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Controlled fermentation of heat-shocked, unsalted and inoculated Moroccan Picholine green olives

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SUMMARY: The present work reports the controlled fermentation of heat-shocked, unsalted and inoculated green olives. The effects of heat-shock (60, 70 and 80 °C three times for 5 min), inoculation with the oleuropeinolytic strain of *L. plantarum* FSO175 (*L.p*-FSO175) and the addition of Cell-Free Supernatant of *C. pelliculosa* L18 (CFS of *C.p*-L18) on the fermentation process of unsalted green olives were examined. The results showed a drastic reduction in the initial indigenous *Enterobacteria*, and an improvement in the acidification of heat-shocked olives at 70 and 80 °C, when compared to 60 °C. The inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 enhanced the fermentation and preservation of unsalted green olives, indicated by a significant decrease in pH, increase in free acidity and total disappearance of *Enterobacteria*. The heat-shock treatment at high temperature (80 °C), inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 led to the best reduction in bitterness, and favorable color changes (L, a, and b) in fermented olives. This sequential method led to more appreciated sensory characteristics (mainly bitterness and color) of fermented olives, lower spoilage incidence in olives, and reduced fermentation time to 50 days, and therefore may be suitable to control the fermentation of unsalted green olives of the Moroccan picholine variety.

KEYWORDS: *C. pelliculosa; Fermentation; Heat-shock; L. plantarum; Olives; Un-salted.*

RESUMEN: ***Fermentación controlada de aceitunas verdes picholine marroquíes sometidas a choque térmico e inoculadas sin sal.*** El presente trabajo reporta la fermentación controlada de aceitunas verdes sometidas a choque térmico, sin salar e inoculadas. Se estudian los efectos del choque térmico (60 °C, 70 °C y 80 °C tres veces durante 5 min), la inoculación con cepa oleuropeinolítica de *L. plantarum* FSO175 (*L.p*-FSO175) y la adición de sobrenadante libre de células de *C. pelliculosa* L18 (CFS de *C.p*-L18), sobre el proceso de fermentación de aceitunas verdes sin salar. Los resultados mostraron la drástica reducción de las enterobacterias autóctonas iniciales, y la mejora de la acidificación de las aceitunas sometidas a choque térmico de 70 °C y 80 °C, en comparación con 60 °C. La inoculación con *L.p*-FSO175 y la adición de CFS de *C.p*-L18 mejoró la fermentación y conservación de las aceitunas verdes sin salar, indicada por una disminución significativa del pH, aumento de la acidez libre y desaparición total de enterobacterias. El choque térmico a alta temperatura (80 °C), la inoculación con *L.p*-FSO175 y la adición de CFS de *C.p*-L18 condujeron a una mejor reducción del amargor y cambios de color favorables (L, a y b) en aceitunas fermentadas. Este método secuencial, que permitió apreciar las características sensoriales (principalmente amargor y color) de las aceitunas fermentadas, y una menor incidencia de deterioro en las aceitunas, y redujo el tiempo de fermentación a 50 días, puede ser adecuado para controlar la fermentación de aceitunas verdes sin salar de Marruecos, variedad picholine.

PALABRAS CLAVE: *Aceitunas; C. Pelliculosa; Choque térmico; Fermentación; L. Plantarum; Sin sal.*

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1. INTRODUCTION

Fermented green olives are produced according to traditional and industrial processes. The traditional process is based on the natural lactic fermentation of directly brined green olive fruits (Bautista-Gallego *et al.,* 2010). The industrial process is based on the chemical debittering of olives with sodium hydroxide to eliminate the bitterness due to oleuropein (OLP), followed by washing with tap water and brining at 10-12% NaCl, where a natural fermentation process takes place (Garrido-Fernández *et al.,* 1997; Gómez *et al.,* 2006).

These processes have many drawbacks. The traditional process is characterized by a long (6-8 months) and non-controlled fermentation process (Ciafardini and Zullo, 2019), normally leading to fermented olives (end product) of variable quality and associated with high olive spoilage incidence (Fernández-Diez *et al.,* 1985). The industrial process, based on alkali treatment of olives, is characterized by the production of large amounts of wastewater which is rich in phenolic compounds and sodium hydroxide, constituting a serious environmental problem (Arroyo-López *et al.,* 2008). The development of a controlled bioprocess may overcome, or at least reduce, the impact of these drawbacks.

Lactic acid bacteria (LAB) with oleuropeinolytic activity are frequent in natural fermented olive brines (Ghabbour *et al.,* 2011). In a previous work, *L.p*-FSO175, used as an autochthonous oleuropeinolytic starter, allowed the control of olive fermentation in brine with low salt content (5%), but led to fermented olives with some bitterness (Ghabbour *et al.,* 2016). Recently, other authors demonstrated the strong oleuropeinolytic activity of *L. plantarum* starter in low-salt green olive brine (Pino *et al.,* 2019), which may reduce the bitterness in fermented olives. *L.p*-FSO175 was used in this work and demonstrated its high oleuropeinolytic activity in the absence of sodium chloride (Ghabbour *et al.,* 2020), indicating its possible use in biological debittering and fermentation of unsalted green olives.

Candida yeast species (i.e. *Candida pelliculosa*) are dominant in naturally fermented olives, and can lead to various olive spoilages (Arroyo-López *et al.,* 2008; Arroyo-López *et al.,* 2012a). Therefore, their use as starter culture in olive fermentation is not suitable; however, their cell-free supernatant (CFS) can be used as a source of nutrients for LAB (Arroyo-López *et al.,* 2008; Arroyo-López *et al.,* 2012b; Sidari *et al.,* 2019) and enzymes, particularly beta-glucosidase and esterase, highly valued in biological debittering of olives, due to their involvement in the biodegradation of oleuropein (Rodriguez-Gómez *et al.,* 2012, Anagnostopoulos *et al.,* 2017).

Several studies reported the benefits of heat-shock to olives prior to brining in order to improve their fermentation process, via the eradication of undesirable microbiota (Chorianopoulos *et al.,* 2005), and the increase in permeability of fruit (Balatsouras *et al.,* 1983). On the other hand, fermented olives are naturally processed in brine which is rich in sodium chloride (5-10%) (Garrido-Fernández *et al.,* 1997; Gómez *et al.,* 2006). A high consumption of sodium chloride is associated with risk of diseases for consumers (i.e. blood pressure, heart and kidney diseases). That is why the World Health Organization (WHO) set a global goal of reducing salt intake by 30% before 2025, and recommends to reduce salt intake for adults to less than 5g per day (World Health Organization, 2013). Several studies reported the replacement of NaCl with KCl and $CaCl_2$ (Mateus *et al.,* 2016; Zinno *et al.,* 2017), and other authors studied table olive processing at reduced NaCl concentrations (Pino *et al.,* 2018; Pino *et al.,* 2019). The production of unsalted fermented green olives is of great interest to overcome the drawbacks of chemicals (NaCl and NaOH) for human health and the environment.

The objective of this work is to control the fermentation process of unsalted and non-alkali treated green olives, based their prior heat-shock (60, 70 and 80 °C tree times for 5min), followed by the addition of oleuropeinolytic agents (strain of *L.p*-FSO175 and CFS of *C.p*-L18).

2. MATERIALS AND METHODS

2.1. Inoculum preparation

The strain used in this work (*L.p*-FSO175) was isolated from natural fermented green olives and selected for its oleuropein biodegradation capacity (Ghabbour *et al.,* 2011). The *L.p*-FSO175 strain was reactivated twice in de Man Rogosa and Shar-

pe (MRS) broth (BIOKAR, FRANCE) at 30 ºC for 18 hours before use, then transferred twice to modified MRS broth containing 1% oleuropein (OLP) as a sole carbon source and incubated at 30 °C for 18 hours. The culture in the presence of OLP was made to induce the production of enzymes involved in the biodegradation of oleuropein (i.e. β-glucosidase and esterase). An aliquot of this overnight culture was centrifuged at 12.000g/12min at 4 °C (Hermle Labnet Z216MK), and the pellet obtained was washed twice with sterile physiological saline solution and re-suspended in the same solution at 8 Log cfu/mL concentration.

2.2. Preparation of cell-free supernatant

The *C. pelliculosa* L18 strain used in this work was isolated from naturally-fermented green olives and selected for its positive oleuropeinolytic activity (Rokni *et al.,* 2021). The *C. pelliculosa* L18 strain was subcultured twice in modified Yeast Extract Glucose (YEG) broth, containing 1 % oleuropein as a sole carbon source for 4 days of incubation at 25 °C. The culture obtained was centrifuged twice at 12000g/12min at 4 °C (Hermle Labnet Z216MK). The CFS of *C.p*-L18 was sterilized using sterile 0.22 µm filters (ISOLAB, Germany) before use.

2.3. Preparation and inoculation of the olives

Fresh green olive fruits of the Moroccan Picholine variety were purchased from the Oujda market area (East of Morocco). The olives were sorted manually to obtain fruits with uniform color and maturity degree. The olives were separated into 3 lots of 7 Kg, and each one was subdivided into 4 sub-lots designated a, b, c and d. The olives were then placed in 2-L flasks at a ratio of 800 g olives to 650 mL tap water. The trials of the first lot (designated a-6, b-6, c-6 and d-6) were heat-shocked at 60 °C three times for 5min. The trials of the second lot (designated a-7, b-7, c-7 and d-7) were heat-shocked at 70 °C three times for 5min. The trials of the third lot (designated a-8, b-8, c-8 and d-8) were heat-shocked at 80 °C three times for 5min. After each heat-shock, the water was changed, except for the third treatment which was kept for olive fermentation.

The olives were cooled at room temperature and then inoculated (2%, v/v) with an overnight culture of *L.p*-FSO175 and/or added with (2%, v/v) sterile CFS *C.p*-L18. The assays were carried out as follows: trials (a-6, a-7 and a-8) were not inoculated, they were used as controls; trials (b-6, b-7 and b-8) were inoculated with *L.p*-FSO175; trials (c-6, c-7 and c-8) were added with CFS *C.p*-L18, and the trials (d-6, d-7 and d-8) were inoculated with *L.p*-FSO175 and added with CFS *C.p*-L18. All the assays, made in duplicate, were incubated at 30 °C. Water samples were taken aseptically during the fermentation process, and subjected to physico-chemical and microbiological analyses.

2.4. Physico-chemical analysis

The physico-chemical parameters analyzed in de-bittering waters were pH, free acidity, total sugars and total polyphenols. The pH was measured using a pH meter type Crison pH 2000 after calibration at pH 4 and 7. The free acidity was determined using NaOH (0.1N) and phenolphthalein as indicators. The results were expressed as percent of lactic acid. The soluble sugar contents were determined using the method of (Ashwell, 1957), based on the measurement of the green color developed by the reaction of soluble sugars with anthrone in the presence of sulphuric acid. The green color developed was measured at 630 nm. The results obtained were expressed in grams of total sugars per 100 mL olive water. The total polyphenols were determined as described by Marigo (1973) based on the measurement of the blue color developed during the reaction of the phenolic fraction with the Folin Ciocalteu reagent after neutralization with 20% sodium carbonate. The blue color developed was measured at 760 nm after incubation at 40 °C/20 min. The polyphenol contents in the olive water was determined by reference to a standard range prepared with gallic acid under the same conditions as those of the samples. The results were expressed in mM and they were the average of 3 measurements.

2.5. Microbiological analysis

The samples taken from the trials at the 1st, 4, 8, 15, 22, 29, 36, 43 and 50th day of the fermentation process were subjected to successive decimal dilutions in a sterile saline solution. From the decimal dilutions, each microbial group was inoculated in its specific medium, using the pour-plate method. The

lactic acid bacteria (LAB) were determined on de Man Rogosa & Sharpe Agar (MRS) pH 5.7 ± 0.1 (Biokar, France) containing cycloheximide (0.01%), after 48 hours of incubation at 30 °C. The yeasts and moulds were enumerated on Potato-Dextrose-Agar (PDA) (Biokar, France) acidified with lactic acid (0.1N) to pH 3.5, after 48-72 hours of incubation at 25 °C. The *Enterobacteria* were enumerated on Deoxycholate Lactose Agar (DCL) pH 7.3 ± 0.2 (Biokar, France), after 48 h incubation at 37 °C. The results were the average of 3 measurements.

2.6. Color evaluation of the olives

In order to evaluate the effects of heat-shock and inoculation on color changes in fermented olives, olive samples were carefully taken on the 1st, 22nd and 50th day of fermentation. The fruit samples were subjected to color measurements using BYK-Gardner Model 9000 Color View Spectrophotometer (Silver Spring, MD, USA) and expressed in terms of the CIE L*, a*, b* parameters. The results were the average of 3 measurements.

2.7. Examination of sensory characteristics and spoilage of fermented olives

At the end of the fermentation process, the organoleptic properties of fermented olives were assessed for color, flavor, crunchiness, bitterness and acidic by 20 trained panellists composed of teachers and doctorate degree students in our university. The sensorial evaluation was recorded using a line scale ranging from 0 (no perception) to 10 (extreme) as described by (Meilgaard *et al.,* 1991). The fermented olives were also evaluated for the presence of spoilage indicators such as white spots, gas pockets, softening and off-odor formation, and the results were expressed in %.

2.8. Statistical analysis

The statistical analysis performed using the STATGRAPHICS Centurion XVII package (Stat point Technologies, Inc., Virginia, USA) was used for all calculations. The Two-Way ANOVA were carried out for heat-shock and inoculation. The least significant difference (LSD) values were calculated at the 5% probability level to determine which levels of the factors influenced the dependent variables analyzed.

3. RESULTS

3.1. Physicochemical analysis

3.1.1. pH and free acidity

The pH and free acidity changes during the fermentation process of unsalted green olives are presented in (Figures 1, 2), and their mean values are presented in Table 1. Whatever the heat-shock applied, the pH showed a slight decrease from 5.5 to 4.4 in un-inoculated olives (control) (Figure 1a); While, in olives inoculated with *L.p*-FSO175, with or without the addition of CFS of *C.p*-L18, the pH decreased progressively from 4.5 to stabilize at the end of fermentation at around 3.8-3.9 and 3.7, respectively (Figure 1b, Figure 2d).

The free-acidity values obtained showed a continuous increase during the fermentation of all assays (Figures 1 and 2). At the end of fermentation, the highest free-acidity value (0.94%) was obtained for

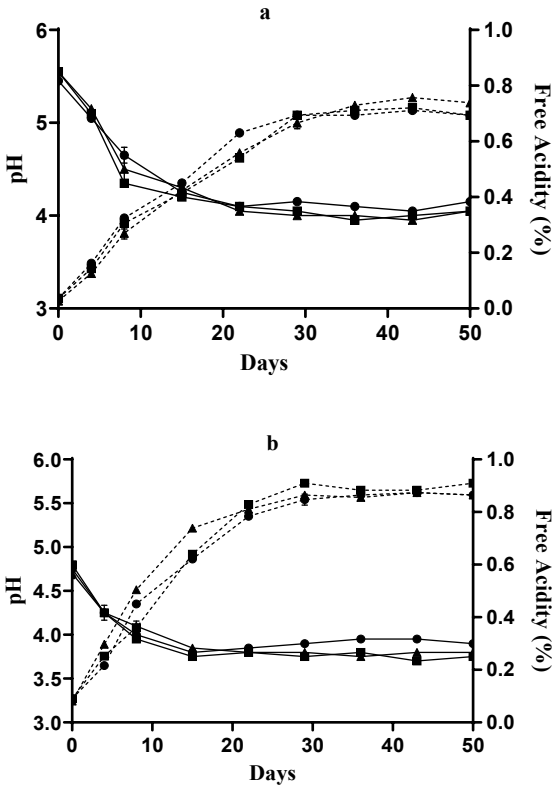

Figure 1. Evolution of pH (—) and free acidity (%) (----), during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (a): un-inoculated olives (control), (b): inoculated olives with *L.p*-FSO175. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

Table 1. Mean values and mean squares from the analyses of variance of pH, Free Acidity (% of lactic acid), Sugars and polyphenols (mM), LAB, Yeast and *Enterobacteria* biomass (Log CFU/mL)), during the controlled fermentation of heat-shocked (60 °C, 70 °C, 80 °C), un-salted and inoculated Moroccan green olives. a: un-inoculated olives (control), b: inoculated olives with *L.p*-FSO175, c: olives added with CFS of *C.p*-L18, d: olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18).

Factors			pH	Free acidity	Sugars	Polyphenols	LAB	Yeasts	Entero
Heat-shock	60 °C		4.06 a	0.60 ab	4.70 c	32.33 b	7.87 b	7.11 a	0.35 a
	70 °C		4.01 c	0.61 a	5.03 b	38.91 a	8.28 a	6.86 b	0.29 b
	80 °C		4.02 b	0.59 b	5.68 a	38.86 a	8.25 a	6.72 c	0.28 b
Inoculation	a		4.40 a	0.48 d	6.90 a	38.84 a	6.71 d	6.26 d	0.41 a
	b		3.99 b	0.64 b	4.44 c	35.81 c	9.25 a	6.74 c	0.29 b
	c		3.87 c	0.57 c	5.23 b	37.22 b	7.38 c	7.19 b	0.30 b
	d		3.85 d	0.71 a	3.98 d	34.93 d	9.19 b	7.39 a	0.26 c
Source of variation		Df							
Heat-shock		2	0.094	0.002	27.004***	1548.690***	5.809*	4.114	0.163
Inoculation		3	5.291***	0.778***	133.467***	237.420***	133.745***	20.301***	0.322
Heat-shock * Inoculation		6	0.018	0.012	0.565	9.482	0.670	0.293	0.009
Repetition		2	0.001	0.000	0.016	0.016	0.037	0.017	0.120
Residual		310	0.143	0.081	2.694	26.313	1.531	1.646	0.251

Means values in each column followed by the same letter are not significantly different according to LSD test at $p < 0.05$.

Df: Degrees of freedom, *Significant at 0.05 probability level, **Significant at 0.01 probability level; ***Significant at 0.001 probability level.

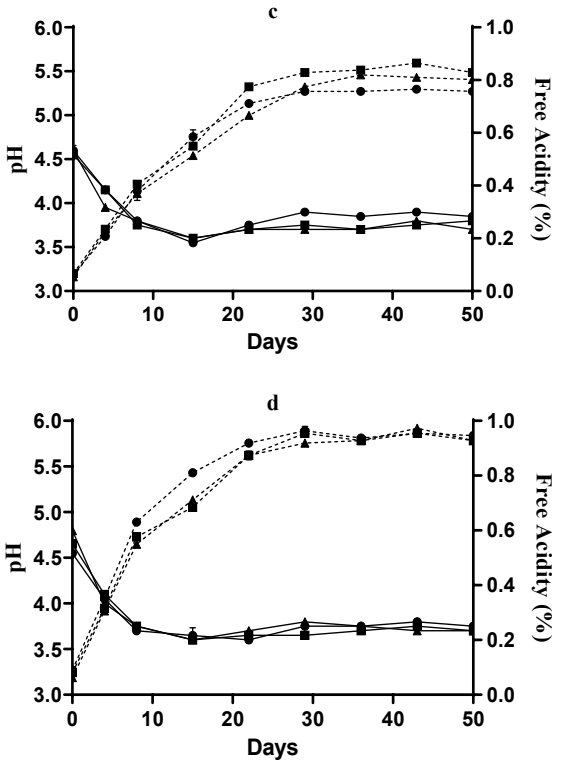

Figure 2. Evolution of pH (—) and free acidity (%) (----), during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (c): olives added with CFS of *C.p*-L18, (d): inoculated olives with *L.p*-FSO175 and added with CFS of *C.* L18. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18 (assay d), followed by that obtained (0.9%) with inoculation with *L.p*-FSO175 (assay b), then that (0.8%) obtained with the addition of CFS of *C.p*-L18 (assay c). The lowest acidity value (0.7%) was observed for un-inoculated olives (control).

Significant differences ($p < 0.05$) were obtained for pH and free acidity values between the assays, due to the effects of heat-shock and inoculation (Table 1). A high mean value for free acidity (0.64%) and low mean value for pH (3.99) were obtained in olives inoculated with *L.p*-FSO175 (b), compared to un-inoculated olives (control, a), with free acidity and pH values of 0.48% and 4.40, respectively. The inoculation with *L.p*-FSO175 and CFS of *C.p*-L18 addition (assay d) led to a substantial mean value for free acidity (0.71%) and lower mean value for pH (3.85). Thus, the highest mean value for free acidity (0.84%) and the lowest mean value for pH (3.81) were obtained, respectively, on the 43rd and the 15th days of processing. From these results, it can be observed that the increase in heat-shock led to a decrease in pH and increase in free acidity. Indeed, the inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 led to the obtention of the lowest mean value for pH (3.85) and highest mean value for free acidity (0.71%) (Table 1).

The results of the combined ANOVA (Table 1) showed the predominant effects of inoculation (more than 95%), respectively, on the variance in pH and free acidity. The influence of heat-shock was less than 1%.

3.1.2. Total sugars

The results of total sugar contents obtained for the olives are reported in (Figures 3 and 4). The sugar contents showed a progressive increase during the 20th day of the fermentation to achieve values ranging from 6-10 mM in all assays. They then decreased until the end of the process to reach 5-6 mM in un-inoculated olives (assay a) and less than 4mM in inoculated olives (assays b, c, and d).

The results of the combined ANOVA (Table 1) showed that the predominant effects of inoculation were of about 80% on the variance in sugars. The influence of heat-shock was less than 16%. According to the LSD test at $p < 0.05$, the accumulation of sugars in olive water was significantly dependent on heat-shock and inoculation (Table 1). The heat-shock at (70 and 80 °C) increased the mean value of total sugars significantly (5-5.6mM), when compared to that of 60 °C (4.7mM). At the same time, the olives inoculated with *L.p*-FSO175 and CFS of *C.p*-L18 (assay d) showed a decrease in sugars to obtain the lowest mean value of (3.97 mM), followed by that (4.44 mM) obtained in presence of *L.p*-FSO175 (assay b). The un-inoculated olives (assay a) and the olives added with CFS of *C.p*-L18 (assay c) showed high mean values for total sugars, which were 6.91 mM and 5.23 mM, respectively.

During the first 3 weeks of fermentation, the increase in temperature of heat-shock of olives led to significant increase in total sugars, and the highest mean value for sugars was obtained with heat-shock at 80 °C (Table 1).

3.1.3. Polyphenols

The results of polyphenols contents are reported in Figures 3 and 4. The results showed the same trend as sugars but with higher values in the un-inoculated olives, when compared to inoculated olives. They increased rapidly during the first week of fermentation to reach 44 mM in olives inoculated with *L.p*-FSO175 (assay b), and those added with CFS of *C.p*-L18 (assay c); 41 mM were

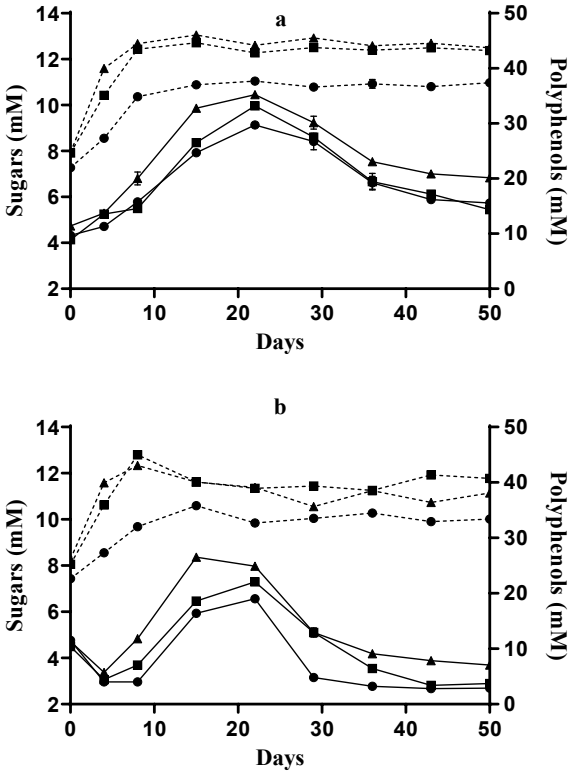

Figure 3. Total polyphenol (----) and total sugar (—) contents (mM) during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (a): un-inoculated olives (control), (b): inoculated olives with *L.p*-FSO175. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

obtained in the presence of *L.p*-FSO175 and CFS of *C.p*-L18 (assay d), and 45 mM was obtained in un-inoculated olives (assay a) (Figures 3a and 3b, (Figures 4c and 4d). After their maximum values, the polyphenol contents showed a slight decrease to stabilise at the end of fermentation, at around of 40 mM and 43 mM, respectively in assays (b, c, d) and (a). The heat-shock (70 and 80 °C) increased significantly ($p < 0.05$) the total polyphenols (38.9 mM), when compared to (32.33 mM) at 60 °C (Table 1). However, the inoculation with *L.p*-FSO175 and CFS of *C.p*-L18 (assay d) decreased the polyphenol content to the lowest mean value (34.93 mM), followed by that (35.81 mM) obtained for olives inoculated with *L.p*-FSO175 (assay b). Higher mean values for polyphenols (38.85 mM and 37.22 mM) were observed, respectively, for un-inoculated olives (assay a) and olives added with CFS of *C.p*-L18 (assay c).

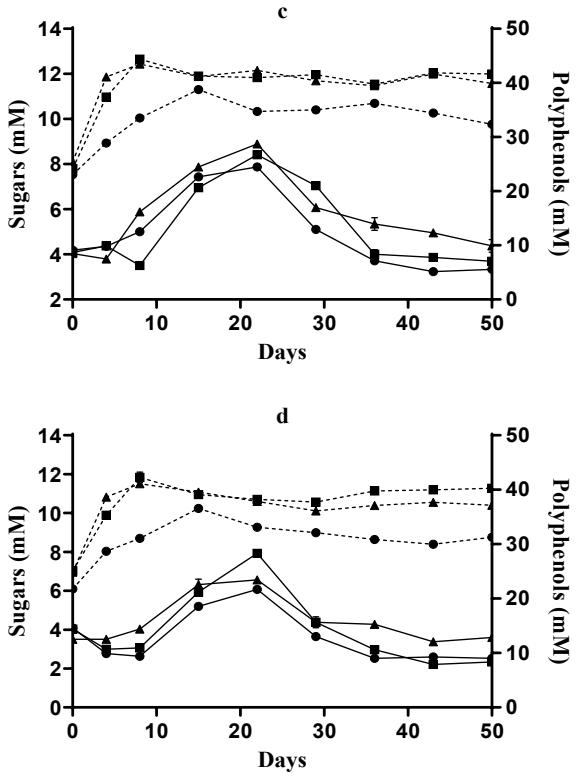

Figure 4. Total polyphenol (….) and total sugar (—) contents (mM) during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (c): olives added with CFS of *C.* L18, (d): inoculated olives with *L.p*-FSO175 and added with CFS of *C.p*-L18. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown in bars.

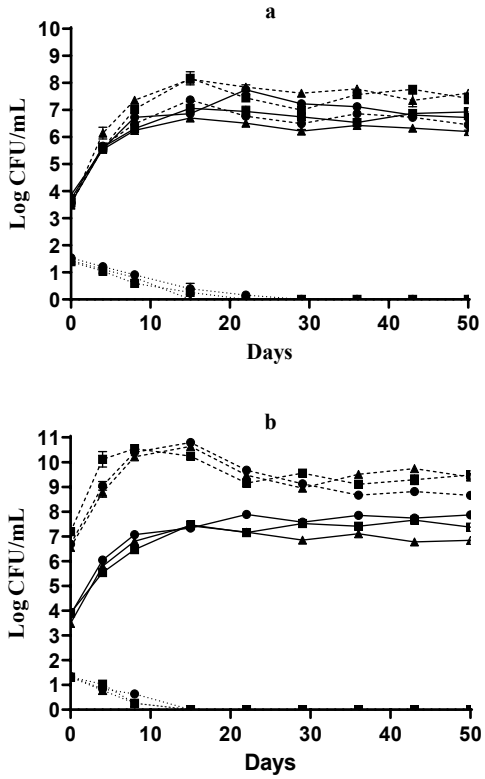

Figure 5. Evolution of LAB (----), Yeasts (—) and *Enterobacteria* (……) populations, during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (a): un-inoculated olives (control), (b): inoculated olives with *L.p*-FSO175. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown on the bars.

During the first 8 days of fermentation, the increase in temperature of heat-shock led to a significant increase in polyphenol content, and the highest polyphenol content was observed with heat-shock at 80 °C. The results of the combined ANOVA (Table 1) showed the predominant effects of Heat-shock of about 86% on the variance in polyphenols. The influence of inoculation explained less than 13%.

3.2. Microbiological analysis

3.2.1. Lactic acid bacteria (LAB)

The results of the microbial population of LAB in olive assays are reported in (Figures 5 and 6), and their mean values are presented in Table 1. In the olives inoculated with *L.p*-FSO175 (Figures 5b and 6d), the LAB population showed significant growth during the first 15 days of fermentation, from 7 Log CFU/mL to about 10 Log CFU/mL, followed by a very slight decrease to 9-10 Log CFU/mL at the end of the process. In the olives added with CFS of *C.p*-L18 (Figure 6c), LAB population showed an important growth from 3-4 Log CFU/mL to reach 8 Log CFU/mL and stabilize at this level until the 50th day of the process. However, in the un-inoculated olives (Figure 5a), the natural LAB population showed an increase from 3-4 Log CFU/mL to 7 Log CFU/mL during the first 15 days, followed by a slight decrease to stabilize at the end of the fermentation process between 6 and 7 Log CFU/mL, respectively, in assays treated at 60 °C and (70, 80 °C).

Significant differences ($p < 0.05$), were observed in LAB biomass due to the effect of heat-shock and inoculation (Table 1). Both heat-shock temperatures (70 and 80 °C) increased the LAB biomass significantly ($p < 0.05$) when compared to that obtained with 60 °C. A high increase in LAB growth was observed during the first 20 days of fermentation.

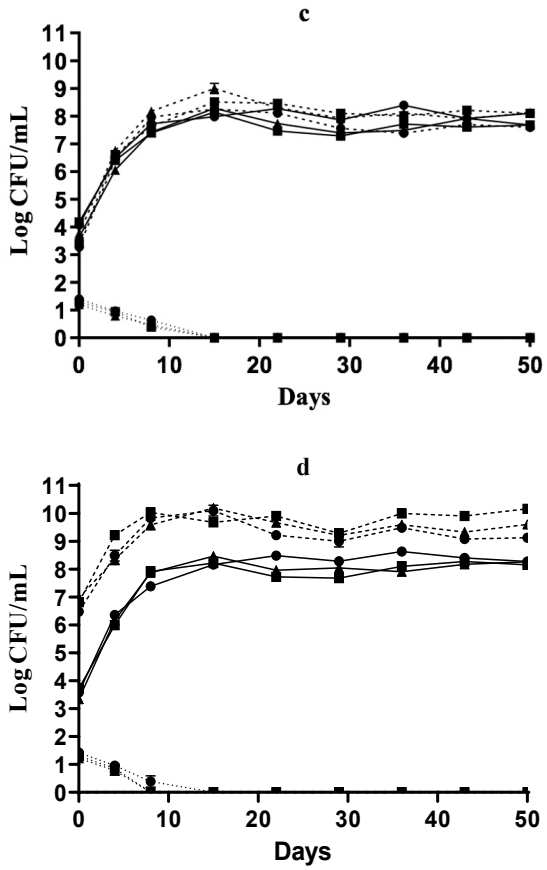

Figure 6. Evolution of LAB (----), Yeast (—) and *Enterobacteria* (……) populations, during the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan green olives. (c): olives added with CFS of *C.* L18, (d): inoculated olives with *L.p*-FSO175 and added with CFS of *C.p*-L18. Heat-shock 60 °C (—●—), heat-shock 70 °C (—■—), heat-shock 80 °C (—▲—). Data are the mean of three measurements and Standard error is shown on the bars.

The highest mean value for LAB count (9.25 Log CFU/mL) was obtained in olives inoculated with *L.p*-FSO175 (assay b), followed by that (9.2 Log CFU/mL) obtained in the presence of inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (assay d), and finally by that obtained (7.38 Log CFU/mL) in the presence of CFS of *C.p*-L18. The lowest mean value for LAB count was obtained in un-inoculated olives (assay a) (6.71 Log CFU/mL). The results of the combined analyses (Table 1) revealed that LAB biomass was highly influenced by the inoculation effect, which explained about 95% of the total variance observed. However, the impact of heat-shock on LAB biomass was lower than about 4%.

3.2.2. Yeasts and molds

The results from the microbial population of yeasts and molds in olives assays are reported in Figures 5 and 6, and their mean values are presented in Table 1. This population increased progressively during the first 15 and 22 days of the fermentation process, respectively, in the assays treated at (70 and 80 °C) and at 60 °C. In assays supplemented with CFS of *C.p*-L18, with or without inoculation with *L.p*-FSO175 (Figures 6, c and d), the yeasts and molds population showed significant growth during the first 15 days of fermentation, from 4 Log CFU/mL to about 8 Log CFU/mL, followed by a slight decrease to stabilize at 7 Log CFU/mL. In olives inoculated with *L.p*-FSO175, the natural yeast and mold population showed a progressive growth to reach the maximum of 7-8 Log CFU/mL (Figure 5b), which remains lower than that obtained in olives added with CFS of *C.p*-L18 (Figure 6c), and clearly higher than that (6-6.5 Log CFU/mL) observed in un-inoculated assays (Figure 5a).

The results of the combined ANOVA (Table 1) showed that the variance in yeast biomass was predominantly affected by inoculation (82%). However, even the effect of heat-shock was lower and explains less than 16%, the increase in temperature of heat-shock olives significantly decreased ($p < 0.05$) the mean value of the yeast and mold biomass (Table 1). The lowest mean value for the yeast and mold count (6.72 Log CFU/mL) was obtained with heat-shock at 80 °C; while the highest one (7.11 Log CFU/mL) was observed with heat-shock at 60 °C. However, with regard to inoculation, the assays (b, c and d) (table 1), showed the highest and significant mean values for yeasts and molds compared to that found in the un-inoculated olives (assays a) (6.27 Log CFU/mL). The inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (assay d) showed the highest mean value for yeasts and molds (7.39 Log CFU/mL), followed by that obtained in the presence of CFS of *C.p*-L18 (assay c) (7.2 Log CFU/mL) which was higher than that obtained with inoculation with *L.p*-FSO175 (b) (assay 6.75 Log CFU/mL).

3.2.3. Enterobacteria

The *Enterobacteria* population presented in (Figures 5 and 6) was initially low and in all olive, assays did not exceed 1.4 Log CFU/mL at the beginning of the fermentation. This population decreased rapid-

ly during the first week and disappeared completely on the 8th day of fermentation in heat-shocked olives at (70 and 80 °C) and inoculated with *L.p*-FSO175 and supplemented with CFS of *C.p*-L18 (assay d) (Figure 3d). With other treatments (*L.p*-FSO175 (assay b) or the addition of CFS of *C.p*-L18 (assay c)), the disappearance of this population was observed on the 15th day of the process. In the un-inoculated and heat-shocked (at 60 °C) olives, this population showed a delay to disappear and were totally eliminated on the 22nd and 30th day of the fermentation process, respectively (Figure 5a).

The results of the combined ANOVA (Table 1) showed that the variance in *Enterobacteria* was affected with the inoculation of about 36%, while the effects of heat-shock explained less than 18%. A significant difference ($p < 0.05$) in mean value for *Enterobacteria* counts was found between heat-shocked olives at 60 °C and heat-shocked at (70 and 80 °C). However, no significant difference was found between *Enterobacteria* counts obtained at 70 or 80 °C (Table 1).

In the presence of inoculation with *L.p*-FSO175, significantly ($p < 0.05$) lower mean values for *Enterobacteria* counts were found in olives (assays b, c and d) when compared to that of un-inoculated olives (assay a) (0.41 Log CFU/mL). The inoculation with *L.p*-FSO175 and the addition of CFS of *C.p*-L18 (assay d) allowed the reduction of *Enterobacteria* counts to reach the lowest mean value (0.26 Log CFU/mL); while no significant difference was observed in the mean value for *Enterobacteria* counts between olives inoculated with *L.p*-FSO175 (assay b) (0.29 Log CFU/mL) and olives added with CFS of *C.p*-L18 (c) (0.3 Log CFU/mL).

3.3. Sensorial and organoleptic analysis

3.3.1. Olives color changes

The mean values for the CIE *l *a *b parameters of olive color are represented in (Table 2). The olives from all the assays showed high lightness (*l) in the range 62-74, which is generally associated with better color. The increase in temperature of heat-shock olives increased the lightness (*l), yellowness (*b), but decreased the greenness (*a). Heat-shock at 80 °C led to a significant increase in the mean values for (*l = 69.61), (*b= 51.19) and decrease in greenness (*a =

Table 2. Mean values and mean squares from the analyses of variance for olives color basing CIE*l*a*b parameters (*l =lightness, *a = greenness, *b = yellowness) during the controlled fermentation of heat-shocked (60 °C, 70 °C, 80 °C), un-salted and inoculated Moroccan green olives. a: un-inoculated olives (control), b: inoculated olives with *L.p*-FSO175, c: olives added with CFS of *C.p*-L18, d: olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18).

Factors			CIE *l*a*b parameters		
			*l	*a	*b
Heat-shock	60 °C		67.64 b	1.97 a	48.91 c
	70 °C		68.52 b	1.36 b	49.45 b
	80 °C		69.61 a	1.11 b	51.11 a
Inoculation	a		65.82 c	0.98 c	48.17 d
	b		71.41 a	2.45 a	49.43 b
	c		68.37 b	1.01 c	48.75 c
	d		68.77 b	1.47 b	52.94 a
Source of variation		Df			
Heat-shock		2	35,205	7,081	47,328
Inoculation		3	141,252	12,740	124,049
Heat-shock * Inoculation		6	235,947	9,067	132,858
Repetition		2	6,228	0,062	6,766
Residual		94	141,512	10,390	119,117

Means values in each column followed by the same letter are not significantly different according to LSD test at $p < 0.05$. Df: Degrees of freedom, *Significant at 0.05 probability level, **Significant at 0.01 probability level; ***Significant at 0.001 probability level.

1.11). Meanwhile, heat-shock at 60 °C led to the lowest mean values for the CIE *l *b parameters and the highest mean value for greenness (*a) (Table 2).

With regards to inoculation, a significant ($p < 0.05$) improvement in all CIE *l *a *b parameters was observed. The best improvement in color was observed in olives inoculated with *L.p*-FSO175 (assay b) (*l=71.71, *a=2.45 and *b=49.43) and in olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18 (assay d) (*l=68.77, *a=1.47 and *b=52.94) (Table 2). The best olive color was obtained at the end of the fermentation process (50th day), indicated by the best values for lightness (*l=74.08), greenness (*a=3.76) and yellowness (*b=57.54).

The combined analysis of variance (Table 2) showed the dominant effect of inoculation compared to that of heat-shock on all parameters of CIE*l*a*b, presenting about 32% of the observed variance for greenness (*a), 28% for yellowness (*b) and about 25% for lightness (*l). The influence of heat-shock was of about 6, 18 and 11%, respectively, on the variance for lightness (*l), for greenness (*a) and for yellowness (*b). An important effect due to the interaction (heat-shock * inoculation) was observed on the variance for lightness (*l) (42%), for greenness (*a) (23%) and for yellowness (*b) (30.9%).

3.3.2. Sensory characteristics of fermented olives

The mean values measured for the sensory characteristics of un-salted fermented green olives are reported in Table 3. Significant acceptability and palatability were determined by the panellist for all sensory characteristics due to the effects of inoculation and heat-shock. All the sensory characters showed significant differences ($p < 0.05$), due to the increase in heat-shock temperature (Table 3). Lowest mean values for all sensory characters were obtained with heat-shock at 60 °C, while, an acceptable sensory characteristic was found by the panellists for heat-shocked olives at 70 and 80 °C. Heat-shock at 80 °C led to a significant ($p < 0.05$) increase in the scores for acidic (7.1), bitterness (6.68) and color (6.8). A significant difference ($p < 0.05$) was obtained for bitterness between olives heat-shocked at (70 and 80 °C) and those treated at (60 °C). However, no significant difference was observed between heat-shocked olives at 70 and 80 °C.

Table 3. Mean values and mean squares from the analyses of variance of Sensory Characteristics (Color, Flavor, Crunchiness, Bitterness and Acidness) of un-salted fermented olives, under various effects of Heat shock (60 °C, 70 °C, 80 °C) and Inoculations. a: un-inoculated olives (control), b: inoculated olives with *L.p*-FSO175, c: olives added with CFS of *C.p*-L18, d: olives inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18).

Factors			**Color**	**Flavor**	**Crunchiness**	**Bitterness**	**Acidness**
Heat-shock	60 °C		4.58 c	4.86 c	5.80 c	5.30 b	6.17 c
	70 °C		6.46 b	6.61 a	7.26 a	6.57 a	6.42 b
	80 °C		6.80 a	6.17 b	6.92 b	6.68 a	7.10 a
Inoculation	a		4.90 c	4.58 c	5.70 c	5.18 d	5.28 d
	b		6.25 ab	6.36 a	7.26 a	6.95 a	7.50 a
	c		6.23 b	6.52 a	6.90 b	6.12 c	6.62 c
	d		6.42 a	6.07 b	6.78 b	6.50 b	6.85 b
Source of variation		Df					
Heat-shock		2	113.662***	66.354***	46.912***	47.512***	18.462***
Inoculation		3	29.811***	47.166***	27.248***	33.848***	52.015***
Heat-shock * Inoculation		6	33.206***	27.387***	27.856***	19.223***	18.106***
Repetition		19	0.319	0.311	0.267	0.226	0.437
Residual		209	0.245	0.221	0.238	0.279	0.292

Means values in each column followed by the same letter are not significantly different according to LSD test at $p < 0.05$.

Df: Degrees of freedom, *Significant at 0.05 probability level, **Significant at 0.01 probability level; ***Significant at 0.001 probability level.

The un-inoculated olives (assay a) showed the lowest values for all characteristics, compared to inoculated ones (assays b, c and d). According to the LSD test significant differences were observed at $p < 0.05$ for all the characteristics analyzed due to the effect of inoculation. The lowest mean values for all scores were observed in the un-inoculated olives. However, the highest scores for all sensory characterteristics were obtained in the presence of inoculation with *L.p*-FSO175 (assay b), followed by inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (d). The inoculation with CFS of *C.p*-L18 (c) showed the highest score for flavor.

The results of the combined analysis of variance (Table 3) showed the predominant effect of heat-shock, which presented about 46% for crunchiness, flavor, and bitterness and 64% for color; while the acidic variance was affected by the effect of inoculation (58%). The inoculation effect presented about 33% of the variance for flavor and bitterness, and only slightly influenced the variance in color (16.8%). Indeed, the interaction between heat-shock and inoculation showed significant effects at $p < 0.001$ on the variance in all characteristics analysed and explained about 19% for color, flavor, bitterness acidic and 27% for crunchiness.

3.3.3. Examination of olive spoilage

The results of the assessment of fermented olive spoilage showed that the main olive spoilages recorded are gas pocket, softening, off-odors and lactic spots. The un-inoculated, heat-shocked olives at 60 °C (assay a) showed the highest gas pocket incidence at about 19%, along with the presence of softening and off-odors. However, the lowest rate of spoiled olives was observed in olives inoculated with *L.p*-FSO175, added or not with CFS of *C.p*-L18), and a low level of gas pocket spoilage (5% and 3.34%) was obtained in olives inoculated with L.p-FSO175 and treated at 70 and 80 °C, respectively. No off-odor was detected by panellists for heat-shocked olives at 70 or 80 °C, inoculated with *L.p*-FSO175, and/or supplemented with CFS of *C.p*-L18.

4. DISCUSSION

In a previous work, the controlled fermentation of Moroccan Picholine variety green olives, using the oleuorpeinolytic strain of *L.p*-FSO175, showed some bitterness in fermented olives (Ghabbour *et al.,* 2016). To overcome this problem and in order to improve both the bio-debittering and fermentation processes, the green olives were subjected to heat-shock (60, 70, 80 °C) tree times for 5 min, followed by inoculation with *L.p*-FSO175 and/or addition of CFS of *C.p*-L18.

The results showed that the highest mean values for sugars and polyphenols (5.6 mM and 38.9 mM, respectively), obtained in the olives heat-shocked at 80 °C, could be attributed to the increase in permeability of olive pulp due to heat-shock (Balatsouras *et al.,* 1983), facilitating the release of nutrients into the olive water. These results indicate the beneficial double technological roles of heat-shock: firstly, by providing sugars for the microorganisms, including LAB starter (Argyri *et al.,* 2014), and secondly by eliminating high amount of polyphenols (mainly oleuropein) by osmosis, thus accelerating the debittering process (Valenčić *et al.,* 2010).

It should be emphasized that the high temperatures of heat-shock (70 and 80 °C) significantly increased the LAB growth (to 8-10 Log CFU/mL) on the 15th day of fermentation. In fact, the heat-shock permitted the dominance of LAB during the first days of the fermentation process, through providing high amounts of nutrients, mainly sugars, polyphenols, and vitamins. In addition, when the inoculation was added, more significant ($p < 0.05$) differences in LAB were obtained compared to un-inoculated olives. The highest mean values for LAB were obtained, respectively, in olives inoculated with *L.p*-FSO175 (9.25 Log CFU/mL) and with *L.p*-FSO175 and added with CFS of *C.p*-L18 (9.2 Log CFU/mL). This result is similar to that reported by (Saravanos *et al.,* 2008).

In the presence of *L.p*-FSO175 and CFS of *C.p*-L18, lower mean values for sugars and polyphenols were observed in the olive water (3.98 mM and 34.94 mM, respectively), and in the presence of *L.p*-FSO175, the mean values obtained were (4.44 mM and 35.81 mM, respectively); while, the highest mean values for sugars and polyphenols were found in un-inoculated olives (6.9 mM and 38.85 mM, respectively). These results mean that high sugar contents were metabolized by the *L.p*-FSO175 starter culture and as a result, the lowest values for pH (3.7-3.8) and highest values for free acidity (of about 1%) were found. The latter is related to the accumulation

of organic acids produced by inoculum through the consumption of the sugars present in olives (Chorianopoulos *et al.,* 2005; Panagou *et al.,* 2008). Similar results for brine acidity (1.0-1.2%) and low pH values (3.8-3.9) were reported in heat-shocked olives which were fermented by *L. plantarum* (Etchells *et al.,* 1966).

The increase in temperature of heat-shock significantly decreased ($p < 0.05$) the yeast and mold population, and the lowest mean value (6.72 Log CFU/mL) was obtained for heat-shocked olives at 80 °C. Similar results were reported by Chorianopoulos *et al.,* (2005), who attributed the decrease in yeasts and molds to heat-shock. However, in the presence of *L.p*-FSO175 and CFS of *C.p* L18 (assay d), a significant ($p < 0.05$) increase in yeasts and molds was observed, allowing the highest mean value for yeasts and molds (7.39 Log CFU/mL), followed by that (7.2 Log CFU/mL) obtained in the presence of CFS of *C.p*-L18 (assay c). The presence of this population in all heat-shocked olives is due to the autochthonous yeasts and molds coming from the epidermis of the fruits themselves (Arroyo-Lopez *et al.,* 2012b; Pereira *et al.,* 2015). The olives added with CFS of *C.p*-L18 (assay c) showed significant ($p < 0.05$) differences in yeast and mold growth compared to un-inoculated olives. This result could be related to the wealth of nutrients in CFS of *C.p*-L18 (amino acids, fatty acids, vitamins and salts) which are necessary for yeasts and molds to grow. The persistence of the yeast and mold population during this process is of double technological importance. Firstly, they promote LAB growth (starter and/or autochthonous), providing them with the essential nutrients for their growth (Arroyo-López *et al.,* 2008; Arroyo-Lopez *et al.,* 2012b; Sidari *et al.,* 2019). Secondly, they improve the biological debittering process of fruits, based on their esterase and β-glucosidase activities (Rodriguez-Gomez *et al.,* 2012; Anagnostopoulos *et al.,* 2017).

The results obtained in this work showed that the increase in temperature of heat-shock led to a significant ($p < 0.05$) decrease in *Enterobacteria*, and a low count of this population was observed at the beginning of fermentation. Compared to 70 and 60 °C, heat-shock at 80 °C resulted in the lowest mean value for *Enterobacteria*. This finding indicates the beneficial effect of heat-shock on olives to give another advantage to the starter culture to dominate the fermentation process by eliminating most of the competitive and interfering microbiota (Etchells *et al.,* 1966; Balatsouras *et al.,* 1983; Chorianopoulos *et al.,* 2005).

In addition, inoculation with *L.p*-FSO175 and the addition of CFS of *C.p*-L18 significantly decreased the *Enterobacteria* count compared to un-inoculated olives, leading to the lowest mean value for this population (0.26 Log UFC/mL) (assay d). The earlier disappearance of *Enterobacteria* on the 8th day of fermentation was obtained in heat-shocked olives at (70 and 80 °C) and inoculated with *L.p*-FSO175 and added CFS of *C.p*-L18 (assay d). However, the un-inoculated and heat-shocked (at 60 °C) olives showed the highest mean value for *Enterobacteria* (0.41 Log UFC/mL), and their disappearance was obtained, with a delay, on the 22nd and 30th day of the fermentation process. The reduction in *Enterobacteria* can be attributed to antibacterial and antifungal compounds produced by *L.p*-FSO175, such as organic acids, hydrogen peroxide and bacteriocins (Abouloifa *et al.,* 2021). Furthermore, their total disappearance, during the first week of process, can also be related to the inhibitory effect of phenolic compounds (Landete *et al.,* 2008; Segovia-Bravo *et al.,* 2009). The safety of the final product can be ensured by the combination of two hurdles, namely acidity (pH) and antimicrobial compounds. However, a heat sterilization of fermented olives, packed in hermetically sealed containers, is mandatory to ensure their safety during storage.

With regard to color, the results showed that as the temperature of heat-shock increased, better color was obtained. It was demonstrated that the increase in olives storage temperature improved olive color (Rodríguez-Gómez *et al.,* 2014). The heat-shock at 80 °C showed a significant ($p < 0.05$) increase in mean values for lightness (*l = 69.61), yellowness (*b= 51.19) and decrease in greenness (*a = 1.11). This finding may be explained by the thermal inactivation of polyphenol oxidase (PPO) after heat-shock at 80 °C, thus preventing the browning of olive fruits (Whitaker and Lee, 1995). However, in olives heat-shocked at 60 °C, lowest color scales for CIE *L *a *b parameters were observed. This result may be explained by the increase in oxidation of polyphenols caused by the persistence of PPO activity in the raw material, favored by the pH of the olive water (4.5), which falls in the optimum pH range (pH 4-7) of PPO (Ben-Shalom *et al.,* 1977). Heat-shock at 60

°C seems to be insufficient to deactivate the PPO. This result is in agreement with previous works, reporting that short-term treatments (few minutes) at 70 or 90 °C, are generally sufficient to destroy all of the PPO activity in plants (Yemenicioğlu and Cemeroğlu, 2003).

The best color improvement (*l=71.71, *a=2.45 and *b=49.43) was observed in olives inoculated with *L.p*-FSO175 (assays b), followed by (*l=68.77, *a=1.47 and *b=52.94) inoculated with *L.p*-FSO175 and added with CFS of *C.p*-L18 (assay d). These results can be attributed to the favorable effect of lower pH and high acidity on the color parameter, through the diffusion of organic acids in fermented olives (Garrido Fernandez *et al.,* 1997b). Moreover, the inoculation with *L.p*-FSO175 and/or addition of CFS of *C.p*-L18 combined with heat-shock at 60 °C allowed significant improvement in color, meaning that the polyphenol oxidation observed in un-inoculated heat-shocked olives at 60 °C was overcome by the high acidification assured by the inoculum. The PPO involved in polyphenol oxidation was reported to be inhibited at pH lower than 4 in heat-shocked olives at 60° (Nicolas *et al.,* 1994). Thus, inoculation with *L.p*-FSO175 and heat-shock at 60 °C can be sufficient to avoid the enzymatic browning of fermented olives.

The results of sensory attributes of un-salted fermented green olives, indicate that the heat-shock at 80 °C permitted significant ($p<0.05$) improvement of acidness (7.1), bitterness (6.68) and colour (6.8). Significant ($p<0.05$) differences in values of bitterness were obtained between olives heat-shocked at (70 °C and 80 °C) and that treated at 60 °C, which may be linked with the effect of heat-shock in increasing release of polyphenols from olives observed in this work. These results indicate the beneficial effects of heat-shock at (70 °C and 80 °C) to promote the debittering of un-salted green olives, by increasing the permeability of olives pulp (Balatsouras *et al.,* 1983), and facilitating the diffusion of polyphenols and other nutrients from olives by osmosis, and consequently increasing debittering process (Valenčić *et al.,* 2010). On the other hand, the best appreciations of colorness by the panellists due to heat-shock of olives by high temperatures (70 °C and 80 °C), were confirmed by CIE*L*a*b parameters.

The inoculation of olives with *L.p*-FSO175 led to significant ($p<0.05$) appreciation of all sensory attributes. Previous works reported the improvement of sensory attributes of fermented olives by using an oleuropeinolytic strain of *L. plantarum* as debittering and fermenting agent (Tataridou and Kotzekidou, 2015, Ghabbour *et al.,* 2016). Thus, the reduction of bitterness in olives inoculated with *L.p*-FSO175 can be attributed to the high oleuropeinolytic activity of this strain, particularly in the absence of salt (Ghabbour *et al.,* 2020). The reduction of bitterness observed in olives added with the CFS of *C.p*-L18 can be related to the β-glucosidase enzyme induced in *C. pelliculosa* L18 by oleuropein (Rokni *et al.,* 2021). The effectiveness of some *Candida* species starters in bio-debittering was reported (Ciafardini and Zullo, 2019) to have led to lower concentrations of polar phenolic compounds in olive brines. The oleuropeinolytic activity of *L.p*-FSO175 and the CFS of *C.p*-L18, is of great interest because it contributes to the high accumulation of hydroxytyrosol in fermented olives (Chytiri *et al.,* 2020), which is highly desired to improve their nutritional value (Bertelli *et al.,* 2020).

The results of the analysis of spoilage in fermented olives showed that the increase in temperature due to the heat-shock (70 °C and 80 °C) of olives led to a low incidence of gas pockets (less than 5%) in fermented olives, which can be due to gas-producing microorganisms (Asehraou *et al.,* 2000). In the presence of inoculation with *L.p*-FSO175 with or without the addition of CFS of *C.p*-L18, the lowest rate of gas pockets was observed and no off-odors were detected by panellists. These results should be explained by an adequate balance in the olives' microbiota (LAB > yeasts) to reduce tissue damage (Golomb *et al.,* 2013). The benefits of inoculation with *Lactobacillus* starters in improving the hygienic quality of fermented olives was reported (Peres *et al.,* 2008, Hurtado *et al.,* 2010, Aponte *et al.,* 2012, Ghabbour *et al.,* 2016).

From all the obtained results, compared to 60 and 70 °C, the heat-shock of olives at 80 °C showed its advantage in improving the physicochemical, microbiological and the sensory properties of un-salted fermented olives. In addition, the inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 (assay d) showed the best improvement in the fermentation profile of un-salted olives, compared to the olives inoculated with *L.p*-FSO175 (b) or added with CFS of *C.p*-L18 (assay c). These results indicate that the heat-shock of olives at 80 °C, followed by

inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18, could be a suitable process for the controlled fermentation of un-salted green olives. This process is characterized by a high decrease in pH (< 3.8) and increase in acidity (> 0.8%), and by an early disappearance of *Enterobacteria* (first week), and a reduced fermentation time (50 days). Furthermore, this process allows the production of un-salted green olives with considerable antioxidants (Bertelli *et al.,* 2020), and probiotic and organoleptic properties (Abouloifa *et al.,* 2019; Abouloifa *et al.,* 2020).

5. CONCLUSIONS

The present study reports the controlled fermentation of heat-shocked, un-salted and inoculated Moroccan Picholine green olives for the first time. The results showed that heat-shock at 80 °C improved the fermentation profile of un-salted olives by increasing the release of nutrients (i.e. sugars and polyphenols), reducing the bitterness and improving the color of fermented olives. The inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18 enhanced the fermentation process, indicated by a high decrease in pH, high increase in free acidity, and rapid disappearance of *Enterobacteria* obtained on the 8th day of fermentation. This process produced a substantial improvement in sensory attributes (bitterness, color and acidity) and reduced the spoilage incidence in fermented olives. Based on these results, the heat-shock of olives at 80 °C, followed by their inoculation with *L.p*-FSO175 and addition of CFS of *C.p*-L18, can be appropriate for the biological debittering and fermentation of un-salted Moroccan Picholine green olive variety. Furthermore, this process presents promising prospects not only for consumers by providing salt-free olives, but also by protecting environment, by releasing chemical-free wastewater (NaCl and NaOH).

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Lipid profile, volatile compounds and oxidative stability during the storage of Moroccan *Opuntia ficus-indica* seed oil

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SUMMARY: The fatty acids, sterol, tocopherol and volatile compositions of Moroccan cold-pressed cactus (*Opuntia ficus-indica*) seed oil were studied. The most abundant fatty acid, tocopherol and sterol were linoleic acid (60.6%), γ-tocopherol (533 mg/kg) and β-sitosterol (6075 mg/kg), respectively. In this study, 23 volatile compounds were identified with perceivable odor attributes for 14 compounds. The oxidative quality of cactus seed oil was monitored over 4 weeks at 50 °C. Increases in PV, K232 and FFA were detected during the first two weeks as well as a decrease in the induction time; whereas no change was reported for the K270 values. The amount of total phenolic content increased until it reached 0.3 mg/kg and then decreased by the end of the storage period; while tocopherols started to decrease after the first week. The fat-free residue extracts showed a very strong effect to reduce the oxidation of linoleic acid. Consequently, the extracts were significantly more effective to bleach β-carotene in the β-carotene-linoleic acid assay in comparison with the control.

KEYWORDS: *β-Sitosterol; γ-Tocopherol; Opuntia ficus-indica; Oxidative stability; Peroxide value.*

RESUMEN: ***Perfil lipídico, compuestos volátiles y estabilidad oxidativa durante el almacenamiento del aceite de semilla de*** **Opuntia ficus-indica** ***marroquí.*** Se estudiaron los ácidos grasos, esteroles, tocoferoles y la composición volátil del aceite de semilla de cactus marroquí (*Opuntia ficus-indica*) prensado en frío. Los ácidos grasos, tocoferoles y esteroles más abundantes fueron el ácido linoleico (60,6%), γ-tocoferol (533 mg/kg) y β-sitosterol (6075 mg/kg), respectivamente. En este estudio, se identificaron 23 compuestos volátiles con atributos perceptibles para 14 de ellos. La oxidación del aceite de semilla de cactus fue monitoreada durante 4 semanas a 50°C. Se observó un aumento en el PV, K232 y FFA durante las dos primeras semanas y una disminución en el tiempo de inducción, mientras que no se apreciaron cambios para los valores de K270. La cantidad de fenoles totales aumentó hasta alcanzar 0,3 mg/kg y luego disminuyó al final del almacenamiento, mientras que los tocoferoles comenzaron a disminuir después de la primera semana. Los extractos de residuos libres de grasa mostraron un efecto muy fuerte para reducir la oxidación del ácido linoleico. En consecuencia, los extractos fueron significativamente más efectivos para blanquear el β-caroteno en el ensayo de β-caroteno-ácido linoleico en comparación con el control.

PALABRAS CLAVE: *β-Sitosterol; Estabilidad oxidativa; γ-Tocoferol; Índice de peróxido; Opuntia ficus-indica.*

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1. INTRODUCTION

Recently, the requirements for natural ingredients, healthy foods and nutraceuticals had increased (Matthäus and Özcan, 2011). Plants are viable sources to satisfy this necessity because they contain a large number of bioactive compounds with several positive effects on human nutrition, cosmetics, medicine or pharmacy.

A fruit with multiple properties such as *Opuntia ficus-indica* (OF) is beneficial to the production of nutraceuticals functional foods due to the presence of essential components, such as amino acids, fatty acids, carbohydrates, minerals, vitamins and soluble fibers (Chahdoura *et al.,* 2017). The cactus plant, which belongs to the Cactaceae family, is native to Mexico. Several parts of the plant have been used in human nutrition for thousands of years. In the 16th century the plant was introduced into the Mediterranean basin as well as South and North Africa (Matthäus and Özcan, 2011).

Interest in *Opuntia ficus-indica* (OF) has increased in many countries as a result of its socio-economic and ecological benefits such as overcoming desertification and erosion, use in animal feed and cosmetic products (Taoufik *et al.,* 2015). The seeds in OF represent about 10% of the fruit and are discarded as waste after the production of cactus juice. (Gharby *et al.,* 2013).

The oil yield from OF seeds in Morocco ranged from 5.4 – 9.9% (Taoufik *et al.,* 2015) and this was similar to the results reported in different studies that used solvent extraction (Salvo *et al.,* 2002; Ramadan and Mörsel, 2003). *Opuntia ficus-indica* seed oil (OFSO) has been characterized to have a very high content of unsaturated fatty acids, in which linoleic acid is recorded as the major fatty acid (Salama *et al.,* 2020). A low amount of saturated fatty acids (palmitic and stearic) was detected in OFSO (Salama *et al.,* 2020). The oil also contains important compounds like sterols and tocopherols (El Mannoubi *et al.,* 2009). Regarding pharmacological activity, OFSO showed relevant antioxidant and antimicrobial activities (Ramírez-Moreno *et al.,* 2017), α-glucosidase inhibitory activity, and cytotoxicity against human tumor cell lines, in addition to anti-inflammatory and analgesic activities (Chahdoura *et al.,* 2017).

Therefore, the purpose of this study was to analyze the physicochemical parameters, fatty acid composition, tocopherols and phytosterols in OFSO from Morocco. In addition, the volatile compounds and key odorants of Moroccan OFSO were determined by using GC-MS olfactometry. The oxidative stability of OFSO was monitored over 4 weeks at 50 °C to reveal changes in peroxide value, acidity, UV absorptions, total phenolic content, total tocopherols and induction time, all of which could give an overview on the impact of storage on the quality attributes of the oil.

2. MATERIALS AND METHODS

2.1. Materials

Opuntia-ficus indica seeds and its cold-pressed seed oil were purchased from two local coops in Tiznit, Morocco, and Marrakech, Morocco, respectively. The seeds were identified by Professor Fennane (Scientific Institute, Mohammed V University, Rabat) (Nounah *et al.,* 2021). The two coops collected the fruits from the two areas (Tiznit and Marrakech) in 2017. The cactus fruit was peeled and the seeds were manually collected, sundried and cold-pressed using a KOMET D85-type screw press. OF seeds were used for phenolic compound extraction and cold-pressed OFSO was used for oil parameters.

2.2. Methods

2.2.1. Storage of OFSO at 50 °C for one month

Brown bottles were filled with OFSO and tightly closed. To accelerate lipid oxidation, the bottles were put in an air oven at 50 °C for four weeks. After each week, corresponding samples were removed from the oven for analysis. Peroxide value (PV), free fatty acid (FFA), specific extinction coefficient (K232 and K270), total phenolic content (TPC), total tocopherols and induction period (IP) were measured to determine the oxidative stability of OFSO.

2.2.2. Determination of peroxide values (PV)

In brief, 5 g from the oil were dissolved in a mixture of acetic acid-isooctane (60:40 v/v). Zero point five milliliters of potassium iodide (saturated), were put in the previous solution and shaken for 60 sec. After that, 100 mL of boiled Millipore water were added and titration was done to the solution by pH metric titration with alcoholic (KOH) = 0.1 mol/L.

The peroxide value was determined using METHROM Titrando 888 and expressed as mEq of active oxygen/kg oil.

2.2.3. Determination of free fatty acid (FFA)

After the dissolution of 10 g from the oil in 100 mL of diethyl ether/ethanol (1:1 v/v), the oil was titrated with potassium hydroxide (ethanolic KOH) of known titer (0.1N). METHROM Titrando 888 with Tiamo software was used to measure the % FFA.

2.2.4. UV absorption analysis

Measuring the absorbance at around 232 nm and 270 nm allows the detection and evaluation of the primary and secondary oxidation products. OFSO (250 mg) was put into a graduated flask (25 mL) and diluted with spectrophotometric grade cyclohexane (25 mL). The sample was homogenized by using a vortex (30 seconds), and then the resulting solution was placed into a quartz cuvette. A double beam spectrophotometer was used to determine absorbances at 232 and 270 nm using pure cyclohexane as a blank. The specific extinctions were measured according to the following equation:

$$K\lambda = \frac{E\lambda}{C \times S}$$

Where: Kλ: specific extinction at a wavelength λ; Eλ: absorption or extinction at a wavelength λ; C: the solution concentration (g/100 mL). S: quartz cell length (cm).

2.2.5. Oxidative stability (Rancimat analysis)

To study the oxidative stability of the sample, a Metrohm® 743 Rancimat was used. The oxidation process was tested for 3.6 g of OFSO by exposure to 110 °C in a heating block with an air flow rate of 20 L/hour. The volatile components formed as oxidation products were captured in distilled water and the change in conductivity as a result of oil degradation was determined automatically by the instrument. With the steep increase in conductivity the sample had lost the resistance to oxidation and the inflection point of the curve was calculated. The oxidative stability of the samples was given as induction time in hours.

2.2.6. Determination of fatty acid composition

Methods DGF C-VI 10a (00) and C-VI 11d (19) (DGF 2013) were used to measure the fatty acid composition as follows: in a tube, one drop from the oil sample was dissolved in n-heptane (1 mL). Then 50 mg of CH_3ONa were added and vortexed for 1 min. After that, distilled water (0.1 mL) was added and the tube was centrifuged for 5 min at 3000 rpm. Afterwards, the aqueous bottom phase was eliminated carefully. After adding HCl (1 mol with methyl orange (Merck, Darmstadt, Germany)) (50 µL) again the lower phase was removed. Another centrifugation (with the same parameters) was done after adding $NaHSO_4$ (20 mg) (monohydrate, extra pure). The upper phase from the tube (n-heptane) was transferred to a vial and injected into a GC (HP5890, Agilent Tech. Waldbronn, Germany) with CP-Sil 88 capillary column, (100 m, 0.25 mm, 0.2 µm for length, ID and film thickness, respectively). The injector temperature was 250 °C and the oven program started from 155 °C, heated to 220 °C (1.5 °C/min), and held at isotherm for 10 min. Detection was done by a flame ionization detector (FID) monitored at 250 °C. Other parameters were as follow: 36 mL/min for carrier gas (hydrogen); the gas flow was 1.1 mL/min; split ratio 1:50; 30 mL/min for detector gases (hydrogen), air (300 mL/min) and nitrogen 30 mL/min; the injection volume was 1 µL. After automatic integration of the peak areas by the software, the fatty acids methyl esters (FAME) were reported as weight percent by direct internal normalization.

2.2.7. Determination of sterol composition

The determination of the sterol composition was made according to method DGF F-III 1 (98) (DGF 2013) and described as follows: cholestan-3-ol as internal standard and 5 mL ethanolic KOH were added to 250 mg OFSO and refluxed for 15 min. Solid-phase extraction was used to isolate the unsaponifiable matter by using a column of Al_2O_3. The sterol fraction was separated from unsaponifiable matter by thin-layer chromatography with n-heptane and distilled diethyl ether (50:50 v/v) as mobile phase. The sterols were silylated using a silylating agent (MSHFBA, with 50 uL methylimidazole dissolved in the MSHFBA-vial). The sterol fraction composition was illustrated by GLC, on a SE 54 CB column (Macherey-Nagel, Duren, Germany; 50 m,

0.32 mm, 0.25 µm for length, ID and film thickness, respectively). The other parameters were set as follows: the temperature of the injection and detection adjusted to 320 °C, the carrier gas was hydrogen, the program temperature, 245–260 °C (5 °C/min). Standard compounds such as β-sitosterol, campesterol, stigmasterol separated from rapeseed oil or Δ7-avenasterol, Δ 7-stigmasterol, and Δ 7-campesterol isolated from sunflower oil were used to identify the peaks. For the first time, the other sterols were reported by GC-MS and then identified in the samples by comparison with the retention time.

2.2.8. Determination of tocopherol composition

Method DGF F-II 4a (00) (DGF 2013) was used to determine the tocopherol composition as follows: 150 mg of oil sample were dissolved in 1 mL of n-heptane. After filtration (1.0 µm and 0.45 µm), the oil sample was directly used for HPLC analysis as follows: low-pressure gradient system (Merck-Hitachi), with a pump (L-6000), F-1000 Merck Hitachi fluorescence spectrophotometer detection system and a ChemStation software for integration. 20 µL of the sample were injected by a Merck 655-A40 autosampler onto the HPLC column (Diol phase 25 cm × 4.6 mm ID). The separation was performed with n-heptane and tert-butyl methyl ether (482:18 mL v/v) as mobile phase at a flow rate of 1.3 mL/min.

2.2.9. GC-MS-olfactometry analysis

Three grams of OFSO were put into a 20 mL headspace vial. The vial was purged with a stream of helium (10 PSI, 20 mL/min) at 80 °C for 20 min using a PTA 3000 dynamic headspace system, and the volatile compounds were trapped with an online Tenax trap cooled to -35 °C using a Peltier element as a cooling device. Afterwards the trap was heated for 10 min at 200 °C to transfer the volatiles via an uncoated fused silica transfer line at 200 °C to the GC system. As GC System a Trace 1300 Series GC equipped with a CPSil 19 fused silica capillary column (14% cyanopropyl-phenyl 86% dimethylpolysiloxane, 60 m, 0.32 mm ID, 1 mm film thickness) was used for the separation of volatile compounds. The temperature program was 40 °C for 5 min, then heated at 3 °C/min to 245 °C, and finally held at isotherm for the least 10 min. Olfactometry volatiles were divided in a ratio of 1:3 between the MS (Thermo Scientific, Darmstadt, Germany) and the olfactometry detection port (ODP).

Through a transfer line set at 200 °C, one part of the volatiles reached the MS ionization source (electronic ionization (EI) set to 230 °C in positive mode). The other volatiles were moved to the ODP set at 260 °C. For each new sequence, a blank consisting only of air was used to ensure good reproducibility of the retention times (DGF 2013).

GC-olfactometry was done by two test persons who noted the retention time of the olfactory perceptions, its intensity and aroma description. The lists of aroma profiles were compared to the chromatograms of the samples and the olfactory impressions were assigned to the corresponding peaks. Peak identification was performed by comparing mass spectra information with the NIST 2005 database as well as known data from the internal library of the instrument. Chemical standards were injected into the GC-MS system to validate some identifications.

2.2.10. Determination of the total phenolic compounds (TPC)

Extraction of phenolic compounds was done according to Owon *et al.* (2021) with some modifications. One gram of the OFSO was extracted by a mixture of methanol-water (80:20 v/v) using an ultrasonic bath for 30 min. After centrifugation for 5 min at 3000 rpm the supernatant was filtered into a 50-mL round-bottom flask. The extraction was repeated twice before removing the solvent by a rotary evaporator at 40 ºC.

The residue was dissolved with 1 mL from the same solvent (ultrasonic bath), transferred into a 5-mL volumetric flask and the 50-mL flask was washed twice. The volumetric flask was filled with 0.3% MeOH-HCl and 2 mL of Na_2CO_2 (2%) were added to a 100-µL aliquot. Fifteen microliters of Folin-Ciocalteu (Merck, Darmstadt, Germany) reagent were added after 2 min.

The absorbance was measured after a further 30 min using a spectrophotometer (U 2000, LTD, Hitachi Tokyo, Japan) at 750 nm wavelength. Total phenolic content was expressed as gallic acid (mg) equivalents GAE/g extract.

2.2.11. Determination of OFS antioxidant activity with the ß-carotene bleaching method

The ß-carotene/linoleic acid assay was used to evaluate the antioxidant activity of OFS. Linoleic acid (40 mg) and Tween-20 (400 mg) were mixed in a flask with 1 mL of a mixture of 12 mg ß-carotene dissolved in 2 mL of chloroform. The chloroform was evaporated at 40 °C (rotary evaporator, IKA, model RV 10 CS93) and an emulsion was formed by adding 100 mL distilled water and mixing with a vortex. 0.2 mL of the OFS extract were added to 5 mL of the emulsion in a tube and the absorbance was immediately measured against a blank (the emulsion without ß-carotene) at 470 nm with a spectrometer (Analytik Jena Specord 250). Afterwards the absorbance of the solution was measured every 15 min for one hour and finally after 120 min. The tubes were incubated at 40 °C in a water bath (HAAKE C 10) between measurements.

2.3. Statistical analyses

Values were determined in three replicates (means ± SD). One-way ANOVA was used to analyze mean values at the 0.05 significance level.

3. RESULTS AND DISCUSSION

3.1. Physicochemical parameters and fatty acids composition

The oil extracted from OFS was a yellow-green oil that had a slightly fruity taste. The results in Table 1 show that very low acidity of about 0.4% (calculated as oleic acid) was reported for OFSO. The PV of OFSO reached about 3.5 meqO_2/kg and extinction coefficients (K232 and K270) were determined to be 2.83 and 0.35, respectively.

The oxidation of lipids is a significant cause of quality degradation in edible oil. Oxidative stability refers to the resistance of the oil to oxidation during processing and storage. The time it takes to reach the critical point of oxidation, whether it is a sensorial transition or a sudden acceleration of the oxidative phase, can be expressed as resistance to oxidation (Silva *et al.,* 2001).

Oxidative stability as a marker of quality has been extensively evaluated by accelerated methods such as the Rancimat method, which uses elevated temperatures and airflow. The induction period (IP) at 110 °C of OFSO was reported to be 6.9 h. The results showed that the oxidative stability of OFSO was higher than that of sunflower (5.3h) (Almoselhy, 2021). However, it was less than moringa (10 h), olive (23 h) and argan (31 h) oils (Gharby *et al.,* 2013; Salama *et al.,* 2020). Generally, this difference is related to the number of antioxidants and the percentage of polyunsaturated fatty acids. The higher the percentage of polyunsaturated fatty acids, the lower the oxidative stability.

OFSO consisted of different fatty acids which are also typical for other seed oils, including saturated fatty acids such palmitic and stearic acids, as well as oleic, vaccenic and linoleic acids, representing the unsaturated fraction (Table 2). The data shows that OFSO contained SFA and PUFA of about 16.0 and 82.7%, respectively. Therefore, the calculated oxidizability (COX) value was determined by the percentage of unsaturated C18 fatty acids as follows:

$$COX = (1\,[18{:}1\%] + 10.3\,[18{:}2\%] + 21.6\,[18{:}3\%])/100$$

In this work, the COX value of OFSO was 6.45, which was greater than that for fresh palm, peanut, camellia and moringa oils (1.61, 4.63, 1.77 and 0.82 respectively) (Xu *et al.,* 2015; Salama *et al.,* 2020). On the other hand, the ratio of PUFA/SFA was 3.80 in OFSO. It is known that the COX value and the ratio of PUFA/SFA are considered as tendency parameters of oils to undergo oxidation (Méndez *et al.,* 1996). Linoleic, oleic and palmitic acids were the most abundant fatty acids in OFSO (60.6, 16.3 and 11.8%, respectively), while stearic and vaccenic acids were found in lower amounts (3.5 and 4.6%, respectively). This result makes OFSO belong to the group of oleic-linoleic acid oils and agrees with the results reported by Özcan and Al Juhaimi (2011); Ciriminna *et al.* (2017); Gharby *et al.* (2021) who showed that oleic and linoleic acids ranged from 17.61 to 25.52% and from 57.98 to 61.80%, respec-

Table 1. Physicochemical parameters of OFSO

Parameters	Value
Free fatty acid (oleic acid %)	0.40±0.04
Peroxide value (meqO_2/Kg oil)	2.1±0.3
K232	2.83±0.01
K270	0.35±0.04
Induction period (h)	6.85±0.02

Values are the mean of three values ± standard deviation.

Table 2. Fatty acid composition of OFSO

Fatty acid	Value (%)
Myristic	0.1±0.0
Palmitic	11.8±0.0
Stearic	3.5±0.0
Arachidic	0.4±0.0
Behenic	0.2±0.0
Lignoceric	0.1±0.0
Palmitoleic	0.6±0.0
Elaidic	0.1±0.0
Oleic	16.3±0.0
Vaccenic	4.6±0.0
Linoleic	60.6±0.1
Linolenic	0.2±0.0
Gondoic	0.2±0.0
SFA	16.00
MUFA	21.84
PUFA	60.87
Total unsaturated fatty acids (USFA)	82.71
PUFA/SFA	3.80
Total fatty acids	98.71
Cox value	6.45

SFA: Total saturated fatty acids, MUFA: Mono unsaturated fatty acids. PUFA: Polyunsaturated fatty acids, USFA: Total unsaturated fatty acids. COX: Calculated oxidizability value.

Values are the mean of three values ± standard deviation.

tively. The fatty acid composition of OFSO was similar to those of grape seed and sunflower oils, which also belong to the oleic-linoleic acid oils (Özcan and Al Juhaimi, 2011).

Linolenic acid was detected at a low level in OFSO (0.2%). This small amount of linolenic acid helps in detecting adulteration of this oil with other oils if other cheaper oils with higher amounts of linolenic acid were added. Palmitoleic, arachidic, gadoleic and behenic acids were also reported in OFSO in very small amounts. Ciriminna *et al.* (2017) reported a higher amount of stearic acid (3.92%) in comparison to the present results. The observed difference may be due to the difference in fruit maturity and geographical origin. The dietetic quality of OFSO is high as the total unsaturated fatty acids/total saturated fatty acids ratio is approximately 5.2, which is similar to that of argan oil (Gharby *et al.,* 2013).

3.2. Sterols and tocopherols composition

Sterols constitute the major part of the unsaponifiable matter in many oils. The phytosterol content can play a role in detecting adulterations in vegetable oils (Taoufik *et al.,* 2015). It has been shown that phytosterols lower blood LDL cholesterol by around 10–15% as part of a healthy diet (Ramadan and Mörsel, 2003). Table 3 shows the sterol composition measured in this study. β-sitosterol represented the highest amount of all phytosterols in OFSO (6075 mg/kg). This high amount of β-sitosterol in OFSO makes it very useful in inhibiting dietary cholesterol absorption (Taoufik *et al.,* 2015). Other sterols were determined in OFSO such as campesterol (877 mg/kg), Δ-5-avenasterol (411 mg/kg), sitostanol (266 mg/kg) and cholesterol (189 mg/kg). The total sterol amount of OFSO (855 mg/100 g) was lower compared to the Tunisian (1606 mg/100 g) and German (933 mg/100 g) OFSO. β-sitosterol was the predominant component in all samples, varying from about 71.6% in the Tunisian samples to 72.3% in German samples (Ramadan and Mörsel, 2003; El Mannoubi *et al.,* 2009).

Table 3. Sterol and tocopherol compositions of OFSO

Compound	Value (mg/kg)
β-Sitosterol	6075±38
Campesterol	877±38
Δ-5-Avenasterol	411±11
Sitostanol	266±26
Cholesterol	189±3
Δ-7-Avenasterol	180±5
Δ-7-campesterol	133±21
Stigmasterol	124.9±0.01
Δ-7-Stigmasterol	111±26
Δ-5-2,4-stigmastadienol	103.0±0.9
Δ-5-2,3-stigmastadienol	101±2
Total sterol	8554±80
α-tocopherol	11±0
γ-tocopherol	533±3
δ-tocopherol	4±0
β-tocotrienol	7±0
γ-tocotrienol	4±0
Total tocopherol	559±3

Values are the mean of three values ± standard deviation.

Tocopherols are natural antioxidants that have some antioxidant properties. OFSO contains large amounts of tocopherols and tocotrienols (Taoufik *et al.,* 2015). In our study, the vitamin E active compounds identified were α, γ, δ-tocopherols and β, γ-tocotrienols while β-tocopherol was absent in the seed oil. Table 3 shows the tocopherol content in OFSO. γ-tocopherol was the major tocopherol in OFSO, accounting for 533 mg/kg (95.28%), whereas α-tocopherol, ß-tocotrienol, δ-tocopherol and γ–tocotrienol accounted for 11, 7, 4, and 4 mg/kg, respectively. These findings were close to the results reported by Ramadan and Mörsel (2003), who found that γ-tocopherol, α-tocopherol and δ-tocopherol represented 330, 56 and 5 mg/kg. The higher biological activity of α-tocopherol in comparison to the other tocopherols makes it interesting for human consumption, but γ-tocopherol shows the highest antioxidant capacity (Gharby *et al.,* 2013). The high antioxidant activity of the tocopherols against the oxidation of polyunsaturated fatty acids helps to enhance oil quality by preserving it from rancidity during storage and prolonging the shelf-life (Matthäus and Özcan, 2011). The amount of tocopherols in Moroccan OFSO (559 mg/kg) is much higher than that of the Tunisian (447 mg/kg) and the German OFSO (403 mg/kg), which were solvent extracted oils (Ramadan and Mörsel, 2003; El Mannoubi *et al.,* 2009). In comparison with olive (220 mg/kg) and sunflower oils (490 mg/kg), OFSO had higher amounts of tocopherols, but it was lower than the amounts in soybean (650 mg/kg) and argan oils (850 mg/kg) (Gharby *et al.,* 2013).

3.3. Volatile compound composition

The volatile compounds present in trace amounts in the oil are of great interest because they mainly reflect the quality of the oil and influence consumer acceptance. The combination of these substances in a specific concentration ratio gives a characteristic flavor to the oil which in many cases is experienced as a unique sensation (Morales and Przybylski, 2013). The analysis of Moroccan OFSO volatile compounds and key odorants was done using GC-MS-olfactometry.

The results are reported in Table 4. In this work 23 volatile compounds were identified, 14 of them with perceivable odor attributes. The volatile composition of OFSO was mainly characterized by alkanes, aldehydes and alcohols. Most of the volatile compounds, aroma-active or not, were crucial to the final smell of OFSO. A previous study was done to determine the volatile composition of the whole fruit of three varieties of *Opuntia ficus-indica* (Oumatou *et al.,* 2016). In this study, the authors used Solid-Phase Microextraction (SPME) connected with GC-MS. The study recorded 46 volatile compounds. The most considerable compounds in the three varieties were n-hexanol and 2-hexanal. In addition, nonanal, 1-octanol and n-hexanol were found in the fruit (Oumatou *et al.,* 2016) and the seed oil of the present investigation.

From our results, 3-methylbutanal and 2-methylpropanal were determined in the volatile composition of OFSO. Hu *et al.* (2014) found these two aldehydes as typical volatile compounds in rapeseed, sunflower, and soybean oils. Indeed, these two aldehydes are also known as products of microorganism activities such as bacteria or fungi (Bonte *et al.,* 2017).

Table 4. Volatile compounds of OFSO

VOLATILE COMPOUNDS	ODOR IMPRESSION
Acetaldehyde	Sweet - fruity - roasted
Ethyl ether	
2-methyl pentane	
3-methyl pentane	
n-hexane	Burnt – Gas
2-methyl propanal	Moldy – sweet
Cyclohexane	
2,2,4 trimethyl pentane	Cheese
3-methyl Butanal	Detergent - chocolate
2-methyl Butanal	
Pentan-1-ol	
Formic acid	
Acetic acid	Vinegar
Acetoin	Fatty- pleasant buttery
1 butanol,3-methyl-acetate	Burnt
1-Hexanol	grille - green – cut grass
[R- (R; R)]-2,3-Butanediol	
Benzaldehyde	mint - sweet - orange - nutty
Hexanoic acid	Wood-like - cheese - fermented – medicine
1-Octanol	Stinky
Benzeneacetaldehyde	
Nonanal	Detergent, soap, lemon, Sweet, Fatty
phenylethyl Alcohol	Nutty, rye, gum

The pleasant buttery odor indicates the existence of acetoin (3-hydroxy-2-butanon) in OFSO. Peroxidation reaction products can be found as by-products from the degradation of linoleic acid (Bonte *et al.,* 2017). In the case of OFSO, alkanes (propane and butane), aldehydes (propanal, butanal, nonanal), and alcohols (propanol, butanol, pentanol) were found as secondary products of degradation.

3.4. Oxidative stability of OFSO for one month at 50 ºC

Many factors such as temperature, light and oxygen availability reduce the organoleptic and nutritional assets through oxidation. In this study, the influence of temperature on OFSO was evaluated. OFSO was placed in brown closed flasks to eliminate the effect of the unrestrained access of oxygen and light and stored in the oven at 50 °C. The evolution of the oxidative state was reported by PV, FFA, the UV absorptions (K232 and K270) and induction period (IP). Also, total phenolic content and total tocopherols were estimated (Figure 1a, 1b, 1c, 1d). Fatty substances can be oxidized in the presence of oxygen and certain factors (e.g.: temperature). This oxidation, called autooxidation, leads initially to the production of hydroperoxides by fixing oxygen to the unsaturated fatty acids (Mohdaly *et al.,* 2010). To estimate the first stages of this oxidation, PV was measured. PV is a simple and commonly used method for determining the oxidation and rancidity of oil caused by unsaturated fatty oxide saturation.

Temperature had a remarkable effect on the PV of OFSO. Three phases were observed, and the first one showed a very strong increase during the first week to reach the highest value of about 8.3 meqO_2/kg oil. Due to the oxygen impermeability of the container, the available oxygen was consumed and the production of further peroxides was avoided. In the second phase, a decrease in PV to reach 6.9 meqO_2/kg oil by the end of the third week was noticed (Figure 1a). Although the level of PV had decreased, this did not reflect the quality of the oil, because in general oil oxidation goes through two stages, a first with the production of hydroperoxides and a second stage with a proliferation of hydroperoxides which are transformed into secondary

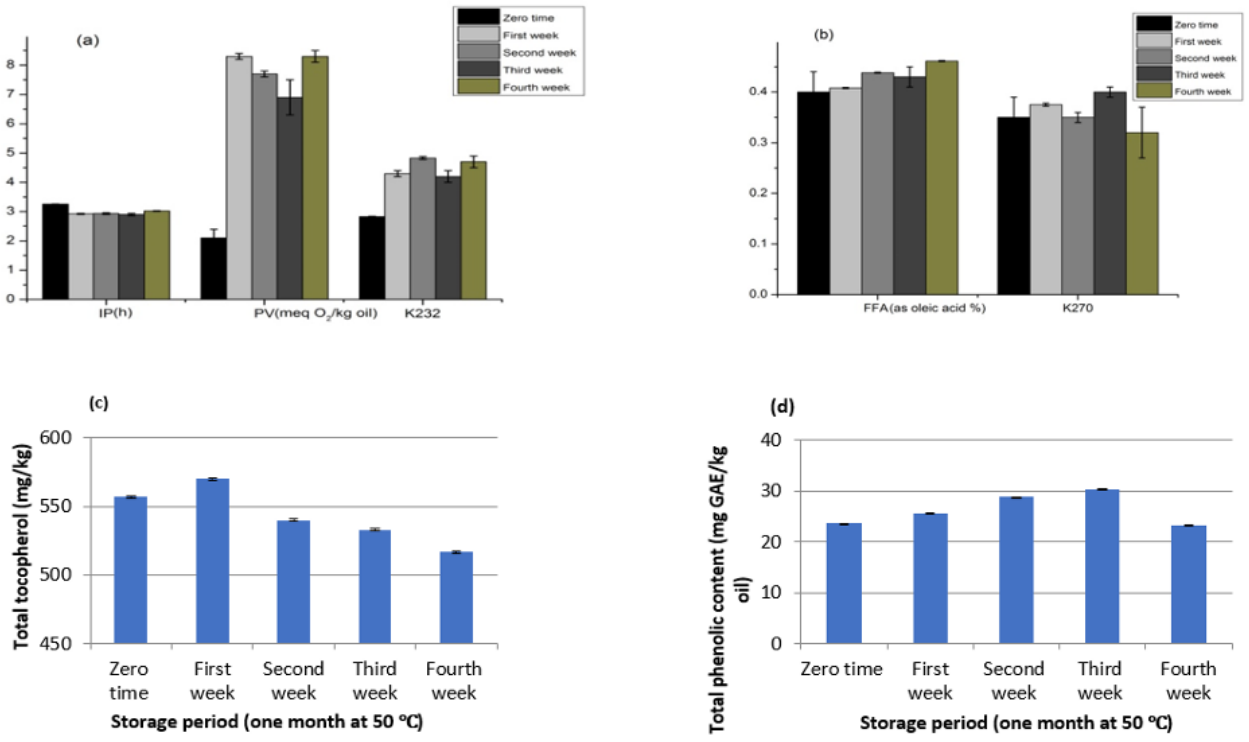

Figure 1. Effect of storage period of OFSO for one month at 50 °C on, (a): Induction period (IP) (h), Peroxide value (PV) (meqO_2/Kg oil), and K232, (b): Free fatty acid (FFA) (as oleicacid %) and K270, (c): Total tocopherol (mg/kg), (d): Total phenolic content (mg GAE/kg oil). Values are the mean of three values ± standard deviation.

products. In the third phase, PV started to increase again and reached about 8.3 meqO_2/kg oil. This rapid increase in PV may be due to the high content of linoleic acid.

The IP of OFSO was significantly affected by temperature during the storage period. It could be observed from the results that the oil lost around 30 min of resistance after one week of storage at 50 °C and IP became almost stable during the next three weeks with no significant differences between them (Figure 1a).

Spectrophotometric examination in the ultraviolet range can indicate oil quality. The absorption at the mentioned wavelengths was due to the existence of conjugated dienes and trienes resulting from the oxidation process. These results were expressed as extinctions E, which is typically denoted by K232 and K270 values referring to conjugated dienes and compounds of secondary oxidation (ketones, aldehydes, etc.). Figure 1a shows the evolution of K232 values during storage for 4 weeks at 50 °C. The K232 value of OFSO at the initial analysis was 2.83 and after one month of storage at 50 °C, the K232 reached 4.7. During the first two weeks of storage, an increase in the absorbance values as a result of peroxide formation and the existence of fatty acid oxidation products could be noticed. After the second week, as the oxygen available was depleted, and the amount of compounds measured at 232 nm was seen to remain relatively constant. This measure confirmed what was found previously for PV.

The evolution of the formation of secondary oxidation products was followed by measuring the absorbance at 270 nm under storage for one month at 50 °C. The storage led to little or no effect on the K270 values (Figure 1b). This result could be explained by the fact that the production of secondary oxidation products had not started yet and oxidation stopped at the production of hydroperoxides. Salama *et al.* (2020) showed that the value for K270 during the storage period of cactus oils (one month) increased and then started to decrease and after that, although it increased again by the end of the storage period. This finding was also observed by Torres *et al.* (2006), who found that the K232 and K270 values for soybean and jojoba oils increased, then decreased and by the end of storage period (240 h) increased again.

The percentage of FFA gives information on the alteration of oil by hydrolysis. FFA occurs in fats because of enzymatic hydrolysis by lipases, metal ions promoting degradation of free radicals or at an elevation of temperature (Goswami *et al.,* 2013). The changes in FFA values during storage at 50 °C are depicted in Figure 1b. The initial FFA value was seen to be 0.40% (calculated as oleic acid), which increased slowly and steadily to 0.46% after a storage period of one month. OFSO showed good resistance to the hydrolyzation of triglycerides during one month of storage at 50 °C.

The total amount of tocopherols decreased by the end of the storage period (517 mg/kg) in comparison with time zero (Figure 1c). After the first week, the value of total tocopherols started to decrease until it reached the lowest amount by the end of the storage period at about 517 mg/kg. The same results were found by Lerma-Garcia *et al.* (2009) who found that the amount of tocopherols (181 mg/kg at zero time) decreased greatly after the third week of storage until the end of the storage period (25 mg/kg) (7 weeks at 60 ºC).

Total phenolic content from OFSO at time zero was 23.6 mg GAE/kg oil (Figure 1d). During the storage period, the total phenolic content increased until it reached 30.3 mg GAE/kg oil in the 3rd week. By the end of the storage period, the total amount of phenolic compounds decreased to 23.3 mg GAE/kg oil. This agreed with Nounah *et al.* (2021), who reported that the exposure of OFS to high temperature (roasting at 110 ºC) for a certain time caused an increase in total polyphenol content. Also, Prabakaran *et al.* (2018) reported that significant increases among different storage times were observed after 12, 24, and 48 weeks of storage of raw soybean flour. Among the three-storage times, the raw soybean flour stored at the 45 ºC had the highest total phenolic content. The raw soybean flour for 48 weeks had the highest total phenolic acid content in all three storage times. Therefore, the increase in total phenolic content in OFSO under higher temperatures and longer storage periods could be explained by the degradation of complex polymerized phenolic structures into simple phenolic structures.

3.5. Antioxidant activity of OFS by β-carotene bleaching method

The results of the antioxidant activity of the extract from OFS by β-carotene linoleic acid are

shown in Fig. 2. In the absence of an antioxidant, carotene decolorizes rapidly because the free linoleic acid radicals attack the double bonds of the β-carotene, causing it to lose its double bonds and, as a result, its orange color. By scavenging the linoleate free radical and any other free radicals produced inside the system, the existence of a phenolic antioxidant will limit the degree of β-carotene destruction.

A reduction in absorbance of β-carotene in the presence of methanolic extract obtained from cactus seeds in the coupled system of β-carotene and linoleic acid was observed (Figure 2). The antioxidant activity of the methanolic extract from OFS was compared to a synthetic antioxidant (TBHQ) which was added to foods to prevent or delay oxidation and to a control sample without any added antioxidant. By using a concentration of 10 mg/mL it was noticed that TBHQ possessed a more powerful antioxidant effect than the extract from OFS. In addition, it was reported that the control sample, without the addition of extract solution, oxidized most rapidly. OFS methanolic extract was markedly effective in inhibiting linoleic acid oxidation and subsequently reduced the bleaching of ß-carotene in comparison with the control.

Many studies showed a good correlation between the antioxidant activity and OFS content of bioactive compounds. The results of the present work are in accordance with those of Zeghad *et al.* (2019), who demonstrated that OFS extracts have potent antioxidant capacities.

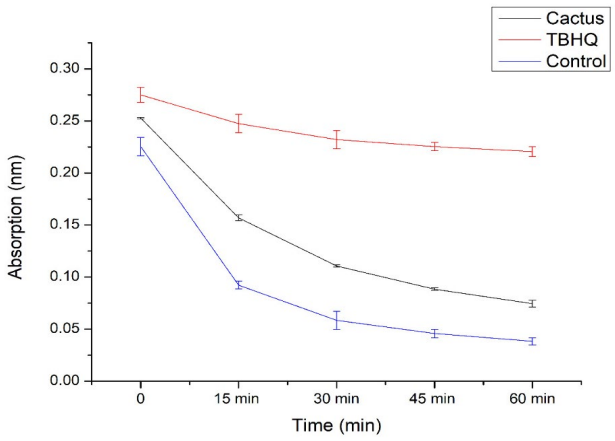

FIGURE 2. Antioxidant activity of OFS extract (10 mg/mL) using the ß-Carotene/linoleic acid bleaching method against TBHQ and control. Values are the mean of three values ± standard deviation.

4. CONCLUSIONS

Nowadays, cold-pressed cactus seed oil is becoming more and more popular in the cosmetic industry, but it can also be interesting for human nutrition. It has a good fatty acid profile with low acidity and peroxide value. Cactus seed oil is a very rich source of tocopherols and phytosterols. The storage of the oil at 50 °C for 4 weeks served to make a determination regarding the oxidative stability of the oil. The high content of linoleic acid is likely to be responsible for the oxidative sensitivity of cactus seed oil. Therefore, special care, such as storage under an inert atmosphere in dark packaging should be taken into consideration for prolonged storage. Indeed, an additional storage experiment considering different oxidation factors and storage facilities is needed in order to determine the shelf-life of cactus seed oil more in detail. The methanolic extract from OFS has a potent antioxidant activity which may be used in order to improve the stability of cactus seed oil and extend its storage time.

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The intensity of the cluster drop affects the bioactive compounds and fatty acid composition in hazelnuts

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SUMMARY: This study was conducted to determine how the intensity of the cluster drop effects nut traits, bioactive compounds, and fatty acid composition in Tombul, Palaz and Kalınkara hazelnut cultivars. The cluster drop significantly affected bioactive compounds and fatty acid composition while it did not affect the traits of the nuts. As cluster drop intensity increased, nut traits and bioactive compounds in all cultivars increased. Strong cluster drop intensity determined the highest total phenolics, total flavonoids, and antioxidant activity. Except for the Kalınkara cultivar, a low amount of linoleic acid was detected while high amounts of oleic and stearic acid were determined in slight cluster drop intensity. As cluster drop intensity increased, palmitic acid increased. Principal component analysis showed that the slight and intermediate drop intensity were generally associated with kernel length, oleic, linoleic, stearic, palmitoleic, 11-eicosenoic and arachidic acids. In contrast, strong intensity was associated with nut and kernel weight, kernel ratio, kernel width, kernel thickness, kernel size, bioactive compounds, and palmitic acid. As a result, the bioactive compounds and fatty acid composition, which are important for human health, was significantly affected by cluster drop intensity.

KEYWORDS: *Antioxidant; Cluster drop; Hazelnut; Nut traits; Oleic acid; Phenolics.*

RESUMEN: ***Influencia de la intensidad de la caída del racimo sobre los compuestos bioactivos y la composición de ácidos grasos en la avellana.*** El estudio se realizó para determinar el efecto de la intensidad de la caída de los racimos en las características de las avellanas, los compuestos bioactivos y la composición de ácidos grasos en cultivares de avellanas Tombul, Palaz y Kalınkara. La caída del racimo afectó significativamente a la composición de bioactivos y ácidos grasos, mientras que no afectó a las características de la avellana. A medida que aumentaba la intensidad de la caída de los racimos, aumentaban los compuestos bioactivos en todos los cultivares. La fuerte intensidad de caída de los racimos determinó que los fenoles totales, los flavonoides totales y la actividad antioxidante fueran más altos. Excepto para el cultivar Kalınkara, con un bajo contenido de ácido linoleico, un alto contenido de los ácidos oleico y esteárico se determinó en una ligera intensidad de caída de racimos. A medida que aumentaba la intensidad de la caída de los racimos, aumentaba el ácido palmítico. El análisis de componentes principales mostró que la intensidad de caída leve e intermedia generalmente se agrupaba con la longitud del grano, los ácidos oleico, linoleico, esteárico, palmitoleico, 11-eicosenoico y araquídico. En contraste, la intensidad fuerte se agrupó con el peso de la avellana y el grano, la proporción del grano, el ancho del grano, el grosor del grano, el tamaño del grano, los compuestos bioactivos y el ácido palmítico. Como resultado, la composición de compuestos bioactivos y ácidos grasos, que es eficaz para la salud humana, se vio significativamente afectada por la intensidad de la caída del grupo.

PALABRAS CLAVE: *Ácido oleico; Antioxidante; Avellana; Caída de racimo; Fenoles; Rasgos de la avellana.*

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1. INTRODUCTION

Hazelnut constitutes a significant part of the daily diet in developed and developing countries as well as being widely used in the confectionery, ice cream, baking, and chocolate industries. Hazelnut is rich in dietary fiber, lipids, fatty acids, micro-macro mineral elements, vitamins (Balta *et al.,* 2006; Alasalvar *et al.,* 2010; Turan, 2019), phytosterols and phytostanols, squalene, and phenolic compounds (Yılmaz *et al.,* 2019; Di Nunzio, 2019). It stands out as an antioxidant source in preventing diseases such as cardiovascular, neurodegenerative, inflammatory, colon cancer, and type-2 diabetes (Di Nunzio, 2019; Yılmaz *et al.,* 2019). Considering that the consumption of fruits with rich nutritional content is recommended to increase the body's resistance to pandemic diseases such as Covid-19 (Muscogiuri *et al*., 2020), hazelnut fruit stands out as a significant source of nutrients.

The primary factors affecting nut quality and bioactive contents in hazelnuts are genetic structure, ecology, climate, fertilization, pruning, harvest time, and diseases and pests (Balta *et al.,* 2006; Cristofori *et al.,* 2015; Turan, 2019). Irrigation is another significant factor which affects the yield and quality characteristics of hazelnuts. In recent years, the drought caused by global climate change has negatively affected the yield and quality of hazelnuts, like many other fruit species. Water availability was reported by many researchers as a factor that directly affects the yield and quality of hazelnuts (Bignami *et al.,* 2011; Bostan and Tonkaz, 2013).

When the climate data belonging to recent decades are examined, it is estimated that the temperature will increase by 1.5 °C on average (Arora, 2019), while precipitation will decrease by about 30% (Lorite *et al.,* 2018) worldwide until 2030. Hazelnut growing in Turkey generally takes place on sloping lands with no irrigation, thus precipitation provides the water requirement for the plants. Therefore, obtaining a high yield and quality product strictly depends on sufficient and regular precipitation. In the case of a water deficit, a significant cluster drop occurs, resulting in a decrease in yield and quality (Bignami *et al.,* 2011; Milosevic and Milosevic, 2012; Bostan and Tonkaz, 2013).

The cluster drop in hazelnut is a phenomenon that continues from the fruit set to ripening (Milosevic and Milosevic, 2012). The cluster drop intensity changes depending on ecological factors, variety, pollen source, incompatibility, cultural practices, diseases and pests, as well as water deficit (Bignami *et al.,* 2011; Bostan and Tonkaz, 2013). Milosevic and Milosevic (2012) reported the occurrence of nut cluster drop density at three different levels: slight (< 10%), intermediate (10-20%), and strong (> 20%).

To date, many studies have been conducted to determine the effects of factors such as cultivar (Köksal *et al.,* 2006; Alasalvar *et al.,* 2010), genotype, echography, cultural practices (Balta *et al.,* 2006; Yılmaz *et al.,* 2019; Balik, 2021), harvest time, maturity level (Cristofori *et al.,* 2015), storage, drying methods (Turan, 2019), altitude, and direction (Balta *et al.,* 2018) on the bioactive compounds and fatty acids in hazelnuts. However, there is no study in the literature on the changes in bioactive compounds and fatty acid composition depending on the cluster drop intensity in hazelnuts.

The main aim of this study is to determine the changes in nut traits, bioactive compounds, and fatty acid composition of Turkish hazelnut cultivars (Tombul, Palaz and Kalınkara) depending on the cluster drop intensity.

2. MATERIALS AND METHODS

2.1. Plant materials

This research was carried out at commercial orchards (40°54'38.6"N latitude, 37°48'19.3"E longitude, 245 m altitude) in the Ordu province on the prominent hazelnut cultivars of Turkey, which are Tombul, Palaz, and Kalınkara, in two consecutive growing seasons, 2019 and 2020.

Trial orchards were established as multi-stemmed (7-9 stems per system) training systems and planted at distances between 4 m × 3 m and within rows. Standard cultural practices, such as fertilization, pruning and weed control were performed regularly, except for irrigation. During the research, branch thinning was carried out in the winter period and suckering was carried out twice during the vegetation period. Chemical control was carried out against the diseases and pests of nut weevil (*Curculio nucum*), green shield bug (*Palomena prasina*), and powdery mildew (*Erysiphe corylacearum*). Weeding was performed twice a year before harvest. A total of 250 g $NH_4H_2PO_4$ (monoammonium phosphate), 100 g K_2SO_4 (potassium sulphate), and 500 g N (ni-

trogen) were supplied per system. In addition, foliar fertilizer was applied twice a year. There weren't any nutrient deficiency symptoms in the leaf or fruit during the growing season.

At harvest time (10-15 August), all clusters on the plants were harvested, separated from husks, and dried naturally (under sunlight) until the moisture content decreased to 6%. No rainfall was observed during drying and the weather conditions were as follows: the temperature was 23.9 °C and 23.8 °C, precipitation was 0 mm and 0 mm, and hours of sunshine were 10.9 h and 10.7 h in 2019 and 2020, respectively (TSMS, 2021). The samples were stored in ambient conditions (at 22-24 °C and 70-80% RH) until analysis.

The precipitation and temperature values of the study area are presented in Figure 1 (TSMS, 2021).

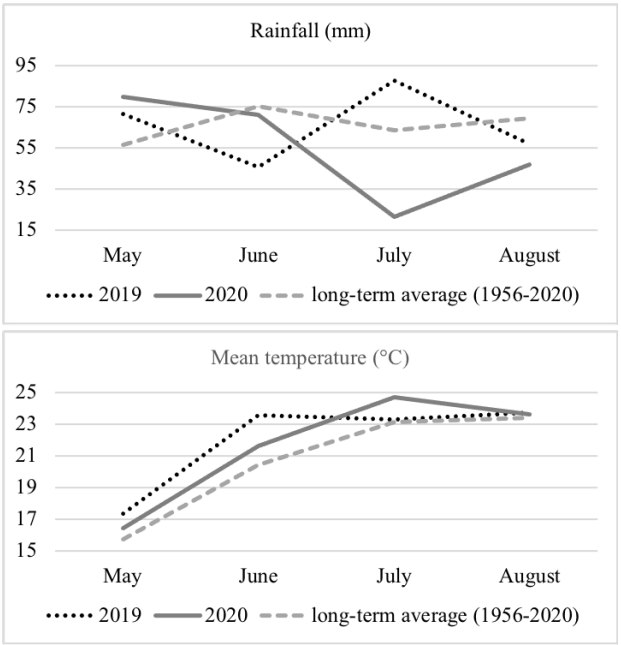

Figure 1. Rainfall (mm) and mean temperature (° C) between May and August

2.2 Experimental design

Fifty plants were marked for each of Tombul, Palaz and Kalınkara hazelnut cultivars as a result of observations made for many years in orchards with the same conditions, altitude and direction. Cluster drop was recorded in the marked plants after the fruit set (beginning of June). In light of the observations, it was determined that cluster drop in the orchards was different. In orchards where the cluster drop was observed, 15 plants were selected (total of 45 per cultivar) in 3 replicates for each cultivar to monitor this phenomenon.

2.3. Cumulative drop ratio

The cumulative drop ratio (%) was calculated by counting the dropped clusters at 20-day intervals from the fruit set (beginning of June) to harvest (beginning of August), 4 times in total. The equation of '∑dropped clusters/total clusters x 100' was used in the calculation. Then, the sampling was made in 15 plants with slight (< 10%), intermediate (10-20%) and strong (> 20%) drop density (Table 1) according to the classification by Milosevic and Milosevic (2012) in each cultivar.

Table 1. Cumulative cluster drop ratio (%) of investigated hazelnut cultivars (two-year average)

Cultivars	Cluster drop intensity	26 June	16 July	05 August
Tombul	Slight	1.9±0.35	5.9±1.27	6.8±1.18
	Intermediate	4.8±0.87	12.0±2.65	15.7±2.19
	Strong	10.2±1.84	26.5±6.37	33.7±4.71
Palaz	Slight	3.6±0.71	7.1±1.43	8.9±1.34
	Intermediate	5.0±0.90	15.0±3.30	17.5±2.45
	Strong	12.0±2.16	24.0±5.28	32.0±6.08
Kalınkara	Slight	2.6±0.47	6.5±1.49	7.8±1.17
	Intermediate	5.7±1.03	14.3±3.14	18.6±2.60
	Strong	8.9±1.60	24.4±5.38	30.0±4.20

Values are mean ± standard deviation (n=3)

2.4. Nut traits

Fifty nuts were used for nut and kernel traits in each treatment. Nut weight (g) and kernel weight (g) were measured with digital balance (Radwag, AS/220/C/2, Poland) to an accuracy of 0.01 g. Shell thickness (mm) and kernel dimension (mm) (width, thickness and length) were measured with a digital caliper (Mitutoyo, CD-15CP, Japan) to an accuracy of 0.01 mm. The kernel ratio (%) was calculated by the equation of 'kernel weight/nut weight × 100' as previously reported. Kernel size (mm) was calculated as the geometric mean of kernel size (width, thickness and length) (Balta *et al.,* 2018).

2.5. Bioactive compounds

Bioactive compounds were determined as total phenolics, total flavonoids and antioxidant activity (FRAP and DPPH assays). Bioactive compounds were detected in defatted hazelnut samples. The defatting process was performed according to the Soxhlet extraction method (Firestone, 1997).

For the detection of bioactive compounds, 1 g defatted hazelnut sample was accurately weighed and extracted with 10 ml methanol. The obtained solution was centrifuged in a cooler-type device for 30 min at 12,000 rpm, at 4 °C. The resultant filtrate was used for determining the total phenolics, total flavonoids and antioxidant activity.

2.5.1. Total phenolics

The total phenolic content was determined using the Folin-Ciocalteu reagent (Merck, Germany). The prepared samples were measured at a wavelength of 760 nm in a spectrophotometer (Shimadzu, Japan). The results were expressed as mg·100 g^{-1} as gallic acid equivalent (GAE) (Ozturk *et al.,* 2018).

2.5.2. Total flavonoids

Total flavonoid content was determined according to the method of Ozturk *et al.* (2018). Absorbance values were determined in a spectrophotometer (Shimadzu, Japan) at a wavelength of 415 nm. The results were expressed as mg·100 g^{-1} in terms of quercetin equivalents (QE).

2.5.3. Antioxidant activity (FRAP and DPPH assays)

The antioxidant activity was measured according to FRAP [ferric ions (Fe^{+3}) reducing antioxidant power assay] (Benzie and Strain, 1996) and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assays (Blois, 1958). Prepared samples were measured in a spectrophotometer (Shimadzu, Japan) at 700 nm for the FRAP assay and at 517 nm for the DPPH assay. The results were expressed as mmol 100 g^{-1} in terms of trolox equivalents (TE).

2.6. Fatty acid composition

0.1 g of hazelnut oil was accurately weighed in a test tube and 1 mL potassium methylate and 4 mL hexane were added. The resultant mixture was shaken for 30 seconds and 0.5 mL H_2SO_4 were added. The resultant supernatants were diluted with hexane and filtered through a 0.45 µm filter. A GC (gas chromatography system) (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and capillary column (0.25 mm × 0.20 µm, 100 m) was used to analyze samples for their fatty acid compositions. The column temperature was programmed as follows: held at 140 °C for 5 min, raised to 240 °C at a rate of 4 °C/min and held at 240 °C for 15 min. The injector and detector temperatures were 250 °C. Nitrogen was used as carrier gas. At a flow rate of 3 mL/min. The injection volume was 1 mL with a split ratio of 1:100. Fatty acid peaks were identified based on standard FAMEs (fatty acid methyl esters) by comparing retention times. Results were expressed as percentages of relative areas of identified fatty acids (Sengul, 2019). The obtained fatty acid composition was used to calculate the fatty acids in terms of: saturated fatty acid (SFA) (palmitic, stearic and arachidic), monounsaturated fatty acid (MUFA) (palmitoleic, oleic and 11-eicosenoic) and polyunsaturated fatty acid (PUFA) (linoleic and linolenic).

2.7. Statistical analysis

The data were subjected to ANOVA by using SPSS 23.0 (SPSS Inc. Chicago, USA) software. Differences among means were determined with the LSD multiple-comparison test at $p < 0.05$. PCA (Principal Components Analysis) and component biplot analysis were performed using JMP 10 (trial) software.

3. RESULTS AND DISCUSSION

3.1. Nut traits

Nut weight, kernel weight, kernel ratio and shell thickness are significant quality characteristics in hazelnuts (Balta *et al.,* 2018). A high kernel ratio is a desirable characteristic for the hazelnut industry. Small and medium-sized nuts are important for the confectionery industry while large nuts are suitable for in-shell marketing. In addition, a thinner shell is a desired characteristic for in-shell marketing (Guler and Balta, 2020). The effect of the cluster drop intensity on nut weight, kernel weight, kernel ratio and shell thickness in the hazelnut cultivars was insignificant ($p > 0.05$). However, an increase in cluster drop intensity caused an increment in nut weight, kernel weight and kernel ratio in all cultivars. The highest nut and

Table 2. Nut weight, kernel weight, kernel ratio shell thickness and kernel size according to intensity of cluster drop in different hazelnut cultivars

Cultivars	Cluster drop intensity	Nut weight (g)	Kernel weight (g)	Kernel ratio (%)	Shell thickness (mm)	Kernel width (mm)	Kernel thickness (mm)	Kernel length (mm)	Kernel size (mm)
	Slight	1.75±0.10 a	0.92±0.05 a	52.3±0.94 a	1.02±0.04 a	12.53±0.30 a	11.01±0.79 a	14.75±0.13 a	12.67±0.28 a
Tombul	Intermediate	1.77±0.15 a	0.93±0.09 a	52.4±0.64 a	0.95±0.06 a	12.26±0.28 a	11.73±0.54 a	14.49±0.30 a	12.77±0.30 a
	Strong	1.83±0.07 a	0.96±0.04 a	52.5±0.35 a	1.00±0.05 a	12.57±0.15 a	11.86±0.08 a	14.27±0.27 a	12.86±0.02 a
Significance		ns	ns	ns	ns	ns	ns	ns	ns
LSD (0.05)		0.22	0.12	1.40	0.10	0.50	1.10	0.48	0.48
	Slight	1.82±0.02 a	0.92±0.03 a	50.3±1.12 a	1.13±0.13 a	14.17±0.21 a	12.03±0.87 a	12.70±0.38 a	12.93±0.22 a
Palaz	Intermediate	1.87±0.10 a	0.94±0.06 a	50.5±0.82 a	1.06±0.05 a	14.44±0.28 a	12.50±0.31 a	12.17±0.31 a	13.00±0.17 a
	Strong	1.92±0.03 a	1.00±0.02 a	52.0±0.63 a	1.05±0.06 a	14.55±0.21 a	12.72±0.27 a	12.01±0.48 a	13.10±0.13 a
Significance		ns	ns	ns	ns	ns	ns	ns	ns
LSD (0.05)		0.12	0.08	1.76	0.18	0.48	1.11	0.80	0.35
	Slight	1.97±0.12 a	1.01±0.09 a	51.3±2.56 a	1.10±0.13 a	12.08±0.60 a	11.50±0.44 a	16.70±0.72 a	13.24±0.41 a
Kalınkara	Intermediate	1.99±0.10 a	1.06±0.09 a	53.1±1.79 a	1.23±0.14 a	12.53±0.37 a	11.86±0.20 a	16.53±0.30 a	13.49±0.26 a
	Strong	2.11±0.05 a	1.15±0.06 a	54.7±1.57 a	1.11±0.07 a	13.03±0.82 a	11.62±0.53 a	16.43±0.73 a	13.54±0.24 a
Significance		ns	ns	ns	ns	ns	ns	ns	ns
LSD (0.05)		0.19	0.16	4.04	0.24	1.24	0.83	1.24	0.63

The differences among mean values shown on the same line with the same letter are not significant ($p < 0.05$). Differences were determined using the LSD test. * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$ and ns: not significant. n= 300 for the nut and kernel traits (three replicates × five plants for each replicate × twenty nuts)

kernel weights were detected for the Kalınkara cultivar, while the lowest nut and kernel weights were found for the Tombul cultivar. The Palaz cultivar had intermediate nut and kernel weight. The Kalınkara cultivar had the highest kernel ratio, while Palaz cultivar had the lowest kernel ratio. The lowest and highest shell thickness were measured in the Tombul and Kalınkara cultivars, respectively (Table 2). Milosevic and Milosevic (2012) reported that in Tonda Gentile Romana, Nocchione and Istarski Duguljasti hazelnut cultivars, as the cluster drop intensity increased, the nut weight, kernel weight, kernel ratio and shell thickness also increased. However, they reported that the increase in these traits was not statistically significant. In previous studies, the highest nut weight and kernel weight were reported for the Kalınkara cultivar, and the lowest for the Tombul cultivar. On the contrary, the highest kernel ratio and thinnest shell was recorded for the Tombul cultivar (Balik *et al.,* 2016).

The effect of cluster drop intensity on kernel dimensions was insignificant in all cultivars ($p > 0.05$). Although not statistically significant, the kernel size of all cultivars increased with the increment in cluster drop intensity. The Kalınkara cultivar had the largest kernel size, followed by the Palaz and Tombul cultivars. The kernel size of all cultivars was above 12.5 mm (Table 2), meaning that they were all suitable for marketing (Yılmaz *et al.,* 2019). Milosevic and Milosevic (2012) reported that in Tonda Gentile Romana, Nocchione and Istarski Duguljasti hazelnut cultivars, as the cluster drop intensity increased, the kernel size increased. However, they reported that the increase in nut size was not statistically significant. In addition, Balik *et al.* (2016) reported the highest kernel size for the Palaz cultivar, followed by Kalınkara and Tombul cultivars.

The results were similar to reports on the same cultivars by different researchers in terms of nut weight, kernel weight, kernel ratio, shell thickness and kernel size. It has been reported that the nut and kernel traits of hazelnuts can be also be influenced by ecological conditions, and cultural and technical practices (Balta *et al.,* 2018; Guler and Balta, 2020; Bak and Karadeniz, 2021).

3.2. Bioactive compounds

Phenolic compounds play a significant role in reducing the risks of disease in human beings. The

antioxidant properties of phenolic compounds are effective against many pathological problems associated with oxidative stress damage. In addition, bioactive compounds in plants have anti-inflammatory, antiulcer, antiallergic, antimicrobial, antithrombotic, antiatherogenic and anticarcinogenic effects (Di Nunzio, 2019). Ecological conditions, cultivar (Yılmaz *et al.,* 2019), maturity level, and cultural practices (Cristofori *et al.,* 2015) affect the bioactive compounds in hazelnuts. In addition, stressors such as drought, low and high temperature, pathogenic attack, and exposure to ultraviolet light cause an increase in bioactive compounds (Naikoo *et al.,* 2019).

In this study, total phenolics were significantly affected by the cluster drop intensity in all hazelnut cultivars ($p < 0.05$). However, the difference between slight and intermediate cluster drop intensity in Tombul and Kalınkara cultivars was insignificant in terms of total phenolic content. Total phenolics increased with the increment in cluster drop intensity in all cultivars. The highest total phenolic content was determined for the Palaz cultivar, followed by the Kalınkara and the Tombul cultivars (Table 3). The highest total phenolic content was reported for the Palaz cultivar by Balik (2021), while it was determined for the Tombul cultivar by Karaosmanoglu and Ustun (2021). Balık (2021) detected the lowest total phenolic content in the Kalınkara cultivar.

Flavonoids are a significant group of polyphenols with antioxidant properties (Di Nunzio, 2019). The total flavonoid content in all cultivars was significantly affected by cluster drop intensity ($p < 0.05$). The total flavonoid content increased with an increase in cluster drop intensity except for the Tombul cultivar. However, the difference between the slight and intermediate drop intensity in terms of total flavonoid content in the Kalınkara cultivar was insignificant. The Palaz cultivar had the highest total flavonoids, while the Tombul cultivar had the lowest total flavonoids (Table 3). Balık (2021) reported the highest total flavonoid content in the Tombul cultivar (34.0 mg·100 g^{-1}), followed by Palaz (13.2 mg·100 g^{-1}) and Kalınkara (12.6 mg·100 g^{-1}) cultivars.

Antioxidants are effective against the formation of the free radicals in the body, preventing the occurrence and progression of oxidative stress-induced diseases. The hazelnut is a natural source of antioxidants (Contini *et al.,* 2011), with high antioxidant activity. According to the FRAP assay, cluster drop intensity affected the antioxidant activity of all cul-

Table 3. Total phenolics, total flavonoids and antioxidant activity (FRAP and DPPH) according to intensity of cluster drop in different hazelnut cultivars

Cultivars	Cluster drop intensity	Total phenolics (mg GAE·100 g^{-1})	Total flavonoids (mg QE·100 g^{-1})	FRAP (mmol TE·100 g^{-1})	DPPH (mmol TE·100 g^{-1})
	Slight	43.5±0.10 b	4.4±0.09 b	0.35±0.02 b	1.35±0.04 b
Tombul	Intermediate	44.6±0.93 b	3.9±0.15 c	0.39±0.02 b	1.50±0.01 a
	Strong	62.6±1.38 a	5.8±0.21 a	0.67±0.03 a	1.51±0.01 a
Significance		***	***	***	***
LSD (0.05)		1.92	0.31	0.05	0.05
	Slight	87.1±0.59 c	5.6±0.34 c	0.99±0.04 c	1.64±0.01 b
Palaz	Intermediate	136.3±1.77 b	14.1±0.58 b	1.72±0.03 b	1.64±0.01 b
	Strong	193.4±2.95 a	17.8±0.55 a	2.86±0.07 a	1.90±0.06 a
Significance		***	***	***	***
LSD (0.05)		4.02	1.00	0.09	0.07
	Slight	78.3±1.13 b	7.5±0.34 b	0.56±0.03 b	1.64±0.06 b
Kalınkara	Intermediate	80.0±0.79 b	7.8±0.06 b	0.58±0.02 b	1.90±0.01 a
	Strong	109.8±0.88 a	11.5±0.55 a	1.20±0.03 a	1.91±0.01 a
Significance		***	***	***	***
LSD (0.05)		1.82	0.76	0.05	0.08

The differences among mean values shown on the same line with the same letter are not significant ($p < 0.05$). Differences were determined using the LSD test. * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$ and ns: not significant. n= 9 for the bioactive compounds (three replicates × three different measurements for each replicate)

tivars ($p < 0.05$). However, the difference between the slight and intermediate drop intensity in terms of antioxidant activity in Tombul and Kalınkara cultivars was insignificant. Antioxidant activity increased with the increase in cluster drop intensity for all cultivars. The highest antioxidant activity was determined for the Palaz cultivar, followed by Kalınkara and Tombul cultivars (Table 3).

The antioxidant activity determined by the DPPH assay was affected by the cluster drop intensity for all cultivars ($p < 0.05$). However, the difference between the intermediate and strong cluster drop intensity in Tombul and Kalınkara cultivars as well as slight and intermediate cluster drop intensity in the Palaz cultivar was insignificant in terms of the antioxidant activity. The increase in cluster drop intensity increased the antioxidant activity for all cultivars. The Kalınkara cultivar had the highest antioxidant activity, while Tombul cultivar had the lowest (Table 3).

In previous studies, according to FRAP and DPPH assays, the highest antioxidant activity was reported for the Tombul cultivar (1.22 mmol·100 g^{-1} and 0.25 mmol·100 g^{-1}, respectively); while the lowest antioxidant activity was found for the Kalınkara cultivar (2.05 mmol·100 g^{-1} and 0.24 mmol·100 g^{-1}, respectively). In the Palaz cultivar, antioxidant activity was recorded as 1.28 mmol·100 g^{-1} and 0.24 mmol·100 g^{-1}, respectively (Balik, 2021).

The results for the bioactive compounds were generally similar to those previously reported for the same cultivars (Balik, 2021; Karaosmanoglu and Ustun, 2021). However, there were also some differences between this study and previous ones. The differences were thought to be due to ecological conditions (Yılmaz *et al.,* 2019; Balik, 2021), maturity level (Cristofori *et al.,* 2015), and cultural practices (Yılmaz *et al.,* 2019). In addition, as a general phenomenon, the increase in cluster drop intensity increased bioactive compounds in all cultivars. Similarly, higher total phenolics and antioxidant activity were reported in fruits of date palms with drops (Othmani *et al.,* 2020). Total phenolics, total flavonoids and antioxidant activity were reported to be higher in nuts of plants which were exposed to drought stress (Bignami *et al.,* 2011; Shahi *et al.,* 2020). This phenomenon is related to the production of the reactive oxygen species and the increase in secondary metabolites resulting from the defense response of plants against stress (Rejeb *et al.,* 2014). Even though the genetic structure is the primary factor in accumulating secondary metabolites in plants, the ecological factors also have a significant effect. Some climatic factors such as temperature, light and precipitation affect the accumulation of the phenolic compounds (Dumas *et al.,* 2003). Bioactive compounds in plants increase as a defense mechanism against temperature stress (Naikoo *et al.,* 2019; Shahi *et al.,* 2020). In the present study, the temperature values were higher than the long-term average (mean 2.5 °C); while the precipitation values were lower (about 39%) during nut development, between May and August (Figure 1). This situation caused the high phenolic and antioxidant accumulation in plants with strong cluster drop intensity and exposure to drought stress.

3.3. Fatty acid compositions

The hazelnut, a rich source of fatty acids (Contini *et al.,* 2011) with significant amounts of MUFAs (Alasalvar *et al.,* 2010; Karaosmanoglu and Ustun, 2021), effectively improves cholesterol balance and triglyceride levels and reduces the risk of atherosclerosis and coronary heart disease. The major fatty acids in hazelnuts with high MUFA content is oleic acid, constituting approximately 80% of total fatty acids (Balta *et al.,* 2006; Köksal *et al.,* 2006; Alasalvar *et al.,* 2010), followed by linoleic, palmitic and stearic acid, respectively. The fatty acid composition in hazelnuts is affected by many factors, such as ecological condition, genetic structure, location, cultural practices (Balta *et al.,* 2006; Köksal *et al.,* 2006; Balik, 2021), early harvest, storage, drying methods (Turan, 2019) and maturity level (Cristofori *et al.,* 2015), as well as drought, which is one of the ecological factors that causes significant changes in fatty acid composition (Hamrouni *et al.,* 2011; Xu *et al.,* 2011).

The oleic acid contents in all cultivars were significantly affected by drop intensity ($p < 0.05$). However, the differences between slight and strong drop intensities in the Tombul cultivar, slight and intermediate drop intensities in the Palaz cultivar, and intermediate and strong drop intensities in the Kalınkara cultivar were not statistically different. The highest oleic acid was determined in the Palaz cultivar, followed by Tombul and Kalınkara cultivars (Table 4). Balik (2021) reported the highest oleic acid content in the Palaz cultivar (82.39%) and the lowest in the

Table 4. Fatty acids composition (%) according to intensity of cluster drop in different hazelnut cultivars

Cultivars	Cluster drop intensity	Oleic	Linoleic	Palmitic	Stearic	Palmitoleic	11-eicose-noic	Arachidic	Oleic/ Linoleic	∑SFA	∑PUFA	∑MUFA
	Slight	82.45±0.84 a	9.13±0.08 c	5.75±0.14 b	2.60±0.09 a	0.07±0.00 a	nd	nd	9.0±0.02 a	8.3±0.23 a	9.1±0.08 c	82.5 ±0.84 a
Tombul	Intermediate	80.17±0.79 b	11.42±0.12 a	6.04±0.16 ab	2.29±0.07 b	0.07±0.00 a	nd	nd	7.0±0.01 c	8.3±0.23 a	11.4±0.12 a	80.2 ±0.79 b
	Strong	81.40±0.82 ab	9.91±0.09 b	6.11±0.16 a	2.50±0.08 a	0.07±0.00 a	nd	nd	8.2±0.01 b	8.6±0.24 a	9.9±0.09 b	81.5±0.82 ab
Significance		*	***	*	**	ns	-	-	***	ns	***	*
LSD (0.05)		1.63	0.20	0.31	0.16	0.0	-	-	0.02	0.47	0.20	1.63
	Slight	83.55±0.88 a	9.44±0.05 b	4.58±0.11 c	2.08±0.07 a	0.09±0.00 b	0.15±0.00 a	0.07±0.01 a	8.8±0.05 a	6.7±0.18 c	9.5±0.05 b	83.8±0.88 a
Palaz	Intermediate	81.90±0.85 ab	10.61±0.07 a	5.22±0.14 b	1.99±0.07 a	0.17±0.01 a	0.07±0.00 b	0.02±0.01 b	7.7±0.03 b	7.2±0.21 b	10.7±0.07 a	82.1±0.86 ab
	Strong	80.37±0.82 b	10.55±0.07 a	6.79±0.22 a	2.08±0.07 a	0.09±0.00 b	0.07±0.00 b	0.02±0.01 b	7.6±0.03 c	8.9±0.29 a	10.6±0.07 a	80.5 ±0.82 b
Significance		*	***	***	ns	***	***	***	***	***	***	**
LSD (0.05)		1.70	0.13	0.32	0.14	0.01	0.01	0.01	0.07	0.46	0.13	1.71
	Slight	77.66±1.55 b	15.64±0.31 a	4.38±0.22 c	1.88±0.11 c	0.14±0.01 a	0.18±0.00 a	0.11±0.00 a	5.0±0.00 c	6.4±0.33 b	15.6±0.31 a	78.0±1.57 b
Kalınkara	Intermediate	78.57±1.57 ab	13.88±0.28 b	4.95±0.25 b	2.60±0.16 a	0.06±0.02 b	0.09±0.02 b	0.06±0.02 b	5.7±0.00 b	7.6±0.42 a	13.9±0.28 b	78.7±1.58 ab
	Strong	81.39±1.63 a	10.31±0.21 c	6.05±0.30 a	2.24±0.13 b	0.05±0.01 b	0.07±0.01 b	0.04±0.01 b	7.9±0.00 a	8.3±0.45 a	10.3±0.21 c	81.5±1.63 a
Significance		*	***	***	**	***	***	**	***	**	***	*
LSD (0.05)		3.16	0.54	0.52	0.27	0.03	0.03	0.03	4.87	0.81	0.54	3.18

The differences among mean values shown on the same line with the same letter are not significant ($p < 0.05$). Differences were determined using the LSD test. * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$ and ns: not significant; nd: not detected. n= 9 for the fatty acid composition (three replicates × three different measurements for each replicate)

Tombul cultivar (78.19%). It was reported at 76.76% in the Kalınkara cultivar. Köksal *et al.* (2006) reported the highest oleic acid content in the Kalınkara cultivar (78.9%), followed by the Tombul (77.8%) and Palaz (77.6%) cultivars.

The linoleic acid was significantly affected by cluster drop intensity in all cultivars ($p < 0.05$). Besides, the difference between intermediate and strong drop intensities in the Palaz cultivar was insignificant. The Kalınkara cultivar had the highest linoleic acid, while the Tombul cultivar had the lowest (Table 4). Similarly, the highest linoleic acid was reported for the Kalınkara cultivar by different researchers (9.13-13.41%). The lowest was recorded for the Palaz cultivar (5.91-7.56%) (Alasalvar *et al.,* 2010; Balik, 2021).

The palmitic acid content in all cultivars was significantly affected by the cluster drop intensity ($p < 0.05$). Palmitic acid increased as the cluster drop intensity increased in all cultivars. The highest palmitic acid content was determined in the Tombul cultivar followed by Palaz and Kalınkara cultivars (Table 4). Similarly, Balik (2021) reported the highest oleic acid content in the Tombul cultivar (7.71%), followed by the Palaz (7.34%) and Kalınkara (6.58%) cultivars. On the contrary, Köksal *et al.* (2006) reported the highest palmitic acid content in the Kalınkara cultivar (5.71%) and the lowest in the Palaz cultivar (4.87%).

The cluster drop intensity did not affect the stearic acid content in the Palaz cultivar; whereas the the Tombul and Kalınkara cultivars were significantly affected ($p < 0.05$). The Tombul cultivar had the highest stearic acid content, while the Palaz cultivar had the lowest (Table 4). Alasalvar *et al.* (2010) determined that the highest stearic acid content was detected in the Tombul cultivar (3.24%). The lowest was recorded for the Kalınkara cultivar (2.08%). On the contrary, Köksal *et al.* (2006) reported the highest stearic acid content in the Kalınkara cultivar (2.42%) and the lowest in the Tombul cultivar (1.75%).

The palmitoleic acid content in the Tombul cultivar was not significantly affected by the drop intensity; whereas the Palaz and Kalınkara cultivars ($p < 0.05$) were significantly affected. The highest level of palmitoleic acid was determined in the Palaz cultivar followed by Kalınkara and Tombul cultivars (Table 4).

11-eicosenoic and arachidic acid were not detected in the Tombul cultivar and were significantly altered by the cluster drop intensity in the Palaz and

Kalınkara cultivars ($p < 0.05$). The Kalınkara cultivar had the highest 11-eicosenoic acid content, whereas Tombul cultivar had the lowest 11-eicosenoic acid content. The highest arachidic acid was determined in the Kalınkara cultivar, followed by the Palaz and Tombul cultivars (Table 4). In previous studies, the highest levels of palmitoleic and arachidic acid were reported for the Palaz cultivar (0.29% and 0.18%, respectively), while the lowest was detected for the Tombul cultivar (0.16% and 0.12%, respectively). Also, the Kalınkara cultivar had the highest 11-eicosenoic acid (0.20%) content. The lowest 11-eicosenoic acid was reported for the Tombul cultivar (0.16%) (Alasalvar *et al.,* 2010).

The difference in oleic/linoleic ratio depending on the cluster drop intensity was significant in all cultivars ($p < 0.05$). While the highest oleic/linoleic acid ratio was determined in the slight cluster drop intensity in the Tombul and Palaz cultivars, it was determined in the strong cluster drop intensity in the Kalınkara cultivar. Depending on the cultivars, the highest oleic/linoleic acid ratio was recorded for the Tombul cultivar, while the lowest ratio was determined in the the Kalınkara cultivar (Table 4). According to different researchers, the oleic/linoleic ratio was reported to be the highest in the Tombul cultivar and the lowest in the Kalınkara cultivar (Alasalvar *et al.*, 2010; Balik, 2021).

The saturated fatty acid (SFA) in the Tombul cultivar was not affected by drop intensity, whereas in the Palaz and Kalınkara cultivars it was significantly affected ($p < 0.05$). In all cultivars, the highest SFA values were recorded for the strong cluster drop intensity and increased as the cluster drop intensity increased. While the Tombul cultivar had the highest SFA value, the Palaz cultivar had the lowest value (Table 4). In previous studies, the highest SFA was reported for the Palaz cultivar by Karaosmanoglu and Ustun (2021), while it was determined in the Kalınkara cultivar by Balik, (2021).

The polyunsaturated fatty acid (PUFA) in all cultivars was significantly affected by drop intensity ($p < 0.05$). Whereas the highest PUFA was determined in the intermediate cluster drop intensity in Tombul and Palaz cultivars, it was determined in the slight cluster drop intensity in the Kalınkara cultivar. The highest PUFA was recorded for the Kalınkara cultivar, while the lowest was determined in the Tombul cultivar (Table 4). On the contrary, Alasalvar *et al.* (2010) determined that the highest PUFA was detected in the Palaz cultivar. The lowest level was recorded for the Kalınkara cultivar. In addition, Karaosmanoglu and Ustun (2021) reported a higher PUFA content in the Tombul cultivar than in the Palaz cultivar.

The difference in monounsaturated fatty acid (MUFA) depending on the cluster drop intensity was significant in all cultivars ($p < 0.05$). While the highest MUFA was determined in the slight cluster drop intensity in Tombul and Palaz cultivars, it was determined in the strong cluster drop intensity in the Kalınkara cultivar. Whereas the Tombul cultivar had the highest MUFA value, the Kalınkara cultivar had the lowest value (Table 4). Similarly, Alasalvar *et al.* (2010) reported the highest MUFA for the Tombul cultivar, followed by Palaz and Kalınkara cultivars. Also, Karaosmanoglu and Ustun (2021) reported higher PUFA for the Palaz cultivar than the Tombul cultivar.

The findings of the fatty acids composition in the study are generally similar to previous reports on the same cultivars by different researchers (Köksal *et al.,* 2006; Alasalvar *et al.,* 2010; Balik, 2021; Karaosmanoglu and Ustun, 2021). Fatty acid composition is affected by many factors such as the genetic structure, ecological condition, location, cultural practices (Balta *et al.,* 2006; Balik, 2021), early harvest, storage, drying methods (Turan, 2019) and maturity (Cristofori *et al.,* 2015).

Previous studies stated that the fatty acid composition changes with an increment in unsaturated fatty acid content in plants exposed to drought stress (Xu *et al.,* 2011). On the contrary, Hamrouni *et al.* (2011) reported a decrease in unsaturated fatty acid and an increase in saturated fatty acid content under drought stress. Similar results were reported in studies that determined the change in fatty acid composition due to water stress in hazelnuts (Bignami *et al.,* 2011; Bostan, 2020). Many researchers reported that drought stress stimulates a wide range of physiological and biochemical responses such as changes in fatty acid composition, wax biosynthesis and osmoprotectant synthesis in plants (Xu *et al.,* 2011). In addition, it has been reported that drought stress and fatty acid composition are related, and the unsaturated fatty acid content that increases in the adaptation process of the plant to drought stress maintains the stability and fluidity of the cellular membranes in

the plant (Xu *et al.,* 2011). In the current study, the temperature values were higher (mean 2.5 °C), while the precipitation values were lower (about 39%) than the long-term average during the nut development period (Figure 1). The cluster drops resulting from this situation significantly affected the fatty acid composition of the cultivars in agreement with Xu *et al.* (2011) by having higher oleic and stearic acid and lower linoleic acid in the kernels of low cluster dropped plants except for the Kalınkara cultivar. Palmitic acid increased as the cluster drop intensity increased.

3.4. Principle component analysis

In the Tombul cultivar, the first two components explained 61.6% of the data. PC1 was related to nut weight, kernel weight, kernel thickness, kernel size, total phenolics and antioxidant activity (FRAP and DPPH) and explained 33.6% of the data. PC2 was mainly related to shell thickness, kernel width, total flavonoids, oleic acid, linoleic acid and stearic acid, and explained 28.0% of the data. There was a high positive relation from nut weight to kernel weight, total phenolics to total flavonoids, total phenolics to FRAP, total flavonoids to FRAP, oleic acid to stearic acid. According to the PCA results, the slight cluster drop intensity was grouped in terms of kernel length, oleic and, stearic acid. The intermediate cluster drop intensity was grouped by linoleic acid while the strong cluster drop intensity was grouped by nut weight, kernel width, kernel size, total phenolics, total flavonoids, antioxidant activity and, palmitic acid (Figure 2).

In the Palaz cultivar, the first two components explained 72.3% of the variability in the data. PC1 explained 56.6% of the data and related to nut weight, kernel weight, kernel width, kernel thickness, kernel length, kernel size, kernel ratio, total phenolics, total flavonoids, antioxidant activity (FRAP and DPPH), oleic, linoleic, palmitic, 11-eicosenoic and, arachidic acids. PC2 was defined by stearic and palmitoleic acids and explained 15.7% of the data. A significant highly positive relation was also computed from nut weight to kernel weight, total phenolics to total flavonoids, total phenolics to FRAP, total flavonoids to FRAP and arachidic acid to 11-eicosenoic acid. According to the PCA results, the slight cluster drop intensity was grouped by kernel length, oleic, arachidic and 11-eicosenoic acid. The intermediate cluster drop intensity was grouped by linolenic and palmitoleic acid and the strong cluster drop intensity was grouped by kernel weight, kernel ratio, kernel size, kernel width, total phenolics, total flavonoids, antioxidant activity and palmitic acid (Figure 3).

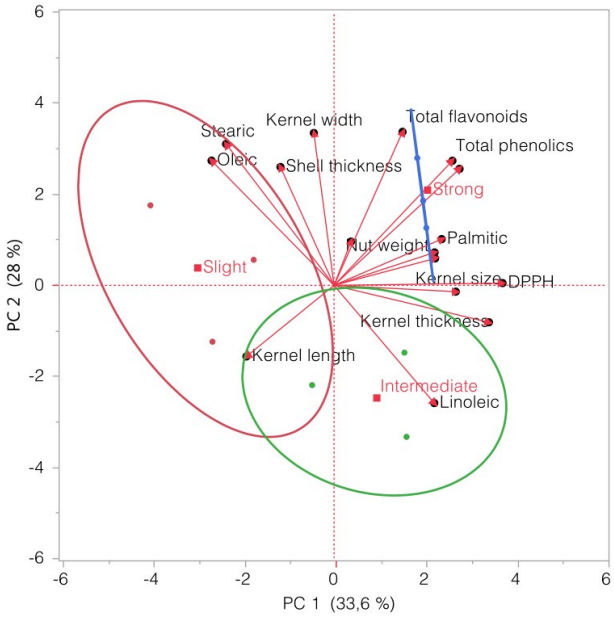

FIGURE 2. Relationships amongst nut traits, bioactive compounds and fatty acids composition in Tombul cultivar in terms of cluster drop intensity

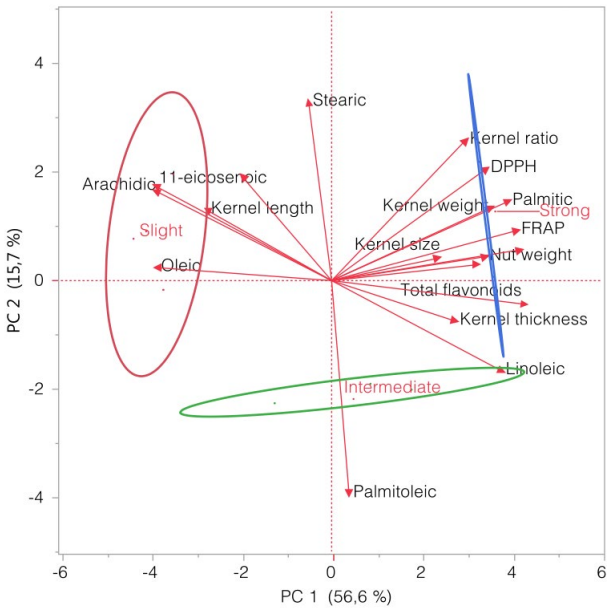

FIGURE 3. Relationships amongst nut traits, bioactive compounds and fatty acids composition in Palaz cultivar in terms of cluster drop intensity

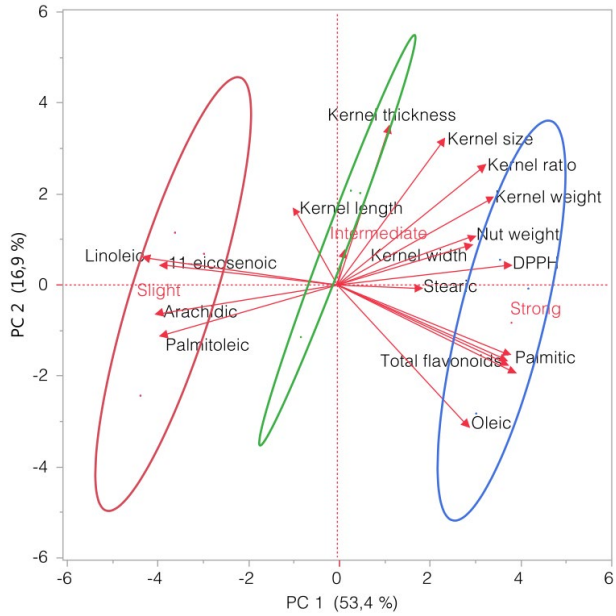

FIGURE 4. Relationships amongst nut traits, bioactive compounds and fatty acids composition in Kalınkara cultivar in terms of cluster drop intensity

In the Kalınkara cultivar, the first two components explained 70.3% of the data. PC1 was related to nut weight, kernel weight, kernel width, kernel ratio, total phenolics, total flavonoids, antioxidant activity (FRAP and DPPH), oleic, linolenic, palmitic, 11-eicosenoic, palmitoleic and arachidic acids, and explained 53.4% of the data. PC2 explained 16.9% of the data and was mainly related to kernel thickness, kernel length and kernel size. There was a highly positive relation from nut weight to kernel weight, kernel weight to kernel ratio, total phenolics to total flavonoids, total phenolics to FRAP, total flavonoids to FRAP, oleic acid to palmitic acid, 11-eicosenoic to palmitoleic and arachidic acid to palmitoleic acid. According to PCA, the slight cluster drop intensity was grouped by linoleic, palmitoleic, arachidic and, 11-eicosenoic acid. The intermediate cluster drop intensity was grouped by kernel length and thickness while the strong cluster drop intensity was grouped by kernel weight, kernel ratio, kernel width, kernel size, total phenolics, total flavonoids, antioxidant activity, oleic and palmitic acid (Figure 4).

In this study, the results from the principal component and correlation analyses supported each other. The properties in the PC1 and PC2 components of all cultivars were highly correlated with each other (Figures 2, 3, and 4). Many researchers have confirmed such a relationship in hazelnuts (Balta *et al.,* 2006; Yılmaz *et al.,* 2019, Bak and Karadeniz, 2021).

4. CONCLUSIONS

Nut traits were not found to be significantly affected by the cluster drop intensity in this study. However, bioactive compounds and fatty acid composition were significantly altered by the intensity of cluster drop. Bioactive compounds of the cultivars were enhanced by increasing cluster drop intensity. In addition, the severity of drop intensity affected the oleic/linoleic acid balance, and slight cluster drop intensity mostly caused higher oleic acid content, except for the Kalınkara cultivar. In general, the slight and intermediate cluster drop intensities were effective on fatty acids, whereas the strong cluster drop intensity was effective on nut traits and bioactive compounds. As a result, it has been determined that the cluster drop intensity significantly affects bioactive compounds and fatty acid composition, which is beneficial for human health. Also, the results of this study showed the possible effects of the upcoming dangers of global climate change (global warming) on hazelnuts and will be useful for future studies.

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Changes in the quality parameters of *Cephalaria syriaca* L. seed oil after the refining process

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SUMMARY: The present study has determined that the crude-oil refining process from the *Cephalaria syriaca* (CS) seed, which could be a new vegetable oilseed source, changed its physical and chemical quality properties (except specific gravity and refractive index). It was also determined that the dominant saturated and unsaturated fatty acids in the crude and refined oils were myristic (21.06–11.80%), palmitic (10.8–8.91%), stearic (2.26–2.70%), oleic (29.17–34.24%) and linoleic (35.56–40.57%). The vitamin E values of the crude and refined CS seed oils were 51.95–50.90 mg/kg, respectively. The oxidative stability values for crude and refined CS seed oils were 2.32–2.69 h, respectively. β-sitosterol and campesterol were the predominant sterols. As a result of the refining process, although magnesium, potassium, iron and copper decreased, the ratios of sodium, aluminum, calcium, chromium, strontium, rubidium, and barium increased. The results provide preliminary data for the future consumption of CS oil in particular for refined CS seed oil.

KEYWORDS: Cephalaria syriaca *seed; Crude and refined vegetable oil; Physico-chemical; Quality properties; Food.*

RESUMEN: ***Cambios en los parámetros de calidad del aceite de semillas de* Cephalaria syriaca L. *tras un proceso de refinación.*** El presente estudio ha determinado que el proceso de refinación de aceite crudo de la semilla de *Cephalaria syriaca* (CS), que podría ser una nueva fuente de oleaginosas vegetales, modificó sus propiedades de calidad física y química (excepto la gravedad específica y el índice de refracción). También se determinó que los ácidos grasos saturados e insaturados dominantes en los aceites crudos y refinados eran mirístico (21,06–11,80%), palmítico (10,8–8,91%), esteárico (2,26–2,70%), oleico (29,17–34,24%), y linoleico (35,56–40,57%). Los valores de vitamina E de los aceites de semillas CS crudos y refinados fueron 51,95–50,90 mg/kg, respectivamente. Los valores de estabilidad oxidativa de los aceites de semilla CS crudos y refinados fueron de 2,32–2,69 h, respectivamente. El β-sitosterol y el campesterol fueron los esteroles predominantes. Como resultado del proceso de refinación, aunque disminuyeron el magnesio, potasio, hierro y cobre, aumentaron las proporciones de sodio, aluminio, calcio, cromo, estroncio, rubidio y bario. Los resultados proporcionan datos preliminares para el consumo futuro de aceite CS, en particular como aceite de semilla CS refinado.

PALABRAS CLAVE: *Aceite vegetal crudo y refinado; Alimento; Parámetros de calidad; Propiedades físico-químicas; Semilla de* Cephalaria syriaca.

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1. INTRODUCTION

Climate change, rapid population growth, and other similar factors have increased people's demand for food, and this situation has prompted people to seek new food sources. Consumable vegetable oilseeds, which are important to human nutrition, are among these sources. *Cephalaria syriaca L.* (CS) seeds, which are widely distributed throughout the world and can be an alternative vegetable oilseed source, are part of the Dipsacaceae family. It has been reported that 94 Cephalaria species are found in Europe, Eastern Mediterranean, East Asia, and North and Central African countries. This plant grows spontaneously in inefficient, clay and loamy soils and is a single-year weed that is resistant to cold (Göktürk and Sümbül, 2014). It is also found as a weed in wheat cultivation areas. It has been reported that the pelemir seed is generally used as pelemir seed flour after its oil is removed (Karahan and Kılınççeker, 2019). It has been reported that CS seeds, which are similar to wheat seeds, have high protein (14.21%), fat (22.28%) and fiber contents (9–30%). CS seed oil is greenish-yellow with a distinctive fragrance, and it has been reported to be rich in saturated fatty acids, such as myristic, palmitic, and stearic and in oleic and linoleic unsaturated fatty acids (Yıldırım *et al.*, 2019). Kavak and Baştürk (2020) have identified 30 different volatile compounds dominated by aldehydes and alcohols. In addition, there are studies with positive and negative results on various antibacterial, antifungal, antioxidant, and cytotoxic properties (Kırmızıgül *et al.,* 2012; Pasi *et al., 2*009). These studies focused on the seed flour, which is generally obtained from defatted seeds. It has been stated that the reason for removing the oil was its bitter taste.

In evaluating CS seed oil in terms of vegetable oil technology and nutrition, we found that a detailed study of the composition of the crude and refined CS oil has not been conducted, and that those that were conducted generally focused on the properties and fatty acid composition of the crude oil. In the present study, the physicochemical composition of crude oil from CS seed, which is important in terms of vegetable oil technology, was determined and, different from previous studies on this subject, this CS oil was refined under in vitro conditions. Thus, the importance of the crude and refined CS oils in terms of the quality parameters of vitamin E, oxidative stability, fatty acids, sterol and mineral substance compositions, vegetable oil technology and nutritional properties were determined.

2. MATERIALS AND METHODS

2.1. Material

The seeds of the CS plant used in this study were obtained from the Tekirdağ Province, Turkey, in June 2021 and crude CS oil was extracted using a solvent in the first stage and then refined by neutralizing, bleaching, winterizing, and deodorizing, and the composition of fatty acids, vitamin E values, and sterol and mineral compositions were determined in addition to the physicochemical quality parameters using the methods noted below.

2.2. Methods

2.2.1. Refining process

The chemical refining process was carried out as neutralization, bleaching, winterization and deodorization, respectively. In order to remove gummy matter and soap compounds, CS crude oil was first treated with water, then phosphoric acid/sodium hydroxide at 75 °C was applied and the oil separated to remove impurities (soap stock) to obtain neutralized CS oil. In the bleaching stage, neutralized CS oil was processed with activated carbon and diatomaceous earth under 710 mm Hg vacuum at 90 °C for 40 minutes for the separation of color substances. During the winterization stage, CS oil, which was cooled to 5-6 °C and treated with perlite, was filtered and purified from waxy substances, and volatile components such as aldehydes and ketones were removed by applying deo-dorization at 220 °C under 3-4 mBar. Finally, refined CS oil was obtained by cooling it to below 40 °C using a plate heat exchanger (De Greyt, 2013).

2.2.2. Physico and chemical methods

Specific gravity, viscosity, flash point, peroxide value, free fatty acidity, iodine value, color, saponification value, unsaponifiable matter, oxidative stability and amount of sterols were analyzed according to AOAC (2000), Lazaridou *et al*. (2004) and Knothe (2006).

2.2.3. Vitamin E analysis

The analytical method developed by Arnaud *et al*. (1991) was used, and the reading process was done in HPLC. The properties of the phase and column used are given below.

Mobile phase: 970 ml hexane + 30 ml 1-4 diox-ane. Column: MAXSIL 5 SILICA 250*4.00 mm 5 micron P/NO OOG-0053-DO Phenomenex or Licrosorb S160-5 micron 25 cm x 4.6 mm. Wavelength in fluorescence detector ex:293, em:326. Flow Rate: 1ml/min. Loop: 20 µl.

2.2.4. Analysis of fatty acid composition

The fatty acid compositions of the oil samples were determined according to AOCS Method No: Ce 1-62 (1998). FAMEs were analyzed on a GC-2025 series gas chromatograph (Shimadzu, Japan), equipped with a hydrogen flame ionization detector (FID) and a fused silica capillary column (60 m × 0.25 mm id), coated with 0.20 µm RTX-2330. Analysis conditions were as follows: colon: 180 °C, injection: 200 °C, detector: 200 °C, gas flow rates with carrier gas (N_2): 30 ml/min, combustible gas (H_2): 28 ml/min. Dry air: 220 ml/min. Injection amount: 1 µl. The injector and FID were set at 260 °C. Commercial mixtures of fatty acid methyl esters standard (FAME) mix (Merck-USA) were analyzed under the same operating conditions to determine relative area percentage.

2.2.5. Sterol analysis

The sterol compositions of the oil samples were determined according to Lechner *et al*. (1999). Commercial mixtures of sterol standard mix (Merck-USA) were used.

GC (Gas chromatography) operating conditions were as follows:
Instrument: GC 2025 (Shimadzu-Japan)
Column: Fused silica capillary column (RTX-2330) (60 m × 0.25 mm i.d.; film thickness 0.20 micrometer). Detector: Flame ionizing detector. Carrier gas: Nitrogen. Split ratio: 50:0. Flow rate: 0.80 ml/min. Injection block temperature: 280 °C. Detector temperature: 290 °C. Column temperature: 260 °C. Injection volume: 1 µl.

2.2.6. Oxidative stability analysis

In this study, the induction time was determined with a Metrohm 743 Rancimat device (Methrom) and rancimat device by using 3 grams of CS seed oil obtained from each period for oxidative stability. Oxidative stability was measured at an airflow rate of 10 l/hour set at 110 degrees. Conductivity of 0.055 µs ultra-pure water was used in the study (Coppin and Pike, 2001).

2.2.7. Determination of mineral contents

The mineral matter compositions of the oil samples were determined by pre-burning, according to Skujins (1998). Inductively Coupled Plasma Atomic Emission was used for mineral matter analyses. Results were calculated as ppm.

Working conditions of ICP-AES Instrument: Alet: ICP-AES (Varian-Vista), Plasma gas (Ar): 20-22.5 l/min in 1.5 l/min, Auxiliary gas (Ar): 0.50-2.25 l/min in 0.75 l/min Signal washout: Four orders (10.000 x)

2.2.8. Statistical Analysis

The data obtained in the study were analyzed using the SPSS (Statistical Package for Social Sciences) Windows 22.0 program. In the evaluation of the data, as descriptive statistical methods, mean standard deviation and quantitative continuous data were compared between two independent groups, One-Way Anova test and Duncan's multiple comparison test were used (Nelson, 1987).

3. RESULTS AND DISCUSSION

As seen in Table 1, the fat content of CS seeds was 20.91%. Some researchers have reported that the oil content of CS seeds is within the range of 11–34% (Hallen *et al*., 2004). Others have reported that these values may vary according to various conditions, such as soil, climate, and geographical location (Sezgin *et al.,* 2017; Kavak and Baştürk, 2020).

In vegetable oil technology, the % oil content of some seeds is specified as follows; 22–36% for sunflower seeds, 25–37% for safflower and 22-49% for rapeseed (Gökalp *et al*., 2001). The fat content in CS seeds, compared to that in other vegetable oilseeds, was the same or higher than that in cottonseed and corn (18–20%) seeds.

As seen in Table 1, the physical properties of CS oil, such as density, refractive index, and viscosity values were 0.9258 g/cm^3, 1.4708, and 84.4 mPain crude CS seed oil, respectively. These values were

Table 1. Physico-chemical quality parameters of crude and refined oils from CS.

Parameter	Crude Oil	Refined Oil
Acidity (%)	0.40±0.01*[a]	0.19±0.02[b]
Peroxide (%)	4.93±0.10[a]	3.77±0.15[b]
Specific Gravity (g/cm^3)	0.925±0.003[a]	0.926±0.005[a]
Viscosity	84.40±0.25[a]	76.90±0.50[b]
Refractive Index	1.4708±0.002[a]	1.4707±0.006[a]
Iodine Number	89.45±0.90[b]	103,308±1.20[a]
Saponification Number (mg KOH/g)	196.98±1.40[a]	151.60±1.32[b]
Unsaponifiable Substances (g/100g)	1.05±0.06[a]	0.77±0.08[b]
Vitamin E (mg/100 g)	51.90±1.15[a]	50.95±0.91[b]
Flash Point (°C)	238±2.02[b]	282±2.66[a]
Oxidative Stabilite (h)	2.32±0.19[b]	2.69±0.12[a]
Total Sterol amount (g/kg)	4.91±0.23[a]	4.03±0.14[b]

determined to be 0.9263 g/cm^3, 1.4707, and 76.9 mPa, respectively, in refined CS seed oil. According to these results, the change in the specific gravity and refractive index of CS oil was not statistically significant at $p < 0.05$ as a result of the refining process, while a significant change was found in viscosity values. Yazıcıoğlu *et al*. (1978) reported that the specific gravity and refractive index values of crude CS oil is 0.9229 g/cm^3 and 1.4706, respectively. The specific gravity value is one of the properties that provides general information about the sources of oils. Accordingly, it was observed that the specific gravity and refractive index values of the crude and refined oils from CS seeda were comparable to those of other vegetable oils (0.910–9.930 g/cm^3 and 1.4670–1.4750, respectively).

Viscosity is resistance to flow and is also an important parameter in vegetable oil technology. Often, the viscosity of oils containing high molecular weight fatty acids is higher than that of low molecular weight oils. There was no reported value related to the viscosity of CS seed and refined oils, which was observed to be comparable to that of canola oil (78.8 cP) compared to other vegetable oils (Noureddidini *et al*., 1992). In general, it was determined that the physical properties of CS seed oil after the refining process were statistically different from before the refining process, except for its specific gravity and refractive index ($p < 0.05$) (Table 1).

As seen in Table 1, the chemical properties of CS seed oil, such as free fatty acidity, peroxide, iodine number, saponification number, and unsaponifiable substance number values, were 0.40%, 4.93 meqO_2/kg, 89.45, 196.98 mg KOH/g, and 1.05 g/100 g in crude CS oil, respectively, while these values were 0.19%, 3.72 meqO_2/kg, 103.308, 151.6 mg KOH/g, and 0.77, respectively, in refined CS oil. With the refining process, it was determined that the free fatty acidity and peroxide values of crude CS oil decreased and the iodine value increased. Kavak and Baştürk (2020) have determined that the free fatty acidity and peroxide values of CS seed crude oil obtained from different locations is 0.27–0.83% and 2.46–5.39 meqO_2/kg, respectively. It was observed that the crude and refined free fatty acidity and peroxide values of CS seed oil were lower than those of sunflower, corn, and canola seed crude oils, which are commonly used vegetable oils.

Yazıcıoğlu *et al*. (1978) have reported that in CS crude oil, the iodine value is 88.4, saponification number is 192 mg KOH/g, and unsaponifiable substance amount is 1.24 g/100 g. The iodine value in sunflower, corn, canola, hazelnut, and cotton varies between 99 and 141.29. The iodine value in refined CS seed oil is comparable to that in other vegetable oils. On the other hand, the saponification number values in vegetable oils such as sunflower, corn, canola, hazelnut and cotton were between 187 and 195 mg/g. It was determined that the saponification values in CS seed crude oil were comparable with those in other vegetable oils, and that in the refined CS seed oil they were lower than in other vegetable oils. In terms of unsaponifiable substance, crude and refined oils from CS seeds could be a new source for food and industrial use.

As seen in Table 1, the vitamin E values of crude and refined oils from CS seeds were 51.95 and 50.90 mg/kg, respectively. It was also determined that vitamin E decreased as a result of the refining process. Kavak and Baştürk (2020) have stated that the vitamin E values in the crude oil from CS seeds, which they collected from different locations, were between 54.0 and 467.0 mg/kg. The vitamin E amounts in other refined vegetable oils have been reported in corn (3.11–4.46 mg/kg), soybean (1.19–1.42 mg/kg), sunflower (9.52–11.4 mg/kg), and canola (3.82–4.95 mg/kg) (Castelo-Branco *et al*., 2016). It was determined that the vitamin E contents in the crude and

Table 2. Color values for CS crude and refined oils.

Oil	Red	Yellow	Blue	Dark
Crude Oil (1[u])	2.8±0.1*[a]	70.0±0.2[a]	0	0
Refined Oil (5.25[u])	1.3±0.1[b]	12.3±0.1[b]	0	0

CS: *Cephalaria syriaca L.* seed; * Mean ± standard deviation; One-way ANOVA analysis was determined (n=3) by Duncan's multiple range tests.
Degree of significance: [a-b]p-value < 0.05;
In each column, means with different letters are significantly different.

Table 3. Fatty acid compositions of CS crude and refined oils.

Fatty acids	Crude Oil	Refined Oil
Myristic	21.06±1.25*[a]	11.80±1.05[b]
Palmitic	10.80±1.02[a]	8.91±0.40[b]
Palmitoleic	0.28±0.05[a]	0.23±0.03[b]
Margaric	0.07±0.01[a]	0.06±0.02[b]
Heptadecanoic	0.08±0.02[a]	0.03±0.01[b]
Stearic	2.26±0.45[b]	2.70±0.80[a]
Oleic	29.17±2.05[b]	34.24±2.25[a]
Linoleic	35.56±2.50[b]	40.57±2.75[a]
Linolenic	0.17±0.03[b]	0.24±0.04[a]
Arachidic	0.32±0.02[b]	0.51±0.05[a]
Eicosanoic	0.16±0.04[b]	0.27±0.07[a]
Behenic	0.02±0.01[b]	0.37±0.10[a]
Erucic	0.03±0.01[b]	0.05±0.02[a]
Lignoceric	0.03±0.02[a]	0.02±0.01[b]

CS: *Cephalaria syriaca L.* seed; * Mean ± standard deviation; One-way ANOVA analysis was determined (n=3) by Duncan's multiple range tests. Degree of significance: [a-b]p-value < 0.05; In each column, means with different letters are significantly different.

refined oils from CS seeds were higher than in other vegetable oils, and in this respect, it was considered a good source for oxidation resistance, oil stability, and health.

The color values for CS seed oil are provided in Table 2. The redness, yellowness, and blueness values of the crude oil obtained from these seeds varied at 2.8 R, 70.0 Y, 0 B (1''), respectively; while the redness, yellowness and blue values of refined rosemary seed oil were 1.3 R, 12.3 Y, 0 B (5,25''), respectively. As a result of the refining process, the level of redness and yellowness statistically decreased ($p < 0.05$) after bleaching. Kavak and Baştürk (2020) have found that the L*, a*, and b* values of crude oil from CS seeds is 18.63/24.87, -1.01/2.37, and 4.73/13.32, respectively. There have been no studies on the color values of refined oil from CS seeds. Compared to other vegetable oilseeds, it was determined that the color values for crude and refined CS seed oil were as good as those of edible vegetable oils, such as sunflower, canola, and hazelnut (Duman and Özcan, 2020).

As seen in Table 3, 14 types of fatty acid components were determined in the crude and refined CS oil—myristic (21.06–11.80%), palmitic (10.8–8.91%), stearic (2.26–2.70%), oleic (29.17–34.24%), linoleic (35.56–40.57%), linolenic (0.17–0.24%), arachidic (0.32–0.51%), and erucic (0.03–0.05%). In other studies on CS crude oil, the fatty acids were myristic (14.60–17.30%), palmitic (9.41–23.81%), oleic (28.10–33.20%), and linoleic (10.28–36.9%). Sarikahya *et al*. (2013), Sarikahya *et al.* (2015), Bretagnolle *et al*. (2016) and Kavak and Baştürk (2020) reported that CS crude oil had similar properties to those of babassu oil (11–27%) and coconut oil (16–21%) in terms of myristic acid composition and to corn oil in terms of the composition of other fatty acids. In studies on the physico-chemical changes that occur during the refining stages of various oils (crude and refined canola, cotton seed, peanut, sunflower, soybean oils), it has been reported that there may be changes in the composition of saturated and unsaturated fatty acids with tendencies to increase and decrease. It has been stated that this may be due to the type of refining process (physical or chemical refining) and the variety of parameters in the stages (El-Mallah *et al*., 2011; Mohdaly *et al*., 2017; Shah *et al*., 2018; Özcan *et al*., 2021).

As seen in Table 4, the total sterol values for crude and refined CS oil was 4.91–4.03 g/kg, which were close to sunflower, corn, canola, hazelnut and cotton seed oils (2–5 g/kg) in terms of total sterol amount (Lavedrine *et al*., 1997; Westrate and Meijer, 1998; Cercaci *et al*., 2003; Yıldırım *et al*., 2019). Examining the nutritional and crude and refined CS oil in terms of sterol composition, these amounts found in CS crude and refined oils showed that β-sitosterol and campesterol were dominant and that these sterol values were 71.41–67.65% and 15.91–16.06%, respectively. It was determined that CS oil was similar to palm kernel oil in terms of sterol composition. As a result of the refining process, it was determined that while the β-sitosterol, Δ-stigmasterol, and stigmasterol levels in CS oil decreased, the level of

Table 4. Sterol compositions of CS crude and refined oils.

Sterol Composition (%)	Crude Oil	Refined Oil
β-Sitosterol	71.41±2.25*[a]	67.65±2.00[b]
Brassicasterol	nd	nd
Campesterol	15.91±0.90[b]	16.06±0.75[a]
Δ-7-Stigmasterol	1.08±0.04[a]	0.87±0.08[b]
Cholesterol	nd	nd
Stigmasterol	1.80±0.01[a]	1.72±0.02[b]

CS: *Cephalaria syriaca L.* seed; nd: Not determined; * Mean ± standard deviation; One-way ANOVA analysis was determined (n=3) by Duncan's multiple range tests. Degree of significance: [a-b]p-value < 0.05; In each column, means with different letters are significantly different.

campesterol increased. These substances, which are among the most important minor components in oils, play an important role in lowering serum cholesterol levels.

As seen in Table 5, 11 minerals were detected in the crude and refined CS oils. The dominant minerals were sodium, magnesium, potassium, calcium, and iron (Na, Mg, K, Ca, and Fe, respectively). As a result of the refining process, although the mineral substances of Mg, K, Fe, and copper (Cu) decreased, the ratio of Na, aluminum (Al), Ca, chromium (Cr), strontium (Sr), rubidium (Rb), and barium (Ba) increased. In the literature, there is no information on the mineral composition of CS crude or refined oils. In this respect, our data are the first on this subject. On the other hand, in terms of mineral substance composition, it was determined that CS oils contained lower amounts of minerals at the ppm level compared to that of olive oil (Sayago *et al*., 2018). According to these results, crude and refined CS oils, which have minerals which are beneficial for human nutrition, can be consumed for the development of bones and teeth and for muscle and nervous system functions. In addition, refined CS oil appears to be a good source of micro and macro minerals as a food ingredient for human nutrition.

Table 5. Mineral matter compositions of CS crude and refined oils.

Mineral (ppm)	Crude Oil	Refined Oil
Sodium	11.56±0.10*[b]	47.10±0.80[a]
Magnesium	26.96±1.10[a]	14.18±1.01[b]
Aluminum	3.58±0.08[b]	4.32±0.05[a]
Fluorine	52.40±1.25[a]	34.66±1.10[b]
Calcium	27.60±1.10[b]	35.93±1.15[a]
Chromium	0.01±0.01[b]	0.06±0.01[a]
Manganese	0.03±0.01	nd
Iron	4.25±0.09[a]	2.65±0.06[b]
Copper	1.46±0.02	nd
Strontium	0.19±0.04[b]	0.32±0.03[b]
Barium	nd	0.05±0.01

CS: *Cephalaria syriaca L.* seed; nd:Not determined; * Mean ± standard deviation; One-way ANOVA analysis was determined (n=3) by Duncan's multiple range tests.
Degree of significance: [a-b]p-value < 0.05; In each column, means with different letters are significantly different.

4. CONCLUSIONS

As can be seen in the literature, this research is the first work on the refining of crude CS oil. The results obtained from the present study provide important data for future consumption of CS oil, especially for its refined product. It was determined that all physical and chemical properties (except specific gravity and refractive index) in the crude oil from CS seeds changed as a result of the refining process ($p < 0.05$). It was also determined that CS seeds, in addition to having an average oil ratio comparable to that of other oilseeds, such as sunflower, corn, canola, hazelnut oils, can be refined. It can be a new source of oilseed raw material. In addition to the determined quality properties of the refined oil from CS seeds, it was determined that it is a good source of new crude materials in terms of vitamin E, essential fatty acids, minerals, and sterol composition for use in cosmetics and human nutrition. In future studies, it is recommended that biotechnological studies be conducted on the seeds to reduce the toxicity of myristic acid.

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Fatty acid profile and rheological behavior of annatto seed oil (*Bixa orellana*), cupuassu seed fat (*Theobroma grandiflorum*), and their blends

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SUMMARY: Annatto seed oil (ASO) and cupuassu seed fat (CSF) were combined at the ratios: 30:70, 50:50, and 70:30 (% w/w). Their fatty acid profile, nutritional quality, FTIR (Fourier Transform Infrared) spectra, and rheological behavior were evaluated. ASO increased the content of polyunsaturated fatty acids in the blends; whereas CSF conferred higher contents of monounsaturated fatty acids. The blends exhibited low atherogenicity and thrombogenicity indices, suggesting nutritional advantages. The Newtonian fluid behavior and FTIR results suggested that mixing ASO and CSF at different proportions did not affect the functional groups. ASO showed an activation energy value which indicated that this fat viscosity was more sensitive to temperature changes. The Newtonian model proved to be suitable to describe the behavior of samples, according to statistical fit parameters R^2, χ^2, and RSS. The resulting blends presented improved physicochemical properties and nutritional attributes, indicating their feasibility for the development of new products.

KEYWORDS: *Activation energy; Amazonian matrices; Nutritional quality; Viscosity.*

RESUMEN: ***Perfil de ácidos grasos y comportamiento reológico de aceite y grasa de semillas de achiote (*Bixa orellana*), y de cacao blanco (*Theobroma grandiflorum*) y sus mezclas.*** Se combinaron aceite de semilla de achiote (ASO) y grasa de semilla de cacao blanco (CSF) en las proporciones: 30:70, 50:50 y 70:30 (% p/p), respectivamente. Se evaluó su perfil de ácidos grasos, calidad nutricional, espectros FTIR (Fourier Transform Infrared) y comportamiento reológico. ASO incrementó el contenido de ácidos grasos poliinsaturados en las mezclas, mientras que CSF confirió mayores contenidos de ácidos grasos monoinsaturados. Las mezclas exhibieron bajos índices de aterogenicidad y trombogenicidad, lo que sugiere ventajas nutricionales. El comportamiento del fluido newtoniano y los resultados de FTIR sugirieron que mezclar ASO y CSF en diferentes proporciones no afectó a los grupos funcionales. ASO mostró un valor de energía de activación que indicó que la viscosidad de esta grasa era más sensible a los cambios de temperatura. El modelo newtoniano demostró ser adecuado para describir el comportamiento de las muestras, según los parámetros de ajuste estadístico R^2, χ^2 y RSS. Las mezclas resultantes presentaron propiedades fisicoquímicas y atributos nutricionales mejorados, lo que indica su viabilidad para el desarrollo de nuevos productos.

PALABRAS CLAVE: *Calidad nutricional; Energía de activación; Matrices amazónicas; Viscosidad.*

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1. INTRODUCTION

The current demand for oils and fats, with emphasis on healthy foods and new uses, has accelerated the search for alternative sources of these lipids. However, many oils and fats with interesting characteristics and unique compositions have not yet been fully considered for product formulation due to the scarcity of information about their properties, as is the case of some Amazonian matrices (Serra *et al.,* 2019).

Bixa orellana L., also known as annatto, is a species native to tropical America. In Brazil, it is native to the North and Northeast regions and occurs mainly in the states of Amazonas, Pará, Paraíba, Piauí, Maranhão, Ceará, and Bahia (Stringheta *et al*., 2018). Annatto is an important source of natural dyes, such as fat-soluble bixin and water-soluble norbixin, with colorations ranging from yellow to dark red. These dyes are widely used in the food, pharmaceutical, and cosmetic industries, due to their high stability, coloring capacity, and lack of toxicity. In addition, the oil extracted from annatto seeds has been arousing the interest of many industrial sectors due to the presence of δ-tocotrienol (Costa *et al*., 2013), which are antioxidant compounds used in the treatment of cardiovascular diseases and cancer, and are also effective in preventing lipid oxidation (Costa *et al*., 2013; Shen *et al.,* 2021).

Cupuassu (*Theobroma grandiflorum*) is a fruit which is native to the Brazilian Amazon and has high agro-economic potential. Its pulp presents wide acceptance in the national and international markets for its unique characteristics such as flavor, aroma, and texture. It is also worth mentioning that a natural fat with chemical and sensory characteristics similar to those of cocoa butter is obtained from cupuassu seeds (Bezerra *et al*., 2017). Cupuassu fat has been considered an excellent source of monounsaturated fatty acids (MUFA) because it has a great content of oleic acid in its composition. These fatty acids have greater oxidation stability when compared to polyunsaturated fatty acids (PUFA), in addition to reducing the levels of LDL cholesterol and lowering the risk of coronary heart disease (Bezerra *et al*., 2017; Serra *et al.*, 2019).

Knowledge of the composition and physicochemical properties of oils and fats and their blends is essential for product development, as well as for designing and improving industrial processes. Iodine value (IV) measures the degree of unsaturation of fatty acids present in oils and fats. Since the melting point and oxidative stability of oils and fats are related to the degree of unsaturation, IV can provide an estimation of these properties. The higher this value, the higher the degree of unsaturation and the greater the susceptibility to oxidation (Dymińska *et al.*, 2016).

Nutritional quality indexes are often used to evaluate oils and fats because they allow a better understanding of the health effects of fatty acids (Attia *et al*., 2015). Atherogenicity index (AI) and thrombogenicity index (TI) are directly related to the stimulation of platelet aggregation (Ulbricht and Southgate, 1991). Therefore, lipids with lower AI and TI values have greater potential to prevent coronary heart diseases.

The most important factors for the utilization of oilseed matrices in industries are quality, stability, and nutritional and functional characteristics of the oils and fats obtained. Although pure oil (as example, palm, sesame, canola, soy and olive oil) is not suitable for direct use in the development of new products (Hashempour-Baltork *et al.,* 2016), blends of oils and fats have been widely applied, due to changes in their natural physicochemical characteristics. Thus, by simply combining different matrices, it is possible to obtain products with interesting technological, nutritional, and functional properties (Bezerra *et al.,* 2017).

In this context, considering the little information available and the increasing demand for new alternative lipid sources, the present study aimed to obtain blends of different proportions of annatto seed oil (ASO) and cupuassu seed fat (CSF), as well as to determine their fatty acid profiles, nutritional quality indices, Fourier Transform Infrared (FTIR) spectra, and rheological behavior.

2. MATERIALS AND METHODS

2.1. Materials and blend preparation

The ASO sample was provided by *Gran Oils* Industry (São Paulo, Brazil) and The CSF sample was provided by *Amazon Oil* Industry (Ananindeua, Pará, Brazil). For blend preparation, ASO and CSF samples were previously heated at 40 ºC, in order to facilitate the homogenization process.

2.2. Determination of fatty acid composition

The fatty acid profiles of ASO, CSF, and their blends were determined by gas chromatography (GC) (CP 3380, Varian Inc., USA) equipped with a flame ionization detector (FID) and a CP-Sil 88 capillary

column (60 m x 0.25 mm x 0.25 mm) (Varian Inc., USA). Fatty acid methyl esters were prepared based on the method proposed by Rodrigues *et al.* (2010) and the operating conditions were helium as carrier gas at a flow rate of 0.9 mL·min^{-1}, FID at 250 °C, and injector split ratio of 1:100 at 245 °C. The column temperature was programmed at 80 ºC for 4 min, with a subsequent increase to 220 ºC at 4 ºC·min^{-1}. Individual fatty acid peaks were identified by comparison of retention times of the gas chromatography with those of known mixtures of fatty acid standards (74X Nu-check-prep, Inc., USA) run under the same operating conditions.

2.3. Iodine and saponification values

Iodine and saponification values were obtained according to methods Cd 1c-85 and Cd 3-94 (AOCS, 2004), respectively, based on the fatty acid compositions.

2.4. Lipid quality indexes

The nutritional quality of ASO, CSF, and their blends was evaluated using six nutritional indicators based on fatty acid compositions. Atherogenicity (*AI*) and thrombogenicity (*TI*) indexes and the ratio of polyunsaturated to saturated fatty acids (*P/S*) were determined according to Ulbricht and Southgate (1991) using Equations 1, 2, and 3, respectively. The ratio of hypocholesterolemic/hypercholesterolemic fatty acids (*h/H*) was determined according to Santos-Silva *et al.* (2002), using Equation 4. And the nutritive value index (*NVI*) was determined according to Chen *et al.* (2016), using Equation 5.

$$AI = \frac{[C12:0 + 4 \times (C14:0) + C16:0]}{[\sum \omega 6 + \sum \omega 3 + \sum MUFA]} \quad (1)$$

$$TI = \frac{(C14:0 + C16:0 + C18:0)}{[(0.5 \times \sum MUFA) + (0.5 \times \sum \omega 6) + 3 \times \sum \omega 3) + (\sum \omega 3 / \sum \omega 6)]} \quad (2)$$

$$P/S = \frac{\sum PUFA}{\sum SFA} \quad (3)$$

$$h/H = \frac{C18{:}\omega 91 + C18{:}2\ \omega 6 + C20{:}4\ \omega 6 + C18{:}3\ \omega 3 + C20{:}5\ \omega 3 + C22{:}5\ \omega 3 + C22{:}6\ \omega 3}{C14{:}0 + C16{:}0} \quad (4)$$

$$NVI = \frac{(C18{:}0 + C18{:}1)}{C16{:}0} \quad (5)$$

2.5. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

The spectra of ASO, CSF, and their blends were obtained with a Fourier transform infrared spectrophotometer (Shimadzu Corporation IR Prestige 21 Cat. No. 206-73600-36- Kyoto-Japan), at the 4000 to 600 cm^{-1} regions, using 32 scans with spectral resolution of 4 cm^{-1}. *OriginPro* v8.0 software was used to graphically plot the spectra obtained, with the aim of evaluating possible changes in functional groups.

2.6. Rheological measurements

The flow behaviors of ASO, CSF, and their blends were determined using a programmable rotational viscometer (DV-II+, Brookfield Engineering Laboratories, USA) equipped with a DIN-87 spindle. The viscometer was coupled to an ultra-thermostatic bath (SL 152/10, Solab, Brazil) for temperature control. Measurements were performed at temperatures of 30, 40, and 50 ºC, with shear rates ranging from 26 to 155 s^{-1}. Shear stress (τ) and viscosity (η) were obtained using *WinGather* software (version 1.1, Brookfield Engineering Laboratories, USA).

2.6.1. Rheological modeling

Oils and fats generally exhibit a linear relationship between shear stress and strain rate, and the Newtonian model is the most commonly used to describe their flow behaviors. Therefore, the data obtained experimentally were fitted to the Newtonian rheological model (Equation 6).

$$\tau = \eta \dot{\gamma} \quad (6)$$

Where τ = shear stress (Pa); η = viscosity (Pa.s); $\dot{\gamma}$ = shear rate (s^{-1}).

The fitting of data to the Newtonian model was performed by nonlinear regression analysis using

OriginPro software (version 8.0, OriginLab, USA). The coefficient of determination (R^2), the reduced chi-square value (χ^2), and the residual sum of squares (RSS) were the parameters used to evaluate the fits.

2.6.2. Influence of temperature on rheological behavior

The Arrhenius equation (Equation 7) was used to evaluate the influence of temperature on the viscosity (η) of the samples. The fitting of data to the Arrhenius model was performed by nonlinear regression analysis using *OriginPro* software (version 8.0, OriginLab, USA).

$$ln\,\eta = ln\,A + \frac{E_a}{RT} \quad (7)$$

Where η = viscosity (mPa.s); A = pre-exponential factor (Pa.s); E_a = activation energy (kJ mol^{-1}); R = universal gas constant (8.314 J/mol K); T = absolute temperature (K).

3. RESULTS AND DISCUSSION

3.1. Fatty acid composition, iodine value, and saponification value

The fatty acid composition, iodine and saponification values, and nutritional quality indexes of ASO, CSF, and their blends are presented in Table 1. The results indicate that ASO is rich in *PUFA* (49.25%) and *MUFA* (31.02%), whereas CSF presents high contents of saturated (42.56%) *SFA* and *MUFA* (40.60%). The main fatty acids present in ASO were α-linolenic (49.25%), oleic (31.02%), and palmitic (12.73%). Silva *et al*. (2008) obtained similar contents for oleic (33.9%) and palmitic (16.4%) acids using this same method of analysis. However, in their work, linoleic (34.3%) was the main *PUFA* in ASO.

Some differences were also observed in the fatty acid composition of ASO described by Rao *et al.* (2015). They obtained lower contents of oleic (17.5%) and linolenic (15.1%) acids, and greater concentrations of palmitic (26.9%) and stearic (10.8%) acids. Costa *et al.* (2013) suggest that the variations in the fatty acid profile of ASO can be attributed to the diversity of the species studied.

Regarding CSF, the main fatty acids present were oleic (40.60%), stearic (31.78%), α-linolenic (11.56%), and palmitic (7.51%). Previous studies confirm that oleic acid is the major compound found in CSF (41.60-43.17%), followed by stearic (31.60-33.91%), α-linolenic (11.37-11.50%), and palmitic (7.37-7.50%) acids (Bezerra *et al.,* 2017; Serra *et al.,* 2019). The high content of oleic acid characterizes CSF as a good source of *MUFA*. Bhattacharjee *et al*. (2020) highlighted the role of oleic acid in reducing the risk of cardiovascular diseases, decreasing inflammatory responses, and regulating serum cholesterol levels. It also has the advantage of having greater oxidative stability compared to *PUFA*.

The main fatty acids present in the blends were oleic (33.89-37.71%), α-linolenic (22.90-38.02%), stearic (14.27-24.28%), and palmitic (9.08-11.16%). Increasing the proportion of ASO in the blends promoted an increase in the content of *PUFA* and a consequent reduction in *SFA*. On the other hand, the samples with greater amounts of CSF also presented higher contents of *MUFA*, which makes the blends more interesting to human nutrition due to the beneficial effects of these fatty acids on the prevention of cardiovascular diseases, besides conferring greater stability (Bezerra *et al.,* 2017).

As expected, ASO showed a higher iodine value (*IV*) (155.52 g $I_2$100 g^{-1}) than CSF (74.30 g I_2 100 g^{-1}) because it contains a greater content of unsaturated fatty acids. Thus, the use of CSF may have contributed to increasing the oxidative stability of the blends.

The saponification value (*SV*) is considered a measurement of the chain length of all fatty acids present in the oil or fat (Sivakanthan *et al.,* 2019). The *SV* of ASO (193.49 mg KOH g^{-1}) was higher than that of CSF (190.92 mg KOH g^{-1}).

3.2. Nutritional quality indexes

Atherogenicity and thrombogenicity indexes lower than 1.0 and 0.5, respectively, are recommended in terms of human health (Fernandes *et al.,* 2014). ASO and CSF showed the same *AI* result (0.16), which indicates that these samples have high and equivalent contents of anti-thrombogenic fatty acids (Table 1). As expected, CSF showed a *TI* (0.66) higher than ASO (0.12), because it presents a greater content of *SFA*, especially stearic acid (31.78%). In addition, ASO presented a great concentration of α-linolenic acid (49.25%), which is a *PUFA* that reduces platelet aggregation potential. The blends showed *AI* and *TI* lower than the recommended values, which is very

Table 1. Fatty acid composition (%), iodine and saponification values, and nutritional quality indices of annatto seed oil (ASO), cupuassu seed fat (CSF), and their respective blends.

Fatty acids	ASO	ASO:CSF (% w/w)			CSF
		70:30	50:50	30:70	
Lauric acid (C12:0)	nd	0.20 ± 0.00^{d}	0.33 ± 0.00^{c}	0.46 ± 0.01^{b}	0.65 ± 0.02^{a}
Myristic acid (C14:0)	0.12 ± 0.01^{e}	0.17 ± 0.00^{d}	0.20 ± 0.00^{c}	0.23 ± 0.00^{b}	0.28 ± 0.00^{a}
Palmitic Acid (C16:0)	12.73 ± 0.07^{a}	11.16 ± 0.10^{b}	10.12 ± 0.09^{c}	9.08 ± 0.10^{d}	7.51 ± 0.10^{e}
Stearic Acid (C18:0)	6.88 ± 0.17^{e}	14.27 ± 0.01^{d}	19.28 ± 0.01^{c}	24.28 ± 0.02^{b}	31.78 ± 0.05^{a}
Oleic acid (C18:1, ω-9)	31.02 ± 0.10^{e}	33.89 ± 0.14^{d}	35.79 ± 0.12^{c}	37.71 ± 0.17^{b}	40.60 ± 0.23^{a}
Linoleic acid (C18:2, ω-6)	nd	1.58 ± 0.01^{d}	2.64 ± 0.01^{c}	3.70 ± 0.02^{b}	5.28 ± 0.03^{a}
α-Linolenic acid (C18:3, ω-3)	49.25 ± 0.10^{a}	38.02 ± 0.02^{b}	30.46 ± 0.01^{c}	22.90 ± 0.01^{d}	11.56 ± 0.01^{e}
Arachidic acid (C20:0)	nd	0.09 ± 0.00^{d}	0.15 ± 0.00^{c}	0.20 ± 0.01^{b}	0.29 ± 0.01^{a}
Behenic acid (C22:0)	nd	0.62 ± 0.00^{d}	1.03 ± 0.00^{c}	1.44 ± 0.01^{b}	2.05 ± 0.02^{a}
∑Saturated	19.73 ± 0.12^{e}	26.51 ± 0.11^{d}	31.11 ± 0.10^{c}	35.69 ± 0.14^{b}	42.56 ± 0.19^{a}
∑Monounsaturated	31.02 ± 0.10^{e}	33.89 ± 0.14^{d}	35.79 ± 0.12^{c}	37.71 ± 0.17^{b}	40.60 ± 0.23^{a}
∑Polyunsaturated	49.25 ± 0.10^{a}	39.60 ± 0.03^{b}	33.10 ± 0.02^{c}	26.60 ± 0.03^{d}	16.84 ± 0.04^{e}
Iodine value (g I_2·100 g^{-1})	155.52	131.35	115.04	98.74	74.30
Saponification value (mg KOH·g^{-1})	193.49	192.72	192.23	191.71	190.92
Atherogenicity index (AI)	0.16	0.16	0.16	0.16	0.16
Thrombogenicity index (TI)	0.12	0.16	0.24	0.35	0.66
Ratio of polyunsaturated to saturated fatty acids (P/S)	2.50	1.49	1.06	0.75	0.40
Hypocholesterolemic/hypercholesterolemic fatty acids (h/H)	6.25	6.49	6.68	6.91	7.37
Nutritive value index (NVI)	2.98	4.31	5.44	6.83	9.64

Triplicate means in the same row followed by different letters are significantly different by Tukey's test ($p \leq 0.05$). nd - not detected. Nutritional indexes determined by indirect method

desirable from a human health perspective (Wołoszyn *et al.,* 2020).

The *P/S* ratio is frequently used to evaluate the nutritional quality of oils and fats. A balanced intake of polyunsaturated and saturated fatty acids is beneficial for controlling serum cholesterol levels. A *P/S* ratio higher than 0.45 is recommended in human diets for the prevention of coronary heart disease and some chronic illnesses such as cancer. The *P/S* ratios of all samples were consistent with the recommended value, except for CSF (*P/S* = 0.40) and the most favorable result was observed for ASO (2.50). In addition, all blends showed *P/S* results which are beneficial to human health and increasing the proportion of *ASO* improved the nutritional quality of the blends.

The value of the *h/H* ratio (hypocholesterolemic/hypercholesterolemic – *h/H*) ranged from 6.25 to 7.37, with the highest value for CSF and the lowest for CSO. The mixtures presented values within this range, and as the concentration of CSF in the mixture increased, this ratio increased. This relationship responds directly to the characteristics of the studied fats. The higher the value of *h/H*, the greater the relationship with risk factors, contributing to the increase in cholesterol, so this is an important measurement in the characterization of fats and their mixtures. It is important to have a low *h/H* ratio in the diet to reduce negative prothrombotic effects caused by increased n–6 linoleic acid concentration in the diet. Despite the limitations, compared to the *PUFA/MUFA*, the *h/H* ratio may more accurately reflect the effect of the fatty acid composition on cardiovascular diseases (Chen and Liu, 2020). Values as low as 0.21 can be found for algae and close to 11.0 to 15.0 for camelina oil (*Camelina sativa*) (Chen and Liu, 2020).

CSF exhibited a greater nutritional value index (*NVI*) value (9.64) than ASO (2.98), which was due to the greater proportion of stearic (*C18:0*) and oleic (*C18:1*) acids present in CSF. Furthermore, it was

also observed that the addition of CSF to the blends directly influenced the *NVI*, which ranged from 4.31 to 6.83.

3.3. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

The spectra obtained in the region of 4,000 to 500 cm^{-1} for ASO, CSF, and their blends are presented in Figure 1. The absorption bands of highest intensity were identified in the region of 2937 to 2843 cm^{-1} and can be attributed to axial deformation vibrations of C-H bonds present in methyl (CH_3) and methylene (CH_2) groups, and double bonds (=C-H).

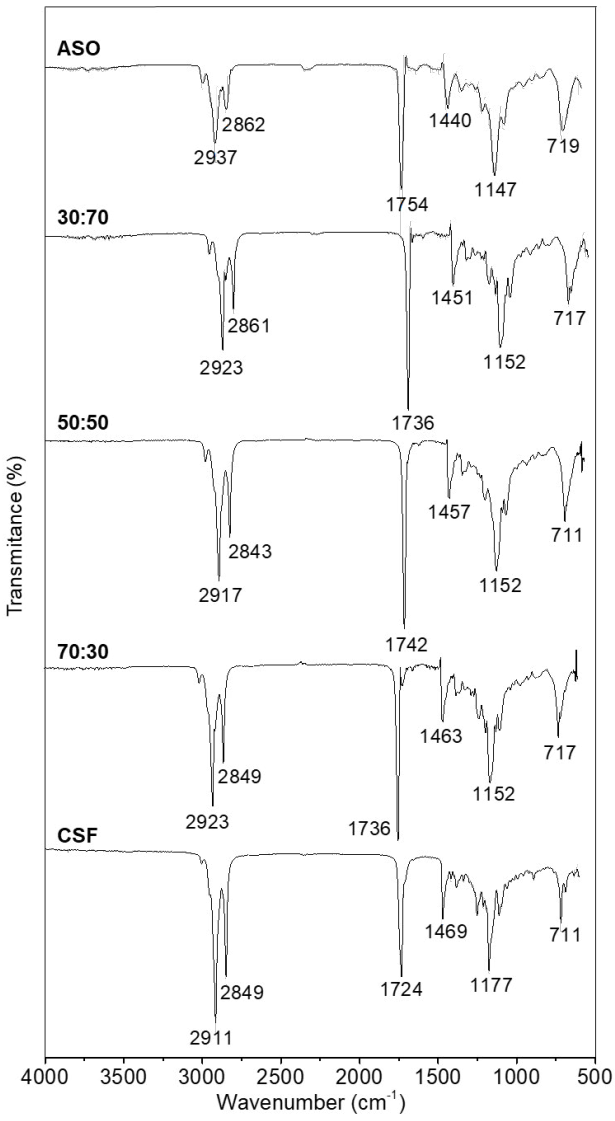

FIGURE 1. Infrared vibrational spectra of annatto seed oil (ASO), cupuassu seed fat (CSF), and their respective blends.

The band that appears in the 1754 - 1724 cm^{-1} region refers to axial deformation vibrations of the carbonyl group (C=O) present in triacylglycerol esters. The bands with intermediate intensity, which appear in the region of 1469 to 1440 cm^{-1}, originate from angular deformation vibrations of C-H bonds present in methyl and methylene groups. The absorption bands which refer to the axial deformation vibrations of C-O bonds of esters which constitute the triacylglycerols are in the 1177 - 1147 cm^{-1} region, which contains part of the "fingerprint" region of the compounds. (García-González *et al.,* 2013). Finally, the peaks at 719 and 711 cm^{-1} can be assigned to the benzene ring.

The results obtained are consistent with the limits reported in other studies (Bitencourt *et al*., 2018). In addition to the bands identified in this work, a set of absorption bands in the regions of 3338 cm^{-1}, 1612 cm^{-1}, and 1562 cm^{-1} was also found. These data are related to the identification of trans-bixin (carotenoid) present in the oil (Rao *et al.,* 2014).

Regarding the spectra of the blends, it was observed that the vibration bands of the bonds involved are very close and did not undergo relevant shifts with respect to wavelength, indicating the absence of chemical interactions between oil and fat (Silva-Júnior *et al*., 2008). These results suggest that combining ASO and CSF in different proportions did not affect the functional groups, which is expected since it is just a mixture and there is no reaction.

3.4. Rheological behavior

The rheological curves of shear stress versus shear rate of the pure samples and their blends are presented in Figure 2. All samples showed a linear relationship between shear stress and shear rate, i.e., they exhibited Newtonian fluid behavior (Silva *et al.,* 2017). It can be noted that, for a constant strain rate, there is a decrease in shear stress values with increasing temperature (Figure 2). This effect can be associated with the structural collapse of molecules that constitute the samples, which occurs due to the action of hydrodynamic forces generated, promoting the alignment of molecules (Alparslan and Hayta, 2002).

The Newtonian fluid behavior was confirmed through the parameters of experimental data that were fitted to the Newtonian model, as can be seen in Table 2. R^2, χ^2, and RSS values confirm the excellent fit of data to the Newtonian model, which indicates that this model is able to accurately predict the rhe-

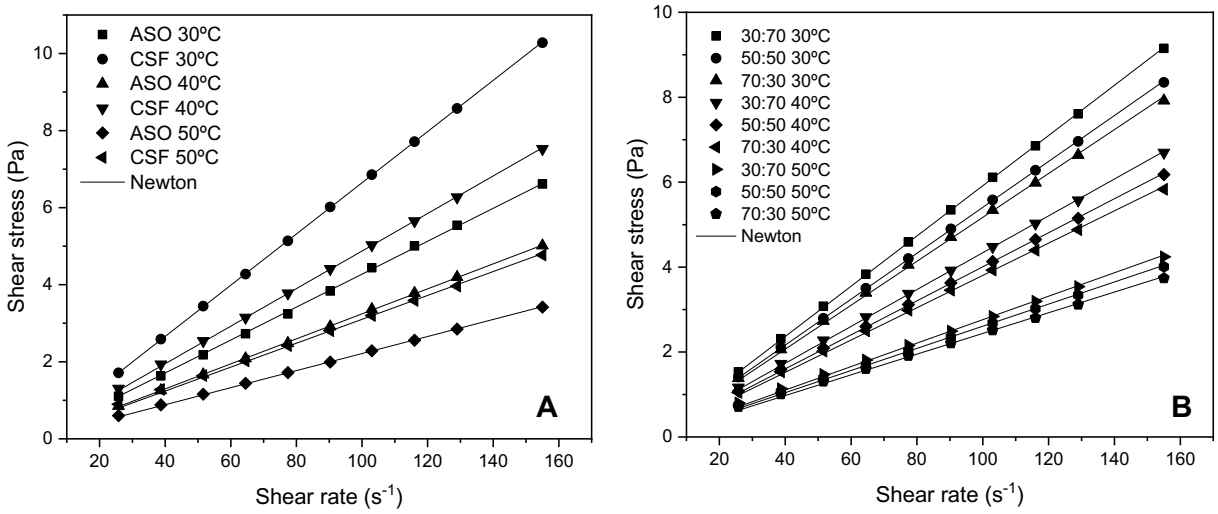

FIGURE 2. Relationship between shear stress and shear rate of pure samples (A) and blends (B), with curves fitted to the Newtonian model.

TABLE 2. Parameters of the Newtonian model fitted to the experimental data of annatto seed oil (ASO), cupuassu seed fat (CSF), and their respective blends.

Sample	T (ºC)	Parameters			
		η (Pa s)	R^2	χ^2	RSS
ASO	30	0.0427^{g}	0.9996^{d}	$0.0013^{c,d}$	0.0118^{g}
	40	0.0324^{j}	$0.9998^{a,b,c}$	0.0003^{e}	0.0026^{m}
	50	0.0221^{o}	$0.9997^{b,c,d}$	0.0003^{e}	0.0023^{n}
ASO:CSF (70:30)	30	0.0517^{d}	0.9993^{e}	0.0031^{a}	0.0281^{a}
	40	0.0380^{i}	0.9990^{f}	$0.0023^{a,b}$	0.0210^{b}
	50	0.0244^{n}	0.9979^{i}	$0.0020^{b,c}$	0.0175^{c}
ASO:CSF (50:50)	30	0.0541^{c}	$0.9999^{a,b}$	0.0003^{e}	0.0028^{l}
	40	0.0401^{h}	$0.9997^{b,c,d}$	$0.0007^{d,e}$	0.0063^{j}
	50	0.0261^{m}	0.9986^{g}	$0.0015^{b,c,d}$	0.0135^{f}
ASO:CSF (30:70)	30	0.0592^{b}	$0.9999^{a,b}$	0.0003^{e}	0.0031^{k}
	40	0.0434^{f}	$0.9997^{c,d}$	$0.0009^{d,e}$	0.0080^{h}
	50	0.0276^{l}	0.9983^{h}	$0.0020^{b,c}$	0.0181^{d}
CSF	30	0.0665^{a}	1.0000^{a}	$0.0002^{d,e}$	0.0015^{o}
	40	0.0487^{e}	$0.9998^{a,b}$	0.0007^{d}	0.0065^{i}
	50	0.0310^{k}	0.9985^{g}	$0.0023^{a,b}$	0.0208^{c}

R^2: Coefficient of determination; χ^2: Reduced Chi-squared; RSS: residual sum of squares. Triplicates mean values with the same letters on the same column do not significantly differ among themselves ($p \leq 0.05$) according to Tukey's test.

ological behavior of ASO, CSF, and their blends, in the temperature range studied.

The Newtonian fluid behavior was confirmed through the parameters of experimental data that were fitted to the Newtonian model because all samples showed high R^2 values (> 0.998) and low χ^2 (< 0.003) and RSS (< 0.028) values, as can be seen in Table 3. R^2, χ^2, and RSS values confirm the excellent fit of data to the Newtonian model, which indicates that this model is able to accurately predict the rheological behavior of ASO, CSF, and their blends, in the temperature range studied.

Table 3. Parameters of Arrhenius model fitted to the data of pure samples and blends.

Sample	A (Pa s)	Ea (kJ mol^{-1})	R^2	χ^2
ASO	0.0673	78.99	0.9711	0.0032
30:70	0.0894	84.47	0.9632	0.0054
50:50	0.0816	82.60	0.9656	0.0046
70:30	0.0929	86.45	0.9635	0.0052
CSF	0.0841	82.03	0.9634	0.0054

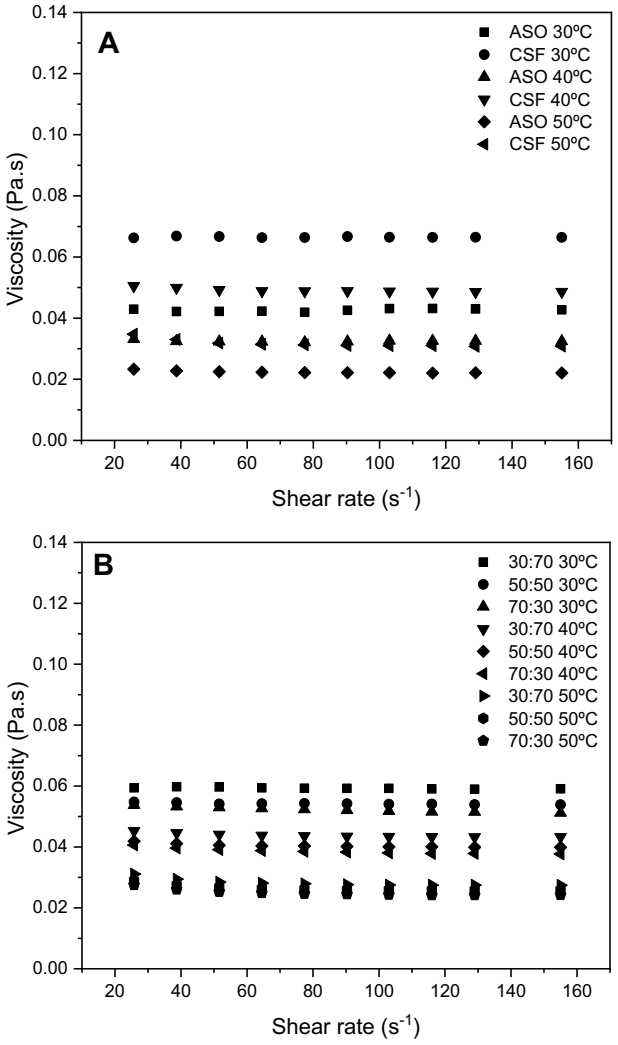

Figure 3. Relationship between viscosity and shear rate of pure samples (A) and blends (B).

Previous studies have attributed the Newtonian fluid behavior, characteristic of oils and fats, to the long-chain molecules that constitute them (Santos *et al*., 2004). Santos *et al*. (2005) studied the rheological behavior of traditional vegetable oils and their blends and found similar results.

As shown in Figure 3, viscosity remained constant regardless of the shear rates tested, whereas the increase in temperature caused a gradual reduction in viscosity of all samples. The effect of temperature on the viscosity of oils and fats can be attributed to the reduction of intermolecular interactions that occur due to increased thermal molecular motion (Santos *et al.,* 2005).

The viscosity of all samples was inversely proportional to the SV and directly proportional to the IV. This behavior occurs because the viscosity of vegetable oils depends on the fatty acid composition, which increases according to their chain size and decreases with unsaturation (Santos *et al.,* 2004). As expected, ASO presented lower viscosity compared to CSF at all temperatures studied because ASO has a greater content of unsaturated fatty acids (Figure 3 B). Besides, the increase in ASO caused a reduction in the viscosity of the blends. (Figure 3 A).

From an industrial point of view, the decrease in viscosity facilitates the material flow and heat exchange during processing. The lower the viscosity of a fluid, the lower the pressure drop during flow, decreasing power costs with pumping and, consequently, energy costs (Braga *et al.,* 2013).

The Arrhenius equation (Equation 5) adequately represented the effect of temperature on the apparent viscosity of the samples, showing R^2 values higher than 0.96, for both pure samples and mixtures, as can be seen in Figure 4 and Table 4.

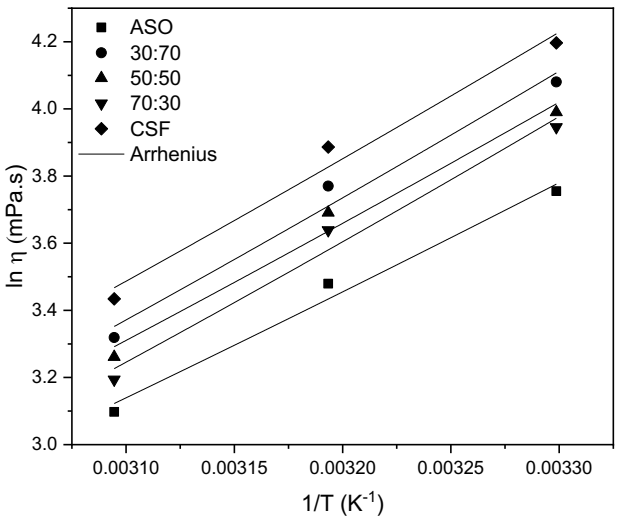

Figure 4. Influence of temperature on the viscosity of pure samples and blends.

The activation energy (E_a) indicates the sensitivity of a material to temperature changes. ASO showed lower E_a (78.99 kJ/mol) than CSF (82.03 kJ mol^{-1}), which indicates that the fat viscosity is more sensitive to temperature changes (Steffe, 1996). The blends exhibited higher E_a results than the pure samples, indicating that the combinations of oil and fat increased their sensitivity to temperature changes.

Regardless of the method of obtaining or using vegetable oils, the knowledge of thermophysical properties, such as viscosity, is of fundamental importance for the achievement of the stages of equipment and process design or even for product specification. In the cosmetics, food and pharmaceutical industries, knowledge of such properties is necessary for the design and development of calculations, equipment and processes involving the production and formulation of new products.

The mixture of fats had a positive effect on viscosity, allowing flexibility in their applications, in addition to providing nutritional properties. While cupuassu seed fat has been used to replace cocoa fat (Medeiros *et al.,* 2006), presenting a higher percentage of saturated fatty acids, annatto fat has more advantages in terms of unsaturated fatty acids, which gives the characteristic viscosity properties to two pure fats. The behavior of the mixture both contributed to a better distribution of the fatty acid profile and allowed an important variation in the viscosity of the fats.

4. CONCLUSIONS

The blends of ASO were demonstrated to have improved nutrition and functionality compared to the individual samples. Greater amounts of ASO increased PUFA content in the blends, whereas greater concentrations of CSF conferred higher MUFA levels. AI and TI results were relatively low, and the blends with greater fat concentrations also showed higher h/H values, suggesting that these samples contain great amounts of fatty acids, which is considered beneficial for human health.

Regarding the spectra of the blends, it was observed that the vibration bands did not undergo a relevant shift in relation to wavelength, indicating that the different combinations did not affect their functional groups.

All samples exhibited Newtonian fluid behavior. The Newtonian model satisfactorily described their rheological behavior in the temperature range studied, according to parameters R^2, χ^2, and RSS.

The blends of ASO and CSF have interesting characteristics, with notable improvements in their nutritional, and functional properties, making them valuable raw materials for the development of new products in the food, pharmaceutical, and cosmetic industries. For example, protective agents and carriers of fat-soluble vitamins, cosmetic emulsions and creams, among other products

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CONFLICT OF INTEREST

The authors declare complete absence of conflict of interest between them and the companies *Gran Oils* Industry (São Paulo, Brazil) and *Amazon Oil* Industry (Ananindeua, Pará, Brazil).

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The influence of microwave roasting on bioactive components and chemical parameters of cold pressed fig seed oil

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SUMMARY: The effect of microwave roasting process on the compositional parameters and bioactive contents of fig seed oil were investigated. Fig seeds were ground and roasted in a microwave oven at 350, 460 and 600 Watt for 5 and 10 minutes and the roasted seeds were processed to obtain oil. The results showed that peroxide, K_{232} and K_{270} values were adversely affected by roasting. Fig seed oil was a prosperous source of γ-tocopherol and significant losses were observed due to microwave pre-treatment. The major fatty acids in fig seed oil were linolenic, linoleic and oleic acids; whereas the major triacylglycerols were LnLO, LnLnL, LnLnLn and LnLnO, according to fatty acid profile. The most abundant sterol in the fig seed oil samples was β-sitosterol with 3235.90 to 3625.62 mg/kg, followed by Δ5- and Δ7-avenasterols. The principal component analysis and agglomerative hierarchial clustering served to differentiate between intense and mild microwave-treated oils as well as the unroasted samples.

KEYWORDS: *Fig seed oil; Microwave; Sterols; Tocopherols; Triacylglycerols*

RESUMEN: ***Influencia del tostado por microondas en los componentes bioactivos y los parámetros químicos del aceite de semilla de higo prensado en frío.*** Se investigó el efecto del proceso de tostado por microondas sobre los parámetros de composición y contenido bioactivo del aceite de semilla de higo. Las semillas de higo se molieron y tostaron en un horno de microondas a 350, 460 y 600 vatios durante 5 y 10 minutos a continuación se obtuvo el aceite. Los resultados han demostrado que los valores de peróxido, K_{232} y K_{270} se vieron afectados negativamente por el tostado. El aceite de semilla de higo es una buena fuente de γ-tocoferol y se observaron pérdidas significativas mediante el pretratamiento con microondas. Los principales ácidos grasos del aceite de semilla de higo fueron los ácidos linolénico, linoleico y oleico; mientras que los principales triacilgliceroles fueron LnLO, LnLnL, LnLnLn y LnLnO que ratificaron el perfil de ácidos grasos. El esterol más abundante de las muestras de aceite de semilla de higo fue el β-sitosterol que varió de 3235,90 a 3625,62 mg/kg, acompañado de Δ5-avenasterol y Δ7-avenasterol. El análisis de componentes principales y la agrupación jerárquica aglomerativa permitieron la diferenciación de aceites tratados con microondas intensos y suaves, así como las muestras sin tostar.

PALABRAS CLAVE: *Aceite de semilla de higo; Esteroles; Microondas; Tocoferoles; Triacilgliceroles.*

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1. INTRODUCTION

Ficus carica L., generally known as fig, is one of the oldest cultivated fruit trees and is a member of the Moraceae botanical family. It is grown in warm and dry climates, and its natural areas are the Mediterranean coast and western Asia. Major producers are Turkey, Egypt, Morocco, Spain, Greece, California, Italy, Brasil, Algeria and Iran (Nakilcioğlu-Taş, 2018). The tree is mainly cultivated for its fig fruit, which is consumed either as fresh, dried, canned or as jam, juice and puree. Fresh and dried fruits are principal sources of minerals, vitamins, dietary fiber, amino acids, organic acids and sugar (Solomon *et al*., 2006). Fig fruits have also been denoted to be fine sources of phenolics that contribute to the nutritional quality and antioxidant capacity of the fruit (Veberic *et al*., 2008).

The fig holds a vast number of small seeds that vary greatly in amount depending on the volume and the maturity of the fruit. The seeds are located within the interior part of the fruit as a mass with a jelly-like flesh (Badgujar *et al*., 2014). A considerable amount of waste containing large amounts of seeds is generated when figs are used for the production of juice and puree. The obtained by product is commonly utilized for recovering fig seed oil. The seeds contain notable amounts of oil, which may reach up to 30% in dried figs (İçyer *et al*., 2017).

Fig seed oil is distinguished by its high linolenic acid content, while oleic, linoleic, stearic and palmitic acids were also described to be present in the oil (Joseph and Raj, 2011). The replacement of saturated fatty acids in the human diet with polyunsaturated ones was recommended by WHO/FAO (WHO, 2008). Fig seed oil can be incorporated into common dietary items as a good source of plant-based polyunsaturated fatty acid. Apart from the fatty acid profile, fig seed oil is also a valuable resource of γ-tocopherol and other bioactive compounds, such as α-tocopherol and phytosterols (Güven *et al*., 2019).

Microwave pre-treatment is a desirable technique used to produce oil from seeds. The technique has several advantages such as short processing time, low energy consumption, higher yield and better retention of nutraceuticals in the resultant oil (Azadmard-Damirchi *et al.*, 2011) when compared to conventional methods. A number of works have been performed to observe the influence of microwave pre-treatment before the extraction of oil from pumpkin (Ali *et al.*, 2017), sunflower (Goszkiewicz *et al*., 2020), chia seed (Ozcan *et al*., 2019), pomegranate (Đurđević *et al*., 2017), black cumin (Bakhshabadi *et al*., 2017), Camellia (Ye *et al*., 2021) and other seeds. Of course, each oil obtained from various oilseeds has its own characteristic triacylglycerol profile and all oils respond differently to thermolytic and oxidative reactions that take place during heating processes due to their different chemical composition. There is no information available on the impact of microwave roasting prior to oil extraction on the quality, stability or chemical composition of fig seed oil. Some reports have been published on the chemical profile of fig seed oil (Duman and Yazıcı, 2018; Güven *et al*., 2019), although the information regarding the impact of microwave pre-treatment on the oil composition is lacking and needs to be investigated. Because no research on oil extraction from fig seeds with the use of microwave application wase detected by the authors, The effective parameters including microwave power and time were determined in the present work. Hence, the aim of the present study was to investigate the changes in composition (fatty acid and triacylglycerol profiles) and bioactive compounds (sterols and tocopherols) in fig seed oil due to microwave roasting before mechanical extraction. Since the intensity of heat affects the composition of oil, various power settings (350, 460 and 600 W) and radiation times (5 and 10 min) were taken into consideration. The results of this study may be used to evaluate the feasibility of using microwave pre-treatment as an improvement method for the manufacture of cold-pressed fig seed oil.

2. MATERIALS AND METHODS

2.1. Materials

Fig seeds were kindly supplied by Egesia (Aydın, Turkey). Potassium hydroxide, β-sitosterol, pyridine, sodium hydroxide, ethyl alcohol, and chloroform were purchased from Merck (Darmstadt, Germany). Diethyl ether, methanol, isopropyl alcohol, isooctane, acetonitrile, methyl orange, *n*-hexane, hydrochloric acid, acetone, 5α-cholestan-3β-ol, silica gel, α-, β-, δ- and γ-tocopherol standards were purchased from Sigma-Aldrich (St- Louis, ABD). 37 fatty-acid methyl ester mix, N,O-Bis (trimethylsilyl) and trifluoroacetamide with trimethylchlorosilane were purchased from Supelco (Bellefonte, USA).

2.2. Methods

2.2.1. Microwave pre-treatment and pressing of the fig seeds

Fig seeds were first ground in a grinder (Sinbo, Turkey) and then roasted at 350, 460 and 600 Watt for 5 and 10 minutes in a microwave oven (Arçelik, Turkey). The seeds were cooled to room temperature after roasting. The pressing process was performed with a laboratory scale (single head, 2 hp, 12 kg seed/h capacity, 1.5 kW power) screw press (Koçmaksan KMS 10, Turkey). After pressing, centrifugation was applied to obtain a clearer oil. The fig seed oil samples were kept at 4 ºC in nitrogen atmosphere until analyses.

2.2.2. Fat content

The oil content in the seeds was measured by soxhlet extraction in accordance with AOCS Official Methods Am 2-93. *n*-hexane was the extraction solvent.

2.2.3. Peroxide value and spectrophotometric extinction coefficients at 232 and 270 nm (K_{232} and K_{270})

Peroxide value, spectrophotometric extinction coefficients at 232 and 270 nm were measured by AOCS Official Methods Cd 8-53 and Ch 5-91 (AOCS, 2003), respectively.

2.2.4. Fatty acid profile

The percentages of fatty acids were determined by preparing their corresponding methyl esters according to the method established by International Union of Pure and Applied Chemistry (IUPAC, 1987). The esters were analyzed with a gas chromatography instrument (GC 2010, Shimadzu, Japan) fitted with a flame ionization detector. The separation of the peaks was achieved with a DB-23 column (60 m length x 0.25 mm internal diameter and 0.25 µm film thickness) (J&W Scientific). The column, injector and detector temperatures were 195, 230 and 240 °C, respectively. Nitrogen was the carrier gas (1.0 ml/min).

2.2.5. Triacylglycerol profile

The triacylglycerol profile of the fig seed oils was determined using AOCS Official Method Ce 5b-89 (AOCS, 2003). The oil sample (0.5 g) was dissolved in acetone and analyzed using HPLC (Shimadzu, Japan) fitted with a differential refractometer detector (RID). Chromatographic elution was achieved using an ACE 5 C18 column (4.6 mm ×250 mm, 5µm particle size, ACE, Scotland). The mobile phase consisted of acetone/acetonitrile (1:1) at a flow rate of 1.5 ml/min. The column temperature was 30 °C and volume of injection was 10 µl. Triacylglycerol peaks were defined by matching with those in the literature (Holčapek *et al*., 2005).

2.2.6. Sterol profile

The sterol profile of oil samples was determined in accordance with AOCS Ch 6–91 (2003). The sterols were first silanized and then quantitatively detected with a gas chromatography instrument (GC 2010, Shimadzu, Japan) fitted with a flame ionization detector. Chromatographic seperation was performed using a HP-5 column (Chrom Tech., USA) with 30 m length, 0.25 µm film thickness and 0.25 mm internal diameter. Nitrogen was the carrier gas at a flow rate of 0.8 mL/min. The injector, detector and column temperatures were 280, 290 and 260 °C, respectively.

2.2.7. Tocopherol profile

The tocopherol composition of the fig seed oil was detected using an HPLC instrument (Shimadzu, Kyoto, Japan) equipped with an InertSustain NH_2 column. The column was 250 mm in length, with 4.6 mm internal diameter and 5 µm particle size (GL Sciences, Japan). The mobile phase was composed of *n*-hexane:isopropyl alcohol (99.5:0.5) in an isocratic system. The injection volume was 20 µl and the flow rate was 1.2 ml/min. Tocopherol homologues were determined at 290 nm. α-, and γ- tocopherol standards were used to prepare the standard curves.

2.2.8. Statistical analysis

The SPSS software, version 15.0 (SPSS Inc., Chicago, USA) was used for statistical evaluation. Differences were calculated by one-way ANOVA procedure and Duncan's multiple range test was used to compare the significance of differences at $p < 0.05$. Data were also analyzed with multivariate tests (PCA and AHC) using XLSTAT, version 2020 (Addinsoft, USA).

3. RESULTS AND DISCUSSION

The oil content in the fig seeds was 22.18% on wet weight basis, similar to the findings of former works (Nakilcioğlu-Taş, 2019).

The changes in lipid oxidation parameters (peroxide value and specific ultraviolet absorbances) as a result of the microwave process are presented in Table 1. Peroxide value, indicator of primary oxidation products, increased by ascending microwave power. Significant differences were determined in peroxide values of increasing radiation times at 460 and 600 W power settings. The K_{232} value increased significantly due to microwave roasting, although no significant differences were determined among the three power settings and two process times. The K_{270} value, which shows the secondary oxidation products (aldehydes and ketones), was detected to increase with ascending power settings.

Tocopherols are antioxidants which are naturally present in edible oils and play important roles as Vitamin E for human health. The influence of microwave power settings on the tocopherol composition of the fig seed oil is given in Table 1. Fig seed oils contained α- and γ-tocopherols varying in 101.62-114.07 mg/kg and 3888.22-4132.09 mg/kg, respectively. Güven *et al*. (2019) reported 4267 mg/kg of γ-tocopherol; and Baygeldi *et al*. (2021) described 314.61±51.53 mg/100 g of γ-tocopherol, 7.40±0.26 mg/100 g of d-tocopherol and 3.71±0.62 mg/100 g of α-tocopherol for fig seed oil. Microwave pre-treatment caused significant losses in both tocopherols, possibly due to thermo-induced oxidation and degradation of tocopherols (Ji *et al.*, 2019), similar to the findings reported for poppy seeds (Ghafoor *et al*., 2019) and pumpkin seeds (Yoshida *et al*., 2006). In the current work, the highest tocopherol loss was determined at the highest microwave power setting (600 W) at the longer period of roasting (10 min). Moreover, roasting time was found to be statistically important on both α- and γ- tocopherol content at 600 W power setting.

The changes in fatty acid composition due to microwave roasting are shown in Table 2. The major fatty acid was linolenic acid, ranging from 45.06-46.01%, slightly higher than the findings of previous works (İçyer *et al*., 2017; Duman and Yazıcı, 2018; Baygeldi *et al.*, 2021). Extended roasting time seemed to decrease the linolenic acid content in the samples. Linoleic acid was the other leading fatty acid, and varied from 27.99-28.75% and found to be the lowest at 600 W and 10 min of microwave heating. Oleic acid was the predominant monounsaturated fatty acid (16.63-16.98%) and it slightly decreased due to the microwave process. Modest changes were observed in palmitic, stearic and arachidic acids; whereas myristic, palmitoleic, heptadecanoic, heptadecenoic, gadoleic acids were determined to remain unchanged. C 16:1 and C 18:0 were high and negatively correlated (r = -0.93). Previous works have reported either decreases in unsaturated fatty acids due to degradation (Fathi-Achachlouei *et al*., 2019; Suri *et al*., 2020), or statistically constancy in fatty acid profile (Güneser and Yılmaz, 2017) for different types of oils.

Table 1. Peroxide value, UV spectrophotometric indices and tocopherol contents of oils obtained from fig seeds roasted at different microwave setting and times

Microwave power (W)	Time (min)	Peroxide value (meqO_2/kg oil)	K_{232}	K_{270}	α-tocopherol (mg/kg)	γ- tocopherol (mg/kg)
Control		1.06±0.00^{A}	1.86±0.31^{A}	0.36±0.03^{A}	114.07±0.89^{A}	4057.08±102.74^{BC}
350	5	1.06±0.00^{A}	2.27±0.14^{B}	0.41±0.08^{AB}	108.46±2.91^{B}	4079.05±157.60^{BC}
	10	1.06±0.00^{A}	2.30±0.16^{B}	0.41±0.01^{AB}	108.99±3.82^{B}	4132.09±57.57^{C}
460	5	1.41±0.50^{B}	2.60±0.18^{B}	0.49±0.09^{BC}	107.14±1.96^{B}	3955.39±141.13^{AB}
	10	2.13±0.00^{C}	2.46±0.15^{B}	0.47±0.03^{AB}	108.09±1.61^{B}	4125.55±71.23^{BC}
600	5	1.42±0.00^{B}	2.41±0.15^{B}	0.58±0.12^{C}	108.86±1.51^{B}	4070.05±92.80^{BC}
	10	3.02±0.25^{D}	2.58±0.28^{B}	0.51±0.03^{BC}	101.62±2.29^{C}	3888.21±87.35^{A}

The results are presented as mean±standard deviation (n=4). Means in the same column with different letters are significantly different according to Duncan's multiple range test ($p < 0.05$).

Table 2. Fatty acid composition of oils obtained from fig seeds roasted at different microwave settings and times (%)

Fatty acids	Control	350 W		460 W		600 W	
		5 min	10 min	5 min	10 min	5 min	10 min
C 14:0	0.01±0.00^{A}	0.01±0.01^{A}	0.01±0.00^{A}	0.01±0.00^{A}	0.01±0.00^{A}	0.01±0.00^{A}	0.01±0.00^{A}
C 16:0	6.46±0.04^{A}	6.53±0.16^{AB}	6.46±0.09^{A}	6.49±0.13^{A}	6.44±0.07^{A}	6.50±0.05^{A}	6.66±0.09^{B}
C 16:1	0.33±0.12^{A}	0.38±0.10^{A}	0.47±0.25^{A}	0.33±0.03^{A}	0.48±0.21^{A}	0.43±0.06^{A}	0.35±0.12^{A}
C 17:0	0.03±0.00^{A}	0.03±0.01^{A}	0.03±0.01^{A}	0.03±0.01^{A}	0.03±0.01^{A}	0.03±0.01^{A}	0.03±0.00^{A}
C 17:1	0.02±0.01^{A}	0.02±0.00^{A}	0.02±0.00^{A}	0.02±0.01^{A}	0.02±0.00^{A}	0.02±0.00^{A}	0.02±0.01^{A}
C 18:0	2.23±0.10^{B}	2.19±0.16^{AB}	2.13±0.09^{AB}	2.24±0.10^{B}	2.04±0.17^{A}	2.15±0.03^{AB}	2.26±0.08^{B}
C 18:1	16.98±0.25^{B}	16.71±0.11^{AB}	16.65±0.15^{A}	16.86±0.24^{AB}	16.63±0.13^{A}	16.98±0.26^{B}	16.89±0.03^{AB}
C 18:2	28.53±0.18^{BCD}	28.28±0.19^{ABC}	28.10±0.49^{AB}	28.75±0.06^{D}	28.21±0.45^{ABC}	28.59±0.09^{CD}	27.99±0.07^{A}
C 18:3	45.22±0.49^{A}	45.66±0.37^{AB}	45.95±0.52^{B}	45.06±0.14^{A}	46.01±0.64^{B}	45.10±0.36^{A}	45.60±0.15^{AB}
C 20:0	0.07±0.02^{AB}	0.05±0.02^{AB}	0.04±0.02^{A}	0.08±0.01^{A}	0.04±0.04^{A}	0.05±0.02^{AB}	0.05±0.01^{AB}
C 20:1	0.13±0.03^{A}	0.13±0.01^{A}	0.14±0.05^{A}	0.14±0.02^{A}	0.11±0.03^{A}	0.14±0.01^{A}	0.12±0.01^{A}

The results are presented as mean±standard deviation (n=4). Means in the same line with different letters are significantly different according to Duncan's multiple range test ($p < 0.05$).

Triacylglycerols are the major components and represent 95-98% of edible oils. The determination of the triacylglycerol composition has critical importance to understanding the characteristics of oils. The change in triacylglycerol composition in fig seed oils by microwave application is given in Table 3. The major triglycerides identified were LnLO (oleolinoleolinolenin), LnLnL (linoleodilinolenin), LnLnLn (trilinolenin) and LnLnO (oleodilinolenin). In addition, LnLL (dilinoleolinolenin), LnLP (palmitolinoleolinolenin), SLLn (stearolinoleolinolenin), LLO (oleodilinolein), LnLnP (palmitodilinolenin), LLP (palmitodilinolein), LOP (palmitooleolinolein), LOO (dioleolinolein), LnOO (dioleolinolenin), LLL (trilinolein), LnLnS (stearodilinolenin), SLO (stearooleolinolein), OOO (triolein) and SOLn (stearooleolinolenin) were determined in descending order. The triacylglycerol composition of fig seed oils was in good agreement with their fatty acid profile. The major triglyceride, LnLO, varied from 13.00-13.54% and the increase in process time caused a slight decrease in the LnLO ratio at 460 and 600 W power settings. The other three crucial triacylglycerols, namely LnLnL, LnLnLn and LnLnO, ranged from 13.07-13.29%, 10.82-11.19% and 10.32-10.74%, respectively. Various results have been reported for the triacylglycerol composition of different matrices in response to microwave roasting, prior to oil extraction (Yoshida *et al*., 2006, Ali *et al*., 2017). In the current study; LOP, LLO and LnLL were determined not to be affected significantly by microwave pre-treatment. Although remarkable discrepancies were determined between the triacylglycerol percentages of unroasted and microwave roasted fig seed oils, different microwave power settings and radiation times did not have a clear influence on the rates of the remaining triacylglycerol. LnLnLn was determined to be highly correlated with C18:3 ($r = 0.947$).

Phytosterols are valuable components not only for their ability to lower serum cholesterol levels but also for their anti-ulcerative, anti-inflammatory, anti-bacterial and antitumour properties in humans (Moreau, 2003). The changes in the sterol distribution of fig seed oils by microwave roasting are shown in Table 4. The major sterols were β-sitosterol, Δ7-avenasterol and Δ5-avenasterol. In addition, 24-methylene-cholesterol, campestanol, campesterol, Δ7-campesterol, stigmasterol, sitostanol, clerosterol, Δ7-stigmastenol, Δ5-24-stigmastadienol were detected in small amounts. The total sterol contents in oil samples varied between 4859.96-5281.90 mg/kg and the microwave pre-treatment was found to be statistically insignificant on total sterols. Güven *et al*. (2019) reported 6516.20 mg/kg of total sterols for fig seed oils. A number of works have been published indicating the enrichment in phytosterol content in the oil due to microwave roasting (Azadmard-Damirchi *et al*., 2010), however, there are also reports showing a reduction in

Table 3. Triacylglycerol profile of oils obtained from fig seeds roasted at different microwave settings and times (%)

Triacylglycerols	Control	350 W		460 W		600 W	
		5 min	10 min	5 min	10 min	5 min	10 min
LnLnLn	10.99±0.16^{AB}	11.00±0.16^{ABC}	11.17±0.09^{BC}	10.82±0.06^{A}	11.19±0.17^{A}	10.82±0.03^{A}	11.03±0.11^{BC}
LnLnL	13.07±0.13^{A}	13.13±0.08^{A}	13.29±0.04^{B}	13.10±0.07^{A}	13.22±0.15^{AB}	13.11±0.10^{A}	13.07±0.07^{A}
LnLL	8.13±0.32^{A}	8.07±0.33^{A}	8.06±0.43^{A}	8.27±0.19^{A}	8.28±0.26^{A}	7.96±0.20^{A}	8.36±0.18^{A}
LnLnO	10.61±0.45^{A}	10.74±0.47^{A}	10.59±0.23^{A}	10.69±0.23^{A}	10.32±0.34^{A}	10.35±0.19^{A}	10.36±0.05^{A}
LnLnP	5.36±0.13^{BC}	5.31±0.07^{ABC}	5.41±0.05^{C}	5.36±0.05^{BC}	5.23±0.11^{AB}	5.19±0.10^{A}	5.40±0.02^{C}
LLL	2.23±0.38^{BC}	1.84±0.06^{A}	1.95±0.32^{AB}	1.98±0.17^{AB}	2.40±0.06^{C}	2.09±0.25^{ABC}	2.37±0.13^{C}
LnLO	13.16±0.40^{AB}	13.54±0.13^{B}	13.41±0.29^{B}	13.46±0.18^{B}	13.00±0.19^{A}	13.54±0.21^{B}	13.01±0.13^{A}
LnLP	7.59±0.63^{AB}	8.05±0.17^{B}	7.84±0.61^{B}	7.68±0.42^{AB}	7.38±0.27^{AB}	7.63±0.53^{AB}	6.99±0.23^{A}
LnLnS	1.64±0.66^{AB}	1.24±0.17^{A}	1.48±0.42^{A}	1.62±0.42^{AB}	1.90±0.28^{AB}	1.76±0.55^{AB}	2.23±0.16^{B}
LLO	5.85±0.09^{A}	5.87±0.14^{A}	5.86±0.14^{A}	6.00±0.19^{A}	5.98±0.18^{A}	6.02±0.07^{A}	5.86±0.08^{A}
LnOO	2.93±0.23^{BC}	2.54±0.08^{A}	2.64±0.23^{A}	2.71±0.11^{AB}	2.77±0.10^{ABC}	2.65±0.28^{A}	3.05±0.09^{C}
LLP	3.28±0.09^{A}	3.49±0.16^{AB}	3.43±0.13^{AB}	3.44±0.12^{AB}	3.47±0.30^{AB}	3.58±0.15^{B}	3.35±0.16^{AB}
SLLn	6.21±0.24^{AB}	6.35±0.04^{B}	6.22±0.25^{AB}	6.20±0.19^{AB}	6.04±0.35^{AB}	6.20±0.10^{AB}	5.93±0.09^{A}
LOO	2.93±0.11^{AB}	2.95±0.03^{AB}	2.89±0.08^{A}	2.96±0.01^{AB}	2.93±0.05^{AB}	3.04±0.12^{B}	2.89±0.06^{A}
LOP	3.28±0.16^{A}	3.29±0.09^{A}	3.21±0.11^{A}	3.19±0.09^{A}	3.21±0.12^{A}	3.31±0.17^{A}	3.29±0.09^{A}
SOLn	0.79±0.04^{A}	0.69±0.05^{AB}	0.61±0.07^{B}	0.67±0.12^{AB}	0.69±0.07^{AB}	0.75±0.10^{A}	0.74±0.09^{A}
OOO	0.66±0.02^{AB}	0.67±0.01^{AB}	0.68±0.02^{AB}	0.66±0.04^{A}	0.69±0.01^{AB}	0.69±0.04^{AB}	0.70±0.03^{B}
SLO	1.28±0.11^{AB}	1.22±0.05^{A}	1.27±0.07^{AB}	1.20±0.07^{A}	1.29±0.02^{AB}	1.31±0.05^{AB}	1.38±0.08^{B}

The results are presented as mean±standard deviation (n=4). Means in the same line with different letters are significantly different according to Duncan's multiple range test ($p < 0.05$).

Table 4. Sterol content of oils obtained from fig seeds roasted at different microwave settings and times (mg/kg)

Sterols	Control	350 W		460 W		600 W	
		5 min	10 min	5 min	10 min	5 min	10 min
24-methylene cholesterol	2.93±0.04^{A}	2.73±0.28^{A}	2.81±0.07^{A}	2.91±0.63^{A}	2.88±0.49^{A}	2.85±0.41^{A}	2.92±0.71^{A}
Campesterol	162.46±20.97^{A}	156.25±4.22^{A}	151.02±12.57^{A}	144.04±5.31^{A}	160.54±14.06^{A}	147.96±12.50^{A}	144.61±7.38^{A}
Campestanol	0.84±0.31^{A}	0.78±0.25^{A}	0.87±0.17^{A}	1.00±0.08^{A}	0.98±0.09^{A}	1.09±0.22^{A}	1.21±0.39^{A}
Stigmasterol	127.28±18.29^{B}	121.31±4.98^{AB}	117.53±10.77^{AB}	109.90±4.82^{A}	122.52±10.23^{AB}	113.03±8.15^{AB}	108.72±6.37^{A}
Δ-7-campesterol	5.01±2.23^{A}	3.71±0.61^{AB}	3.66±0.11^{AB}	4.07±0.41^{AB}	3.65±0.18^{AB}	3.47±0.70^{A}	4.25±0.62^{AB}
Clerosterol	20.34±2.04^{A}	22.36±1.94^{A}	21.03±3.46^{A}	24.42±0.71^{A}	20.44±0.92^{A}	20.89±3.95^{A}	20.98±5.50^{A}
β-sitosterol	3538.03±290.18^{A}	3522.73±95.20^{A}	3395.31±320.98^{A}	3235.90±98.36^{A}	3625.62±301.81^{A}	3300.68±264.15^{A}	3250.98±153.31^{A}
Sitostanol	7.87±2.21^{A}	10.58±4.11^{A}	8.66±0.72^{A}	15.09±9.66^{A}	11.49±4.18^{A}	11.19±2.08^{A}	12.48±3.15^{A}
Δ-5-avenasterol	1041.41±52.49^{A}	1053.03±18.89^{A}	1007.04±86.23^{A}	969.62±39.14^{A}	1078.90±87.42^{A}	981.80±68.97^{A}	999.73±55.94^{A}
Δ-5,24 stigmastadienol	87.44±8.78^{A}	85.31±1.06^{A}	84.91±4.98^{A}	80.44±2.40^{A}	90.69±11.76^{A}	85.34±7.70^{A}	83.52±4.76^{A}
Δ-7-stigmastenol	68.46±27.44^{A}	52.81±1.96^{B}	48.48±3.18^{B}	47.34±1.64^{B}	58.19±6.67^{AB}	47.97±2.42^{B}	54.04±3.20^{B}
Δ-7-avenasterol	219.06±2.88^{A}	236.18±4.74^{AB}	223.64±19.59^{AB}	225.24±16.40^{AB}	249.14±20.81^{B}	218.91±9.07^{A}	237.03±15.56^{AB}
Total sterols	5281.90±427.17^{A}	5267.78±117.03^{A}	5064.94±454.57^{A}	4859.96±172.21^{A}	5425.04±451.54^{A}	4935.18±368.04^{A}	4920.47±245.30^{A}

The results are presented as mean±standard deviation (n=4). Means in the same line with different letters are significantly different according to Duncan's multiple range test ($p < 0.05$).

sterol amount (Zhou *et al*., 2016) for various types of oils. β-sitosterol, which accounted for 66.08-67.02% of all sterols, showed a similar trend to total sterols and was not affected by microwave radiation. Δ5-avenasterol was the second most abundant sterol and accounted for 19.74-20.31%. Different microwave power settings and roasting periods were found to be ineffective on Δ5-avenasterol content. The third major sterol was Δ7-avenasterol, ranging from 4.16-4.81% of the sterol fraction and the highest value was obtained at 460 W power and 10 min duration. Campesterol and stigmasterol were the other two sterols and varied from 2.94-3.07% and 2.21-2.40%, respectively. Campesterol was not affected by microwave roasting as in 24-methylene cholesterol, sitostanol, campestanol, clerosterol and Δ5,24 stigmastadienol. Stigmasterol and the remaining individual sterols were found to be only slightly affected by radiation.

In the current work, the principal component analysis (PCA) was performed to provide an overview of the microwave pre-treatment, oil quality and chemical parameters. To perform PCA, the data were displayed in a matrix. The variables which had Kaiser–Meyer–Olkin measurement of sampling adequacy indexes lower than 0.5 were eliminated and the remaining variables were PV, K_{232}, K_{270}, α-tocopherol, γ- tocopherol, C20:0, C18:2, C18:1, C18:3, C18:0, LnLnLn, LnLL, LnLnO, LLL, LnLO, LnLP, LnLnS, LnOO, SLLn, OOO, SLO, campesterol, stigmasterol, campestanol, β-sitosterol, Δ-7-avenasterol, Δ-5,24-stigmastadienol, Δ-7 campesterol and total sterols. The factor score plot is shown in Figure 1. The first two principal components explained 67.59% of the variance (Factor 1: 38.60%, Factor 2: 28.99%). F1 showed high and positive correlations with PV, LnLnS, campestanol and negative correlations with α-tocopherol, LnLP and SLLn. F2 is positively correlated with LnLnLn, β-sitosterol, Δ-5,24-stigmastadienol, total sterols and negatively correlated with LnLO. The factor score plot showed that oils obtained by microwave radiation at 460 and 600 W for 5 and 10 minutes showed a positive correlation; whereas the oils obtained from treatment at 350 W (5 and 10 min) and unroasted seeds had negative correlations with F1. Additionally, fig seeds that were roasted for 10 min at 460 W power setting had a positive correlation and seeds that were processed for 5 min at 460 and 600 W power had negative correlations with F2.

Agglomerative hierarchial clustering (AHC) is an unsupervised method that is used to acquire clusters in terms of their closeness. In the current work, AHC was carried out to reveal the discrimination of the fig seed oils according to microwave treatment. A dendrogram derived from AHC, where 2 main clusters can be observed, is given in Figure 2. In the first cluster, 350 W- 5 min and 350 W- 10 min formed a couple and were sur-

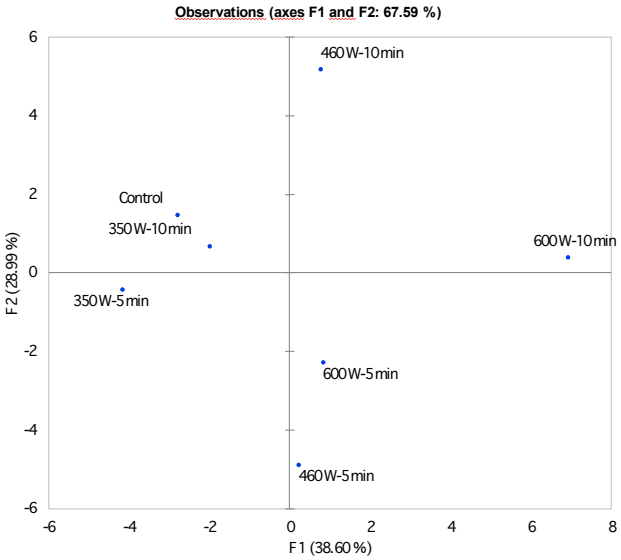

FIGURE 1. PCA score plot presenting the relations between unroasted and MW roasted (350, 460 and 600 W for 5 and 10 minutes) fig seed oils

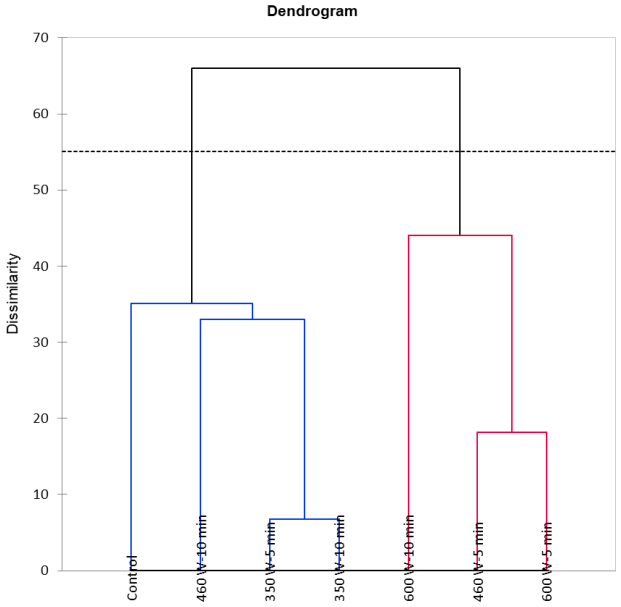

FIGURE 2. AHC dendrogram presenting the closeness of unroasted and MW roasted (350, 460 and 600 W for 5 and 10 minutes) fig seed oils

rounded by 460 W-10 min. This group was conjoined with the unroasted sample, verifying their position on the PCA score plot. In the second cluster 460 W- 5min and 600 W- 5 min were determined to be closely related and surrounded by 600 W- 10 min.

4. CONCLUSIONS

This work reports the changes in chemical composition and bioactive contents in fig seed oil due to microwave pre-treatment. Fatty acid and triacylglcerol profiles were significantly affected by microwave application, however, a certain pattern was not observed due to increasing power and radiation times. Phytosterols were determined to be protected in the oil rather than a possible degradation; whereas significant losses were detected for both α- and γ- tocopherols. PCA and AHC analyses served to differentiate intense and mild microwave-treated oils as well as the unroasted samples. Although a considerable number of works have been published about the effect of microwave pre-treatment on the composition of different vegetable oils, this is the first report evaluating the influence of roasting on cold-pressed fig seed oil. Fig seed oil was determined to be an excellent source tocopherol and the proper selection of processing method can significantly improve the nutritive value of the resultant oil. The findings of the work can contribute to further projects on fig seeds and oils for better evaluation of these nutrient-rich underused seeds. Also, more work should be performed to clarify the other compositional parameters of fig seed oil.

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Optimization of low thermal treatments to increase hydrophilic phenols in the Alperujo liquid fraction

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SUMMARY: Hydrophilic phenols are the main bioactive compounds in alperujo. Among them, 3,4-Dihydroxyphenylglycol (DHPG), Hydroxytyrosol (HT) and Tyrosol (Ty), are the most relevant and deeply studied. These compounds exhibit high antioxidant capacity and a wide range of health benefits as well as technologically promising properties. Given that, their recovery represents an attractive opportunity to valorize this by-product. In this work low thermal treatments were applied to alperujo in order to obtain phenol-enriched liquid fractions. Optimization assays combining different levels of temperature (30 to 90 ºC), time (60 to 180 min) and water content (70 to 90%), followed by response surface methodologies were performed. The results indicated that by applying optimal conditions, is possible to obtain theoretical yields of Total phenols, DHPG, HT and Ty of 2.4, 957.8, 3.4 and 6.4 times greater, respectively, than raw dry alperujo. Interestingly, all the evaluated conditions can be reproduced with low investment in a standard olive oil industry.

KEYWORDS: *Alperujo; Hydroxytyrosol; Olive mill by products; Phenolic compounds; Thermal treatments; TPOMW.*

RESUMEN: ***Optimización de tratamientos a baja temperatura para incrementar fenoles hidrofílicos en la fracción líquida de Alperujo.*** Los fenoles hidrofílicos representan los principales compuestos bioactivos del alperujo. Los más relevantes son 3,4-Dihidroxifenilglicol (DHFG), Hidroxitirosol (HT) y Tirosol (Ti). Estos compuestos presentan alta capacidad antioxidante, beneficios para la salud e importantes propiedades tecnológicas, por ello su recuperación representa una alternativa para la valorización de este subproducto. En este trabajo, se aplicaron al alperujo tratamientos térmicos para obtener fracciones líquidas enriquecidas con compuestos fenólicos. Se realizaron ensayos combinando niveles de temperatura (30 ºC a 90 ºC), tiempo (60 min a 180 min) y humedad del alperujo (70 % a 90 %), seguidos de metodologías de superficie de respuesta. Los resultados indicaron que, mediante la aplicación de las condiciones óptimas, es posible obtener rendimientos teóricos de fenoles totales, DHFG, HT y Ti, 2.4, 957.8, 3.4 y 6.4 veces superiores a los obtenidos a partir del alperujo inicial. Es destacable que las condiciones establecidas, se pueden reproducir con bajo costo en una industria olivícola estándar.

PALABRAS CLAVE: *Alperujo; Compuestos fenólicos; Hidroxitirosol; Subproductos olivícolas; Tratamientos térmicos.*

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1. INTRODUCTION

Argentina is the main olive oil producer among South American countries. Olive production in Argentina is mainly located in the central western region in the provinces of San Juan, Catamarca, La Rioja, Mendoza and Córdoba (Gómez del Campo *et al.*, 2010). Olive oil extraction in Argentina is generally carried out with continuous two-phase centrifugation systems. These systems generate a semisolid waste made of olive pulp, stone and vegetation water commonly called "alperujo" or two-phase olive-mill waste (TPOMW) (Alburquerque *et al.*, 2004).

Different technologies have been developed in Mediterranean countries to dispose or reuse alperujo. Secondary oil extraction followed by electrical energy cogeneration represents the main reuse alternatives in leading olive oil producing countries. Nevertheless, other technologies such as composting, gasification, anaerobic digestion, extraction of added-value products, animal feed and soil amendment also present a promising horizon (Morillo *et al.*, 2009; Roig *et al.*, 2006).

The total amount of alperujo generated in the San Juan province amounts to 60.000 to 80.000 t per year (Monetta *et al.*, 2019), however, it is still considered as a residue. Different reports have evaluated the effect of alperujo as olive soil amendment under local conditions (Monetta *et al.*, 2012) which gave rise to recommendations under conservationist management. This practice represents a simple alternative to disposing olive mill by-products, but still presents several limitations and it is not a way to take advantage of all alperujo constituents.

Virgin olive oil is well known for its nutraceutical qualities, most of them provided by the presence of phenolic bioactive compounds. Hydrophilic phenols are the most abundant bioactive compounds of olives and virgin olive oil, although tocopherols and carotenes are also present. The prevalent classes of hydrophilic phenols found in virgin olive oil are phenolic alcohols and acids, flavonoids, lignans and secoiridoids (Servili *et al.*, 2009).

Interestingly, due to their hydrophilic properties, during the malaxation of milled olives, only 2% of phenolic compounds pass through the oil, with the remaining 98% being retained in the alperujo and usually discarded with it (Owen *et al.*, 2003). Among the phenols found in alperujo, 3,4-Dihydroxyphenylglycol (DHPG), Hydroxytyrosol (HT) and Tyrosol (Ty), are the most relevant and deeply studied. These compounds exhibit high antioxidant capacity (Rubio-Senent *et al.*, 2013) and a wide range of health benefits like anti-inflammatory, anti-modulatory, anti-platelet, anti-cancer, anti-viral, anti-microbial or phyto-regulatory properties, among others (Fernández-Prior *et al.*, 2021; Lama-Muñoz *et al.*, 2021) as well as technologically promising properties which aid in the formulation of safer and healthier foods (Balzan *et al.*, 2021; Bartella *et al.*, 2021; Bermúdez-Oria *et al.*, 2019; Munekata *et al.*, 2020). Therefore , they are generally required as additives and inputs in cosmetic, pharmaceutical, agricultural, nutraceutical, and animal feed and food industries.

Different reports describe the extraction of hydrophilic phenolic compounds from olive by-products by employing multistage technologies. The methods employed usually consist of initial pretreatments to enhance the solubilization of water-soluble phenols (Lama-Muñoz *et al.*, 2011; Niknam *et al.*, 2021), followed by a second stage focused on the recovery of the phenolic fraction (Fernández-Prior *et al.*, 2020; Gil and Tuberoso, 2021; Rubio-Senent *et al.*, 2017). Among different approaches, high-pressure thermal treatments have been implemented at industrial scale (Lama-Muñoz *et al.*, 2019). By this technology, alperujo is exposed to high-pressure steam (150 - 170 ºC, 1.2 MPa) during short time periods (15 to 90 minutes). This procedure serves to relax the structure of organic matter, induce the auto-hydrolysis of complex molecules and favor the solubilization of simple phenolic compounds. After thermal treatment, a solid-liquid separation is performed by three-phase centrifugation systems, and a phenol-enriched liquid fraction is obtained. This procedure has been reported as highly efficient to obtain phenol-enriched liquid fractions, although the investment required for applying high temperatures and pressure levels represents an obstacle for scaling up the process at industrial scale in Argentina, as well as in other regions where the alperujo-associated industry is not as developed as in main olive oil producing countries.

Based on the same principles of hydrothermal treatments described, but with the aim of finding a solution to the high investment required, the aim of the present work was to determine the optimal conditions required to obtain high quality phenol-enriched liquid fractions applying low thermal treatments. Treatments were performed combining temperature (30 to 90 ºC), time (60-180 min) and alperujo water content (70 to 90 %). Liq-

uid fractions were obtained by centrifugation, and then total phenolic compounds, DHPG, Ty and HT levels were determined. A two-stage experimental program was developed. A factorial assay towards the selection of relevant variables was carried out, followed by response surface methodologies to determine the optimum operating conditions required for the recovery of total as well as individual phenols of interest.

2. MATERIALS AND METHODS

2.1. Origin and characterization of raw material

For experimental assays, 20 kg of fresh alperujo were obtained from a continuous two-phase centrifugation system (Oliomio 200 Eco, Toscana Enologica Mori, Italy) placed in the olive oil extraction plant of INTA EEA San Juan, Argentina. Milled olive fruits were from the Arbequina cultivar at a maturity index of 4. The raw material was homogenized and stored in individual pots of 1 kg at -20 °C. Before starting the assays, 3 individual samples of alperujo were subjected to the following chemical determinations: pH, water content, organic matter content, total soluble phenols, DHPG, HT and Ty.

2.2. Analytical determinations

Raw alperujo was characterized according to the following determinations: Water content was measured gravimetrically at 70 ºC (Martinez *et al*., 2021); pH was determined by potentiometric determination in 1:5 (w/v) water extract (Martinez *et al*., 2021); total organic matter and ashes were determined by loss on ignition at 550 ºC for 24 h (Martinez *et al*., 2021). Total organic carbon was calculated from organic matter according to (Navarro *et al*., 1993). The phenolic content in alperujo was determined in methanolic extract (methanol:water 80:20 (v/v) using 2 mL per gram of fresh alperujo) by a modified version of the method described by (Singleton and Rossi, 1965) with Folin-Ciocalteu reagent and measured in a Shimadzu 1240 UV–Visible spectrophotometer at 725 nm. The results were expressed as mg of caffeic acid per kg of initial sample. The individual phenols of interest (DHPG, HT and Ty) were measured in methanolic extract (explained above) by high-performance liquid chromatography (HPLC Hewlett-Packard series 1100), using a Kinetex® EVO 5 µm C18 (250 × 4.6 mm) Phenomenex® column. The analysis was performed at room temperature, the elution was at a flow rate of 1.0 mL/min, with a mobile phase A of acetonitrile and B ultrapure water, using the following gradient over a total run time of 55 min: 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min, 95% A at 52 min until the run was completed. The chromatograms were analyzed at 280 nm by integration peaks at different wavelengths, according to calibrations performed with external standards. The results were expressed in mg/Kg or mg/L.

For determinations in liquid fractions, total soluble solids were determined by a portable refractometer (Atago, JPN), and expressed as Brix degrees. Total soluble phenols were determined in 1/10 water dilutions by the colorimetric method described above. The results were expressed as mg of caffeic acid/L of liquid fraction. The individual phenols of interest (DHPG, HT and Ty) were measured following the protocol described above.

2.3. Selection of relevant variables

In order to evaluate the relevant conditions required to increase the total phenolic concentration in the alperujo liquid fraction a factorial assay was performed. Closed plastic tubes containing 40 g of alperujo were positioned in thermostatic baths at two temperature levels (30 and 70 ºC) and two exposure times (60 and 180 min). In addition, a control treatment of untreated alperujo was included. Three replicates of each treatment were performed in parallel. Once treatments were applied, samples were centrifuged at 3500 g for 20 min to separate the solid and liquid phases. After centrifugation, weight, total soluble solids and total soluble phenols were determined in liquid phases. The percent of phenols recovered was calculated by the following equation:

$$\%\ Phenols\ recovered = \frac{T\ liquid\ phase\ PC\ \left(\frac{mg}{Kg}\right) \cdot\ T\ liquid\ phase\ weight\ (g).100\ \%}{Control\ liquid\ phase\ PC\left(\frac{mg}{Kg}\right) \cdot Control\ liquid\ phase\ weight\ (g)}$$

Where: "T liquid phase PC" represents the phenolic content in the liquid phases obtained after treatment application. "T liquid phase weight" represents the weight of the liquid phase obtained after treatment application. "Control liquid phase PC" represents the phenolic content in liquid phases obtained without treatment application. "Control liquid phase weight" represents the weight of the liquid phase obtained without treatment application.

2.4. Optimization of variables

For the development of optimization studies, Box-Behnken experimental design followed by response surface methodology were applied. Box-Behnken is a second-order design based on three-level incomplete factorial designs. It is used to measure the combined effect of the factors under study, reducing the number of experiments, improving the performance of time and resources (Box and Behnken, 1960). The response surface methodology (RSM) is a technique to model and analyze statistical problems. The main objective is to optimize the response surface which is influenced by various parameters, quantifying the relationship among them and plotting the response (Montgomery and Wiley, 1984). A total of fifteen experimental trials combining three factors at three levels were analyzed: temperature (50 , 70 and 90 ºC), exposure time (30 , 75 and 120 min) and alperujo water content (70 , 80 and 90%). Trials were performed in closed plastic tubes containing 100 g of alperujo in thermostatic baths. As performed in a previous stage, once trials were conducted, phase separation was accomplished by centrifugation at 3500 g for 20 min. Liquid phases were weighed, and total phenols, DHPG, HT and Ty concentrations were determined. Then the content of phenols recovered was calculated considering both concentration in liquid phase and amount of liquid phase recovered by the following equation:

$$Phenols\ recovered\ (mg) = liquid\ phase\ phenolic\ content\ \left(\frac{mg}{Kg}\right) \cdot\ liquid\ phase\ weight\ (Kg)$$

2.5. Statistical analysis and experimental design

Relevant variable selection was performed under factorial design. Analysis of variance (ANOVA) was achieved using Infostat Professional Software 2.0 version (UNC). For comparison of means LSD Fisher ($p \leq 0.05$) was employed.

Variable optimization was performed in the Box-Behnken design according to Response Surface methodology, using Design Expert software 7.0.0 (Stat-Ease Inc., Minneapolis, USA). Statistical differences were established at $p \leq 5\%$. Model significance, polynomic equation, data normality and surface response graphs were determined for each variable. Model efficiency was determined by R2 and adjusted R2. The response surface statistical model responded to the equation adjusted by three factors (temperature, water content and time) and four response variables (Total phenols, DHPG, HT and Ty). The optimal levels of each response variable were obtained and the polynomic equation was determined.

3. RESULTS AND DISCUSSION

3.1. Raw material characterization

As shown in Table 1, the chemical composition of the alperujo employed in the assays was similar to others employed in different reports (Alburquerque *et al.*, 2004; Morillo *et al.*, 2009). It was characterized by high water content and slightly acidic pH. Regardless of the water content, it was mainly composed of organic matter and a minor proportion of mineral ashes. Among organic constituents, the total phenolic content (7233.8 mg/Kg) was in the lower limit compared to other reports, presenting values ranging from 6000 to 26000 mg/Kg of raw dry alperujo (Alburquerque *et al.*, 2004; Rubio-Senent *et al.*, 2017). As stated, the amount and profile of phenolic compounds present in olives is highly variable and dependent mainly on cultivar and maturity stage as well as agronomic and climatic conditions (Obied *et al.*, 2008). In the present work, the alperujo employed belonged to the Arbequina cultivar, grown under intensive drip irrigated system, harvested at a maturity index of 4 and immediately milled. Arbequina represents one of the most abundant olive cultivars in Argentina (Gómez del Campo *et al.*, 2010) and generally the olive oils obtained with this cultivar are characterized by low phenolic content (Monasterio *et al.*, 2017). Regarding the individual phenols of inter-

Table 1. Main chemical parameters of raw alperujo

Parameter	Mean value *
pH	5.04 ± 0.05
Water content (%)	65.6 ± 0.4
Organic matter (%)	32.9 ± 2.0 (95.6)
Total Organic Carbon (%)	17.2 ± 1.1 (48.2)
Ash (%)	1.5 ± 0.5 (4.4)
Total phenols (mg/kg)	2485.0 ± 318.2 (7233.8)
DHPG (mg/kg)	0.3 ± 0.1 (0.9)
HT (mg/kg)	472.2 ±18.4 (1372.7)
Ty (mg/kg)	80.5 ± 8.5 (234.0)

* Mean value ± SD of three independent determinations. Numbers between brackets indicate values in raw dry alperujo.

est, Hydroxytyrosol was the main hydrophilic phenolic compound observed representing almost 20% of the total phenol content, while tyrosol and DHPG together did not reach 4%. These results agree with previous data (Rubio-Senent *et al.*, 2013) and support the significance of applying pre-treatments in order to induce phenolic compound hydrolysis and increase the total amount of hydrophilic forms.

3.2. Relevant variables selection

Table 2 shows the effect of thermal treatments performed on alperujo samples (40 g) on the weight of liquid fraction recovered as well as the total soluble solids and total phenols. As shown, the weight of the liquid fraction reached its highest value when thermal treatments were performed at 70 ºC, without presenting statistical differences between 60 and 180 minutes. On the other hand, the weight of the liquid fraction obtained by treatments achieved at 30 ºC did not present differences compared to untreated control samples. In addition, the level of total soluble solids and total phenols presented a similar pattern, showing an increase when thermal treatments were performed at 70 ºC and remaining similar to untreated alperujo in treatments carried out at 30 ºC (Table 2). These results agree with previous data which suggest that thermal treatments provoke the relaxation of organic matter constituents allowing the exit of intracellular water and favoring the hydrolysis of complex molecules and the solubilization of lower compounds such as phenols, sugars, organic acids and proteins, among others (Rodríguez *et al.*, 2007).

Taken together, both the increase in liquid fraction weight and the total phenol content in these fractions, the amount of recoverable phenols increased to 136-138% with respect to the control when thermal treatments at 70 ºC where applied (Figure 1). The observed increase in the total amount of recoverable phenols is lower than that observed in other reports where thermal treatments were performed at higher temperatures (Lama-Muñoz *et al.*, 2011; Rubio-Senent *et al.*, 2013). As these authors explain, the importance of the application of a heat treatment is to achieve a solubilization of high value-added components, such as phenols, and to enable phase separation. Obviously, the higher the treatment temperature, the higher

Table 2. Thermal treatments applied and main parameters of liquid fractions obtained

Sample	Temperature (ºC)	Exposure Time (min)	Weight of liquid fraction (g)*	Total soluble solids (ºbrix)*	Total phenols (mg/kg)*
Alperujo	RT	untreated	14.5 ± 2.3 a	7.0 ± 0.1 a	4046 ± 261 a
Alperujo	30	60	14.6 ±1.6 a	7.2 ± 0.2 b	4522 ± 271 b
Alperujo	30	180	14.4 ±1.2 a	7.2 ± 0.1 b	4215 ± 337 ab
Alperujo	70	60	16.7 ±0.5 b	7.4 ± 0.2 c	4867 ± 299 c
Alperujo	70	180	16.4 ±0.8 b	7.5 ± 0.2 c	4898 ± 219 c

* Mean values ± SD of three independent determinations. ANOVA analysis was performed. Significant differences in the same column are indicated by different letters. LSD Fisher ($p \leq 0.05$) was used for comparison of means.

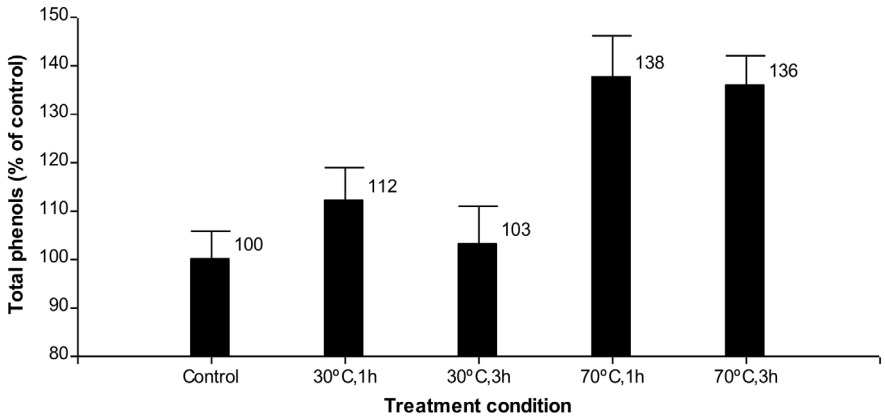

Figure 1. Total phenol content in liquid fractions of alperujo obtained by each treatment. Results are expressed as % of control treatment. Numbers in each column indicate average value of three independent assays. Error bars indicate standard deviation.

the solubilization of phenols and the better the phase separation, but also the higher the formation of degradation metabolites, such as furfurans from sugars, and the higher the investment and operating costs. For this reason, the application of thermo-malaxation is sought to improve the use of alperujo, a first step being the use of temperatures of up to 70 ºC which solubilize phenols and allow an adequate separation of phases, even if a quantity of solids in suspension is reached, which implies an intermediate sedimentation step prior to the extraction of phenols from the liquid phase (Fernández-Prior *et al.*, 2020).

3.3. Optimization of variables

Table 3 summarizes the experimental design conditions applied as well as the values of response variables (Total phenols, DHPG, HT and Ty) expressed as mg in liquid fractions obtained from 100 g of initial sample. Response surface methodology was used to optimize each response variable considering the linear, quadratic, and cross-product interactions of the independent variables at the 95% confidence level. The analysis of the variance (ANOVA) for each response variable is shown in Table 4. As shown, Total phenols, DHPG and Hydroxytyrosol described quadratic response surface models with high R2 and R2 adjusted values. Temperature, water content and the quadratic effect of water content were highly desirable (p-value ≤ 0.05) for Total phenols and HT, while Temperature and its quadratic effect were significant for DHPG. On the other hand, the interaction between independent variables and Tyrosol was explained by a linear model, with temperature and water content as the significant variables.

The polynomial that describes the relationships between the process variables and each response variable are represented by the following equations:

$$\text{Total phenols (mg)} = -6077.87870 - 1.77437*T + 1.87793*t + 163.71542*WC + 0.018556*T*t + 0.027625*T*WC - 0.010944*t*WC - 0.00414583*T^2 - 0.015436*t^2 - 1.05683*WC^2$$

$$\text{DHFG (mg)} = 52.40351347 - 2.171123359*T - 0.08614005*t + 0.479013698*WC + 0.000984705*T*t + 0.011767141*T*WC - 0.000356229*t*WC + 0.010037259*T^2 + 0.000388239*t^2 - 0.008143276*WC^2$$

$$\text{HT (mg)} = -1626.094951 - 3.87111763*T - 0.870032674*t + 47.36861411*WC + 0.007666309*T*t + 0.013617516*T*WC + 0.013152993*t*WC + 0.021685848*T^2 - 0.005201345*t^2 - 0.316310484*WC^2$$

$$\text{Ty (mg)} = -41.12670915 + 0.220703203*T + 0.010423401*t + 0.432320791*WC$$

Where: "T" represents temperature; "t" represents time; "WC" represents water content.

Table 3. Experimental assay and response variables of Box Behnken design. Response variables indicate the contents in total phenols, DHPG, HT and Ty, in liquid fractions obtained after each run

Experimental design				Response variables				
Run	Sample weight (g)	Temperature (ºC)	Time (min)	Water content (%)	Total phenols (mg)	DHPG (mg)*	HT (mg)*	Ty (mg)*
1	100	70	75	80	349.0	0.7	62.6	14.1
2	100	70	75	80	348.5	0.5	87.0	11.4
3	100	90	75	70	286.9	6.6	72.9	7.6
4	100	90	120	80	384.4	12.1	101.6	16.9
5	100	50	75	70	269.2	1.3	55.0	3.8
6	100	50	75	90	190.5	ND	26.2	11.3
7	100	70	120	70	256.2	1.0	36.0	6.2
8	100	90	30	80	323.9	8.4	88.0	15.1
9	100	70	75	80	357.2	0.2	76.1	7.3
10	100	70	120	90	143.4	0.2	19.2	13.8
11	100	70	30	90	182.9	0.2	18.2	ND
12	100	90	75	90	230.3	ND	55.0	16.3
13	100	50	30	80	286.3	0.2	58.9	3.6
14	100	70	30	70	276.0	0.4	58.7	2.7
15	100	50	120	80	280.0	0.4	44.8	1.9

* DHPG, HT and Ty indicate Dihydroxyphenylglycol, Hydroxytyrosol and Tyrosol, respectively.

Table 4. Analysis of variance (ANOVA) for phenolic compounds in the liquid fractions of alperujo from optimization assays

Source	Sum of Squares	gl	Mean Square	F ratio	P value	Model	R^2	R^2 adj.
Total phenols								
A-Temperature	4975.0	1.0	4975.0	7.8	0.0380*	Quadratic	0.95	0.87
B-Time	3.3	1.0	3.3	0.0	0.9457			
C-Water content	14552.2	1.0	14552.2	22.9	0.0049*			
AB	1115.6	1.0	1115.6	1.8	0.2423			
AC	122.1	1.0	122.1	0.2	0.6793			
BC	97.0	1.0	97.0	0.2	0.7119			
A^2	10.2	1.0	10.2	0.0	0.9043			
B^2	3607.7	1.0	3607.7	5.7	0.0628			
C^2	41239.3	1.0	41239.3	65.0	0.0005*			
Total error	47.7	2.0	23.9					
Dihydroxyphenylglycol								
A-Temperature	99.5	1.0	99.5	210.6	0.0007*	Quadratic	0.99	0.97
B-Time	2.5	1.0	2.5	5.4	0.1031			
C-Water content	0.3	1.0	0.3	0.6	0.4904			
AB	3.1	1.0	3.1	6.7	0.0818			
AC	7.4	1.0	7.4	15.6	0.0289			
BC	0.1	1.0	0.1	0.2	0.6726			
A^2	40.7	1.0	40.7	86.2	0.0026*			
B^2	1.6	1.0	1.6	3.3	0.1666			
C^2	1.7	1.0	1.7	3.5	0.1562			
Total error	0.1	2.0	0.1					
Hydroxytyrosol								
A-Temperature	2200.6	1.0	2200.6	19.6	0.0069*	Quadratic	0.94	0.82
B-Time	61.0	1.0	61.0	0.5	0.4944			
C-Water content	1354.8	1.0	1354.8	12.1	0.0178*			
AB	190.4	1.0	190.4	1.7	0.2497			
AC	29.7	1.0	29.7	0.3	0.6292			
BC	140.1	1.0	140.1	1.2	0.3149			
A^2	277.8	1.0	277.8	2.5	0.1766			
B^2	409.6	1.0	409.6	3.6	0.1145			
C^2	3694.2	1.0	3694.2	32.9	0.0023*			
Total error	298.8	2.0	149.4					
Tyrosol								
A-Temperature	155.9	1.0	155.9	20.1	0.0012*	Linear	0.79	0.73
B-Time	1.5	1.0	1.5	0.2	0.6704			
C-Water content	126.4	1.0	126.4	16.3	0.0024*			
Total error	23.3	2.0	11.6					

* Statistically significant (p-value ≤ 0.05)

As can be seen in Figure 2, all the response variables increased with temperature. Regarding time and water content, these factors presented particular patterns for each response variable. Total phenols and HT showed maximum levels when time and water content were set at central points (Figure 2 A, B, E, F). Tyrosol was greater when the water content was set at the lowest level without being highly affected by time (Figure 2 G, H), while DHPG was not highly affected by time or water content (Figure 2 C, D). Based on the different models and equations, the optimal conditions and the higher values for the different response variables are summarized in Table 5.

Table 6 shows the theoretical levels of the different compounds evaluated (total phenols, DHPG, HT and Ty) expressed as concentrations in liquid fractions obtained and in raw dry alperujo. As can be observed, the concentration of total phenols solubilized reached 3804 mg/kg liquid fraction, and 17434 mg/kg raw dry alperujo. The values obtained are interesting and similar to those obtained experimentally by other authors (Lama-Muñoz *et al.*, 2011). The comparison of theoretical values obtained for each response variable (Table 6) with the initial alperujo content (Table 1) indicates increases of 2.4, 957.8, 3.4 and 6.4 folds in initial total phenols, DHPG, HT and Ty, respectively, from raw dry alperujo. These results confirm that applying low thermal treatments to alperujo is possible to obtain phenol-enriched fractions. The observed increase in the content of hydrosoluble phenols supports the hypothesis that thermal treatments provoke the hydrolysis of complex hydrophobic phenolic compounds and favor the solubilization of hydrophilic molecules. The results show that the extraction of phenols is temperature and time dependent, and that for the tested conditions the solubilized amount of the main phenols in free form can justify their extraction as mentioned by other authors (Fernández-Prior *et al.*, 2020). Likewise, at these temperatures it is necessary to continue studying the solubilization of polymerized or more complex phenols that could increase with subsequent chemical or enzymatic hydrolysis processes by the addition of acids or enzymes or by the action of those already present during a certain storage time, which will also help to separate suspended solids that hinder the application of chromatographic processes to obtain final phenolic extracts.

4. CONCLUSIONS

In this work low thermal treatments were applied to alperujo in order to obtain phenol-enriched liquid fractions. All temperatures employed were under 90 ºC with the aim to evaluate conditions that could potentially be reproduced with low investment in a standard olive oil industry. The relevant variable selection stage indicated that the treatment of alperujo at 70 ºC during 1 to 3 h was effective to obtain liquid fractions with higher total soluble solids and phenolic contents than raw alperujo. Furthermore, the optimization tri-

Table 5. Theoretical optimal conditions for each response variable

Response variable	Temperature (ºC)	Time (min)	Water content (%)	Value* (mg)	D - Value
Total phenols	90.0	87.2	78.18	380.4	0.98
Dihydroxyphenylglycol	89.9	117.7	85.74	12.3	1.00
Hydroxytyrosol	90.0	82.0	78.52	101.5	1.00
Tyrosol	88.2	81.1	88.37	17.4	1.00

*Values obtained from 100 g of initial sample.

Table 6. Theoretical content of total phenols, Dihydroxyphenylglycol, Hydroxytyrosol and Tyrosol for optimal conditions obtained by response surface methodology

Compound	Liquid fraction (mg/Kg)	Raw dry alperujo (mg/Kg)
Total phenols	3804	17434
Dihydroxyphenylglycol	123	862
Hydroxytyrosol	1015	4727
Tyrosol	174	1495

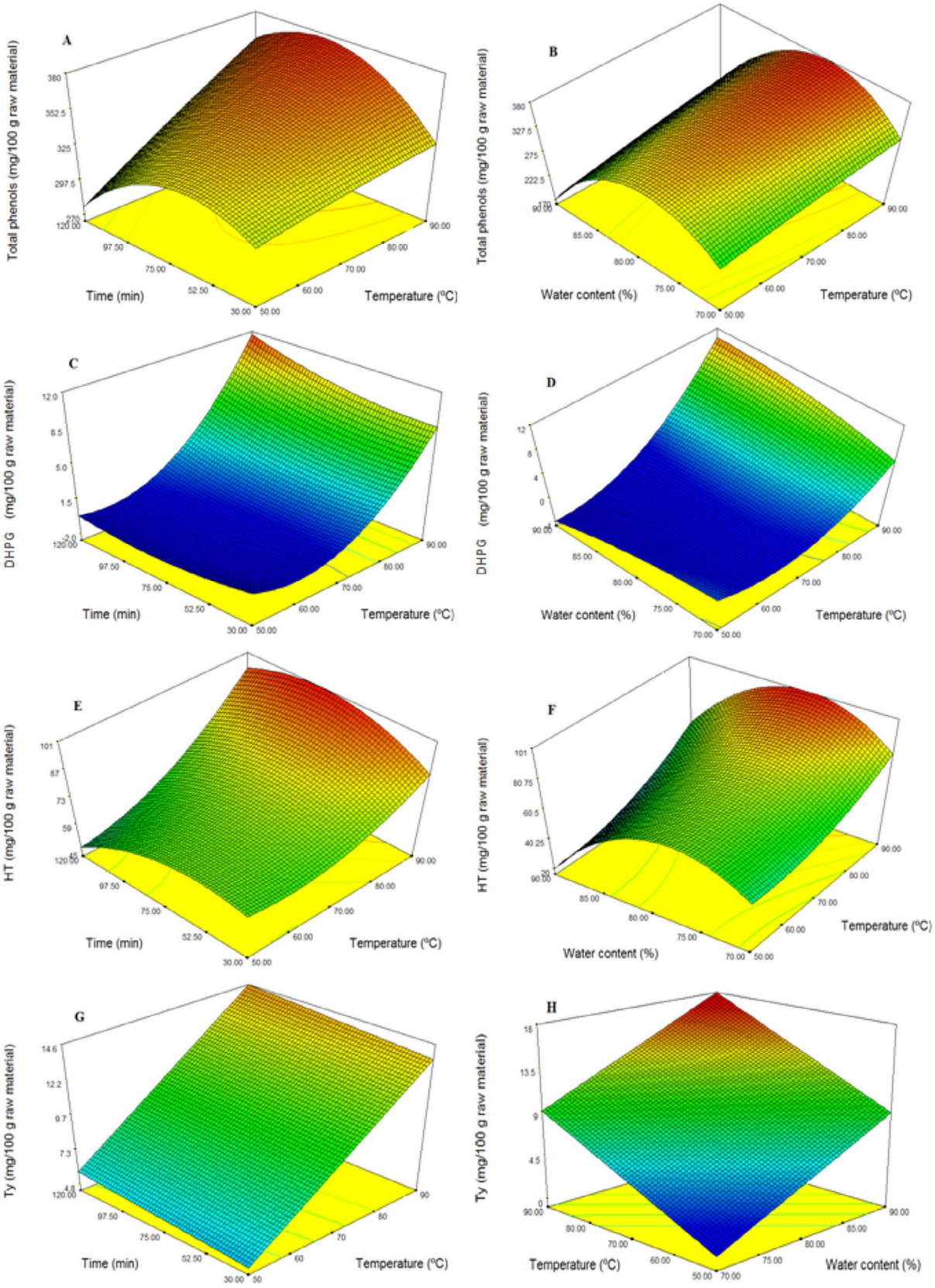

FIGURE 2. Response surface methodology graphs (temperature/time and temperature/water content) for each response variable. (A-B) Total phenols, (C-D) Dihydroxyphenylglycol, (E-F) Hydroxytyrosol, (G-H) Tyrosol.

al indicated that regarding the phenols of interest, not only temperature, but also alperujo water content and time were significant to obtain higher yields of each particular compound. Theoretical equations showed that by applying optimal conditions, it is possible to obtain yields of total phenols, DHPG, HT and Ty of 2.4, 957.8, 3.4 and 6.4 times greater with respect to raw dry alperujo. Therefore, the application of a thermal treatment adaptable to the olive oil industry promotes the obtaining of a liquid fraction from which it is possible to extract high added-value components, such as phenols, and thus improve the use of the main by-product from this industry.

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Extraction of bioactive lipids from *Pleuroncodes monodon* using organic solvents and supercritical CO_2

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SUMMARY: A huge volume of *Engraulis ringens* (Peruvian anchoveta) is caught together with the species *Pleuroncodes monodon* (munida), whose potential bioactive lipids are not commercially exploited. In the present study, lipid with carotenoid pigment (astaxanthin) and essential fatty acids (EPA+DHA) were obtained from munida lipids extracted with hexane:isopropyl alcohol (He-I), acetone (Ac), ethanol (Et) and supercritical CO_2 + ethanol (SC-CO_2-Et). The functional quality of the fatty acids was determined by atherogenicity index (AI), thrombogenicity index (TI) and the hypocholesterolemia:hypercholesterolemia (H:H) ratio. The highest astaxanthin (ASTX) contents (4238.65 and 4086.71 µg/g lipid) corresponded to extractions using Ac and SC-CO_2-Et. EPA+DHA ranged from 31.15 to 31.85% and the functional quality ranges were between 0.56-0.61 (AI), 0.19-0.21 (TI) and 1.73-1.81 (H:H). Consequently, SC-CO_2-Et extraction would be advisable because of its low environmental impact. The IA and IT quality indexes suggest that the consumption of munida lipids would be healthy, although the H:H ratio shows the opposite.

KEYWORDS: *Astaxanthin; EPA+DHA; Fatty acid profile; Functional quality index; Munida; Red squat lobster.*

RESUMEN: ***Obtención de lípidos bioactivos de* Pleuroncodes monodon *utilizando solventes orgánicos y CO_2 supercrítico.*** Las enormes capturas de *Engraulis ringens* (anchoveta Peruana) son acompañadas por la especie *Pleuroncodes monodon* (munida) cuyo potencial en lípidos bioactivos no es aprovechado comercialmente. En el presente estudio se obtuvo lípidos con pigmentos carotenoides (astaxantina) y ácidos grasos esenciales (EPA+DHA) a partir de lípidos de munida extraídos con hexano:alcohol isopropílico (He-I), acetona (Ac), etanol (Et) y CO_2 supercrítico + etanol (SC-CO_2-Et). La calidad funcional de los ácidos grasos fue evaluada mediante índices de aterogenicidad (AI), trombogenicidad (TI) y la relación hipocolesterolemia:hipercolesterolémica (H:H). Los mayores contenidos de astaxantina (ASTX) (4238.65 y 4086.71 µg/g de lípido) fueron obtenidos utilizando Ac y SC-CO_2-Et. En todas las muestras EPA+DHA osciló entre 31.15 y 31.85% y los rangos de índices de calidad funcional fueron: 0.56-0.61 (AI), 0.19-0.21 (TI) y 1.73-1.81 (H:H). Se concluye que la extracción SC-CO_2-Et sería recomendable por su bajo impacto al medio ambiente. Los índices de calidad AI y TI sugieren que el consumo de lípido de munida podría ser saludable, aunque la relación H:H muestra lo contrario.

PALABRAS CLAVE: *Astaxantina; Camaroncito rojo; EPA+DHA; Índice de calidad funcional; Munida; Perfil de ácidos grasos.*

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1. INTRODUCTION

The "munida" or red squat lobster (*Pleuroncodes monodon*), is a decapod marine crustacean with an elongated body, belonging to the family Munididae (Santamaría *et al*., 2018). In the Peruvian sea its large biomass accompanies the anchoveta (*Engraulis ringens*), Castillo *et al*. (2020) make estimates of 2,201,712 and 1,687,044 t in summer and spring, 2019, respectively. Despite the abundant biological information on munida, studies on the extraction and quantification of its bioactive components are required in terms of value generation and commercial use.

Marine lipids are known to be the main source of polyunsaturated fatty acids (PUFA), especially ω-3 fatty acids (eicosapentaenoic acid EPA; 20:5 ω-3 and docosahexaenoic acid DHA; 22:6 ω-3) which are considered essential because of their significant influence on biochemical and physiological processes involved in human health (Narayan *et al*., 2006).

The biological functionality of edible oils is assessed by indexes based on the fatty acid contents, on the AI pro-atherogenic and anti-atherogenic fatty acids ratio, on the TI or ratio of saturated (pro-thrombogenic) and unsaturated (anti-thrombogenic) fatty acids, and H:H index, correlating unsaturated and saturated fatty acids (Chen and Liu, 2020).

Besides of the referred lipids, marine crustaceans are a source of pigments such as astaxanthin (ASTX), a 40-carbon ketocarotenoid (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) belonging to the xanthophyll family (Núñez-Gastélum *et al*., 2016). Natural ASTX has been referred to as a supercarotenoid with high levels of health protection and anti-inflammatory effects among other benefits (Capelli, 2018). In addition, it has a high antioxidant capacity associated with reduced risk of oxidative stress-generated diseases, such as cardiovascular diseases (Régnier *et al*., 2015).

Regarding the extraction of ASTX by solvents, it is known that its high polarity favors the process. Routray *et al*. (2019) used different organic solvents of medium polarity and their mixtures and concluded that hexane was not a good option, although its combination with acetone improved extraction efficiency. An alternative method is the supercritical fluid extraction (SFE) which offers technological and ecological advantages as well as obtaining analytes without exposure to oxygen or thermal damage. Efficient extractions of phospholipids and glycolipids from *Farfantepenaeus paulensis* were conducted using supercritical CO_2 + 15% ethanol (Sánchez-Camargo *et al*., 2012).

The objective of this study is focused on the extraction of lipids from munida using solvents hexane + isopropyl alcohol, acetone, absolute ethanol and Supercritical CO_2 + ethanol and the evaluation of the quality of their bioactive lipid components (ASTX, EPA and DHA).

2. MATERIALS AND METHODS

2.1. Characteristics of the raw material

Munida specimens were frozen on board inmediately after caught by the scientific research vessel "Humboldt" belonging to Instituto de Mar del Perú (IMARPE) in June 2019 at the area 18°6' 20.401" S & 70°48' 14.4" W, in front of Caleta Vila Vila (Tacna), 3.5 nm off the southern coast of Peru. Samples were placed in thermal boxes to maintain cooling until arrival to the Bioactive Compounds Laboratory of Instituto Tecnológico de la Producción (ITP). The size distribution of the specimens ranged from 10 to 16 mm cephalothorax length, mean of 12.9 ± 1.2 mm, mode of 13 mm; female specimens represented 54.4% of total samples with a mode of 13 mm, while males registered 14 mm.

2.2. Sample preparation

50 kg of "munida" were placed in a cold air dryer (CV-20AN, ASAHI, Japan) at 21 °C for 27 h, then crushed in an analytical mill (A11 basic, IKA, USA) and sieved to obtain a homogeneous material between 0.50 and 0.85 mm particle size. The munida meal (MM) was packed in vacuum-sealed bags and kept at -18 °C until analysis.

2.3. Proximal chemical composition

Moisture, fat, ash and protein contents were determined by duplicate determinations of fresh munida and MM according to FAO (1986) methodologies.

2.4. MM lipid extraction methods

Four different lipid extraction procedures were performed using a mixture of hexane + isopropyl

alcohol 60:40 (v/v) (He-I), acetone (Ac), absolute ethanol (Et) and Supercritical CO_2 + ethanol as cosolvent (SC-CO_2-Et).

He-I: The technique described by Sachindra *et al*. (2006) was followed. A mixture 60:40 hexane ACS (Fermont, Mexico) with HPLC grade isopropyl alcohol (Fisher Scientific, Spain) was used to dissolve 8 g MM sample in 50 mL tubes with 40 mL, vortexed for 2 min, sonicated at 25 °C for 10 min and centrifuged (Centrifuge 5804 R, Eppendorf, Brazil) for 40 min at 3200 g at 4 °C after 5 min resting time. The extract was filtered through Whatman Nº 42 filter paper and the residue was subjected to further extraction following the same procedure.

Ac: 8 g MM were placed in 50 mL tubes with 40 mL of Ac (ACS, Merck, 99.5% purity), vortexed for 2 min, sonicated at 25 °C for 10 min and centrifuged for 40 min at 3200 g at 4 °C after 5 min resting time. The extract was filtered using Whatman Nº 42 filter paper and the residue was treated with two additional extractions.

Et: According to the Dalei and Sahoo (2015) methodology 10 g MM were thoroughly homogenized with 100 mL Et (ACS Sharlau, Spain) for 1 hour using a magnetic stirrer. The extract was filtered through Whatman Nº 42 filter paper. Solid recovery was performed on the residue by 4 extractions until the filtrate was colorless.

SC-CO_2-Et: A multi-solvent extractor Model 2802.000 (Top Industrie, France) equipped with a CO_2 pump (HPFlow Pump 50 - 100), co-solvent pump (90-2491 REV L, SSI), chiller (PCPR 13.02-NED, National Lab), reactor (ø 163 x 353 mm) and a stainless-steel separator (ø 78 x 278 mm) to receive the lipid were used (Barriga-Sánchez *et al.,* 2022). Pressure was manually controlled by a back pressure regulator.

The extraction of 35 g MM by SC-CO_2-Et was performed following the reference parameters reported by Sánchez-Camargo *et al*. (2012), 200 bar, temperature 50 °C and a solvent ratio 85/15 (CO_2/ethanol) for 2 h. Evaporation of the solvent was carried out using a rotary evaporator (Laborota 4003, Heidolph) at 40 °C and the residue was stored in Ultrapure nitrogen atmosphere (Linde Peru) at -19 °C, until further analysis.

Analyses were conducted in three replicates.

2.5. Lipid yield in MM

The MM lipid yield was obtained by calculations according to Equation 1.

$$Yield\ (\%) = \frac{MM\ lipid\ weight}{MM\ weight} x100 \quad (1)$$

2.6. Thin layer chromatography (TLC)

The methodology of Núñez-Gastélum *et al*. (2016) was applied in all samples. 1 g lipid was dissolved in 1 mL ACS petroleum ether (Tedia, USA) vortexing for 1 min. 5 µL of each sample were placed on a silica gel 60 F_{254} plate (Merck, Germany) pre-dried at 110 °C for 2 h. The plate was placed in a chamber saturated with 50 mL acetone: hexane (25:75, v/v) as the mobile phase.

Bands were visualized under a 254 nm UV TLC lamp (Merck) and identified by comparing the Retention Factor (Rf) value with the standard ASTX by applying Equation 2. Tests were conducted in triplicate.

$$Rf = \frac{\text{Solute migration distance}}{\text{mobile phase migration distance}} \quad (2)$$

2.7. Determination of total carotenoids expressed as ASTX

Total carotenoids expressed as ASTX were determined in all samples according to the methodology of Sánchez-Camargo *et al*. (2011). A standard solution of ASTX (98.6%, Dr Ehrenstorfer) was prepared by diluting 1 to 5 µg/mL of ASTX standard in hexane. 50 mg lipid sample were diluted to 10 mL in hexane. The absorbance value of each solution and the sample were measured at 472 nm (highest absorbance observed) using a UV-200 Spectrophotometer (Shimadzu, Japan) with hexane as the calibration blank**.** Carotenoids were expressed as µg ASTX/g lipid and µg ASTX/g MM.

2.8. Fatty acid chromatography

Fatty acids were determined as described by Prevot and Mordret (1976). A gas chromatograph with a FID detector (Autosystem XL, Perkin Elmer, USA) equipped with a Supelcowax 10 column (Merck, Germany) (30 m × 0.25 mm id; film thickness: 0.25

μm) was used. Peak areas were calculated using Total Chrom Navigator software (Version: 6.2.0.0.0:B27, 2001, USA), and each fatty acid percentage was calculated by comparing the individual peak area with the fatty acid total area. The fatty acid peaks were identified by comparison with the retention times of the standard F.A.M.E. Mix C4-C24 (Supelco, Sigma-Aldrich Inc, USA).

2.9. Functional quality of MM lipid

The fatty acid profile of the MM lipid was used to determine its functional quality by means of the AI and TI according to equations 3 and 4, respectively (Ulbricht and Southgate, 1991). The H:H was evaluated in accordance with equation 5 as defined by (Santos-Silva *et al*., 2002).

$$AI=\frac{(C12:0)+4(C14:0)+(C16:0)}{(\sum MUFA)+(\sum \omega\text{-}6)+(\sum \omega\text{-}3)} \quad (3)$$

$$TI=\frac{(C14:0)+(C16:0)+(C18:0)}{0.5(\sum MUFA)+0.5(\sum \omega\text{-}6)+3(\sum \omega\text{-}3)+(\frac{\sum \omega\text{-}3}{\sum \omega\text{-}6})} \quad (4)$$

$$H:H=\frac{(C18:1\omega\text{-}9)+(C18:2\omega\text{-}6)+(C20:4\omega\text{-}6)+(C18:3\omega\text{-}3)+(C20:5\omega\text{-}3)+(C22:5\omega\text{-}3)+(C22:6\omega\text{-}3)}{(C14:0)+(C16:0)} \quad (5)$$

Where: C12:0 (lauric acid); C14:0 (myristic acid); C16:0 (palmitic acid); C18:0 (stearic acid); C18:1 ω-9 (oleic acid); C18:2 ω-6 (linoleic acid); C18:3 ω-3 (linolenic acid); C20:4 ω-6 (arachidonic acid); C20:5 ω-3 (eicosapentaenoic acid); C22:5 ω-3 (docosapentaenoic acid); C22:6 ω-3 (docosahexaenoic acid); MUFA (monounsaturated Fatty Acids).

2.10. Statistical analysis

Minitab version 17 was used for analysis of variance and Tuckey's comparison test for lipid yield data, ASTX contents and fatty acid profile obtained for each extraction procedure.

3. RESULTS AND DISCUSSION

3.1. Proximal Chemical Composition (PCC)

Table 1 shows similar fat content in munida fresh samples to the data obtained by Albrecht-Ruiz and Cueto (2006) and *P. planipes* fresh samples (Fonseca-Rodríguez and Chavarría-Solera, 2017) and meal data (Civera *et al*., 2000) showing the higher yield oil in munida as an advantage.

Fat is one of the most variable components in marine animals and is influenced by biotic and abiotic factors (age, catching area, time and depth of capture). Bascur *et al*. (2017) investigated the effect of seasonal variations and food availability to which *P. monodon* ovigerous females were exposed during their reproductive period (February to December) and during winter. The results indicated that these organisms adjusted their biochemical processes to ensure their survival and that of their embryos.

3.2. Thin Layer Chromatography (TLC)

Table 2 shows that ASTX (Rf 0.53) was identified in all *P. monodon* oil extracts. The values obtained were 0.63 and 0.81, which would evidence the esterified form of this molecule (monoesters and

Table 1. Munida proximal chemical composición (g/100g sample)

	Moisture	Fat	Protein	Ashes
Fresh munida (*P. monodon*)[1]	73.57 ± 0.07	6.06 ± 0.08	10.69 ± 0.11	7.59 ± 0.09
Fresh munida (*P. monodon*)[2]	74.2	6.50	10.60	4.70
Fresh munida (*P. planipes*) [3]	83.12 ±1.66	1.16 ± 0.28	13.52 ± 1.15	1.51 ± 0.44
Munida meal (MM)[1]	9.17 ± 0.02	23.16 ± 0.26	34.5 ± 0.12	15.15 ± 0.06
P. planipes meal [3]	7.83 ± 1.44	8.04 ± 1.42	40.45 ± 2.56	39.00 ± 1.55

Values in the Table are mean ±SD of duplicate analyses. [1]Results obtained in the present study, [2] Albrecht-Ruiz and Cueto, (2006); [3]Civera *et al.,* (2000).

Table 2. Retention factors (Rf) of munida *(P. monodon*) lipid extracted with different solvents

Sample	Rf
ASTX standard	0.53
Lipid extracted by Et	0.53, 0.63, 0.81 y 0.99
Lipid extracted by Ac	0.53, 0.63, 0.81 y 0.99
Lipid extracted by He-I	0.53, 0.63, 0.81 y 0.99
Lipid extracted by SC-CO_2-Et	0.53, 0.63, 0.81 y 0.99

Et = absolute ethanol, He-I = hexane and isopropyl alcohol 60:40 (v/v), Ac = acetone, SC-CO_2-Et = Supercritical CO_2 + ethanol.

diesters) as typical forms which are characteristic in crustaceans (Hornero-Méndez, 2019). The Rf values obtained also agree with the results obtained by Dalei and Sahoo (2015) in crustacean shell residues. These authors also refer to the fact that Rf 0.99 evidences the presence of β-carotene, a molecule that would also be present in the munida lipids.

3.3. Lipid extraction yield

The highest efficiency in lipid extraction from the MM sample was obtained using Et as solvent. Likewise, Xie *et al*. (2018) reported the advantage of Et in SC-CO_2 extraction compared to 3 solvents per step working with krill meal. This result could be explained by the SC-CO_2 increased polarity and its ability to dissociate protein-phospholipid complexes (Hardardottir and Kinsella, 1988). However, Ali-Nehari *et al*. (2012) reported higher efficiency in krill meal lipid extraction using hexane (16.2% lipids) compared to SC-CO_2-Et (12.2%), although Xie *et al*. (2017) reported higher yields with Et (16.33%) in comparison to hexane yields (12.18%), explaining that alcoholic solvents are more efficient for krill meal lipid extraction.

3.4. Content of carotenoids expressed as ASTX

Carotenoid values expressed as ASTX and extracted under the conditions established in the present study ranged from 2998.01 to 4238.65 µg /g lipid. The highest values were obtained using Ac and SC-CO_2-Et (Table 3). The higher efficiency of Ac compared to Et for extracting ASTX from crustacean lipids has been demonstrated by Dalei and Sahoo (2015) and Xie *et al*. (2018). The lower polarity of Ac facilitates its penetration through the hydrophobic mass surrounding the pigment and favors its miscibility (Dalei and Sahoo, 2015).

All ASTX values obtained in MM by means of the proposed treatments exceeded those reported in residues of *Farfantepenaeus paulensis*, a species belonging to the genus *Penaeus*, (1074 µg ASTX/g lipid) (Sánchez-Camargo *et al*., 2011) and those obtained in krill oil extracted with SC-CO_2-Et (86.2 µg ASTX/g lipid) and hexane (103.2 µg ASTX/g lipid) by Ali-Nehari *et al*. (2012). These results suggest not only the affinities of the solvent and extraction conditions but that munida would represent a source of higher contents of ASTX compounds compared to similar species.

Typically, the choice of solvent is made according to the polarity of the target compound. Routray *et al*. (2019) reported improved ASTX extraction efficiency when using hexane combined with other solvents, although in the present work the use of He-I mixture extracted the lowest ASTX values indicating that isopropyl alcohol did not improve ASTX extraction efficiency (Table 3).

The results of SC-CO_2-Et extraction are in agreement with Routray *et al*. (2019) research data on the significant improvement in this extraction technology to recover ASTX using Et as cosolvent. Also,

Table 3. Lipid yields (%) in munida meal (MM) and ASTX in munida (*Pleuroncodes monodon*) lipid as extracted by different solvents

Extraction methods	Yield (g lipids/100 g MM)	Content of carotenoids (µg ASTX /g lipid)
He-I	17.29 ± 0.45 ^{c}	2998.01 ± 81.54 ^{c}
Ac	14.71 ± 0.29 ^{d}	4238.65 ± 21.04 ^{a}
Et	22.93 ± 0.71 ^{a}	3443.23 ± 126.30 ^{b}
SC-CO_2-Et	18.90 ± 0.36 ^{b}	4086.71 ± 80.11 ^{a}

He-I = hexane and isopropyl alcohol 60:40 (v/v), Ac = acetone, Et = absolute ethanol, SC-CO_2-Et = Supercritical CO_2 + ethanol. Data are shown as mean ± standard deviation. Different letters in the same column indicate significant difference ($p < 0.05$). Tukey test ($p < 0.05$) was used for the comparison of means. All experiments were carried out in duplicate.

Sánchez-Camargo *et al*. (2011) reported 15% Et as co-solvent to substantially improve ASTX extraction, and highlighted the advantage of the solubilization of polar compounds such as phospholipids and glycolipids.

According to Capelli (2018) the recommended daily intake of ASTX (4 mg) can be provided by one gram of munida lipid obtained by Ac and SC-CO_2-Et extractions (4238.65 and 4086.71 µg/g lipid, respectively); nevertheless, considering Ac toxicity, the use of SC-CO_2-Et is considered the best extraction option for safety concerns among the extraction methods evaluated.

3.5. Fatty acids

Table 4 shows the fatty acid profile of munida lipids obtained by the different lipid extraction methods. Among the saturated fatty acids (SFA) C16:0 represented the highest percentage in all the extracts while C18:1 ω-9 was the most abundant among monounsaturated fatty acids (MUFA) and its quantity was not affected by the extraction method applied.

A predominant presence of long-chain PUFA acids was observed, and ranged from 40.79 to 41.51% with high values of EPA and DHA. No significant

Table 4. Fatty acids content in munida (*Pleuroncodes monodon*) lipid (%) extracted with different solvents

Fatty acids	Extraction Procedures			
	He-I	Ac	Et	SC- CO_2-Et
C14:0 (Myristic)	4.04±0.17 ^{b}	4.38±0.15 ^{ab}	4.48±0.03 ^{a}	4.38±0.00^{ab}
C14:1 (Myristoleic)	0.19±0.00	Nd	Nd	Nd
C15:0 (Pentadecaenoico)	0.41±0.01 ^{a}	0.42±0.01 ^{a}	0.42±0.00 ^{a}	Nd
C16:0 (Palmitic)	21.76±0.40 ^{b}	21.99±0.23 ^{ab}	21.95±0.02 ^{ab}	22.71±0.01 ^{a}
C16:1 (Palmitoleic)	7.14±0.16 ^{c}	7.80±0.10 ^{a}	7.54±0.01 ^{ab}	7.19±0.03 ^{bc}
C17:0 (Heptadecaenoic)	2.76±0.01 ^{ab}	2.79±0.13 ^{ab}	2.60±0.11 ^{b}	2.96±0.02 ^{a}
C17:1 (Cis-10-Heptadecenoic)	0.84±0.02 ^{a}	0.17±0.01 c	0.36±0.00 ^{b}	0.16±0.00 ^{c}
C18:0 (Estearic)	2.96±0.01 ^{a}	2.69±0.01 ^{b}	2.67±0.01 ^{a}	2.92±0.02 ^{a}
C18:1 ω-9 (Oleic)	12.92±0.05 ^{a}	13.15±0.03 ^{a}	12.89±0.02 ^{a}	12.9±0.02 ^{a}
C18:1 ω-7 (Vaccenic)	4.36±0.04 ^{a}	4.40±0.01 ^{a}	4.36±0.02 ^{a}	4.46±0.01 ^{a}
C18:2 ω-6 (Linoleic)	1.43±0.00 ^{a}	1.38±0.00 ^{b}	1.43±0.00 ^{a}	1.38±0.01 ^{b}
C18:3 ω-6 (γ-Linolenic)	0.74±0.01 ^{b}	0.81±0.00 ^{a}	0.73±0.01 ^{b}	0.74±0.01 ^{b}
C18:3 ω-3 (α-Linolenic)	0.99±0.00 ^{a}	1.00±0.00 ^{a}	1.00±0.02 ^{a}	0.89±0.00 ^{b}
C18:4 ω-3 (Stearidonic)	5.71±0.35 ^{a}	5.81±0.00 ^{a}	5.81±0.02 ^{a}	5.36±0.01 ^{a}
C20:0 (Arachidic)	0.47±0.00 ^{a}	0.46±0.00 ^{a}	0.38±0.00 ^{b}	0.47±0.02 ^{a}
C20:1 ω-9 (Eicosaenoic)	0.92±0.00 ^{a}	0.96±0.02 ^{a}	0.83±0.00 ^{b}	0.85±0.01 ^{b}
C20:2 (Eicosadienoic)	0.26±0.06 ^{a}	Nd	Nd	0.22±0.01 ^{b}
C20:3 ω-6 (Eicosatrienoic)	Nd	Nd	Nd	0.66±0.00
C20:3 ω-3 (Eicosatrienoic)	0.64±0.01^{b}	0.58±0.01^{c}	0.70±0.03^{a}	Nd
C20:4 ω-6 (Araquidonic)	0.31±0.01	Nd	Nd	Nd
C20:5 ω-3 (EPA)	11.32±0.07 ^{b}	11.26±0.07 ^{b}	11.82±0.02 ^{a}	11.31±0.01 ^{b}
C22:6 ω-3 (DHA)	19.83±0.27 ^{a}	19.96±0.22 ^{a}	20.04±0.04 ^{a}	20.43±0.01 ^{a}
SFA	32.39±0.60 ^{a}	32.73±0.24 ^{a}	32.51±0.16 ^{a}	33.44±0.03 ^{a}
MUFA	26.38±0.15 ^{a}	26.48±0.05 ^{a}	25.98±0.03 ^{b}	25.56±0.02 ^{c}
PUFA	41.23±0.75 ^{a}	40.79±0.30 ^{a}	41.51±0.13 ^{a}	41.00±0.05 ^{a}
∑ ω-3	38.48±0.70 ^{a}	38.60±0.30 ^{a}	39.36±0.13 ^{a}	37.99±0.04 ^{a}
EPA + DHA	31.15±0.35 ^{a}	31.21±0.29 ^{a}	31.85±0.06 ^{a}	31.75±0.03 ^{a}

Et = absolute ethanol, He-I = hexane and isopropyl alcohol 60:40 (v/v), Ac = acetone, SC-CO_2-Et = Supercritical CO_2 + ethanol. Data are shown as mean ± standard deviation. Different letters in the same row indicate significant difference ($p < 0.05$), Tukey test ($p < 0.05$) was used for the comparison of means. All experiments were carried out in duplicate.
Nd: no detected.

differences ($p > 0.05$) regarding the extraction methods were observed. The values reported in the present study exceeded those obtained by Xie *et al*. (2017) using Ac as solvent in three krill species as well as by Ali-Nehari *et al*. (2012) in krill oil. The later one found higher efficiency by using SC-CO_2-Et compared to hexane. These high long-chain PUFA contents are considered essential for membrane fluidity and inflammatory mediator functionality and show potential benefits in neuronal development and cardiovascular health (Janssen and Kiliaan, 2014).

The high C16:0 and C20:5 ω-3 values in munida lipid samples were similar to those reported by Ali-Nehari *et al*. (2012) in oily extracts of krill, in residues of *Farfantepenaeus paulensis* (Sánchez-Camargo *et al*., 2011) and in *Litopenaeus vannamei*; while the C22:6 ω-3 contents were higher in the samples of the present study.

The EPA+DHA contents in *P. monodon* showed no significant differences, and the values obtained by the extraction techniques ranged from 31.15 to 31.85%. ω-6 fatty acids were not detectable in some cases, as in the case of C20:3 ω-6 and C20:4 ω-6. C18:2 ω-6 was the omega-6 fatty acid with the highest content. The results obtained in the present study show similarity to those reported by Ali-Nehari *et al*. (2012) and Xie *et al*. (2017) for krill oil.

3.6. Functional quality of MM lipid

No differences were observed among the AI values of the MM lipids obtained with all the solvents used in the present study (Table 5), although these values were lower than those calculated from studies carried out by Xie *et al*. (2017) and Sánchez-Camargo *et al*. (2012) for *Euphausia superba* and *Penaeus paulensis* respectively. This would be advantageous in the case of munida, considering that Turan *et al*. (2007) refer to AI values and also thrombogenicity indexes (TI) close to zero, which are considered favorable for preventing coronary heart disease.

The AI and TI values obtained in the present study are slightly higher than those obtained by Lopes *et al*. (2014) for grape pomace oil (AI= 0.18-0.32, TI= 0.06-0.17). Studies by Pinto *et al*. (2020) on *Endopleura uchi* oil reported AI values similar to those obtained in our work, although their TI values were higher (AI=0.44, TI=1.32) than ours.

Regarding the use of solvents, the highest H:H ratio was observed in the extracted munida lipids using the He-I mixture (1.81). This value exceeded those obtained by Xie *et al*. (2017) and Xie *et al*. (2018), although it was lower than that obtained by Sánchez-Camargo *et al*. (2012) for *Penaeus paulensis* when using SC-CO_2-Et. Low H:H values are considered unfavorable as they may induce an increase in cholesterolemia (Santos-Silva *et al*., 2002); while high values‒like 2.66 in uxi (*Endopleura uchi*) are recommended by Pinto *et al*. (2020).

Our results suggest that the SC-CO_2-Et lipid extraction method applied in Peruvian marine species of commercial importance contributes to quantifying carotenoid pigments and essential fatty acids (EPA and DHA) among other bioactive comounds. *Argopecten purpuratus* (scallops), *Romaleon setosum*, *Cancer porteri*, *Platymera gaudichaudii*, *Paralomis longipes* (crabs) *Loxechimus albus* (sea urchin) and many other fish species represent a promising dietary source.

Table 5. Functional Quality Indexes in munida lipid compared to lipid data from other species data

Solvent	Crustacean	AI	TI	H:H
He-I[1]	*Pleuroncodes monodon*	0.56	0.20	1.81
Ac[1]		0.59	0.20	1.77
Et[1]		0.59	0.19	1.78
SC-CO_2-Et[1]		0.61	0.21	1.73
Ethanol[2]	*Euphausia superba*	1.96	0.24	1.32
Acetone[2]		2.85	0.31	1.14
Ethanol[3]	*Euphausia superba*	1.24	0.17	1.64
Supercritical CO_2 + ethanol[4]	*Penaeus paulensis*	0.97	0.40	2.16

[1]Our data; calculated based on the results of: [2] Xie *et al*. (2017), [3]Xie *et al*. (2018), [4]Sánchez-Camargo *et al*. (2012). He-I = hexane and isopropyl alcohol 60:40 (v/v), Ac = acetone, Et = absolute ethanol, SC-CO_2-Et = Supercritical CO_2 + ethanol, AI: atherogenicity index, TI: thrombogenicity index, H:H: Hypocholesterolemia: hypercholesterolemia ratio.

4. CONCLUSIONS

Higher ASTX contents were obtained from munida using Ac and SC-CO_2-Et, followed by Et extractions. On the other hand, the contents of SFA, PUFA, omega-3, EPA+DHA in munida lipids with all extraction solvents showed no significant differences. PUFA were the most predominant in the lipid. Among SFA and MUFA, C16:0 and C18:1 ω-9 were prevalent. The sum of C20:5 ω-3 and C22:6 ω-3 varied from 31.15 to 31.85%.

The functional quality indexes AI and TI for munid lipid were favorable; while the H:H values were low. The results of the present study suggest that it is an important source of lipids which contain ASTX, EPA and DHA. The extraction of munida lipids with Et or SC-CO_2-Et in further studies are suggested for possible application in the food industry.

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CONFLICTS OF INTERESTS

No potential conflict of interest was reported by the authors**.**

DECLARATION OF ETHICS

The authors hereby declare their agreement with this publication and their contributions to justify their authorship; that there is no conflict of interest; and that they have complied with all relevant ethical and legal requirements and procedures. All sources of funding are fully and clearly detailed in the acknowledgement section. The respective signed legal document is on file with the journal.

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Oxidative stability and compositional characteristics of oil from microwave irradiated black cumin seed under accelerated oxidation condition

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SUMMARY: The present work evaluated the impact of microwave pre-treatment on the storage stability, fatty acids and triacylglycerol contents in black cumin seed oil (BCO) during storage at 62 ºC. During storage, the oxidative indicator values (free acidity, peroxide value, *p*-anisidine value, TOTOX, specific extinctions and thiobarbituric acid) for the oils increased faster in untreated oil samples than in the microwaved samples. The degradation rate of polyunsaturated fatty acids (PUFAs) and triacylglycerol species (LLL and OLL) during storage were higher in untreated samples compared to treated ones, indicating that oxidation proceeded more slowly in the treated samples. During storage, the generation of hydroperoxides, their degradation and the formation of secondary oxidation products as investigated by FTIR, were lower in the treated oils. In conclusion, microwave pre-treatment prior to oil extraction reduced the oxidative degradation of oil samples, thereby increasing the storage stability of BCO.

KEYWORDS: *Black cumin seed oil; Fatty acids; Microwave pretreatment; Oxidative stability*

RESUMEN: ***Estabilidad oxidativa y composición del aceite de semillas de comino negro, irradiadas con microondas, en condiciones de oxidación acelerada*.** En el presente trabajo se evaluó el impacto del pretratamiento de las semillas de comino negro con microondas sobre la estabilidad durante el almacenamiento, los ácidos grasos y las especies de triacilgliceroles del aceite de las semillas de comino negro (BCO) durante el almacenamiento a 62 ºC. Durante el almacenamiento de los aceites, los indicadores oxidativos (acidez libre, peróxidos, *p*-anisidina, TOTOX, extinciones específicas y ácido tiobarbitúrico) aumentaron más rápidamente en los aceites de semillas sin tratar que en los de las muestras tratadas con microondas. La degradación durante el almacenamiento de los ácidos grasos poliinsaturados (PUFA) y las especies de triacilgliceroles (LLL y OLL) fue mayor en las muestras no tratadas en comparación con las tratadas, lo que indica que la oxidación avanzó más lentamente en las muestras tratadas. Durante el almacenamiento, la generación de hidroperóxidos, su degradación y la formación de productos de oxidación secundarios investigados por FTIR, fueron menores en los aceites tratados. En conclusión, el pretratamiento con microondas de las semillas antes de la extracción del aceite redujo la degradación oxidativa de los aceites, lo que aumentó la estabilidad de almacenamiento de BCO.

PALABRAS CLAVE: *Aceite de semilla de comino negro; Ácidos grasos; Estabilidad oxidativa; Pretratamiento de microondas*

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1. INTRODUCTION

Nigella sativa Linn. is an annual herb cultivated mostly in South and Southwest Asia. The used part is the seed known as black cumin seed, which is utilized worldwide for edible and medicinal applications. The seed contains a high amount of oil which may play a significant role in health and nutrition because of its fatty acid composition (FAC), polyphenol compounds, volatile oil and other important phytochemicals (tocols, sterols and polar lipids) (Ramadan, 2013; Piras *et al.,* 2013). The black cumin seed and its oil (BCO) have medicinal and therapeutic benefits (Mazaheri *et al.,* 2021). The consumption of BCO obtained from pre-treated seeds has a wide range of possible applications in the pharmaceutical and food industries. BCO is dominated by unsaturated fatty acids, especially PUFAs (59.7%), followed by monounsaturated fatty acids (24.1%), and saturated fatty acids (16.1%) (Kiralan *et al.,* 2020). The quality of oil highly depends on its processing methods or conditions.

Several pre-treatment processes for seeds, such as freeze-thaw, infrared, UV irradiation, rapid gas decompression, ultrasonic baths, and microwave, are applied for edible seeds to enhance the extraction of bioactive phytochemicals, and accessibility of favorable nutraceuticals (Zhang *et al.,* 2020; Fathi-Achachlouei *et al.,* 2019; Kiralan *et al*., 2016). Among them microwave irradiation is used as an impressive technique in the food industry which may results in various physicochemical alterations such as oxidation stability, flavor, fatty acid concentration, tocols, antioxidative status, bioactivity, color and nutritional properties (Ali *et al.,* 2017a; Ali *et al.,* 2017b; Fathi-Achachlouei *et al.,* 2019; Karrar *et al.,* 2020). Microwave irradiation has gained in popularity, because it needs very short processing time compared to traditional heating methods (Đurđević *et al.,* 2017). The microwave irradiation of black cumin seed can affect its phytochemical composition, quality and oxidative stability, and understanding the impact of microwave irradiation on oilseeds is of great importance. To optimize BCO yield and further increase its quality and oxidative stability, optimum time and microwave power combination needs to be established. To date, few research works have been determined the oxidation degradation and physicochemical characteristics of microwave roasted BCO without applying any heat or storage treatment of the seed oil (Mazaheri *et al.,* 2019; Suri *et al.,* 2019; Bakhshabadi *et al.,* 2017). This work has therefore been considered to determine the impact of microwave irradiation prior to oil extraction on the oxidative stability, fatty acids and triacylglycerol contents in BCO under accelerated oxidation conditions.

2. MATERIALS AND METHODS

2.1. Materials

Black cumin seed (2.5 kg) was bought from Rajshahi, Bangladesh. The seed was cleaned, dried in the shade at ambient temperature, and preserved at 4 °C in a refrigerator. The chemicals and solvents used were of analytical grade. Thioburbituric acid was product of HiMedia Laboratories (Mumbai, India). Acetic acid and standards were bought from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals or reagents were purchased from Merck (Darmstadt, Germany or Mumbai, India) unless otherwise stated.

2.2. Pre-treatment and oil extraction

The cumin seed samples were spread on the Pyrex petri dishes (12 cm diameter) set on a turntable plate of the microwave oven (MS3042G, LG, China). After covering the dishes, the samples were then microwaved at a frequency 2450 MHz (capable of producing 580 W power) for 1, 2, and 3 min depending on trial results. After pre-treatment, the samples were allowed to cool to 25 °C and thoroughly mixed. Oils from untreated and treated whole black cumin seeds were obtained by pressing using a locally-made mechanical pressing machine at room temperature (27 °C). The temperature of the outflowing oil was around 40 °C. After filtering to remove particles, the oils were weighed and stored in capped glass bottles at a temperature below −15 °C for analysis.

2.3. Accelerated oxidation of oil samples

The untreated or treated BCOs (75 g) were placed in 100-mL glass beakers, and beakers were put in an incubator at 62 °C for accelerating oil oxidation. The oils were withdrawn at regular intervals of 0, 7, 14, and 21 days.

2.4. Oxidative indices

The American Oil Chemists' Society (AOCS, 1987) methods were applied to estimate free fatty acids (FFA) (method Ca 5a-40), peroxide value (PV)

(method Cd 8-53), and thiobarbituric acid value (TBA) (method Cd 19-90). Following the PORIM (PORIM, 1995) test methods, specific extinctions (method p2.15) at 233 and 269 nm ($E^{1\%}_{233}$ and $E^{1\%}_{269}$) and *p*-anisidine value (*p*-AV) (method p2.4) of the oils were estimated using a spectrophotometer (T 60, PG Instruments, Leicestershire, UK). The oxidative value was determined by Holm's equation: TOTOX = 2PV + *p*-AV (Wai *et al.,* 2009).

2.5. Color development

The absorbance of a 5.0% (w/v) oil solution in chloroform was computed at 420 nm with a spectrophotometer (T 60U, PG Instruments, Leicestershire, UK) indicating an index of color formation, (Yoshida *et al.,* 1999).

2.6. Fatty acid composition (FAC)

The FAC was estimated after the preparation of methyl esters using the PORIM (PORIM, 1995) test method p3.4. A gas chromatography (Clarus 590 GC PerkinElmer, USA) equipped with a flame ionization detector was used to determine the FAC oil samples. Helium gas was passed (2 mL/min) as carrier gas. Fatty acids were separated on a 0.25 mm i.d. × 30 m × 0.25 µm capillary column (Elite-FFAP). Analysis was carried out at an initial oven temperature 120 °C which was raised to 240 °C at 4 °C/min. The injector and detector temperatures were controlled at 120 °C and 250 °C, respectively. The peaks were identified by comparison with the standards (methyl arachidate, methyl behenate, methyl decanoate, methyl *cis*-13-docosenoate, methyl dodecanoate, methyl linoleate, methyl linolenate, methyl myristate, methyl octanoate, methyl oleate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl tetracosanoate) (Sigma-Aldrich Co., St. Louis, MO, USA).

2.7. Triacylglycerol (TAG) molecular compounds

The concentrations in molecular TAG compounds present in seed oils were determined by a HPLC system (Agilent 1260 Infinity, USA) equipped with a column (50 mm x 4.6 mm i.d x 2.7 µm) packed with Poroshell 120 EC-C18 (Agilent, USA) and evaporative Light Scattering Detector (ELSD). The solvent system, acetone/acetonitrile (65:35, v/v) was used as a mobile phase at a flow rate of 1 mL/min. The concentrations in TAG species were determined by using standards (POL, OOL, POO, OLL, PLL, MOL, OOO, PLP, POP, SOO and POS, where P- palmitic, M- myristic, O- oleic, L- linoleic) (Sigma-Aldrich Co., St. Louis, MO, USA).

2.8. FT-IR spectroscopy

The FTIR spectra of oils were measured by a Fourier Transform Spectroscopy (IRAffinity- 1S, Shimadzu Corporation, Kyoto, Japan) furnished with a high-sensitivity pyroelectric detector (deuterated L-alanine doped triglycine sulphate). Samples were applied to a sodium chloride cell and periodic scans (15 scans and 4 cm^{-1} resolution) were performed in the spectral range of 850-4000 cm^{-1}. The spectra were computed as absorbance values at each data point.

2.9. Statistical analysis

The data were declared as the mean and standard deviation (SD) of triplet determinations. One way analysis of variance (ANOVA) was performed, and mean values were compared at $p < 0.05$ significance level by Duncan's multiple range test using IBM SPSS 22 statistics.

3. RESULTS AND DISCUSSION

The moisture content in black cumin seed was 5.50% (DM), which reduced to 4.10, 2.60 and 1.10% (DM) with treatment times 1, 2 and 3 min respectively. The quantities of crude oil ranged from 27.65% (DM) for fresh sample to 35.54% (DM) for the 3-min microwaved one. The oil content increased with increasing pre-treatment times. Mazaheri *et al.* (2019) and Bakhshabadi *et al.* (2017) also reported that the oil yield in black cumin seed increased with increasing seed pre-treatment times. The microwave pre-treatment of seeds enhanced extraction efficiency and mass transfer coefficients of the seeds due to the severely ruptured cell membranes. A permanent pore was formed in the seed which allows oil migration through the permeable cell walls (Azadmard-Damirchi *et al.,* 2010).

3.1. Oxidative indices

The concentrations of FFA in the oil samples increased significantly ($p<0.05$) as a result of increasing accelerated storage time (Figure 1a). This

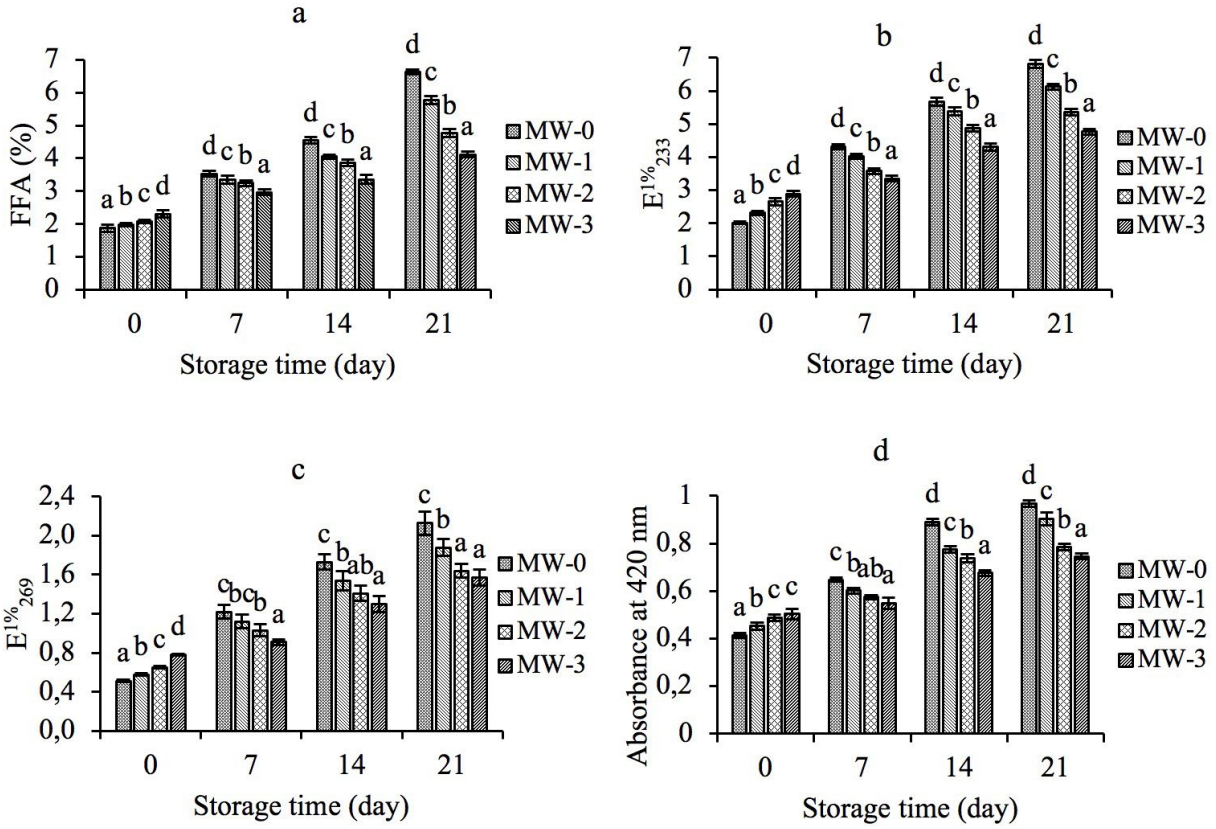

FIGURE 1. Changes in FFA (a), specific extinction at 233 nm, (b) specific extinction at 269 nm (c), and color value (d) of untreated and microwave pre-treated (MW-1, pre-treated for 1 min; MW-2, pre-treated for 2 min; and MW-3, pre-treated for 3 min) black cumin seed oils during storage. Each value is the mean ± standard deviation of triplicate determinations. Mean values were compared by Duncan's multiple range test. Values in each storage time grouped with different letters on bars are significantly different ($p < 0.5$).

hydrolytic degradation was noted to be biggest in the untreated sample (6.62%) with the lowest in the 3 min microwaved sample (4.12%) at the end of 21 days of storage. It also indicated that the higher initial FFA content in the sample microwaved for 3 min, did not affect the hydrolytic degradation of BCO during storage at 62 °C. Microwave pre-treatment had a significant impact on ultraviolet absorptions at 233 ($E^{1\%}_{233}$) and 269 nm ($E^{1\%}_{269}$) in the oils (Figures 1b and 1c). The absorptions were significantly ($p<0.05$) enhanced for all the oils throughout the storage treatments. At the end of 21 days of storage, the concentrations in conjugated dienes and trienes were the greatest in the untreated samples, with the lowest found for the 3-min microwaved samples. The lower values for absorptions indicate better storage stability of treated samples compared to untreated samples. In addition, the concentration of conjugated diene was higher than triene in all oils expressed by the biggest value for $E^{1\%}_{233}$ at 233 nm. Ali *et al.* (2017a) also followed a similar trend for pumpkin seed oil. Pre-treatment and storage conditions employed in this research affected the formation of color in the oils (Figure 1d). Browning substances were generated during microwave irradiation and storage treatment which resulted in a significant ($p < 0.05$) increment in absorbance at 420 nm. The absorbance values limited from 0.41 to 0.50 at 420 nm, were enhanced markedly ($p <0.05$) during incubation and these increments were detected to be higher in the untreated oils. The formation of Maillard reaction products at the storage temperature might be responsible for the color increments in the oils during storage. However, longer seed pre-treatment time had a bigger impact on the further reduction in oxidative stability. Thus, the present findings support the earlier work done by Ali *et al.* (2017b), where they indicated that oil color increased with increasing seed pre-treatment time or heating time of a groundnut oil sample.

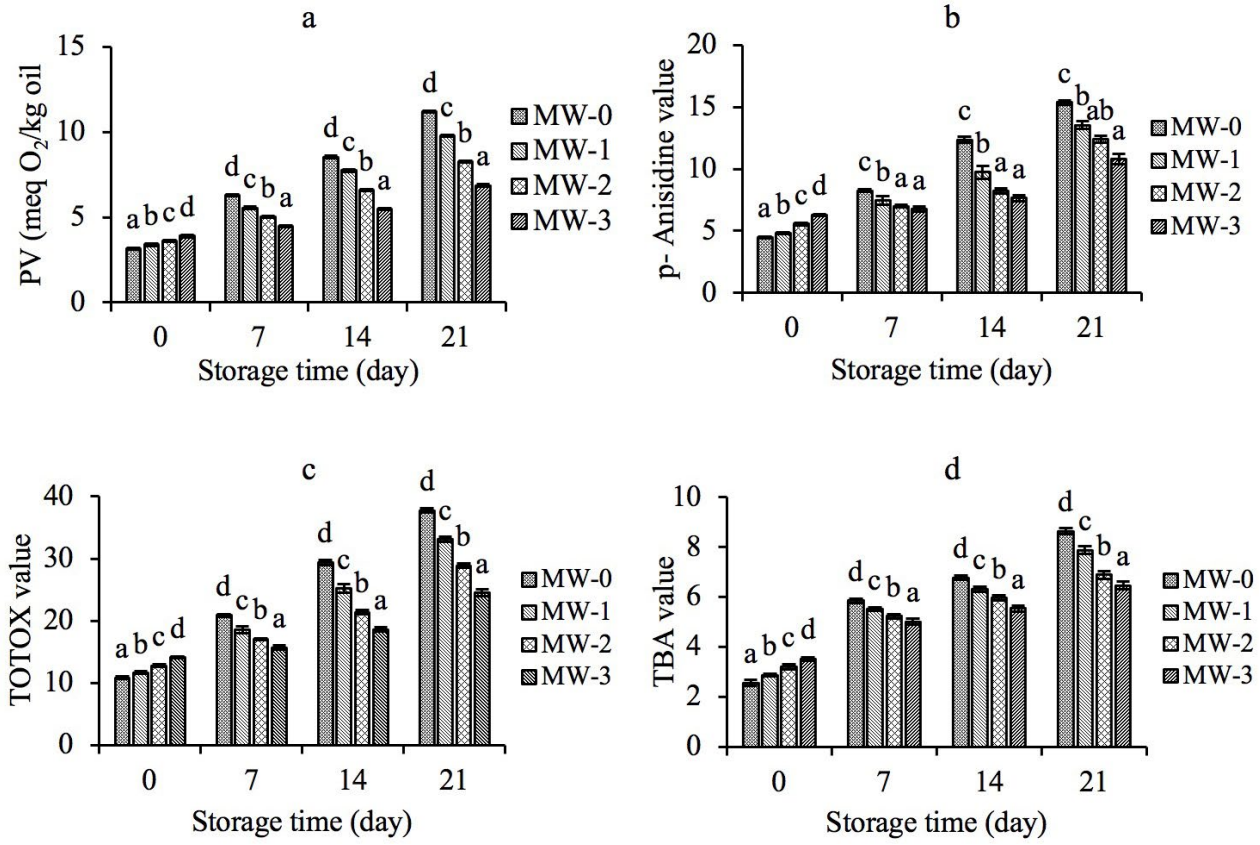

FIGURE 2. Changes in peroxide value (a), *p*-Anisidine value (b), TOTOX value (c), and TBA value (d) of untreated and microwave pre-treated (MW-1, pre-treated for 1 min; MW-2, pre-treated for 2 min; and MW-3, pre-treated for 3 min) black cumin seed oils during storage. Each value is the mean ± standard deviation of triplicate determinations. Mean values were compared by Duncan's multiple range test. Values in each storage time grouped with different letters on bars are significantly different ($p < 0.05$).

Figure 2a indicates the formation of unstable oxidative substances determined by peroxide value (PV) which was found to be faster in the raw sample than the treated samples during the storage oxidation. Oils from pre-treated samples (6.88 meq O_2/kg) had the lowest concentrationa in hydroperoxide than that of the raw one (11.20 meq O_2/kg) at the end of 21 days incubation. Ali *et al.* (2017b) also found a slower increment in PV for treated groundnut seed oil compared to the unroasted sample during thermal oxidation. In addition, at the initial phase of the storage period, samples from the microwave pre-treated oils had slightly higher PV (3.40-3.90 meq O_2/kg); while in the untreated oils it was relatively low (3.17 meq O_2/kg). The higher value could be the result of the exposure time at an elevated temperature during the microwave pre-treatment of the seed samples. Microwave pre-treatment itself was reported to cause slight oil oxidation during prolonged heating (Anjum *et al.,* 2006). The PV and *p*-AV are normally used to determine the degree of lipid oxidation. The pre-treatment of seeds decreased the *p*-AV significantly ($p < 0.05$) in BCO compared to the raw samples under accelerated oxidation conditions (Figure 2b). Ali *et al.* (2017b) reported similar results after 9 h heating of oil extracted from microwaved groundnut seed. In the present work, the *p*-anisidine values (*p*-AVs) from the lowest to the highest (10.77 to 15.36), were followed in oils pre-treated at 3, 2, 1 and 0 min after 21 days of accelerated storage. This indicates an extended shelf-life of oils produced from microwaved seeds, probably through the formation of Maillard reaction products (MRPs). Figure 2c shows the marked differences in total oxidation values (TOTOX) in the BCOs under storage conditions. The oil samples of untreated seeds displayed the highest TOTOX values, which revealed that the microwave irradiation reduced the formation of oxidative products during storage at 62 °C. The changes in the thiobarbituric acid (TBA) level of treated BCOs were significantly lower ($p<0.05$)

than that of the untreated seed oil (Figure 2d). Before the storage of oils under accelerated conditions, the TBA values at 0, 1, 2 and 3 min for pre-treated samples were 2.56, 2.87, 3.21 and 3.52, respectively, and after 21 days, the TBA values were increased to 8.64, 7.88 6.89 and 6.47, respectively. This reveals that the oil samples from untreated seeds were more susceptible to oxidation at storage temperature than the oil samples from treated seeds. In addition, a sharp increase in TBA values was detected at an earlier stage of storage followed by a decrease. This can be due to the volatilization of secondary oxidation products or their breakdown.

3.2. Fatty acid composition

The changes in fatty acid composition (FAC) in the oil may indicate its stability, physical properties and nutritional attributes. The dominant fatty acids in BCO were mainly oleic, linoleic and palmitic acids with percentages of 23.25, 57.94 and 13.43, respectively, and myristic, palmitoleic, stearic, linolenic, behenic and lignoceric present in concentrations of less than 1% (Table 1). Saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in fresh BCO amounted to 15.01, 23.46 and 58.51% of the total fatty acids, respectively. The FAC suffered small changes upon microwave pre-treatment. During pre-treatment, the concentration in C18:2 decreased slightly; whereas the concentrations of C16:0 and C18:0 increased slightly. The PUFA degradation may cause this trend, and Ali *et al.* (2017a) saw a similar trend in the case of pumpkin seed oil during thermal oxidation. As can be seen in Table 2, the relative content in PUFA was reduced; while that of SFA or MUFA increased in BCOs during storage treatment. Ali *et al.* (2017b) followed a similar trend in groundnut oil during heating at 170 °C. However, the changes in fatty acid concentrations were lower in microwaved samples than in untreated ones during storage at 62 °C. Suri *et al*. (2019) also reported that dry air roasting slightly influenced the FAC of BCO. The greatest reduction in PUFA was recorded for fresh BCO (7.90%) and the lowest for 3-min microwaved sample (3.20%) after 21 days of storage. The present data indicate that the change in FAC in the pre-treated samples was smaller compared to the raw sample; this indicates the higher tendency of fresh sample towards the generation of oxidation products and polymerized compounds by oxidation degradation of PUFAs. In addition, the ratio of PUFA to SFA (P/S) of all samples decreases with increasing storage time, which serves to realize the status of oxidative tendency of lipids (Lee *et al.,* 2007). In this regard, the pre-treated samples showed the lowest P/S ratio change (decrease) which indicates that oxidative reactions progressed more rapidly in raw samples than in microwaved samples during storage.

Table 1. Fatty acid composition (%) of untreated and microwave pre-treated black cumin seed oils before storage

	Pre-treatment time (min)			
Fatty acids	**0**	**1**	**2**	**3**
Myristic acid (C14:0)	0.17±0.01^{a}	0.17±0.02^{a}	0.16±0.01^{a}	0.16±0.01^{a}
Palmitic acid (C16:0)	13.43±0.21^{c}	13.03±0.15^{b}	12.52±0.19^{a}	14.19±0.18^{d}
Palmitoleic acid (C16:1)	0.21±0.02^{c}	0.22±0.02^{c}	0.18±0.01^{b}	0.15±0.01^{a}
Stearic acid (C18:0)	0.50±0.03^{ab}	1.20±0.10^{c}	0.47±0.05^{a}	0.61±0.04^{b}
Oleic acid (C18:1)	23.25±0.14^{a}	23.36±0.18^{a}	23.39±0.22^{a}	24.31±0.43^{b}
Linoleic acid (C18:2)	57.94±0.39^{b}	57.24±0.42^{b}	55.92±0.37^{a}	56.09±0.32^{a}
Linolenic acid (C18:3)	0.57±0.01^{b}	0.55±0.02^{ab}	1.45±0.03^{c}	0.51±0.04^{a}
Behenic acid (C22:0)	0.57±0.02^{a}	0.58±0.03^{a}	1.29±0.02^{b}	0.56±0.09^{a}
Lignoceric acid (C24:0)	0.34±0.02^{a}	0.42±0.02^{b}	1.91±0.03^{c}	0.46±0.02^{b}
∑Saturated fatty acids	15.01	15.40	16.35	15.98
∑Monounsaturated fatty acids	23.46	23.57	23.57	24.46
∑Polyunsaturated fatty acids	58.51	57.79	57.37	56.69

Each value is the mean ± standard deviation of triplicate determinations. Mean values were compared by Duncan's multiple range test. Values within a row with the same letters are not significantly different at $p < 0.05$

Table 2. Saturated, monounsaturated, and polyunsaturated fatty acids of untreated and microwave pre-treated black cumin seed oils during storage

Pre-treatment time (min)	Storage time (days)	Fatty acid composition (%)			
		Saturated fatty acids	Monounsaturated fatty acids	Polyunsaturated fatty acids	P/S
0	0	15.01 (100)	23.46 (100)	58.51 (100)	3.9
	7	15.19 (101.2)	24.01 (102.3)	57.91 (99.00)	3.8
	14	19.24 (128.2)	23.53 (100.3)	57.23 (97.8)	3.0
	21	19.71 (131.3)	23.56 (100.4)	53.87 (92.1)	2.7
1	0	15.40 (100)	23.58 (100)	57.79 (100)	3.8
	7	15.41 (100.1)	26.73 (113.4)	55.28 (95.7)	3.6
	14	15.78 (102.5)	25.30 (107.3)	55.23 (95.6)	3.5
	21	18.26 (118.6)	23.74 (100.7)	55.03 (95.2)	3.0
2	0	16.35 (100)	23.57 (100)	57.37 (100)	3.5
	7	17.82 (109.0)	23.63 (100.3)	55.68 (97.1)	3.1
	14	18.76 (114.7)	23.71 (100.6)	55.41 (96.6)	3.0
	21	19.02 (116.3)	23.54 (99.9)	55.16 (96.1)	2.9
3	0	15.98 (100)	24.46 (100)	56.60 (100)	3.5
	7	16.14 (101.0)	25.72 (105.2)	55.13 (97.4)	3.4
	14	16.36 (102.4)	26.67 (109.0)	54.74 (96.7)	3.3
	21	17.37 (108.7)	26.68 (105.0)	54.48 (96.8)	3.1

Each value is the mean of triplicate determinations. Number in parenthesis is relative % of saturated, monounsaturated, and polyunsaturated fatty acids based on the initial saturated, monounsaturated, and polyunsaturated fatty acid content before oxidation. P/S- ratio of polyunsaturated to saturated fatty acids.

3.3. Triacylglycerol (TAG) composition

During the accelerated oxidation test, the changes in concentration in TAG species (P, palmitic; M, myristic; O, oleic; L, linoleic) from BCO determined by HPLC are given in Figures 3 and 4. The major TAG species were LLL (21.89%), PLL (18.75%), OLL (17.22%), POL (13.22%), OOL (9.21 %) and PLP (5.06%). The species OOO, POO, and LOS were present as minor components (< 4%). However, microwave pre-treatment did not inflict changes (with few exceptions) in the TAG species of BCOs because only a few species possessing more than four double bonds were present in the TAGs. The percentages of LLL, OLL, PLL, OOL and POL in BCO decreased whilst, in most case, the percentages of PLP, OOO, and POO remained unaltered or slightly increased with increasing storage time. At the end of 21 days of storage, the changes in concentration in those TAG species were significantly lower ($p < 0.05$) in 3-min pre-treated samples than untreated ones. The changes in POL were not significant during storage. The most significant reduction was found in LLL (Figure 3a) among all the species. The percentage of this TAG in untreated and 3-min treated samples reduced from 21.89 to 15.57% and from 20.38 to 19.41%, respectively, after 21 days of storage at 62 °C. This reduction might be due to a decrease in the concentration of C18:2 by the oxidative process. In this work, good agreement between the fatty acid and TAG compositions was also noted.

3.4. Evaluation by FT-IR

The most significant spectral changes occurring in BCOs during accelerated storage conditions are shown in Figure 5. The detected functional groups responsible for the IR absorption peak (Ali *et al.,* 2017b; Lerma-Garcia *et al.,* 2010; Guillen *et al.,* 1997): 3008 (C–H stretching vibration of cis-double bond); 2928 and 2854 (Asymmetric and symmetric stretching vibration of CH_2, resp.); 1745 (C=O stretching vibration); 1465 (bending vibrations of the CH_2 and CH_3); and 1163 (C–O stretching vibration). The absorbance of *cis*-double bond at 3008 cm^{-1} (shoulder) suffers a slow shifting toward higher values during storage.

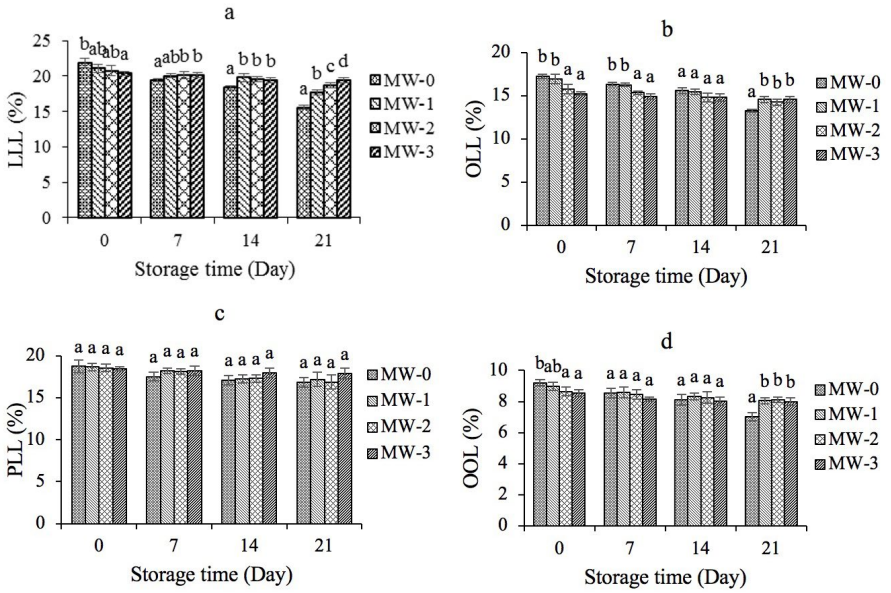

FIGURE 3. Changes in triacylglycerol composition of untreated (MW-0) and pre-treated (MW-1, pre-treated for 1 min; MW-2, pre-treated for 2 min; and MW-3, pre-treated for 3 min) black cumin seed oils during storage. (a) LLL, (b) OLL, (c) PLL, and (d) OOL. Each value is the mean ± standard deviation of triplicate determinations. Mean values were compared by Duncan's multiple range test. Values in each storage time grouped with different letters on bars are significantly different ($p < 0.05$).

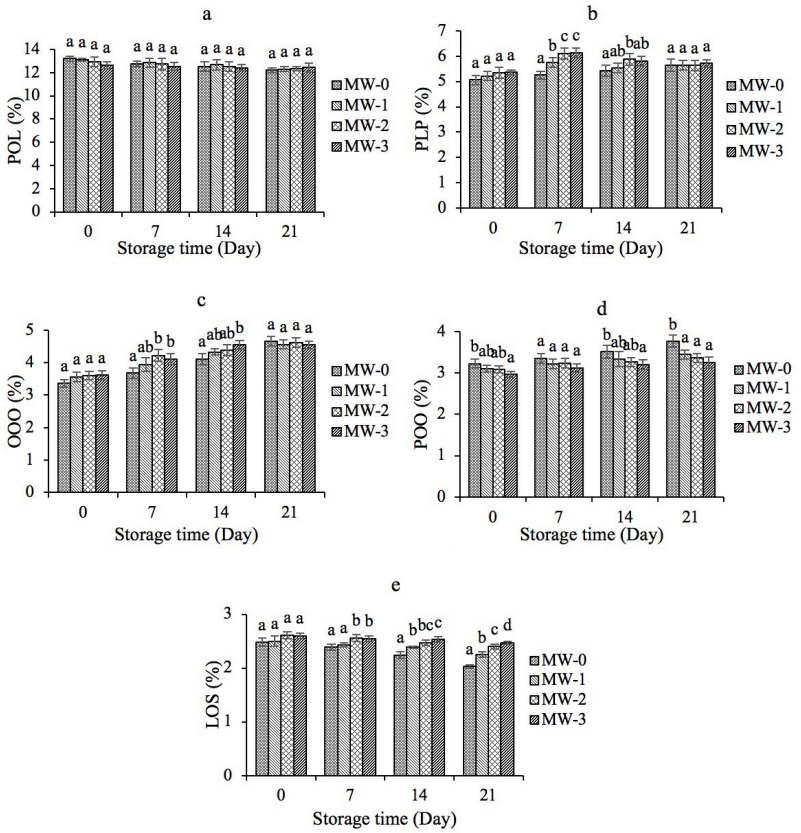

FIGURE 4. Changes in triacylglycerol composition of untreated (MW-0) and pre-treated (MW-1, pre-treated for 1 min; MW-2, pre-treated for 2 min; and MW-3, pre-treated for 3 min) black cumin seed oils during storage. (a) POL, (b) PLP, (c) OOO, (d) POO, and (e) LOS. Each value is the mean ± standard deviation of triplicate determinations. Mean values were compared by Duncan's multiple range test. Values in each storage time grouped with different letters on bars are significantly different ($p < 0.05$).

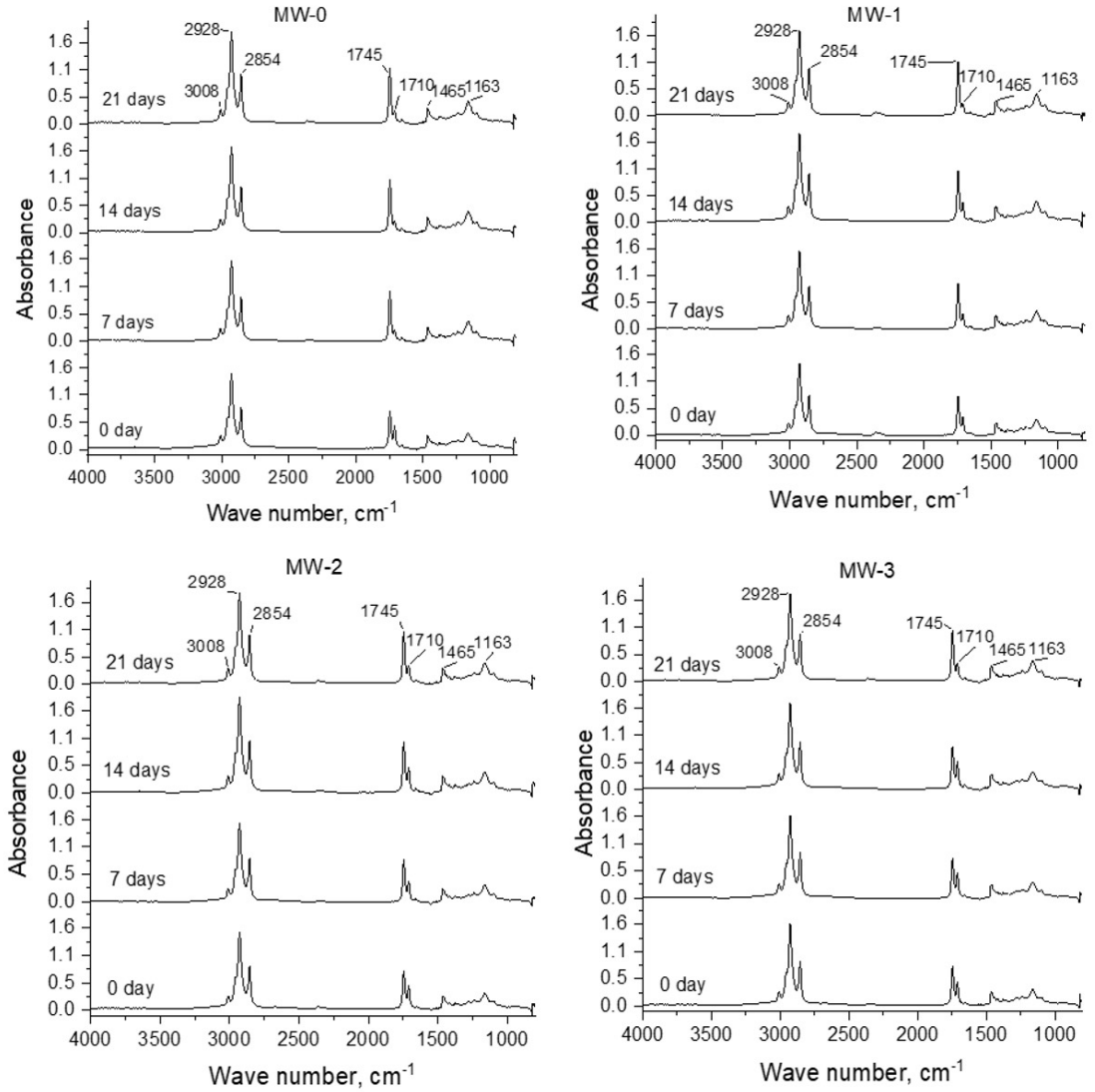

FIGURE 5. Changes in FT-IR spectra of black cumin seed oils extracted from untreated and pre-treated black cumin seeds during storage. a) 0 days of storage b) 7 days of storage c) 14 days of storage, and d) 21 days of storage.

This increment can be attributed to the formation of free radicals under accelerated oxidation conditions which initiate a primary oxidation reaction in unsaturated fatty acids (Moharam *et al.,* 2010*;* Belitz and Grosch*,* 1999). This interpretation agrees with that reported in the literature (Farag *et al.,* 1992). The bands at 2928 and 2854 cm^{-1} enhanced their intensity (absorbance) because of the surrounding chemical changes taking place due to the oxidation process. The vital peak at 1745 cm^{-1} corresponds to the carbonyl substances generated from the decomposition of hydroperoxide during accelerated oxidation (Smith *et al.,* 2007); the absorbance of it increased with oxidation time. The intensity of a weak peak near 1465 cm^{-1} increased with the oxidation treatment. The intensity of the peak at 1163 cm^{-1} related to the proportion in the sample of saturated acyl groups (Guillen *et al.,* 1997), showed similar alterations under storage conditions, and increased its intensity. A similar trend was followed for the peaks at 2927, 2854, 1745, 1465 and 1161 cm^{-1} by Valdés *et al.* (2015) for almonds during storage at 62 °C. In this research, the peak intensities of raw samples were greatly shifted compared to microwaved seed oils during storage, which indicates a clear impact of pre-treatment of BCOs. During storage, the intensities of absorbance of almost all peaks increased and these increments were bigger in fresh oils, which is attributed to the oxidative reactions

proceeding more rapidly in the fresh oils than in the microwaved ones. Similar results from IR data were reported by Jan *et al*. (2019) upon pan and microwave roasting of black cumin seeds. The results from the change in FTIR spectra are also in accordance with those shown in the changes in oxidative indices.

4. CONCLUSIONS

The present data reflects the promising impacts of microwave irradiation on the oxidation stability and compositional changes in black cumin seed oil. Oxidative indices indicate higher tendency to generate volatile and non-volatile oxidation products in the untreated oil samples compared to microwaved oil samples during storage. The exposure of black cumin seed to microwaves caused no major change in the concentration in fatty acids in the oils. During the oxidation treatment at 62 °C, both microwaved and untreated seed oils become oxidized with the decomposition of PUFA and generation of some unexpected and harmful substances. However, the slower degradation rate of PUFA in microwaved samples during treatment probably provided protection against the oil oxidation process. In conclusion, the changes in oxidation parameters, FAC, TAGs and FTIR spectral data at 21 days of storage were more evident for untreated black cumin seeds, which indicate a higher extent of oxidative compounds compared to those found in microwaved samples. Finally, the difference in quality or stability may illustrate the importance of Maillard reaction products generated by the action of microwave in seed samples.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare there are no conflicts of interest.

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The effects of processing aids and techniques on olive oil extractability and oil quality indices

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SUMMARY: This study was conducted to investigate the effects of processing aids and techniques such as talcum powder (2% w/w), calcium carbonate (2% w/w), warm water dipping (45 °C), combined treatment (warm water dipping+2% calcium carbonate) and control (without adding processing aid) on extractability and quality of 'Tarom 7' olive oil as randomized complete block design with three replicates. The results showed that there were no significant differences in the carotenoid content, K_{232}, fatty acid profile or the Cox's value in the oil obtained from untreated and treated fruits with processing aids. The highest chlorophyll content (0.84 mg/kg), total phenolic content (236.94 mg/kg), paste extractability (8.5%) and the lowest peroxide values (0.32 meqO_2/kg), K_{270} (0.38) were obtained from the oil extracted with 2% talc powder. According to the results, it can be suggested that the 2% talc powder treatment could have a positive effect on olive oil quality and paste extractability.

KEYWORDS: *Oil yield; Olive oil; Processing aids; Profile fatty acids; Qualitative characteristics.*

RESUMEN: ***Efectos de los coadyuvantes tecnológicos y técnicas sobre la extractabilidad e índices de calidad del aceite de oliva.*** Este estudio se llevó a cabo para investigar los efectos de los coadyuvantes del procesamiento y técnicas, como talco (2 % p/p), carbonato de calcio (2 % p/p), inmersión en agua tibia (45 °C), tratamiento combinado (inmersión en agua tibia + carbonato de calcio al 2%) y control (sin adición de coadyuvante) sobre la extractabilidad y calidad del aceite de oliva 'Tarom 7' en un diseño de bloques completos al azar con tres repeticiones. Los resultados mostraron que no hubo diferencias significativas en el contenido de carotenoides, K_{232}, perfil de ácidos grasos y el valor de Cox del aceite obtenido de frutos no tratados y tratados con coadyuvantes de procesamiento. El mayor contenido de clorofila (0,84 mg/kg), contenido de fenoles totales (236,94 mg/kg), extractabilidad de la pasta (8,5%) y los valores más bajos de peróxidos (0,32 meqO_2/kg) y K_{270} (0,38) se obtuvieron para el aceite extraído con 2 % de talco. De acuerdo con los resultados, se puede sugerir que el tratamiento con talco al 2% podría tener un efecto positivo sobre la calidad del aceite de oliva y la extractabilidad de la pasta.

PALABRAS CLAVE: *Aceite de oliva; Características cualitativas; Coadyuvantes de elaboración; Perfil de ácidos grasos; Rendimiento de aceite.*

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1. INTRODUCTION

Olive trees, *Olea europaea*, are one of the most important commercial fruit crops in the world which originated from the eastern Mediterranean region (Khaleghi *et al*., 2015). The olive oil is the main product of olive trees and plays a key role in human health, due to its high contents in antioxidants and monounsaturated fatty acids (Altieri *et al*., 2015).

Reviews have shown that only 70-80 % of the oil located in the vacuoles of pulp cells can be extracted during the oil extraction process (Aguilera *et al*., 2010; Caponio *et al*., 2016). The rest of the oil (20-30 %) remains in the form of microgels and emulsion within the vegetable water (Espínola *et al*., 2009; Caponio *et al*., 2014; Al-Okaby *et al*., 2015).

It has been reported that most olive oil-producing countries (such as Spain and Italy) have utilized processing aids (co-adjuvant agents and techniques) to improve oil extraction efficiency (up to 10 to 30%) and to reduce the loss in oil in pomace (García *et al*., 2005; Cruz *et al*., 2007; Canamasas and Ravetti, 2011). Although talc and micronized talc powder (Fernández-Valdivia *et al*., 2008), specific enzymes (Sharma and Sharma, 2007; Najafian *et al*., 2009), common salt (NaCl, KCl), calcium carbonate (Espínola *et al*., 2009) and hot water dipping (García *et al*., 2005; Al-Rousan, 2017), as processing aids and techniques, have been commonly applied in the olive oil industry, nowadays, in Europe, only the use of hydrate magnesium silicate (natural talc) and kaolinitic clays are permitted (Vidal *et al*., 2018). Generally, these aids have improved oil extractability from the olive paste by breaking down the oil-water emulsion (Sadkaoui *et al.,* 2016). Researchers have stated that the effects of processing aids on oil extractability and quality could be different among olive cultivars (Peres *et al*., 2014; Al-Rousan, 2017). So, Espínola *et al*. (2009) reported that the oil extracted from 'Picual', 'Hojiblanca' and 'Arbequina' cultivars treated with 2% calcium carbonate was 1.08, 1.19 and 1.11 times greater than those of untreated extraction, respectively. Several studies have indicated that the application of processing aids enhanced oil extractability by 80.6 to 92.8%, leading to an increase in oil extraction yield from 18.9 to 22.1% (Cruz *et al*., 2007; Carrapiso *et al*., 2013; Caponio *et al*., 2014).

However, no report indicating the negative impacts of processing aids on organoleptic and chemical characteristics of oil upon adding the processing aids has been published (Carrapiso *et al*., 2013; Espínola *et al*., 2015). The sensory analysis results published by Vidal *et al*. (2018), indicated that the bitter, fruity and spicy attributes of olive oil were not significantly altered by using talc. Also, Espínola *et al*. (2009) stated the flavor or taste of olive oil was not changed by adding calcium carbonate. García *et al.* (2005) showed that panel tests of olive oil obtained by heating fruit was no different from that of untreated but the studies demonstrated that the concentration and type of processing aids imposed significant impacts on oil quality. So, Fernández-Valdivia *et al.* (2008) showed that the chlorophyll and carotenoid contents in olive oil did not change upon adding talc powder at either 1 or 2%. Also, the results reported by Cruz *et al.* (2007) and Moya *et al.* (2010) revealed that processing aids would not alter the chlorophyll content in the olive oil. At the same time, Al-Rousan (2017) found that the chlorophyll pigments were higher in the oil obtained from pre-heated fruits than those of untreated. In addition, Moya *et al.* (2010) reported that the amount of extinction coefficient (K_{270}) decreased by adding talc powder. The results published by Caponio *et al.* (2014) showed that adding talc powder at 1 and 2% during the malaxation stage to the 'Coratina' olive cultivar lowered the peroxide value of the extracted oil. García *et al*. (2005) reported that total phenol content was lower in the oil extracted with heated fruit compared to non-heated fruit.

One of the major problems in olive production areas in the Southwest of Iran is the high temperature during the accumulation of oil in the fruit, which causes the amount of oil produced, in particular, 'Tarom 7' cultivar of olive, to be reduced. Considering that there is no study on the effect of the processing aids on increasing oil extractability and improving the chemical properties of oil of 'Tarom 7' olive cultivar, in Iran, the present study aimed to investigate the effects of processing aids on the oil extractability and quality of olive cv. Tarom 7.

2. MATERIALS AND METHODS

2.1. Site of experiment and sampling method

This study was conducted on 15-year-old olive trees of cv. 'Tarom 7' grown with 5×6 m between and within rows in the olive orchard collection at

Shahid Chamran University of Ahvaz, located in the western area of Karun River in Ahvaz city, Iran (31°20´ N, 48°41´ E, 22 m above sea level).

From each tree, 3 kg of healthy fruits were harvested by handpicking in early November, 2016. Fruits of 15 trees were picked according to the maturity index of 4.2 (Espínola *et al*., 2009). Harvested fruits were immediately transferred to the physiology laboratory of horticulture and divided into 5 groups of 9 kg (with three replicates each including 3 kg).

2.2. Oil extraction

30 kg of healthy olive fruits were harvested and divided into 5 groups of 6 kg (three repetitions of 2 kg each). Each group was considered as a treatment. For the extraction of olive oil, an Abencor system (Commercial Abengoa S.A., Sevilla) was used. First, the olive fruits were crushed with a hammer mill, and the paste was malaxed for 30 minutes in the thermoheater at 25 ºC. Then the paste was centrifuged at 5000 rpm for 30 minutes. Finally, the oil samples were separated and stored at 4 ºC in the dark.

2.3. Processing aids and techniques

2.3.1. Solid aids

Talc powder (2% w/w) and calcium carbonate (2% w/w) were added to the paste at the beginning of the malaxation stage. For each replicate, 1.96 kg paste was taken from olive fruits crushed with a hammer mill then 40 g talc powder or calcium carbonate were added at the beginning of the malaxation stage.

2.3.2. Warm water dipping

Healthy olive fruits were immersed in a thermostatic water bath at 45 ° C for 5 min prior to the beginning of the oil extraction process.

2.3.3. Warm water dipping and solid aid

First, fruits were treated with warm water dipping (45 ° C for 5 min) before oil extraction, and then, 1.96 kg paste was taken from olive fruits crushed for each replicate and 40 g calcium carbonate were added to the paste at the beginning of malaxation. Olive oil extracted without any treatments was considered as control.

2.4. Determination of oil quality parameters

2.4.1. Free acidity

Free acidity (% oleic acid per 100 g oil) was determined as described by the European Community Reg. 2568/91 (EEC, 1991). 50 mL of ethanol: chloroform (50:50) were added to 10 g of oil. Next, the solution was titrated with 0.1 N KOH in the presence of phenolphthalein as indicator. Finally, the acidity was calculated according to equation (1)

$$\%\ \text{Free Fatty Acid} = \frac{(\text{mL of titrant})(\text{Nof titrant})(\text{Mwt. of fatty acid})}{(\text{sample wt.})(10)} \quad (1)$$

Where N is normality; Mwt is the molecular weight of oleic acid (282); and M is molarity.

2.4.2. Chlorophyll and carotenoid contents

The chlorophyll and carotenoid contents in the oil were determined using a spectrophotometer (UNICO UV-2100, manufactured in USA) as described by Mínguez-Mosquera *et al*. (1991). For this purpose, 1 g of the olive oil was dissolved in 10 mL of isooctane solution and the absorption spectrum of the solution was captured at wavelengths of 670 nm and 470 nm for the chlorophyll and carotenoid, respectively. Subsequently, the chlorophyll and carotenoid contents of the oil were evaluated in mg/kg of oil using Equations (2) and (3), respectively.

$$\text{Chlorophyll (mg/kg)} = (A670\times 10^6)/(613 \times 100 \times d) \quad (2)$$

$$\text{Carotenoid (mg/kg)} = (A470\times 10^6)/(2000 \times 100 \times d) \quad (3)$$

Where A is the absorption number and d is cell thickness.

2.4.3. UV extinction coefficient

To evaluate the specific extinction coefficient (K_{232} and K_{270}), 250 mg of oil were diluted with 25 mL of cyclohexane (of spectrophotometry grade) and then homogenized with a vortex for 30 seconds. The absorption of the solution was then determined at wavelengths of 232 nm and 270 nm

by a spectrophotometer (UNICO UV-2100, manufactured in the USA) according to the European Commission Regulation EEC/2565/91 (EEC, 1991).

2.4.4. Peroxide value

Peroxide value (milliequivalents of active oxygen per kilogram of oil) was determined according to AOCS (1998) method cd8-53.

Briefly, 30 mL of acetic acid–chloroform solution (3:2 v/v) were added to 5 g of oil. Then, 0.5 mL of saturated potassium iodide solution (KI) was added to the solution and the mixture was left for 1 minute. Subsequently, 30 mL of distilled water were added immediately.

The solution was titrated with 0.1 N sodium thiosulfate until the yellow iodine color almost disappeared. Next, a few drops of starch were added to the solution before being titrated with a 0.02 N thiosulfate solution. Finally, the corresponding peroxide value was obtained from Equation (4).

$$\text{Peroxide value (meq}O_2\text{/kg)} = \frac{(S-B)\times N\times 1000}{\text{mass of sample, g}} \quad (4)$$

Where B is the volume of titrant (mL of blank), S is the volume of titrant, mL of sample, N is the normality of the sodium thiosulfate solution.

2.4.5. Total phenolic content

The total phenolic content in the oil was obtained according to Montedoro *et al*. (1992) using the Folin–Ciocalteu reagent. Total phenolic content was calculated in mg of gallic acid per kg of olive oil. In this method, 2 g of olive oil and 2-3 drops of TWEEN® 20 were added to 10 mL of a methanol: water solution (80:20). The solution was centrifuged at 5000 rpm for 10 minutes, the supernatant was collected and the residual solution was once more added with 10 mL of the methanol: water solution and re-centrifuged under the same conditions before collecting the supernatant. In the next step, 1 mL of the obtained solution was combined with 1 mL of the methanol: water solution and 5 mL of double-distilled water. Then, 0.5 mL of the Folin–Ciocalteu reagent and 2 mL of 15% sodium carbonate were added to the solution. Finally, 1.5 mL of double-distilled water were added to the obtained solution and the mixture was subjected to vortex for 30 seconds. The resultant mixture was left in the dark for 2 hours and then the absorbance of the solution was read at 765 nm by a spectrophotometer (UNICO UV-2100, manufactured in the USA), and total phenol content was obtained from the following equation.

$$\text{Total phenol content}\left(\frac{\text{mg of gallic acid}}{\text{kg of oil}}\right) = \frac{\text{Galic acid (mg/ml)} \times \text{V (ml)} \times 1000}{\text{W (g)}} \quad (5)$$

Where V is the volume of the solution, W is the weight of the oil sample.

2.4.6. Fatty acid profile

The Fatty acid composition was determined according to European Official Methods of Analysis (EEC, 1991). 100 mg oil sample were dissolved in 10 mL n-hexane with 100 µL 2 N methanolic potassium hydroxide solution. Then the sample was vigorously shaken for 30 seconds and centrifuged for 15 minutes. The supernatant phase was used for chromatographic analysis. Chromatographic analyses were performed on Young Lin ACME 6000 (manufactured in South Korea) equipped with a flame ionization detector (FID), a split/splitless injector, and a BPX-70 capillary column (100 m ×0. 25 mm ID×0.2 µm film thicknesses, SGE, Australia). Helium was used as carrier gas. The temperatures of the injector, oven, and detector were set at 230, 200, and 280 °C, respectively.

2.4.7. Cox value

The cox value or oxidation index was obtained based on the content in 18-carbon fatty acids, according to the following equation:

$$\text{Cox value} = \frac{[(\%C18{:}1)1 + (\%C18{:}2)10.3 + (\%C18{:}3)21.6]}{100} \quad (6)$$

Where C18:1, C18:2, and C18:3 are the oleic acid, linoleic acid, and linolenic acid contents, respectively (Fatemi and Hammond, 1980).

2.5. Statistical analysis

These experiments were conducted with a completely randomized design in three replicates. The data were subjected to analysis of variance (ANOVA) using SAS Ver. 9.1 Software. Mean comparison of data was performed using Duncan's multiple range test at 5% significance level.

3. RESULTS

3.1. Chlorophyll and carotenoid contents

As shown in Table 1, a significant difference was observed in chlorophyll content among all treatments. The oil extracted with 2% talc powder (0.84 mg/kg) and 2% calcium carbonate (0.83 mg/kg) exhibited the highest chlorophyll content, while the lowest chlorophyll content (0.4 mg/kg) was related to the combined treatment (warm water dipping+2% carbonate calcium). In addition, chlorophyll contents in the oil extracted with warm water dipping, 2% calcium carbonate, and 2% talc powder were 0.7, 0.83, and 0.84 mg/kg, respectively, which were 1.22, 1.45, and 1.47 times greater than that of the control treatment (0.57 mg/kg). This result showed that there was no significant difference in chlorophyll content between the extracted control oil and combined treatments. Although the carotenoid value ranged from 0.22 to 0.32 mg/kg, the amount of carotenoid was not different among treatments. The processing aids and techniques used did not have significant effect on the carotenoid content (Table 1).

3.2. K_{232} and K_{270} extinction coefficients

No statistically significant difference in the K_{232} extinction coefficient was found between the oil extracted from untreated (control) and treated fruits with processing aids and techniques (Table 1).

Furthermore, the results showed that the processing aids and techniques had a statistically significant effect on the K_{270} extinction coefficient. The lowest value for the K_{270} extinction coefficient (0.33) was observed in the oil extracted with warm water dipping, which did not show a significant statistical difference between the oils extracted with either 2% talc powder (0.38) or the control treatment (0.37). The highest value for the K_{270} extinction coefficient

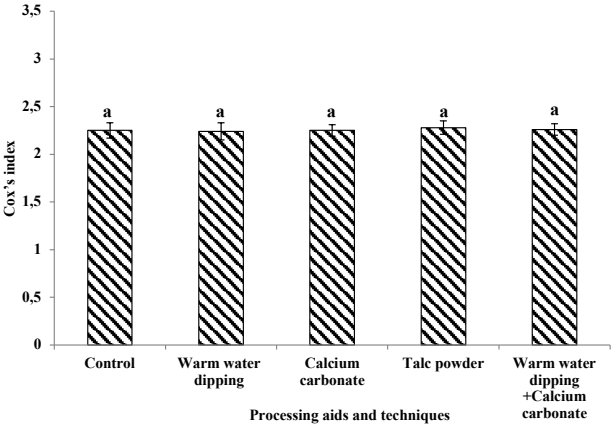

Figure 1. Effect of processing aids and techniques on Cox index. Values (Mean±Standard Deviation) with different letters are significantly different ($p < 0.05$) using Duncan's multiple range test. Number of replicates = 3

Table 1. Effect of different processing aids and techniques on `Tarom 7' olive oil quality indices

Oil quality indices	Processing aids treatments				
	Control	Warm water dipping (45 °C)	Calcium carbonate (2 % w/w)	Talc powder (2% w/w)	Warm water dipping (45 °C)+ Calcium carbonate (2% w/w)
Chlorophyll (mg/kg)	0.41±0.19^{c}	0.70±0.09^{b}	0.83±0.09^{a}	0.84±0.11^{a}	0.57±0.06^{c}
Carotenoid (mg/kg)	0.25±0.07^{a}	0.22±0.12^{a}	0. 31±0.10^{a}	0.32±0.11^{a}	0.32±0.18^{a}
Free acidity (% oleic acid)	0.35±0.01^{a}	0.32±0.01^{b}	0.26±0.01^{c}	0.31±0.01^{b}	0.25±0.01^{c}
K_{270}	0.37±0.06^{b}	0.33±0.07^{b}	0.44±0.02^{a}	0.38±0.05^{b}	0.45±0.03^{a}
K_{232}	2.79±0.06^{a}	2.79±0.07^{a}	2.74±0.05^{a}	2.73±0.08^{a}	2.79±0.08^{a}
Peroxide value (meq O_2/kg oil)	0.40±0.01^{a}	0.37±0.01^{b}	0.29±0.01^{d}	0.32±0.01^{c}	0.32±0.01^{c}
Total phenol content (mg/kg)	174.44±2.03^{d}	181.38±12.51^{c}	222.77±3.08^{b}	236.94±4.21^{a}	193.86±12.38^{c}

Values (Mean± Standard Deviation) in the same row with different superscripts are significantly different ($p < 0.05$) using Duncan's multiple range test.
Number of replicates = 3

(0.45) was related to the combined treatment (warm water dipping+2% carbonate calcium), which indicated no significant difference with the 2% calcium carbonate treatment (0.41) (Table 1).

Moreover, the value for the K_{270} extinction coefficient in the oils treated with 2% calcium carbonate was approximately 18.91% higher than that of the control treatment. The increased rate of the K_{270} extinction coefficient in fruits treated with warm water dipping+2% carbonate calcium was 21.62% compared to the untreated fruits (Table 1).

3.3. Free acidity

A significant difference was observed in free acidity between treated fruits treated with processing aids and untreated fruits. The highest and lowest values for free acidity were obtained for the control (0.35%) and combined treatment (warm water dipping+2% carbonate calcium) (0.25%), respectively (Table 1).

Moreover, the results showed that free acidity was the same in warm water dipping treated and untreated fruits. Also, there was no significant difference in the free acidity in the oil obtained from talc powder and combined treatments (Table 1).

Based on the results, the acidities of the extracted oils with warm water dipping, 2% calcium carbonate, combined treatment (warm water dipping+2% carbonate calcium), and 2% talc powder were 8.57, 25.71, 25.71, and 11.42% higher than that of the extracted oil under control treatment, respectively (Table 1).

3.4. Total phenol content

The data showed that the processing aid treatments had a significant effect on total phenol content. The highest total phenol content (236.94 mg/kg of oil) was related to the oil extracted with 2% talc treatment; while the oil extracted with the combined treatment (warm water dipping+2% carbonate calcium) showed the lowest total phenol content (174.44 mg/kg of oil). Compared to the control treatment, the 2% talc and 2% calcium carbonate treatments exhibited 22.24 and 14.93% higher total phenolic contents, respectively; while the oil extracted with warm water dipping or under the combined treatment (warm water dipping + 2% carbonate calcium) showed lower values for total phenolic content by 6.42 and 10%, respectively.

3.5. Peroxide value

As shown in Table 1, the results indicated that the application of the processing aids tended to attenuate the peroxide value of the extracted oil, as compared to the control treatment. Accordingly, the highest peroxide value (0.40 meqO_2/kg) was that of the oils obtained from untreated fruits; while this index was minimal (0.29 meqO_2/kg) for the oil extracted with 2% calcium carbonate. The peroxide values for the oils obtained with warm water dipping, 2% calcium carbonate, combined treatment (warm water dipping + 2% carbonate calcium), and 2% talc powder were found to be 7.5, 27.5, 20, and 12.5% lower than that of the extracted oil under control treatment, respectively.

3.6. Profile of fatty acids and Cox value

The results indicated that the oils extracted from fruits which were treated and untreated with processing aids showed the same values for palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), oleic acid/linoleic acid ratio (C18:1/C18:2), monounsaturated fatty acids-to-polyunsaturated fatty acids ratio (MUFA/PUFA), unsaturated fatty acids-to-saturated fatty acids ratio (UFA/SFA), and the Cox value; whereas the value for palmitic acid (C16:0) was different between the oils extracted with processing aids and that of the control treatment (Table 2; Figure 2).

The highest and lowest contents in palmitic acid (16.30 and 16.03%, respectively) were related to 2% calcium carbonate and warm water dipping treatments, respectively.

3.7. Paste extractability (%)

The impact of the use of processing aids and technique treatments on paste extractability is shown in Figure 2. A significant difference in paste extractability was found between the control treatment and the treatment with processing aids. The use of processing aids and technique treatments improved paste extractability. The lowest (4.07%) and the highest (9.4%) improvement in paste extractability were related to control and 2% talc powder treatments, respectively. In addition, no significant difference in paste extractability was observed between fruits treated with calcium carbonate (2%) and

TABLE 2. Effect of different processing aids and techniques on the fatty acid composition of `Tarom 7' olive oil.

Fatty acid composition (%)	Processing aids treatments				
	Control	Warm water dipping (45 °C)	Calcium carbonate (2% w/w)	Talc powder (2% w/w)	Warm water dipping (45 °C)+ Calcium carbonate (2% w/w)
Palmitic acid (C16:0)	16.26±0.12[a]	16.03±0.11[b]	16.30±0.19[a]	16.08±0.15[b]	16.09±0.08[b]
Palmitoleic acid C16:1)	1.67±0.31[a]	1.58±0.23[a]	1.59±0.21[a]	1.58±0.19[a]	1.59±0.23[a]
Stearic acid (C18:0)	2.2±0.35[a]	2.22±0.20[a]	2.18±0.18[a]	2.15±0.15[a]	2.19±0.31[a]
Oleic acid (C18:1)	54.3±0.71[a]	54.07±1.37[a]	53.59±0.98[a]	54.53±1.01[a]	53.95±0.96[a]
Linoleic acid (C18:2)	12.95±0.61[a]	12.87±0.40[a]	13.15±0.71[a]	13.26±0.81[a]	13.16±0.92[a]
Linolenic acid (C18:3)	1.72±0.31[a]	1.73±0.22[a]	1.68±0.09[a]	1.71±0.12[a]	1.70±0.26[a]
SFA[c]	18.46±0.35[a]	18.25±0.42[a]	18.48±0.32[a]	18.23±0.52[a]	18.28±0.62[a]
MUFA[d]	54.97±1.31[a]	55.65±1.02[a]	55.18±1.32[a]	56.11±1.41[a]	55.54±0.01[a]
PUFA[e]	14.67±0.91[a]	14.60±0.52[a]	15.18±0.62[a]	14.97±0.71[a]	14.86±0.01[a]
MUFA/PUFA[f]	3.81±0.40[a]	3.82±0.22[a]	3.72±0.33[a]	3.74±0.62[a]	3.73±0.01[a]
UFA/SFA	3.82±0.62[a]	3.84±0.42[a]	3.78±0.34[a]	3.91±0.22[a]	3.85±0.01[a]
Oleic/Linoleic	4.19±0.31[a]	4.21±0.28[a]	4.07±0.21[a]	4.11±0.31[a]	4.11±0.01[a]

[a-b] Values (Mean± Standard Deviation) in the same row with different superscripts are significantly different ($p < 0.05$) using Duncan's multiple range test.
[c]: Saturated fatty acids. [d]: Monounsaturated fatty acids. [e]: Polyunsaturated fatty acids. [f]: Monounsaturated fatty acids / Polyunsaturated fatty acids. Number of replicates = 3

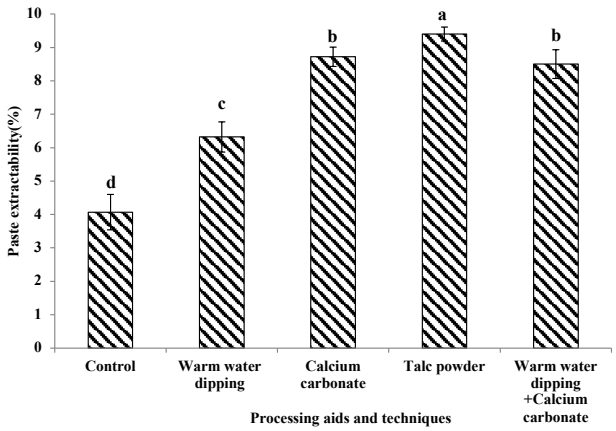

FIGURE 2. Effect of processing aids and techniques on paste extractability (%)
Values (Mean±Standard Deviation) with different letters are significantly different ($p < 0.05$) using Duncan's multiple range test. Number of replicates = 3

combined treatment. Paste extractability was lower in warm water dipping (6.32%) compare to calcium carbonate (2%) and combined treatment.

4. DISCUSSION

4.1. Chlorophyll and carotenoid content

Chlorophyll and carotenoid are the main pigments in olive oil. These pigments play an important role in oxidative activity (Khaleghi *et al*., 2015). Our findings showed that chlorophyll content was affected by the type of processing aids. The amount of chlorophyll was higher in oil extracted from fruits treated with processing aid in comparison to those of untreated fruits. The highest chlorophyll content (0.84 mg/kg) was obtained from oil extracted with 2% talc powder; while the lowest chlorophyll content (0.57 mg/kg) was observed in the control treatment. Results showed there was no significant difference in carotenoid content between control treatment and processing aid treatments. These findings were in agreement with the results reported by several authors who indicated that chlorophyll pigment contents were significantly increased by the addition of processing aids such as pre-heated treatment, or talc powder (García *et al*. 2005; Criado *et al*., 2007; Caponio *et al*., 2014; Al-Rousan, 2017). Reviews showed that a warm water dipping treatment could inactivate enzymes, especially lipoxygenase, which was important to pigment degradation (García *et al*., 2005). Cruz *et al*. (2006) stated that salt aids could increase the solubility of pigments in oil. Our results are not in agreement with the results of Moya *et al*. (2010) for picual olive, Cruz *et al*. (2007), Espínola *et al*. (2015), or Peres *et al*. (2014), who believed that the processing aids imposed no significant effect

on not only chlorophyll pigment and carotenoid contents but also on the xanthophyll content. It seems that this inconsistency could be attributed to genetic and physiological differences between the studied cultivars or differences in the type of processing aids.

4.2. K_{232} and K_{270} extinction coefficients

According to published reports, the extinction coefficient provides a measurement of secondary oxidation processes in the oil that lead to the formation of conjugated dienes (K_{232}), aldehydes, and ketones (K_{270}) (Moya *et al*., 2010; Carrapiso *et al*., 2013; Caponio *et al*., 2014). Our findings revealed no significant difference among the considered treatments in terms of the K_{232} index, but rather significant differences in K_{270}, so that the combined treatment (warm water dipping + 2% calcium carbonate) and also the 2% calcium carbonate treatment could further inhibit the formation of aldehydes and ketones, as compared to other treatments. These results were in agreement with the reports submitted by Moya *et al*. (2010). Also, Caponio *et al*. (2014) and Carrapiso *et al.* (2013) showed that talc powder increased the value of K_{270}, whereas K_{232} was not affected by the addition of talc. These published results are similar to the present study.

4.3. Free acidity

Previous studies have shown that oil acidity is a result of the formation of free fatty acids upon the activity of a particular type of enzyme to decompose triglycerides (Cruz *et al*., 2007; Moya *et al*., 2010). Our findings showed that processing aids tend to affect the activity of the enzyme involved with the process of triglyceride decomposition so that the combined treatment (warm water dipping + 2% calcium carbonate) could further retard the activity of this enzyme.

However, these findings were not in agreement with the results of García *et al*. (2005), Fernández-Valdivia *et al*. (2008), Moya *et al*. (2010), Carrapiso *et al*. (2013), and Caponio *et al*. (2014), who concluded that processing aids such as hot water dipping, talc, and micro talc did not affect free fatty acids. Canamasas and Ravetti, (2014) found that oil extracted with Talc (2.0%) and microtalc had the lower acidity (free fatty acid) than the control. These results are in agreement with the present study. Farag *et al*. (1997) demonstrated that some processing aids such as pre-heated treatment (microwave heating) could reduce the amount of free fatty acids by reducing lipase activity.

4.4. Total phenol content

The results of the present experiment showed that the processing aids affected the release of phenolic compounds and their transmission into the oil phase. Contrary to the pigments which develop in particular parts of the fruit, phenolic compounds are found in most parts of fruit in various forms, i.e. water-soluble or fat-soluble. The processing aids significantly affected the solubility and release of these compounds. Among other treatments, the 2% talc powder imposed the largest impacts on the solubility and release of these compounds.

Although the results of this study were inconsistent with previous reviews about exposing olive fruits of ‘carrascina’, ‘Galega’, ‘Cobrançosa’ and ‘Vulgar’cultivras to warm water and talc (Carrapiso *et al*., 2013; Peres *et al*., 2014), our findings were in agreement with those of Ben-David *et al*. (2010), Caponio *et al*. (2014) and Al-Rousan (2017) on ‘Barnea’, ‘Nabali Baladi’, ‘Nabali Muhassan’ olive cultivars who reported that the total phenolic content f the olive oil was enhanced in the presence of processing aids.

Servili *et al*. (2003) also indicated that pre-heated treatment reduced total phenol compounds in the olive oil by increasing polyphenol oxidase and peroxidase activities.

4.5. Peroxide value

According to existing reports, peroxide value represents the primary oxidation of the oil and serves as an important factor in determining the quality of olive oil, indicating whether the oil is healthy or rather spoiled. When olive oil or olive fruit is exposed to free air where it comes in contact with oxygen in the presence of adverse temperature conditions, primary oxidation of the oil or fruit begins; this process then contributes to increased peroxide content in the oil and the formation of free radicals in the oil (Moya *et al*., 2010; Peres *et al*., 2014). The processing aids tend to inhibit this oxidation process and control the peroxide value of the oil by controlling the oxygenated free radicals. The results of this study showed that the 2%

calcium carbonate lowered the peroxide value of the extracted oil. This finding was in agreement with the findings of Caponio *et al*. (2014), who reported that the presence of processing aids tends to decrease the peroxide value of the extracted oil from the 'Coratina' olive cultivar, which is rather inconsistent with the reports published by Cruz *et al*. (2007).

The difference in the results could possibly be due to the type of cultivar, concentration, and type of processing aids.

4.6. Profile of fatty acids and Cox's value

The results of this study indicated that the profile of fatty acids and Cox value were not affected by processing aids. these findings were similar to the results of Ben Brahim *et al*. (2015), who stated there was no significant difference between the effects of the calcium carbonate (1.5%) treatment and the control treatment on the contents of palmitic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), polyunsaturated fatty acids, (PUFA), and unsaturated fatty acids (UFA) as well as the MUFA/PUFA ratio in the 'Chemlali' olive cultivar.

4.7. Paste extractability (%)

According to the results, adding processing aids increased paste extractability. While paste extraction rate was lower in untreated fruits with processing aids. In fact, the highest paste extractability was related to the 2% talc powder treatment. The results of this research were in agreement with the reports published by Carrapiso *et al*. (2013), Koprivnjak *et al*. (2016), Caponio *et al*. (2014), and Moya *et al*. (2014), who observed that physical processing aids tended to enhance the efficiency of the oil extraction process. Vidal *et al.* (2018) reported that an increase greater than 2% of talc proportion can have a negative effect on extraction efficiency. Canamasas and Ravetti, (2014) found that talc powder and calcium carbonate improved paste extractability from 8.2 to 10.2 and 10.7% for the 'Arbequina'olive cultivar, respectively. Cruz *et al*. (2007) indicated that pre-heating the fruit caused cell walls to soften, and release oil from the cells. Other researchers believed that talc powder and calcium carbonate, as solid aids, could help to break oil-water emulsions and improve paste extractability (Canamasas and Ravetti, 2014).

5. CONCLUSIONS

In this experiment, the processing aids and techniques used could facilitate the process of oil extraction and enhance the efficiency of the process compared to the control by contributing to the coalescence of fine and coarse oil droplets and altering the cell wall and membrane to release larger amounts of oil content from the cells and other fruit components. According to the results, the value of oil extracted with 2% talc powder was greater than the other treatments. Furthermore, the values for chlorophyll content and total phenol content in oils extracted with 2% talc powder were higher than the control and other treatments. The amount of acidity and peroxide value were significantly reduced in oils obtained by adding 2% talc powder compared to the control treatment. Therefore, the best processing aid used in this study was the 2% talc powder treatment, which was able to improve oil extraction yield and oil quality parameters.

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Preparation of human milk fat substitute and improvement of its oxidative stability

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SUMMARY: 1,3-Dioleoyl-2-palmitoylglycerol (OPO) was synthesized by enzymatic interesterification using palm stearin rich in tripalmitin (PPP) and ethyl oleate. Enzymatic interesterification parameters such as temperature, water content, enzyme load, and substrate molar ratio were optimized. High contents of C52 (primarily OPO and its isomeric compounds) production (46.7%) and *sn*-2 palmitic acid (PA) content of 75.3% were detected. In addition, OPO-human milk fat substitute (HMFS) was blended with coconut, soybean, algal and microbial oils at a weight ratio of 0.70:0.18:0.11:0.004:0.007 to simulate fatty acids in human milk fat (HMF) according to the mathematical model. The main and important fatty acids in the Final-HMFS were within the ranges of those present in HMF. The Final-HMFS could promote the absorption of fats and minerals and the development of retina tissues in infants. The mixture of L-ascorbyl palmitate (L-AP) and vitamin E (VE) resulted in a synergistic antioxidant effect both in OPO-HMFS and OPO-HMFS emulsions. This finding has great significance in improving the quality and extending shelf-life of HMFS.

KEYWORDS: *1,3-dioleoyl-2-palmitoylglycerol; Enzymatic interesterification; Human milk fat substitutes; Oxidative stability; Physical blending.*

RESUMEN: ***Preparación de sustitutos de grasa de leche humana y mejora de su estabilidad oxidativa.*** Se sintetizó el 1,3-dioleoil-2-palmitoilglicerol (OPO), utilizando estearina de palma rica en tripalmitina (PPP) y oleato de etilo, mediante interesterificación enzimática. Se optimizaron los parámetros de la interesterificación enzimática, como la temperatura, el contenido de agua, la carga de enzimas y la relación molar del sustrato. Se lograron altos rendimientos de C52 (principalmente OPO y sus isómeros, 46,7%) y un contenido de ácido palmítico (PA) en *sn*-2 del 75,3%. Además, el sustituto graso de leche humana OPO (HMFS), se mezcló con aceites de coco, soja, algas y microbianos, en una proporción en peso de 0,70:0,18:0,11:0,004:0,007 para simular los ácidos grasos de la leche humana (HMF) de acuerdo con un modelo matemático. Los ácidos grasos principales e importantes en HMFS-Final estaban casi dentro de los rangos de los presentes en HMF. El HMFS-Final podría promover la absorción de grasas y minerales y el desarrollo de los tejidos de la retina en los bebés. La mezcla de palmitato de L-ascorbilo (L-AP) y vitamina E (VE) resultó tener un efecto antioxidante sinérgico, tanto en la emulsión OPO-HMFS como en la OPO-HMFS. Este hallazgo tiene una gran importancia para mejorar la calidad y prolongar la vida útil de HMFS.

PALABRAS CLAVE: *1,3-dioleoil-2-palmitoilglicerol; Estabilidad oxidativa; Interesterificación enzimática; Mezcla física; Sucedáneos de grasa de leche humana.*

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1. INTRODUCTION

Human milk is recognized as the gold standard of infant nutrition because it provides not only the energy and optimum balanced nutrition for growing infants, but also immunological protection to newborns (Wang *et al*., 2019). A population-based birth cohort study of neonates from Brazil showed that the durations of breastfeeding positively correlated with the intelligence quotient (IQ), educational attainment, and income of adults (Victoria *et al*., 2015). Thus, breastfeeding has not only clear short-term benefits, but also influences long-term consequences on human capital. However, the rate of breastfeeding continues to decline because of medical or personal reasons. In these situations, some infants are partly or even fully fed infant formulas. Due to the demand for high-quality infant formulas in recent years, extensive simulation studies on the composition and distribution of HMF fatty acids have been undertaken.

Human milk contains about 2 to 6% fat, of which more than 98% is in the form of triacylglycerols (TAGs), providing approximately 50–60% of the dietary energy required for infants (Wang *et al*., 2019). Commercial infant formulas are usually manufactured from cow milk and vegetable oils which may have similar fatty acid (FAs) compositions to human milk, but quite different position distribution in TAGs. In human milk, saturated fatty acids (SFAs), predominantly PA (20-30% of total FA), are largely located at the sn-2 position (70% of all PA); whereas the sn-1 and sn-3 positions are occupied by unsaturated fatty acids (USFAs), such as oleic acid (OA) (Ghide and Yan, 2021). Thus, the characteristic component of human milk TAGs is OPO. However, cow milk and vegetable oils generally contain SFAs esterified to the sn-1 and sn-3 positions and USFAs at sn-2 position. These differences not only affect the nutrition and digestion of fats, but also cause stool hardness and constipation due to the preferential hydrolysis of PA in the sn-1,3 positions by pancreatic lipase to form calcium soap which is water-insoluble and can hardly be absorbed in the intestine of babies (Lee and Chang, 2021). In contrast, when PA is esterified to the sn-2 position, the 2-monoacylglycerol formed is readily absorbed (Ghide and Yan, 2021), therefore, the kinetics of hydrolysis of different TAGs may induce a different digestive process.

The synthesis of OPO mainly takes place in two steps: the first step is the synthesis of TAGs enriched in PA at position 2 by chemical or enzymatic methods. Zou *et al*. (2012b) produced TAGs enriched in PA at position 2 by chemical interesterification of palm stearin and sodium methoxide as catalyst. Jimenez *et al*. (2010) synthesized TAGs enriched in PA at position 2 by enzymatic interesterification of palm stearin and Novozym 435 as catalyst. In the second step, OPO was obtained by the synthesis of TAGs enriched in PA at position 2 with acyl donors using sn-1,3 regiospecific lipase. Esteban *et al*. (2011) obtained TAGs with 66% PA at sn-2 position and 67.5% OA at sn-1,3 positions, by acidolysis of PA-enriched TAGs and several OA-rich free fatty acid fractions in solvent-free media at 50 °C. Besides, OPO was also obtained by enzymatic interesterification. For instance, Lee *et al*. (2010) synthesized a high OPO content (31.43%) from a PPP-rich fraction and ethyl oleate by a lipase-catalyzed interesterification.

HMF contains long-chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA, 0.2~0.4 mol%, Table 3), arachidonic acid (ARA, 0.3~0.6 mol%, Table 3), which are crucial for infant growth and development (Qin *et al*., 2014, Haddad *et al*., 2012). Specifically, DHA and ARA are present in large amounts in the membranes of the brain and retina (Hoffman *et al*., 2009). Appropriate amounts of these fatty acids play a complex role in the development and function of the neuronal and retinal tissues after birth (Hoffman *et al*., 2009). They can be synthesized from linolenic and linoleic acid precursors by adults but infants lack this capacity and for this reason an adequate supply of LC-PUFAs are recommended in infant formulas (Ab *et al*., 2017).

When preparing HMFS products, the oxidative stability of HMFS must be acceptable during processing and storage. Some unsaturated fatty acids in HMFS, especially DHA and ARA, are easily oxidized to form lipid hydroperoxides and other small oxidation compounds that produce off-flavors via further decomposition. Researchers have reported that the ingestion of a diet high in polyunsaturated fatty acids which are insufficiently protected by antioxidants may increase the risk of atherosclerosis (Sakai *et al*., 1995). Oxidation reduces the nutritional value and safety of HMFS and in order to prevent the oxidation of oil, different methods have been developed to protect against the lipid oxidation effect, although adding antioxidants remains the most effective one.

The aim of this work was to produce HMFS containing OPO and LC-PUFAs, whose structures and contents are highly similar to HMF's. The first step involved enzymatic interesterification of the PPP-rich fraction with ethyl oleate to prepare OPO-HMFS and the second step involved the blending of the OPO-HMFS with other oils in order to adjust the contents of LC-PUFAs to simulate the fatty acid profiles in HMF based on some mathematical model. In order to improve the yield of OPO and reduce production costs, the interesterification reaction conditions were optimized and a linear restraint function was established to calculate the physical mixing ratio of oils in the second step. Improvement in the oxidative stability of HMFS-Emulsion by adding antioxidants was also achieved.

2. MATERIALS AND METHODS

2.1. Chemicals and enzymes

Lipozyme RM IM (*Rhizomucormiehei* immobilized in an ion exchange resin) and Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on a silica support) were purchased from Novozymes A/S (Bagsvaerd, Denmark). Pancreatic lipase powder was purchased from Sigma-Aldrich (Shanghai, China). Palm stearin (PS), coconut oil (CO) and soybean oil (SO) were donated by Shanghai Kerry Oils & Grains Industries Co., Ltd. (Shanghai, China). Algal Oil (AO) and microbial oil (MO) were purchased from Fuxing Biotechnology Co., Ltd. (Wuhan, Hubei). The fatty acid profiles of CO, SO, MO and AO are shown in Table 1. Analytical or chromatographic-grade reagents were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other reagents were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

2.2. Preparation of PPP-rich fraction

Palm stearin was mixed with acetone (1:5 w/v) and placed in a vacuum drying oven at 30 °C for 4 h. The PPP-rich fraction was collected after filtration and the removal of acetone was carried out in a vacuum-rotary evaporator at 60 °C and 1.33 × 103 Pa for 1 h (Zou *et al*., 2012b). The percentage of PPP in the sample increased from 52.8 to 85.4%.

2.3. Lipase-catalyzed synthesis of OPO

The synthesis of OPO was carried out in a solvent-free system. The PPP-rich fraction was mixed acyl (oleic acid or methyl oleate or ethyl oleate) donors at substrate molar ratios of 1:8 and then lipases (10% based on the weight of the reaction mixture) were add-

Table 1. Fatty acid profiles (%) of coconut, soybean, algal, microbial oil

Fatty acid	CO		SO		AO		MO	
	total	sn-2	total	sn-2	total	sn-2	total	sn-2
C8:0	7.9±0.4	1.6±0.1	-	-	-	-	-	-
C10:0	8.1±0.3	3.0±0.1	-	-	-	-	-	-
C12:0	48.9±0.7	80.5±0.9	-	-	-	-	-	-
C14:0	18.4±0.6	8.2±0.6	-	-	8.7±0.4	15.1±0.6	-	-
C16:0	8.3±0.6	1.1±0.1	11.8±0.2	1.4±0.1	32.7±0.6	19.1±0.4	9.2±0.1	6.5±0.5
C18:0	2.2±0.1	-	3.3±0.1	-	0.7±0.5	1.1±0.2	5.1±0.3	4.4±0.5
C18:1	4.7±0.2	4.4±0.2	21.3±0.4	22.8±0.5	1.4±0.3	4.7±0.2	11.7±0.4	17.9±0.6
C18:2	1.5±0.1	1.2±0.1	54.8±0.3	70.6±0.8	0.4±0.1	-	9.4±0.4	15.6±0.6
C18:3	-	-	7.7±0.2	5.2±0.2	0.3±0.1	0.3±0.1	5.1±0.3	6.7±0.2
C20:0	-	-	0.3±0.1	-	0.4±0.2	-	6.6±0.1	-
C20:4n-6	-	-	-	-	-	-	48.1±0.6	45.6±0.8
C22:0	-	-	0.8±0.1	-	-	-	4.8±0.2	3.3±0.1
C22:5n-3	-	-	-	-	7.1±0.2	8.1±0.2	-	-
C22:6n-3	-	-	-	-	48.3±0.7	51.6±0.9	-	-

-: compound not detected because its content was too low. CO: Coconut Oil; SO: Soybean Oil; AO: Algal Oil; MO: Microbial Oil. Results are expressed as mean ± standard deviation (n = 2)

ed to start the reaction in a shaking water bath at 55 °C and stirred at 200 rpm. The synthesis conditions were selected on the basis of previous research presented in this paper.

2.4. Fatty acid composition analysis

The fatty acid composition of the products was analyzed through GC-MS using a Shimadzu GC2010A (Kyoto, Japan) gas chromatography instrument coupled with a GCMS-QP2010 quadrupole mass spectrometer (Shimadzu) and a Rtx®-Wax capillary column, 30 m length, 0.25 mm i.d and 0.25 μm film, consisting of cross-bond polyethylene glycol (Restek). The GC analysis method was reported by Zou *et al*. (2012a).

2.5. Analysis of C52 content by GC-FID

C52, PPP and diacylglycerols (DAGs) in the reaction products were detected through GC using an Agilent 8890A GC platform equipped with a flame ionization detector and a fused silica capillary column DB-1HT (15 m length × 0.25 mm internal diameter × 0.25 μm film thickness, Agilent, Santa Clara, USA). The GC analysis method was reported by Liu *et al*. (2020).

2.6. Sn-2 Positional analysis of TAGs by pancreatic lipase

The hydrolysis of TAGs and determination of FA composition and sn-2 FA composition in TAGs were carried out according to the method described by Lee *et al*. (2010).

2.7. Molecular distillation

KDL1, UIC GmbH (Alzenau, Germany) was used to purify TAGs in the products under the following conditions: the operating pressure was set at 1×10^{-3} mbar, the rotor velocity at 300 min^{-1}, and the feed flow rate at 1.5 mL/min (feed temperature was 60 °C). When the heating oil temperature was increased to 210 °C, ethyl oleate, DAGs and free fatty acids were separated from the product.

2.8. OPO-HMFS blending with selected oils to prepare Final-HMFS

OPO-HMFS TAGs are the main components in the fat in infant formula, and are rich in OA and PA. Nevertheless, the contents in other major fatty acids, such as linoleic acid, lauric acid and some LC-PUFAs should also be within the ranges of their corresponding fatty acids in HMF. According to the fatty acid composition and distribution of typical human milk fat, a linear restraint function for the development of HMFS formula was established as follows:

$$\sum_{i=1}^{5} x_i = 1 \quad (i = 1, 2, 3, 4, 5) \quad (1)$$

$$\sum_{i=1}^{5} a_{i1} x_i < 20 \quad (2)$$

$$19.6 < \sum_{i=1}^{5} a_{i2} x_i < 29.0 \quad (3)$$

$$26 < \sum_{i=1}^{5} a_{i3} x_i < 40.6 \quad (4)$$

$$7.1 < \sum_{i=1}^{5} a_{i4} x_i < 20.1 \quad (5)$$

$$0.5 < \sum_{i=1}^{5} a_{i5} x_i < 3 \quad (6)$$

$$0.2 < \sum_{i=1}^{5} a_{i6} x_i < 0.4 \quad (7)$$

$$0.35 < \sum_{i=1}^{65} a_{i7} x_i < 0.6 \quad (8)$$

$$5 < \left[\sum_{i=1}^{5} a_{i4} x_i\right] : \left[\sum_{i=1}^{5} a_{i5} x_i\right] < 15 \quad (9)$$

$$y = c_2 x_2 + c_3 x_3 + c_4 x_4 + c_5 x_5 \quad (10)$$

i is the serial number of the selected oils and x_1, x_2, x_3, x_4 and x_5 represent the OPO-HMFS, coconut oil, soybean oil, algal oil and microbial oil, respectively; a_{i1}, a_{i2}, a_{i3}, a_{i4}, a_{i5}, a_{i6} and a_{i7} are the contents in lauric acid (C12:0)+myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6) , Linolenic acid (C18:3n-3), docosahexaenoic acid (DHA) and arachidonic acid (ARA) in the selected oils, respectively. c_2, c_3, c_4 and c_5 represent the marketing cost of coconut, soybean, algal and microbial oils, respectively.

2.9. Oil properties analysis

2.9.1 DSC melting profile

The melting profiles of oil samples were determined by differential scanning calorimetry (DSC) using a DSC Q2500 according to the method Cj1-94 (AOCS, 2009).

*2.9.2 Acid value (AV), peroxide value (PV) and p-anisidine value (*p*-AV)*

The AV, PV and *p*-AV were determined according to methods 5a-40 Cd, 8b-90 and p 2.4 (AOCS, 1997), respectively.

2.9.3 Induction period (IP)

The oxidative stability was determined in terms of induction period (IP) using the Rancimat method (Olajide *et al*., 2020). 3 Grams of sample with and without added antioxidants were carefully weighed into Rancimat tubes and subjected to accelerated oxidation at 100 °C under an air flow of 20 L/h.

2.9.4 Emulsion preparation and oven test

For convenience, infant formula liquid milk is also popular in the market. Most papers only tested the oxidative stability of HMFS itself, but did not evaluate the oxidation stability of HMFS in emulsion. HMFS-in-water emulsions (500 g) were prepared as mixtures of purified HMFS, Tween 80 and a phosphate buffer solution (pH 7.0) according to methods described by Chen *et al*. (2020). These samples were divided into experimental groups (0.02% antioxidants added) and a control group (no antioxidant added). Particle size distributions of the emulsions were measured by Mastersizer 2000 (triplicate, at room temperature) according to the method by Zou *et al*., (2012a). The average emulsion droplet size 14.90±0.76 nm was similar to HMF, and there was no visible significant change in the state of the emulsions over the course of the oven test. The emulsions were held in an oven at 63 ± 1 °C and the induction period of the samples was dependent on the oil reaching a peroxide value of 80 meq O_2/kg oil (Shi *et al*., 2017; Olajide *et al*., 2020).

2.10. Statistical analysis

All assays were carried out in duplicate or triplicate and the data are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out using IBM SPSS 22.0, followed by Duncan's multiple range test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Influence of reaction time and acyl donors on the contents in C52 and *sn*-2 PA

The whole process for obtaining OPO consisted of two reactions: first, obtaining TAGs rich in tripalmitin (PPP) from palm stearin and second, obtaining OPO by interesterification or acidolysis of TAGs enriched in PA at position 2 and acyl (oleic acid or methyl oleate or ethyl oleate) donors, catalyzed by sn-1, 3 specific lipases.

Acyl donors are important raw materials for the preparation of OPO. Srivastava, *et al*., (2006) reported that methyl ester is a better acyl donor than free acid for producing HMFS. Three kinds of oleic acyl donors (oleic acid, methyl and ethyl oleate) were compared to investigate their influence on the contents in C52 and sn-2 PA in our study. The initial rate of C52 synthesis using methyl or ethyl oleate with TAGs rich in PPP was higher than the oleic acid tested (Figure 1A). This was mainly due to better solubility of methyl or ethyl oleates in solvent-free systems than oleic acid. It should be noted that PPP with a high melting point (about 60 °C) hardly melt into liquid at the optimal reaction temperature of lipase, so organic solvent must always be added to the reaction system to reduce the PPP dissolution temperature and the viscosity of the reaction system so as to ensure the high activity of the lipase. However, large scale use of solvents may bring potential safety hazards. Methyl or ethyl oleates can act as both substrate and solvent to accelerate the dissolution of PPP at appropriate temperatures. There

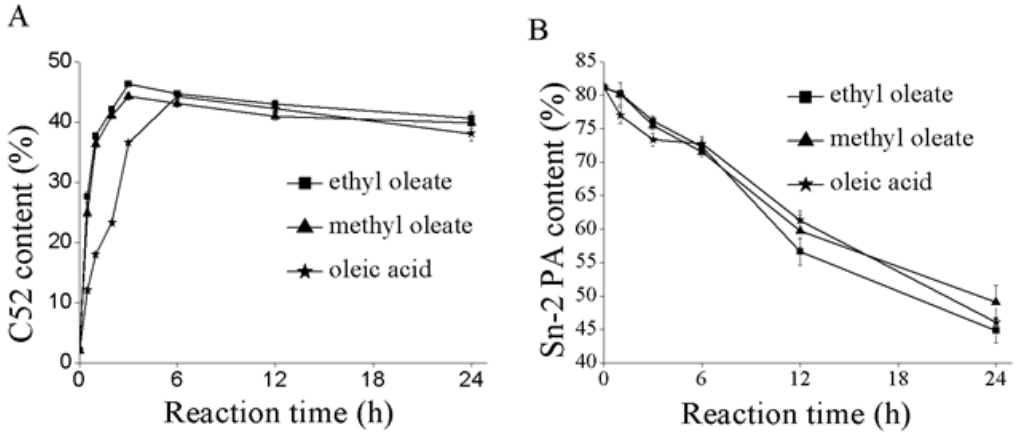

FIGURE 1. Influences of reaction time and acyl donors on the content in C52 (A) and sn-2 PA (B) using sn-1,3 specific lipases. Values are mean ± standard deviation (n=2).

was no significant difference in the initial rate of C52 synthesis between methyl or ethyl oleate use. Ethyl oleate was eventually selected because ethanol hydrolyzed by ethyl oleate in the interesterification reaction is much safer compared to methanol. Moreover, the boiling point of ethyl oleate (220 °C) is lower than that of oleic acid (241 °C) at the same pressure, hence easier to remove during the purification of OPO. When the heating oil temperature of molecular distillation was increased to 210 °C, the by-products of interesterification reaction including DAGs, MAGs and ethyl oleate were almost completely removed. The purified product did not contain ethyl oleate and only a small amount of DAGs (1.6 ± 0.2%, Table 2). The C52 content increased with time, but was practically constant from 6 h and its maximum content of over 40% was similar to all the acyl donors tested.

The content in PA at the sn-2 position decreased with the extension of reaction time (Figure 1B). Although Lipozyme RM IM exhibited the sn-1, 3-specificity, TAG with OA at the sn-2 position was possible to produce due to acyl-migration in the form of DAGs. DAGs are inevitable intermediates in lipase-catalyzed interesterification and they could be converted from 1,2-DAGs and 1,3-DAGs (Xu *et al*., 1998). The thermal instability of these DAGs may lead to acyl transfer as reaction time increases, reducing the yield of OPO (Xu *et al*., 1998). The percentage content of PA in the sn-2 position, determined by GC analyses, represents acyl migration. The acyl migration rates of oleic acid, methyl oleate and ethyl oleate in 3 h were 7.8, 5.7 and 5.1%, respectively. Compared to oleic acid, the PA content at the sn-2 position decreased more slowly in a short reaction time (3 h) with ethyl oleate tested, thus showing a lower acyl migration rate, which may be related to the polarity difference between oleic acid and ethyl oleate (Figure 1B). Li *et al*. (2010) have reported that organic solvents with low polarity could inhibit the acyl-migration of sn-2 fatty acids in TAGs. To obtain a higher C52 and sn-2 PA contents in short reaction times, ethyl oleate was selected to be the most ideal acyl donor.

TABLE 2. The composition of fatty acids and acylglycerols of the OPO-HMFS under optimum conditions

Fatty acid (area%)	Total	Sn-2 position	Sn-1,3 position
C16:0	33.6±1.1	75.3±1.5	12.8
C18:0	6.9±1.3	11.1±1.1	4.7
C18:1	53.3±0.7	9.5±0.2	75.2
C18:2	6.2±1.2	4.1±1.1	7.3
Acylglycerols (area%)	C52	PPP	DAGs
	46.7±0.8	2.9±1.2	1.6±0.2

Results are expressed as mean ± standard deviation (n = 3). Operational conditions: temperature, 55 °C; substrate molar ratio, 1:10 (PPP-rich fraction/ ethyl oleate); enzyme load, 10 wt %; water content, none; reaction time, 3 h. C52: primarily OPO and its isomeric compounds; PPP: tripalmitin; DAG: diacylglycerols. Fatty acid content at sn-1,3 positions of TAGs is determined by equation:

$$\text{FA at sn} - 1,3 \text{ positions (mol\%)} = \frac{3 * \%\text{total FA} - \%\text{FA at sn} - 2 \text{ position}}{2}$$

3.2. Optimization of interesterification conditions

3.2.1. Effect of temperature on the contents of C52 and sn*-2 PA*

Higher temperature decreases the viscosity of substrates and increase mass transfer, which in turn reduce reaction time to equilibrium, but also enhance the rate of occurrence of acyl-migration and other negative impacts, such as fat oxidation. Previous studies show that substrate could be dissolved at low temperature in solvent system, which partitions in a more stable environment (Esteban *et al*., 2011; Wei *et al*., 2015). However, the addition of solvents is harmful to the environment and/or economy. Solvent-free is a much safer, much more environmentally-friendly and economical industrial application. Thus, all the experiments in this study were based on a solvent-free system.

The reaction accelerated as temperature increased from 50 to 55 °C and the C52 content increased with it (Figure 2A). However, the C52 content, when above 55 °C, changed only slightly. At 55 °C, the C52 content was 45.4% and PA content at the sn-2 position was 75.2% at the end of the 3-h reaction. Compared to other temperatures, the products at 50 °C were characterized by higher PA content (i.e. 77.0%) at the sn-2 position; while the C52 content was only 39.8%, which may be due to lower catalytic efficiency. Moreover, higher temperature could decrease the sn-2 PA content significantly and might cause adverse consequences on enzyme properties and so, 55 °C was selected as the most ideal reaction temperature.

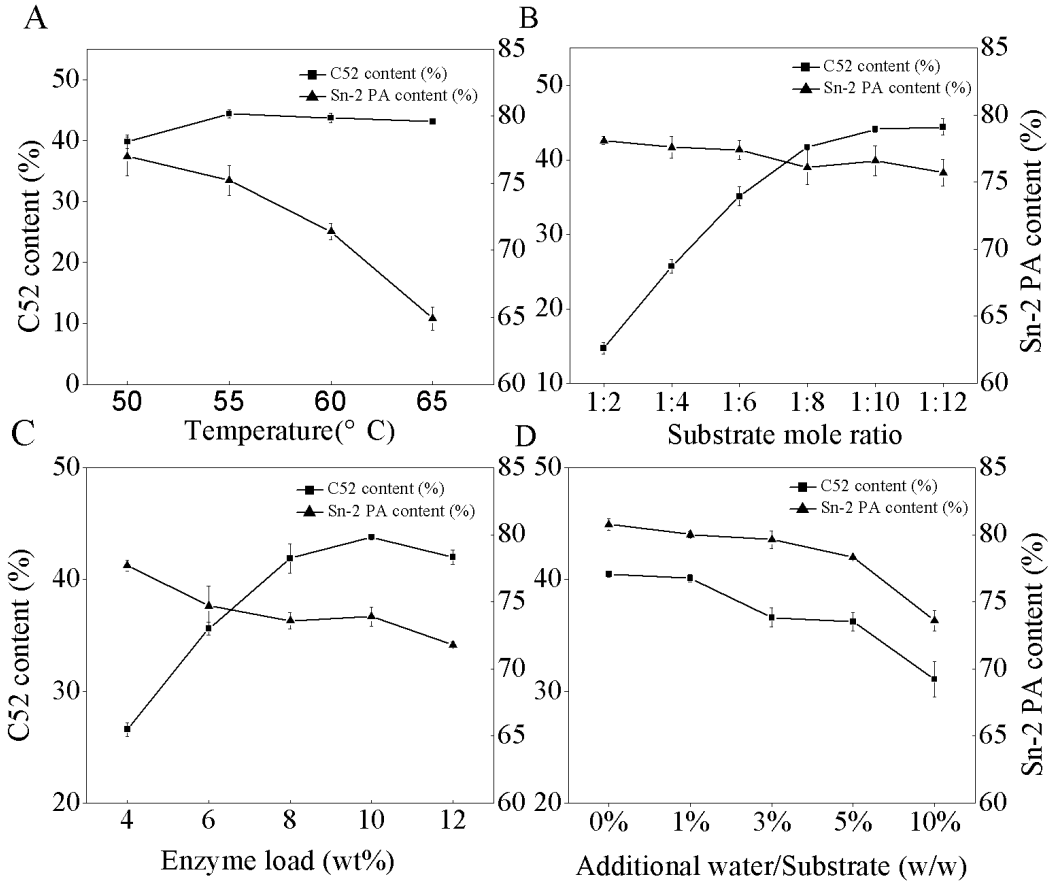

FIGURE 2. Influences of various parameters on the content in C52 and sn-2 PA in the interesterification of PPP-rich fraction with ethyl oleate using Lipozyme RM IM: (A) Effects of temperature. Reaction conditions: substrate molar ratio, 1:10 mol/mol; enzyme load, 10 wt %; 3h; water content, none. (B) Effects of substrate molar ratio. Reaction conditions: enzyme load, 10 wt %; 55 °C; 3 h; water content, none. (C) Effects of enzyme load. Reaction conditions: substrate molar ratio, 1:10 mol/mol; 55 °C; 3 h; water content, none (D) Effects of water content. Reaction conditions: substrate molar ratio, 1:10 mol/mol; enzyme load, 10 wt %; 3h. Values are mean ± standard deviation (n=2).

3.2.2 Effect of substrate ratio on the contents of C52 and sn-*2 PA*

According to the reaction formula, OPO can be synthesized under ideal conditions by maintaining the ratio of the PPP-rich fraction to ethyl oleate at 1:2. However, the interesteri- fication reaction is reversible, and the feedback regulation of the product will inhibit the reaction. To obtain the highest OPO yield, the effect of substrate ratio was compared in this experiment. From Figure 2B, the C52 content in the enzymatic products at the substrate ratios of 1:2, 1:4, 1:6, 1:8, 1:10 and 1:12 were 14.7, 25.7, 35.1, 41.7, 44.1 and 44.4%, respectively. The C52 content between the molar ratios of 1:10 and 1:12 were nearly identical, indicating that increasing the amount of ethyl oleate continuously would not necessarily improve the OPO yield. At the same time, using high amounts of ethyl oleate would mean a limitation for industrial production of OPO-rich HMFS. In addition, no significant difference in the sn-2 PA content with all the molar ratios tested indicated that the amount of ethyl oleate had very little influence on the acyl migration reaction. Thus, to improve catalytic and economic efficiency, an optimum molar ratio of 1:10 between the PPP-rich fraction and ethyl oleate is required.

3.2.3 Effect of enzyme load on the contents in C52 and sn-*2 PA*

Enzyme load is related to reaction rate. Higher enzyme load will accelerate the reaction rate and improve the incorporation rate of the acyl donor in the interesterification reaction. When the enzyme load was less than 10%, the content in C52 increased as enzyme load increased and reached its highest point (43.8%) at 10%, but later decreased as enzyme load increased (Figure 2C). Meanwhile, the content in sn-2 PA decreased slowly and continuously as the enzyme load increased. The sn-2 PA content was only 71.8% when the enzyme load reached 12%. These findings are understandable because the water content in the Lipozyme RM IM may have promoted the amount of DAG produced from TAGs, thereby enlarging the possibility of acyl-migration. An enzyme load of 10% (w/w) generated the C52 content (43.8%) and sn-2 PA content (73.9%) after 3 h, showing preferable yield and catalytic efficiency.

3.2.4 Effect of water content on the contents in C52 and sn-2 PA

Researchers have found that the water content in the synthetic reaction of TAG favors the content in DAG, which is due to hydrolytic side reactions (Zou *et al*., 2012b). Moreover, the presence of water on the surface of lipase is important for maintaining its activity and the flexibility of its protein structure. With increased water content in the system, the yields of C52 and sn-2 PA decreased consistently (Figure 2D). The content in C52 was 44.9% in the system without water, which was 3.6% higher than that in the system with 10% water content. Similarly, the content in sn-2 PA was 77.1% in the system without water, which was 7.8% higher than that in the system with 10% water content. A similar study was also reported in a study by Liu *et al*., (2020). The results indicated that high moisture in the reaction system can accelerate the hydrolysis of TAGs to DAGs and cause more acyl-transfer, reducing the content in sn-2 PA. It has been speculated that there are some hydrophilic groups on the surface of Lipozyme RM IM itself, which maintains its activity. As a result, the reaction was carried out in the absence of water.

Overall, the optimal OPO synthetic conditions were as follows: temperature, 55 °C; substrate molar ratio, 1:10 (PPP-rich fraction/ethyl oleate); enzyme load, 10 wt %; water content, none; reaction time, 3 h. Under these conditions, using molecular distillation, the composition of fatty acid and acyl glycerol of the OPO-HMFS are shown in Table 2. The OA and PPP contents in the OPO-HMFS were 53.3 and 2.9%, respectively, showing that OA was effectively incorporated into the substrate. A relatively small amount of PA on the TAGs migrated from the sn-2 to sn-1, 3 positions, since 75.3% of PA can be observed at the sn-2 position of OPO-HMFS.

3.3. OPO-HMFS blending with selected oils to prepare Final-HMFS.

Compared to the fatty acid composition and location distribution of HMF (PA, 19.6% - 29.0%; OA, 26.0% - 40.6%; linoleic acid, 7.1% - 20.1%; myristic acid+ lauric acid < 20.0%; linoleic acid/ linolenic acid, 5-15; DHA, 0.2%-0.4% and ARA, 0.3%-0.6%), the synthesized OPO-HMFS contained a smaller amount of linoleic acid and no medium-chain fatty acids or LC-PUFAs, but was characterized by much higher PA content (33.6%). In order

Table 3. Theoretical and Determined Values (area %) of the Final Product Obtained under Optimum Blending Conditions and Typical fatty acid composition (mol %) of human milk fat

Fatty acid	Theoretical values		Determined Values		HMF	
	total	sn-2	total	sn-2	total	sn-2
C8:0	1.6	1.3	2.0±0.1	1.2±0.2	0.1~0.6	0.0~0.7
C10:0	1.3	0.5	1.0±0.1	0.8±0.1	1.9~3.8	0.1~2.1
C12:0	8.1	10.8	9.6±0.4	12.5±0.1	1.0~9.4	0.9~13.2
C14:0	3.2	1.5	5.4±0.2	2.8±0.3	2.1~10.4	5.4~16.9
C16:0	26.4	49.0	26.2±0.9	47.9±0.5	19.6~29.0	42.5~59.9
C18:0	5.6	7.8	4.1±0.1	3.4±0.3	4.2~8.7	0.9~2.9
C18:1	39.6	17.2	39.5±0.7	20.0±0.6	26.0~40.6	5.4~18.4
C18:2	12.3	10.6	10.6±0.3	10.5±0.5	7.1~20.1	2.6~15.5
C18:3	1.2	0.7	0.8±0.3	0.3±0.1	0.5~3.0	0.1~1.8
C20:4n-6	0.4	0.3	0.4±0.0	0.3±0.1	0.3~0.6	0.0~1.8
C22:5n-3	0.1	0.1	0.1±0.0	0.1±0.0	0.0~0.3	0.0~0.7
C22:6n-3	0.2	0.2	0.3±0.0	0.2±0.1	0.2~0.4	0.5~1.5

Results are expressed as mean ± standard deviation (n = 3). Theoretical values: Calculated from Matlab R2018b software. Determined values: OPO-HMFS was blended with coconut, soybean, algal and microbial oils at a weight ratio of 0.70:0.18:0.11:0.004:0.007. HMF (human milk fat): Typical fatty acid composition (mol %) of human milk fat was adapted from previous studies (Qin *et al*., 2014, Haddad *et al*., 2012).

to add LC-PUFAs into infant formula, many studies used the acidolysis or interesterification method to prepare structural lipids containing LC-PUFAs, which was not efficient and thus increased production costs (Hoffman *et al*., 2009). Consequently, in our study, OPO-HMFS was physically blended with CO, SO, AO, and MO to adjust the contents in major and important fatty acids (ARA and DHA) to the proper ranges and maintain regional specificity of fatty acids like HMF.

Based on the fatty acid profiles of HMF, OPO-HMFS contained a smaller amount of linoleic acid (6.2±1.2%, Table 2) and no medium chain fatty acids or LC-PUFAs; therefore, CO, SO, AO, and MO, which are rich in lauric acid (48.9%), linoleic acid (54.8%), DHA (48.3%), and ARA (48.1%), respectively, were selected to blend with the OPO-HMFS to supplement the lack in fatty acids in OPO-HMFS (Tables 1 & 2). These selected oils are common in the market with low cost and convenient for industrialized production. To guarantee good quality and lower production costs of the Final-HMFS, Matlab R2018b was used to optimize the blending process. The final desirable formula constituted OPO-HMFS/CO/SO/AO/MO at a weight ratio of 0.70:0.18:0.11:0.004:0.007. The determined and theoretically evaluated fatty acid compositions and positional distributions of the final product under this blending ratio are shown in Table 3. The contents in PA and sn-2 PA were 26.2 and 47.9%, and the contents in LC-PUFA including ARA and DHA were 0.4 and 0.3%, respectively. The main and important fatty acids were almost within the ranges of these in HMF (Table 3), indicating that the fatty acid composition of the final product was highly similar to HMF and may be used as a fat substitute in infant formula. Besides, researches have reported differences between the fatty acid compositions of HMF, especially LC-PUFAs, at different stages of lactation (Sala-Vila *et al*., 2005; Haddad *et al*., 2012) and different proportions of fatty acids can also be formulated through the established model to meet the nutritional needs of infants in different stages of lactation.

3.4. Oil properties analysis

3.4.1. DSC melting curve

The melting curves of OPO-HMFS, Final-HMFS and palm stearin were determined by DSC. A lower melting temperature was observed for the OPO-HMFS product (18.1 °C) compared to palm stearin (57.6 °C), which contributed to the high content in OA and low content in PA in the product. The melting

temperatures of both OPO-HMFS and Final- HMFS (14.3 °C) were lower than the normal human body temperature (about 37.5 °C) depicting that they are suitable to be used as fat base materials in infant formulas. The melting range of HMFS is important for its digestion and absorption. The melting range of Final-HMFS was relatively narrower than that of OPO-HMFS (Figure 3), showing that the content in the high melting point component in OPO-HMFS was higher than that of Final-HMFS.

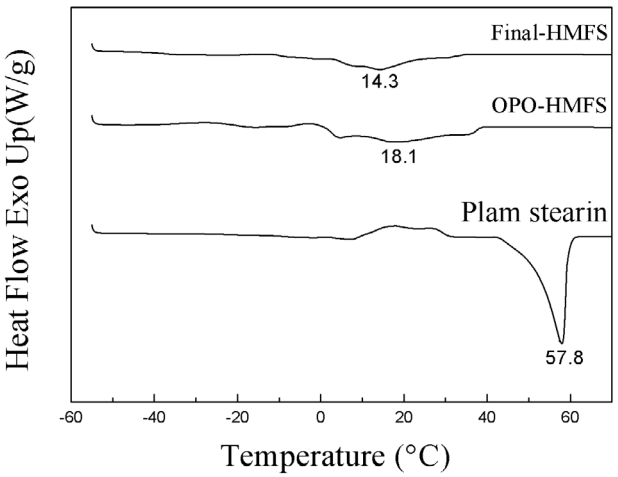

Figure 3. DSC Melting curves of OPO-HMFS, Final-HMFS and Palm stearin.

3.4.2. AV

Table 4 summarizes the basic properties of palm stearin, OPO-HMFS and Final-HMFS. TAGs hydrolyze to release free fatty acids during heating or the action of lipase, which influences the stability of oil. AV can be used to indicate the degree of oil hydrolytic rancidity and refining. The undetectable AV of OPO-HMFS showed that molecular distillation was an effective method to remove free fatty acid or fatty acid ethyl ester. The AV of the Final-HMFS was only 0.3 mg KOH/g, indicating that the selected oil blended with OPO-HMFS was of good product quality.

Table 4. AV (mg KOH/g), PV (meq O_2/Kg oil), *p*-AV and IP (h) of samples

Samples	Palm stearin	OPO-HMFS	Final-HMFS
AV	0.4±0.1	-	0.3±0.1
PV	3.8±0.2^{a}	1.1±0.3^{b}	3.4±0.4^{a}
p-AV	3.2±0.3^{a}	0.9±0.1^{b}	3.0±0.4^{a}
IP	16.8±0.7^{a}	4.1±0.9^{c}	6.3±0.6^{b}

-: compound not detected because its content was too low. Results are expressed as mean ± standard deviation (n = 3). Means in the same row with different letters are significantly different according to Duncan's multiple range test ($P < 0.05$). OPO-HMFS was synthesized using palm stearin enriched in tripalmitin and ethyl oleate by enzymatic interesterification under optimum conditions. Final-HMFS was obtained by blending OPO-HMFS with coconut, soybean, algal and microbial oils at a weight ratio of 0.70:0.18:0.11:0.004:0.007.

3.4.3. PV and p-*AV*

PV and *p*-AV were used to evaluate the degree of oil oxidation and deterioration. PV represents the main products of oil oxidation, like hydroperoxides, while *p*-AV represents the small molecular compounds such as aldehydes and ketones produced by further decomposition of peroxides. High PV and *p*-AV indicate high oxidation of oil samples. From the Table 4, the PV and *p*-AV of OPO-HMFS (1.1 meq O_2/kg oil and 0.9, respectively) were quite low compared with palm stearin (3.8 meq O_2/kg oil and 3.2, respectively), thus implying the purification process had also removed the majority of peroxides. The PV (3.4 meq O_2/kg oil) and *p*-AV (3.0) in Final-HMFS was higher than that in OPO-HMFS but it was still within an acceptable range.

3.4.4. Oxidative stability of HMFS

The rancimat assay characterizes the oxidative stability of oils. The oxidative stability indexes of palm stearin, OPO-HMFS and Final-HMFS were measured by the Rancimat test and expressed as IP (Table 4). Higher IP reflects a longer shelf-life of products. The IP value of the OPO-HMFS (4.1 h) and Final-HMFS (6.3 h) were lower compared to palm stearin (16.8 h). For one thing, USFAs in HMFS were more easily oxidized than SFAs in palm stearin; for another thing, the reason may be due to the loss in tocopherols and β-carotene in the process of refining OPO-HMFS by molecular distillation, thus reducing antioxidant capacity. The decrease in oxidative stability after molecular distillation is in agreement with several similar studies (Nielsen *et al*., 2006; Qin *et al*., 2014). Fat oxidative rancidity is due to the long-term storage of HMFS under unfavorable conditions, which usually requires some protection from antioxidants.

The efficacy of β-carotene, L-AP, VE and TBHQ (*tert*-butylhydroquinone) as antioxidants was eval-

uated using the Rancimat test at 100 °C, and concentrations at 0.02% (w/w) under air saturation conditions. The results were expressed as induction period (IP) corresponding to the oxidative stability of OPO-HMFS (Figure 4). The IPs of the blank, β-carotene, L-AP, VE and TBHQ were 3.8, 5.6, 7.8, 8.1, and 12.7 h, respectively. This indicates that the antioxidant activities of the antioxidants decreased as follows: TBHQ > VE ≈ L-AP > β-carotene. Although TBHQ has strong antioxidant activity compared to other antioxidants, it is a synthetic antioxidant. The existence of a double bond in β-carotene can prevent oil from being oxidized, but the effect is not profound. VE, which contains hydroxyl groups, can inhibit lipid oxidation by capturing free radicals, which is a natural antioxidant and a nutritional component contained in vegetable oil. L-AP is the only unnatural antioxidant allowed to be added to baby food in China. In some instances, combined

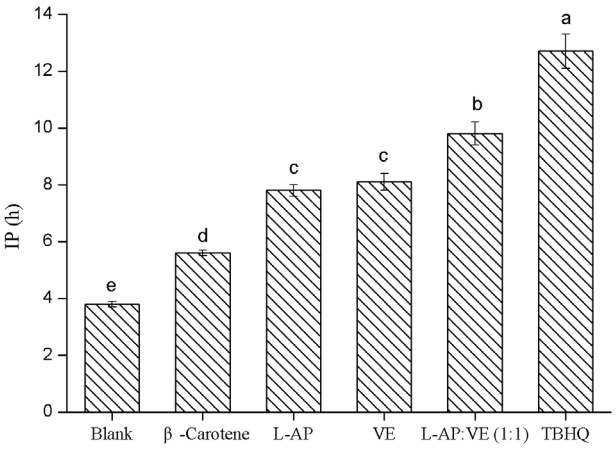

Figure 4. IPs of OPO-HMFS spiked with 0.02% (w/w) of antioxidants and without antioxidant. Values are mean ± standard deviation (n=3). Different letters are significantly different at P < 0.05 according to Duncan's test.

antioxidants have greater antioxidant activities than their individual effects. The IP of the mixture of VE and L-AP (at a weight ratio of 1:1) is 9.8 h, which is higher than that of VE or L-AP alone, indicating that L-AP has a synergistic effect with VE. When VE and L-AP are used together, L-AP could regenerate the phenoxyl radical of VE back to active VE. The synergistic effect of VE and L-AP cannot only enhance the oxidative stability of OPO-HMFS, but also reduce cost because L-AP is low-priced.

3.4.5. Oxidative stability of OPO-HMFS-Emulsion in oven test

HMFS are widely used in emulsions because of low water solubility. Emulsion can be used as an excellent carrier for wrapping, protecting and transporting HMFS, so that it can reach the designated position in the gastrointestinal tract digestion process (Hu, *et al*., 2003). Nevertheless, the oxidation of HMFS in emulsion is inevitable. In this study, PV was used to evaluate the oxidative stability of OPO-HMFS-Emulsion during the oven test at 63 ± 1, for 21 days. As shown in Figure 5, the PV of the blank group was 2.7±0.5 meq/kg on the first day, which then began to accelerate until it reached a maximum of 118.6±1.9 meq/kg after 21days of storage. In contrast, the PV of HMFS containing the TBHQ, VE, L-AP, mixture of VE and L-AP increased slightly until day 9, and then gradually increase dynamically with storage time. The changes in PV in the samples spiked with TBHQ, VE and L-AP showed that the addition of antioxidants was better at preventing oxidation of the emulsions and they were 20.4, 70.2, and 80.5 meq/kg on the last day, respectively. This indicates that the antioxidant activities of the antioxidants in emulsions decreased in the following order: TBHQ > VE > L-AP. Similarly, the results from Figure 5 showed that the mixture of VE and L-AP resulted in a synergistic antioxidant effect. Besides, the OPO-HMFS-Emulsion oxidative stability of L-AP and VE at a ratio of 2:3 was greater

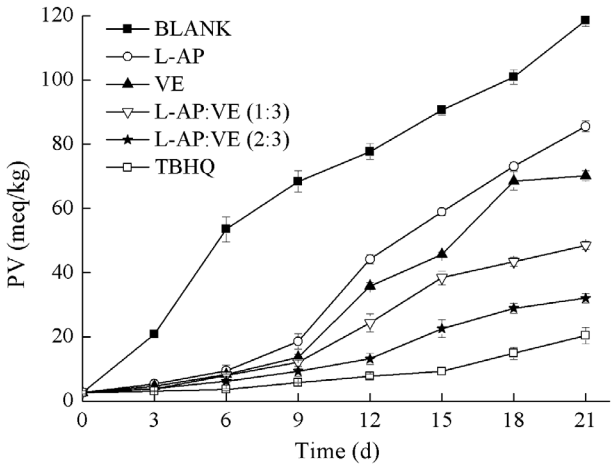

Figure 5. Changes in peroxide value of OPO-HMFS-Emulsion spiked with 0.02% (w/w) of antioxidants and without antioxidant during oven test at 63 ± 1, for 21 days. Values are mean ± standard deviation (n=2).

than that of ratio 1:3. Although increasing the proportion of L-AP in mixed antioxidants can improve the oxidative stability of OPO-HMFS emulsion, the proportion of VE in it should be kept at a higher level because VE also plays an important role in improving immunity and promoting the growth of infants.

4. CONCLUSIONS

This study produced HMFS which contained OPO and LC-PUFAs, whose content and structure of fatty acids are highly similar to HMF. The enzymatic interesterification conditions for OPO-HMFS (enzyme load, substrate molar ratio, temperature, water content) were optimized. A high content in C52 production (46.7%) and a sn-2 PA content of 75.3% were achieved. Moreover, OPO-HMFS was blended with coconut oil, soybean oil, algal oil and microbial oil at weight ratios of 0.70:0.18:0.11:0.004:0.007 to simulate the fatty acid profiles of HMF according to some mathematical models. The main and important fatty acids were closely within the ranges naturally found in HMF. The mixture of L-AP and VE resulted in a synergistic antioxidant effect both in OPO-HMFS and OPO-HMFS-Emulsion.

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Extraction of oil, carotenes and tocochromanols from oil palm (*Elaeis guineensis*) fruit with subcritical propane

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SUMMARY: This work aims to screen the extraction of oil and bioactive compounds including carotenes and tocochromanols from oil palm fruit with subcritical propane and without using a cosolvent. The overall extraction curves of palm oil with subcritical propane were studied and compared to those extracted with supercritical carbon dioxide. Carotenes and tocochromanols were evaluated not only in the extracted oil, but also in the oil of residual fiber in order to calculate the efficiency to recover these valuable compounds. The experimental results showed that oil yield of up to 70 % could be obtained within 120 minutes with subcritical propane at 50 bar and a flow rate of 35 $kg \cdot h^{-1} \cdot kg^{-1}$. It was also shown that compressed propane is an excellent solvent for the extraction of oil enriched in carotenes and tocochromanols. Subcritical propane extraction can be used as an alternative process for the simultaneous recovery of these valuable minor components from palm fruit.

KEYWORDS: *Carotenoids; Palm oil; Subcritical propane extraction; Tocochromanols.*

RESUMEN: ***Extracción de aceite, carotenos y tococromanoles del fruto de palma aceitera (*Elaeis guineensis*) con propano subcrítico.*** Este trabajo tiene como objetivo evaluar la extracción de aceite y compuestos bioactivos, incluidos los carotenos y tococromanoles, del fruto de la palma aceitera mediante propano subcrítico sin usar codisolventes. Se estudiaron las curvas generales de extracción de aceite de palma con propano subcrítico y se compararon con las extraídas con dióxido de carbono supercrítico. Se evaluaron carotenos y tococromanoles no solo en el aceite extraído, sino también en el aceite de fibra residual para calcular la eficiencia de recuperación de estos valiosos compuestos. Los resultados experimentales mostraron que se podía obtener un rendimiento de aceite de hasta el 70 % en 120 minutos con propano subcrítico a 50 bares y un caudal de 35 $kg \cdot h^{-1} \cdot kg^{-1}$. También se demostró que el propano comprimido es un excelente solvente para la extracción de aceite enriquecido en carotenos y tococromanoles. La extracción con propano subcrítico se puede utilizar como un proceso alternativo para la recuperación simultánea de estos valiosos componentes menores del fruto de la palma.

PALABRAS CLAVE: *Aceite de palma; Carotenoides; Extracción subcrítica con propano; Tococromanoles.*

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1. INTRODUCTION

Palm oil is a liquid which is extracted from the fleshy mesocarp of the fruits of the palm tree, *Elaeis guineensis,* which typically contain 45 to 55% oil (Tan and Nehdi, 2012). According to Phoon *et al.* (2018), **c**rude palm oil contains carotenoids, ca. 500–700 $mg \cdot kg^{-1}$, mainly in the form of alpha- and beta-carotenes, and ca. 1000–1200 $mg \cdot kg^{-1}$ tocopherols and tocotrienols (the whole group called tocochromanols). Carotenoids and tocochromanols are interesting valuable bioactive minor compounds. Many studies have reported that carotenes can provide support for the prevention and control of diseases caused by vitamin A deficiency (Strobel *et al.*, 2007). Alpha-tocopherol, known as vitamin E, and gamma-tocotrienol are strong antioxidants. The combined effects of the properties of carotenes and tocochromanols give palm oil a higher natural oxidative stability compared to many other edible oils. Therefore, palm oil has become the starting material to produce natural carotenes and tocochromanols (Abu-Fayyad and Nazzal, 2017; Ghazali *et al.*, 2022; Hoe *et al.*, 2020; Iftikhar *et al.*, 2017).

Supercritical extraction has been proven to be a modern separation technique applied in edible oil processing. Supercritical carbon dioxide ($SCCO_2$) is the most commonly studied fluid. However, propane is also an interesting fluid because it is non-toxic with low critical pressure (P_c = 42.5 bar). Because the critical temperature of propane is rather high (T_c = 96.7 °C), this fluid is preferably used at subcritical conditions (Brunner, 1994). It was reported that subcritical propane has been successfully used to extract oil from pequi (*Caryocar coriaceum*) pulp (Pessoa *et al.*, 2015), inajá (*Maximiliana maripa*) pulp (Turola Barbi *et al.*, 2019), baru (*Dipteryx alata vogel*) seeds (Fetzer *et al.*, 2018), foxtail millet bran (Shi *et al.*, 2015), kiwi fruit seeds (Coelho *et al.*, 2016), macauba pulp (Trentini *et al.*, 2017), flaxseed (Piva *et al.*, 2018), pumpkin seeds and peel (Cuco *et al.*, 2019). With the palm fruit (*Elaeis guineensis*), the subcritical propane extraction of oil using ethanol as cosolvent had been performed in a few studies (da Silva *et al.*, 2018; Jesus *et al.*, 2013). However, the determination of oil, carotenes, and tocochromanol contents in the pure subcritical propane extract and the residual fibers of palm fruit within a single run has not been reported.

In this context, the study aimed at extracting oil from the palm mesocarp by means of pure subcritical propane and compared with $SCCO_2$ extraction. In addition, samples of extracted oil and the oil of residual fibers were analyzed for their contents in carotenes and tocochromanols to evaluate the efficiency of using the compressed propane to recover these valuable minor compounds.

2. MATERIALS AND METHODS

2.1. Materials

Palmitic acid (> 99%) and squalene (GC grade) came from Merck (Germany). Monopalmitin (99%), dipalmitin (99%) and tetradecane (99%) were supplied by Sigma (USA). Pyridine (99.8%), hexane (> 95%), acetone (> 99.8%), acetonitrile (HPLC grade) and N-methyl-N-trimethylsilyl-trifluoroacetamide were purchased from Fluka (Switzerland), Lab-Scan (Ireland), Riedel-de Häen (Germany), Prolabo (France), and Macherey-Nagel (Germany), respectively.

Ripe palm fruits (*Elaeis guineensis*) were from Carotech (Malaysia). The fruits were separated into skin, mesocarp (pulp), and kernel. The yellow part of the mesocarp was investigated in this work. The average particle size of the pulp ready for extraction was about 1 mm x 2 mm x 6 mm (Phan Tai and Brunner, 2019).

2.2 Equipment and experimental procedure

A standardized supercritical extraction system developed at the Institute of Thermal Separation Processes, Hamburg University of Technology was used as described in previous research (Phan Tai and Brunner, 2019). Fluid, propane or carbon dioxide (99.95% purity) was delivered from the reservoir tank by a Maximator pump (max. 600 bar) to the 100 mL steel extractor cell, which was loaded with 14.5 g of palm mesocarp for each run. A specific flow rate of 35 $kg \cdot h^{-1}$ of gas per kg of sample was used and the pressure and temperature were monitored. The extracts were collected continuously in 10-mL glass vials, used as sample collectors at atmospheric pressure. Duplicate runs were carried out for each experimental condition with a reproducibility of ± 5%.

2.3. Analytical method

2.3.1. High-performance liquid chromatography (HPLC) analysis

A Gynkotek HPLC system equipped with a RF 1002 Fluorescent detector was used for the analysis. Tocopherols and tocotrienols in the oil samples were separated on a LiChrosorb Diol 5 µm column (250 mm x 4.6 mm). The mobile phase was hexane (96%) and butyl-methyl-ether (4%) at a flow rate of 1300 µL.min^{-1}. The injection volume was 20 µL. External standard curves were used to determine tocochromanol contents in the oil samples.

2.3.2. Gas chromatography (GC) analysis

A capillary gas chromatograph system (Hewlett Packard HP 5890A) was used to analyze monoacylglycerols (MAGs) and diacylglycerols (DAGs). The stationary phase was a J & W Scientific fused silica (DB-5ht) column (30m×0.25mm i.d. with 0.1-µm coating). The carrier gas was nitrogen (2 L.min^{-1}). The oven temperature was programmed as followed: 120 °C, 2 min constant; 10 °C·min^{-1} to 220 °C; 5 °C·min^{-1} to 360 °C; 360 °C, 10 min constant. Injection volume was 1 µL at a split ratio of 1:20. For a better peak recording, sample compounds were silylated with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA). For the quantification of MAGs and DAGs, monopalmitin and dipalmitin were used as reference standards, respectively.

2.3.3. Soxhlet extraction

A Soxhlet method was used to determine the oil contents in fresh palm mesocarp and its fibers after extraction. Hexane was used as extraction solvent. The extraction time was 8 hours (Phan Tai and Brunner, 2019).

2.3.4. Spectrometer

A UV-Vis spectrometer (UV-120-02 from Shimadzu) was used to determine the concentrations of carotenes in the analyzed samples. For each measurement, an amount of 10 to 20 mg oil sample was diluted with a 2-mL mixture of acetone and hexane (30:70 by Vol. %). The absorbance was recorded at the wavelength of 450 nm and compared to the standard curve prepared by the same treatment of a series of known amounts of β-carotene.

2.4. Statistical analysis

Statistical analysis was performed with JMP® version 10 software (SAS, USA). Data were expressed as the mean of triplicate measurements. One-way analysis of variance (ANOVA) and Tukey test ($P < 0.05$) were carried out to test any significant differences between means.

3. RESULTS AND DISCUSSION

3.1. Characteristics of palm mesocarp

The composition of mesocarp varies with the size and age of the palm fruit. Table 1 presents the average composition of palm mesocarp used as material input for the extraction in this study. The results show that palm mesocarp is a good source to extract oil and valuable minor compounds like carotenes or tocochromanols. The concentrations of these components are in agreement with those reported by Phoon *et al.* (2018). However, the total mono and diacylglycerol contents in the studied palm fruit is rather high compared to 5% reported elsewhere (Tan and Nehdi, 2012). This can be attributed to the enzymatic hydrolysis of the oil under the influence of an endogenous lipase in the pulp (Doye R. Abigor, 1985) after long transportation and preservation of the palm fruit. The difference may be also due to the analysis method, maturation stage, and environmental growth variation of the palm fruit.

Table 1. Composition of palm fruits used in this work

Component	Concentration*
Total oil	45.1 ± 0.9%
Carotenes	450 ± 14 mg·kg^{-1}
Tocochromanols	800 ± 40 mg·kg^{-1}
Monoacylglycerols	3.0 ± 0.1%
Diacylglycerols	7.0 ± 0.2%

*: Average values of triplicate analyses ± standard deviation.

3.2. Course of extraction of palm oil

The course of a solid extraction of oil can be represented by an overall extraction curve, in which the amount of extract accumulated during the course of the extraction is plotted as a func-

tion of time. Figure 1 shows the extraction curves of palm oil with subcritical propane in comparison with $SCCO_2$. According to Brunner (1994), the first part of the overall extraction curve is linear, corresponding to a constant extraction rate. The gradient of this part may represent the equilibrium solubility of the extract in supercritical fluid. However, the straight line of the overall extraction curve could correspond to a constant mass transfer resistance. In the second part, the extraction rate is declining and the graph approaches a limiting value where all the extractible substances are removed from the input material.

The results show that free oil was more soluble in subcritical propane than in $SCCO_2$. However, extraction with $SCCO_2$ gave better total oil yields after 45 minutes when the available oil near the palm surface was depleted. Within the study conditions, palm oil could be recovered by up to 80% after 120 minutes with $SCCO_2$ at 400 bar and 70% with subcritical propane at 50 bar. The difference in oil recovery can be attributed to the structural change in palm fibers during the process. It was proven that $SCCO_2$ can affect the cellulose structure by increasing the accessible surface area of the cellulosic substrates (Kim and Hong, 2001; Putrino *et al.*, 2020). Lau *et al.* (2006) reported a palm oil yield of 77.3% obtained with $SCCO_2$ at 300 bar and 80 °C for 8h. In another research, it was shown that flaxseed oil extraction yields using subcritical propane were lower compared to the result from using $SCCO_2$ (Piva *et al.*, 2018).

3.3. Solubility of palm oil in subcritical propane and $SCCO_2$

The loading of solvent during the extraction can also be obtained from the first part of palm oil extraction curves. This value is commonly considered as apparent solubility and calculated from the extraction when palm oil is easily accessible throughout the fixed bed (at constant extraction rate). In the case of palm oil, $SCCO_2$ at 400 bar only had a loading capacity of 1.7 - 2.7%, while subcritical propane at 50 bar can reach an oil loading of up to 4.5% depending on the extraction condition as described in Figure 2. The same phenomena were also reported by Zanqui *et al.* (2016) in which the extraction of Sacha inchi (*Plukenetia volubilis* L.) oil using subcritical propane was faster compared to $SCCO_2$ due to the higher solubility of lipids in propane.

3.4. Extraction of carotenes and tocochromanols

Palm oil is a very good source of carotenes and tocochromanols, which are interesting valuable minor compounds in supercritical fluid extraction. The concentrations of carotenes and tocochromanols in extracted palm oil by compressed propane at 50 bar are presented in Figure 3. It was observed that the concentration of these compounds varied moderately during extraction time with the subcritical propane. Using subcritical propane can co-extract these minor compounds with concentrations in the same range as a normal pressed palm oil. This is in agreement with

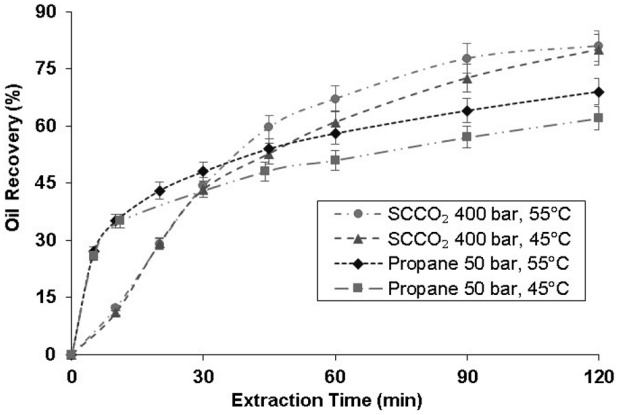

FIGURE 1. Extraction of mesocarp with subcritical propane and $SCCO_2$ at 35 $kg \cdot h^{-1} \cdot kg^{-1}$. Bars represent the experimental standard deviation of duplicates.

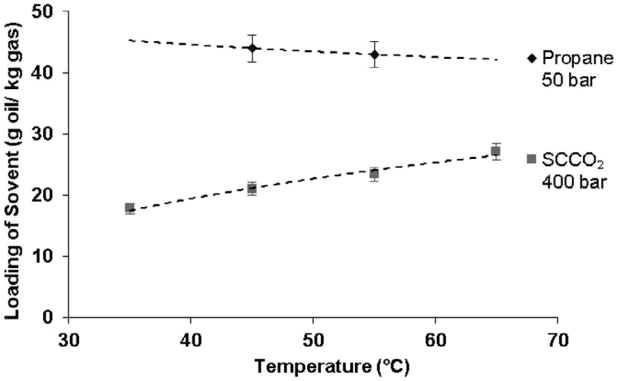

FIGURE 2. Palm oil loading capacity of subcritical propane and $SCCO_2$. Data points represent mean values and standard deviation (n=2).

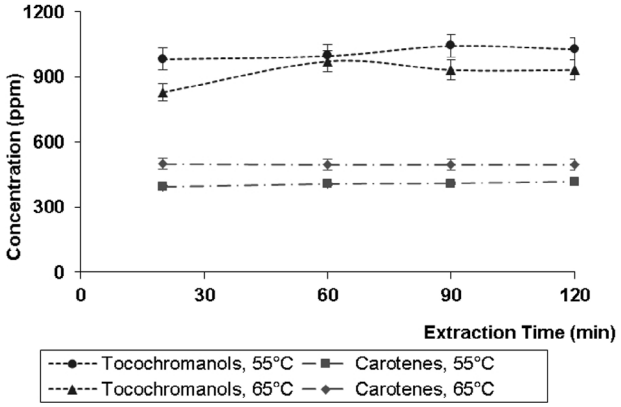

FIGURE 3. Carotenes and tocochromanols as extracted with propane at 50 bar and 35 kg·h^{-1}·kg^{-1}. Bars represent the experimental standard deviation of triplicates.

Trentini *et al.* (2017) regarding extraction from macauba pulp, who assert that propane is more efficient to extract active compounds like tocopherols and carotenoids compared to CO_2. In the extraction of oil from perilla, a higher concentration in tocopherols was determined in oil resulting from compressed propane extraction compared to the classical Soxhlet method (Silva *et al.*, 2015).

Palm oil is a complex product consisting of many components as presented in Table 1. Therefore, the concentration in carotenes and tocochromanols of extracted palm oil depends not only on their solubilities in the solvent but also on the solubilities of other compounds like mono, di- or triglycerols at the same time. A decrease in tocochromanol concentration was observed when the temperature was increased from 55 to 65 °C. A rather high temperature may decrease the content in tocochromanols because these compounds are sensitive to temperature. In contrast, it was observed that the concentration of carotenes increased with an increase in temperature. The condition of less solubility of tocochromanols in extracted palm oil can be more favourable for the solubility of other compounds like carotenes. As a result, the concentration in carotenes increased from ca 400 mg·kg^{-1} to 500 mg·kg^{-1} when temperature increased from 55 to 65 °C. Zanqui *et al.* (2016) also showed that temperature can influence the lipid composition of Sacha inchi oil extracted by subcritical propane. The subcritical propane of *Maximiliana maripa* pulp at 40 °C and 60 bar also provided fast extractions and high yields of oil enriched in beta-carotene (Turola Barbi *et al.*, 2019).

It was reported that a high amount of carotenes and tocochromanols remained in the palm residue from extraction with $SCCO_2$ or screw pressing (Birtigh *et al.*, 1995). However, subcritical propane stands out as a good solvent to recover these valuable compounds. As shown in Figure 4, there is a slight difference in tocochromanol and carotene concentrations in the oil extracted from palm mesocarp by subcritical propane and those of oil in the palm residue of extraction. To objectively evaluate the efficiency of recovery of tocochromanols and carotenes, a relative comparison of the concentrations of these compounds in the extracted oil and residue oil was used. Enrichment factor K of a component, as described in a previous study (Phan Tai and Brunner, 2019), is defined as the following equation (Eq. 1). As a result, extraction with a higher value of K(X) provides better potential to recover component X.

$$K(X) = \frac{\text{Concentration of component X in the extracted oil}}{\text{Concentration of component X in the residue oil}} \quad \text{Eq. 1}$$

The results in Table 2 show that carotenes and tocochromanols can be recovered effectively by sub-

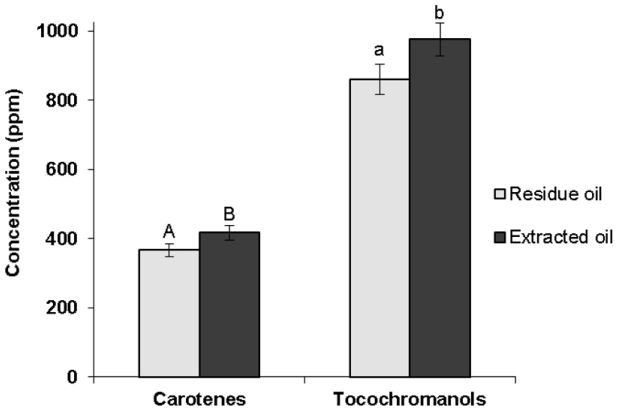

FIGURE 4. Concentration in carotenes and tocochromanols in extracted and residue oils by subcritical propane extraction at 50 bar, 55 °C, 35 kg·h^{-1}·kg^{-1}. Bars with different lower-case and upper-case letters are significantly different by the Tukey test ($P < 0.05$) within the same group (n=3).

TABLE 2. Enrichment factors of carotenes and tocochromanols with subcritical propane.

Temperature	K (carotenes)	K (tocochromanols)
45 °C	1.24	0.79
55 °C	1.14	1.13
65 °C	1.27	1.03

critical propane. The recovery efficiency of these minor compounds by using subcritical propane was better compared to other extraction methods by using $SCCO_2$ or traditional crew pressing. Phan Tai and Brunner (2019) reported that enrichment factors K(carotenoids) and K(tocochromanols) of palm oil extracted by $SCCO_2$ at 400 bar and temperature of 45–65 °C was only around 0.90–1.19 and 0.54–0.86, respectively. This also agrees with a previous study which confirmed that compressed propane has higher solvating power compared to $SCCO_2$, which results in a reduction in the consumption of solvent, higher efficiency and shorter extraction time (Silva *et al.*, 2015). It was also reported that subcritical propane extraction was a suitable and selective method for the extraction of the foxtail millet bran oil in view of smaller times and lower pressures employed compared to $SCCO_2$ and revealed the possible high content in carotenoids and highest tocopherol content obtained (Shi *et al.*, 2015).

Moreover, it was reported that the extraction of minor compounds from the palm-pressed fiber is not very practical (Chuang and Brunner, 2006). Therefore, the results of this study prove that using subcritical propane as an extraction solvent will bring more benefit because of better recovery of these valuable compounds. The subcritical propane extraction of palm oil, simultaneously recovering its high contents in carotenes and tocochromanols from the palm fruits appears as a promising alternative separation technique for palm oil processing.

4. CONCLUSIONS

Subcritical propane extraction has been proven as an alternative separation technique for palm oil processing. The preliminary study shows that it is possible to extract palm mesocarp directly by subcritical propane without using cosolvent with the aim of recovering valuable minor compounds like carotenes and tocochromanols. Compressed propane at a pressure of 50 bar and flow rate of 35 $kg \cdot h^{-1} \cdot kg^{-1}$ can be used to recover up to 70% palm oil after 120 minutes. Carotene and tocochromanol concentrations in the extracted oil reached the same levels as in commercial palm oil. Moreover, recovery efficiencies of carotenes and tocochromanols were much higher in the case of extraction with subcritical propane than with $SCCO_2$.

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Changes in the essential oil content and composition of *pelargonium graveolens* l'hér with different drying methods

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SUMMARY: In this study, the effect of various drying methods (fresh plant, shade-drying, sun-drying, and oven-drying at 30 and 60 °C) on the essential oil (EO) composition of rose-scented geranium were determined. Essential oil samples were extracted by hydrodistillation and analyzed by GC and GC-MS systems. The highest EO contents were obtained in the fresh plant (1.98%), followed by shade-drying (1.34 %) and oven-drying at 30 °C (1.20 %). The main components were citronellol (23.99-39.87%), geraniol (4.15-17.09%), menthone (4.48-8.34%), linalool (1.96-7.42%), β-caryophyllene (2.63-4.32%), geranyl tiglate (0.99-4.52%), citronellyl butyrate (0.53-5.31%) and cis-rose oxide (0.71-3.15%). The drying methods showed a marked impact on the constituents of the EO samples. The results demonstrated that drying the aerial parts of fresh geranium, and shade-drying and oven-drying at 30 °C were the best optimal methods to obtain the highest oil yield, and citronellol, geraniol, and linalool contents in the oil.

KEYWORDS: *Rose-scented geranium; Essential oil yield; Chemical composition; Drying methods; Citronellol; Geraniol*

RESUMEN: ***Cambios en el contenido y composición del aceite esencial de* pelargonium graveolens *l'hér con diferentes métodos de secado.*** Se estudió el efecto de varios métodos de secado (planta fresca, secado a la sombra, secado al sol y secado en horno a 30 y 60 °C) sobre la composición del aceite esencial (AE) de geranio con aroma a rosas. Los aceites esenciales de las muestras fueron extraídos por hidrodestilación y analizados mediante GC y GC-MS. Los mayores contenidos de AE los obtuvo la planta fresca (1,98%), seguido del secado a la sombra (1,34 %) y secado en estufa a 30 °C (1,20 %). Los principales componentes fueron citronelol (23,99-39,87 %), geraniol (4,15-17,09 %), mentona (4,48-8,34 %), linalol (1,96-7,42 %), β-cariofileno (2,63-4,32 %), geranil tiglato (0,99-4,52 %), butirato de citronelilo (0,53-5,31 %) y óxido de cis-rosa (0,71-3,15 %), los métodos de secado mostraron un marcado impacto en los constituyentes de las muestras de EO. Los resultados demostraron que el secado de las partes aéreas del geranio fresco, y el secado a la sombra y el secado en horno a 30 °C fueron los mejores métodos óptimos para obtener el mayor rendimiento de aceite y contenido de citronelol, geraniol y linalool en el aceite.

PALABRAS CLAVE: *Composición química; Genario; Geranio perfume a rosa; Rendimiento de aceite esencial; Secado.*

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1. INTRODUCTION

The genus *Pelargonium* is cultivated around the world for the production of essential oils and absolutes. *Pelargonium graveolens* L'Hér is an aromatic and hairy shrub from which oil is obtained from its leaves, flowers, and stems. Various factors such as cultivar, oil distillation method, distilled part of the plant, age of the material, growing location and seasonal changes in the region, as well as harvest season and time, affected the final essential oil composition of rose-scented geranium (Verma *et al.,* 2013; Szutt *et al.,* 2019). The main constituents of the essential oils of *P. graveolens* were reported as geraniol (14.1-34.6%), citronellol (15.2-31.3%), linalool (2.9-9.2%), citronellyl formate (4.4-9.2%), isomenthone (4.5-6.6%), 10-epi-γ-eudesmol (4.7-6.7%) and geranyl formate (3.8-6.2%) by Verma *et al.* (2013) citronellol (20.9-39.5%), geraniol (10.9-26.5%), linalool (2.9-14.2%), isomenthone (7.4-9.4%), citronellyl formate (5.5-9.1%) and 10-epi-γ-eudesmol (5.2-9.0%) by Singh *et al.* (2018); citronellol (22.3%), geraniol (15.5%), geranyl acetate (13.1%), limonene (9.3%), phenyl ethyl alcohol (5.9%) and linalool (5.6%) by Szutt *et al.* (2018); citronellol (27.0%), geraniol (20.7%), 10-*epi*-γ-eudesmol (13.1%), citronellyl formate (6.4%) and linalool (5.7%) by Ben ElHadj *et al*. (2020). According to ISO 4371-2012, *P. graveolens* essential oil from different geographical origins should have citronellol (18-43%), geraniol (5–20%), linalool (2-–11%), citronellyl formate (4-12%), isomenthone (4-10%), geranyl formate (1–8%), (*Z*)-r*ose* oxide (0.4-3.5%), menthone (0.0-2.5%) and geranyl tiglate (0.7-2.0%) as the main components (ISO, 2012). Considered one of the top 20 oils in the world, the essential oil of rose-scented geranium was extensively used as a flavoring agent in the food, soaps and beverages industry, cosmetic, perfumery, aromatherapy, traditional medicine, and pharmaceutical industries. Rose-scented geranium is famous for its strong rose-like pleasant fragrance; it is cultivated due to its high-value essential oil used in herbal medicine and aromatherapy and the production of high-quality perfumes and cosmetics. Also, *P. graveolens* essential oils or/and extracts are well known for their sensory attributes and pharmacological properties, antioxidant, antibacterial, antifungal, antimicrobial, insecticidal, allelopathic, anti-aflatoxin, anti-urease, anti-tyrosinase, therapeutic, repellent, fumigant and photoprotective effects (Lohani *et al.,* 2019; Ben ElHadj *et al.*, 2020; Kujur *et al.,* 2020). Different drying methods have been developed for the quality product and high-quantity products, and it has been observed that the essential oil content and components depend on the drying conditions, drying method, and plant species (Özgüven *et al.*, 2019). A previous report showed that drying methods and temperature had a significant effect on quality indicators such as organoleptic and sensory properties, oil content, and composition in medicinal and aromatic plants such as *Laurus nobilis* (Sekeroglu *et al.,* 2007), *Mentha longifolia* (Saeidi *et al.,* 2016), *Mentha pulegium* (Ahmed *et al.,* 2018), *Ocimum americanum* (Bhatt *et al.,* 2018), *Thymus daenensis* (Mashkani *et al.,*2018), *Lippia citriodora* (Aghdam *et al.,* 2019), *Lavandula angustifolia* (Sałata *et al.,* 2020) and *Dracocephalum moldavica* (Morshedloo *et al.,* 2021). In most cases, it has been reported for many plants that increasing drying temperature lowers the essential oil content, while keeping the temperature below 30-35 °C preserves more aromatic compounds (Mashkani *et al.,* 2018; Sałata *et al.,* 2020). Drying techniques affect the essential oil yield and composition, so it is very important to determine an appropriate drying method to achieve higher active substances in medicinal and aromatic plants. The drying methods may differ from one aromatic herb and spice to another. For medicinal and aromatic plants which are sensitive to the drying process, optimum drying is required to obtain a high-quality product, as some bioactive compounds change during the drying process. Therefore, the optimization of quality requires studying each specific pre-drying and drying method for each type of herb (Thamkaew *et al.,* 2021). Although a large number of herb-drying studies have been conducted in recent years, as far as we know, studies on the effectiveness of different drying methods on the quantity, quality, and composition of the essential oil of rose-scented geranium are scarce. The present study aimed to determine the influence of different drying methods, which included fresh plants (control), sun-drying, shade-drying, oven-drying at 30 °C, and oven-drying at 60 °C on dry herbage yield and essential oil content and composition of rose-scented geranium.

2. MATERIALS AND METHODS

2.1. Sample preparation

Pelargonium graveolens was grown in the Burhaniye Aromatic Plants Field Station, Balıkesir Metropolitan Municipality Rural Services Department during the 2019 growing season. The fresh aerial parts of *Pelargonium graveolens* (Geraniaceae) which were used in this research were harvested at the flowering phase during a one-year vegetative cycle. The harvested plants were then randomly divided into five groups containing three sets of 700 g of fresh weight in each method. While one of the sets was used as a fresh sample, different drying methods were applied to the others, including shade-drying, sun-drying, oven-drying at 30 °C, and oven-drying at 60 °C. The initial moisture content was determined at 105 °C for 7 h in the oven until there was no change in weight in two measurements.

2.2. Drying methods

The samples were divided into five batches containing 700 g of fresh weight in 3 replicates for each method. The methods were shade-drying at room temperature of 20-25 °C, sun-drying under direct sunlight at 24-27 °C, oven-drying at 30 °C, and oven-drying at 60 °C. In all drying methods, drying was continued until final moisture content reached approximately 10% on a wet basis. Then, when the constant weight was reached, they were ready for essential oil extraction. Shade-drying was carried out at a dark and dry room temperature under natural airflow, without exposure to direct sunlight, 5 cm layer thickness, and shelves on top of each other. For the sun-drying method, a clean white cloth was laid on a cage net 20 cm above the ground in an open area and the samples were dried under direct sunlight by mixing regularly. For the oven-drying method, samples were dried in a laboratory oven (Venticell, Germany) and two temperatures of 30 and 60 °C were used.

2.3. Extraction of essential oils and analysis

To obtain essential oil, 400 g of plant samples, which were subject to different drying methods, were used. The samples included in each application were divided into four as 100 g each. Three of these four samples were used in three replicates to obtain the essential oil. One was reserved as a spare. In each repetition of each method, 100 g of plant samples were ground to obtain essential oil, and immediately after grinding, using 400 mL of distilled water, it was distilled with a Clevenger device (S-H LTD., Ankara, Turkey) for three hours. The essential oil samples obtained were stored at 4 °C in the dark until analysis.

The essential oil analyses and identification were performed using Gas Chromatography-Mass Spectrometry analyses (GC/MS). GC/MS analyses were carried out on an Agilent 7890A GC system equipped with a J&W DB-Wax fused silica capillary 122-7061 column (250 °C: 60 m x 250 µm x 0.15 µm), and 5975C model MS and flame ionization detector (FID) were used simultaneously. The initial temperature of the column was kept at 50 °C, held for 1 min, and gradually increased from 25 °C/min to 200 °C, and then reached 230 °C at 3 °C/min, held for 15 min. The injection volume was 1 µL neat with a split ratio of 50:1. Helium was the carrier gas, used at a constant pressure of 10 psi and a flow rate of 1.0 mL/min. The compounds were identified using the Whiley and NIST Mass Spectral Library data of the GC/MS system, and by comparing the MS and retention index data with the mass spectral literature data (Adams, 2007). The percentages of each component were reported as raw percentages based on total ion current without standardization of each drying method. Changes in the essential oil composition of *Pelargonium graveolens* using different drying methods is summarized in Table 1.

2.4. Statistical analysis

The data were analyzed using the analysis of variance in SPSS. The mean of the main constituents for the essential oil values was compared using Duncan's multiple range test at 1% confidence interval. In order to visually evaluate the changes in terpene classes according to drying methods, a PCA biplot consisting of drying methods and terpenes was created. The biplot suggested by Yan and Rajcan (2002), was also applied to investigate variations within the different studies based on multi-traits data. The PCA biplot, the correlation coefficient between any two terpene classes is approximated by the cosine of the angle between their vectors. Acute angles indicate positive correlations, obtuse angles indicate negative correlation, and right angles indicate no correlations between two

Table 1: Chemical composition of essential oils obtained from aerial part of *Pelargonium graveolens* subjected to different drying methods (n=3)

RI[a]	Constituent[c]	Fresh plant	Methods[b]			
			Shade-drying	Sun-drying	Oven-drying at 30 °C	Oven-drying at 60 °C
1136	α-Pinene	0.48 ± 0.04	0.54 ± 0.03	0.85 ± 0.01	0.61 ± 0.05	0.20 ± 0.04
1161	2H-Pyran	0.07 ± 0.01	0.06 ± 0.01	ND	0.06 ± 0.01	ND
1168	Pentanoic acid. 4-methyl, methyl ester	0.10 ± 0.00	0.08 ± 0.02	0.16 ± 0.01	0.22 ± 0.03	ND
1169	Cyclopentasiloxane. decamethyl-	0.03 ± 0.02	0.06 ± 0.03	0.03 ± 0.01	ND	0.39 ± 0.09
1173	β-Pinene	0.05 ± 0.02	ND	0.19 ± 0.03	ND	ND
1175	Myrcene	0.16 ± 0.02	0.15 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
1177	α-Phellandrene	0.08 ± 0.01	0.04 ± 0.00	0.06 ± 0.01	0.10± 0.01	ND
1182	2-Butenoic acid. 2-methyl, methyl ester (E)	0.76 ± 0.02	0.20 ± 0.05	1.12 ± 0.06	0.70 ± 0.03	ND
1188	Limonene	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	ND
1192	Sabinene	0.08 ± 0.01		0.08 ± 0.00	0.12 ± 0.01	ND
1200	β-Ocimene. (E)-	0.17 ± 0.01	0.29 ± 0.02	0.03 ± 0.02	0.18 ± 0.01	ND
1209	β-Ocimene. (Z)-	0.18 ± 0.01	0.2 ± 0.01	0.21 ± 0.01	0.17 ± 0.01	ND
1210	p-Cymene	0.22 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	0.21 ± 0.01	0.15 ± 0.02
1211	Heptadecanoic acid. methyl ester	0.04 ± 0.01	0.04 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	ND
1214	Terpinolene	0.05 ± 0.01	0.07 ± 0.00	0.07 ± 0.01	ND	ND
1218	Cyclohexasiloxane	0.08 ± 0.01	0.27 ± 0.01	0.37 ± 0.03	0.08 ± 0.01	1.51 ± 0.07
1227	6-Methyl-5-hepten-2-one	0.12 ± 0.01	ND	ND	0.14 ± 0.01	ND
1240	*cis*-Rose oxide	0.71 ± 0.01 d	2.00 ± 0.29 c	3.15 ± 0.02 a	1.04 ± 0.12 d	2.46 ± 0.17 b
1254	Pentadecane	0.09 ± 0.01	ND	0.09 ± 0.00	ND	0.35 ± 0.01
1260	Linalool oxide	0.09 ± 0.24	ND	ND	ND	0.29 ± 0.01
1267	2-Ethyl-1-hexanol	5.4 ± 0.17	1.61 ± 0.40	4.02 ± 0.20	3.34 ± 0.48	1.43 ± 0.07
1281	Menthone	5.51 ± 0.11 b	4.48 ± 0.15 c	8.34 ± 0.30 a	6.52 ± 0.19 b	4.55 ± 0.03 c
1283	Linalool	6.27 ± 0.38 b	7.42 ± 0.44 a	2.88 d ± 0.07 d	3.78 ± 0.19 c	1.96 ± 0.01 e
1289	β-Bourbonene	1.22 ± 0.09 c	0.95 ± 0.05 d	1.83 ± 0.08 b	1.34 ± 0.16 c	2.23 ± 0.13 a
1291	α-Gurjunene	0.27 ± 0.01	ND	ND	ND	0.27 ± 0.01
1292	Aromadendrene	0.28 ± 0.00	ND	0.46 ± 0.01	0.32 ± 0.01	0.56 ± 0.01
1301	Isopulegol	0.07 ± 0.02	0.32 ± 0.01	0.09 ± 0.01	0.12 ± 0.02	0.06 ± 0.01
1303	α-Bergamotene	ND	ND	0.19 ± 0.01	ND	0.12 ± 0.01
1305	α-Guaiene	0.12 ± 0.01	ND	ND	0.13 ± 0.01	ND
1308	Citronellyl formate	ND	ND	ND	ND	4.94 ± 0.02
1310	β-Copaene	ND	ND	ND	ND	0.31 ± 0.1
1312	β-Elemene	0.97 ± 0.04	ND	0.65 ± 0.01	0.82 ± 0.06	0.25 ± 0.02
1316	β-Caryophyllene	4.32 ± 0.18 a	3.36 ± 0.2 b	3.04 ± 0.02 c	4.27 ± 0.05 a	2.63 ± 0.03 c
1327	α-Elemene	0.52 ± 0.03	0.49 ± 0.04	0.18 ± 0.02	ND	0.32 ± 0.02
1334	γ -Muurolene	0.73 ± 0.03	0.04 ± 0.01	0.49 ± 0.01	ND	1.52 ± 0.14
1327	trans-Muurola-3.5-diene	0.38 ± 0.01	ND	0.21 ± 0.01	0.23 ± 0.01	ND
1329	Germacrene D	0.19 ± 0.01	ND	0.20 ± 0.01	0.19 ± 0.01	0.15 ± 0.01
1333	Alloaromadendrene	0.12 ± 0.01	0.72 ± 0.01	ND	0.38 ± 0.01	0.35 ± 0.01
1334	Valencene	0.91 ± 0.02	0.16 ± 0.01	ND	1.04 ± 0.01	ND
1335	Geranyl formate	ND	0.45 ± 0.04	ND	ND	0.79 ± 0.02
1336	Neral	0.45 ± 0.05	0.27 ± 0.01	ND	0.43 ± 0.01	0 ± 0
1339	α-Terpineol	0.61 ± 0.01	0.72 ± 0.02	0.26 ± 0.01	0.39 ± 0.02	0.24 ± 0.01
1342	α-Humulene	1.15 ± 0.06	0.86 ± 0.02	0.85 ± 0.02	1.14 ± 0.03	0.73 ± 0.04
1343	Isoledene	0.54 ± 0.01	0.46 ± 0.01	0.28 ± 0.01	0.65 ± 0.02	ND
1348	Viridiflorene	3.16 ± 0.1 a	2.22 ± 0.05 c	2.69 ± 0.26 b	3.31 ± 0.01 a	3.28 ± 0.09 a
1356	Citronellol	33.06 ± 0.77 b	39.87 ± 0.23 a	27.84 ± 1.21 c	34.67 ± 0.46 b	23.99 ± 0.48 d
1364	Bicyclogermacrene	0.6 ± 0.03	0.33 ± 0	0.29 ± 0.01	0.47 ± 0.01	ND
1366	β-Selinene	ND	ND	ND	ND	0.64 ± 0.01
1367	δ-cadinene	1.17 ± 0.01 c	1.06 ± 0.02 c	1.71 ± 0.11 b	0.05 ± 0.23 d	3.30 ± 0.05 a
1369	*cis*-Muurola-3.5-diene	ND	0.18 ± 0.01	0.21 ± 0.02	1.36 ± 0.20	ND
1371	Nerol	1.31 ± 0.02	1.03 ± 0.01	1.30 ± 0.03	1.17 ± 0.01	1.23 ± 0.02
1372	γ -*Cadine*ne	0.18 ± 0.01	0.15 ± 0.01	0.98 ± 0.02	0.20 ± 0.01	ND

RI[a]	Constituent[c]	Fresh plant	Methods[b]			
			Shade-drying	Sun-drying	Oven-drying at 30 °C	Oven-drying at 60 °C
1374	Geranyl isobutyrate	ND	0.22 ± 0.01	0.10 ± 0.02	ND	0.44 ± 0.01
1377	Geranyl propionate	ND	ND	0.22 ± 0.01	ND	1.10 ± 0.03
1381	α-Cubebene	0.15 ± 0.01	0.17 ± 0.01	0.21 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
1387	Geraniol	16.26 ± 0.07 a	17.09 ± 0.12 a	11.18 ± 0.59 c	14.05 ± 0.26 b	4.15 ± 0.05 d
1398	Aromadendrene. dehydro	0.08 ± 0.00	0.12 ± 0.02	0.09 ± 0.00	0.06 ± 0.01	0.3 ± 0.02
1399	(+)-Calamenene	0.32 ± 0.00	0.33 ± 0.02	0.46 ± 0.01	0.45 ± 0.01	0.95 ± 0.01
1410	Geranyl isovalerate	0.25 ± 0.01	0.22 ± 0.00	0.28 ± 0.04	0.30 ± 0.03	1.29 ± 0.01
1428	2-Phenylethyl alcohol	0.80 ± 0.06	0.28 ± 0.01	1.25 ± 0.04	0.76 ± 0.01	0.14 ± 0.01
1435	2.6-Octadiene. 2.6-dimethyl	0.41 ± 0.02	0.36 ± 0.01	0.46 ± 0.01	0.38 ± 0.01	1.14 ± 0.04
1442	α-Calacorene	0.06 ± 0.01	0.05 ± 0.00	0.21 ± 0.01	0.10 ± 0.01	0.37 ± 0.02
1446	10-epi-cubebol	0.06 ± 0.00	ND	ND	0.06 ± 0.01	ND
1449	5.11-Epoxycadin-1(10)-ene	0.27 ± 0.03	0.26 ± 0.01	0.90 ± 0.01	0.41 ± 0.01	1.35 ± 0.05
1452	(E.Z)-α-Farnesene	ND	0.15 ± 0.01	ND	0.06 ± 0.01	ND
1454	Alloaromadendrene oxide-(1)	ND	ND	0.22 ± 0	0.12 ± 0.01	0.21 ± 0.01
1459	Furopelargone A	0.14 ± 0.01	0.09 ± 0.00	0.36 ± 0.02	0.23 ± 0.01	ND
1472	Citronellyl butyrate	0.66 ± 0.06 bc	0.53 ± 0.02 c	0.81 ± 0.01 bc	1.06 ± 0.08 b	5.31 ± 0.11 a
1485	Geranyl butyrate	ND	0.27 ± 0.02	0.32 ± 0.01	ND	0.77 ± 0.02
1490	Caryophyllene oxide	0.40 ± 0.01	0.55 ± 0.02	1.40 ± 0.03	0.58 ± 0.01	0.76 ± 0.03
1493	Bicyclogermacrene	0.07 ± 0.22	ND	ND	ND	0.28 ± 0.00
1509	Ledol	0.23 ± 0.01	0.27 ± 0.01	0.60 ± 0.01	0.27 ± 0.00	0.79 ± 0.01
1511	1.10-di-*epi*-Cubenol	0.75 ± 0.01	0.57 ± 0.01	1.19 ± 0	1.37 ± 0.05	0.46 ± 0.01
1517	Cubenene	ND	ND	0.20 ± 0.01	ND	0.67 ± 0.01
1526	Cadina-1.4-diene	ND	0.43 ± 0.01	0.42 ± 0.01	ND	0.76 ± 0.01
1528	1-*epi*-Cubenol	0.35 ± 0.02	0.57 ± 0.02	0.45 ± 0.05	0.38 ± 0.03	0.51 ± 0.04
1531	Methyl cinnamate	ND	ND	0.4 ± 0.01	ND	ND
1537	Geranyl tiglate	1.17 ± 0.25 c	1.68 ± 0.07 b	0.99 ± 0.04 c	1.75 ± 0.15 b	4.52 ± 0.03 a
1541	Globulol	0.32 ± 0.01	ND	0.28 ± 0.02	ND	0.17 ± 0.01
1544	Hexahydrofarnesylacetone	ND	ND	0.88 ± 0.01	ND	1.36 ± 0.01
1554	α-Eudesmol	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.00	0.09 ± 0.02	ND
1564	Spathulenol	0.52 ± 0.02	0.36 ± 0.01	0.98 ± 0.01	0.55 ± 0.02	1.69 ± 0.02
1575	Cedrol	ND	ND	0.73 ± 0.01	ND	ND
1583	Farnesol 2	ND	ND	0.19 ± 0.01	ND	0.12 ± 0.01
1588	Geranyl acetate	0.06 ± 0.01	0.07 ± 0.01	0.13 ± 0.01	0.08 ± 0.01	0.16 ± 0.01
1596	tau-Cadinol	ND	ND	0.55 ± 0.01	ND	0.24 ± 0.01
1598	*trans*-Cadina-1(6).4-diene	0.38 ± 0.02	0.15 ± 0.01	0.22 ± 0.01	ND	ND
1616	2-Phenylethyl tiglate	0.51 ± 0.02 e	1.34 ± 0.11 c	1.74 ± 0.29 b	0.96 ± 0.15 d	3.43 ± 0.05 a
1641	Isospathulenol	0.09 ± 0.25	0.14 ± 0.01	0.24 ± 0.02	0.16 ± 0.01	ND
1652	α-Cadinol	0.40 ± 0.03	0.39 ± 0.01	0.55 ± 0.07	0.75 ± 0.06	0.65 ± 0.04
1678	1.4-Benzenedicarboxylicaciddimethyl ester	1.38 ± 0.17	ND	1.18 ± 0.01	1.07 ± 0.01	0.32 ± 0.03
1723	Caryophylladienol I	ND	ND	0.25 ± 0.01	ND	0.31 ± 0.01
1757	Caryophylla-3.8(13)-dien-5β-ol	ND	ND	0.24 ± 0.01	0.26 ± 0.01	0.48 ± 0.01
	Monoterpenes (M)	66.37 ± 1.17 b	75.19 ± 2.72 a	57.62 ± 0.74 c	64.26 ± 1.22 b	40.53 ± 0.8 d
	Monoterpenes hydrocarbons (MH)	2.03 ± 0.03 b	1.99 ± 0.06 b	2.58 ± 0.06 a	2.09 ± 0.08 b	1.63 ± 0.04 c
	Oxygenated monoterpenes (OM)	64.34 ± 0.38 b	73.2 ± 1.32 a	55.04 ± 0.57 c	62.17 ± 2.01 b	38.9 ± 3.04 d
	Sesquiterpenes (S)	22.8 ± 0.41 c	18.29 ± 0.69 d	27.25 ± 0.97 b	24.27 ± 0.35 c	41.69 ± 0.45 a
	Sesquiterpenes hydrocarbons (SH)	18.88 ± 0.37 b	14.25 ± 0.56 c	18.62 ± 0.31 b	18.74 ± 0.75 b	29.19 ± 0.31 a
	Oxygenated sesquiterpenes (OS)	3.92 ± 0.05 d	4.04 ± 0.06 d	8.63 ± 0.26 b	5.53 ± 0.20 c	12.56 ± 0.33 a
	Others (O)	9.94 ± 0.16 c	4.45 ± 0.49 e	12.09 ± 0.29 b	8.28 ± 0.69 d	14.41 ± 0.13 a
	Total (%)	99.11 ± 0.33	98.23 ± 0.47	96.96 ± 0.29	96.81 ± 0.19	96.63 ± 0.29

[a]RI: retention Index, [b]: Averages of the same linear values (each section separately) followed by same letter did not differ significantly from Duncan's multiple range tests at 0.01% significance. [c]: Mean value ± standard deviation, and the mean values of the components of each drying method were based on the average of three replicates. ND: not detected.

classes. The length of the vector describes the discriminating ability of the terpene class. A terpene class with a short vector indicates that the class is not associated with other classes, lacks variation, or is not useful for drying method discrimination (Akçura, 2011). A PCA biplot analysis was performed using GGE biplot software (Yan, 2001).

3. RESULTS AND DISCUSSION

The results showed that the drying methods had a significant effect on EO content (Figure 1). Fresh plant and samples dried by shade-drying and by oven at 30 °C showed high EO contents (1.98, 1.34, and 1.20%, respectively). In contrast, low essential oil content of 0.70 and 0.42% was obtained from sun-drying and oven-drying at 60 °C. The methods fresh sample, shade-drying, and oven-drying at 30 °C resulted in higher EO, while increasing temperature (from 30 to 60 °C) showed a decrease in EO content. Similarly, Çalışkan *et al.* (2017) in *Mentha piperita*, found higher essential oil content in shade-drying and oven-drying at 38 °C than sun-drying. Although not as much as the fresh plant material, both shade-drying at ambient temperature and oven-drying at 30 °C gave feasible results in terms of oil yield. The changes in essential oil content during the drying process depended on temperature, time, and drying method. Similarly, Sourestani *et al.* (2014) in *Agastache foeniculum*, found higher essential oil content at room temperature (25 °C) than oven-drying at 40 °C. Shade-drying and oven-drying (at 30 °C) methods are considered efficient to achieve the best EO quality and quantity (Saeidi *et al.,* 2016). Some studies pointed out that increased drying temperature can damage glandular trichomes, decomposition of some essential oil components through high-temperature autoxidation and hydro peroxidation (Turek and Stintzing, 2006), and accelerated evaporation and decomposition of essential oil components (Mashkani *et al.,* 2018) can cause a decrease in essential oil content.

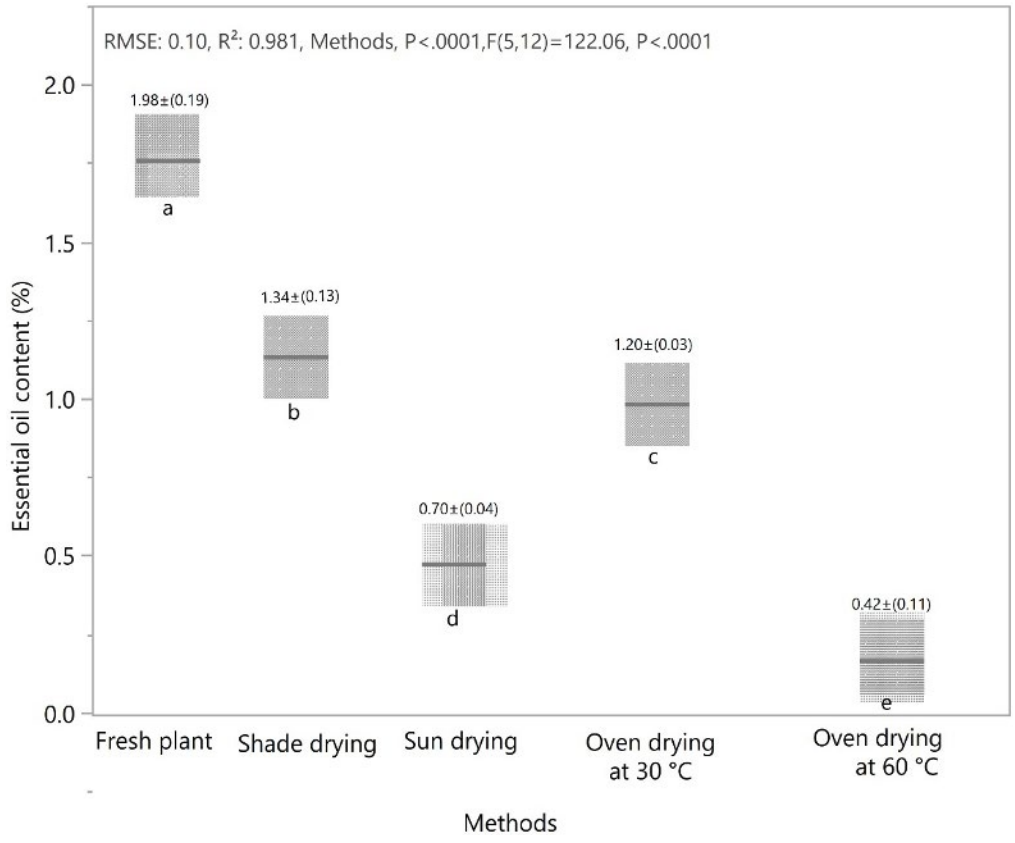

FIGURE 1. The comparison mean of the essential oil content (%) of *Pelargonium graveolens* changes according to the different drying methods (n=3). Results are expressed as means ± standard error indicated on the box plot. Means of essential oil content followed by similar letters in boxess are not significantly different at 1% probability level by the LSD test (LSD value= 0.1932). The results of the essential oil content for each drying method are based on the average of three replicates.

Ninety-three components were identified in EO samples of rose-scented geranium by using different drying methods comprising 96.63 to 99.11% of total EO (Table 1). The majority of them consisted of oxygenated monoterpenes and sesquiterpene hydrocarbons. The main components in the EO in all drying methods were citronellol (24.0-39.9%), geraniol (4.2-17.1%), menthone (4.5-8.3%), and linalool (2.0-7.4%). Other main components in the oil were found to be β-caryophyllene, *cis*-rose oxide, geranyl tiglate, citronellyl butyrate, viridiflorene, 2-phenylethyl tiglate, β-bourbonene, δ-cadinene and nerol. These results are consistent with previous reports (Juliani *et al.,* 2006), which demonstrated that commercial geranium oils are characterized by high citronellol (19-45%) and lower amounts of geraniol (less than 24%) and linalool (less than 14%) as the main components.

Citronellol and geraniol, which are oxygenated monoterpene, reached their highest values under natural shade-drying conditions; whereas the lowest value was achieved by artificial oven-drying at 60 °C. Since high temperatures cause a large loss in citronellol, geraniol, and linalool contents, these should be considered to be compounds which are sensitive to direct sunlight, high and low temperatures. The results demonstrated that the aerial parts of geranium with sun-drying, oven-drying at 30 °C, and oven-drying at 60 °C presented decreased citronellol content by about 12.03, 5.20, and 15.88% as compared to the shade-drying, with geraniol content by 5.91, 3.04, and 12.94% and linalool content by 4.54, 3.64, and 5.46%, respectively. Oxygenated monoterpenes with sweet rose-like (citronellol) and flowery rose-like (geraniol) odor in geranium oil are important reasons for the demand for perfumery.

The highest percentage of δ-cadinene, citronellyl butyrate, geranyl tiglate and 2-phenylethyl tiglate were obtained from samples dried in an oven 60 °C;, while the highest amount of linalool and β-caryophyllene were obtained from the sample dried by shade ambient temperature and the fresh sample. Increasing the drying temperature from 30 to 60 °C significantly reduced the contents in citronellol, geraniol, β-caryophyllene, menthone, and linalool in the dried aerial parts of geranium; whereas the contents in citronellyl butyrate, geranyl tiglate, 2-phenylethyl tiglate, δ-cadinene and spathulenol increased. Citronellyl formate, β-copaene, and β-selinene were detected only in oven-dried samples at 60 °C. The sun-drying method had a stimulative effect on some other compounds' biosynthesis and accumulation such as α-pinene, cis-rose oxide, menthone, δ-cadinene, γ-cadinene, and caryophyllene oxide. The drying method affected the geranium's chemical profiles and caused significant changes in the contents in citronellol, geraniol, linalool, menthone, and β-caryophyllene, which are the main compounds in the EO.

Factors such as plant species, drying method, drying conditions and time, amount of water evaporated during drying, temperature, the chemical structure of the compounds, oxidation, chemical reactions, degradation, isomerization, cyclization, dehydrogenation, glycoside hydrolysis, autoxidation of terpenoids, esterification and/or other processes could significantly change the chemical profiles of EO and some of the EO compounds may be lost, reduced and/or increased (Ahmed *et al.,* 2018; Bhatt *et al.,* 2018; Beigi *et al.,* 2018; Özgüven *et al.,* 2019; Thamkaew *et al.,* 2021). One of the most important chemical changes is due to the autoxidation of oil components that affect the deterioration process of terpenoids, and increasing drying temperature and exposure to direct sunlight causes further loss in aroma components and degradation of aroma quality (Başer and Demirci, 2011; Thamkaew *et al.,* 2021). Compared to shade drying, especially in an oven at 60 °C and sun drying, the volatile profile of the EO changed due to the formation of secondary aroma compounds such as terpene esters, sesquiterpenes, alcohols, aldehydes, and others. During drying, the EO composition and content increased, decreased and the production of new compounds occurred. During the drying process, the EO compositions of the plants changed, which may be a result of the release of components from the rupture of their cell walls, oxidation reactions, or hydrolysis of glycosylated volatile compounds (Xing *et al.,* 2018).

Among the compounds identified in EO, the percentage of oxygenated monoterpenes (OM) was the highest, ranging from 38.9 to 73.2%. EO extracted from plants dried at 30 and 60 °C and sun-dried contained more sesquiterpenes and fewer monoterpenes compared to fresh samples and drying in shady, natural conditions. Sun and oven drying most reduced the contents in compounds from the OM groups, while fresh and drying in shady, nat-

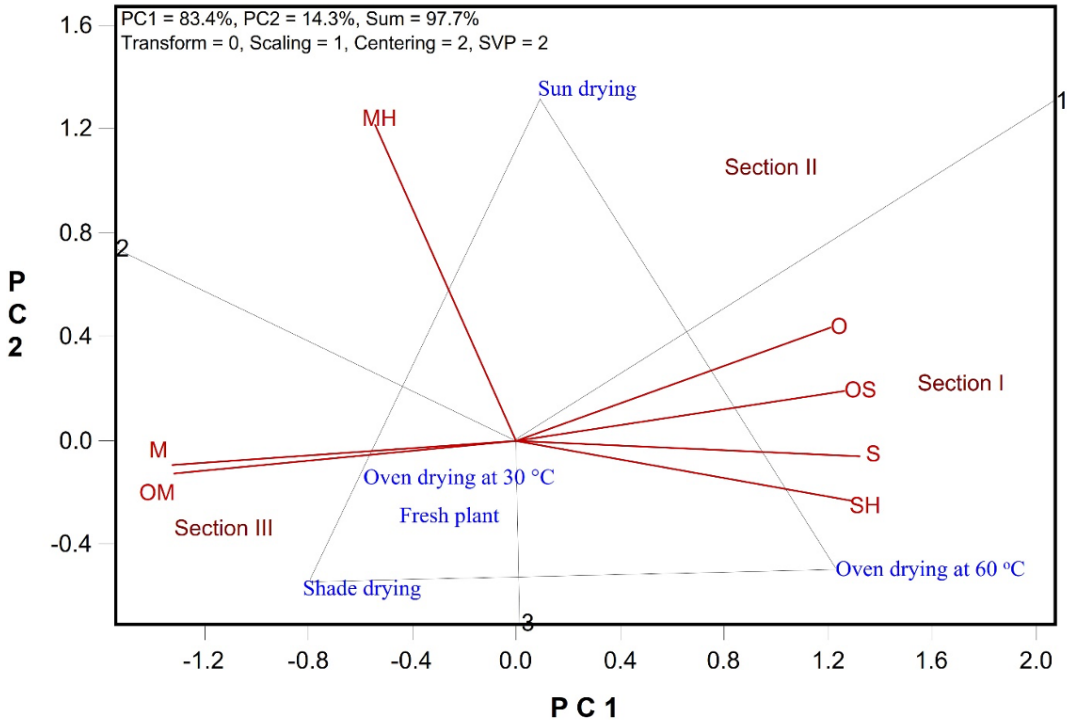

FIGURE 2. PCA biplot of the changes in active ingredient groups of geranium essential oil according to drying methods and the relations between groups. M: Monoterpenes, MH:Monoterpenes hydrocarbons, OM: Oxygenated monoterpenes, S: Sesquiterpenes, SH: Sesquiterpenes hydrocarbons, OS: Oxygenated sesquiterpenes, O: Others

ural conditions reduced the contents in compounds from the SH and OS groups. Drying of *Pelargonium graveolens* in the shade was most suitable for a high percentage of OM. Aghdam *et al.* (2019), who found high monoterpene content in fresh lemon verbena plants and sesquiterpene content in oven drying, presented similar conclusions.

Drying in direct sun resulted in a reduction in the contents in EO, citronellol, geraniol, linalool, and β-caryophyllene compared to fresh samples, shade-drying, and oven-drying at 30 °C, so it may not be an appropriate drying method for geranium. The shade-drying method preserved the EO content and the major volatile components in geranium better than the oven-drying and sun-drying methods. However, although the drying time of shade-drying is longer than sun-drying, it can provide advantages in terms of preserving light-sensitive substances and minimizing light-dependent chemical reactions such as oxidation. In terms of EO content and components, it was found that drying with hot air at 60 °C is not appropriate, but low drying temperature (30 °C) is appropriate for oil content and preservation of heat-sensitive compounds in geranium.

The PCA-biplot created to visually evaluate the changes in terpene classes according to drying methods is shown in Figure 2. The PCA biplot in this study captured 97.7% of the variations due to drying method and drying method by terpene group interactions. Polygons were created to evaluate drying methods in the biplot, and drying methods are presented with vectors. Terpenes, which had a positive relationship with drying methods, were located close to each other. In the three sections on the biplot, drying methods and terpene classes, which are positively related, formed three groups. Oxygenated sesquiterpenes (OS), sesquiterpenes (S), sesquiterpenes hydrocarbons (SH) and others (O) increased with oven-drying at 60 °C, monoterpenes (M) and oxygenated monoterpenes (OM) increased with sun-drying, monoterpenes hydrocarbons (MH) increased with shade-drying, fresh plant and oven-drying at 30 °C methods (Figure 2).

4. CONCLUSIONS

The quality of medicinal dried herbs is defined by the content in bioactive compounds. The drying methods had a significant impact on the essential oil con-

tent and composition, which is the quality indicator of *Pelargonium graveolens* L'Hér. While high temperature reduced the rate of EO in oven-drying, fresh plant and shade-drying were found to be more appropriate in terms of oil content and components compared to the other methods. A significant difference was noted in the percentage of main constituents such as citronellol, geraniol, linalool, β-caryophyllene geranyl acetate, geranyl tiglate, citronellyl butyrate and viridiflorene between the different natural and artificial drying methods, as well as between them and fresh samples. While the highest oxygen monoterpene contents were identified in shade-dried and fresh plants, oven-drying had a stimulating effect on the biosynthesis and accumulation of sesquiterpene compounds. The data can be used by pharmaceutical and perfumery industries in their post-harvesting programs. The results of this study showed that drying *Pelargonium graveolens* in natural shade is more suitable for high oil yield and oxygenated monoterpene content while drying this plant in the oven at 30 °C can be recommended to shorten the drying process.

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RESEARCH DATA POLICY DATA AVAILABILITY

The authors declare no conflict of interest relating to the article.

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