Variations among groups were determined by TUKEY HSD test using STATISTICA software (STATISTICA Six Sigma, version 7).

3. RESULTS AND DISCUSSION

3.1. Total lipid content

The values for seasonal and monthly variation in the total lipid amount of *G. rufa* during the sampling period for Garip and Ilıcalar are given in Table 1. The average total lipid content per individual was determined for proportional values (%).

The PERMANOVA results obtained were used to identify similarities in total lipid content, within and among seasons, genders and stations. In the PERMANOVA analysis, the total lipid data were factored by season and gender at the stations. Also, the data were factored by the stations during the sampling period.

According to the ANOVA results, seasonal differences were the most significant between autumn-summer at the Garip station and between autumn-summer and autumn-spring at the Ilıcalar station ($p < 0.05$) (Table 1). In addition, there were no significant differences between gender groups in terms of total lipid content at the Ilıcalar station ($p < 0.05$). Similarly, the ANOSIM-R results showed that there was no separation between gender groups (ANOSIM-R= -0.003) at the Ilıcalar station. However, despite the fact that the seasonal groups were separated, there was strong overlapping between groups (ANOSIM-R=0.49) at the Ilıcalar station. Season and gender groups were barely separated (ANOSIM-R=0.23; ANOSIM-R=0.10, respectively) at the Garip station. Therefore, seasonal difference in the total lipid content was more significant at the Ilıcalar station than at the Garip station.

The lipid content of *G. rufa* varied between 1-6% at the Ilıcalar station and 0.89-5% at the Garip station. The highest value was detected in autumn (5.26, 4.80%, respectively). Olgunoglu *et al.* (2014) determined the highest energy value for *Silurus triostegus* to be in the winter months when the lipid content was at its highest. Considering that the energy levels are related to the lipid content, it can be said that the energy value reached its highest level in the autumn months for *G. rufa*.

It is well known that fish lipids are generally affected by many factors such as age, seasonal change, nutrition, gender, reproductive cycle and geographical location (Všetičková *et al.*, 2020). In the present study, adult fish specimens were used during the sampling. Therefore, it is thought that the change in total lipid content was more affected by the temperature changes in both locations. The temperature values throughout the year in the Ilıcalar station (8-26 °C) are always higher than at the Garip station (0.5-21°C) (Table 4). Bauer and Schlott (2009)

Table 1. Average total lipid amount (%) of *G. rufa* at Garip and Ilıcalar stations during the sampling period

	SPRING (n=6)			SUMMER (n=9)			AUTUMN (n=8)			WINTER (n=2)		
	March	April (n=3)	May (n=3)	June (n=3)	July (n=3)	August (n=3)	September (n=3)	October (n=2)	November (n=3)	December	January** (n=1)	February** (n=1)
GARIP (n=25)		2.74 ± 1.81^{ab}			1.56 ± 1.35^{b}			4.80 ± 1.47^{a}			1.92 ± 0.03^{ab}	
	-	4.17 ± 1.40^{abc}	1.30 ± 0.25^{bcd}	0.56 ± 0.36^{d}	3.22 ± 0.85^{abcd}	0.89 ± 0.44^{cd}	4.35 ± 2.33^{abc}	4.80 ± 0.92^{ab}	5.24 ± 1.05^{a}	-	1.90	1.94
	SPRING (n=13)			SUMMER (n=9)			AUTUMN (n=10)			WINTER (n=7)		
	March (n=5)	April (n=5)	May (n=3)	June (n=3)	July (n=3)	August (n=3)	September (n=3)	October (n=4)	November (n=3)	December (n=4)	January (n=3)	February
ILICALAR (n=39)		2.93 ± 1.89^{b}			3.03 ± 2.12^{b}			5.26 ± 1.35^{a}			3.64 ± 0.86^{ab}	
	2.48±1.83	4.25±2.18	1.47±0.71	1.06±0.31	3.06±1.78	4.97±2.63	6.31±0.71	5.04±1.34	4.49±1.52	3.47±0.42	3.87±1.35	-

Means followed by different letters and letter groups in the same row for months and seasons are significantly different ($p < 0.05$, n=2-5 for months; n=2-13 for seasons) values are means±SD. Means followed by different letters (a, b, c) and letter groups (ab, abc, cd, bd, bd) in the same row for months and seasons are significantly different (ANOVA-TUKEY HSD Test, $p < 0.05$, n=2-3 for months; n=2-9 for seasons) values are means±SD. **: n=1 (Tukey test was not used in the differences of January and February for Garip Station during the sampling period)

found that the average lipid content ranged from 2.7 to 6.9% in fish from three carp farms. However, the authors emphasized that the lipid content in the diet also affected the lipid content in the fish. Similarly, Varga *et al.* (2013) studied carp from different cultures in different parts of Hungary with the same diet, and found that environmental factors significantly (P=0.001) affected their lipid contents.

In a study conducted by Akpinar (1999), in which the fatty acid changes in the lipids of *Cyprinion macrostomus* fed and starved at two different temperatures (24 and 35 °C) in Kangal Fish Hot Spring (Sivas) were investigated, it was determined that food intake was better in fish fed and starved at 24 °C. Some fatty acids which were not in the food were detected in their lipids, and 24 °C was the threshold temperature for this. Also, Akpinar (1999) indicated that high temperature is significantly effective in nutrition and that the food consumed could be used at a minimum level for *Cyprinion macrostomus* in hot spring waters. It is understood that if the temperature is lowered (24 °C), the food is utilized better by the fish and their lipid metabolism becomes accelerated. The Ilıcalar station is a location where water transport is provided to the hot spring area located in the region. However, considering the total lipid values, *G. rufa* had a higher average total lipid content in the fish caught at the Ilıcalar station (4%) than at the Garip station (3%), which has colder waters. However, in the present study, the temperature was below this value (24 °C) at both stations throughout the year. It is thought that the high lipid content of the fish from the Ilıcalar station may have been due to the nutrient content in this location.

When the total lipid content of *G. rufa* was factored by season and gender, regardless of the station during the sampling period, it was observed that the difference was significant only for season (ANOSIM-R=0.26). The pair-wise test results of PERMANOVA revealed that they were significant in terms of lipid content between summer and autumn, and spring and autumn (P_{perm}=0.001). The differences between male and female fish were not significant (P_{perm}=0.12).

Lipids are transported from the muscles to the gonads for the development of the gonads in the reproduction period. The total lipid contents in the muscle are remarkably affected by season, especially during the reproduction period (Všetičková *et al.*, 2020).

The spawning period of *G. rufa* females peaks in the middle of spring and decreases slowly from the end of May to November. On the other hand, it peaks in April in *G. rufa* males. This is due to the increase in gonad weight, which indicates that the breeding season declines after April (Abedi *et al*., 2011). The lipid content in the muscles of females decreases to a minimum in the spawning period. However, in males, the lowest lipid content is in the post-spawning period (Všetičková *et al*., 2020). The seasonal ANOSIM-R value was found to be 0.26 for both genders, which means that there was a slight change in the fatty acid composition in male and female individuals. However, there was a significant difference between spring and autumn in females (P_{perm}= 0.001) and between autumn and winter in males (P_{perm}= 0.004, respectively). Thus, the most effective factor on the change in lipid content of *G. rufa* is seasonal changes.

3.2. Fatty acid composition

First, the effects of season and gender on the change in the fatty acid composition of *G. rufa* were investigated and second, their dietary fatty acid composition was determined for different locations (Garip and Ilıcalar stations) and regardless of these locations. Different statistical analysis methods were used, such as ANOSIM-R, P_{perm}, SIMPER, PCO and Tukey tests.

3.2.1. Factors influencing the fatty acid composition of G. rufa according to different locations

The effect of location difference was observed to be significant, albeit only slightly, in the change in the fatty acid composition of *G. rufa* (ANOSIM-R=0.27). The season factor was partially significant in the fatty acid composition of the fish at both stations. However, seasonal variation was more prominent at the Ilıcalar station (ANOSIM-R=0.21) than at the Garip station (ANOSIM-R=0.07). The ANOVA test (TUKEY HSD) results showed that monthly and seasonal changes were significant for total polyunsaturated fatty acids (∑PUFA), 18:0, 22:6ω3 (docosahexaenoic acid, DHA), 20:1ω9 and 20:4ω6 (arachidonic Acid, ARA) at the Ilıcalar station (Tables 2 and 3) and total monounsaturated fatty acids (∑MUFA), 14:0, 20:5ω3 (eicosapentaenoic acid, EPA), 18:1ω9 and 18:0 at the Garip station (Tables 2 and 3). The pair-wise test results, PERMANOVA, revealed that the difference in FA composition was significant between spring and winter at the Ilıcalar station, and between spring and autumn in the Garip station (P_{perm}=0.002). The SIMPER results revealed that the average highest similarity was in winter at the Garip and Ilıcalar stations with close values (88, 89%, respectively). The gender difference in fatty acid composition was not significant at the stations because gender formed barely-separated groups.

Figure 2 shows a two-dimensional configuration plot of the PCO analysis of a resemblance matrix of fatty acids in *G. rufa* collected from different locations (Garip and Ilıcalar). The fish samples from the Ilıcalar station were characterized by 18:1ω9, EPA, DHA and 18:0 fatty acids; whereas those from the Garip station were characterized by 18:1ω9 and 22:1ω9. The stations formed separate but overlapping groups for fatty acid composition (ANOSIM-R=0.27), and differences between the stations were not significant.

The major SFA in all factor groups were 16:0 and 18:0 (Table 2). Similarly, these two fatty acids were reported by Guler *et al.* (2008) as the major fatty acids in *Cyprinus carpio*, ranging from 14.6 to 16.6% in all seasons. Misir *et al.* (2013) reported that 16:0 was the prominent SFA contributing to approximately 60% of ∑SFA, followed by 18:0 for *Chalcalburnus tarichi*. 18:1ω9 was the main MUFA in all muscle tissues of nine freshwater fish species collected from the Tigris River (Turkey). In addition, 18:1ω9 was recorded as the predominant fatty acid in *Cyrinus carpio* for all seasons (15.1–20.3%) (Guler *et al.,* 2008). The SIMPER results also showed that the FAs which contributed the most to the similarity between the Garip and Ilıcalar stations were 18:1ω9 (28%), 16:0 (22%) and DHA (9%), respectively. It was determined that 18:1ω9 was the most significant contributor to FAs for all seasons and genders at both stations (Table 3).

Oleic acid, 18:1ω9 is the subrate for two important desaturases, Δ12 and Δ15, which are only available from primary producers. These enzymes enable the conversion of 18:1ω9 to 18:2ω6 (linoleic acid, LNA) and 18:3ω3 (linolenic acid, LA). Animals obtain these two essential fatty acids from their diet rather than replacing other fatty acids (Dalsgaard *et al*., 2003). It is known that *G. rufa* prefers phy-

Table 2. Fatty acid composition of *G. rufa* at The Garip station during the sampling period (% Total FAME)

Fatty Acids	April (n=3)	May (n=3)	**SPRING (n=6)**	June (n=3)	July (n=3)	August (n=3)	**SUMMER (n=9)**	September (n=3)	October (n=2)	November (n=3)	**AUTUMN (n=8)**	January (n=1)**	February (n=1)**	**WINTER (n=2)**
14:0	1.33±0.54^{a}	1.10±0.17^{a}	**1.21±0.38^{b}**	0.93±0.31^{a}	1.29±0.17^{a}	2.28±0.72^{ab}	**1.50±0.42^{b}**	1.96±0.41^{ab}	2.30±0.74^{ab}	3.40±0.87^{b}	**2.57±0.91^{a}**	1.66	1.18	**1.42±0.34^{ab}**
16:0	16.84±1.27	18.57±1.28	**17.71±1.48**	20.42±1.40	19.26±2.65	20.93±1.56	**20.20±1.84**	13.80±12.07	18.18±0.04	21.54±2.41	**17.80±7.50**	19.70	19.03	**19.37±0.48**
i16:0	0.35±0.09	0.48±0.05	**0.43±0.14**	0.40±0.39	0.28±0.08	0.59±0.36	**0.42±0.34**	0.30±0.12	0.18±0.06	0.22±0.04	**0.24±0.15**	0.25	0.49	**0.37±0.21**
18:0	3.78±0.84^{bc}	5.08±0.44^{ab}	**4.43±0.93^{ab}**	6.58±1.08^{a}	4.05±0.38^{bc}	3.74±0.73^{bc}	**4.79±1.51^{a}**	3.61±0.42^{bc}	2.95±0.18^{bc}	2.92±0.41^{c}	**3.19±0.48^{b}**	3.81	3.32	**3.57±0.35^{ab}**
i17:0	0.81±0.17	0.61±0.53	**0.71±0.37**	0.82±0.12	0.75±0.42	0.65±0.56	**0.74±0.30**	1.00±0.19	0.87±0.19	1.04±0.19	**0.98±0.18**	0.88	0.59	**0.74±0.20**
17:0	1.03±0.22	1.00±0.11	**1.02±0.16**	1.06±0.22	0.79±0.08	0.65±0.56	**0.83±0.35**	1.22±0.43	0.88±0.19	1.10±0.21	**1.09±0.30**	0.97	1.07	**1.02±0.07**
∑SFA	24.69±1.71	27.52±2.21	**26.11±2.29**	31.75±1.18	26.94±2.42	30.35±0.85	**29.66±2.40**	22.76±10.94	26.14±1.16	31.18±2.00	**26.70±7.19**	27.69	26.67	**27.18±0.78**
∑FA*	0.55±0.14	0.68±0.09	**0.60±0.12**	1.54±0.57	0.52±0.22	1.51±0.66	**1.18±0.56**	0.87±0.10	0.78±0.20	0.96±0.25	**0.63±0.20**	0.42	0.99	**0.69±0.29**
16:1ω11	1.41±0.65	1.34±0.10	**1.38±0.42^{ab}**	1.25±0.41	0.79±0.56	1.17±0.68	**1.07±0.53^{b}**	2.16±0.86	1.24±0.57	2.02±0.27	**1.88±0.66^{a}**	0.14	1.70	**0.92±1.11^{ab}**
16:1ω9	5.56±1.35^{ab}	5.55±0.89^{ab}	**5.56±1.02^{ab}**	4.18±1.25^{b}	4.77±0.39^{b}	6.98±1.75^{ab}	**5.31±1.68^{b}**	6.86±1.50^{ab}	6.32±1.99^{ab}	9.55±2.15^{a}	**7.74±2.20^{a}**	9.01	5.76	**7.39±2.30^{ab}**
16:1ω7	0.37±0.06	0.44±0.05	**0.40±0.06**	0.29±0.19	0.52±0.45	0.67±0.70	**0.49±0.45**	0.22±0.20	0.41±0.01	0.52±0.19	**0.38±0.26**	-	1.01	**0.50±0.71**
17:1	0.48±0.38	0.39±0.27	**0.43±0.31**	-	0.92±0.08	0.71±0.64	**0.54±0.53**	0.93±0.36	-	1.49±0.47	**0.91±0.69**	1.02	-	**0.51±0.72**
18:1ω11	0.55±0.22	1.20±1.79	**1.00±0.96**	0.89±0.23	0.44±0.38	0.64±0.52	**0.66±0.40**	0.28±0.25	0.18±0.01	0.19±0.17	**0.22±0.17**	0.68	0.37	**0.52±0.22**
18:1ω9	32.71±2.51^{a}	26.25±6.91^{ab}	**29.48±5.84**	16.29±1.81^{b}	37.51±4.21^{a}	28.67±5.14^{ab}	**27.49±9.86**	33.44±6.65^{a}	35.40±2.35^{a}	32.46±7.04^{a}	**33.57±5.39**	29.80	26.05	**27.93±2.65**
18:1ω7	0.91±0.77	0.41±0.11	**0.66±0.56**	2.34±2.05	0.39±0.15	0.56±0.46	**1.10±1.41**	1.34±1.27	0.41±0.01	0.26±0.23	**0.70±1.28**	-	-	**-**
20:1ω9	0.66±0.39	1.27±0.19	**0.26±0.27^{b}**	-	-	-	**-**	2.15±1.99	2.34±0.18	1.61±0.31	**2.00±1.13^{a}**	-	-	**-**
∑MUFA	43.50±1.43^{a}	36.89±4.89^{ab}	40.22±4.98^{ab}	25.50±2.15^{b}	46.00±4.14^{a}	39.91±4.85^{ab}	37.15±9.69^{b}	48.32±10.51^{a}	46.82±0.62^{a}	48.47±7.81^{a}	47.87±7.04^{a}	41.33	**35.72**	**38.28±3.89^{ab}**
MUFA*	0.85±0.52	0.04±0.02	**1.05±0.64**	0.26±0.24	0.66±0.18	0.51±0.05	**0.47±0.18**	0.94±0.14	0.51±0.14	0.37±0.19	**0.47±0.26**	0.68	0.83	**0.51±0.21**
16:2ω4	1.10±0.91	0.96±0.07	**1.03±0.76**	0.78±0.05	0.50±0.36	1.81±1.03	**0.96±0.85**	1.14±0.71	1.16±0.15	0.54±0.48	**0.92±0.57**	0.81	0.43	**0.62±0.27**
18:2ω6	2.18±0.26	2.32±0.22	**2.25±0.23**	2.11±0.07	1.79±0.03	1.61±1.41	**1.84±0.74**	2.35±1.25	2.54±0.38	1.65±0.62	**2.14±1.13**	1.97	1.87	**1.92±0.07**
18:3ω6	0.77±0.24	0.11±0.09	**0.44±0.17**	0.91±0.58	-	0.71±0.65	**0.54±0.43**	0.81±0.63	0.80±0.02	0.39±0.01	**0.65±0.40**	0.17	0.77	**0.47±0.42**
18:3ω4	0.68±0.28	0.24±0.14	**0.46±0.21**	0.41±0.04	0.41±0.26	0.43±0.33	**0.42±0.21**	0.41±0.01	0.19±0.10	0.73±0.33	**0.47±0.29**	0.38	0.95	**0.67±0.40**
18:3ω3	8.16±0.19	7.76±1.46	**7.96±0.96**	6.32±0.10	8.88±0.65	10.86±2.73	**8.69±2.42**	9.77±1.55	9.06±0.62	5.05±4.65	**7.82±3.50**	7.42	7.26	**7.34±0.11**
20:3ω6	0.54±0.11	0.51±0.14	**0.52±0.11**	1.00±0.61	0.56±0.40	0.40±0.30	**0.66±0.57**	0.41±0.36	0.52±0.18	0.53±0.18	**0.48±0.24**	0.59	0.21	**0.40±0.27**
20:4ω6	1.79±0.31^{bc}	2.83±0.25^{ab}	**2.31±0.62^{ab}**	4.09±0.37^{a}	1.61±0.17^{bc}	1.78±0.39^{bc}	**2.49±1.23^{a}**	1.46±1.05^{bc}	0.87±0.08^{c}	1.04±0.37^{c}	**1.15±0.65^{b}**	2.03	2.70	**2.36±0.47^{ab}**
20:3ω3	0.94±0.05	1.04±0.21	**0.99±0.15**	1.05±0.29	1.17±0.05	1.05±1.01	**1.09±0.53**	0.44±0.38	1.35±0.13	0.90±0.53	**0.84±0.54**	0.78	0.88	**0.83±0.07**
20:4ω3	0.50±0.17	0.35±0.31	**0.43±0.24**	0.43±0.38	0.52±0.46	0.68±0.61	**0.54±0.44**	0.84±0.63	0.63±0.15	0.37±0.25	**0.61±0.53**	0.39	1.03	**0.71±0.45**
20:5ω3	5.74±1.21^{ab}	8.17±2.36^{ab}	**6.96±2.14^{ab}**	10.33±0.22^{b}	4.62±0.14^{a}	5.17±2.30^{a}	**6.71±2.96^{a}**	3.93±1.94^{a}	3.06±0.95^{a}	3.45±1.72^{a}	**3.53±1.48^{b}**	5.61	7.21	**6.41±1.13^{ab}**
21:5ω3	0.64±0.22	0.40±0.35	**0.52±0.29**	0.82±0.37	0.27±0.03	0.38±0.27	**0.49±0.37**	0.30±0.11	0.61±0.44	0.35±0.14	**0.39±0.24**	0.26	0.83	**0.55±0.41**
22:5ω3	0.93±0.19	1.02±0.13	**0.98±0.15**	1.01±0.32	0.56±0.52	0.52±0.46	**0.69±0.45**	0.82±0.32	1.06±0.02	0.55±0.49	**0.78±0.38**	1.15	1.07	**1.11±0.05**
22:6ω3	6.59±1.11^{bc}	8.93±2.00^{ab}	**7.76±1.93**	12.55±0.55^{a}	5.10±0.47^{c}	3.69±0.94^{c}	**7.11±4.17**	4.67±1.77^{c}	3.83±0.75^{c}	4.12±1.31^{c}	**4.25±1.26**	7.84	11.72	**9.78±2.75**
∑PUFA	**31.80±3.14^{abc}**	**35.59±2.98^{ab}**	**33.67±3.43**	**43.13±0.72^{a}**	**26.98±1.54^{bc}**	**29.74±5.44^{abc}**	**33.28±7.83**	**28.92±7.18^{abc}**	**27.04±0.55^{abc}**	**20.35±8.64^{c}**	**25.43±7.15**	**30.98**	**37.61**	**34.52±4.52**
PUFA*	1.23±0.24	0.95±0.50	**1.06±0.51**	1.32±0.64	1.00±0.51	**0.65±0.59**	**1.05±0.86**	1.57±1.12	1.36±0.45	0.68±0.59	**2.03±0.67**	1.58	0.68	**1.37±0.91**
Bacterial	3.50±0.91	3.05±1.21	**3.29±0.99**	3.60±0.28	3.30±0.23	3.87±0.77	**3.59±0.55**	4.57±0.94	2.65±0.58	4.91±1.12	**4.22±1.30**	3.42	3.14	**3.28±0.19**
DHA/EPA	1.16±0.08	1.10±0.06	**1.13±0.07**	1.21±0.03	1.11±0.14	0.77±0.24	**1.03±0.24**	1.26±0.23	1.27±0.15	1.30±0.33	**1.28±0.22**	1.40	1.63	**1.51±0.16**
Zooplankton	0.66±0.49	0.32±0.25	**0.49±0.69**	-	-	-	**-**	2.15±1.99	2.34±0.18	1.61±0.31	**2.00±1.13**	-	-	**-**
Terrestrial	10.34±0.07	10.08±1.68	**10.21±1.07**	8.43±0.13	10.67±0.68	12.47±1.51	**10.53±1.94**	12.12±2.68	11.61±1.00	6.70±5.05	**9.96±4.10**	9.40	9.14	**9.27±0.18**
∑ω3/∑ω6	4.12±0.58	4.57±0.76	**4.35±0.65**	3.92±0.42	5.20±1.16	6.60±3.80	**5.24±2.67**	3.72±0.33	3.93±0.46	3.99±2.36	**3.87±1.29**	4.66	5.42	**5.04±0.53**
16:1ω7/16:0	0.02±0.00	0.02±0.00	**0.02±0.00**	0.01±0.01	0.03±0.02	-	**0.02±0.03**	0.02±0.00	0.02±0	0.03±.0.02	**0.01±0.01**	-	0.05	**0.03±0.04**

Means followed by different letters (a, b, c) and letter groups (ab, abc, bc) in the same row for months and seasons are significantly different (ANOVA-TUKEY HSD Test, $p < 0.05$, n=2-3 for months; n=2-9 for seasons) values are means±SD. **: n=1 (Tukey test was not used in the differences of these months during the sampling period). *: Minor FAs with mean proportion ≤ 0.5 in all the sampling periods, Inluded in this fraction were FAs: ι15:0, 15:0, ι17:0, 19:0, 20:0 from SFA, 14:1, 15:1, 18:1ω6, 18:1ω5, 22:1ω5, 22:1 9, 24:1 from MUFA, 18:2, 18:4ω3, 20:2ω6, 20:2 from PUFA ι: indicates iso-branched FAs.

TABLE 3. Fatty acid composition of *G. rufa* at the Ilıcalar station during the sampling period (% Total FAME)

Fatty Acids	March (n=5)	April (n=5)	May (n=3)	SPRING (n=13)	June (n=3)	July (n=3)	August (n=3)	SUMMER (n=9)	September (n=3)	October (n=4)	November (n=3)	AUTUMN (n=10)	December (n=4)	January (n=3)	WINTER (n=7)
14:0	2.73±1.96	2.43±1.72	1.09±0.37	**2.23±1.65**	0.83±0.43	2.03±0.64	3.88±3.51	**2.25±2.36**	3.33±0.65	2.46±0.62	4.86±3.50	**3.43±2.03**	2.89±0.41	1.27±0.23	**2.20±0.92**
i16:0	0.31±0.14	0.34±0.10	0.37±0.33	**0.34±0.19^{b}**	0.67±0.16	0.76±0.63	0.67±0.82	**0.70±0.56^{a}**	0.21±0.08	0.16±0.05	0.23±0.08	**0.20±0.07^{b}**	0.16±0.02	0.20±0.08	**0.18±0.06^{b}**
16:0	18.42±3.10	18.39±0.70	17.25±1.39	**18.14±1.99**	18.67±0.84	23.54±3.40	20.16±3.70	**20.79±3.54**	21.85±2.86	18.96±1.43	19.43±3.61	**19.97±2.67**	19.36±2.25	21.33±0.82	**20.20±1.97**
i17:0	0.41±0.38	0.85±0.21	0.87±0.08	**0.69±0.44**	0.83±0.04	0.51±0.37	0.54±0.41	**0.63±0.32**	0.33±0.26	0.79±0.10	1.20±0.50	**0.78±0.46**	0.76±0.06	0.67±0.13	**0.72±0.10**
17:0	1.23±0.48	1.46±0.65	0.92±0.06	**1.25±0.51**	1.07±0.09	0.91±0.38	1.02±0.30	**1.00±0.27**	0.57±0.53	0.84±0.21	1.47±0.74	**0.95±0.58**	0.61±0.44	1.08±0.25	**0.81±0.42**
18:0	3.86±0.94 ^{ab}	4.81±1.59 ^{ab}	4.43±0.69 ^{ab}	**4.36±1.19^{ab}**	6.10±1.25^{a}	5.47±3.04 ^{ab}	2.64±0.37 ^{ab}	**4.74±2.46^{a}**	3.21±0.75 ^{ab}	2.66±0.72^{b}	3.11±0.52^{ab}	**2.96±0.65^{b}**	3.67±0.88^{ab}	4.08±1.02^{ab}	**3.85±0.88^{ab}**
∑FA*	**0.67±0.19**	**0.92±0.10**	**0.86±0.27**	**0.53±0.20**	**0.56±0.40**	**0.64±0.40**	**0.72±0.53**	**0.64±0.39**	**0.81±0.07**	**0.82±0.09**	**1.26±0.59**	**1.23±0.85**	**0.71±0.19**	**0.92±0.72**	**0.90±0.48**
∑SFA	**27.63±5.10**	**29.20±2.85**	**25.79±1.76**	**27.54±3.71**	**28.74±0.30**	**33.,86±0.96**	**29.64±3.70**	**30.74±3.15**	**30.31±3.31**	**26.70±1.81**	**31.56±9.32**	**29.52±5.21**	**28.16±2.01**	**29.55±0.79**	**28.86±1.67**
16:1ω11	1.02±0.78	0.78±0.62	0.90±0.53	**0.90±0.63**	0.76±0.39	0.74±0.53	0.98±0.71	**0.83±0.49**	0.90±0.21	0.93±0.88	1.86±0.60	**1.20±0.93**	1.44±0.78	0.84±0.56	**1.19±0.71**
16:1ω9	7.77±2.41	5.81±2.17	6.14±1.97	**6.64±2.24**	4.83±2.91	7.76±3.36	8.18±1.88	**6.92±3.04**	9.79±1.25	8.38±1.43	7.73±1.22	**8.61±1.45**	7.64±2.05	5.22±0.41	**6.60±1.96**
16:1ω7	1.06±0.79	0.65±0.57	0.29±0.28	**0.72±0.65**	0.25±0.22	0.34±0.32	0.79±0.34	**0.46±0.39**	1.68±0.79	1.38±1.01	1.54±0.60	**1.52±1.41**	1.53±0.77	0.14±0.11	**0.93±0.92**
17:1	1.60±0.37^{a}	1.21±0.90^{ab}	-	**1.08±0.85**	-	0.36±0.31^{ab}	0.87±0.02 ^{ab}	**0.41±0.41**	0.71±0.15 ^{ab}	0.94±0.12 ^{ab}	1.56±0.98 ^{ab}	**1.06±0.59**	0.92±0.42^{a}	0.51±0.42^{ab}	**0.74±0.45**
18:1ω11	0.22±0.20	1.26±1.04	0.78±0.09	**0.75±0.78**	0.98±0.10	0.65±0.53	0.37±0.18	**0.66±0.41**	0.29±0.19	0.30±0.14	0.19±0.10	**0.26±0.16**	0.55±0.53	0.41±0.10	**0.49±0.39**
18:1ω9	26.35±6.45	19.79±11.11	27.87±8.33	**24.18±8.94**	23.54±4.40	26.22±11.8	34.13±8.73	**27.96±9.54**	30.59±4.17	34.32±4.10	23.17±15.43	**29.86±9.31**	35.25±1.73	32.63±7.35	**34.13±4.63**
18:1ω7	0.44±0.36	1.31±1.59	1.52±1.62	**1.02±1.28**	2.06±1.37	2.21±1.64	0.67±0.20	**1.65±1.31**	1.11±0.43	0.95±0.58	0.21±0.16	**0.78±0.58**	1.31±0.44	2.22±1.28	**1.70±0.94**
20:1ω9	2.54±1.19^{a}	1.91±1.10^{ab}	-	**1.71±1.36**	-	-	-	-	1.50±1.31^{ab}	0.30±0.21^{b}	2.48±0.57^{a}	**1.31±1.18**	2.39±0.37^{a}	1.94±0.19^{ab}	**2.20±0.37**
MUFA*	**0.54±0.22^{b}**	**0.84±0.69^{b}**	**0.15±0.14^{b}**	**0.57±0.51**	**0.60±0.36^{b}**	**1.19±1.17^{ab}**	**0.13±0.08^{b}**	**0.63±0.98**	**0.55±0.23^{b}**	**0.91±0.79^{b}**	**3.60±2.62^{a}**	**1.36±0.92**	**0.70±0.55^{b}**	**1.54±1.24^{ab}**	**0.61±0.57**
∑ MUFA	**41.54±7.89**	**33.56±11.32**	**37.65±6.08**	**37.58±9.10^{a}**	**32.99±5.45**	**39.47±11.3**	**46.12±6.97**	**39.53±9.73^{ab}**	**47.14±2.47**	**48.41±2.92**	**42.34±7.85**	**45.96±8.97^{ab}**	**51.73±1.21**	**45.45±4.48**	**48.59±4.23^{a}**
16:2ω4	0.39±0.23	0.69±0.50	0.77±0.15	**0.59±0.36**	0.80±0.14	0.72±0.72	0.66±0.11	**0.73±0.16**	0.52±0.50	0.77±0.49	1.17±0.65	**0.82±0.55**	0.89±0.14	0.54±0.31	**0.74±0.45**
16:3ω3	0.81±0.59^{ab}	0.94±0.44^{a}	0.08±0.06^{b}	**0.69±0.56^{a}**	-	-	0.36±0.27 ^{ab}	**0.12±0.^{27b}**	0.24±0.18 ^{ab}	0.33±0.28 ^{ab}	-	**0.19±0.29^{b}**	0.45±0.12^{ab}	0.06±0.04^{b}	**0.29±0.24^{ab}**
18:2a	0.40±0.18	0.26±0.12	0.85±0.71	**0.45±0.39**	0.46±0.07	0.50±0.03	0.39±0.15	**0.45±0.10**	0.60±0.39	0.37±0.09	0.47±0.25	**0.47±0.24**	0.83±0.23	0.46±0.39	**0.67±0.38**
18:2ω6	2.69±0.90	2.85±1.99	1.69±0.30	**2.52±1.36^{a}**	1.73±0.35	2.05±0.43	2.24±0.71	**2.01±0.54^{ab}**	1.87±0.17	1.59±0.31	2.61±1.36	**1.98±0.81^{ab}**	0.81±0.76	1.84±0.10	**1.25±0.88^{b}**
18:3ω6	0.49±0.23	0.60±0.22	0.64±0.25	**0.57±0.22**	0.20±0.17	0.36±0.32	0.57±.29	**0.38±0.34**	0.45±0.05	0.42±0.17	0.43±0.25	**0.43±0.16**	0.25±0.20	0.52±0.36	**0.37±0.31**
18:3ω4	0.51±0.43	0.51±0.45	0.15±0.13	**0.43±0.44**	0.39±0.34	0.24±0.11	0.52±0.35	**0.39±0.29**	0.44±0.25	0.24±0.21	0.58±0.32	**0.41±0.27**	0.59±0.45	0.39±0.18	**0.51±0.42**
18:3ω3	7.36±2.34	7.68±5.13	7.94±0.57	**7.62±3.27^{a}**	6.47±0.56	6.70±0.99	10.20±2.91	**7.79±2.54^{a}**	6.46±0.16	7.95±0.58	8.91±4.97	**7.79±2.58^{a}**	3.64±2.49	3.94±1.14	**3.77±1.89^{b}**
20:2ω6	1.10±0.75^{a}	0.54±0.26^{ab}	0.66±0.21^{ab}	**0.78±0.54^{a}**	0.42±0.36^{ab}	0.15±0.13^{b}	0.26±0.23^{ab}	**0.28±0.25^{b}**	0.22±0.20^{b}	0.37±0.07^{ab}	0.43±0.07^{ab}	**0.34±0.14^{b}**	0.24±0.17^{b}	0.32±0.27^{ab}	**0.28±0.20^{b}**
20:3ω6	0.85±0.41	0.82±0.25	0.72±0.22	**0.81±0.30**	0.88±0.86	0.51±0.14	0.51±0.39	**0.63±0.53**	0.45±0.39	0.69±0.21	0.67±0.26	**0.61±0.28**	0.53±0.28	0.47±0.11	**0.50±0.21**
20:4ω6	1.92±1.00^{ab}	2.98±0.96^{a}	2.41±0.23^{ab}	**2.44±0.94^{a}**	3.24±0.75^{a}	1.79±1.18^{ab}	0.80±0.27^{b}	**1.94±1.31^{ab}**	1.07±0.58^{b}	0.95±0.26^{b}	1.11±0.46^{b}	**1.03±0.39^{b}**	1.17±0.21^{b}	2.02±0.85^{ab}	**1.54±0.68^{ab}**
20:3ω3	1.12±0.72	1.32±0.29	0.84±0.35	**1.13±0.53**	0.94±0.21	0.88±0.39	0.95±0.41	**0.92±0.32**	0.94±0.20	0.96±0.28	0.97±1.09	**0.96±0.55**	0.87±0.22	0.82±0.08	**0.85±0.16**
20:4ω3	0.64±0.29	1.19±0.45	0.94±0.48	**0.92±0.45**	1.22±0.62	0.77±0.49	0.59±0.24	**0.86±0.47**	0.49±0.37	0.58±0.46	0.91±0.77	**0.65±0.60**	0.62±0.45	0.18±0.12	**0.43±0.43**
20:5ω3	4.46±2.76	5.86±2.63	6.98±2.24	**5.58±2.59^{a}**	7.29±1.23	4.30±2.72	2.37±0.98	**4.65±2.64^{ab}**	2.88±0.57	3.15±1.08	2.69±0.19	**2.93±0.72^{b}**	3.55±0.30	4.85±2.04	**4.11±1.38^{ab}**
21:5ω3	0.33±0.14^{b}	0.50±0.14^{ab}	0.64±0.22^{ab}	**0.47±0.20**	1.00±0.60^{a}	0.35±0.26^{ab}	0.15±0.14^{b}	**0.50±0.46**	0.37±0.18^{ab}	0.61±0.23^{ab}	0.26±0.04^{b}	**0.43±0.22**	0.24±0.19^{b}	0.40±0.23^{ab}	**0.31±0.20**
22:5ω3	0.67±0.28	0.93±0.26	0.98±0.28	**0.84±0.29**	1.30±1.13	0.89±0.70	0.56±0.16	**0.92±0.71**	0.59±0.20	1.08±0.03	1.03±0.61	**0.92±0.38**	0.73±0.39	0.98±0.24	**0.84±0.34**
22:6ω3	6.61±3.25^{abc}	9.09±2.5^{abc}	10.14±1.7^{ab}	**8.38±2.89^{a}**	11.32±3.63^{a}	5.74±3.09 ^{abc}	2.92±1.05^{c}	**6.66±4.93^{ab}**	4.24±0.64^{abc}	4.36±1.40^{bc}	3.57±0.76^{bc}	**4.09±1.00^{b}**	3.85±1.68^{bc}	6.78±3.6 ^{abc}	**5.11±2.84^{ab}**
PUFA*	**0.48±0.41**	**0.48±0.25**	**0.13±0.13**	**0.65±0.61**	**0.61±0.42**	0.73±0.85	0.19±0.07	0.50±0.58	0.74±0.25	0.47±0.32	0.29±0.26	0.47±0.32	0.85±0.71	0.43±0.22	0.99±0.85
∑PUFA	30.83±7.48^{ab}	37.24±9.53^{a}	36.56±4.9^{ab}	34.87±7.9^{a}	38.27±5.20^{a}	26.67±10.33^{ab}	24.24±5.69^{ab}	29.73±9.62^{ab}	22.55±1.14^{ab}	**24.89±2.2^{ab}**	**26.10±8.73^{ab}**	**24.52±4.20^{b}**	**20.11±1.2^{b}**	**25.00±4.5^{ab}**	**22.56±3.82^{b}**
Bacterial	4.22±1.07^{ab}	5.00±1.60^{ab}	2.85±0.22^{b}	**4.20±1.40**	3.13±0.28^{ab}	3.34±1.63^{ab}	3.82±1.95^{ab}	**3.43±1.40**	2.64±0.65^{b}	3.67±0.33^{ab}	6.37±2.44^{a}	**4.17±1.99**	3.06±0.32^{b}	3.62±0.14^{ab}	**3.30±0.39**
DHA/EPA	1.66±0.41	1.68±0.46	1.51±0.28	**1.63±0.38**	1.56±0.42	1.34±0.25	1.26±0.18	**1.39±0.29**	1.52±0.43	1.53±0.80	1.33±0.27	**1.47±0.53**	1.07±0.37	1.40±0.43	**1.21±0.40**
Zooplankton	2.66±1.24^{ab}	1.91±1.10^{ab}	-	**1.76±1.43**	0.20±0.16^{b}	0.73±0.27^{ab}	-	**0.31±0.30**	1.50±1.31^{ab}	0.37±0.09^{b}	4.65±2.34^{a}	**1.99±1.14**	2.39±0.37^{ab}	2.36±0.77^{ab}	**2.38±0.52**
Terrestrial	10.05±3.17	10.54±7.10	9.63±0.31	**10.14±4.50^{a}**	8.21±0.90	8.75±0.96	12.44±3.60	**9.80±2.95^{ab}**	8.33±0.28	9.53±0.83	11.53±5.25	**9.77±3.30^{b}**	4.45±3.18	5.78±1.22	**5.02±2.46^{a}**
∑ω3/∑ω6	3.14±0.41	3.43±0.57	4.65±0.51	**3.75±0.52**	4.63±0.49	4.07±0.31	4.19±0.59	**4.29±0.45**	4.08±1.22	4.37±0.17	3.07±0.76	**3.84±0.94**	4.94±1.40	3.30±0.54	**4.12±0.97**
16:1ω7/16:0	0.06±0.04	0.04±0.03	0.02±0.02	**0.04±0.03**	0.01±0.01	0.02±0.02	0.04±0.02	**0.02±0.02**	0.08±0.04	0.07±0.05	0.09±0.06	**0.08±0.08**	0.08±0.04	0.01±0.01	**0.05±0.05**

Means followed by different letters (a,b) and letter groups (ab) in the same row are significantly different for months and seasons (ANOVA-TUKEY HSD Test, $p < 0.05$, n=3-5 for months; n=9-13 for seasons) values are means±SD. *: Minor FAs with mean proportion ≤ 0.5 in all the sampling periods, Inluded in this fraction were FAs: ι15:0, 15:0, ι17:0, 19:0, 20:0 from SFA, 14:1, 15:1, 18:1ω6, 18:1ω5, 22:1ω5, 22:1 9, 24:1 from MUFA, 18:2, 18:4ω3, 20:2ω6, 20:2 from PUFA ι: indicates iso-branched FAs.

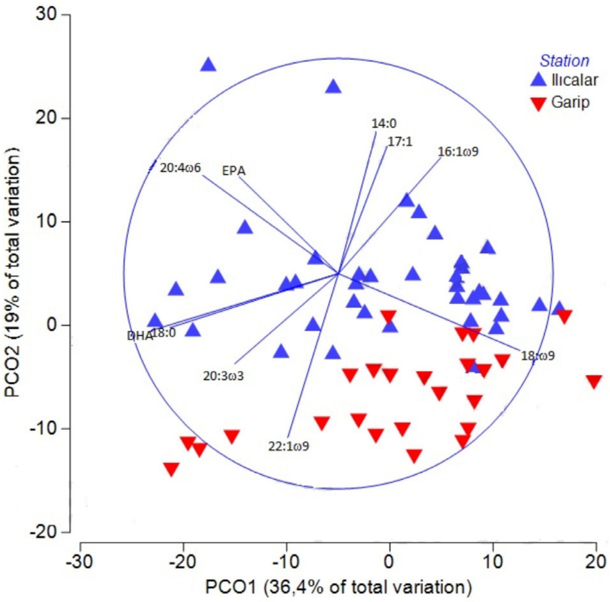


Figure 2. Two-dimensional configuration plot of a PCO analysis of a resemblance matrix of fatty acids of *G. rufa* at the Garip (n=25) and Ilıcalar (n=39) Stations. The lower tringular matrix was created using by Bray-Curtis similarity coefficients. Pearson correlation > 0.60.

toplankton in its diet especially, Bacillariophyceae members from Chrysophyta (Demirci *et al*., 2016). Yalcin-Ozdilek and Ekmekci (2006) reported that Chrysophyta members were abundant in all seasons in the diet of *G. rufa* in the Asi River, Turkey. Moreover, 18:1ω9 is used as a characteristic fatty acid marker for Cryptophyceae, together with Dinophyceae and Chlorophyta (Dalsgaard *et al.,* 2003). The percentage of abundance of other types of food in the diet of *G. rufa* varies depending on the season (Yalcin-Ozdilek and Ekmekci, 2006).

Guler *et al*. (2008) reported that the PUFA content in *Cyprinus carpio* fillets differed in each season and was 39% in spring, 43% in summer and 36% in autumn. They indicated that DHA was the major PUFA for *Cyrinus carpio* in summer and winter. DHA plays an important role in adaptation processes. When fish are exposed to low water temperatures, PUFA content increases (Lavens *et al*., 1999). Similarly, the highest ∑PUFA content in *G. rufa* (34%; 35%, respectively) was observed to be found in the lowest temperatures detected during the sampling period, which were in winter (8.03 °C) at the Garip station and in spring (12.63 °C) at the Ilıcalar station. The PUFA content was higher in the fish samples from the Garip station (Table 2) than from the Ilıcalar station (Table 3). This is thought to be related to temperature because the temperature of the Ilıcalar station was higher than the Garip station during the sampling period (Table 4).

The fatty acid marker for diatoms (Bacillariophyceae) is EPA and for dinoflagellates (Dinophyceae) is DHA (Viso and Marty, 1993). EPA and DHA contents vary among/within species depending on environmental factors such as diet and habitat, as well as whether the fish are wild or farmed (Tocher, 2010). The fact that 18:1ω9, EPA and DHA are the most abundant fatty acids may indicate that *G. rufa* prefers the members of Cryptophyceae, Dinophyceae, and Chlophyta as food sources in the Murat River. Moreover, the 16:1ω7/16:0 ratio has been used to

Table 4. Chlorophyll-a concentration (µg/L) and water temperature (ºC) at the stations during the sampling period

	SPRING			SUMMER			AUTUMN			WINTER		
	March	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**	**January**	**February**
							GARIP					
Chl-a		**0.10**			**5.81**			**1.98**			**2.55**	
	0.09	0.10	0.10	3.28	4.60	9.54	1.97	3.46	0.52	1.96	3.95	1.75
Temperature		**11.13**			**21.76**			**12.67**			8.03	
	8.20	8.6	16.6	20.12	25.1	20.05	0.50	17.00	20.50	9.60	7.40	7.10
							ILICALAR					
Chl-a		**1.77**			**4.18**			**0.91**			**1.19**	
	0.86	1.03	3.42	1.71	0.41	10.72	0.31	1.02	1.41	1.20	1.15	1.21
Temperature		**12.63**			**23.57**			**21.73**			**16.67**	
	11.00	8.10	18.80	23.7	25.5	21.50	21.50	21.00	22.70	20.5	15.50	14.00

infer a diatom-dominated food chain base (Auel *et al*., 2002). While 16:1ω7 is found in cyanobacteria, dinoflagellates, and a specific isomer, the *trans* one, is found in bacteria, 16:1ω7 is most prevalently associated with diatoms (Parrish, 2013). The 16:1ω7/16:0 ratio varied from 0.02 to 0.08% (summer-autumn) at the Ilıcalar station and 0.01-0.03% (autumn-winter) at the Garip station. These results showed that *G. rufa* at consumed more diatoms the Ilıcalar station than those at the Garip station during the sampling period. However, 18:1ω9, EPA and DHA were the most abundant dietary fatty acids in *G. rufa*. Yalcin-Ozdilek and Ekmekci (2006) showed that *G. rufa*'s main diets primarily comprised diatoms found in the Chrysophyta. However, the fatty acid composition detected in the present study indicated there were more dinoflagellates than diatoms in *G. rufa's* diet. Freshwater fish cannot synthesize certain fatty acids, especially C_{18} PUFA, such as 18:2ω6, 18:3ω6, although they can directly ingest many LC-PUFAs such as ARA, DHA, EPA from their prey (Tocher, 2010). Dietary EPA, DHA, and ARA improve reproductive success and increase the quality of broodstock eggs (Mazorra *et al*., 2003). They are critical to the general health of organisms and most consumers synthesize them inefficiently from their precursors (e.g., 18:3ω3 or ALA and 18:2ω6 or LNA). Therefore, EPA, DHA, and ARA are considered essential dietary FAs in aquatic ecosystems (Dalsgaard *et al*., 2003; Parrish, 2009). 18:3ω3 is also higher in freshwater herbivorous Cypriniformes, including *G. rufa*. 18:3ω3 was one of the highest PUFA at both stations during the sampling period (Tables 2 and 3). It is stated in many studies that terrestrial plants abundantly synthesize 18:3ω3 and 18:2ω6, which are also used as dietary marker in the fatty acid composition of aquatic organisms (Parrish, 2013). ARA was more prominent in the fish from the Ilıcalar station than those from the Garip station for PUFA in the PCO analysis (Figure 2). Freshwater fish have relatively high contents of 18:2ω6 and ARA, which are indicative of freshwater algae and terrestrial dietary sources (Parzanini *et al*., 2020). These fatty acids were present in significant percentages in the fatty acid composition of *G. rufa*. From the fatty acid values, it was deduced that *G. rufa* also preferred diets of terrestrial origin.

C_{13}, C_{15}, C_{16} and C_{17} SFA and MUFA and their isomers, as well as 18:1ω6 characterize bacterial fatty acids (Dalsgaard *et al*., 2003; Parrish, 2013). Therefore, the presence of these fatty acids in fish tissue may indicate a bacterial diet. The results of the present study showed that *G. rufa* fed on bacteria (Tables 2 and 3). However, terrestrial markers outnumbered both bacterial and zooplanktonic markers at both stations during the sampling period (Tables 2 and 3).

The C_{20} and C_{22} group zooplankton fatty acids were in very small pergentages in *G. rufa*, which were found in a smaller amount in the fish samples from the Garip station than in those from the Ilıcalar station. These fatty acids characterize copepod species and are used in the analysis of marine fish to reveal nutritional relationships (Iverson *et al.,* 2009). These fatty acids were observed in *G. rufa* in winter as well as other seasons at the Ilıcalar station, and not in winter at the Garip station. *G. rufa* preferred zooplankton as food in winter months, because the Chl-a content was found to be lower at the Ilıcalar station (1.19 µg/L) than at the Garip station (2.55 µg/L) in winter. The highest Chl-a content was detected in summer at both stations. It was higher at the Garip station (5.81 µg/L) (Table 2) than the Ilıcalar station (4.18 µg/L) in summer (Table 3). In the periods when Chl-a was abundant (Table 4), *G. rufa* tended toward an herbivorous diet, which is their main diet.

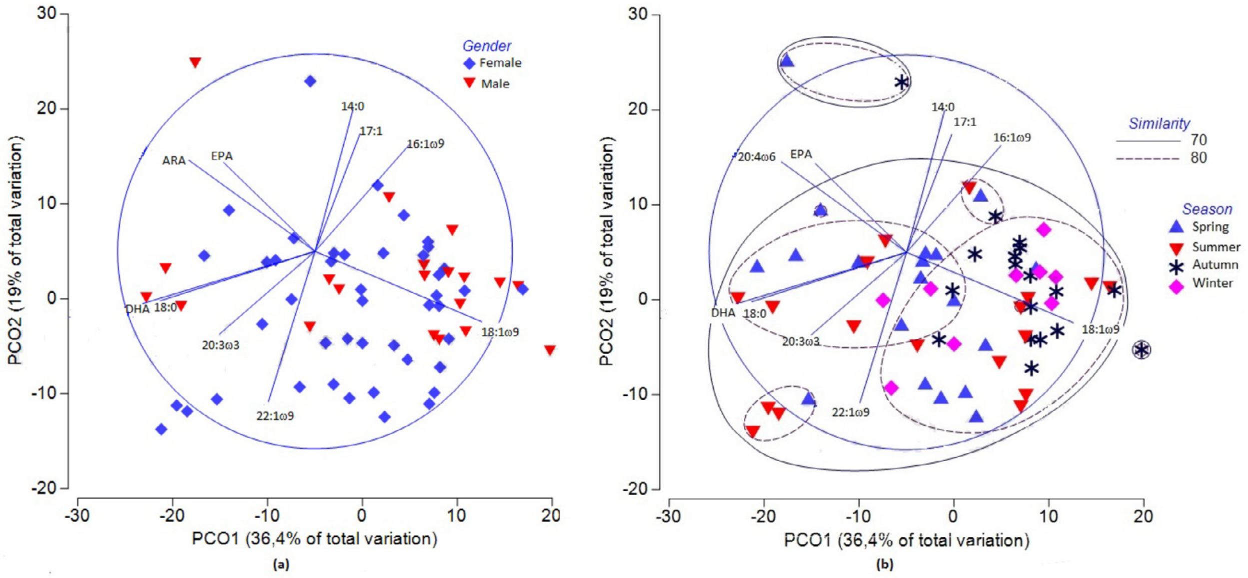
The ω3/ω6 ratio is greater in herbivorous freshwater fish (Parzanini *et al*., 2020). The ω3/ω6 ratio varied between 3-5 at the Ilıcalar station (Table 3) and 4-7 at the Garip station (Table 2). It reached the highest value in August at 6.60 at the Garip station (Table 2). The Ilıcalar station included zooplanktonic FAs at higher levels than the Garip station. Phytoplankton were probably more abundant than zooplankton at the Garip station, and *G. rufa* preferred phytoplankton, which is the primary food source in all seasons at the Garip station.

The results of seasonal changes in dietary fatty acid levels showed that the fatty acid composition of *G. rufa* changed according to the change in nutritional content and variety depending on the season. There are already many approaches and studies showing that fatty acids give clues about the food consumed. All these approaches can provide valuable information about consumer nutrients and food ecology in complex aquatic ecosystems. Each of these approaches is evaluated and used in studies according to the content of the study (Dalsgaard *et al*., 2003). FAs are known indicators of specific food sources, the results can indicate the diet of consumers and are a potentially powerful trophic measures which reflect what is included in an individual's diet over a period of several weeks (Kirsch, 1998). From this point of view, it would not be wrong to say that *G. rufa* predominantly feeds on plants, but can also feed omnivorously. It can be stated that *G. rufa* has a very wide food preference from bacteria to zooplankton. Demirci *et al*. (2016) emphasized similar results in their study on the nutritional characteristics of *G. rufa* from organisms in stomach-intestinal contents. *G. rufa* can feed on various plankton species, although they prefer phytoplankton (Demirci *et al*., 2016). It was also found in the present study that the results of dietary fatty acids indicated the same results.

3.3. Factors affecting the fatty acid composition of *G. rufa*, regardless of location

Figure 3 shows the PCO analysis of seasonal and gender differences in terms of the fatty acid composition, regardless of the stations. The results of the PERMANOVA pair-wise tests revealed that the fatty acid compositions did not differ significantly regardless of the stations during the sampling season. Where several samples represent several seasons at the same time, they were grouped according to their close proximity to the nearest seasons (e.g., warm seasons, cold seasons, hot seasons). In general, spring-summer (hot seasons) and winter-autumn (cold seasons) were located in the same area with 80% similarity. These areas were represented by more samples. However, all seasons were located in the same area with 70% similarity (Figure 3b). The PERMANOVA main test results revealed that the effect of season was significant in the fatty acid composition regardless of the station (P_{perm}=0.001). Almost all seasons were characterized by 18:1ω9 with 80% similarity. The highest difference was detected between autumn and spring (P_{perm}=0.001). However, autumn and winter were characterized by 18:1ω9 with 80% similarity more than the others (Figure 3b).

Figure 3a shows the PCO analysis of the fatty acid composition of the gender groups, regardless of station. The results of the PERMANOVA pair-wise test indicated that the fatty acid composition of females in summer-winter was significantly different from the other seasons (P_{perm}=0.001). Although the difference in the fatty acid composition of males was not as great as in females, the results of spring-winter seasons were different from the other season



(a) (b)

Figure 3. Two-dimensional configuration plot of a PCO analaysis of a resemblance matrix of fatty acids in the seasons and genders. The lower tringular matrix was created using by Bray- Curtis similarity coefficients. Pearson correlation > 0.6. (a): Two-dimensional configuration plot of a PCO analysis of a resemblance matrix of fatty acids in the females (n=44) and males (n=20) of *G. rufa*. (b): Two-dimensional configuration plot of a PCO analaysis of a resemblance matrix of fatty acids in the seasons regardless of location (n= 19,18,18,9 for spring, summer, autumn and winter, respectively).

(P_{perm}=0.003). The gender variable groups formed barely-separated groups for both males and females (ANOSIM-R=0.13; 0.14, respectively). There was no significant seasonal difference in both genders. Females were characterized by 18:1ω9, DHA, EPA and ARA; whereas males were characterized by DHA, 18:0 and 18:1ω9 (Figure 3a). Şen Özdemir and Caf (2018) found similar results for female seahorses. They reported that female seahorses were characterized by EPA, DHA and ARA in multidimensional scaling results and that 18:1ω9 was not the most significant contributor to the fatty acid composition of both seahorse males and females. The only difference was that in their study, DHA replaced 18:1ω9, contrary to the results obtained in the present study. Such differences in freshwater and marine fish are expected since they have fundamental differences such as different feeding habitat (Parzanini *et al.*, 2020). Urquidez-Bejarano *et al.* (2016) reported that 16:0, 18:0, 18:1, ARA and EPA were significantly higher in ripe female gonads than in spent gonads for angelfish. They observed a similar trend in male gonads, and there was no statistically significant difference between genders. Similarly, the difference between males and females in terms of the fatty acid composition of *G. rufa* muscle tissue used in this study were not significant. It is widely known that FAs like EPA, DHA and ARA are involved in numerous physiological processes from growth to reproduction. Therefore, they are vital to consumers including vertebrates like fish species (Paulsen *et al.,* 2014). Also, DHA plays an important role in the female reproductive system. It is transferred from the muscle to the liver and gonads and effects the egg quality and survival of larvae. In addition, a balanced presence of linolenic (18:3ω3) and linoleic (18:2ω3) fatty acids in the feeding of fresh water fish larvae increases the optimal survival rate (Higgs *et al.*, 1992).

CONCLUSIONS

This study provides a first comprehensive report on the total lipid content and FA composition of *G. rufa* according to biotic and abiotic factors (season, gender, location) and determines feeding behavior using dietary fatty acids. In particular, the analysis performed revealed fundamental differences and similarities between/within factor groups. Seasonal differences were more prominent than the other factor groups in terms of both total lipid content and fatty acid composition ($p < 0.05$). In addition, annual average total lipid content was higher in females than males during the sampling period. *G. rufa* was characterized by a high MUFA content, mainly 18:1ω9, for all factor groups during the sampling season. *G. rufa* had high percentages of dietary fatty acids such as EPA, DHA, 18:1ω9, ω3/ω6 and ARA fatty acids, thus indicating herbivorous feeding. The study also showed that although *G. rufa* preferred predominantly phytoplankton, it had a very wide range of food preference from bacteria to zooplankton. Although there were no significant differences between locations, both seasonal and gender differences were more prominent in the different locations. According to the dietary fatty acid results, the diet composition, which changes depending on season rather than location, is a major factor in determining the fatty acid composition of *G. rufa*.

ACKNOWLEDGMENTS

This study includes a part of the project supported by Bingöl University Scientific Research Project Coordination Unit, number BAP-2021-35585. I would like to thank Koyun, M., Caf, F., Kırıcı, M. for their support in collecting the fish samples and Elazig Fisheries Research Institute for providing the oppotunity for Chl-a analysis.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e519
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0223221

Estimation of mass transfer terms in the lycopene recovery employing *Moringa oleifera* Lam oil as solvent

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Submitted: 21 February 2022; Accepted: 1 February 2023; Published online: 10 October 2023

SUMMARY: The aim of this work was to assess the terms associated with mass transfer in the lycopene recovery from the waste of a tomato canning plant. *Moringa oleifera* Lam oil was employed as solvent. An ultrasonic extraction was carried out on skin and seeds. The operational variables were temperature (skin: 45, 60, 75, 90 ºC; seed: 45, 60, 75 ºC), matrix/solvent ratio (m/v) (1:20, 1:25, 1:30), particle size (skin: < 1, 1-2, > 3.15 mm; seed: < 1, 1-2, > 2 mm) and extract separation method (filtration and centrifugation). Kinetic constant, lycopene concentration on the solid surface, volumetric coefficient of mass transfer and effective diffusivity were determined. The more the kinetic constant increased, the higher the volumetric coefficient of mass transfer was. Effective diffusivity increased with temperature. Activation energy values suggest a possible deterioration of lycopene at temperatures higher than the optimum. The use of *M. oleifera* oil as solvent should increase the biological value of the lycopene extracts.

KEYWORDS: *Effective diffusivity; Kinetic constant; Lycopene recovery; Mass transfer coefficient;* Moringa oleifera *seed oil.*

RESUMEN: ***Estimación de los términos de transferencia de masa en la recuperación de licopeno empleando aceite de* Moringa oleifera *Lam como solvente.*** El objetivo del presente trabajo fue evaluar los términos asociados a la transferencia de masa en la extracción de licopeno a partir del residuo de la industria de conservas de tomate. Como solvente se utilizó aceite de *Moringa oleífera* Lam. Se realizó una extracción ultrasónica sobre piel y semillas. Las variables operacionales investigadas fueron, temperatura (piel: 45, 60, 75, 90 ºC; semillas: 45, 60, 75 ºC), relación soluto/solvente (m/v) (1:20, 1:25, 1:30), tamaño de partícula (piel: <1, 1-2, >3.15 mm; semilla: <1, 1-2, >2 mm) y métodos de separación del extracto (filtración y centrifugación). Se determinaron la constante cinética, la concentración de licopeno en la superficie del sólido, el coeficiente volumétrico de transferencia de masa y la difusividad efectiva. A mayor contante cinética, mayor coeficiente volumétrico de transferencia de masa. La difusividad efectiva aumentó con la temperatura. Los valores de energía de activación sugieren un posible deterioro del licopeno a temperaturas superiores a las óptimas. El uso del aceite de *M. oleífera* como solvente debe incrementar el valor biológico de los extractos de licopeno.

PALABRAS CLAVE: *Aceite de semilla de* Moringa oleífera; *Coeficiente de transferencia de masa; Constante cinética; Difusividad efectiva; Extracción de licopeno.*

Citation/Cómo citar este artículo: Sariego Y, Pita A, González M, Acosta G, Zumalacárregui B, Cruz L. 2023. Estimation of mass transfer terms in the lycopene recovery employing *Moringa oleifera* Lam oil as solvent. *Grasas Aceites* **74** (3), e519. https://doi.org/10.3989/gya.0223221

1. INTRODUCTION

The consumption of fruits and vegetables has beneficial effects on health due to the presence of compounds with biological properties. Bioactive compounds, obtained from vegetable by-products, have a considerable interest as dietary supplements or food preservatives (Oreopoulou and Strati, 2011; Nour *et al.*, 2018). The development of functional foods has been explored recently in order to obtain favorable effects on health which go beyond their nutritional value (Waliszewski and Blasco, 2010). Carotenoid pigments are a group of nearly 600 fat-soluble pigments which are responsible for the yellow, orange and red colors in fruits and vegetables (Baranska and Kaczor, 2016). These tonalities are derived from lycopene, which is the main pigment responsible for the red color of tomatoes. It represents approximately, 80 – 90% of the total pigment content (Dolatabadi *et al.*, 2016). In addition to the coloring effect, its antioxidant capacity represents other outstanding property (Waliszewski and Blasco, 2010; Cruz *et al.*, 2013). Lycopene, by acting as a powerful antioxidant, reduces the risk of cardiovascular, inflammatory, neurodegenerative diseases and cancer or mitigates their damages (Waliszewski and Blasco, 2010; Dolatabadi *et al.*, 2016; Rodríguez, 2016). Its absorption depends on food intake and only 10 to 30% is absorbed, resulting in a limited contribution through the diet (Cruz *et al.*, 2013). Its use as a food additive could be a solution for increasing its consumption.

The tomato processing industry generates a large amount of waste (Poojary and Passamonti, 2015; Galanakis, 2015; Nour *et al.*, 2018). A satisfactory solution to this environmental and economic problem would be waste recovery and reuse (Cruz *et al.*, 2016). This means the waste would be converted into the raw matter for the lycopene extraction process with a potential environmental, social and economic impact (Galanakis, 2015; Cruz *et al.*, 2016). However, for its recovery, efficient isolation and protection technologies are required. These technologies should not affect the structure or physiological properties of lycopene and must take into account its sensitivity to oxygen, extreme pH, light and high temperatures (Choksi and Vishal, 2007; Bailey, 2015).

Extraction is a very important stage in the recovery of lycopene (Lianfu and Zelong, 2008; Nour *et al.*, 2018). Bearing in mind the high solubility of lycopene in lipids, an oily solvent should be employed. The extracts obtained can be incorporated tino foods where an oily ingredient is required (Cruz, 2013; Gámez *et al.*, 2016). The recovery of lycopene by employing *M. oleifera* oil as solvent may be a favorable option. The oil obtained from its seeds is composed of a wide variety of unsaturated fatty acids. Among them oleic acid is the predominant one. Values have been reported to range from 65.14 - 73.36%. (Ferrer *et al.*, 2020; Gharsallah *et al.*, 2021). The high resistance of *M. oleífera* oil to oxidation can be attributed to its high level of unsaturated fatty acids (Ferrer *et. al.*, 2020; Özcan, 2020). *M. oleifera* oil also has antioxidant properties due to its phytochemical content. A wide range of total phenolic compound, 48 – 400,17 mg GAE/kg oil, has been reported (Özcan, 2020; Gharsallah *et al.*, 2021). Both unsaturated acids and polyphenols content provide nutritional and antioxidant effects from *M. oleífera* (Ferrer *et al.*, 2020). Moreover, an additive which is free of organic solvent will contribute to obtaining more healthy foods. These aspects will give an additional value to lycopene extracts.

The industrial method most commonly used to obtain this pigment is based on conventional solid–liquid extraction (Rodríguez, 2016; Yilmaz *et al.*, 2017; Hoyos *et al.*, 2022). The simultaneous application of ultrasound as an intensification technology has been studied (Rodríguez, *et al.*, 2014; Yilmaz *et al.*, 2017). A 50% decrease in extraction time (Kumcuoglu *et al.*, 2014) and a yield of up to 87.25% (Rahimi and Mikani, 2019) have been reported with respect to conventional extraction.

The knowledge of the terms associated with mass transfer, such as effective diffusivity, mass transfer coefficient and kinetic constant are required for modelling and process assessment. Although the literature includes papers where those terms are reported, those corresponding to lycopene extraction from *M. oleifera* oil have not been published before. The aim of this work was to assess the terms associated with mass transfer in the lycopene recovery from the waste of tomato canning by employing *M. oleifera* oil as solvent.

2. MATERIALS AND METHODS

2.1. Tomato waste

Tomato waste (*Lycopersicum esculentum* Mill. var. *Amalia*) was obtained from a tomato canning plant. Samples were taken during the tomato harvest season, January to March of 2019 in the western region of Cuba. Waste, composed of skins and seeds, was protected from light and stored at 2 ± 0.5 ºC until the experiments were conducted.

2.2. *Moringa oleifera* seed oil

The oil obtained from *Moringa oleifera* Lam var. *Supergenius*, was the extraction solvent to be employed. It was produced by milling the seeds harvested in the same region and period.

2.3. Procedure for obtaining lycopene

The separation of skins and seeds by means of a flotation-sedimentation process in water was the primary treatment for the industrial waste (Devinder, 2008). Afterwards, the skins and seeds were dried. In order to avoid a possible degradation of lycopene, the experimental material was dried at 55 ºC for 1.5 h in an oven (Incubator, model P/G 2007, R. P. China). The degradation of bioactive compounds has been linked to the drying temperature-time combination (Cruz *et al.*, 2016). The moisture contents of both experimental materials were determined and expressed as percentage (AOAC, 2000).

Ultrasonically-assisted extraction was carried out in a bath with a temperature control (SB-120DT, R. P. China). The equipment was operated at a frequency of 40 kHz and 120 W. These values are within the recommended ranges according the literature (20 – 100 kHz and 100 – 800 W, respectively) (Rodríguez *et al.*, 2014). To avoid an increase in temperature as a consequence of the ultrasonic effect, a cryostat (Ningbo Scientz Biotechnology Co, LTDDC-3006, R. P. China) was included in the system. Prior to the experiments, the operational temperature of the cryostat was defined according to the reactor temperature. In this way, the cryostat operation guaranteed the bath temperature to be ± 1 ºC. A diagram of the experimental system is shown in Figure 1. In all cases the extraction process was carried out in 1 h.

The extracts obtained were centrifuged (Hitachi, SCT15B, Japan) at 3800 g for 10 min. or filtered through a gauze piece. Their absorbance were measured in a AUV-visible spectrophotometer (Rayleigh, model Vis-723G, R. P. China).

2.4. Lycopene quantification

A methodology without organic volatile solvents was employed. Lycopene was extracted and quantified in the oil. Lycopene concentration was measured from a calibration curve ($R^2 = 0{,}998$) of pure lycopene standard (Sigma- Aldrich, USA). The specific extinction coefficient ($A^{1\%} = 3\,465$) was estimated from the curve. Absorbance was measured in an AUV-visible spectrophotometer (Rayleigh, model Vis-723G, R. P. China) with *M. oleifera* oil as blank. Lycopene concentration was measured at the maximal wavelength identified (483 nm) to minimize interference from other carotenoids (Poojary and Passamonti, 2015). Figure 2 shows the UV-vis spectra of lycopene stand-

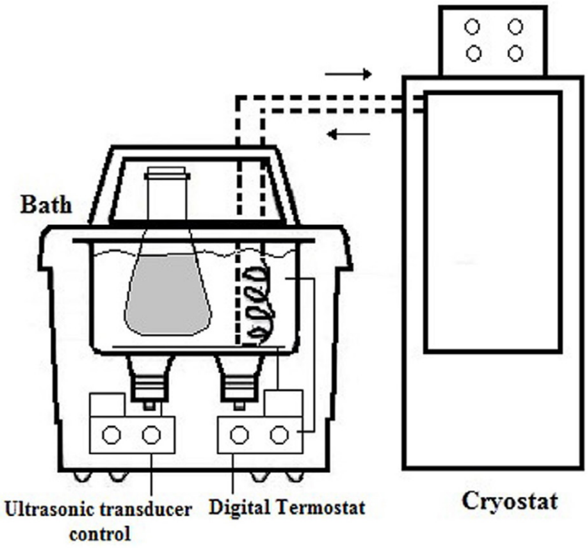

Figure 1. Experimental system.

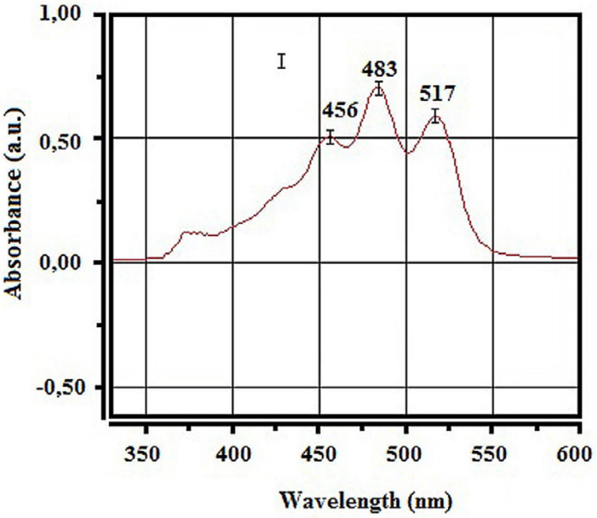

Figure 2. UV-vis spectra of lycopene standard in *M. oleifera* oil.

ard in *M. oleifera* oil. The upper wavelength is explained from the redshift phenomena as a result of the interaction between lycopene and the solvent (Hoyos *et al.*, 2022). Lycopene concentration was expressed as µg/mL (or µg/g dry matter).

2.5. Experimental design

The operational parameters should be previously defined to determine the terms associated with mass transfer. The combining effect of temperature (T) and matrix/solvent ratio (MSR) on the extraction assisted by ultrasound was considered in a first experimental design (skin: $4^1 \cdot 3^1$; seed: 3^2). The levels of the operational parameters for both materials are shown in Table 1.

In the case of skin, for a better detection of the effect of temperature, the design was made at 90 ° C. The particle size ranging 1 – 2 mm was used for both experimental materials.

A second experimental design to assess the influence of particle size (PS) and separation method (SM) was developed at the optimum temperature corresponding to the first design and MSR at 1:25. To guarantee the total imbibition of the experimental materials, the MSR value was selected. Table 1 shows the operational parameter levels for both materials (skin and seed: $2^1 \cdot 3^1$). Filtration, through a gauze piece, and centrifugation were the SM applied to both experimental materials.

The mid-range and the largest PS for both skin and seed corresponded to the waste received. The experimental material was ground in a domestic blade mill (Oster, BVSTBMH23-053, U.K.). The material obtained was sieved for selecting the sizes required.

In all experiments a mass of 0.4 g of skin or seed was weighed (Sartorius, model BS124S, Germany). The experimental designs were carried out for both skin and seed, separately.

2.6. Extraction stages

An exhaustive extraction was developed in irder to determine the extraction stages for each experimental material. It was performed in four stages, on the same sample, at the optimum T, PS of 1-2 mm each , and MSR of 1/25 (m/v). To make the absorbance measurement procedure easier, the last condition was selected. Each extraction stage lasted 80 min. The total extracted lycopene mass was determined from the sum of the extracted masses in each of the successive stages. The yield was obtained from equation 1 (Rodríguez, 2016).

$$Y = \frac{\text{m (extracted mass)}}{\text{M (total mass)}} \cdot 100 \qquad (1)$$

where: Y is the extracted lycopene yield (%); m is the extracted lycopene mass in each stage (µg/g dry matter); M corresponds to the total mass of lycopene extracted (µg/g dry matter).

2.7. Extraction kinetics

Ultrasonic-assisted extractions were developed at different T (45, 50, 55, 60, 70, 75, 80 and 90 °C) for

Table 1. Experimental designs (n =3)

First experimental design			
Skin		Seed	
Temperature (ºC)	Matrix/solvent ratio (m/v)	Temperature (ºC)	Matrix/solvent ratio (m/v)
45	1:20	45	1:20
60	1:25	60	1:25
75	1:30	75	1:30
90			
Second experimental design			
Skin		Seed	
Particle size (mm)	Separation method	Particle size (mm)	Separation method
< 1 1 - 2 > 3.15	Filtration Centrifugation	< 1 1 – 2 > 2	Filtration Centrifugation

80 min. They were developed under the corresponding conditions previously decided by the study about extraction stages. Samples were taken and their absorbance measured every 10 min. The results were fitted to the fundamental leaching equation (equation 2), which corresponds to a first-order kinetic model (Treybal, 1997; Poojary and Passamonti, 2015).

$$C = C_s\left(1 - e^{-k\cdot t}\right) \qquad (2)$$

where: k is the kinetic constant (min^{-1}); Cs is the lycopene concentration on the solid surface (µg/mL); Cis the lycopene concentration(µg/mL); t refers to time (min).

Upon this base, the leaching equation can be expressed as shown in equation 3 (Turhan *et al.*, 2006).

$$C = C_s\left(1 - e^{-\frac{k_L\cdot A}{V}\cdot t}\right) \qquad (3)$$

where: volumetric mass transfer coefficient, $k_L\cdot A$ (m^3/s) can be calculated using this equation. The activation energies were evaluated from the kinetic constants, after being fitted to the Arrhenius model (equation 4).

$$k = k_o \,.\, e^{\frac{Ea}{RT}} \qquad (4)$$

where: k is the kinetic constant (min^{-1}); k_o is the frequency factor (min^{-1}), Ea corresponds to activation energy (J/mol); R is the universal constant of gases (8.31 J/mol·K); Tis temperature (K).

Lycopene diffusivity on *M. oleifera* oil was estimated by applying Ficks' second law and considering diffusion as the controlling mechanism. Due to the irregular shapes of the skin particles, they were assumed to be solids of infinite slab geometry (thickness: 0.091 mm, according to preliminary studies); while seed particles were taken as spheres (diameter: 0.1915 mm, related to a granulometric analysis). In both cases a uniform lycopene content was considered. Equations 5 and 6 show the solutions of the diffusion model for skin and seed, respectively. These equations are simplifications of the differential equation solutions based on a series with an infinite number of terms (Varzakas *et al.*, 2005).

$$\frac{C}{C_0} = \frac{8}{\pi^2}\cdot e^{\left[-\pi^2\frac{D_e}{4L^2}t\right]} \qquad (5)$$

$$\frac{C}{C_0} = \frac{6}{\pi^2}\cdot e^{\left[-\pi^2\frac{D_e}{r^2}t\right]} \qquad (6)$$

where: Cis the lycopene concentration (µg/mL); C_0 is the initial lycopene concentration; D_e refers to effective diffusivity, m^2/s; L is half-thickness of the skin particle, m; r, is the radius of the seed particle, m; tis time, min.

2.8. Statistical analysis

All experiments were carried out in triplicate. The response surface method to establish the optimal operational conditions in the experimental designs was applied. The statistical analysis was performed using Statgraphics Centurion XVII (Statistical Graphics, Rockville, MD, USA).

3. RESULTS AND DISCUSSION

3.1. Lycopene extraction

The experimental results showed good agreement with the models (Table 2). Both parameters, T and MSR, were linked to the lycopene extraction. The effect of these variables on the extraction from skin and seed is shown in Figures 3 A and C, respectively.. An increase in the lycopene concentration was achieved when MSR decreased. The influence of this variable on the extraction from skin was greater than the extraction from seeds due to the higher lycopene content of the skin.

As expected, the extraction depended on T as stated in the literature (Meireles *et al.*, 2012; Rodríguez, 2016). The same temperature-related behavior was detected in the skin and seed experiments. This behavior depended on the value of this variable (quadratic term in the models). The higher the temperature, the higher the extraction, until an inflection point was reached. A later increase in T brought about the opposite effect. The increase in T increased the solubility of the lycopene and decreased the dissolvent viscosity (Devinder, 2008; Rodríguez, 2016). Both effects favor the mass transfer from the solid matrix to solvent. However, the subsequent decrease might be associated with the degradation (oxidation) and/or isomerization reactions of lycopene when the extraction was carried out at temperatures higher than the aforementioned inflection point (Devinder, 2008; Poojary and Passamonti, 2015). It has been reported that lycopene isomerization causes a decrease in the visible-band absorption in UV-Vis spectrophotometric analysis (Poojary and Passamonti, 2015; Rodríguez, 2016). These two effects, to all appear-

Table 2. Statistical models for significant parameters of both experimental designs (p value < 0.05)

	First experimental design			Second experimental design	
Terms	Skin	Seed	Terms	Skin	Seeds
Independent term	6.6190	2.2347	Independent term	2.1019	0.6984
T: Temperature		0.0621	PS: particle size	-0.2638	-0.6214
MSR: matrix/ solvent ratio	-2.2768	-0.3580	SM: separation method	-0.1002	- 0.1285
T^2	-3.2822	-0.2771	PS^2	0.2219	0.5940
SSR^2			PS·SM	0.0680	0.0678
T· SSR	0.8797				
R^2 (%)	91.07	91.95	R^2 (%)	92.86	99.06
Mean absolute error	0.3166	0.0758	Mean absolute error	0.0629	0.0462
Durbin-Watson value	2.2371	2.1605	Durbin-Watson value	3.2354	2.1938
T optimum (ºC)	67 (-0.103)[a]	63(0.112)[a]			
SSR optimum (m/v)	1/20 (-1)[a]	1/20 (-1)[a]			
Optimum concentration (µg//mL)	7.57	2.59			

a: coded variable levels

ances, were less pronounced in seeds as a result of their lower lycopene content Lycopene content in tomato seed is approximately half that of the skin (Bailey, 2015; Baranska and Kaczor, 2016; Yilmaz *et al.*, 2017).

The optimum values for lycopene concentration (Table 2) were 7.57 µg/mL (18.9 mg/kg dry matter) and 2.59 µg/mL (6.5 mg/kg dry matter) for skin and seed, respectively. These were achieved for MSR, 1/20 (m/v) and 67 °C for skin, and 63 °C for seed.

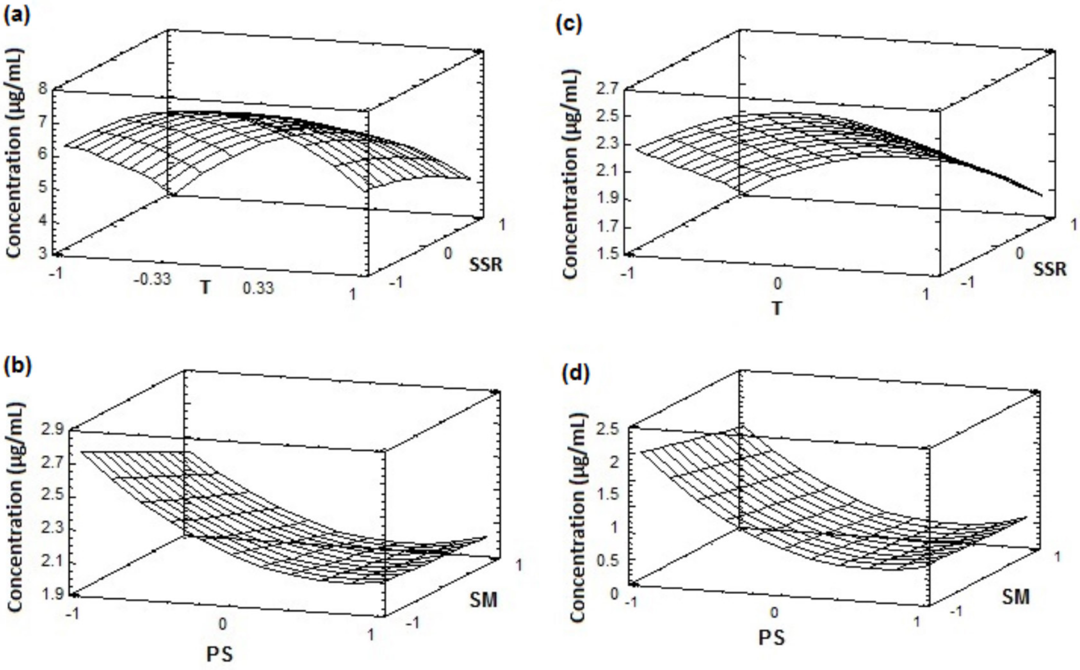

Figure 3. Surface graphics. First experimental design: a) skin; c) seed. Second experimental design: b) skin; d) seed.

The employment of *M. oleifera* oil as solvent in the lycopene extraction shows, in general terms, a similar behavior to the organic solvents described in the literature (Gámez *et al.*, 2016). The ease for lycopene to be dissolved in an oil phase was enhanced with the employment of ultrasound because of its effects up the viscosity values of the oil (Rodríguez, *et al.*, 2014; Yilmaz *et al.*, 2017; Rahimi and Mikani, 2019).

3.2. Extract separation

Table 2 (second experimental design) shows the fitted models. They describe the effect of the researched variables satisfactorily. The effects of PS and SM are shown in Figures 3 B and D for skin and seed, respectively. The lycopene concentration increased when PS decreased. Therefore, the highest concentration was achieved for the smallest size for both experimental materials. The lycopene concentration in the skin extracts, Figure 3 B, did not show an appreciable increasing trend with PS because the difference between their levels at the experimental design was lower than 1 µg/mL. The thinness of the skin could be the cause of these slight differences.

The highest lycopene concentration in both MS was reached for the smallest PS (Figures 3 B and D). This result is due to an apparent higher concentration when filtration was employed. However, errors in the absorbance measurement were detected for the smallest PS when filtration was applied. Very small particles can pass through the gauze leading to distortion in the extract absorbance. Therefore, centrifugation as SM should be used when the smallest PS is employed. At the other PS there were no differences between the SM employed, so filtration could be considered sufficient to separate the solid residues in the extracts obtained. Nevertheless, the decision to use a PS of less than 1 mm will depend on a balance between the higher lycopene recovered and the acquisition and operation costs of the centrifugal equipment. Extraction from the seed would be more complex and expensive compared to the skin, due to the milling and centrifugation to be added as additional steps in the extraction process.

The concentrations obtained in extract separation were smaller than in the extraction stage. A MSR equal to 1/25 (m/v) was applied in the extract separation.

The employment of organic solvents for the conventional extraction (CE) of lycopene was used. A mixture of hexane, acetone and ethanol in different proportions at 50 – 60 ºC has been reported. Contents of 90 and 20 mg/kg dry matter for skin and seed have been considered (Devinder, 2008). However, other authors obtained values close to 20 mg/kg dry matter. A maximum lycopene yield of 20 mg/kg dry matter in the ultrasound-assisted extraction (UAE) from skin and seed with acetone has been reported (Oreopoulou and Strati, 2011). Maximum contents of 90.1 mg/kg dry matter and 79.4 mg/kg dry matter for UAE and CE, respectively, were reported by Kumcuoglu (2014). Concentrations of 76.87 mg/kg dry matter and 57.19 mg/kg dry matter for UAE and CE, respectively, were reported by Yilmaz *et al.* (2017). The lycopene concentration ranged from 34.7 – 40.3 mg/kg dry matter (Poojary and Passamonti, 2015) using a mixture of hexane:acetone, with a lower polarity than ethanol. A comparative study of the CE from tomato waste processing using ethanol and edible soybean oil as solvents has been reported by Gámez *et al.* (2016), with recovery of 86.1 mg/kg dry matter and 25.40 mg/kg dry matter, respectively. The lower lycopene recovery when soybean oil was used can be attributed to its low polarity and consequently it is more selective (Gámez *et al.*, 2016). Nonetheless, the value reported is close to the one obtained in the extraction with *M. oleifera* oil because of the similar polarity of both oils (Gámez *et al.*, 2016; Ferrer *et al.*, 2020; Hoyos *et al.*, 2022). In spite of a smaller lycopene concentration when *M. oleifera* oil was used, its employment can contribute to keeping the pigment stability over time, besides the added value that this solvent represents (Gámez *et al.*, 2016).

It should be taken into account that lycopene content is dependent on genetic, agronomic, climatic factors and the extraction and processing conditions. These factors are present in the different results reported in the literature.

3.3. Extraction stages

Figures 4 A and B show the lycopene extraction yield for skin and seed, respectively. Extraction yields of 54, 28, 12 and 6% in the first, second, third and fourth stages, respectively, were reached from skin. In the extraction from seed, a yield of 78% was obtained in the first stage but 16, 6 and 0% in the second, third and fourth stages, respectively. This behaviour is due to the higher original lycopene con-

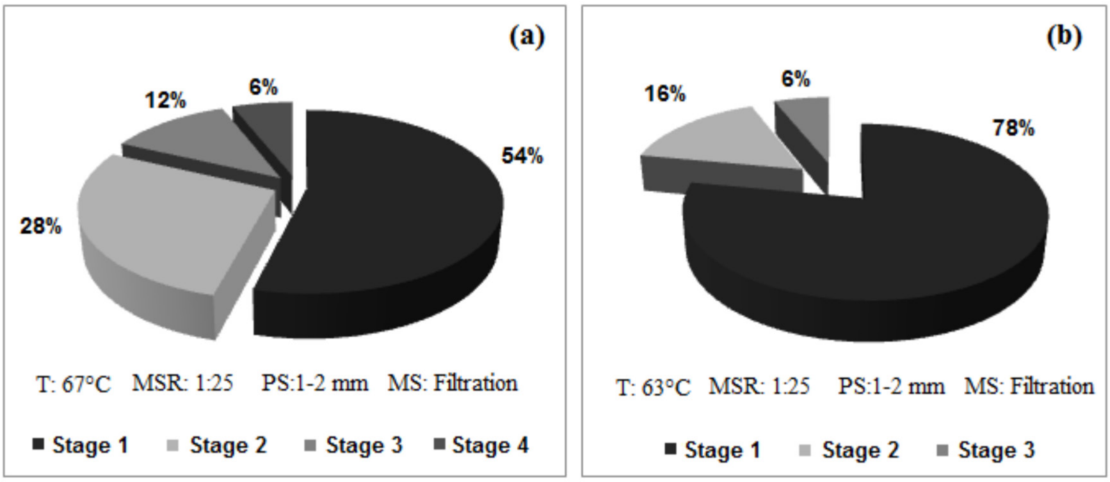

Figure 4. Lycopene yield obtained in each extraction stage for skin (a) and seed (b) (n = 3)

tent in the skin (74.32 µg/g dry matter) than the seed (35.35 µg/g dry matter). It has been proven that the lycopene content in tomato seeds is approximately half of the lycopene content in the skin (Bailey, 2015; Baranska and Kaczor, 2016; Yilmaz *et al.*, 2017). This agrees with previously reported values. The total lycopene masses obtained for both skin and seed are inside the interval for common varieties of red and yellow tomatoes (25 – 150 µg/g dry matter) (Choksi and Vishal, 2007; Bailey, 2015; Rodríguez, 2016; Yilmaz *et al.*, 2017).

3.4. Extraction kinetic

Figures 5 A and D show the behavior of lycopene concentration with extraction time at 45, 70 and 90 ºC for skin and seed, respectively. These temperatures were selected taking into account the lowest temperature (45 ºC), the highest temperature (90 ºC) and a temperature close to the optimum temperature (70 ºC). The profiles show, at first, that lycopene concentration rises rapidly because of the solvent penetration into the solid. A high concentration gradient and a quick mass transfer towards the liquid phase are caused. This increase was gradually diminished with time and the solute transfer from the solid phase was developed more slowly due to the decrease in the concentration gradient among phases (Poojary and Passamonti, 2015; Dolatabadi *et al.*, 2016). A maximum value was obtained at 60 min. and 30 min. for skin and seed (Figures 5 A and D), respectively. These times are similar to those recommended by other authors (Lianfu and Zelong, 2008; Yilmaz *et al.*, 2017). Both times contrast with the CE time of 6 – 6.5 h (data unpublished). As expected, and as stated in the literature (Kumcuoglu, 2014; Rodríguez *et al.*, 2014; Yilmaz *et al.*, 2017) ultrasound application accelerated the extraction process.

Parameters Cs and k, obtained from the fitting of the experimental data to equation 1 are shown in Table 3 for both experimental materials at the studied temperatures. The result demonstrates that the basic leaching equation describes the lycopene extraction from *M. oleifera* oil. A similar fact was reported by Poojary and Passamonti (2015) and Turhan *et al.* (2006). Valdés *et al.* (2015) also reported the satisfactory application of this model to the extraction kinetics of polyphenols from *M. oleifera* leaves. Similar results of the application of this equation for other natural products have been reported in the literature (Pineilo and Sineiro, 2006; Thurhan *et al.*, 2006).

The mass transfer coefficients obtained in this study are similar to ones reported by other authors (Varzakas *et al.*, 2005; Turhan *et al.*, 2006) and they are in correspondence with the behavior of the kinetic constant. In the interval 45 – 70 °C, Cs and k increased with the temperature increment. However, within the range 70 – 90 °C, both parameters decreased although temperature was increased. This behavior of the lycopene concentration (quadratic effect of temperature) in both materials shows what was explained in the first experimental design.

Parameters k, $k_L \cdot A$ and Cs showed a dependence with temperature, although the highest was the last one (Table 3). Firstly, all skin and seed parameters

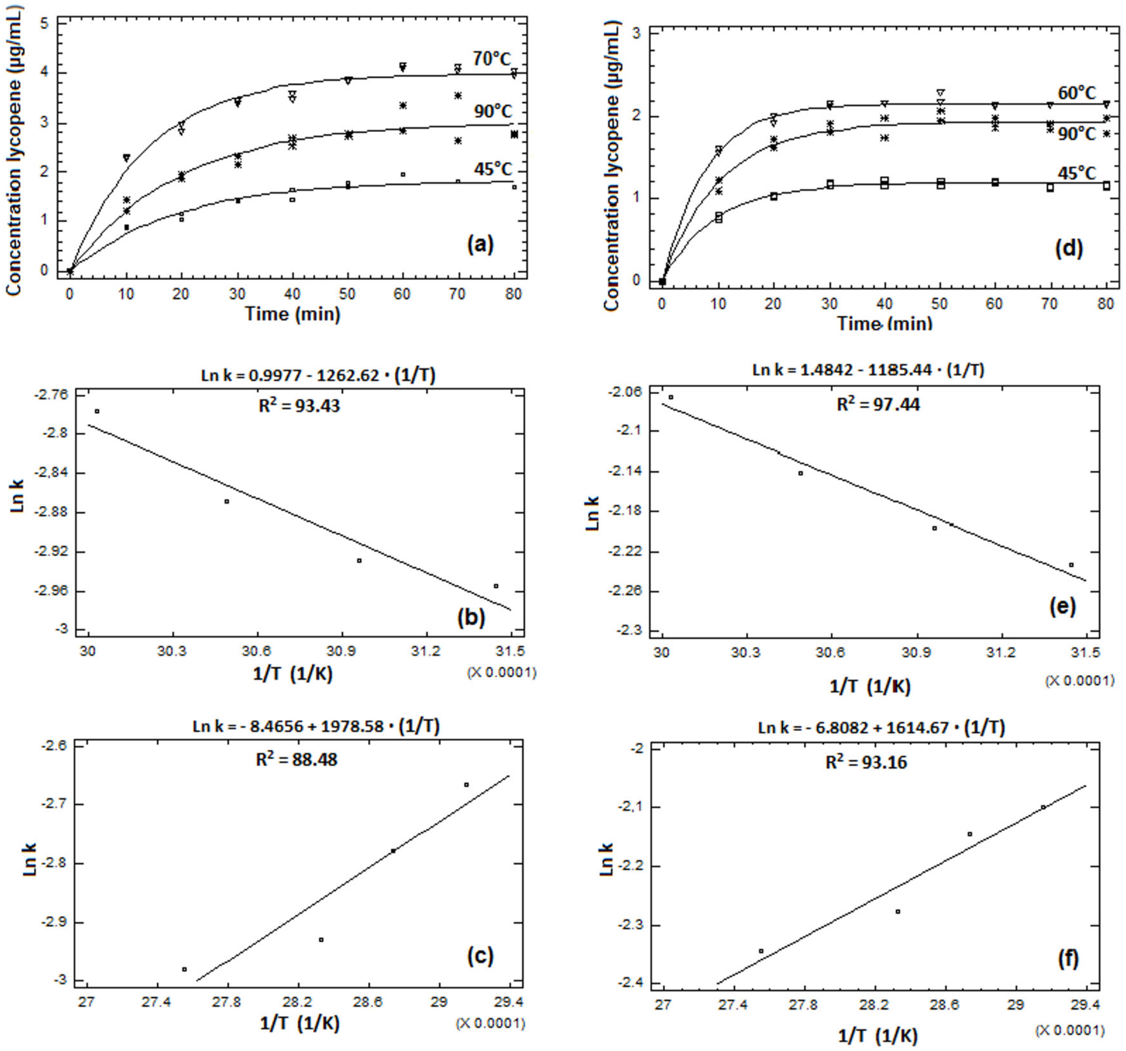

FIGURE 5. Extraction kinetics of lycopene at different temperatures for skin (a) and seed (d). Relationship among the kinetic constant and temperature: below the optimum temperature: (b) skin, (e) seed, and above the optimum temperature: (c) skin, (f) seed.

increased up to 70 °C. This value is close to the optimum extraction temperature (skin: 67 °C; seed: 63 °C). After these temperatures, a decrease in the parameters was obtained. The solubility and diffusivity of the compounds to be extracted were enhanced when temperature was increased. Moreover, the decrease in viscosity with temperature is another aspect that favored extraction. A small increase in the speed and mass transfer coefficient with temperature as a consequence of the resistance among phases has been reported by other authors (Treybal, 1997; Meireles *et al.*, 2012; Valdés *et al.*, 2015). An opposite behavior was seen after 70 ºC. The deterioration of the lycopene with temperature might be related to the decrease in such parameters (Meireles *et al.*, 2012; Rodríguez, 2016).

Although the temperature increased up to 70 ºC, it caused the increase in Cs and k and k_L·A (Table 3), and the highest increase was seen in the last one. As a consequence of the temperature increase, the lycopene leaching speed was increased. Nevertheless, the convective mass transfer showed a lower increase,

After 70 ºC, this behavior changed and it might be associated with lycopene degradation. As expected, and as stated in the literature (Varzakas *et al.*, 2005), the higher the temperature, the higher D_e (Table 3). The De value for the skin was higher than the one presented for the seeds. This could be explained by the structural differences between them. To all appearances, the lycopene transfer through the solid material predominated regarding the behavior

Table 3. Parameters of the kinetic model, volumetric coefficient of mass transfer and effective diffusivity at different extraction temperatures (n = 3)

Temperature (°C)	45	50	55	60	70	75	80	90
Skin								
Cs (µg/mL)	1.8607	1.9820	2.9920	3.9758	4.0042	3.3263	3.1142	3.0297
k (min^{-1})	0.0520	0.0534	0.0567	0.0622	0.0696	0.0621	0.0533	0.0508
R^2 (%)	95.45	96.08	97.22	96.77	97.51	88.49	94.86	92.29
Mean absolute error	0.0856	0.0803	0.1046	0.1605	0.1304	0.2427	0.1723	0.1561
$k_L \cdot A$ (m^3/s)	0.0399	0.0414	0.0423	0.0433	0.0584	0.0359	0.0338	0.0276
$D_e .10^{-11}$ (m^2/s)	3.6619	3.8299	4.8714	4.9050	4.9722	5.0394	5.1401	5.5097
Ea (kJ/mol)	10.49				16.44			
Seed								
Cs (µg/mL)	1.1842	1.3618	1.7832	2.1613	2.1692	2.1717	2.0062	1.9286
k (min^{-1})	0.1067	0.1113	0.1176	0.1268	0.1227	0.1172	0.1026	0.0959
R^2 (%)	98.98	98.43	98.96	99.38	98.87	97.76	98.87	97.29
Mean absolute error	0.0234	0.0320	0.0356	0.0278	0.0493	0.0631	0.0428	0.0663
$k_L \cdot A$ (m^3/s)	0.0399	0.0421	0.0467	0.0499	0.0428	0.0385	0.0351	0.0347
$D_e .10^{-11}$ (m^2/s)	0.9671	1.0971	1.1160	1.1301	1.1810	1.2500	1.3483	1.4600
Ea (kJ/mol)	9.85				13.42			

of Cs. In general terms, D_e values were in the range reported for other agro-food products (Varzakas *et al.*, 2005; Pineilo and Sineiro, 2006).

Although the same behavior with respect to temperature was seen in both skin and seed, a higher increment was obtained in the skin. It might be related to the structural characteristics of these materials. In leaching operations, the leachable solids are contained in a framework of insoluble solids. This restricts the diffusion process and affects the rate of diffusion. This barrier associated with the solid structure provides the dominant resistance (Varzakas *et al.*, 2005). Therefore, the structure of the skin would favor higher lycopene mobility than the seed structure.

Figures 5 B and E show the relation of k with temperature when it was increased up to the optimum value, for skin and seed, respectively. Figures 5 C and F correspond to the temperature variation from the optimum value up to 90 ºC for skin and seed, respectively. In both intervals a good adjustment to the Arrhenius equation (equation 4) was provided.

The knowledge of k, $k_L \cdot A$ and D_e and their relation is very useful information for designing and assessing leaching processes.

The Ea was 10.49 kJ/mol and 9.85 kJ/mol at 45 – 60 °C, for skin and seed, respectively. In the interval 70 – 90 °C, its value was 16.44 kJ/mol and 13.42 kJ/mol for skin and seed, respectively. These values indicate that the lycopene extraction from skin was more dependent on temperature than from seed. The relatively high values for Ea imply that a small temperature change was sufficient to affect the kinetic constant and therefore the speed of the extraction process (Turhan *et al.*, 2006). In both cases, the process was controlled by the diffusion mechanism in the interval of 45 – 70 °C because the activation energy was smaller than 12 kJ/mol. However, in the interval 70 – 90 °C the activation energy was higher than 12 kJ/mol and this is an indicator of chemical processes such as degradation or isomerization (Pineilo and Sineiro, 2006; Turhan *et al.*, 2006; Valdés *et al.*, 2015; Cruz, 2016). This result supports the behavior of Cs, k and $k_L \cdot A$ with temperature taking into account the effect of this parameter on the lycopene concentration.

4. CONCLUSIONS

The experimental values showed good agreement with the basic leaching equation. The optimal

T and MSR (skin: 67 ºC and 1:20; seed: 63 ºC and 1:20) were determined. Moreover, from an operational point of view, PS and SM (skin: 1–2 mm and filtration; seed: < 1 mm and centrifugation) are recommended. The greater the kinetic constant the higher the volumetric coefficient of mass transfer will be. They increased up to the optimum temperature of extraction. A decrease in these parameters with a subsequent increase in temperature was observed. This behavior may be linked to lycopene degradation, which is, in all appearances, the predominant effect of the highest temperatures. The activation energy values support this statement. On the other hand, the effective diffusivity of lycopene in all range of temperature was increased in both materials. The seed showed lower values than the skin taking into account its structural characteristics. The major lycopene concentration was obtained to the optimum values of the investigated variables. The terms associated with mass transfer can be employed for designing and evaluation the lycopene recovery process by employing *M. oleifera* oil as solvent.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e520
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0565221

Rapid detection of argan oil adulteration by frying oils using laser induced fluorescence spectroscopy combined with chemometrics tools

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Submitted: 26 May 2022; Accepted: 07 February 2023; Published online: 10 October 2023

SUMMARY: There is a contentious need for robust and rapid methodologies for maintaining the authenticity of foods. The aim of this study was to detect and quantify argan oil adulteration using Laser Induced Fluorescence (LIF) spectroscopy coupled with chemometric methods. Principal Component Analysis (PCA) and Partial Least Squares Regression (PLSR) were used to assess argan oil authenticity; PCA was used to classify samples according to their quality and the PLS model to determine the amount of adulterants in pure argan oil. The correlation coefficient of the obtained model was about 0.99, with Root Mean Square Error of Prediction (RMSEP) and Standard Error of Prediction (SEP) of 2%. This study demonstrated the feasibility of LIF spectroscopy combined with chemometric tools to identify adulterants in pure argan oil from a percentage of adulteration, of 0.35 % without the need to destruct samples.

KEYWORDS: *Adulteration; Argan oil; Chemometrics; LIF.*

RESUMEN: ***Detección rápida de la adulteración de aceite de argán con aceites de fritura usando espectroscopía de fluorescencia inducida por láser combinada con herramientas quimiométricas***. Existe una necesidad de metodologías sólidas y rápidas para determinar la autenticidad de los alimentos. El objetivo de este estudio es detectar y cuantificar la adulteración del aceite de argán mediante espectroscopia de fluorescencia inducida por láser (LIF) junto con métodos quimiométricos. Se utilizaron el análisis de componentes principales (PCA) y la regresión de mínimos cuadrados parciales (PLSR) para evaluar la autenticidad del aceite de argán. Se utilizó PCA para clasificar las muestras según su calidad y el modelo PLS se aprovechó para determinar la cantidad de adulterantes en el aceite de argán puro. El coeficiente de correlación del modelo obtenido fue de alrededor de 0,99, el error cuadrático medio de la predicción (RMSEP) y el error estándar de predicción (SEP) del 2%. Este estudio demostró la viabilidad de la espectroscopia LIF combinada con herramientas quimiométricas que permiten identificar adulterantes en aceite de argán puro, sin necesidad de destruir muestras, a partir de un porcentaje de adulteración del 0,35 %.

PALABRAS CLAVE: *Aceite de argán; Adulteración; Quimiometría; LIF.*

Citation/Cómo citar este artículo: Farres S, Srata L, Chikri M, Addou S, Fethi F. 2023. Rapid detection of Argan oil adulteration by frying oils using laser induced fluorescence spectroscopy combined with chemometric tools. *Grasas Aceites* **74** (3), e520. https://doi.org/10.3989/gya.0565221

1. INTRODUCTION

Food fraud is when a food is not presented in its authentic form. It can present serious health risks if hazardous materials are added to food products. It can also have an economic impact on consumers for investing in products of inferior quality (Charlebois *et al.,* 2016). Over the last decade, food fraud has been identified as an emerging risk to the food industry and a significant concern to consumers and food regulators (Ulberth, 2020). Among the foods that are subject to fraud is argan oil. This oil is very valuable thanks to its virtues and high price (Ruas *et al.,* 2015). It is one of the oils that has been frequently subjected to adulteration. Sometimes suppliers can adulterate pure argan oil using a low percentage degree of cheap vegetable oils, precisely frying oils, to give acceptable analytical values that can give a favorable quality assessment to the adulterated oil as pure oil. Argan oil is obtained from the fruit of the argan tree. This tree is native to southwestern Morocco, which covers an area of ~829000 ha. Its life span frequently exceeds 200 years (Gonzalez-Fernandez *et al.,* 2020). Moreover, there is a Moroccan regulation, developed in 2003, that manages argan oil according to NM 08.5.090 standards (Moroccan Standard 2003).

This oil is well known for its pharmacological properties and has been used in traditional medicine for centuries. Scientific evidence has inferred from experimental studies that the consumption of argan oil may reduce the risk of disease through a biological mechanism which acts on blood pressure, plasma lipids and antioxidant status (El Midaoui *et al.,* 2016). Thanks to its composition which is rich in antioxidants and monounsaturated and polyunsaturated fatty acids, argan oil could be used in a nutritional prevention setting to prevent the progression of some diseases (ELMostafi *et al.,* 2020). These different actions are ensured by its interesting chemical composition, rich in antioxidants, such as vitamin E, and in particular in gamma tocopherols, as well as the presence of specific polyphenols and sterols (Şekeroğlu *et al.,* 2017).

The demand for high quality and safety in food production obviously calls for high standards of quality and process control (Kharbach *et al.,* 2021), which in turn requires appropriate analytical tools to investigate food. The above discussion clearly gives the importance of finding a way to detect adulteration, so that the quality of argan oil is guaranteed (Kharbach *et al.,* 2021). There are conventional methods used in the detection of adulteration including gas chromatography (Xing *et al.,* 2019), electronic nose and tongue technology (Majchrzak *et al.,* 2018), and UV photo-ionization ion mobility spectrometry (Garrido-Delgado *et al.,* 2018), TR-FTIR spectroscopy (Ozulku *et al.,* 2017), among others. These methods provide high efficiency and sensitivity, and can measure multiple components. However, they are time consuming, expensive and complicated. That is why there is high demand for a sensitive technique that can provide result in less time, without generating waste, at low cost, and without the need for super-qualified personnel. Analytical methods are an important point to consider for both detecting and deterring food fraud (Callao and Ruisánchez, 2018). Thus, the need for rapid, low-cost and confinable analytical methods has motivated the use of spectroscopic techniques associated with chemometrics to characterize oils and fats (Dogruer *et al.,* 2021).

Chemometric methods use multivariate statistic to extract information from complex analytical data (Yang *et al.,* 2005). However, LIF was highlighted as a potential analytical technique for oils and fat characterization because it meets all routine analysis requirements and appears to be the best technique for analyzing oil adulteration. This technique now has the potential to replace or complement classical methods, and a number of works have been reported for detecting vegetable oil adulteration (Addou *et al.,* 2016; Wang and Wan, 2020). LIF is a sensitive, fast, less expensive and non-polluting method of analysis, increasingly used in various fields of product analysis, and food industry to assess quality. It provides information on the presence of fluorescent molecules and their molecular environment within the samples analyzed (Ozaki *et al.,* 2013). Indeed, the fluorescent properties of molecules are very sensitive to changes in their environment. The answer then varies according to the composition and characteristics of the material (Karoui and Blecker, 2011).

In this paper, LIF has been used to determine argan oil adulteration with waste frying oil. A LIF system was assembled in the experiment. Using an Nd: YAG laser beam (532 nm), the fluorescence spectra of mixtures of pure and adulterated argan oil were measured. The identification of several important vegetable oils and the adulterated concentration were achieved by employing PCA (Chikri *et al.,* 2018; Tipping and Bishop, 1999) and PLS model (Wold *et al.,* 2001; Srata *et al.,* 2019). The prediction errors of adulterated concentration were about 2%. The results can provide reference for the quality identification of argan oil, knowing that the adulteration was carried out using two kinds of adulterants.

2. MATERIALS AND METHODS

2.1. Sample preparation

In this work, argan oil, noted AO, was used to assess its adulteration by two commercial vegetable oils noted VO1 and VO2. VO1 is commercial sunflower oil and VO2 is commercial edible oil sold without any indication of its origin. Cheap vegetable oils (VO1 and VO2) were purchased from a market in Oujda (East of Morocco) and pure argan oil (handmade) was obtained from Agadir (south of Morocco).

The samples were divided into two groups. The first one contained samples of AO adulterated by VO1 (54 samples), and the second one contained AO adulterated by VO2 (55 samples). Cheap oils were heated at 200 °C for 30 minutes many times, until the oil had the same color as AO. VO1 and VO2 were heated for the reason that fraudsters use waste frying oil to adulterate argan, since this oil has the same color as argan oil and the detection of adulteration becomes more difficult. The process of adulteration was achieved as follows: AO was adulterated by VO1 from 0 to 31% and by VO2 from 0 to 32%. A total of 109 samples were prepared. The first sample in each group contained 100% AO and the second one contained 99.6% AO and 0.4% adulterant (VO1 or VO2). The procedure was repeated for all samples, increasing the number of drops of adulterant for each sample by one drop. Therefore, for each sample, a drop of pure AO oil was replaced by a drop of adulterant. The drops were added using a micropipette and masses were measured using a digital scale of very high sensitivity. Finally, the prepared samples were homogenized and stored in the dark at ambient temperature.

2.2. LIF analysis

LIF is a physical phenomenon in which a molecule absorbs an amount of the energy from a laser beam. There is thus a transition from a ground state S_0 to an excited state S_1 with a change in the electron orbital. This excited state S_1 has a very short lifetime (a few nanoseconds). Changes in conformation and interactions with surrounding molecules change the molecule from the excited state S1 to low vibrational levels of S_1: this is the internal conversion. In the case of fluorescent molecules, the transition from the excited state S_1 to the ground state S_0 takes place with the release of a photon of lower energy. This phenomenon is laser induced fluorescence, which occur at wavelengths greater than the incident excitation wavelength (Mazouffre 2009). The LIF system used in this work was an assembled system. The spectral measurement of the LIF technique was obtained by irradiating the samples with an Nd: YAG laser beam (532 nm) set at 2 mW, with 3.3 V DC operating voltage and 0.4 A current. During irradiation, the fluorescence emitted by the samples was collected by an optical fiber (2 m long and 400 µm in diameter), equipped with an SMA type connector at each end. The axis of the optical fiber was positioned at an angle of 90° to the direction of the laser beam in order to collect as much fluorescent signal as possible and avoid the detection of photons from the incident beam. The beam was dispersed by a spectrophotometer Avantes, model USB2000, grating 600 lines, blazed at 750 nm, L2 lens, 100 µm slit, equipped with a 2048-pixel charge coupled detector CCD. It consisted of a linear array of silicon (Si) diode and was linked to a computer by a USB connection in order to visualize the fluorescent spectra of each sample. The spectrophotometer used in this work covered the spectral range 500-1000 nm. Note that the spectra were performed in three replicates, under the same conditions. Analyses were made using the average.

2.3. Data processing

The obtained spectral data were converted into Microsoft Office Excel format for Matlab software analysis. The spectral range was reduced from 500-1000 nm to 540-750 nm. Then, to reduce noise and baseline shifts, the spectra were corrected using preprocessing, whose objectives were the attenuation of non-linearity between variables, the elimination of interference and reduction of random noise (Verboven *et al.,* 2012).

Before multivariate data analysis, all LIF spectra were subjected to Savitzky-Golay smoothing (1 point, 2 orders) then the spectra obtained were subjected to a Multiplicative Scatter Correction (MSC) combined with a second derivative for PCA analysis and combined to Baseline for PLS modeling (Savitzky and Golay, 1964; Zheng *et al.,* 2015). MSC is a diffusion correction method that was introduced in 1983 and developed in 1985 (Guidetti *et al.,* 2012). It is based on the idea of correcting the level of dispersion of all sample spectra from an “ideal” spectrum, which is usually the average spectrum. The concept behind MSC is that artifacts or imperfections will be removed from the data matrix (spectra) prior to modeling. The MSC consists of two steps:

- Estimation of the correction coefficients (additive and multiplicative contributions): Each spectrum x_j^{LIF} is then estimated with respect to the average spectrum of all the spectra considered $\bar{\mathrm{x}}^{\overline{\mathrm{LIF}}} = \sum_{\mathrm{j}=1}^{\mathrm{J}} \frac{\mathrm{x}_\mathrm{j}^{\mathrm{LIF}}}{\mathrm{j}}$ by a method of least squares.

$$x_j^{LIF} = a_j + b_j \overline{x^{LIF}} + e_j$$

- Correction of recorded spectrum:

$$\mathrm{x}_{\mathrm{MSC\,j}}^{\mathrm{LIF}} = \mathrm{x}_\mathrm{j}^{\mathrm{LIF}} - \frac{\mathrm{a_j}}{\mathrm{b_j}} = \bar{\mathrm{x}}^{\overline{\mathrm{LIF}}} + \frac{\mathrm{e_j}}{\mathrm{b_j}}$$

e_j: represents the spectrum of the residues
$x_{MSC\ j}^{LIF}$: corrected spectrum
$\mathrm{a_j}$ and $\mathrm{b_j}$ are the correction coefficients that can be estimated by a least squares method.

The first and second derivatives are used for baseline variation reduction and separation of overlapping bands, so hidden bands are enhanced. With regards to baseline preprocessing, most correction methods make the supposition that the observed spectrum is the combination of a useful signal and a signal of uncontrolled variation. Therefore, the correction consists of subtracting the background from the obtained signal.

2.4. Data analysis

The wavelength range used for LIF analysis was reduced to 540 - 750 nm to keep only the part that contains relevant information and to eliminate noise. PCA was exploited to get main information from spectra and reduce the number of variables. Then PLS algorithm was applied on LIF spectra to establish a model that can predict the percentage of adulteration. PLS calibration gives optimum results compared to many other multivariate calibration methods. An important aspect of PLSR is that it collects the relevant spectral information in a few linear combinations of the spectral measurements. These combinations or components can be used to facilitate interpretation of the relationship between concentrations and spectra as well as the relationships among the spectral variables themselves.

The samples were divided into calibration/validation and prediction datasets. The optimum number of latent variables was obtained using the full cross validation method. The prediction performance of each model was evaluated throughout the root mean square error (which represents the standard deviation of the residuals), the prediction standard errors, and the coefficient of determination (R^2) of both calibration and validation data sets. In general, as low as the RMSEC/P and SEC/P values can be, and R^2 as close as possible to 1, the better the model’s predictions will be. Equations corresponding to each parameter are:

$$\mathrm{R} = \frac{\frac{\sum_\mathrm{i}(\mathrm{y_i} - \mathrm{y})(\mathrm{y'_i} - \mathrm{y_i})}{\mathrm{N}-1}}{\sqrt{\frac{\sum_\mathrm{i}(\mathrm{y'_i} - \mathrm{y'})}{\mathrm{N}-1}} - \sqrt{\frac{\sum_\mathrm{i}(\mathrm{y_i} - \mathrm{y})}{\mathrm{N}-1}}}$$

$$\mathrm{RMSEC} = \frac{1}{\mathrm{I_C}} \sum_{\mathrm{i}=1}^{\mathrm{I_C}} (\hat{\mathrm{y}}_\mathrm{i} - \mathrm{y_i})^2$$

$$\mathrm{SEC} = \sqrt{\frac{\sum_{\mathrm{i}=1}^{\mathrm{i_p}} (\hat{\mathrm{y}}_\mathrm{i} - \mathrm{y_i})^2}{\mathrm{I_c} - 1}}$$

$$RMSEP = \frac{1}{I_P} \sum_{i=1}^{I_P} (\hat{y}_i - y_i)^2$$

$$\mathrm{SEP} = \sqrt{\frac{\sum_{\mathrm{i}=1}^{\mathrm{i_p}} (\hat{\mathrm{y}}_\mathrm{i} - \mathrm{y_i} - \mathrm{bias})^2}{\mathrm{I_p} - 1}}$$

$$\mathrm{Bias} = \frac{\sum_{\mathrm{i}=1}^{\mathrm{I_p}} (\hat{\mathrm{y}}_\mathrm{i} - \mathrm{y_i})^2}{\mathrm{I_p}}$$

Where:
$\hat{y}_i$ = the predicted value of the ith observation.
y_i = the measured value of the ith observation.
I_C = number of observations in the calibration set.
I_P = number of observations in the validation set.

3. RESULTS AND DISCUSSION

3.1. Spectral characteristics

Figure 1 shows an overlay of LIF spectra of AO, VO1 and VO2 oils. The spectrum of VO2 oil showed emission at around 650 - 700 nm, This emission was due to the fluorescence of chlorophyll compounds. The AO spectrum showed a fluorescent band around 550 - 625 nm, although this emission was practically absent from the VO1 and VO2 spectra. Figure 2 represents LIF spectra of the same samples after heating VO1 and VO2. The spectra showed the same shape with different intensity. In this figure, the absence of the peak in the red region 650 - 700 nm could be observed, which means the absence of chlorophyll compound emission. It can be concluded from Figures 1 and 2 that heating produced the degradation of the Chlorophyll.

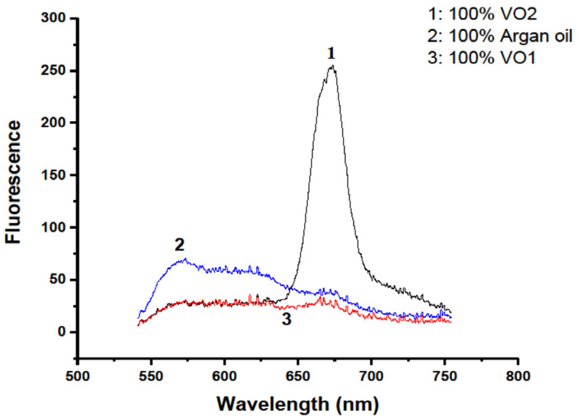

Figure 1. Laser induced fluorescent spectra of pure argan oil, VO1 and VO2 samples before heating without any pretreatments. VO: Commercial vegetable oil.

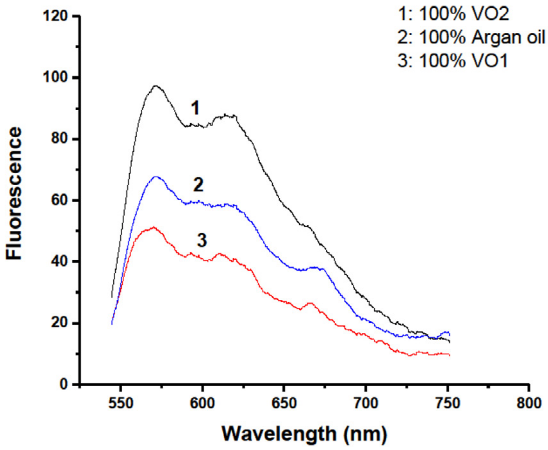

Figure 2. Laser induced fluorescent spectra of argan oil, VO1 and VO2 samples after heating without any pretreatments. VO: Commercial vegetable oil.

Figure 3 shows seven spectra in the spectral range of 544 - 750 nm. The first spectrum correspond to AO's fluorescence and the other spectra represent the fluorescence of the adulterated samples, showing the tendency of the spectra according to the concentrations of adulterant. It can be seen from Figure 3 that the spectra are similar to each other and they are a bit noisy. A vertical baseline shift and intensity difference between the spectra can also be seen. Actually, when an oil sample is adulterated, whether the amount of adulterant is small or large, the whole spectrum of the pure sample is affected. These deformations are corrected using chemometric pretreatments. In order to reduce variability in the spectra and improve the signal-to-noise ratio, the spectra were fitted using smoothing preprocessing and then MSC combined to the second derivative (Savistky - Golay) was applied as a suitable preprocessing for PCA analysis and MSC combined to the baseline for PLS analysis. After the spectra were pretreated, chemometric methods were used to extract hidden information.

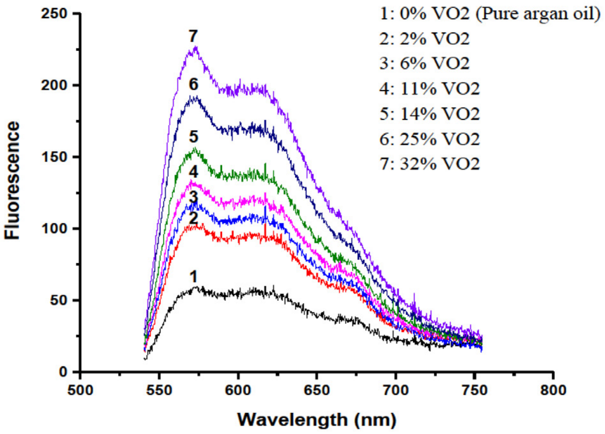

Figure 3. Laser induced fluorescent spectra of some adulterated argan oil samples showing the tendency of spectra according to adulterant concentrations from 0 to 32% in the spectral range 540 –750 nm.

3.2. PCA results

After preprocessing the spectra using smoothing combined to MSC and the second derivative, PCA was performed to explore the similarities and differences in samples, to extract relevant information and reduce the number of variables. Figure 4 presents the score plot of PCA applied to LIF spectra. A total 109 samples were measured in this work. The first two principal components accounted for approximately 73% of the total variability in the data. PC1 explained 72% of the total variance and 1% of the information was explained according to PC2. The interpretation of the results was made via loading plots; they describe how much each variable contributes to a particular principal component. Large loadings (positive or negative) indicate that a particular variable has a strong relationship to a particular principal component. There was a strong relationship between noise in the loadings and the optimal number of components. The concepts of underfitting, overfitting and optimal prediction ability have an important connection to the shape of loading plots, which can be seen when plotting loadings versus wavelength number for all the components computed. For the first few components corresponding to the largest eigenvalues, the shapes of these plots looked like spectra, but as we went towards the components with smaller eigenvalue, we introduced more and more noise (overfitting) and this is sometimes clearly seen as ripples and irregularities in the loading plots. There was a tendency, that close to the optimal number of components, the noise started to become clearly visible in these plots.

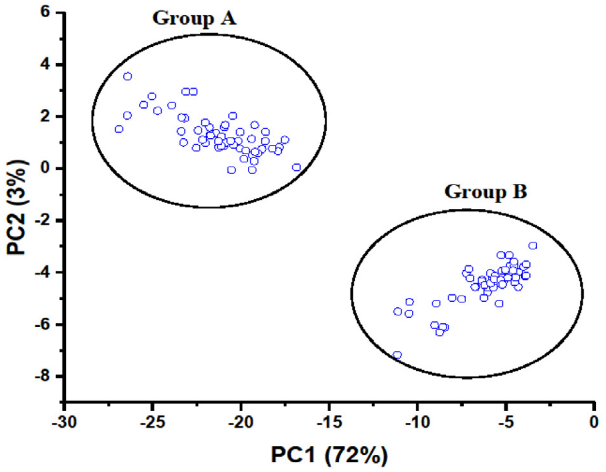

FIGURE 4. Bi-dimensional score plots of PC1 and PC2 vectors of 109 adulterated argan oil samples. Group A: Argan oil samples adulterated by VO1. Group B: samples of Argan oil adulterated by VO2. PC: Principal component, VO: Commercial vegetable oil.

After applying PCA to the pretreated spectra, samples were significantly classified. It can be seen from Figure 4 that PCA can distinguish samples from each other successfully. Adulterated argan oils are different after passing through the PCA process, and it can be seen from the figure that objects were dispersed according to a two-dimensional space (PC1 and PC2) and divided into two sets according to the type of adulterants. The lower right group in the score graph consists of AO samples adulterated with VO2, noted group B and the higher left group consists of AO samples adulterated with VO1, noted group A. By projecting samples according to PC1, it can be easily noticed that the first component contained information which was responsible for the separation between samples; a low separation existed by projecting samples according to PC2. Group B is located in the negative part of PC2 and group A is located in the positive part of PC2. It can be concluded that, by this model, it was possible to determine the type of adulterant for an unknown sample by taking its spectrum and injecting it into this model.

3.3. PLS result

PLS was exploited as a multivariate calibration technique. It constructs a mathematical model based on the features of PCA and multiple regression to find a linear mathematical relationship between two datasets, X (spectra) and Y (level of adulteration) (Morsy and Sun, 2013).

The spectral data were arranged in a 2D matrix (X), the rows of this matrix represent the samples (109 samples) and the columns contain the number of variables. One column vector (Y) containing the concentration of each adulterant was added to this matrix and the data were then analyzed. To make sure that the obtained model was neither over nor underfit, a cross validation using the leave-one-out method was considered. The linearity of the regression model was evaluated by fitting the reference adulteration value against the predicted ones.

The above PCA model successfully identified the type of cheap edible oil added to AO. To further predict the contents of VO1 and VO2 in the blended oil samples, PLS regression was performed. This method has been successfully used in several studies to predict the percentage of adulterants in oils. PLS regression was applied on raw and pretreated spectra and the one that gave the best result was kept. In this work, LIF data were preprocessed by taking a smoothing (1 point) combined to MSC and Baseline. After removing the outliers, the set of samples was randomly divided into three sample sets samples, in which two sets were used as the experimental group, calibration/validation sets, containing almost 85% of the total set of samples (70% calibration, 15 % validation) and one set as the testing group containing the 15% remaining.

The efficiency of the calibration model can be determined generally from three statistical parameters: the correlation (R), the standard error of calibration (SEC) and the mean square error of calibration (RMSEC). a R value greater than 0.9 indicates a good response for the parameter studied and less than 0.7 indicates a poor response. When a new spectrum is inserted into the matrix, it is compared to the spectral basis, the closest spectrum is then used to make a PLS regression.

The graphic display of the calibration/validation and prediction produced using the PLS model with the best performances is shown in Figure 5. The calibration results of 109 samples using PLSR produced excellent overall models with a correlation greater than 0.99 and low values for RMSEC and SEC. The same was true for the predicted model. It gave excellent statistical values with a correlation of 0.92, RMSEP and SEP of about 2%. The results indicate that the method proposed in this work is feasible for the detection and quantification of argan oil adulteration by cheap vegetable oils. Table 1 shows all the statistical results of the calibration and prediction models. A similar study was

Table 1. Results of the PLS regression model for the LIF data matrix.

Calibration			Prediction		
Correlation	SEC	RMSEC	Correlation	SEP	RMSEP
0.99	2.25	2.23	0.92	2.40	2.38

SEC: Standard Error of Calibration, RMSEC: Root Mean Squares Error of Calibration, SEP: Standard Error of Prediction, RMSEP: Root Mean Squares Error of Prediction. Each value in the table represents the result of three repeated measurements.

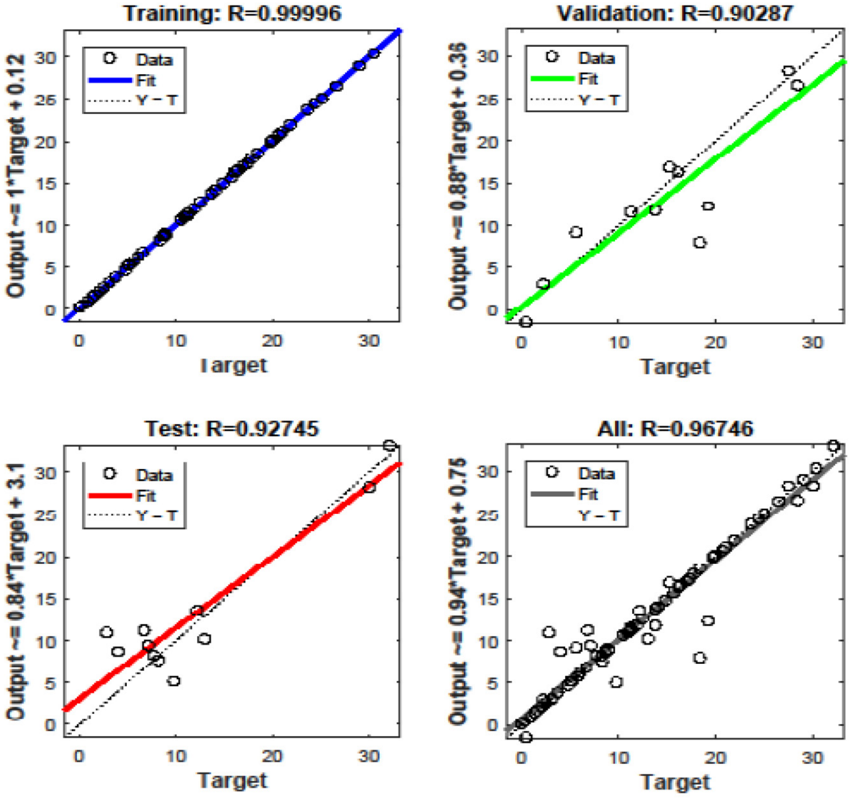

Figure 5. Training, validation and testing models of 109 samples in the spectral range 540 –750 nm. The model shows the best prediction accuracy.

published using near infrared spectroscopy to evaluate the adulteration of argan oil by cheap oils (Farres *et al.,* 2019). When comparing the results obtained in this work to those obtained in the published study, it can be easily seen that the R value is higher and error values are low. Therefore, it can be concluded that LIF spectroscopy was revealed to be most suitable technique to study the adulteration of argan oil, especially since both techniques were carried out on the same samples. Table 2 groups together the results obtained using near infrared spectroscopy (published work) and laser-induced fluorescence spectroscopy.

Table 2. Statistical parameters of PLS regression models for the adulteration of argan oil using two different spectroscopic methods.

	Laser Induced Fluorescence	**Near Infrared Spectroscopy**
Correlation	0.99	0.92
SEC	2.25	3.22
RMSEC	2.23	3.24

SEC: Standard Error of Calibration, RMSEC: Root Mean Squares Error of Calibration.

To prove the efficiency of these models, they were tested on two non-synthetic samples and the results are shown in Figure 6. Two non-synthetically adulterated samples were inserted into the PCA model, and as can be seen from the figure, one of samples was close to group A, and the second one was close to group B. This mean that the first sample was adulterated by VO1 and the second one with VO2.

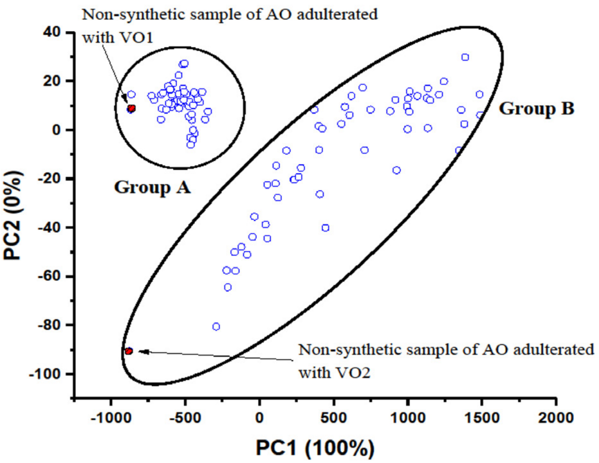

Figure 6. Bi-dimensional score plots of PC1 and PC2 vectors of 111 adulterated argan oil samples indicating the localization of non-synthetic samples.

PC: Principal Component.

4. CONCLUSIONS

Currently, spectroscopy techniques for food authentication are important in the food industry. In this work, it has been demonstrated that LIF spectroscopy in combination with chemometric methods (PCA and PLS) can be used as fast and nondestructive methods for the rapid detection of AO adulteration with different concentrations of cheap vegetable oil, VO1 and VO2. 54 samples were prepared containing AO adulterated by VO1 and 55 samples of AO adulterated by VO2.

PCA was applied to show the existence of spectral differences and discriminate spectral data in relation with the adulteration of argan oil with cheap vegetable oils. Samples were divided into two well-separated groups, easily allowing for the determination of the type of adulterant. It was important to use the combination of smoothing, MSC and second derivative as adequate spectral preprocessing to eliminate noise and any information that could skew the results. Then PLSR model was used to predict the amount of adulterant in argan oil. Before applying PLSR, the spectra were corrected using smoothing combined with MSC and baseline. Analyses were made on 109 samples where 70% were randomly chosen to make the calibration model, 15% for the validation and the remaining 15% for prediction sets. The calibration results produced excellent models with a correlation of 0.99, RMSEC and SEC of about 2% and for the prediction model, the correlation obtained greater than 0.92 RMSEP and about 2% SEP. The obtained models were tested using non-synthetic samples and the results were satisfactory. New samples were successfully classified according to the type of adulterant.

This study provided valuable results which could be applied to consumer protection, because the demand for high quality and safety in food production obviously calls for high standards of quality and process control. Less than 1 second LIF analysis by this model can detect the amount of adulterant in argan oil from 0.35%.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e521
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0452221

Seasonal effect on fatty acid composition in phospholipid classes and triacylglycerols of male *Capoeta umbla*

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Submitted: 30 April 2022; Accepted: 06 February 2023; Published online: 10 October 2023

SUMMARY: The seasonal changes in muscle tissue, total lipids and fatty acid composition of phospholipids (PL), triacylglycerol (TAG), and phospholipid classes of male *Capoeta umbla* were investigated in this study. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) were identified as the major phospholipids (PLs) in the muscle tissue (PS). Triacylglycerols showed high contents of MUFA, 14:0, 16:1n-7, 18:1n-9, 18:2n-6, and 18:3n-3; while phospholipids presented high contents of AA, DHA, and 18:0. Myristic acid, 16:1n-7, 18:1n-9, monounsaturated fatty acids (MUFA), linoleic acid (18:2n-6), and linolenic acid (18:3n-3) were shown to be present in larger concentrations in TAG than in PL classes. In PL classes, 16:0, 18:0, arachidonic acid (20:4n-6) (AA), eicosapentaenoic acid (EPA) (20:5n-3), docosahexaenoic acid (DHA) (22:6n-3) and polyunsaturated fatty acid (ΣPUFA) were higher than the TAG fraction. The fatty acid composition of total lipid and lipid classes (TAG and PL) were affected by the seasonal variations and lipid fraction.

KEYWORDS: Capoeta umbla; *Phospholipid classes; Seasonal fatty acid composition; Triacylglycerol.*

RESUMEN: ***Efecto estacional sobre la composición de ácidos grasos de fosfolípidos y triacilgliceroles de machos de* Capoeta umbla.** En este estudio se investigaron los cambios estacionales en el tejido muscular, la composición total de lípidos y ácidos grasos de fosfolípidos (PL), triacilglicerol (TAG) y clases de fosfolípidos de *Capoeta umbla* machos. Fosfatidilcolina (PC), fosfatidiletanolamina (PE), fosfatidilinositol (PI) y fosfatidilserina (PS) se identificaron como los principales fosfolípidos (PL) en el tejido muscular (PS). Los triacilgliceroles tenían un alto contenido de 14:0, de los MUFA 16:1n-7 y 18:1n-9 y de poliinsaturados 18:2n-6 y 18:3n-3, mientras que los fosfolípidos tenían un alto contenido de araquidónico (20:4n-6) (AA), docosahexaenoico (22:6n-3) (DHA) y 18:0. Se demostró que el ácido mirístico, los monoinsaturados (MUFA) 16:1n-7 y 18:1n-9, el ácido linoleico (18:2n-6) y el ácido linolénico (18:3n-3), están presentes en concentraciones superiores en TAG que en los PL. En las diferentes clases de PL, los ácidos 16:0, 18:0, AA, eicosapentaenoico (20:5n-3) (EPA), DHA y la suma de ácidos grasos poliinsaturados (ΣPUFA) fueron más altos que la fracción TAG. La composición de ácidos grasos de los lípidos totales y las clases de lípidos (TAG y PL) se vieron afectados por las variaciones estacionales y la fracción lipídica.

PALABRAS CLAVE: Capoeta umbla; *Clases de fosfolípidos; Composición estacional de ácidos grasos; Triacilgliceroles.*

Citation/Cómo citar este artículo: Kaçar S, Kayhan Kaya H, Başhan M. 2023. Seasonal effect on fatty acid composition in phospholipid classes and triacylglycerols of male *Capoeta umbla*. *Grasas Aceites* **74** (3), e521. https://doi.org/10.3989/gya.0452221

1. INTRODUCTION

Capoeta umbla, also locally called Siraz or Sarıbalık, is a species belonging to the cyprinidae family, commonly found in the Euphrates-Tigris river system. *C. umbla* is the most commercially valued fish for the local people.

Fish and other aquatic foods are known to be the main sources of polyunsaturated fatty acids (PUFAs). Therefore, humans get most of their eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from eating fish. It has been established that DHA is essential for vision and brain development in babies, Fish consumption is related to the amount of DHA in milk, and fish oil reduces sudden death in people with cardiovascular disease, lowers serum triglyceride levels and total cholesterol, increases HDL cholesterol levels, and reduces the risk of breast, colon, lung and prostate cancer (Lu *et al*., 2021).

It is important to determine the fatty acid composition of the PL and TAG fractions to understand the changes in the fat content of the muscle and to determine the nutritional value of the fish.

The 20-carbon polyunsaturated fatty acids of PL, which are structural components of membranes, play a role as precursor components of eicosanoids, which are biologically and physiologically active molecules. TAGs, which are generally stored in adipose tissue and rich in SFA and MUFA, function as energy reserves (Kaçar *et al*., 2016).

The most abundant phospholipid in cell membranes is phosphatidylcholine, which accounts for 40-50% of total phospholipids. Phosphatidylethanolamine is the second most abundant phospholipid, accounting for 20-50% of the total. These amounts vary depending on the tissue. For example, the amount of PE in the brain is 45% and the amount in the liver is 20%. Phosphatidylserine is a membrane phospholipid which is low in abundance and accounts for only 2-10% of the total phospholipids. Phosphatidylinositol, sphingomyelin, and cardiolipin phospholipids are minor mammalian membrane components. The phospholipid compositions of different mammalian cells and tissues differ significantly. The lipid composition of fish differs depending on several factors, such as their aquatic environment and the biological, physical and chemical properties of this environment. Seasons, migration, sexual maturity and spawning periods, type of species, dietary habits and rearing in aquaculture or natural habitats influence the lipid profiles.

Various investigations into freshwater fish have been carried out, but little information is available on PL and its classes or its TAG fatty acid analysis. (Satar *et al*., 2012; Kaçar *et al*., 2016). Studies on phospholipid classes have generally been carried out in marine fish, although no reports have yet been published about the PL classes of *C. umbla.*

The objective of this study was to determine seasonal changes in the FA composition of triacylglycerol (TAG) and phospholipid classes including phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS).

2. MATERIALS AND METHODS

The study was conducted on *C. umbla*, which were collected in July, November, January and April (39°10’44.68”N, 39°27’43.08”E). The fish were skinned from the area between the dorsal fins and the lateral line of three male and about 5 grams of muscle sample were taken. After determining the wet weights of the muscle samples, they were put into tubes and kept in a chloroform-methanol (2:1v/v) mixture at -80 °C until analysis.

Total lipid extraction was performed according to the method of Bligh and Dyer (1959). Methylation was accomplished by heating with sodium methylate in methanol. Samples containing total muscle lipids were trans-esterified with acidified methanol. The fatty acid composition of the fish was analyzed in triplicate: Auto-oxidation of unsaturated fatty acids was minimized by adding 50 µL of 2% butylated hydroxytoluene in chloroform to each sample during the extraction process, and N_2 was used for the drying process of lipid extracts from tissues.

The method of Vaden *et al*. (2005) was used for the separation of phospholipid classes. Samples containing muscle lipids were transesterified with acidified methanol. The fatty acid methyl esters (FAMEs) were extracted with hexane.

2.1. Gas chromatography analyses

Fatty acid methly esters were analyzed by capillary gas chromatography (SHIMADZU GC 2010 PLUS) with a flame ionization detector (FID), and GC solution (version 2.4) software. The flow rates of compressed air and hydrogen were 400 mL/min and 30 mL/min, respectively. The carrier gas was Helium at a flow rate of 0.5 mL/min. The injection port temperature was 250 °C, the detector temperature 250 °C. One micro-liter was injected through the cold on-column injector. Column (oven) temperature: 170 °C, holding time, 2 minutes; 2 °C/minute to 210 °C, kept constant for 20 minutes; total analysis time: 42 minutes.

A SPSS 16 computer program was used to compare the percentage of fatty acids. A statistical analysis of fatty acid compositions was performed by analysis of variance (ANOVA) and mean comparison was determined according to Tukey's test. Means were obtained in triplicate and statistically significant differences were reported at ($P \leq 0.05$).

3. RESULTS AND DISCUSSION

The captured samples varied between 225 and 290 cm, with weights between 162 and 392 g. Fat content ranged between 1.35% (in November) and 2.46% (in July) (Table 1).

Table 2 shows the seasonal variation in total lipid contents in male *C. umbla.*

Table 1. Lengths, weights and lipid contents of *C. umbla*

	July	November	January	April
Length (cm)*	225±11.01	280±12.20	290±10.40	245±11.14
Weight (gr)*	162±8.13	348±14.44	392±15.18	220±9.15
Total lipids (%)*	2.46±0.16a	1.35±0.02b	1.72±0.06c	1.50±0.07bc

* Means are the average of 3 replicates. Values reported are means ±standard deviation; means followed by different letters in same line are significantly different ($p < 0.05$) according to Tukey's test

Table 2. Fatty acid composition in total lipids of the muscle from male *C. umbla* (%)*

Fatty acids	July	November	January	April
14:0**	1.89±0.06a	2.29±0.06a	4.63±0.19b	2.32±0.12a
15:0	0.21±0.01a	0.27±0.01a	0.27±0.02a	0.22±0.01a
16:0	21.28±1.03a	23.37±1.25a	22.5±1.17a	22.26±1.15a
17:0	0.46±0.02a	0.65±0.03ab	0.38±0.01a	0.82±0.03b
18:0	4.14±0.16a	3.91±0.13a	3.38±0.10a	4.10±0.20a
ΣSFA* **	27.98±1.25a	30.49±1.60a	31.16±1.55a	29.72±1.45a
16:1n-7	18.48±0.72a	11.99±0.72b	15.91±0.85ab	17.15±0.63a
18:1n-9	14.2±0.61a	18.11±0.72b	20.07±1.01b	14.66±0.70a
20:1n-9	1.08±0.04a	2.28±0.15b	2.98±0.18b	0.80±0.03a
ΣMUFA	33.76±1.56a	32.38±1.66a	38.96±1.87b	32.61±1.08a
18:2n-6	1.93±0.04a	2.48±0.09b	2.15±0.11b	2.19±0.11b
18:3n-3	6.63±0.30a	5.86±0.23a	3.27±0.15b	5.67±0.22a
20:2n-6	0.14±0.01a	0.20±0.01b	0.16±0.01a	0.20±0.02b
20:3n-6	0.16±0.01a	0.18±0.01a	0.28±0.02b	0.12±0.01a
20:4n-6	1.0±0.05a	0.99±0.03a	1.29±0.06b	1.30±0.05b
20:5n-3	16.1±0.62a	14.94±0.72ab	12.02±0.55b	14.22±0.88ab
22:5n-3	5.46±0.35a	4.88±0.15ab	3.64±0.26b	5.45±0.33a
22:6n-3	6.75±0.28a	7.51±0.34ab	6.99±0.40a	8.43±0.48b
ΣPUFA	38.17±2.04a	37.04±1.71a	29.8±1.02b	37.58±1.80a
Σn-3	34.94±1.65a	33.19±1.45a	25.92±1.30b	33.77±1.57a
Σn-6	3.23±0.10a	3.85±0.12a	3.88±0.20a	3.81±0.19a
n-3/n-6	10.81	8.62	6.68	8.86

* Means are the averages of 3 replicates. ** Values reported are means ±standard deviation; means followed by different letters in the same line are significantly different ($p < 0.05$) according to Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

The highest saturated fatty acids (SFAs) were found in January (31.16%, prior to reproduction). There were no seasonal changes in total SFA values between July (27.98%) and January (31.16%). The highest concentrations of SFA were 16:0 (21.28-23.37%), and 18:0 (3.38%-4.14%).

Monounsaturated fatty acids (MUFAs) in the muscle tissue increased in January (38.96%, prior to reproduction) and decreased in November (32.38%, post reproduction). The 16:1n-7 ratio was high in July (18.48%) and decreased in November (11.99%). In July, the amount of 18:1n-9 reached its lowest value (14.20%). 18:2n-6, which is one of the n-6 components which are important and essential fatty acids for fish, 18:3n-3 from the n-3 and aracidonic acid (ARA), another important n-6, were detected at a low rate. Among them, 18:2n-6 was found to be 2.48% in November, 18:3n-3 to be 6.63% in July, ARA to be 1.30% in April.

The concentration of PUFA varied seasonally from 29.80% (in January, prior to reproduction) to 38.17% (in July, reproduction). The amount of n-3 PUFAs is much higher than n-6 PUFAs.

In this study, the n-3/n-6 ratios vary for muscle tissue according to season, and are lower in January (6.68), increasing up to 10.81 in July.

The fatty acid results of *C. umbla* male from the Munzur River are compatible with those obtained from *C. umbla* (Yılmaz *et al*., 1995, Aras *et al*., 2009) and other freshwater fish previously taken from different water sources (Uysal *et al*., 2008; Cengiz *et al*., 2012; Çakmak *et al*., 2012; Satar *et al*., 2012; Fallah *et al*., 2013; Kayhan *et al*., 2015; Emre *et al*., 2015; Emre *et al*., 2017; Emre *et al*., 2018; Emre *et al*., 2020). The major fatty acids identified in the fish were 16:0, 18:1n-9, DHA and EPA in all seasons. Linoleic, 18:3n-3, 20:3n-6 and AA were also detected in a much lower percentage.

The levels of SFA, MUFA, and PUFA in fish are not constant and change according to a number of variables. Unsaturated fatty acids (PUFA and MUFA) are often more prevalent than saturated ones in cold water fish. PUFA is less prevalent in tropical species. While PUFAs play a structural role in organs, saturated fatty acids and MUFAs typically serve as energy sources. We can infer that the water source may be the primary factor in this (Kayhan *et al*., 2015). Fish living in cold water, like the Munzur River, are predicted to have higher PUFA ratios. This finding increases the nutritional value of the fish living here. Since total SFA increases the amount of cholesterol in the blood, having more of these components creates negative health effects and reduces the nutritional value of fish.

Because palmitic acid is essential for fatty acid metabolism, nutrients have no effect on the amount of this component, which has the highest percentage of saturated fatty acids. The saturated 16:0 and 18:0, as well as the monounsaturated 16:1n-7 and 18:1n-9, are not essential and can be obtained through food or synthesized from carbohydrates and amino acids. The amounts of these components, which are generally stored and serve to meet energy needs, depend on the organisms that make up the nutrient. It has been seen that the ratio of 16:1n-7 from the monounsaturated FA is higher in some fish. According to Ackman (1989), this feature is unique to freshwater fish. The ratio of 16:1n-7 found in our study was significantly higher than the rates found in *C. umbla* from Hazar Lake (Yılmaz *et al*., 1996), Tuzla Stream, and Tercan Dam Lake (Aras *et al*., 2009). This finding demonstrated that the fatty acid composition of fish is affected by the water source. We believe that fish nutrients are responsible for this effect. Some phytoplankton, zooplankton, and other microorganisms in the water were found to be high in 16:1n-7. Palmitoleic acid serves important nutritional and biological functions because this component is essential for fat synthesis and storage, as well as signaling pathways between organs, cell differentiation, and proliferation. In our analysis, the percentage of 18:2n-6 in muscle total lipids was lower than in other studies (Yılmaz *et al*., 1996; Aras *et al*., 2009) on the same type of fish, while the percentage of 18:3n-3 acid was higher. Fish consume essential fatty acids such as 18:2n-6 and 18:3n-3, which they cannot synthesize, and use these components as precursors in the formation of twenty-carbon PUFAs. Analyses show that the ratio of 18:2n-6 and 18:3n-3 acids are generally low in fish (Aggelousis and Lazos, 1991).

In our study, the percentage of 18:2n-6 in the total muscle lipid was lower than in previous studies on the same type of fish (Yılmaz *et al*., 1996; Aras *et al*., 2009), while the percentage of 18:3n-3 acid was higher. Henderson and Tocher (1987) reported in their study that the 20:3n-6 ratio in many freshwater fish was 1.5%. This component, which could not be determined in previous studies on *C. umbla*

(Yılmaz *et al*., 1996, Aras *et al*., 2009), was found at a rate of less than 1% in the fish species examined in our study. The amount of ARA is high because the solubility of oxygen in hot water is low. Because arachidonic acid is a precursor to eicosanoids, which are vasoconstrictors and stimulate platelet aggregation, only small amounts should be found in fish oils. ARA was found to be lower in the fish species whose total muscle lipids were examined in our study than in the same type of fish previously supplied from other water sources. This could be due to the fact that the Munzur River is colder because the ARA ratio is higher in fish living in warm water environments. The low ARA ratio in Munzur River fish increases the nutritional value of these fish. EPA and DHA were the major fatty acids identified as n- 3 PUFAs in the muscle. Similar results have been reported in *Capoeta capoeta* (Çakmak *et al*., 2012), in *Capoeta damascina* (Fallah *et al*., 2013) and for *Capoeta angorae* (Emre *et al*., 2015). EPA and DHA, which are important n-3 components, have preventive effects on many diseases including cardiovascular disease and cancer. Moreover, EPA is the precursor to eicosanoids, which, unlike ARA, is vasodilator and prevents erythrocyte aggregation. We can say that the fish species we used in our study are rich in EPA and DHA which are n-3 fatty acids.

3.1. Fatty acid composition of the PL fraction

Table 3 shows the PL fatty acid content of male *C. umbla* muscle tissue. The ratio of 16:0 decreased

Table 3. Fatty acid composition in the phospholipid fraction of the muscle from male *C. umbla* (%)*

Fatty acids	July	November	January	April
14:0**	1.09±0.06a	0.83±0.02ab	0.65±0.02b	0.89±0.03ab
15:0	0.16±0.01a	0.09±0.01b	0.21±0.02c	0.15±0.02a
16:0	19.82±0.91a	23.97±1.13b	21.32±1.20a	21.34±1.13a
17:0	0.68±0.03a	1.54±0.05b	0.50±0.02a	0.77±0.03a
18:0	8.36±0.40a	9.16±0.43a	5.73±0.26b	6.79±0.30b
ΣSFA* **	30.11±1.57a	35.59±1.70b	28.41±1.36a	29.94±1.47a
16:1n-7	6.46±0.29a	3.98±0.20b	5.80±0.27a	4.09±0.19b
18:1n-9	12.9±0.58a	12.72±0.67a	12.95±0.55a	8.92±0.41b
20:1n-9	1.27±0.06a	1.60±0.06a	1.62±0.05a	0.60±0.02b
ΣMUFA	20.63±1.10a	18.3±0.86a	20.37±1.02a	13.61±0.75b
18:2n-6	1.16±0.05a	1.47±0.06b	1.60±0.06b	1.04±0.04a
18:3n-3	2.01±0.10a	2.59±0.13a	1.57±0.07b	1.10±0.06b
20:2n-6	0.14±0.01a	0.23±0.02b	0.17±0.01a	0.14±0.01a
20:3n-6	0.25±0.01a	0.25±0.02a	0.33±0.02b	0.16±0.01c
20:4n-6	2.12±0.12a	2.22±0.15a	3.54±0.17b	3.69±0.14b
20:5n-3	18.8±0.75a	18.45±0.93a	19.23±0.83a	23.3±1.20b
22:5n-3	9.62±2.02a	7.14±2.11a	6.92±1.80a	7.53±2.16a
22:6n-3	15.09±0.80a	13.7±0.65a	17.77±0.76b	19.41±0.90b
ΣPUFA	49.19±2.33a	46.05±2.34b	51.13±2.77a	56.37±2.83c
Σn-3	45.52±2.72a	41.88±1.94b	45.49±2.18a	51.34±2.62c
Σn-6	3.67±0.13a	4.17±0.20a	5.64±0.28b	5.03±0.20b
n-3/n-6	12.40	10.04	8.06	10.20

* Means are the average of 3 replicates. ** Values reported are means ±standard deviation; means followed by different letters in the same line are significantly different ($p < 0.05$) according to Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

to a minimum level in July (reproduction period, 19.82%). The highest ΣSFA contents were found in November (35.59%). The ΣSFA amounts decreased prior to the reproduction season (January) in *C. umbla*. Total MUFAs were lowest in April (13.61%). 18:1 n-9 was the most abundant fatty acid in all seasons and ranged from 8.92 to 12.95%. Throughout the year, the PUFAs accounted for 46.05–56.37% of the PL fatty acid composition. The most abundant n-3 PUFA was EPA (18.45-23.3%). This was followed by DHA (13.7-19.41%) and then 22:5n-3 (6.92-9.62%) and n-3/n-6 ratios were found to be 8.06-12.40.

The major fatty acids were in accordance with those obtained from other freshwater fish (Satar *et al.*, 2012; Görgün *et al.*, 2014, Kayhan *et al.*, 2015): 16:0 in SFA, 18:1n-9 in MUFA, and EPA and DHA among PUFAs were found in the PL fraction. Another important finding was that the rate of stearic acid was found to be higher than total lipids. This is an expected result because, despite being a saturated component, 18:0 accumulates more in the PL fraction.

Henderson and Tocher (1987) found that the n-3/n-6 ratio in freshwater fish PL was between 1.6 and 2.0. The most important reason for the high n-3/n-6 ratio, which is used to determine the nutritional value of fish, in the species we studied is that EPA, one of the important n-3 components, was very high, while n-6 fatty acids were very low.

In response to a decrease in ambient temperature, poichlothermic organisms, such as fish, increase the degree of unsaturation of the fatty acids in their membrane lipids. This is an adaptation developed to keep the membranes fluid.

Henderson and Tocher (1987) reported that if the ambient temperature decreases, the degree of unsaturation in fatty acids will increase, and in Farkas *et al.* (1980) the amount of unsaturated fatty acids in the PL fraction will increase. These components with increasing percentages can be monounsaturated or polyunsaturated.

3.2. Fatty acid composition of the TAG fraction

In the muscle TAG fraction, saturated fatty acids 16:0, monounsaturated 16:1n-7 and 18:1n-9, polyunsaturated EPA and DHA were the most abundant fatty acids in the percentage distribution.

The results show that the percentage of FAs varied according to season, ranging from 29.40 to 33.58% SFAs; 22.22% to 30.83% MUFAs, and 38.3% to 48.33% PUFAs.

n-3/n-6 ratios were found to be 8.53 (January) - 9.37 (July) (Table 4).

Although their rates varied in all periods, ΣPUFA was determined the highest in terms of mean values, ΣSFA followed it and ΣMUFA was determined to be the lowest. It was an interesting finding that ΣPUFA had the highest percentage in the muscle TAG of fish, although they had storage lipids. The reason why there is a large amount of PUFA in the TAG fraction is that the water source is very clean, the oxygen content is high, as well as the fact that the nutrients are rich in EPA and DHA. Dominant PUFA, such as EPA and DHA, is either synthesized from the 18:3n-3 acid obtained from food or obtained directly from the food.

In previous studies, it was observed that the ratio of 16:0 was mostly found in the saturated fatty acids in the muscle TAG fraction of fish, (Kozlova and Khotimchenko, 2000; Görgün *et al.*, 2013).

One of the highest fatty acids in the TAG fraction of fish tissues is 16:1n-7. The ratio of this component in fish collected from the same water source was discovered to be quite different. It was proposed that this component, which is supplied by both food and synthesis, is found in high concentrations in the neutral lipids of freshwater fish.

The high ratio of 18:1n-9 and 16:1n-7 in the TAG fraction, in particular, indicates that these components are used more for energy purposes.

The percentage of polyunsaturated dominant components, EPA and DHA, in the fish muscle TAG fraction varied among species (Görgün *et al.*, 2014; Satar *et al.*, 2012; Kaçar *et al.*, 2016). The percentage of EPA and DHA, which are important n-3 components, was higher in *C. umbla* collected from the Munzur River than in many freshwater fish (Satar *et al.*, 2012; Kaçar *et al.*, 2016).

Henderson and Tocher, (1987) stated that in the TAG fraction of fish, MUFA is the highest, followed by SFA and with PUFA as the least. Many studies have found this same order (Kozlova and Khotimchenko, 2000; Görgün *et al.*, 2013; Görgün *et al.*, 2014).

The reason for the presence of MUFA being the highest and PUFA being the lowest in the TAG fraction of fish is that this fraction is rich in monounsaturated fatty acids such as 18:1n-9 and 16:1n-7 and poor in polyunsaturated fatty acids such as EPA and DHA.

Table 4. Fatty acid composition in triacylglycerol fraction of the muscle from male *C. umbla* (%)*

Fatty acids	July	November	January	April
14:0**	1.35±0.07a	2.82±0.12b	1.01±0.08a	2.34±0.15b
15:0	0.16±0.01a	0.27±0.02b	0.27±0.02b	0.24±0.02b
16:0	25.46±1.21a	24.72±1.17a	22.14±1.38a	23.85±1.42a
17:0	0.61±0.03a	0.74±0.03a	0.74±0.03a	0.43±0.02b
18:0	6.0±0.30a	2.67±0.19b	5.24±0.26a	4.48±0.20ab
ΣSFA* **	33.58±1.57a	31.22±1.58ab	29.4±1.51b	31.34±1.99ab
16:1n-7	14.28±0.87a	15.2±0.68a	9.85±0.57b	11.6±0.65b
18:1n-9	12.71±0.67a	13.92±0.63a	10.83±0.54b	11.23±0.57ab
20:1n-9	1.03±0.05a	1.71±0.11b	1.54±0.06b	0.62±0.03c
ΣMUFA	28.02±1.10a	30.83±1.52a	22.22±1.08b	23.45±1.27b
18:2n-6	1.90±0.03a	2.30±0.03b	1.61±0.02c	1.94±0.03a
18:3n-3	5.38±0.25a	13.39±0.32b	2.20±0.13c	3.40±0.15c
20:2n-6	0.15±0.01a	0.17±0.02a	0.13±0.01a	0.18±02a
20:3n-6	0.21±0.02a	0.20±0.01a	0.30±0.02b	0.21±0.02a
20:4n-6	1.43±0.06a	1.07±0.06a	3.03±0.14b	2.07±0.10ab
20:5n-3	17.09±0.78a	10.49±0.57b	19.63±0.98a	22.08±1.18c
22:5n-3	5.91±0.35a	3.56±0.13b	6.45±0.30a	5.85±0.27a
22:6n-3	6.23±0.28a	6.70±0.31a	14.98±0.62b	9.39±0.42ab
ΣPUFA	38.3±1.82a	37.88±1.69a	48.33±2.24b	45.12±2.12b
Σn-3	34.61±1.52a	34.14±1.78a	43.26±2.28b	40.72±2.04b
Σn-6	3.69±0.19a	3.74±0.12a	5.07±0.24b	4.4±0.17ab
n-3/n-6	9.37	9.12	8.53	9.25

* Means are the averages of 3 replicates. ** Values reported are means ±standard deviation; means followed by different letters in the same line are significantly different ($p < 0.05$) according to Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

In our study, unlike other fish species in two seasons, the reason why the TAG fraction had the highest PUFA and the lowest MUFA was that the fish were very rich in EPA (10.49-22.08%) and DHA (6.23-14.98%).

In our study, the n-3/n-6 ratio was significantly higher than in other freshwater fish (Satar *et al.*, 2012; Görgün *et al.*, 2013; Görgün *et al.*, 2014). The most important reason for this is that EPA and DHA, the dominant n-3 components in fish, are relatively high in comparison to other fish, while 18:2n-6, ARA, and 20:3n-6 acids, which are among the n-6 fatty acids, are relatively low.

3.3. Fatty acid composition of PL classes

In the PC fraction, among the saturated FA, 16:0 was found to be the highest (19.66-29.63%), and EPA from the unsaturated FA was found to be 18.66-26.32%, and DHA to be 13.06-19.13%.

It was observed that the ratio of 16:0, and accordingly ΣSFA, decreased in July. It was determined that 18:1n-9 from the monounsaturated FA and ΣMUFA, due to this component, decreased in the months representing spring and EPA, DHA and ΣPUFA only slightly increased compared to other months in the same period. The n-3/n-6 ratios were found to be 8.85-10.10 (Table 5).

16:0 was found to be dominant, as in previous studies (Medina *et al.*, 1995; Kızmaz, 2015).

The rate of EPA from twenty-carbon polyunsaturated FA was found to be significantly higher than in previous studies (Lin *et al.*, 2012; Kızmaz, 2015). As previously stated, these Munzur River fish are extremely high in EPA.

Table 5. Fatty acid composition in phosphatidylcholine of the muscle from male *C. umbla* (%)*

Fatty acids	July	November	January	April
14:0**	0.28±0.01a	0.44±0.02ab	0.68±0.02b	0.76±0.03b
15:0	0.14±0.01a	0.20±0.01b	0.20±0.02b	0.14±0.01a
16:0	19.66±0.92a	28.69±1.22b	29.63±1.34b	23.92±0.65ab
17:0	0.55±0.03a	0.97±0.05b	0.77±0.03ab	0.66±0.03ab
18:0	3.17±0.13a	2.89±0.15a	2.35±0.19b	2.17±0.10b
ΣSFA* **	23.8±1.08a	33.19±1.57b	33.63±1.62b	27.65±1.40a
16:1n-7	4.72±0.23a	4.04±0.24a	5.90±0.27b	4.29±0.21a
18:1n-9	11.94±0.55a	14.06±0.68a	12.09±0.69a	8.48±0.52b
20:1n-9	1.23±0.06a	1.32±0.05a	1.39±0.10a	0.75±0.03b
ΣMUFA	17.89±0.80a	19.42±0.92a	19.38±0.89a	13.52±0.63b
18:2n-6	2.91±0.13a	2.14±0.09a	1.54±0.09b	1.49±0.10b
18:3n-3	1.32±0.06a	3.34±0.14b	1.22±0.11a	1.53±0.10a
20:2n-6	0.21±0.01a	0.19±0.02a	0.14±0.01b	0.18±0.02a
20:3n-6	0.26±0.02a	0.55±0.03b	0.44±0.02b	0.10±0.01c
20:4n-6	2.04±0.14a	1.71±0.08a	2.64±0.10a	3.52±0.15b
20:5n-3	26.32±1.33a	18.66±0.77b	23.29±1.10a	26.11±1.41a
22:5n-3	8.35±0.44a	5.29±0.32ab	4.56±0.25b	6.68±0.36ab
22:6n-3	16.81±0.73a	15.45±0.62a	13.06±0.67b	19.13±0.85c
ΣPUFA	58.22±2.82a	47.33±2.84b	46.89±2.59b	58.74±2.91a
Σn-3	52.8±2.53a	42.74±2.15b	42.13±2.24b	53.45±2.71a
Σn-6	5.42±0.30a	4.59±0.22a	4.76±0.25a	5.29±0.26a
n-3/n-6	9.74	9.31	8.85	10.10

* Means are the averages of 3 replicates. ** Values reported are means ±standard deviation; means followed by different letters in the same line are significantly different ($p < 0.05$) according to Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

The DHA levels in sea fish were found to be higher than in our study (Medina *et al*., 1995; Takama *et al*., 1999). The high content of *C. umbla* with EPA increased the n-3/n-6 ratio in these fish.

In the PC fraction, 16:0 from the saturated FA, 18:1n-9 from the monounsaturated FA, EPA and DHA from the polyunsaturated FA were determined as major components. They were found to be dominant in previously studied sea fish (Takama *et al*., 1999) and in *Alburnus tarichi* (Kızmaz, 2015).

Previous research has found that PUFA is the most abundant and MUFA is the least abundant. (Lin *et al*., 2012; Kızmaz, 2015). In our study, we obtained the same results.

Season, environment, nutritional fatty acids, and temperature have all been reported to influence the phospholipid fatty acid composition.

In the PE fatty acid composition, 16:0 from the saturated FA as in the PC fraction, 18:1n-9 from the monounsaturated FA and EPA and DHA from the polyunsaturated FA were determined as dominant. However, these components were found at different ratios from the PC fraction.

The levels of 16:0, 18:1n-9, EPA, DHA, ΣSFA, ΣMUFA and ΣPUFA in the male muscles were found in the range of 7.15 to 22.66%, 5.38 to 14.64%, 12.80 to 18.81%, 17.16 to 27.53%, 12.93 to 36.12%, 9.94 to 20.54%, 43.27 to 69.14%, respectively (Table 6).

In their studies, Medina *et al*. (1995) and Nadcisa *et al*. (2001) identified 18:0 as the dominant saturated fatty acid. In our study, however, the predominant saturated component was 16:0.

In terms of EPA and DHA composition, the results were similar to previous studies (Lie *et al*., 1992; Medina *et al*., 1995; Nadcisa *et al*., 2001).

Table 6. Fatty acid composition in phosphatidylethanolamine of the muscle from male *C. umbla (*%)*

Fatty acids	July	November	January	April
14:0**	0.49±0.02a	0.24±0.01b	1.51±0.06c	0.79±0.03ac
15:0	0.03±0.01a	0.04±0.01a	0.27±0.02b	0.09±0.01c
16:0	7.15±0.35a	15.37±0.74b	22.66±1.08c	15.49±0.70b
17:0	0.62±0.03a	1.39±0.06b	0.74±0.03a	1.15±0.05b
18:0	4.64±0.21a	9.64±0.40b	10.94±0.51b	8.92±0.47b
ΣSFA* **	12.93±0.64a	26.68±1.25b	36.12±1.69c	26.44±1.24b
16:1n-7	3.49±0.17a	2.55±0.11a	4.37±0.21b	3.06±0.15a
18:1n-9	12.32±0.59a	10.61±0.54a	14.64±0.62a	5.38±0.27b
20:1n-9	2.03±0.10a	1.74±0.08b	1.53±0.06b	1.50±0.05b
ΣMUFA	17.84±0.88a	14.9±0.67b	20.54±0.98c	9.94±0.87d
18:2n-6	1.76±0.06a	1.78±0.05a	1.46±0.06ab	1.18±0.03b
18:3n-3	1.12±0.06a	1.53±0.04b	0.93±0.03a	1.43±0.06b
20:2n-6	0.20±0.01a	0.17±0.01a	0.31±0.02b	0.21±0.01a
20:3n-6	0.33±0.02a	0.30±0.01a	0.31±0.02a	0.32±0.02a
20:4n-6	3.30±0.16a	2.69±0.13a	3.09±0.13a	3.86±0.17a
20:5n-3	18.81±0.76a	17.69±0.69a	12.8±0.60b	18.79±0.92a
22:5n-3	16.09±0.74a	11.44±0.55b	7.21±0.30c	10.2±0.49b
22:6n-3	27.53±1.30a	22.72±1.02b	17.16±0.91c	23.18±1.11b
ΣPUFA	69.14±3.36a	58.32±2.81b	43.27±2.23c	59.17±2.88b
Σn-3	63.55±3.12a	53.38±2.60b	38.1±1.79c	53.6±2.57b
Σn-6	5.59±0.27a	4.94±0.21a	5.17±0.28a	5.57±0.25a
n-3/n-6	11.36	10.80	7.36	9.62

* Means are the average of 3 replicates. ** Values reported are means ±standard deviation; means followed by different letters in same line are significantly different ($p < 0.05$) according to Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

In other studies, the PUFA content was found to be the highest and MUFA to be the lowest, which is similar to our findings (Lin *et al*., 2012; Kızmaz, 2015).

As previously stated, n-6 components were relatively low in the fish used in this study, while n-3 polyunsaturated components were excessively high, so the n-3/n-6 ratio was found to be higher than in previous studies (Lin *et al*., 2012; Kızmaz, 2015).

It was found that 16:0 and ΣSFA ratios increased in January, EPA and ΣPUFA increased in July, but its percentage and n-3/n-6 ratio decreased in January. According to our findings, the amount of muscle PE fatty acid in fish increased and decreased depending on the season.

In the PI fraction, 16:0 and 18:0 from the saturated fatty acids, 18:1n-9 from the monounsaturated, and ARA, EPA and DHA from the polyunsaturated FA were determined in greater abundance in the percentage distribution (Table 7).

Similar to previous studies (Lie *et al*., 1992; Medina *et al*., 1995; Kızmaz 2015), dominant components in PI were found in this study. When compared to the PC and PE classes, the major saturated fatty acid in the PI class was not 16:0, but 18:0, as in other fish species. All these data showed that it was 18:0 in the *sn*-1 position in the PI class.

Another finding from our study was that the ARA ratio found in the PI fraction was significantly higher than the value found in the PC and PE classes. Previously, similar results were discovered in other fish species. Therefore, it was reported that the PI was rich in 18:0 from the saturated and in ARA from the polyunsaturated.

Table 7. Fatty acid composition in phosphatidylinositol of the muscle from male *C. umbla* (%)*

Fatty acids	July	November	January	April
14:0**	0.49±0.02a	0.46±0.03a	0.24±0.01b	0.32±0.01ab
15:0	0.04±0.01a	0.05±0.01a	0.66±0.05b	0.12±0.02c
16:0	8.18±0.34a	8.06±0.29a	8.79±0.32a	11.02±0.56b
17:0	0.59±0.03a	1.34±0.05b	0.74±0.03a	1.45±0.04b
18:0	20.14±1.12a	28.98±1.40a	36.71±1.78b	35.02±1.77b
ΣSFA* **	29.44±1.27a	38.89±1.95ab	47.14±2.36b	47.93±2.23b
16:1n-7	4.20±0.18a	1.42±0.05b	0.94±0.04b	1.46±0.05b
18:1n-9	12.77±0.59a	7.92±0.37b	6.25±0.31b	6.24±0.25b
20:1n-9	1.14±0.04a	0.77±0.03b	0.67±0.03b	0.66±0.04b
ΣMUFA	18.11±0.78a	10.11±0.50b	7.86±0.41b	8.36±0.47b
18:2n-6	0.86±0.04a	0.73±0.03a	0.28±0.01b	0.99±0.04a
18:3n-3	1.01±0.05a	0.48±0.02b	0.20±0.01c	0.33±0.02bc
20:2n-6	0.19±0.01a	0.51±0.02b	0.13±0.01a	0.19±0.01a
20:3n-6	3.95±0.14a	0.63±0.03b	0.20±0.01c	0.55±0.02b
20:4n-6	5.03±0.25a	10.54±0.39b	10.1±0.43b	9.47±0.50b
20:5n-3	19.6±1.05a	20.11±1.11b	13.5±0.69c	16.13±0.85ac
22:5n-3	8.35±0.43a	6.48±0.28a	6.49±0.33a	5.28±0.24b
22:6n-3	13.38±0.61a	11.44±0.53b	14.02±0.67a	10.7±0.50b
ΣPUFA	52.37±2.63a	50.92±2.53a	44.92±2.14b	43.64±2.19b
Σn-3	42.34±2.06a	38.51±1.84ab	34.21±1.71b	32.44±1.62b
Σn-6	10.03±0.55a	12.41±0.61a	10.71±0.67a	11.2±0.53a
n-3/n-6	4.22	3.10	3.19	2.89

* Means are the averages of 3 replicates. ** Values reported are means ±standard deviation; means followed by different letters in the same line are significantly different ($p < 0.05$) according to Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

The reason for the high percentage of ΣSFA in the PI of fish is that this class is rich in the saturated component 18:0. The n-3/n-6 ratio we determined in the PI class of fish in our study was lower than the PC and PE classes.

The high rate of ARA, one of the major n-6 components, is responsible for the low rate of PI subclass in fish.

The ratio of 16:0 acid from the saturated components increased in April along with 18:0, and due to these fatty acids, ΣSFA increased in January and April, 18:1n-9 and ΣMUFA increased in July, ARA and EPA increased in November, DHA increased in January, and the n-3/n-6 ratio increased in July, compared to other periods.

The highest ratio of 16:0 from dominant saturated components was 21.95%, 18:0, 21.01%, 18:1n-9, 16.98%, EPA 9.12%, DHA 10.96%, ΣSFA 47.29%, ΣMUFA 25.22%, ΣPUFA 30.90%, n-3/n-6 4.93 (Table 8).

According to the previous study, PS had the highest percentage of SFA among the classes, and this class contained 16:0 and 18:0 acids in similar proportions, as well as DHA in low proportions. (Medina *et al.*, 1995).

Similar results were found in this study in terms of 16:0 and 18:0 values (16:0 18.67-21.95%, 18:0 15.89-21.01%). Moreover, in our study, the rate of DHA was found to be lower than the PC and PE classes, similar to the previous study (Medina *et al.*, 1995). The percentage of 18:1n-9 increased in April, and EPA and ΣPUFA increased in July.

Table 8. Fatty acid composition in phosphatidylserine of the muscle from male *C. umbla* (%)*

Fatty acids	July	November	January	April
14:0**	2.67±0.06a	1.96±0.07b	1.85±0.04b	2.22±0.10ab
15:0	0.22±0.01a	0.25±0.01a	0.24±0.02a	0.20±0.01a
16:0	20.0±1.04a	20.04±1.10a	18.67±0.81a	21.95±0.94a
17:0	1.55±0.06a	1.49±0.05a	1.30±0.08a	1.91±0.06a
18:0	19.37±0.92a	15.89±0.77b	18.61±0.85a	21.01±1.11a
ΣSFA* **	43.81±2.13a	39.63±1.91b	40.67±2.07b	47.29±2.27c
16:1n-7	9.68±0.57a	5.37±0.35b	7.77±0.38ab	6.97±0.30ab
18:1n-9	14.78±0.71a	15.25±0.87a	15.97±0.68a	16.98±0.70a
20:1n-9	0.76±0.04a	1.10±0.06ab	1.16±0.04b	0.92±0.04ab
ΣMUFA	25.22±1.32a	21.72±1.14b	24.9±1.20a	24.87±1.17a
18:2n-6	1.37±0.03a	1.95±0.06b	2.08±0.06b	1.11±0.05a
18:3n-3	0.76±0.03a	1.28±0.05b	1.06±0.05b	0.50±0.04a
20:2n-6	0.23±0.01a	0.53±0.03b	0.25±0.01a	0.67±0.04b
20:3n-6	0.27±0.01a	0.53±0.03b	0.33±0.01a	0.10±0.01a
20:4n-6	3.34±0.13a	2.20±0.10b	3.60±0.16a	3.15±0.11a
20:5n-3	9.12±0.44a	8.62±0.35a	4.42±0.17b	6.26±0.23ab
22:5n-3	5.31±0.28a	5.01±0.20a	7.17±0.37a	6.45±0.30a
22:6n-3	10.5±0.52a	9.26±0.47a	10.96±0.63a	9.52±0.50a
ΣPUFA	30.9±1.40a	29.38±1.26a	29.87±1.33a	27.76±1.30a
Σn-3	25.69±1.13a	24.17±1.19a	23.61±1.10a	22.73±1.66a
Σn-6	5.21±0.26a	5.21±0.18a	6.26±0.23a	5.03±0.27a
n-3/n-6	4.93	4.63	3.77	4.51

* Means are the average of 3 replicates. ** Values reported are means ±standard deviation; means followed by different letters in the same line are significantly different ($p < 0.05$) according to Tukey's test. *** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

CONCLUSIONS

The highest n-3/n-6 ratio found in the fish's total muscle lipid was 10.81. The n-3/n-6 ratio is used to assess the quality of fish oils. The fact that these values are significantly higher than all other freshwater fish studied in Turkey to date indicates that the nutritional value of these Munzur River fish species is very high. The PL and TAG fatty acid compositions of the fish were found to be different. Monounsaturated fatty acids such as 16:1n-7, 18:1n-9, and thus MUFA and 18:2n-6, 18:3n-3 were found to be more abundant in the triacylglycerols. In PL, unsaturated fatty acids such as 18:0, 20:3n-6, ARA and DHA from the saturated FA were higher. TAG and PL fatty acid compositions were found to vary depending on the season, breeding period, and temperature. Furthermore, it was discovered that different PL classes had different fatty acid compositions based on their biochemical functions.

Acknowledgements

This research was financially supported by the Scientific Research Project of Dicle University (DU-APK-13-FF-75).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical approval

All applicable national guidelines for the care and use of animals were followed.

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GRASAS Y ACEITES 74 (3)
July-September 2023, e522
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1125212

Effects of diets rich in monounsaturated fatty acids on the management and prevention of insulin resistance: A systematic review

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Submitted: 14 November 2021; Accepted: 21 March 2023; Published online: 10 October 2023

SUMMARY: Insulin resistance (IR), which is linked to obesity, is a mechanism associated with metabolic diseases, mainly type 2 diabetes mellitus. Studies have shown that monounsaturated fatty acids (MUFAs) have anti-inflammatory and anti-oxidative properties which positively affect IR. This systematic review examined the effects of MUFAs from different sources on IR in obese or overweight patients with or without metabolic syndrome. A search was carried out in the PubMed/Medline and Bireme/VHL databases, and data from 16 studies were analysed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. The intervention time ranged from 1 day to 5.2 years. All participants were overweight or obese; some had central obesity, a moderate risk of cardiovascular disease, low high-density lipoprotein levels, altered fasting glucose levels, prediabetes or type 2 diabetes mellitus. This systematic review provides evidence that MUFA-rich diets can improve IR.

KEYWORDS: *Metabolic syndrome; Monounsaturated fatty acids; Obesity; Systematic review.*

RESUMEN: ***Efectos de dietas ricas en ácidos grasos monoinsaturados en la gestión y prevención de la resistencia a la insulina: Una revisión sistemática.*** La resistencia a la insulina (RI), que está ligada a la obesidad, es un mecanismo asociado a enfermedades metabólicas, principalmente a la diabetes mellitus tipo 2. Los estudios han demostrado que los ácidos grasos monoinsaturados (AGMI) tienen propiedades antiinflamatorias y antioxidantes, que afectan positivamente a la RI. Esta revisión sistemática examinó los efectos de los AGMI de diferentes fuentes sobre la RI en pacientes obesos o con sobrepeso con o sin síndrome metabólico. Se realizó una búsqueda en las bases de datos PubMed/Medline y Bireme/VHL, y se analizaron los datos de 16 estudios de acuerdo con las pautas Preferred Reporting Items for Systematic Reviews and Meta-Analyses. El tiempo de intervención osciló entre 1 día y 5,2 años. Todos los participantes tenían sobrepeso u obesidad; algunos tenían obesidad central, riesgo moderado de enfermedad cardiovascular, niveles bajos de lipoproteínas de alta densidad, niveles alterados de glucosa en ayunas, prediabetes o diabetes mellitus tipo 2. Esta revisión sistemática proporciona evidencia de que las dietas ricas en MUFA pueden mejorar la RI.

PALABRAS CLAVE: *Ácidos grasos monoinsaturados; Obesidad; Revisión sistemática; **S**índrome metabólico.*

Citation/Cómo citar este artículo: Ramos ELL, Lima MFC, Azevedo ACSF, Lopes MGF, Moreira APB, Souza CT. 2023. Effects of diets rich in monounsaturated fatty acids on the management and prevention of insulin resistance: A systematic review. *Grasas Aceites* **74** (3), e522. https://doi.org/10.3989/gya.1125212

1. INTRODUCTION

Obesity is highly prevalent among adults and increases costs for public health systems worldwide (Bovolini *et al*., 2021). Obesity usually occurs when energy intake exceeds energy expenditure from metabolic and physical activities, leading to fat accumulation, mainly in visceral depots (Crudele *et al*., 2021). Excessive fat accumulation is a risk factor for many chronic diseases, including type 2 diabetes mellitus (T2DM). Although the exact aetiology of obesity is unknown, insulin resistance (IR) is a common factor that links obesity to T2DM and other metabolic disorders (Cho *et al*., 2017). IR is defined as a subnormal response to insulin-stimulated glucose uptake in skeletal muscles and adipose tissue and/or the suppression of hepatic gluconeogenesis. The development of IR is mainly associated with low-grade chronic inflammation induced by pro-inflammatory cytokines (e.g. interleukin-1 beta, interleukin-6, and tumour necrosis factor-alpha) and increased macrophage infiltration into peripheral tissues (Rehman and Akash, 2016). Oxidative stress may also be involved in the development of IR (Furukawa e*t al*., 2004; Hurrle and Hsu, 2017).

Dietary patterns may play a dual role in determining the positive and negative effects of the onset and management of metabolic diseases. A systematic review and meta-analysis showed that altered nutritional habits play an essential role in the risk of IR development (Clifton, 2019). While Western dietary patterns, which are characterized by excessive consumption of ultra-processed foods, soft drinks, carbohydrates, and saturated fatty acids, and low or no physical exercise can lead to the onset of obesity, IR, and T2DM. A balanced diet which is low in sugar, simple carbohydrates, and saturated fatty acids and high in vegetables fiber, monosaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) has positive effects on metabolic risk factors (Clifton, 2019).

The beneficial properties of fatty acids are undervalued. Recent studies indicate that fatty acids, especially MUFAs, are essential for human health. Studies have shown that MUFAs play a pivotal role in health owing to their anti-inflammatory and anti-oxidative properties, which positively affect IR (Clifton, 2019; Ravaut e*t al*., 2021). Interestingly, diet supplementation with olive oil (a component of the MUFA-rich Mediterranean diet) correlates with a low incidence of obesity and metabolic diseases and, therefore, low rates of chronic inflammation and mortality (Ravaut *et al*., 2021). This study aimed to systematically review and summarize the existing evidence on the effect of a MUFA-rich diet on IR in overweight or obese individuals with or without metabolic syndrome (MetS).

2. MATERIALS AND METHODS

2.1. Study design

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.

2.2. Eligibility criteria

We included randomized, controlled studies involving men and women aged 20–65 years who were overweight or obese (body mass index ≥ 25 kg/m^2) and either did or did not have MetS. Studies using MUFA-rich diets (e.g. the Mediterranean diet) or MUFA supplements (e.g. nuts or vegetable oils with emphasis on olive oil [discussion item 4.3]) as the mode of intervention were included. We adopted IR-related biochemical parameters (e.g. blood glucose, insulin, homeostatic model assessment of insulin resistance [HOMA-IR], C-peptide, glucagon-like peptide 1 [GLP-1], and glycosylated haemoglobin levels) as the main outcome measures.

2.3. Exclusion criteria

We excluded intervention studies involving healthy individuals or pregnant women and experimental studies. Articles published more than 10 years ago were also excluded.

2.4. Research methods

PubMed and Bireme/VHL (Virtual Health Library) were searched for articles published between 2010 and 2020. No publication language restrictions were imposed. In both databases, the search was performed by combining keywords related to MUFA (“monounsaturated fatty acids” OR “MUFA”; “extra virgin olive oil” OR “olive oil”; “nuts” OR “nut”; “Mediterranean diet”) and the expected results related to IR assessment (“Insulin Resistance” OR “Insulin Sensitivity” OR “Insulin” OR “HOMA”). Only randomized clinical trials were included (Figure 1).

2.5. Data extraction and management

Three researchers independently selected the articles. Discrepancies were discussed with two other review researchers until a consensus was reached. The following data were extracted from each study: author; publication year; number and characteristics of participants; age group; MUFA intervention duration, quantity, and type; control type; and main results with research outcomes (Table 1).

3. RESULTS

3.1. Literature search

The search identified 124 articles in Pubmed and 21 articles in Bireme: 30 using “monounsaturated fatty acids” OR “MUFA,” 30 using “extra virgin olive oil” OR “olive oil,” 36 using “nuts” OR “nut,” and 49 using “Mediterranean diet.” After excluding duplicate, reviews, and irrelevant articles, 67 were subjected to full-text review, and 16 of them were finally included (Figure 1).

3.2. Study characteristics

Sixteen studies were included in this systematic review. The intervention time ranged from 1 day to 5.2 years. The analysed studies included 1341 participants from countries on all continents except Africa. Nine studies were randomized crossover studies, five were randomized clinical trials, one was a randomized, parallel-arm controlled trial, and one was a nonrandomized clinical trial. Table 1 summarizes the information extracted from the included studies. All participants were overweight or obese. Some had MetS, central obesity, a moderate risk of cardiovascular disease, a low high-density lipoprotein (HDL) level, an altered fasting glucose level, prediabetes, or T2DM.

3.3. Intervention with MUFAs

The interventions proposed by the analysed studies are detailed in Table 1. The main interventions were supplementation with extra virgin olive oil or nuts, MUFA-rich diets including different sources of vegetable oils, and/or a Mediterranean diet.

4. DISCUSSION

This systematic review explores the effects of MUFA-rich diets on IR in adults and older individuals. In most of the included studies, improvements were observed in IR-related parameters (i.e. reduction in blood glucose, insulin, and HOMA-IR levels) after short- or long-term consumption of MUFA-rich diets; whereas the other studies did not report negative results, reinforcing the safety and importance of dietary MUFAs.

4.1. MUFA-rich diets from the consumption of extra-virgin olive oil

One study revealed that short-term extra virgin olive oil consumption, compared with a diet without olive oil consumption, resulted in a 20% decrease in postprandial serum glucose levels and a 40% increase in insulin levels among patients with impaired fasting glucose (Carnevale *et al.*, 2017). The authors suggested that the inhibition of DPP-4 activity is a potential mechanism for GLP-1 and insulin upregulation. A study by D’Amore *et al.* (2016) showed improved blood glucose and insulin sensitivity after short-term consumption of high-polyphenol extra virgin olive oil versus low-polyphenol extra virgin olive oil in individuals without MetS. Polyphenol-rich oil also promoted the transcription of genes and miRNAs involved in anti-inflammatory responses and energy homeostasis. Interestingly, these effects were partially lost in patients with MetS and those consuming low-polyphenol extra virgin olive oil, highlighting the importance of the phenolic fraction and the individual’s health status. In another two studies involving olive oil supplementation, Valente *et al.* (2018) did not observe any effects of short-term consumption of extra virgin olive oil versus coconut oil in overweight women and Galang *et al.* (2020), in a randomized controlled crossover trial, identified significantly increased postprandial glucose levels in patients with type 2 diabetes mellitus, after adding a tablespoon of extra-virgin olive oil.

4.2. MUFA-rich diets from the consumption of nuts

Three studies which examined the effects of nut consumption were included in this review. Hernández-Alonso *et al.* (2017) observed that a 4-month pistachio-enriched diet positively modulated the expression of some insulin sensitivity-related miRNAs in pre-diabetic individuals. In contrast, Liu *et al.* (2017) did not observe any effects of short-term peanut consumption on glycaemic and insulin responses

TABLE 1. Characteristics of studies included in the systematic review

Studies	Country	Design	Population	Duration of intervention	Intervention with MUFA	Secondary intervention/control	Results with MUFA related to insulin resistance
Carnevale *et al.* (2017)	Italy	RCTC	30 women and men	1 day per intervention	Meal with 10g of EVOO	Control meal without EVOO	↓ of postprandial glucose and DPP-4 activity and ↑ insulin and GLP-1
D'Amore *et al.* (2016)	Italy	CT	24 women and men	1 day per intervention	50ml of high-polyphenols EVOO	50ml of low-polyphenols EVOO	Low-polyphenols EVOO: ↑ glucose and insulin sensitivity; transcription of genes and miRNAs involved in anti-inflammatory responses and energy homeostasis
Valente *et al.* (2018)	Brazil	Single-blind RCTC	15 women	1 day per intervention	Breakfasts with 25mL of EVOO	Breakfasts with 25mL of virgin coconut oil	No significant effect between the diets
Galang *et al.* (2020)	Philippines	RCTC	13 women and men	1 day per intervention	Standard breakfast with one tablespoon of EVOO	Standard breakfast without EVOO	↑ postprandial glucose levels
Hernández-Alonso *et al.* (2017)	Spain	RCTC	49 women and men	4 months per intervention	Pistachio-supplemented diet (57 g/day of pistachios)	Isocaloric control diet	↓ circulating miR-192 and miR-375 were positively correlated with glucose, insulin and HOMA-IR
Liu *et al.* (2017)	Australia	RCTC	15 men	1 day per intervention	Peanut meal (shake) with 85 g of ground peanuts	Control meal (shake) matched for energy and macronutrient content	No significant effect between the diets
Abbaspour *et al.* (2019)	USA	RPACT	48 women and men	8 weeks	42.5 g of mixed nuts	69 g of pretzels without salt	↓ glucose ↓ insulin ↓ weight
Montserrat-de la Paz *et al.* (2018)	Spain	RCTC	16 men	1 day per intervention	Fatty meal with MUFA (refined olive oil) + Niacin Fatty meal with MUFA + n-3 PUFA (refined olive oil + fish oil with EPA and DHA) + Niacin	Fatty meal with SFA (milk cream) + Niacina No fat meal + Niacina	↑ insulin secretion (HOMA-β) ↓ HOMA-IR Smaller postprandial C-peptide
Luis *et al.* (2017)	Spain	RCT	361 women and men	3 months	High MUFA hypocaloric diet (46.0% CHO, 34.4% FA, 19.6% PTN), including virgin olive oil	High PUFA hypocaloric diet (45.9% CHO, 34.3% FAT, 19.8% PTN), including sunflower oil	↓ Insulin and HOMA-IR in the AA group (wild-type group)
Lago *et al.* (2016)	Brazil	RCT	18 women	45 days	Rich MUFA-diet (15% of MUFA and 10% of PUFA)	Rich PUFA-diet (15% of PUFA and 10% of MUFA)	↓ fasting glucose; insulin and HOMA-IR in the MUFA-diet group
Chang *et al.* (2018)	Malaysia	Double- blind RCTC	30 women and men	1 day per intervention	High-fat meal with 50.9 g of high oleic sunflower oil (MUFA)	Meal 1: high-fat meal with 50.9g of palm oil (SFA) Meal 2: high-fat meal with 50.9g of sunflower oil (PUFA) Meal 3: Low-fat/high-sucrose	Low-fat/high-sucrose meal induced a greater response for GLP-1, C-peptide, insulin and glucose
Chang *et al.* (2016)	Malaysia	RCTC	47 women and men	6 weeks per intervention	High-fat diet rich in MUFA (49g sunflower oil with 20% MUFA) + Test meal with 51 g fat	High-fat diet rich in SFA + Trial meal containing 51 g of fat Control diet rich in CHO + Test meal with 21 g of fat	Lower curve and peak of C-peptide, insulin and glucose with MUFA-rich meal compared to the control diet
Vafeiadou *et al.* (2015)	United Kingdom	Single-blind RCT	195 women and men	16 weeks	High MUFA diet (refined olive oil and olive oil/rapeseed oil blended spread)	High SFA diet (butter) High n-6 PUFA diet (safflower oil and spread)	No significant effect among the diets
Brassard *et al.* (2017)	Canada	RCTC	64 women and men	4 weeks per intervention	High MUFA diet (refined olive oil)	High SFA diet (cheese) High SFA diet (butter) High PUFA diet (corn oil) Low-fat/high-CHO	No significant effect among the diets
Maiorino *et al.* (2016)	Italy	RCT	215 women and men	1 year	Mediterranean diet (< 50% CHO and > 30% FAT), with 30–50 g of olive oil	Low-fat diet with < 30% FAT and < 10% SFA	↓ HOMA-IR
Maiorino *et al.* (2017)	Italy	RCT	201 women and men	5.2 years	Mediterranean diet (< 50% CHO and > 30% FAT), with 30–50 g of olive oil	Low-fat diet with < 30% FAT and < 10% SFA	↓ HbA1c ↓ HOMA-IR

↓: reduction; ↑: increase; DPP-4: dipeptidyl-peptidase-4; EVOO: extra virgin olive oil; GLP-1: glucagon-like peptide-4; RCT: Randomized Clinical Trial; RCTC: Randomized Clinical Trial Crossover; RPACT: Randomized, Parallel-arm Controlled Trial; FA: Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids; SFA: Saturated Fatty Acids; CHO: Carbohydrate; PTN: Protein.

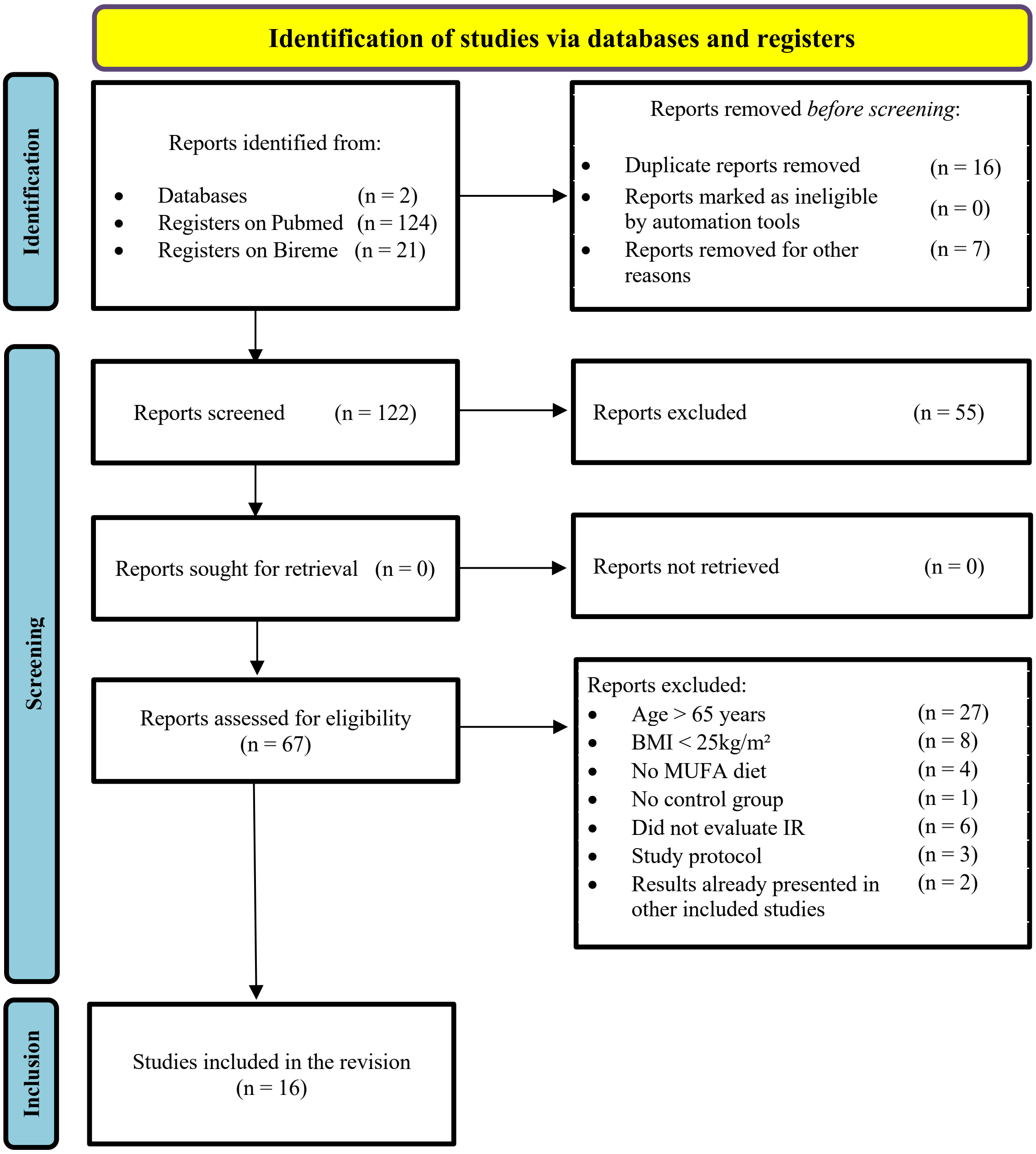

Figure 1. PRISMA Flow Diagram detailing the database searches, the number of abstracts screened and the full texts retrieved. BMI: Body Mass Index; MUFA: Monounsaturated Fatty Acids; IR: Insulin Resistance

in overweight individuals. Unlike previous studies, Abbaspour *et al*. (2019) reported blood glucose and insulin level reductions in overweight individuals after an 8-week diet with mixed-nut supplementation. This beneficial effect on glycaemic control can be attributed, in part, to the replacement of carbohydrates by MUFAs. Notably, the proteins and fibers contained in nuts can also contribute to this hypoglycaemic effect (Azzout-Marniche *et al*., 2014; Weickert and Pfeiffer, 2008). Another finding of this study was body weight reduction in the nut consumption group (Abbaspour *et al*., 2019). According to Qian *et al*. (2016), the effects of a MUFA-rich diet on metabolic risk factors may be partially mediated by changes in body weight. The introduction of a variety of nuts to the diet is interesting, as it can prevent food monotony and maintain consumption. In addition, nuts differ in their nutrient and bioactive compound contents; thus, a combination of nuts provides a greater range of health benefits (Abbaspour *et al*., 2019).

4.3. MUFA-rich diets including different sources of vegetable oils

Seven studies on MUFA-rich diets using different vegetable oil sources were included in this systematic review. Montserrat-de la Paz *et al.* (2018) evaluated the effects of a short-term diet with the three meals containing different sources of fat (saturated fatty acids [milk cream], MUFAs [refined olive oil], MUFAs plus n-3 PUFAs [refined olive oil plus fish oil with EPA and DHA], and no-fat meals) among individuals with MetS. All meals were supplemented with 2 g of niacin. Compared to meals containing saturated fatty acids, meals containing MUFAs improved glycaemia and postprandial insulinaemia and increased C-peptide levels, and showed greater insulin production. The same group of researchers previously demonstrated that the nature of dietary fats in meals influences postprandial triglyceride levels and the control of insulin secretion and sensitivity among individuals with normal or elevated fasting triglyceride levels (López *et al.*, 2008; Lopez *et al.*, 2011). The results of these studies showed improved postprandial beta-cell function and lower IR when the proportion of MUFAs versus that of saturated fatty acids in the diet was increased (Lopez *et al.*, 2011; López *et al.*, 2008).

In a study by Luis *et al.* (2017), obese participants consumed a high-MUFA hypocaloric diet (including virgin olive oil) or a high-PUFA hypocaloric diet for 3 months. In the high-MUFA hypocaloric diet group, individuals with the AA genotype (wild type) showed a reduction in insulin and HOMA-IR levels. This result shows that a single nucleotide polymorphism (SNP) in the brain-derived neurotrophic factor gene (rs10767664 variant) modifies IR after weight loss following a high-MUFA hypocaloric diet. However, the effect of dietary fat composition on the metabolic parameters related to this SNP remains unclear.

Another study which compared the effects of a MUFA-rich diet *versus* a PUFA-rich diet for 45 days in obese women (BMI > 35kg/m^2) was conducted by Lago *et al.* (2016). Although the authors did not identify the MUFA and PUFA sources used, they emphasized that the diet was individualized and had a caloric restriction of about 500 to 1000 kcal per day. At the end of the study, no statistical differences were observed when comparing the PUFA-rich diet and the MUFA-rich diet, but the intragroup evaluation showed a drop in the level of fasting glucose, insulin levels and HOMA-IR, in the MUFA-rich group. These results suggest that the MUFA-rich diet was more efficient in reducing insulin resistance in the studied population.

Two studies did not show the potential benefit of MUFAs versus other fatty acids on IR; however, a high-carbohydrate diet produced interesting results. Chang *et al.* (2018) compared the effects of replacing 7% of one's dietary energy with carbohydrates (simple sugar supplied as sweetened drinks), MUFAs (high-oleic sunflower oil blend), or saturated fatty acids (palm oil) for 6 weeks in individuals with abdominal obesity. The use of MUFAs or saturated fatty acids had no differential impact on glucose and insulin homeostasis and gastrointestinal peptides (GLP-1, GIP, ghrelin, PYY, and CCK) in the fasting or postprandial periods. The diet with refined carbohydrates had greater adverse effects on insulin secretion. In another study, Chang *et al.* (2016) evaluated individuals with MetS who consumed high-fat meals (enriched with saturated fatty acids, MUFAs [high-oleic sunflower oil], or n-6 PUFAs) or low-fat/high-sucrose meals. The short-term consumption of fatty acids (saturated fatty acids, MUFAs, and n-6 PUFAs) resulted in similar insulin, glucose, and C-peptide responses over time (Chang *et al.*, 2016). Compared to high-fat meals (regardless of the fatty acid type), the low-fat/high-sucrose meal induced a greater response for C-peptide (45%), insulin (45%), and glucose (49%).

Two other studies also showed no benefit of MUFAs versus other fatty acids on IR, but positive results were observed for MetS-related parameters. Vafeiadou *et al.* (2015) evaluated the effects of the consumption of three isoenergetic diets rich in saturated fatty acids (butter), MUFAs (refined olive oil and olive oil/rapeseed oil blended spread), or n-6 PUFAs (safflower oil and spread) for 16 weeks in individuals with a moderate cardiovascular disease risk. Replacing saturated fatty acids with MUFAs attenuated nocturnal systolic blood pressure. Regarding the patients' fasting lipid profiles, replacing saturated fatty acids with MUFAs or n-6 PUFAs reduced the total (28.4% and 29.2%, respectively) and low-density lipoprotein (LDL) (211.3% and 213.6%, respectively) cholesterol levels, and the total cholesterol to HDL cholesterol ratio (25.6% and 28.5%, respectively), without significant differences between MUFAs and n-6 PUFAs. Brassard *et al.* (2017) evaluated individuals with abdominal obesity and low HDL cholesterol levels who consumed five isoenergetic diets, each for 4 weeks: 1) rich in saturated fatty acids (cheese), 2) rich in saturated fatty acids (butter), 3) rich in MUFAs, 4) rich in PUFAs, and 5) low-fat, high-carbohydrate. Compared to the diets rich in saturated fatty acids (cheese or butter), the diet rich in MUFAs reduced LDL and the total cholesterol to HDL cholesterol ratio.

4.4. Mediterranean diet

Two studies included in the review evaluated the effects of a Mediterranean diet in patients with T2DM, and long-term follow-ups showed benefits in terms of IR. The Mediterranean diet is one of the most widely described and evaluated diets in scientific literature. It is characterized by a high intake of nuts, vegetables, legumes, fruits, grains, fish, seafood, and extra virgin olive oil and a moderate intake of red wine (Schwingshackl *et al.*, 2020). A large body of observational and experimental evidence suggests that greater adherence to the Mediterranean diet is related to glucose homeostasis, with an emphasis on reduced IR (Roldan *et al.*, 2019; Vitale *et al.*, 2018; Park *et al.*, 2017; Zhong *et al.*, 2016). This beneficial effect is probably related to its contents of MUFAs; PUFAs; fiber; and bioactive compounds such as polyphenols, vitamins, and minerals which result in anti-inflammatory and anti-oxidant actions, GLP agonists, and changes in the intestinal microbiota (Schwingshackl *et al.*, 2020; Martín-Peláez *et al.*, 2020). The PREDIMED study showed that adherence to a Mediterranean diet enriched with either an average of 50 mL/day of virgin olive oil or 30 g/day of nuts for 5 years, compared with a low-fat diet, in participants with MetS decreased xanthine oxidase activity and increased plasma superoxide dismutase and catalase activities (Sureda *et al.*, 2016). Notably, the unique actions of different nutrients or compounds and their derived metabolites can be enhanced by interactions and synergies, making the Mediterranean diet an invaluable tool for the prevention and control of metabolic diseases (Tosatti *et al.*, 2021; Martín-Peláez *et al.*, 2020).

4.5. Limitations

Although this systematic review supports the beneficial role of MUFAs in IR, whether these effects are a primary or secondary outcome of the improvement in obesity-associated chronic inflammation and oxidative stress remain unclear. Another limitation is the diversity of the included publications. Heterogeneity in study characteristics is common in nutritional intervention studies. Therefore, unsurprisingly, the studies selected for the present analysis varied with regard to the type of diets, definition of MUFA-rich diets, study population, intervention duration, and long-term follow-up protocols.

Another consideration is the variation in MUFA levels in the articles reviewed. Use of different MUFA doses is a relevant factor that may have influenced the results. Most dietary guidelines for MUFA consumption are based on the subtraction of recommended saturated and polyunsaturated fat intakes from the total fat intake rather than on evidence for the optimal level of MUFA intake (Liu *et al.*, 2017). A few guidelines have specified quantitative recommendations for MUFA intake, ranging from 10 to 25% of the total caloric value of the diet (Schwingshackl *et al.*, 2021; Sociedade Brasileira de Cardiologia, 2017). Thus, when considering an average requirement of 2000 kcal/day, the minimum recommendation of 10% is equivalent to 22 g of MUFAs. On a daily basis, this MUFA content can be achieved by consuming approximately 27 mL of extra virgin olive oil (Jimenez-Lopez *et al.*, 2020), 67 g of almonds (NEPA, 2011), or 37 g of macadamia nuts (Kris-Etherton, 1999), regardless of food sources.

5. CONCLUSIONS

Our systematic review showed that some improvement in IR-related parameters was observed in most of the evaluated studies, despite the diversity of MUFA interventions, doses, and outcomes. Therefore, individuals with IR may benefit from the consumption of extra virgin olive oil, nuts, a MUFA-rich diet including different sources of vegetable oils, or a Mediterranean diet. However, further studies are necessary to establish dose recommendations.

ACKNOWLEDGMENTS

The authors acknowledge Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq and Universidade Federal de Juiz de Fora.

DISCLOSURE

All authors declare no conflicts of interest regarding the contents of this article.

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International Journal of Fats and Oils

Volumen 74 | Nº 3 | **July-September 2023** | Sevilla (España) | ISSN-L: 0017-3495

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