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Use of molecular methods for the identification of yeast species isolated from fermentations of table olives produced traditionally in Kahramanmaraş

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SUMMARY: In this study, yeast species involved in the naturally fermented green table olive produced in Southern Turkey were investigated. Table olive samples were prepared with regional olive cultivars and traditional methods were employed in the production. Yeasts were isolated from the brines of the samples at the late fermentation stage and identified at the species level by the DNA sequences of the D1/D2 domain of 26S rRNA genes. The D1/D2 domains were amplified by PCR, sequenced and compared to reference sequences deposited in the NCBI database. According to the results, isolated yeasts belonged to the *Candida*, *Debaryomyces*, and *Rhodotorula* genera and salt tolerant species were dominant as the salt content of the brines exceeded 11%. Among the determined species, *Candida oleophila* was the most dominant one and it was thought that isolated strains of *Candida oleophila* may be taken into consideration to be used as starter culture in table olive production.

KEYWORDS: *Fermentation; Molecular identification; Salt; Table olive; Traditional; Yeast*

RESUMEN: ***Uso de métodos moleculares para la identificación de especies de levaduras aisladas de fermentaciones de aceitunas de mesa producidas tradicionalmente en Kahramanmaraş*.** En este estudio, se investigaron las especies de levaduras que intervinieron en la fermentación natural de aceitunas verdes de mesa del sur de Turquía. Las muestras de aceitunas de mesa se prepararon con cultivares de aceitunas regionales y se emplearon métodos tradicionales en la producción. Las levaduras se aislaron de las salmueras de las muestras en la última etapa de fermentación y se identificaron a nivel de especie mediante las secuencias de ADN del dominio D1/D2 de los genes 26S rRNA. Los dominios D1/D2 fueron amplificados por PCR, secuenciados y comparados con las secuencias de referencia depositadas en la base de datos del NCBI. Según los resultados, las levaduras aisladas pertenecen a los géneros *Candida*, *Debaryomyces* y *Rhodotorula*, y las especies tolerantes a la sal fueron dominantes, ya que el contenido de sal de las salmueras superó el 11 %. Entre las especies determinadas, *Candida oleophila* fue la dominante y se pensó que las cepas aisladas de *Candida oleophila* podrían tenerse en cuenta para su uso como cultivo iniciador en la producción de aceitunas de mesa.

PALABRAS CLAVE: *Fermentación; Identificación; Levaduras; Métodos moleculares; Olivas de mesa; Salmueras.*

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1. INTRODUCTION

The table olive is a food product obtained by fermenting the olive fruit, which is characterized by its low sugar content (20-50 g/kg), high oil content (200-350 g/kg) and bitter taste originating from the oleuropein contained in olives. Olive fruits are not suitable for fresh consumption due to their bitter taste and must be processed to remove bitterness (oleuropein) after harvest (Silva *et al*., 2011). Olives are widely produced in the Mediterranean Basin. Turkey is the 4th largest producer in the world (FAO, 2021) and, therefore, this production volume brings an advantage to the production of olive-related products. To benefit from this advantage it is stated that olive varieties should be considered separately and appropriate processing techniques specific to varieties should be developed in order to diversify the products and to improve their quality. (Erten and Tanguler, 2014).

The purpose of table olive production is to remove oleuropein and to preserve the olive fruits via fermentation (Arroyo-López *et al*., 2012a; Leventdurur *et al*., 2016). In the production of naturally fermented (spontaneous) table olives, following the pre-processes (washing, slitting, cracking), olives are left for fermentation in a brine usually containing 8-10% (w/v) salt. The fermentation period takes around 3-9 months. During fermentation, the oleuropein in the olive passes into the brine, the olive becomes less bitter and less prone to spoilage as a result of the activity of microorganisms in fermentation (Kara and Özbaş, 2013).

Table olive fermentation is driven by the natural microbiota of the olive. Although various groups of microorganisms participate in fermentation, the dominant ones are lactic acid bacteria (LAB) and yeasts. LAB carries out the main fermentation in table olive production. With metabolic activities, LAB reduces the pH value by producing lactic acid, suppresses pathogenic and spoilage microorganisms, and also contributes to the debittering of olives. Yeasts participate in the fermentation as secondary microbiota, but they may affect the quality of the product significantly (Silva *et al*., 2011). During fermentation, yeasts produce glycerol, ethanol, higher alcohols, esters, organic acids and other volatiles. These compounds have a direct impact on the flavor of the product. Also, yeasts can suppress the growth of unwanted fungi. In addition, yeasts produce nutrients that can be used by LAB via breaking down complex carbohydrates and proteins, and synthesize various vitamins. Besides, yeasts can hydrolyze the oleuropein with β-glucosidase and esterase activity. Thus, they contribute to LAB development and the debittering of olives (Arroyo-López *et al*., 2012a; Porru *et al*., 2018).

Despite their positive effects on fermentation, yeasts can also cause spoilage if fermentative species become dominant. Yeasts can metabolize lactic acid, which causes an increase in pH and decrease in the protective state of brine. Also yeasts produce a high amount of CO_2 which may lead to gas pocket formation in olive fruits and some yeasts can cause the softening of fruits with their protease, xylanase and pectinase enzymes (Arroyo-López *et al*., 2012a).

As stated above, yeasts play a crucial role in table olive production. Therefore, the identification and determination of the technological properties of the yeast species involved in fermentation are important in terms of improving production techniques, diversifying the products and increasing the quality of the product.

In some studies on the determination of the yeasts involved in olive fermentation, *Saccharomyces cerevisiae, Saccharomyces oleaginosus, Wickerhamomyces anomalus, Candida boidinii, Candida oleophila, Candida diddensiae, Candida quercitrusa, Candida sorbosivorans, Candida hellenica, Pichia galeiformis, Pichia membranifaciens*, *Pichia kluyveri, Pichia guilliermondii, Kluyveromyces lactis, Debaryomyces hansenii, Issatchenkia occidentalis, Torulaspora delbrueckii, Rhodotorula mucilaginosa, Rhodotorula glutinis, Rhodotorula graminis, Zygotorulaspora mrakii, Nakazawaea molendiniolei, Metschnikowia pulcherrima, Yarrowia lipolytica, Yarrowia deformans, Citeromyces nyonsensis* species were identified (Arroyo-López *et al*., 2012b; Hernández *et al*., 2007; Hurtado *et al*., 2008; Deak, 2008; Nisiotou *et al*., 2010; Silva *et al*., 2011; Bautista-Gallego *et al*., 2011; Muccilli *et al*., 2011; Alves *et al*., 2012; Tofalo *et al*., 2013; Pereira *et al*., 2015; Leventdurur *et al*., 2016; Porru *et al*. 2018; Mujdeci *et al*., 2018; Ruiz-Moyano *et al*., 2019).

The content in the brine and the origin of the olive have a significant effect on the diversity of the yeast population in table olives. It has been revealed in various studies that the yeast species and the dom-

inant microbiota vary in table olives with different olive cultivars, with olives obtained from different regions and with the same cultivar in different brine contents (Hurtado *et al*., 2008; Nisiotou *et al*., 2010; Tofalo *et al*., 2013; Pereira *et al*., 2015; Leventdurur *et al*., 2016; Porru *et al*. 2018; Mujdeci *et al*., 2018).

The various species of yeasts involved in fermentation and the variety of metabolic activities of these species will most likely have a different impact on the flavor and quality of the final product. Keeping the fermentation process under control and ensuring the desired quality is important in the production of fermented food. The use of starter cultures in fermented foods is a globally accepted practice as it provides predictable fermentations and standard product quality. Starter cultures are generally obtained from spontaneously fermented foods and selected for their desired metabolic traits. High adaptation to the fermentation environment and becoming dominant in the fermentation are the main criteria taken into consideration in the selection of starters. Therefore, strains that can survive and contribute throughout fermentation have the potential to be used as starter cultures (Corsetti *et al*., 2012; Vinicius De Melo Pereira *et al*., 2020).

The aim of this study is to determine the yeast species involved in the fermentation of traditionally produced green table olives and to specify the dominant species.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Table olive processing and sampling

Five table olive samples (MZ A, MZ B, MZ C, MZ D, MZ E) were obtained from a local producer preparing table olives with regional cultivars (Büyük Topak and Sarı Ulak) according to the traditional method used in Kahramanmaraş (Eastern Mediterranean region of Turkey). The traditional method includes the following steps: (1) washing the olives to remove dirt, (2) slitting the olives, (3) keeping the slit olives in tap water for debittering (tap water was changed at regular intervals until desired debitterness was achieved), (4) placing the debittered olives directly into brine (brine was prepared to contain 15% salt), (5) fermentation (samples in glass containers were kept at room temperature for 3 months). No salt was added to the brine during fermentation. At the end of the 3-month fermentation period, 500 mL brine of the table olive samples was taken and stored at +4 °C until the analysis. Yeasts isolation and chemical analyses were carried out with a maximum delay of 48 hours after sampling.

2.2. Methods

2.2.1. Physicochemical analyses

Salt content, titratable acidity and pH analyses were conducted on the brine samples. Titratable acidity was determined as g/L lactic acid equivalent by titration with N/10 NaOH. Salt content was determined with N/10 $AgNO_3$ solution according to the titration method. The pH values of the brine samples were measured directly with a pH meter without pre-treatment (AOAC, 1990).

2.2.2. Microbiological analyses

For the isolation of yeasts, serial dilutions of the brines of the samples were prepared with sterile physiological saline solution (0.85% w/v NaCl), and plated onto yeast extract peptone dextrose (YPD) agar with chloramphenicol (yeast extract 10 g/L, dextrose 20 g/L, peptone 20 g/L, agar 15 g/L, chloramphenicol 0.1 g/L). Plates were incubated at 25 °C for 4 days. The total number of yeasts (log cfu/mL) was determined by counting the colonies that developed at the end of the incubation, and the colonies were examined morphologically. Colonies which differed morphologically were selected and purified by repeated cultivation in YPD broth (10 g/L yeast extract, 20 g/L dextrose, 20 g/L peptone) and YPD agar media. Pure isolates were stored in 15% glycerol at -20 °C (Silva *et al*., 2011).

2.2.3. Molecular identification

Yeast isolates were identified according to their nucleotide sequences of the D1/D2 domains of the 26S rRNA genes. A small amount of colony was taken with a sterile loop from 24-48 h-old colonies, mixed with 10 µL of sterile distilled water and kept at 95 °C for 15 minutes (da Silva *et al*., 2011).

The obtained colony suspension was used as the template in DNA in PCR studies. NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers were

used for the amplification of the D1/D2 region (Kurtzman and Robnett, 1998)

A PCR reaction mixture was prepared in 40 µL volume containing 1µL template DNA, 4 µL 10× buffer (including 25 mM $MgCl_2$), 1 µL dNTP (2 mM), 1 µL NL1 primer (10 pmol), 1 µL NL4 primer (10 pmol), 1 µL Taq DNA polimerase (10 U/ml) and 31 µL sterile distilled water. PCR conditions were selected as: a single initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min; annealing at 54 °C for 1 min; extension at 72 °C for 1 min; and a single final extension at 72 °C for 10 min. The obtained PCR products were electrophoretically separated in 1% agarose gel and checked under UV after staining with ethidium bromide. The D1/D2 regions of the isolates, determined to be approximately 600 bp in length, were commercially (BM Labosis, Türkiye) sequenced via Sanger dideoxy sequencing. The obtained data were analyzed with the MEGA program and compared with the reference sequences registered in the NCBI database with the help of the BLAST algorithm. Isolates were identified at species level as their closest relative reference (Ozturk, 2015).

3. RESULTS AND DISCUSSION

The results for titratable acidity, pH, salt content and total yeast count in the brine samples were determined in the range of 1.9-2.1 g/L lactic acid, 5.47-5.31 pH, 11.2-11.6% salt content, and 4.1-4.5 log cfu/mL total yeast count, respectively (Table 1).

Salt plays an important role in suppressing spoilage and pathogenic microorganisms in table olive production. During production, the olives are placed in brine with a certain salt content (usually 8%). However, during fermentation, there is an exchange of substances between the olive and the brine and the salt content of the brine decreases. If the salt level in the brine falls below the desired value, the protective/suppressing effect of the salt decreases and unwanted microorganisms may develop in the product. To prevent this situation, the salt level of the brine is controlled and increased, but too much salt can also prevent the growth of the microorganisms that carry out fermentation (Kara and Özbaş, 2013).

In the traditional production method used in this study, brine is prepared with high salt content (15%) and no salt is added to the brine during fermentation. According to the results of the salt analysis, it was determined that the salt content in the brine samples decreased from 15 to around 11% (Table 1). However, this value was higher than the 8% range used mostly in table olive production. Therefore, it can be said that although the amount of salt in the brine decreased, its suppressive effect remained constant.

Total titratable acidity and pH of the samples were determined as 1.98 ± 0.08 (g/L) and 5.39 ± 0.06, respectively (Table 1). When compared to previous studies, it was determined that the total acidity was lower and pH was higher than the values in brines with lower salt concentrations at 90 days of fermentation (Tassou *et al*., 2002; Bleve *et al*., 2015). As the total acidity in brine is composed of organic acids (mainly lactic acid) as a result of microbial activity, the low amount of acid can be explained by the low growth of LAB in the brine considering its high salt content. Another reason may be the debittering process. During debittering, oleuropein passes from the olive into the water, along with the nutrients and microorganisms in the olive, resulting in a decrease in the total number of microorganisms and amount of nutrients. On the other hand, Leventdurur *et al*., (2016) reported that the total acidity of table olives at the end of the 90 days of fermentation showed significant variation according to the region where olives were obtained.

Table 1. Results of chemical and microbiological analyses of brines

Sample	Titratable acidity (g/L)	pH	Salt content (%)	Total yeast count (log cfu/mL)
MZ A	1.9	5.45	11.2	4.5
MZ B	2.0	5.37	11.2	4.5
MZ C	2.0	5.35	11.5	4.3
MZ D	1.9	5.47	11.6	4.1
MZ E	2.1	5.31	11.4	4.5
Mean±SD	1.98 ± 0.08	5.39 ± 0.06	11.38 ± 0.17	4.38 ± 0.17

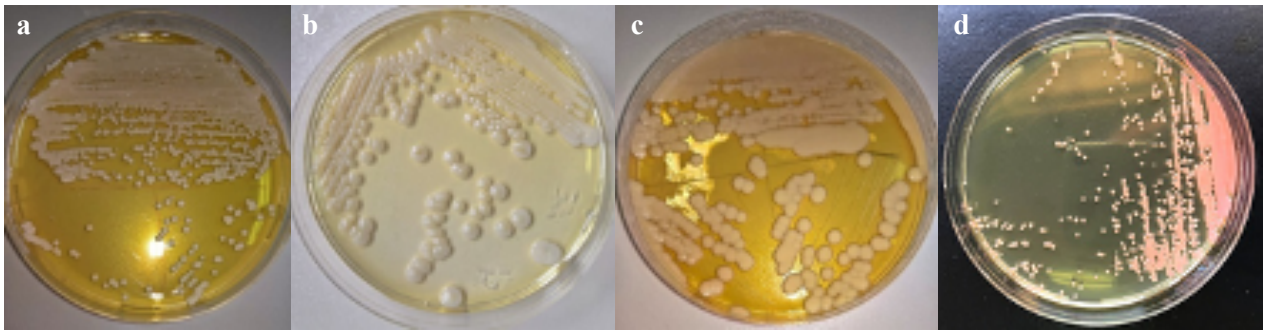

Figure 1. Colony morphology of yeasts isolated from table olive samples (a-MZ 03, b-MZ 02, c-MZ 01, d-MZ R1)

According to the results of the microbiological analysis, the total number of yeasts in the samples was found to be 4.38 ± 0.17 log cfu/ml (Table 1). When compared to the literature, it was determined that the total number of yeasts was lower than the values obtained in the studies on table olives prepared with low-salt brines (Muccilli *et al*., 2011; Tofalo *et al*., 2013; Pereira *et al*., 2015; Leventdurur *et al*., 2016). Likewise, the low total yeast count may result from the salt content in the brine, the production technique, the olive cultivar or its region.

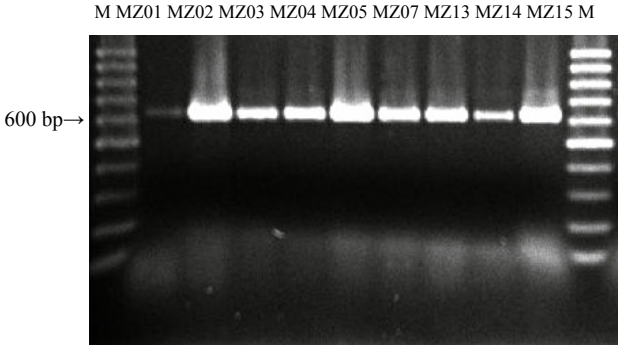

Figure 2. PCR amplification products (620 bp) obtained from pure culture. DNA sizes are numbered according to the M: 100 bp DNA ladder.

At the isolation stage, 49 morphologically different isolates were selected and purified (Figure 1). PCR amplification products were obtained from pure culture. The gel images obtained as a result of the genotypic characterization are shown in Figure 2. Sequences of the D1/D2 region of the isolates were used to compare with the references registered in the NCBI database. The accession numbers for the D1/D2 sequences of isolates also deposited into the NCBI database, are given in Table 3. Comparison results revealed that the isolates belonged to the *Debaryomyces, Candida, Rhodotorula* genus. According to the results obtained, *Debaryomyces hansenii*, *Candida zeylanoides*, *Candida oleophila, Candida diddensiae,* and *Rhodotorula mucilaginosa* species constituted the yeast population in the table olive samples (Table 2).

Among 49 isolates, 20 were identified as *Candida zeylanoides*, 11 as *Debaryomyces hansenii*, 11 as *Candida oleophila*, 5 as *Candida diddensiae* and 2 as *Rhodotorula mucilaginosa* (Table 2).

Debaryomyces hansenii was the second most dominant species among all isolates identified at a rate of 23% (Figure 3). *Debaryomyces hansenii* is an osmotolerant, salt-tolerant species and is frequently

Table 2. Isolates and similarity rates with references registered in the NCBI database

Isolate	Species	Similarity (%)	Reference
MZ 01, MZ 15, MZ 16, MZ 17, MZ 20, MZ 28, MZ 34, MZ 37, MZ 45, MZ 50, MZ 53	Debaryomyces hansenii	99-100	KY512308.1
MZ 02, MZ 04, MZ 07, MZ 11, MZ 18, MZ 21, MZ 24, MZ 27, MZ 31, MZ 35, MZ 49	Candida oleophila	99-100	KY106621.1
MZ 03, MZ 05, MZ 06, MZ 08, MZ 09, MZ 10, MZ 12, MZ 19, MZ 22, MZ 25, MZ 26, MZ 30, MZ 32, MZ 36, MZ 38, MZ 39, MZ 41, MZ 42, MZ 44, MZ 51	Candida zeylanoides	99-100	KC160591.1
MZ 13, MZ 14, MZ 29, MZ 43, MZ 54	Candida diddensiae	99-100	KY106416.1
MZ R1, MZ R2	Rhodotorula mucilaginosa	99	JQ965876.1

Table 3. Accession numbers for the sequences of isolates deposited in the NCBI database

I*	AN**	I*	AN**	I*	AN**	I*	AN**
MZ 01	OQ692035	MZ 14	OQ692049	MZ 27	OQ692065	MZ 41	OQ692081
MZ 02	OQ692058	MZ 15	OQ692036	MZ 28	OQ692040	MZ 42	OQ692082
MZ 03	OQ683789	MZ 16	OQ692037	MZ 29	OQ692050	MZ 43	OQ692051
MZ 04	OQ692059	MZ 17	OQ692038	MZ 30	OQ692076	MZ 44	OQ692083
MZ 05	OQ683790	MZ 18	OQ692062	MZ 31	OQ692066	MZ 45	OQ692043
MZ 06	OQ692069	MZ 19	OQ683792	MZ 32	OQ692077	MZ 49	OQ692068
MZ 07	OQ692060	MZ 20	OQ692039	MZ 34	OQ692041	MZ 50	OQ692044
MZ 08	OQ692070	MZ 21	OQ692063	MZ 35	OQ692067	MZ 51	OQ692084
MZ 09	OQ692071	MZ 22	OQ692073	MZ 36	OQ692078	MZ 53	OQ692045
MZ 10	OQ692072	MZ 24	OQ692064	MZ 37	OQ692042	MZ 54	OQ692052
MZ 11	OQ692061	MZ 25	OQ692074	MZ 38	OQ692079	MZ R1	OQ692033
MZ 12	OQ683791	MZ 26	OQ692075	MZ 39	OQ692080	MZ R2	OQ692034
MZ 13	OQ692048						

I*, isolates
AN**, accession numbers for the sequences of isolates deposited to the NCBI database

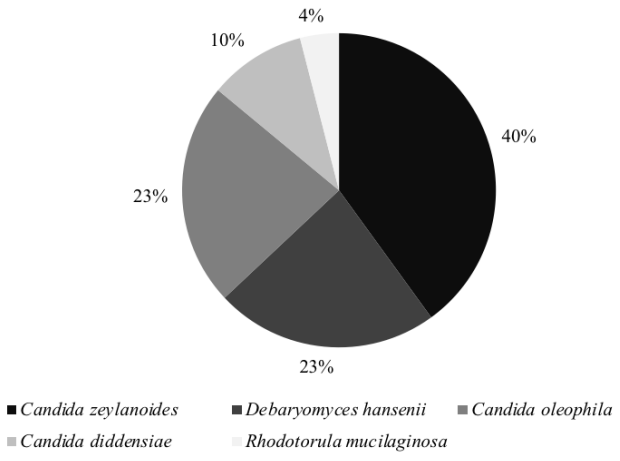

Figure 3. Distribution of isolated yeast species in olive microbiota

isolated from olive brine (Hernández *et al*., 2007; Bautista-Gallego *et al*., 2011; Pereira *et al*., 2015; Bleve *et al*., 2015). *Debaryomyces hansenii* is not usually the dominant species in olive fermentation, but it was reported that it has protease, lipase, esterase, β-glucosidase, catalase activities and it may contribute to the debittering of olives, the development of aroma, and the protection of the product from oxidation (Hernández *et al*., 2007; Ozturk and Sagdic, 2014).

Candida oleophila, as *Debaryomyces hansenii,* constituted 23% of the brine's yeast population (Figure 3). *Candida oleophila* is found in the microbiota of olive fruit, and therefore isolated from spontaneously fermented table olives. Although it is not the dominant species, it was found to have a positive effect on the sensory properties and quality of olives with its lipase, catalase, β-glucosidase activity. Also it can produce B group vitamins during fermentation and contribute to the development of LAB and has an inhibiting effect on some spoiling and pathogenic bacteria (Silva *et al*., 2011; Alves *et al*., 2012; Arroyo-lópez *et al*., 2012a).

Candida diddensiae was detected a rate of 10%, which is a smaller amount than previous species. *Candida diddensiae* is frequently isolated from table olives, as it is one of the dominant species at the beginning of olive fermentations. *Candida diddensiae* has been reported to show high potential for use as a starter for olive fermentation. It was reported to possess protease, lipase, esterase, β-glucosidase activities, good salt tolerance, and the ability to synthesize B group vitamins (Hurtado *et al*., 2008; Muccilli *et al*., 2011; Bautista-Gallego *et al*., 2011).

Rhodotorula mucilaginosa was found to be the yeast species which was present in the smallest amount, constituting only 4% of all isolates. *Rhodotorula mucilaginosa* is frequently isolated from fruits, but found in low amounts in olive fermentation at the initial stage. *Rhodotorula mucilaginosa* is a pigmented yeast with high salt tolerance and esterase activity and reported to cause softening in olives, and to show potential probiotic properties with its high resistance to gastric and pancreatic environ-

ments (Nisiotou *et al*., 2010; Muccilli *et al*., 2011; Alves *et al*., 2012).

Candida zeylanoides was the most dominant species among all isolates with the rate of 40%. *Candida zeylanoides* is a yeast species with high salt resistance, lipase, protease and catalase activities. *Candida zeylanoides* was rarely isolated from olive fermentations. It has been normally usually as the dominant species from meat products and cheese and reported to be effective in the development of meat flavor and can be used as a starter (Fadda *et al*., 2004; Hernández *et al*., 2007; Ozturk and Sagdic, 2014; Ozturk, 2015; Cardinali *et al*., 2021).

The species identified in this study have been reported in previous studies to participate in the table olive fermentations of various olive cultivars (Deak, 2008; Nisiotou *et al*., 2010; Bautista-Gallego *et al*., 2011; Alves *et al*., 2012; Arroyo-López *et al*., 2012b). In fact, only one species (*Debaryomyces hansenii*) was identified as common when compared to the studies on the Gemlik cultivar (Leventdurur *et al*., 2016; Mujdeci *et al*., 2018). But interestingly, as a result of this study, *Candida zeylanoides* was found to be the dominant yeast species in table olive fermentation.

The raw material, processing technique and content in the brine have a significant impact on the diversity of the yeast population in table olives. Olive fermentations are driven mainly by LAB and yeast and the quality of final product depends on the metabolic activities of the mentioned microorganisms. Different species may possess significantly different metabolic properties and the quality of the obtained fermented food may be affected greatly. To ensure reaching the desired quality, the dominant strains of spontaneous fermentations with desired technological traits are used as starter cultures (Corsetti *et al*., 2012; Vinicius De Melo Pereira *et al*., 2020).

According to the results obtained in this study, *Candida zeylanoides* was determined as the most dominant species in the analyzed table olive samples. In light of the information mentioned above, *Candida zeylanoides* is thought to have the potential to be used as a starter in the production of green table olives prepared with high-salt brine.

4. CONCLUSIONS

In this study, yeast species participating in fermentation were determined in table olives produced by traditional method using Büyük Topak and Sarı Ulak olive cultivars. According to the results, strains belonging to Can*dida zeylanoides, Debaryomyces hansenii, Candida oleophila, Candida diddensiae,* and *Rhodotorula mucilaginosa* were identified in the table olive samples. Although the results are in agreement to some extent with previous studies on the subject, some commonly isolated species could not be detected. It is thought that the reasons for this are the differences in olive cultivar and the region of olives, the production technique and the composition of the brine. In addition, only culturable yeast strains were identified. As an interesting result of the study, *Candida zeylanoides*, which is rarely isolated in table olives, was determined as the most dominant yeast. In conclusion, it is thought that the results that can better unveil the diversity of the yeasts species involved in traditional table olive fermentations can be achieved by using culture-independent identification techniques. In addition, it is thought that the *Candida zeylanoides* strains may be considered to be used as starter cultures in the table olives studied, although further studies are needed to determine the technological and metabolic properties of the strains obtained.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Chemical characterization of sunflower oil oxidized by UV and ozone with different degrees of oxidation and study of their antimicrobial action

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SUMMARY: Oxidation by the action of ozone takes place at high rates and involves the reaction of ozone molecules with fatty acid double bonds followed by the formation of stable oxidation products with biological activity. In the present work, a comparative study on sunflower oil oxidized by ultraviolet (UV) light and ozone was carried out. This study involved the chemical characterization of sunflower oil oxidized by UV irradiation and ozonation, in addition to assessing the germicidal activity of oxidized oils obtained under various conditions. The results indicated that under the conditions studied, the increase in the dose of UV irradiation did not produce significant changes in the level of oxidation of the oil. Ozonation promoted the formation of oxygenated compounds at higher rates, increasing in concentration as the applied dosage of ozone increased. The germicidal activity of the oils behaved similarly, with considerably higher activity found in the ozonized oils.

KEYWORDS: *Oxidation; Ozone; Spectroscopy; Sunflower oil; UV irradiation.*

RESUMEN: ***Caracterización química del aceite de girasol oxidado mediante UV y ozono con diferentes grados de oxidación y estudio de su acción antimicrobiana.*** La oxidación por acción del ozono tiene lugar a tasas muy altas e implica la reacción de las moléculas de ozono con los dobles enlaces de los ácidos grasos, seguida de la formación de productos de oxidación estables con actividad biológica. En el presente trabajo se realizó un estudio comparativo del aceite de girasol oxidado por luz UV y por ozono. Este estudio consistió en la caracterización química del aceite de girasol oxidado por irradiación UV y por ozonización. En segundo lugar, se evaluó la influencia en la actividad germicida potencial del producto final obtenido en varias condiciones de ozonización. Los resultados indicaron que, en las condiciones estudiadas, el aumento de la dosis de irradiación UV no produjo cambios significativos en el nivel de oxidación del aceite. La ozonización promovió la formación de compuestos oxigenados en mayor proporción, aumentando su concentración a medida que aumentaba la dosis de ozono aplicada. La actividad germicida de los aceites se comportó de forma similar, encontrándose una actividad considerablemente mayor en los aceites ozonizados.

PALABRAS CLAVE: *Aceite de girasol; Espectroscopía; Irradiación UV; Oxidación; Ozono.*

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1. INTRODUCTION

Vegetable oils are composed of 95% triacylglycerols (TAG). Depending on their origin, they have different compositions of saturated and unsaturated fatty acids, such as oleic, stearic and palmitic acid. These fatty acids are esterified to the hydroxyl residues of the glycerol molecule (Firestone *et al*., 2013).

Ozonized vegetable oils present a more complex composition than the initial oils (Ledea-Lozano *et al.*, 2001). The interaction of ozone with the unsaturated molecules of vegetable oil generates the formation of a complex mixture of chemical compounds including ozonides, peroxides, and aldehydes (Bailey 1978; Soriano *et al*., 2003; De Almeida *et al*., 2016). Ozone reacts with the double bonds present in unsaturated fatty acids to form 1,2,3-trioxolanes or molozonides, which are unstable. In practice, the validity of Criegge's mechanism has been verified, which establishes that in the presence of water, a carbonyl fragment and a terminal hydroxyl hydroperoxide are produced. The hydroxyl-hydroperoxides (HHPs) have high biological activity and provide hydrogen peroxide through their decomposition into a carbonyl derivative. Vegetable oils easily react with ozone, causing the peroxide index to increase to high values. The amount of ozone that reacts with oils depends on the degree of unsaturation of their fatty acids. Ozonized vegetable oils have applications in dermatology and cosmetics, showing a well-known disinfectant and healing effect (Sechi *et al*. 2001; Skalska *et al*., 2009; Valacchi *et al*., 2013; Anzolin 2020). The study of the antimicrobial activity of ozonized oils has revealed that their germicidal character is due to the formation of oxygenated compounds such as hyperoxides, ozonides, aldehydes, and polyperoxides (Bouzid *et al*., 2021). However, the chemical characterization of ozonized vegetable oils is difficult due to the wide variety of oxygenated species they may contain (Ledea-Lozano *et al*., 2019a). Furthermore, vegetable oils also undergo the autooxidation phenomenon, which is the most widespread alteration of oils and fats. This is a complex process that generates a variety of intermediate and final oxygenated compounds such as organic peracids, alcohols, aldehydes, ketones, carboxylic acids, along with combinations of the above. The most widely accepted mechanism is through the free radical mediated by oxygen. The mechanism of free radical formation from fatty acids or acylglycerols is accelerated in the presence of electromagnetic radiation (light), heat, traces of heavy metals (such as iron and copper), and the presence of other active free radicals. (Tenllado van der Reijden, 2013).

The autooxidation mechanism of oils consists of three stages. The first is induction, in which the homolytic breakage of a carbon-oxygen bond occurs, generating a free radical. The second stage is propagation, in which by a reaction between molecular oxygen and the previously formed radical, a peroxide radical is formed. This radical can abstract hydrogen atoms from the carbon chains to form hydroperoxides, generating new free radicals that feed and expand the reaction. The hydroperoxides formed can also decompose, contributing to the propagation of the reaction. Termination reactions occur when free radicals react with each other forming new bonds, stopping to participate in the propagation of the reaction (Villanueva *et al*., 2017).

As already mentioned, edible oil oxidation can be activated by ultraviolet and visible light (Luna *et al*., 2006), which accelerates the formation of free-radicals from fatty acids or acylglycerols. The energy required for hydrogen abstraction depends on its position and proximity to the unsaturated molecules. The hydrogen atoms adjacent to the double bond, especially the one attached to the carbon between the two double bonds, require less energy for abstraction (Choe *et al*., 2006).

This work characterizes sunflower oil oxidized by two routes, the first by photoxidation with UV light and the second by ozone, establishing the main differences in terms of the compounds formed at different applied doses of the oxidizing agent, as well as the germicidal activity they acquire when they are oxidized by one of the selected pathways.

2. MATERIALS AND METHODS

2.1. Sunflower oil

The sunflower oil used in this work was of edible quality and supplied by the Borges Company, Spain.

2.2. UV irradiation of sunflower oil

The irradiation of sunflower oil samples was carried out in a stainless-steel photochemical reactor, equipped with a Philips TUV mercury vapor lamp, 254 nm and 11 w power. The samples were stirred at

200 rpm, the contact time was 2 and 4 h, G UV 2h and G UV 4h.

2.3. Ozonation of sunflower oil

A volume of 40 mL of sunflower oil was ozonized in a bubbling reactor at a controlled temperature of 25.0 ± 0.1 °C. The ozone gas flow rate was 30 L/h and the total ozonation time was 30 min. Ozone was generated through AQOZO equipment (CNIC, Cuba), with an initial ozone concentration of 70 mg/L, measured on an Ultrospec III spectrophotometer (Pharmacia LKB, Sweden). The reaction was carried out in the absence of solvents. The applied ozone doses were 4.3 and 25.8 g /L, for G10 and G30 ozonized oils respectively.

2.4. Determination of the peroxide value (PV)

The sample weight was 0.5 g, which was mixed with a solution of glacial acetic acid:chloroform 3:2 v/v. A volume of 0.5 mL of a potassium iodide saturated solution was added. The mixture was left in the dark for two minutes, then 30 mL of distilled water were added and the mixture was titrated with a sodium thiosulfate solution of 0.1 mol/L. The result is expressed as milligrams of active oxygen per kilogram of sample. The peroxide index value was calculated using the expression:

$$PV = [(M \cdot V)/Pm] \cdot 1000$$

where:
M: Concentration of sodium thiosulfate (0.1 mol/L).
V: Volume of sodium thiosulfate consumed in the titration (mL).
Pm: Weight of the sample (mg)

2.5. Determination of TAG composition

TAG analysis was carried out by gas chromatography on an Agilent 6890 gas chromatograph (Palo Alto, CA) using a Quadrex Aluminium-Clad 400-65HT capillary column (30 m length, 0.25 mm inner diameter, 0.1 μm film thickness: Woodbridge, CT, USA) and a flame ionization detector. The working temperatures were 360 °C in the injector and 370 °C in the detector. The injection split ratio was 1:80, with hydrogen as the carrier gas at a rate of 50 cm/s. The method used was isothermal at 335 °C, with a pressure gradient of 100 to 180 kPa. The peaks were identified by their retention times, which were compared to those of the database obtained from the analyses of oils of known composition.

2.6. Fatty acid determination

Fatty acids were determined as their fatty acid methyl esters (FAMEs) by GC. For this purpose, sunflower oil was transesterified to its corresponding methyl esters by treating 5 mg of sample with 1.5 mL of methanol/toluene/sulfuric acid (88/10/2; v/v/v) for 1 hour at 80 ºC. This determination was made on an Agilent 6890 gas chromatograph (Palo Alto, CA) with a flame ionization detector (FID operating at 200 ºC). The silica gel capillary column used was SP-2380 (30 m x 250 μm x 0,20 μm, Bellefonte, PA). Hydrogen was used as a carrier gas at a rate of 28 cm/s. The injection volume was 1 μL.

2.7. Separation of non-polar and polar triacylglycerol fractions

The separation of the non-polar and polar lipid compounds of the oxidized samples was carried out by adsorption chromatography on a silica gel column, according to the method established by Waltking and Wessels (1981).

The polar lipid content was determined using the following formula:

$$CP\ (\%) = [pM\text{-}pF1]/pM \cdot 100$$

where:
CP- content of polar compounds,
pM- weight of the sample
pF1-weight of the non-polar lipid fraction.

2.8. Molecular exclusion HPLC analysis

The sample preparation consisted of dissolving 10 mg of sample into 1 mL of tetrahydrofuran, which was injected into the HPLC system for molecular exclusion analysis. The HPLC system consisted of a Waters 2695 separation module equipped with a 2420 ELS detector. Two stainless steel columns of 30 cm in length and 7.7 mm internal diameter, packed with a styrene-divinylbenzene copolymer with a particle diameter of 5 μm and different pore

sizes were used. One had a pore size of 10 nm and the other of 50 nm (Waters Associates, USA). The columns were connected in series starting with the 50 nm column. Tetrahydrofuran was used as the mobile phase at a flow rate of 1 mL/min. Stearic acid monoglyceride was used as an internal standard for quantification (1 mg/mL).

2.9. Minimal inhibitory concentration (MIC) of ozonized oils

Antimicrobial activity was determined by the agar dilution method according to CLSI (2012) guidelines; the final inoculum was 103 cfu/mL. Petri plates were prepared with Mueller Hinton agar containing oxidized sunflower oil samples at concentrations ranging from 5 mg/mL to 100 mg/mL. The MIC was defined as the lowest concentration of oils inhibiting visible bacterial growth after incubation for 20 h at 37 °C. Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922 and yeast Candida albicans ATCC10231 were used.

2.10. Statistical analysis

The mean and standard deviation of the three determinations performed in each analysis were given.

3. RESULTS AND DISCUSSION

3.1. Photooxidation of sunflower oil with UV light

The irradiated sunflower oil showed an initial PV of 19.8. Irradiation produced a rapid increase in PV in the first 2 h, reaching values of 95.5 (Table 1). However, this rapid production of peroxides declined at longer exposure times, dropping to a PV of 59.0 after 4 h of exposure to UV radiation. This result shows a double effect of radiation, which induced the formation of peroxides but also destroyed them. Thus, irradiation would cause the decomposition of hydroperoxides resulting in secondary degradation products at long exposure times, producing compounds such as carboxylic acids and aldehydes, which do not contribute to PV value, which tended to decrease, as observed in Table 1.

Table 1. Oxidation parameters and unsaturated fatty acid content in sunflower oils oxidized by UV radiation. Results are the average of three determinations.

			Content (%)	
	PV	**Polar fraction**	**18:1**	**18:2**
Initial oil	19.8	n.d.	52.30 ± 0.01	38.40 ± 0.02
UV2h	95.5	15.3	52.85 ± 0.07	35.75 ± 0.07
UV4h	59.0	11.4	53.00 ± 0.20	35.80 ± 0.42

3.2. Determination of the content of polar lipid compounds in irradiated samples

Fractionation according to the polarity of compounds is one of the techniques used for the characterization of oxidized samples (Ledea-Lozano *et al*., 2005). Its main objective is to quantify the amount of modified triacylglycerols (polar lipid fraction) and those that have not been modified (non-polar lipid fraction). In this study, the results showed that at two hours of irradiation the polar lipid fraction was 15.3%; while at four hours it was 11.4% (Table 1). This result shows that there was degradation of the oxidation products at longer exposure times, in good correlation with the results of PV. Furthermore, photooxidation was mainly affected by linoleic acid. Thus, the results in Table 1 showed that the only fatty acid that showed a decrease in its proportion was that fatty acid with a decrease of approximately 2.6% in the oxidized samples. This result agrees with the higher rates of autoxidation expected for all polyunsaturated fatty acids. The level of linoleic acid in the oils oxidized at 2 and 4 h was the same, indicating that there was no advance in the propagation of the reaction.

3.3. Influence of irradiation on TAG aggregation

The study of the impact of photooxidation on sunflower oil was completed by looking into the composition of the polar lipid fractions by molecular exclusion HPLC. This technique separated glycerolipids of different molecular weights and thus gives information on the formation of dimers and oligomers. These compounds are formed by the condensation of TAGs carrying peroxide derivatives, and their presence alters the oil's physical properties and increases parameters such as oil viscosity. The results of the TAG aggregation of the polar fractions in the irradiated oils are shown in Figure 1. It can be observed that as a result of irradiation, there is no formation of oligomers, but only dimers, and the concentration of dimers was higher (2.41%) at 2 h of irradiation than at 4 h. No significant differences were observed in the

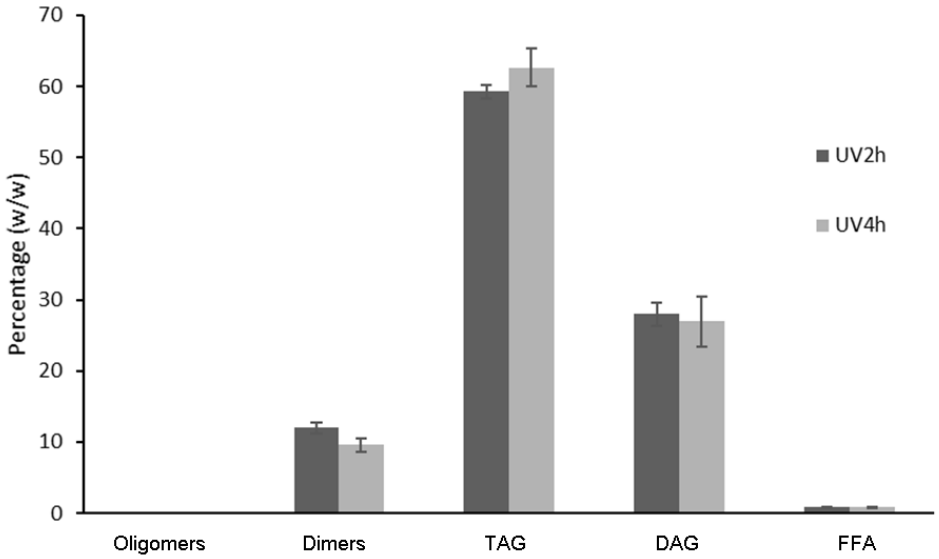

Figure 1. Composition of polar lipid fraction of UV light-irradiated sunflower oil. Data correspond to the average, plus or minus SD, of 3 independent experiments. TAG: altered triacylglycerols; FFA: free fatty acids

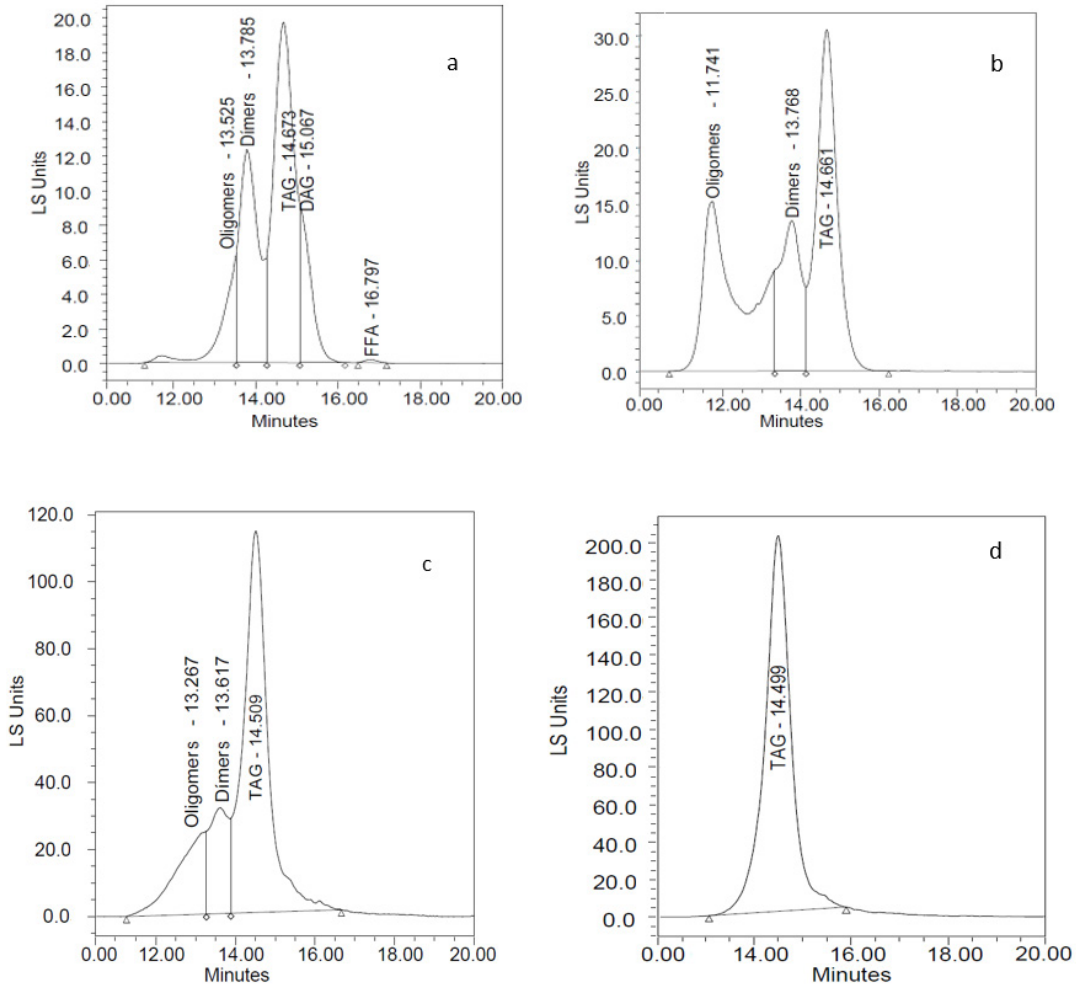

Figure 2. Molecular exclusion HPLC chromatograms from: a) Sunflower oil ozonized with 10% polar lipid compounds; b) Sunflower oil ozonized with 30% polar lipid compounds; c) Sunflower oil ozonized OLEOZON; d) Initial sunflower oil.

other compounds, which were altered TAGs, DAGs, and free fatty acids. The decrease observed in dimer concentration due to the effect of prolonged irradiation was in good agreement with the decrease in peroxides observed in these samples, indicating that at 2 h the termination reactions predominate over those of propagation and no more peroxides with potential biological activity were formed.

3.4. Oxidation parameters of different ozonized sunflower oils

In the present work, 3 ozonized sunflower oils were studied, differing in the dose of ozone applied to them. Therefore, the oils named G10 and G30 are partially ozonized oils, receiving doses of 4.3 and 25.8 g/L ozone. The sample named OLEOZON was the final commercial sample, which received ozone levels of 43 g/L ozone. The oxidative alterations of the oils after reacting with the amounts of ozone are shown in Table 2. When the amount of ozone applied increased, the PV of the oils increased too, with a substantial rise from G10 to G30 (from 49.6 to 159.0). The polar lipid fraction increased accordingly, which showed that ozonation is not subjected to the limitations of photooxidation and is able to increase the amount of peroxides without any apparent limitation. These high levels of oxidation affected the composition of the fatty acids. Therefore, the presence of nonanoic acid in considerable amounts (9:0) in the ozonized samples was remarkable. This fatty acid is not present in the initial oil but accumulated during the ozonation process due to lysis of ozonides formed from oleic acid, and its presence is consistent with previously published results. (Ledea-Lozano *et al*., 2005; Ledea-Lozano *et al.,* 2019). Unsaturated fatty acids decreased in very appreciable amounts. In this case, both oleic and linoleic acid showed substantial decreases in all ozonized oils, although linoleic acid was the one that decreased faster (Table 2). This pattern of decrease in unsaturated fatty acids was expected due to ozone attacks on all the double bonds present in the oil, but there was no preference for those in polyunsaturated fatty acids. In the case of linoleic acid, the possibility of reacting with the added ozone was double, which explains its faster decline. This contrasts with the photooxidation of this oil, which was almost exclusively affected by linoleic acid and in a much lower proportion (Table 1). Changes in the TAG composition observed in the neutral lipid fraction corresponding to ozonized oil samples indicated that those carrying a higher number of double bonds were those that decayed faster throughout ozonation, with important decreases in the most unsaturated LLL and OLL (Table 3). In contrast, the TAGs which displayed a lower level of unsaturation (POO and StOO) experimented an increase in its proportion after the process of ozonation.

Table 2. Oxidation parameters and unsaturated fatty acid content in sunflower oils oxidized by UV radiation. Results are the average of three determinations.

		Content (%)			
	PV	**Polar fraction**	**9:0**	**18:1**	**18:2**
Initial oil	19.8	n.d.	0.00 ± 0.00	52.3 ± 0.04	38.4 ± 0.08
G10	49.6	13.3	0.25 ± 0.07	50.3 ± 0.70	36.3 ± 0.71
G30	159.0	32.2	1.75 ± 0.21	46.3 ± 0.00	29.00 ± 0.21
OLEOZON			3.00 ± 0.14	42.6 ± 0.35	21.5 ± 0.42

Table 3. Triacylglycerol composition of the neutral lipid fraction of the initial sunflower oil and ozonized oils used in this work. TAGs with proportions lower than 2 % were excluded.

	Content (w/w %)								
Sample	**POO**	**POL**	**PLL**	**StOO**	**OOO**	**OOL**	**StLL**	**OLL**	**LLL**
Initial oil	6.2	4.5	5.5	4.3	36.2	8.3	3.3	15.2	11.1
G10	6.6	4.6	5.5	4.5	36.5	8.2	3.3	14.6	10.2
G30	7.8	4.8	5.1	5.4	36.1	7.6	3.3	12.5	7.8

Triacylglycerols were named with 3-letter coding for the fatty acids esterified. P, palmitic, St, stearic, O oleic and L linoleic. Data are the average of 3 determinations. The SDs were under 5% in all cases.

3.5. Characterization of ozonized oil by molecular exclusion HPLC

Previous studies on ozonized oils indicated that the high level of peroxidation induced by this reaction significantly altered the aggregation of TAGs, causing the presence of TAGs oligomers in considerable proportion, which altered the physical properties of the oil, increasing its viscosity (Ledea-Lozano *et al*., 2019b). This effect of ozonation was also observed in this work. Therefore, the initial sunflower oil showed a single peak corresponding to TAGs in the HPLC exclusion system used in this work (Figure 2a), and the samples with different ozone loads, G10 and G30, considerably increased their proportion of TAG dimers and smaller amounts of oligomers began to appear in the oil (Figure 2b and 2c). Finally, OLEOZON oils, which received the highest ozone load, showed a remarkable amount of polymerized TAGs (Figure 2d). The results of the composition of the different ozonized oils are shown in Figure 3, where the formation of considerable amounts of polymeric compounds can be seen, which increased the viscosity of the product. This result is consistent with the previous ones obtained for ozonized oils (Menéndez *et al*., 2008; Uzun *et al*., 2018; Ledea-Lozano *et al*., 2019a). In the case of OLEOZON, it was the oil that received the highest ozone load and contained a very notable proportion of dimers and oligomers, which reached 30% (Figure 3). It is important to note that the proportion of polymerized TAGs corresponds to

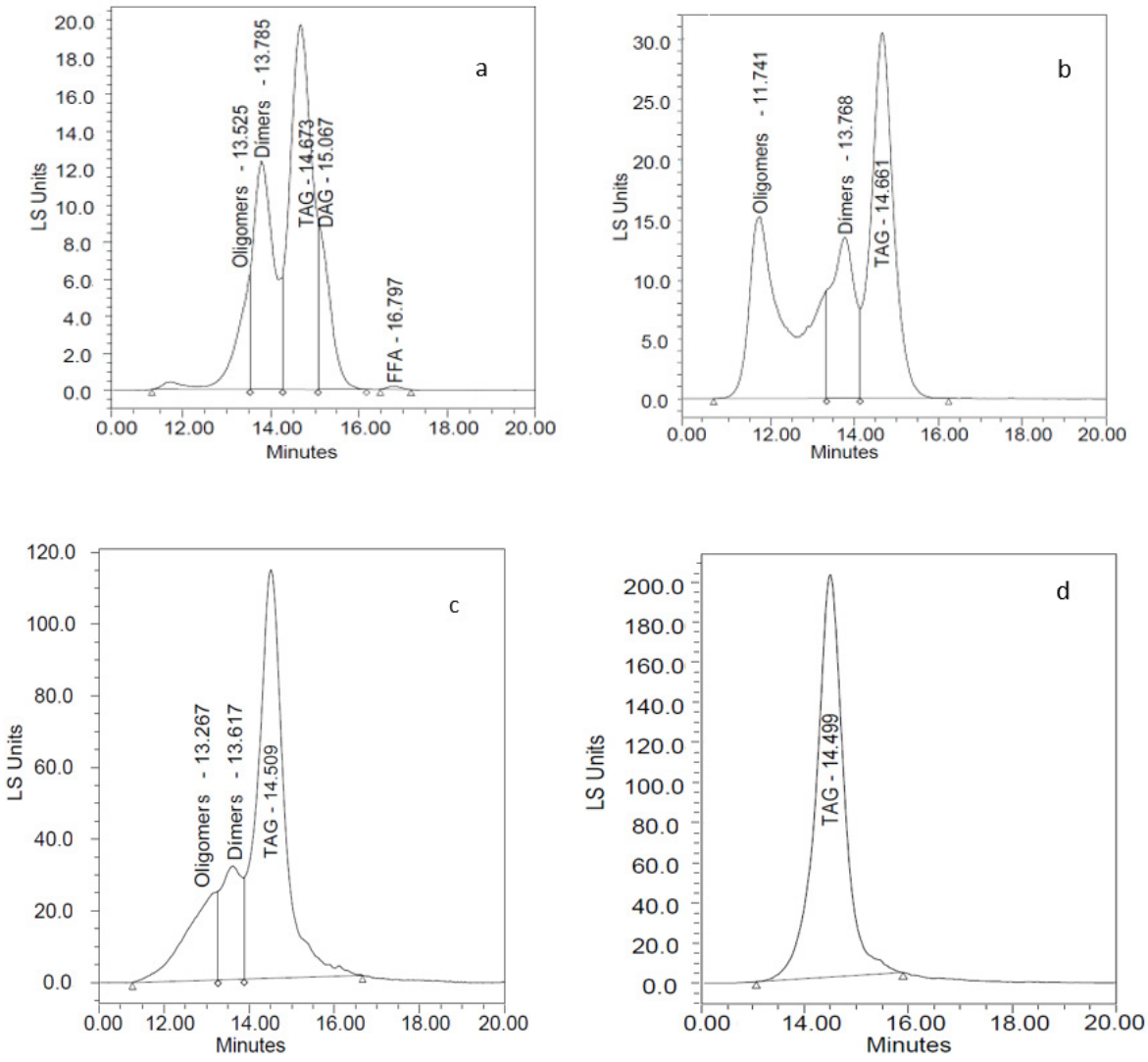

FIGURE 2. Molecular exclusion HPLC chromatograms from: a) Sunflower oil ozonized with 10% polar lipid compounds; b) Sunflower oil ozonized with 30% polar lipid compounds; c) Sunflower oil ozonized OLEOZON; d) Initial sunflower oil.

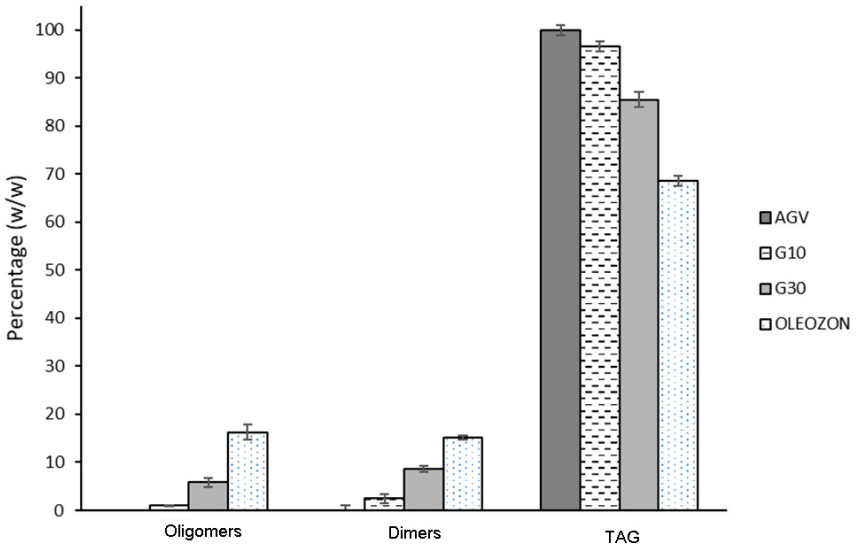

FIGURE 3. Chromatogram of the molecular exclusion HPLC analysis of virgin sunflower oil, partially ozonized sunflower oil and OLEOZON. Data correspond to the average, plus or minus SD, of 3 independent experiments. TAG: triacylglycerols; FFA: free fatty acids

the dose of ozone applied in any case. This result was also reported by other researchers (Guerra-Blanco *et al*., 2021).

3.6. Analysis of the behavior of polymers formed in aged ozonized sunflower oil

A molecular exclusion HPLC analysis of ozonized sunflower oil samples stored at 5 ºC for one and three months (Figure 4) was also carried out. The initial ozonized oil contained 68% non-polymerized TAG and amounts of dimers and oligomers at around 15%. The aged sample showed a significant increase in TAGs, with levels close to 80% at the expense of a notable decrease in the polymerized ones, which remained at around 10% for both groups (dimers and oligomers, Figure 4). This means that during storage, the polymerized TAGs tend to decompose, probably due to the breakage of the peroxide bonds that exist between them. This should involve a loss in viscosity values, which has been previously reported in previous storage trials (Menéndez *et al.*, 2008). Peroxidic species formed during the ozonization of sunflowers are metastable species that tend to decay with storage time. This decay involves loss

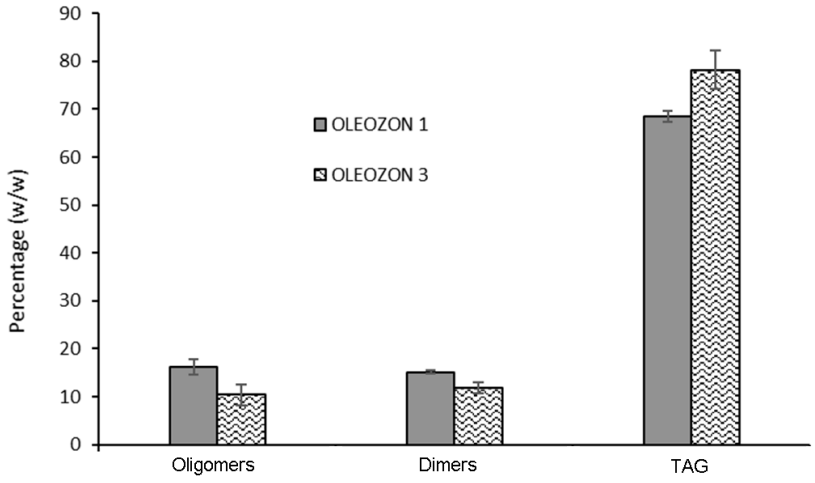

FIGURE 4. Variations in the compositions of TAGs, dimers and oligomers in OLEOZON with one (OLEOZON 1) and three months (OLEOZON 3) of storage from 2 to 8 °C. Data correspond to the average, plus or minus SD, of 3 independent samples. TAG: triacylglycerols; FFA: free fatty acids

in viscosity and, most likely, loss in antimicrobial activity, so it is important to store this product under proper storage conditions in order to avoid these peroxide decompositions, with low temperature storage, and the absence of humidity.

3.7. Determination of the antimicrobial activity of oxidized sunflower oil

The samples irradiated with UV light did not show germicidal activity within the minimum inhibitory concentration (MIC) range established in this work (Table 4), whose upper limit was 100 mg of oxidized oil per mL of sample. MIC values higher than that are not of interest because of the high amount of oil is necessary to obtain any effect. In general, in the case of experiments with UV light, the results indicate that as the irradiation time increases, the peroxidic species formed disappear, so the total amounts of active peroxides accumulated are too low to achieve the necessary inhibitory activity.

Table 4 shows the minimum inhibitory concentration values obtained for ozonized sunflower oil against one Gram-positive bacterium (Staphylococcus aureus ATCC 25923), two Gram-negative (Escherichia coli ATCC 25922 and Pseudomona aeruginosa ATCC 27853) and one yeast (Candida albicans ATCC 10231). The results show that as the applied dose of ozone and the content of polar compounds increased in the ozonized oil, the MIC value decreased, which means that the oil displayed higher antimicrobial activity. This indicated that the peroxides formed during that process are responsible for the biocidal effect and that a longer ozonation times involves the presence of higher amounts of compounds with biocidal activity. This result is consistent with the criterion that the mixture of peroxy compounds formed by ozonation constitutes the active pharmaceutical ingredient in these oils and is in good agreement with previous results (Poznyak *et al*., 2018). The MIC values obtained for each microorganism are related to their morphological and physiological characteristics. Growth inhibition was observed in smaller amounts of ozonized sunflower oil in the case of Gram-positive bacteria and yeast. A greater resistance was observed for the two Gram-negative bacteria. Similar results have been shown for bacteria and yeast treated with ozonized oils (Sechi *et al*., 2001; Ugazio *et al*., 2020; Higa *et al*., 2022). This evaluation shows that even at low doses, ozonized sunflower oil has a remarkable germicidal character that is not present in autoxidized oils.

CONCLUSIONS

Two oxidation methods were applied to sunflower oil, irradiation with UV light and ozonation. The results showed that ozonation supplied a greater formation of peroxidic compounds as the applied dose increased. Irradiation at a higher dose resulted in the decomposition of the peroxidic compounds formed. The study of the polar lipid fraction confirmed this result. The oxidation processes studied induced the polymerization of the triacylglycerols present in sunflower oil, from which dimers, and oligomers were formed. The stored OLEOZON samples showed a decrease in dimers and oligomers and an increase in triacylglycerols between one and three months of storage. The oils irradiated with UV showed no germicidal activity. In the case of ozonized samples, they were able to inhibit the bacteria Pseudomonas

Table 4. Minimum inhibitory concentration values for partially ozonized sunflower and OLEOZON obtained against the studied microorganisms.

	MIC (mg/mL)			
	Staphylococcus aureues ATCC 25923	*Pseudomona aeruginosa* ATCC 27853	Escherichia coli ATCC 25922	*Candida albicans* ATCC 10231
UV2h	nd	nd	nd	nd
UV4h	nd	nd	nd	Nd
G10	44.5	89	89	44.5
G30	22.26	44.5	44.5	11.13
OLEOZON	2.78	5.76	5.76	2.78

nd: inhibition not detected below 100 mg/mL.

Results are the average of 3 determinations. Standard deviation remained below 10% for these values.

aeruginosa, Escherichia coli and Staphylococcus aureus and the yeast Candida albicans, and showed a lower MIC value as the ozone dose applied to the sunflower oil increased.

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DATA AVAILABILITY

The data in this article is available on reasonable demand

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Retarding sunflower oil oxidation during the deep-fat frying of potato chips using micro-encapsulated *Convolvulus arvensis* Linn leaf phenolic extract

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SUMMARY: In this research, the extraction of polyphenols from *Convolvulus arvensis* (CA) leaves was optimized using ethanol (80%) at plant/solvent ratios and extraction times which varied between 1/10 to 1/30 (w/v) and 20 to 120 min, respectively. The extract with the highest polyphenol content was obtained at a ratio of 1/30 and 90 min. At 120 ppm, the preceding extract in either lyophilized (LyCAE) or encapsulated (EnCAE) form was evaluated as an antioxidant during the frying process using sunflower oil in comparison to TBHQ. Frying oil quality indices including refractive index, smoke point, acid value, anisidine value, polar and polymer compounds were monitored throughout frying times. FTIR spectroscopy was used to investigate the changes in *trans*-fatty acids, hydroperoxides and aldehyde contents. The results showed that the phenolic extract, especially in EnCAE form, exhibited superior antioxidant activity over TBHQ, which consequently led to the utilization of this phenolic extract as an antioxidant in frying oils.

KEYWORDS: *Convolvulus arvensis; Deep fat frying; Frying oil quality; FTIR spectroscopy; Micro-encapsulation.*

RESUMEN: ***Retrasar la oxidación del aceite de girasol durante la fritura de patatas utilizando extracto fenólico de hojas de*** **Convolvulus arvensis** ***Linn microencapsulado.*** En esta investigación se optimizó la extracción de polifenoles de hojas de *Convolvulus arvensis* (CA) utilizando etanol (80%) en las relaciones planta/solvente y tiempos de extracción entre 1/10 y 1/30 (p/v) y entre 20 y 120 min, respectivamente. El extracto con mayor contenido en polifenoles se obtuvo con una relación 1/30 durante 90 min. El extracto anterior en forma liofilizada (LyCAE) o encapsulada (EnCAE) se evaluó como antioxidante a 120 ppm durante el proceso de fritura utilizando aceite de girasol, en comparación con TBHQ. Los índices de calidad del aceite para freír, incluidos el índice de refracción, punto de humo, índice de acidez, índice de anisidina, compuestos polares y poliméricos, se monitorearon a lo largo de los tiempos de fritura. Se utilizó espectroscopia FTIR para investigar los cambios en el contenido de ácidos grasos *trans*, hidroperóxidos y aldehídos. Los resultados mostraron que el extracto fenólico, especialmente en forma de EnCAE, mostró una actividad antioxidante superior al TBHQ, lo que condujo a la utilización de este extracto fenólico como antioxidante en los aceites para freír.

PALABRAS CLAVE: *Aceite de fritura; Calidad del aceite de fritura; Convolvulus arvensis; Espectroscopia FTIR; Microencapsulación.*

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1. INTRODUCTION

The frying process is often conducted at high temperatures (170-190 °C), which provides good conditions for the occurrence of oil deterioration reactions such as hydrolysis, oxidation, and polymerization. As a result, many harmful components are generated (Li *et al*., 2021). More than 400 components were identified in frying oils after the frying process, which can be absorbed by the fried food according to their nature and concentration. Due to the dangerous health effects of some of these components, several treatments were investigated to stop or reduce their generation during frying (Choe and Min, 2007). In addition, the reactions that lead to the formation of these compounds also cause changes in the physical and chemical indices of frying oil such as acid value (AV), smoke point (SP), refractive index (RI), total polar compounds (TPCs), total polymers (TPs), para-anisidine value (*p*-AV), conjugated dienes and trienes (K_{232} and K_{270}) (Li *et al*., 2021).

Antioxidants, especially synthetic ones, have been broadly implemented to extend the use-life of frying oil. However, some synthetic antioxidants such as tertiarybutyl hydroquinone (TBHQ) have been strictly regulated (Urbančič *et al.,* 2014). In addition, the long-term intake of synthetic antioxidants has been reported to be toxic or carcinogenic (Li *et al.,* 2021). Moreover, synthetic antioxidants are heat sensitive and the frying temperature (180 °C) can easily destroy or evaporate them (Guo *et al.,* 2016). As a response to the aforementioned drawbacks of synthetic antioxidants, several studies have been performed to examine various plant extracts as a source of natural antioxidants (Farag *et al.,* 2007; Biswal *et al.,* 2021). Recent studies have reported that natural antioxidants exhibited high antioxidant activities and thermal stability which guarantee a longer use-life of the oil (Carelli *et al*., 2005).

The optimal antioxidant concentration of frying oils is crucial (Wang *et al.,* 2020). According to Guo *et al.* (2016), this concentration can only be determined after frying with oils, containing the antioxidants for at least five hours. Indeed, recent studies showed that utilizing these antioxidants, specially plant extracts in the form of micro-capsules or nano-capsules, enhanced their functional characteristics (Biswal *et al.,* 2021). The enhancement in micro-encapsulation techniques (physical, physiochemical or chemical) generated antioxidant microcapsules that have improved the ability to protect the antioxidant against degradation, control its release and mask its taste (Ozkan *et al*., 2019).

Convolvulus arvensis Linn (CA), commonly known as field bindweed, is an annual or sometimes perennial weed native to Europe and Asia and is used for many purposes. Data in the literature show that CA has antibacterial, antidiarrheal, antioxidant, vasorelaxant, immunostimulant, hepato-protective, and diuretic effects (Al-Snafi, 2020). On the other hand, the literature is scarce on the utilization of CA extract as an antioxidant in food systems. Indeed, there is no previous study which investigates the ability of using CA extract to reduce or prevent degradation in frying oils. Therefore, the current study was conducted to optimize the extraction conditions of CA leaf phenolic compounds and identify these compounds present in the extract, in addition to studying the antioxidant activity of the selected extract formulated in two different forms (lyophilized and micro-encapsulated). The effects of formulated CA extracts on the chemical, physical and thermal characteristics of sunflower oil during the deep-fat frying of potato chips were investigated.

2. MATERIALS AND METHODS

2.1. Plant material

Convolvulus arvensis (CA) leaves were collected from the gardens of the Faculty of Agriculture, Cairo University, Egypt. *Convolvulus arvensis* voucher number (000194 WM 194. 09-,05-,02 – 09) was placed in the herbarium of Orman Botanic Garden, Giza, Egypt. Antioxidant-free sunflower oil was kindly supplied by Arma company, 10th of Ramadan, Egypt.

2.2. Chemicals and reagents

Folin–Ciocalteu reagent, Quercetin, *P*-anisidine, Isooctane, Gallic acid, methyl alcohol, silica gel and TBHQ (Tert-Butylhydroquinone) were purchased from Sigma–Aldrich Co., St. Louis, USA.

2.3. Extraction of polyphenols

The plant leaves were dried at 45 °C, milled up to 50 mesh and stored at room temperature. The ex-

traction of polyphenols was performed according to Elsayed *et al.* (2020) with a slight modification. Aqueous ethanol (80%) at solid-to-solvent ratios of 1/10, 1/20 and 1/30 (*w/v*) were used to extract the phenolic compounds using a benchtop lab stirrer (Heidolph, Germany) at maximum speed. Extraction was conducted for 20, 30, 60, 90 and 120 min. The optimum extract with the highest total polyphenols yield was concentrated under vacuum at 45 °C using the EYELA Rotary Evaporator (Tokyo Rikakikai Co., LTD, Japan). The concentrated phenolic extract was divided into two parts. One of them was lyophilized (Edward freeze dryer, England) and the other one was microencapsulated.

2.4. Microencapsulation

Microencapsulation of the optimal CA leaf extract was prepared using the mini spray drier BUCHI B-290 (BÜCHI, Flawil, Switzerland) according to the method described by Sukri *et al.* (2021). Firstly, equal weights of gum Arabic and maltodextrin were dissolved in deionized water with the assistance of vigorous mixing and then the optimal extract was added. When the process was finished the extract represented 20% of the total solid (20g extract/ 80 g encapsulating material). The preceding mixture was magnetically stirred for 5 min before sonication for 20 min using UP200S ultrasound homogenizer (IKA Hielscher GmbH, Berlin, Germany) at 200 W. The spray dryer was operated under the following conditions: mixture flow rate = 8 ml/min, inlet air temperature = 130 °C, outlet temperature = 80 °C and drying air flow rate = 85% of the suction fan controller.

2.5. Total phenolic content (TPC)

The total phenolic content of the plant extracts was determined by Folin-Ciocalteu reagent according to the Elsayed *et al.* (2020) method using UNICO UV-Visible spectrophotometer (UNICO Instruments Co., LTD, U.S.A.) at 750 nm. The results were expressed as mg Gallic acid equivalent per gram plant dry weight (mg GAE/g DW).

2.6. Total flavonoid content (TFC)

The total flavonoid content was determined using the aluminum chloride colorimetric method at 510 nm according to Formagio *et al.* (2014). The results were expressed as milligrams Quercetin equivalent/ gram of dry weight (mg QE/g DW).

2.7. HPLC analysis

An agilent1260 infinity HPLC Series (Agilent, USA) equipped with a Quaternary pump and a Kineted® 5µm EVO C_{18} column 100 mm x 4.6 mm, (Phenomenex, USA) were used to identify phenolic and flavonoid compounds. The separation was achieved using a ternary linear elution gradient with (A) HPLC grade water 0.2% H_3PO_4 (v/v), (B) methanol and (C) acetonitrile at a flow rate of 0.7 mm/min. The detection wavelength was set at 284 nm. The injection volume was 20 µl and the column temperature was held at 30 °C. The compounds were identified by comparing their retention times with those of authentic standards. Calibration curves were used to calculate the concentrations of the compounds.

2.8. Frying process

According to the permissible concentration of TBHQ in vegetable oils (CODEX, 2019 CXS 210-1999) and previous studies (Wang *et al.,* 2020), lyophilized and microencapsulated leaf extracts were separately added to sunflower oil at 120 mg gallic acid equivalent/kg oil and TBHQ was added at the same level (120 ppm). Sunflower oil without added antioxidants was used as a control. Frying was performed in a deep-frying pan. The surface/ oil volume ratio was 0.28 cm^{-1}. One kilogram of oil was heated to 180 ± 10 °C. Fifty g of potato chips were fried for five min every 20 min, which was consecutively repeated for 8 hours per day. This frying process was repeated for four consecutive days with the same oil without replenishment. A 50 ml sample of the oil was collected both in the fresh state and at the end of every frying day. Oil samples were stored at -18 °C for analysis. All the frying processes were done twice.

2.9. Frying oil quality indices

2.9.1. Acid value (AV), smoke point (SP), refractive index (RI), conjugated dienes (K232) and conjugated trienes (K270)

Acid value, smoke point and refractive index were determined according to AOCS (2009). Conjugated dienes (K_{232}) and conjugated trienes (K_{270})

were determined according to the European Commission (2013).

2.9.2. p-*Anisidine value (*p-*AV)*

The *p*-AV was determined according to AOCS (2009). Isooctane was used as a blank. The experiment was performed in triplicate and values were calculated according to the following equation:

$$p\text{-AV} = \frac{25 \times (1{,}2\ A_s - A_b)}{wt}$$

Where (A_s) is the absorbance at 350 nm of the fat solution in isooctane after reacting with the *p*-anisidine reagent, (A_b) is the absorbance of the fat solution without the addition of reagent and (Wt) is the weight of the sample in grams.

2.9.3. Total polar compounds (TPCs %)

One gram of oil was fractionated in a silica gel column chromatography. The PC were recovered with diethyl ether (150 mL) after the non-polar components had been eluted with the same volume of a mixture of petroleum ether (b.p. 40-60 ºC) -diethyl ether (87:13, v/v) according to Waltking and Wessels (1981).

2.9.4. Total polymer content (PCs%)

The polymer content was determined in 1 g of oil by methylation under reflux with a solution of 1% (v/v) concentrated sulfuric acid in methanol, followed by separation of the methanol-insoluble fraction. The insoluble fraction was transferred to a preweighed flask with petroleum ether. The solvent was evaporated to constant weight, according to Pei-Fan and Nawar (1986).

2.10. Infrared spectroscopy (FTIR)

The IR absorption spectra in potassium bromide disks of oil sample were taken in the region (4000-400 Cm^{-1}). A FTIR spectrometer (NICOLET 380 FT-IR, Thermo Scientific, made in China) was used. Data were manipulated by a computer equipped with Professional Windows XP Software.

2.11. Statistical analysis

The data are presented as mean values ± SD. The data were subjected to analysis of variance (ANOVA) followed by Tukey's test using significance level of 0.05 (XLSTAT, Addinsoft, USA).

3. RESULTS AND DISCUSSION

3.1. Phenolic content of *Convolvulus arvensis*

The yield of phenolic compounds was proven to be highly dependent on extraction process parameters. Thus, the effect of plant/solvent ratio and extraction time on the yield of phenolic compounds was investigated. Results in Figure 1(a) illustrate that decreasing plant/solvent ratio caused a significant ($p < 0.05$) increase in the yield of total polyphenol contents (TPC) at various extraction times. Extending extraction time from 20 to 90 min at different plant/solvent ratios (except plant/solvent ratio of 1/10)

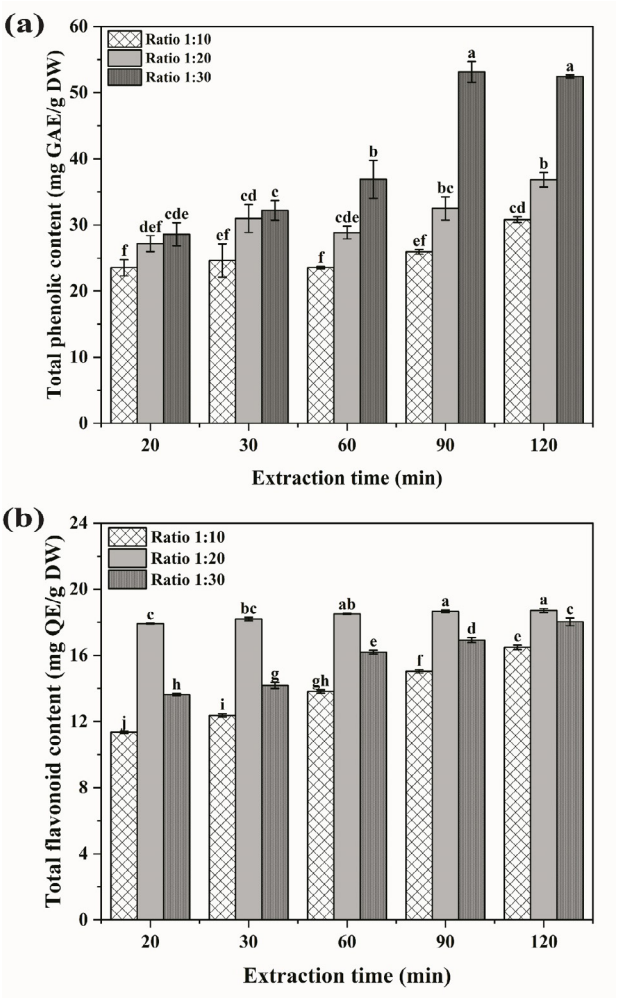

Figure 1. Effect of plant: solvent ratio (w/v) and extraction time on bioactive compound contents in *Convolvulus arvensis* leaves. The results are expressed as mean ± standard deviation (n = 3). Bars with different letters represent significant difference ($p < 0.05$, one-way ANOVA with Tukey test).

caused a significant ($p < 0.05$) increase in the yield of TPC, whereas further increase in extraction time had an insignificant ($p > 0.05$) effect on the yield of TPC. The highest TPC yield was 53.15 ±1.58 mg GAE/g DW, which was obtained at a plant/solvent ratio of 1/30 and extraction time of 90 min. These results agree with those reported by Ćujić *et al.* (2016). They reported that decreasing plant/solvent ratio from 1/10 to 1/80 caused a significant increase in the TPC from 53 to 83 mg GAE/g DW, respectively. They also reported that increasing the extraction time caused an increase in TPC. In a similar trend, Chirinos *et al.* (2007) reported that the TPC of mashua tubers (*Tropaeolum tuberosum*) was increased by increasing extraction time from 0 to 60 min, and a further increase in extraction time from 60 to 120 min had no significant effect on the yield of TPC.

The total flavonoid content (TFC) of CA leaf ethanolic extracts obtained under various extraction conditions are presented in Figure 1(b). What stands out in Figure 1(b) is that the highest yields of TFC were achieved at a plant/solvent ratio of 1/20 for various extraction times. Furthermore, at this plant/solvent ratio (1/20) extending the extraction time to higher than 60 min had no significant ($p > 0.05$) effect on the yield of TFC. In contrast to the TPC extraction pattern, decreasing plant/solvent ratio to 1/30 significantly ($p < 0.05$) reduced the yield of TFC at various extraction times. Moreover, it could be noted that for all plant/solvent ratios, except (1/20), extending extraction time from 20 to 120 min significantly ($p < 0.05$) increased the yield of TFC. The highest TFC yield was 18.72 ± 0.12 mg QE/g DW, which was obtained at a plant/solvent ratio of 1/20 and extraction time of 120 min. Similar results were reported by Chirinos *et al.* (2007), who studied the effect of different extraction times (from zero to 140 minutes) on the yield of TFC. They reported that the TFC yield increased significantly when the extraction time was increased from zero to 60 min, and then it became stable during additional extraction times.

The results indicated that the TPC of the LyCAE was 55.40 ± 0.43 mg GAE/g; whereas it was 9.30 ± 0.22 mg GAE/g for the EnCAE.

3.2. Identification of the phenolic and flavonoid compounds in *Convolvulus arvensis* extract

The phenolic compounds in the optimal ethanolic extract with the highest yield of polyphenols from CA leaves are listed in Table 1. The predominant phenolic compounds were benzoic acid and rosmarinic acid (164.39 and 134.58 µg/g DW, respectively), while quercetin and rutin (173.25 and 155.55 µg/g DW, respectively) were dominant flavonoids.

Elzaawely and Tawata (2012) showed that benzoic acid, syringic acid, *p*-hydroxy benzoic acid, vanillin and ferulic acid were the major identified phenolic acids in the CA leaf acidic ethyl acetate extract. This difference in the phenolic composition of the extracts could be attributed to plant species and solvent type.

Table 1. HPLC analysis of the ethanolic extract of CA leaves extracted with a ratio of 1:30 (w/v) for 90 minutes

Compound name	µg/g dried leaves
Phenolics	
Pyrogallol	0. 50
Quinol	0. 44
Gallic acid	4. 56
p-Hydroxy benzoic acid	32.29
Chlorogenic acid	61.45
Vanillic acid	16.80
Caffeic acid	60.56
Syringic acid	7.62
p-Coumaric acid	73.88
Benzoic acid	164.39
Ferulic acid	6.96
o-Coumaric acid	11.64
Resveratrol	66.29
Cinnamic acid	41.20
Rosmarinic acid	134.58
Flavonoids	
Naringenin	75.04
Myricetin	28.26
Kampherol	69.53
Catechin	8. 97
Rutin	155.55
Ellagic acid	57.12
Quercetin	173.25

The values refer to a single determination

3.3. Frying oil quality indices

3.3.1. Acid value (AV)

The change in AV with frying times is illustrated in Figure 2(a), which clearly shows an increasing trend in the AV of sunflower oils with increasing

frying time for various treatments. Compared to the control sample, sunflower oils containing TBHQ or either LyCAE or EnCAE significantly ($p < 0.05$) exhibited low AV After 32 h of frying. The control sample exhibited the highest AV (2.04 ± 0.02 mg KOH/g oil), whereas the AV of oil samples incorporated with LyCAE and TBHQ were 1.72 ± 0.02 and 1.62 ± 0.01 mg KOH/g oil, respectively. The oil with EnCAE recorded the lowest AV (0.86 ± 0.02 mg KOH/g oil). These results revealed that the TBHQ and EnCAE decreased the AV of frying oil by 20.38 and 57.67%, respectively, after frying for 32 h.

This finding is consistent with that of Asadi and Farahmandfar (2020), who added the phenolic extract of *Teucrium polium* at 200 ppm to canola oil during frying. They found that the *Teucrium polium* extract decreased the acid value by 47.6% after 30 h of frying. The superiority of EnCAE in the reduction of AV could be attributed to the protective effect of the encapsulation process on the stability of antioxidant compounds. According to Munin and Edwards-Lévy (2011), the encapsulation of polyphenols has a good influence on their stability against light and heat.

3.3.2. Smoke point (SP)

The change in smoke point of sunflower oil during frying is shown in Figure 2(b), which reveals that there was a marked decline in the smoke point of sunflower oil during frying for all treatments. At the end of frying (32 h), the control sample exhibited the lowest ($p < 0.05$) smoke point (193.5 ± 0.5 °C); whereas the sample treated with EnCAE exhibited the highest ($p < 0.05$) smoke point (236 ± 5 °C). These results agree with those previously reported by Farag *et al*. (2007). They found that sunflower oil samples enriched with phenolic extract (olive leaf juice extract) at concentrations of 800 and 1600 and 2400 ppm polyphenols showed a higher smoke point than oil samples enriched with BHT and the control ones after frying for 25 h.

3.3.3. Refractive index (RI)

Figure 2 (c) shows that there was a significant ($p < 0.05$) increase in the refractive index during frying for all treatments. It can be clearly noted that after frying for 32 h, the sunflower oil sample containing EnCAE showed the lowest ($p < 0.05$) refractive index

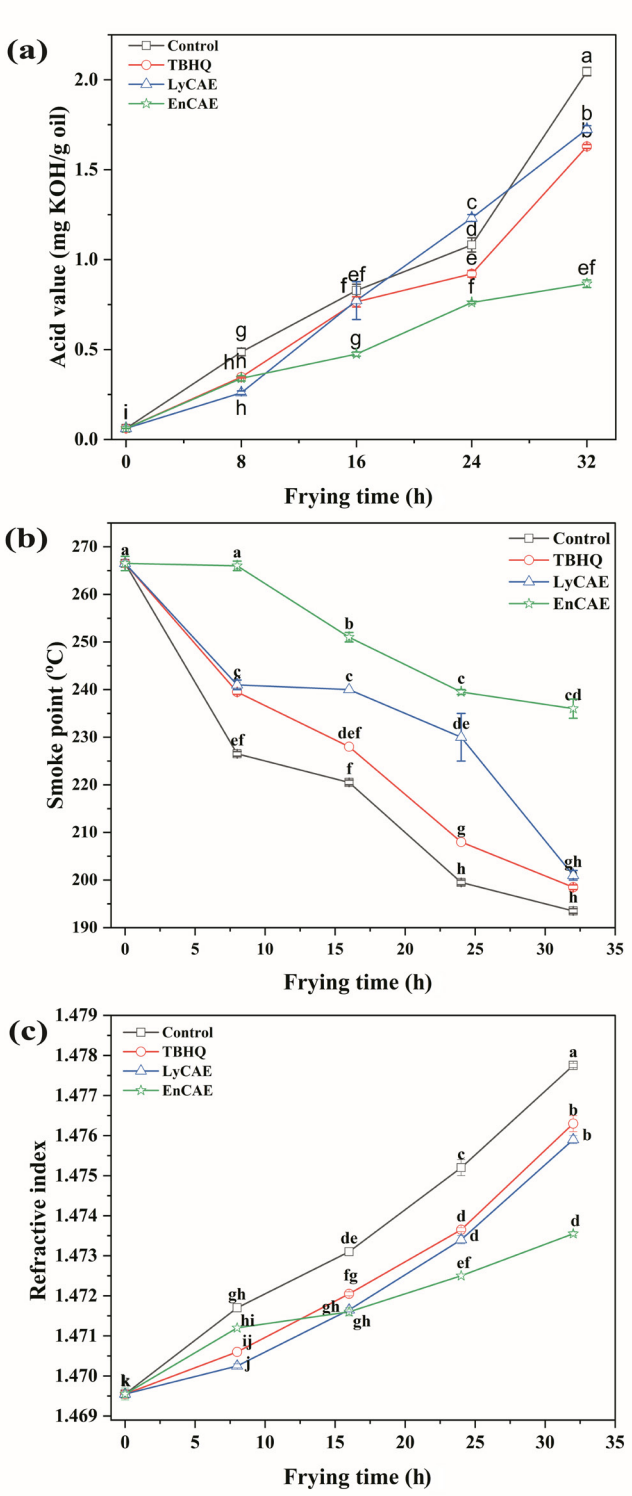

Figure 2. The change in acid value (a), smoke point (b), and refractive index (c) of sunflower oil samples containing TBHQ (TBHQ), lyophilized *Convolvulus arvensis* extract (LyCAE), and microencapsulated *Convolvulus arvensis* extract (EnCAE) throughout the frying process. The results are expressed as mean ± standard deviation (n = 3). Error bars with different letters represent significant difference ($p < 0.05$, one-way ANOVA with Tukey test).

value (1.4736); however, the control sample exhibited the highest ($p < 0.05$) refractive value (1.4778). Moreover, the same data reveal that TBHQ, LyCAE and EnCAE decreased the change in RI of various oil samples by 18.29, 23.17 and 51.22%, respectively. These results are consistent with those of Farag *et al*. (2007), who found that adding phenolic extract (olive leaf juice and pomposia extract, respectively) to frying oils reduced the increase in RI values in comparison to the control sample.

3.3.4. p-*Anisidine value* (p-*AV)*

p-AV is a measure of the level of non-volatile carbonyl secondary oxidation compounds produced during the frying process (Wu *et al.,* 2022). Figure 3(a) shows that sunflower oil samples containing TBHQ, LyCAE and EnCAE significantly ($p < 0.05$) exhibited lower *p*-AV than the control sample after frying for 32 h. TBHQ reduced the increase in *p*-AV by 10.05% compared to the control, while the LyCAE and EnCAE reduced it by 67.49 and 88.07%, respectively. This difference in *p*-AV between LyCAE- and EnCAE-treated samples could be attributed to the high stability of the phenolic extract resulting from the encapsulation process (Munin and Edwards-Lévy, 2011). In a similar trend, Wu *et al.* (2022) found that adding *Camellia oleifera* seed cake into soybean oil reduced its *p*-AV at various frying times in comparison to the control sample.

3.3.5. *Conjugated dienes (K_{232}) and trienes (K_{270})*

After frying for 32 h, it could be clearly noted that there were significant ($p < 0.05$) differences in CD and CT contents among various treatments (Figure 3(b)). The addition of EnCAE to sunflower oil significantly ($p < 0.05$) inhibited the formation of both CD and CT during frying. Moreover, the addition of CAE in either lyophilized or encapsulated form prevented the formation of CD and CT to levels lower than those generated during frying with oil

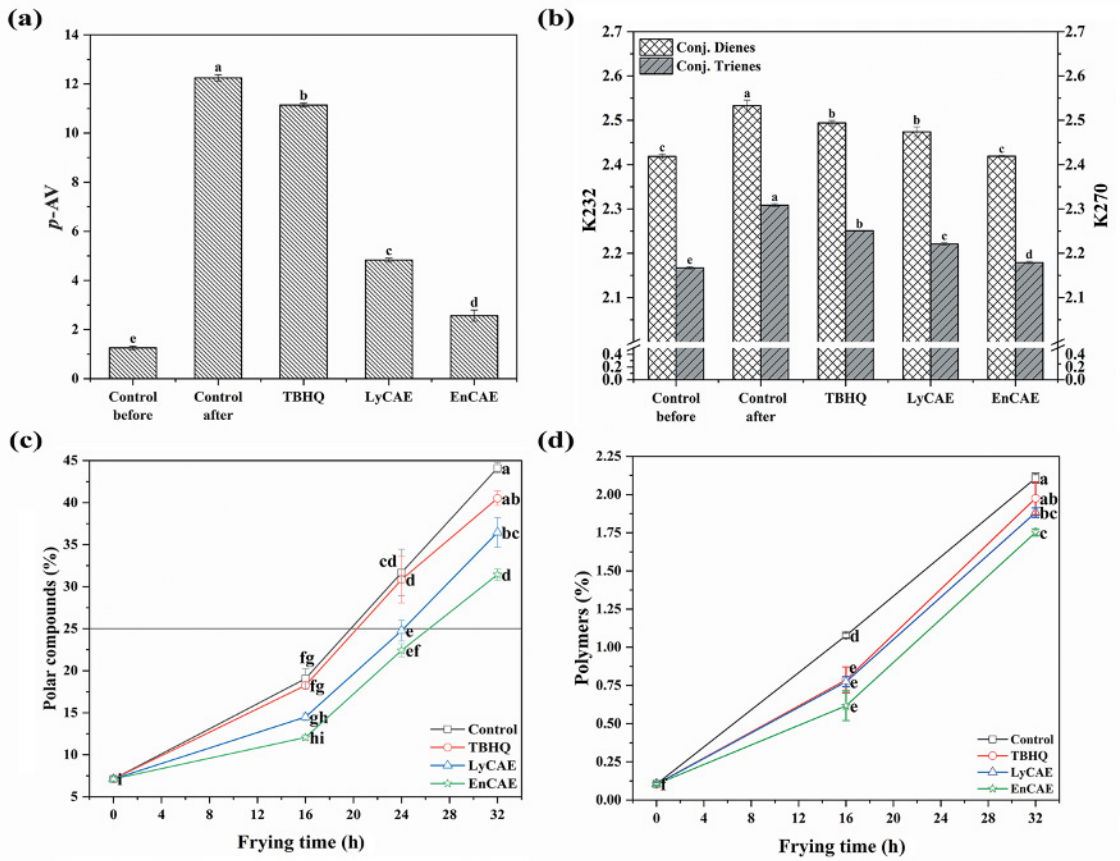

Figure 3. The *p*-anisidine value (a), conjugated dienes and trienes (b) in oil samples after frying for 32 h and the change in polar compound % (C), and polymer % (d) of sunflower oil samples containing TBHQ (TBHQ), lyophilized *Convolvulus arvensis* extract (LyCAE), and microencapsulated *Convolvulus arvensis* extract (EnCAE) throughout the frying process. The results are expressed as mean ± standard deviation (n = 3). Error bars with different letters represent significant difference ($p < 0.05$, one-way ANOVA with Tukey test).

containing TBHQ. This high antioxidant activity of CAE could be attributed to its phenolic compounds, especially rosmarinic acid, which have previously shown protective effects against the oxidation of vegetable oils during frying. Li *et al.* (2021) reported the potent effect of rosmarinic acid in retarding the soybean oil oxidation processes during frying. They also found a negative correlation between the level of rosmarinic acid and the formation of both CD and CT. The EnCAE was superior in preventing the formation of CD and CT. The role of encapsulation is limited to the enhancement of the antioxidant action through controlling the release and protecting the activity of the antioxidants in the frying oil (Sharma *et al.,* 2019).

3.3.6. Total polar compounds (TPCs %)

Due to the severe conditions of the frying process, many complex compounds with high polarity are formed. These compounds can be named and determined as the TPC % value (Wang *et al.,* 2020). Figure 3(c) shows that throughout frying times, oil samples containing various antioxidants exhibited lower TPC % values than the control sample, indicating that these antioxidants play a role in retarding or reducing TP formation during the deep-fat frying of potato chips, as reported by Frankel (2005). Indeed, the addition of CA leaf extract in either LyCAE or EnCAE to frying oils significantly ($p < 0.05$) extended their lifetime to 24 h as the generated TPC % in oil samples containing these antioxidants were acceptable (< 25%) (Firestone, 2007).

Despite all the oil samples exhibiting high TPC % values (> 25%) at the end of the frying process, the oil sample containing EnCAE showed the significantly ($p < 0.05$) lowest TPC % value (31.44 ± 0.68%). The addition of TBHQ, LyCAE and EnCAE to frying oil significantly ($p < 0.05$) inhibited the formation of TPC % in the control sample at the end of the frying process by 8.18, 17.38 and 28.74%, respectively. Our results are consistent with those of Li *et al.* (2021), who found that at the end of frying process, mixing the frying soybean oil with 200 ppm rosemary extract and rosmarinic acid inhibited the formation of TPC % by 19.52 and 24.66%, respectively. Wu *et al.* (2022) found that adding TBHQ or *Camellia oleifera* seed cake phenolic extract (200 ppm) to soybean oil had almost the same effect on the formation of TPC % during frying process. TBHQ and phenolic extract inhibited the formation of TPC% by 19.61 and 19.81%, respectively.

3.3.7. Total polymers (TP %)

During the frying process, monomer free radicals react to generate polymers. The levels of polymer compounds (TP %) is a good measure for frying oil quality since they increase with prolonged frying time. TP % has been proved to be decreased by mixing antioxidants with frying oil, mainly natural polyphenols (Wang *et al.,* 2020).

Based on Figure 3(d), TBHQ, LyCAE and EnCAE all have a good effect on decreasing the formation of polymeric compounds in sunflower oil during frying. After frying for 32 h, the sunflower oil sample containing EnCAE exhibited the lowest significant ($p < 0.05$) TP %, followed by LyCAE, TBHQ, and control samples in the same order. Indeed, EnCAE, LyCAE and TBHQ reduced the formation of TP % by 16.88, 10.70 and 6.26%, respectively, in comparison to the control sample. These results agree with those reported by Wang *et al.* (2020). They found that adding carvacrol methyl ether and TBHQ to sunflower oil reduced the TP % by 60.7 and 39.29%, respectively, during Chinese *youmotou* frying at 180 ^{o}C for 30 h.

3.4. FTIR analysis

The FTIR spectra of fresh oil and various frying oil samples at the end of the frying process were measured in the range of 4000 – 400 cm^{-1}, as shown in Figure 4. IR spectral bands shown at 3470, 1164, and 968 cm^{-1} are assigned to a hydroperoxide group, a carbonyl group, and trans double bonds, respectively, (Guillen and Cabo, 1997*;* Hammad *et al.,* 2021), which were used to assess the effect of the frying process on the quality of oil at the end of the frying process. Considering spectral band of 3470, the control sample showed the highest absorbance value at the end of the frying process; whereas samples containing various antioxidants showed absorbance values closed to those of fresh oil (before frying process).

In addition, control samples at the end of frying process showed the highest absorbance value at 1164 cm^{-1}, which indicates the increase in the formation of the aldehyde functional group in the absence of various antioxidants. The thermal oxidation of oil gener-

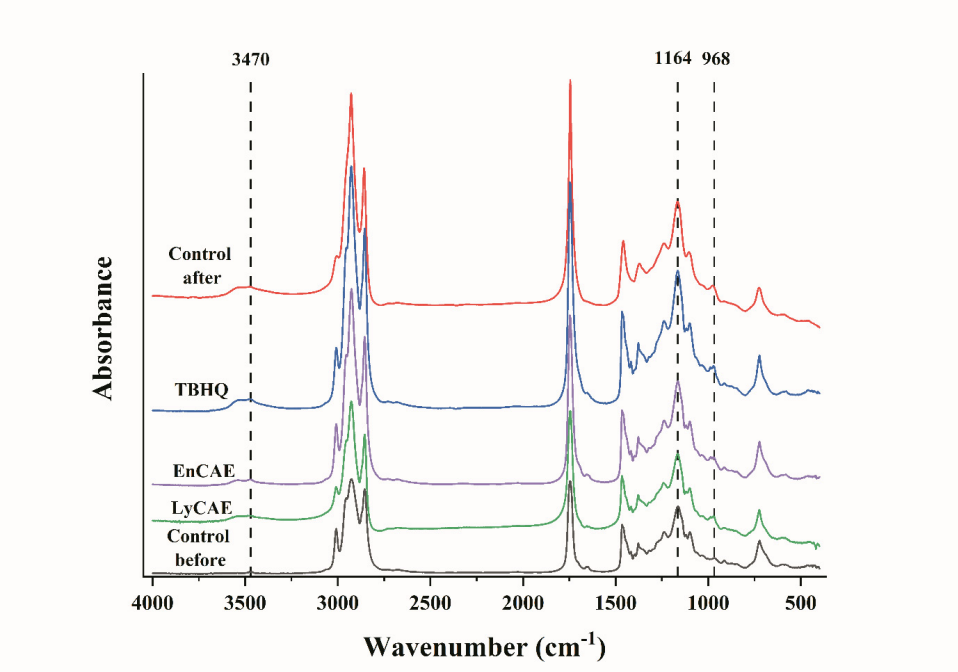

Figure 4. FT-IR spectrum of sunflower oil samples containing TBHQ (TBHQ), lyophilized *Convolvulus arvensis* extract (LyCAE), and microencapsulated *Convolvulus arvensis* extract (EnCAE) after the frying process (32 h). The values refer to a single determination.

ates hydroxyl groups which are represented in peaks in the range of 3000 cm^{-1} to 3600 cm^{-1}, especially after multiple frying cycles. Ahmad *et al. (*2022) reported that the C-O and carbonyl (C = O) bonds of aliphatic esters are responsible for the spectrum peaks near 1150 cm^{-1} and 1750 cm^{-1}, respectively. On the other hand, frying oil containing EnCAE exhibited the lowest absorbance value followed by LyCAE and TBHQ in the same order. At IR spectral band of 968 cm^{-1}, frying oil containing EnCAE exhibited the lowest absorbance value followed by TBHQ and LyCAE in the same order, which indicates the inhibiting effect of these antioxidants on the formation of trans double bonds. The absorbance of oils at 968 cm^{-1} reflects the trans double bond C-H (Guillen and Cabo, 1997). Moreover, multiple frying at various intervals may make oxygen more availableonce the frying oil cools, which could lead to similar peaks in the edible oils (Ahmad *et al.,* 2022).

4. CONCLUSIONS

According to the results, the process variable that had the greatest impact on the yield of the phenolic compounds was the plant/solvent ratio. The maximum TPC yield (53.15 mg GAE/g DW) was obtained by extracting with 80% ethanol for 90 minutes at a plant/solvent ratio of 1/30 (w/v). The HPLC analysis of the extract showed benzoic acid and rosmarinic acid as the main polyphenols while quercetin and rutin were the major flavonoids. This study set out to assess the feasibility of utilizing CA leaf extract as a natural antioxidant to prevent sunflower oil degradation during the deep-fat frying of potato chips in comparison to synthetic TBHQ. At a concentration of 120 ppm, TBHQ, lyophilized and encapsulated CA leaf extract (LyCAE and EnCAE) were individually added to sunflower oil. The findings clearly indicate that both LyCAE and EnCAE exhibited superior antioxidant activity over TBHQ regarding the physical, chemical, and thermal properties of oil. The excessive antioxidant activity of CA leaf extract may be attributed to its high content of rosmarinic acid. In addition, the encapsulation of CA leaf extract promoted its influence much more. This can be due to the protection and enhancement of the polyphenols due to encapsulation. CA leaf extract is a promising antioxidant for oil, particularly during frying. Hence, it can be used as a natural additive in frying oils.

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The effect of harvest time on the volatile compounds and bioactive properties of the flowers, leaves, and stems of *Echinacea Pallida* and its utilization to improve the oxidative stability of vegetable oils

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SUMMARY: The present study was undertaken to investigate the effect of harvest time on the bioactive properties of *Echinacea pallida* and to determine the antioxidant effect of its extract in vegetable oils. *E. pallida* was harvested in June, 2009, June, 2010 and August. 2010. Total phenolic content and antioxidant activity analyses of the plant extracts obtained with three different solvents were carried out using spectrophotometric methods. It was determined that harvest time and solvent type had significant effects on bioactive properties. In addition, the effect of *E. pallida* extract on the oxidative stability of vegetable oils was determined by the rancimat method. The extract (2000 ppm) obtained by ethanol (100%) showed similar oxidative stability on sunflower and canola oils compared to BHA (100 ppm). The GC-MS results revealed various volatile compounds such as bornyl acetate, caryophyllene E, musk ambrette, germacrene D, α-muurolol, musk ambrette, imidazo (1,2-a) pyrimidine, 1-pyrrolidino-1-cyclohexene, 2,3,5,6-tetrahydro-1H-pyrrolizine, pyrazine, and benzenaminium.

KEYWORDS: *Bioactive properties;* Echinacea pallida; *Harvest time; Rancimat; Volatile compounds*

RESUMEN: ***Efecto del tiempo de cosecha sobre los compuestos volátiles y las propiedades bioactivas de flores, hojas y tallos de*** **Echinacea Pallida** ***y su utilización para mejorar la estabilidad oxidativa de aceites vegetales.*** El presente estudio se realizó para determinar el efecto del tiempo de cosecha sobre las propiedades bioactivas de *Echinacea pallida* y el efecto antioxidante de su extracto en aceites vegetales. *E. pallida* se cosechó en junio de 2009, junio de 2010 y agosto de 2010. Los análisis de contenido fenólico total y actividad antioxidante de los extractos de plantas obtenidos con tres solventes diferentes se realizaron utilizando métodos espectrofotométricos. Se determinó que el tiempo de cosecha y el tipo de solvente tenían efectos significativos sobre las propiedades bioactivas. Además, se determinó el efecto del extracto de *E. pallida* sobre la estabilidad oxidativa de aceites vegetales mediante el método rancimat. El extracto (2000 ppm) obtenido con etanol (%100) mostró una estabilidad oxidativa similar en los aceites de girasol y canola en comparación con BHA (100 ppm). Los resultados de GC-MS mostraron la presencia de compuestos volátiles específicos, como el acetato de bornilo, cariofileno E, ambreta de almizcle, germacreno D, α-muurolol, ambreta de almizcle, imidazo (1,2-a) pirimidina, 1-pirrolidino-1-ciclohexeno, 2,3,5,6-tetrahidro-1H-pirrolizina, pirazina y bencenaminio.

PALABRAS CLAVE: *Compuestos volátiles;* Equinacea pallida; *Propiedades bioactivas; Rancimat; Tiempo de cosecha.*

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1. INTRODUCTION

The *Echinacea*, a member of the *Asteraceae* family, is an herbaceous perennial plant that is commonly called purple coneflower. For centuries, *Echinacea*, being native to North America, has been utilized externally in traditional cures for burns and wounds and internally for the treatment of colds, coughs, and headaches. *Echinacea* is mainly cultivated in America, Canada, Europe, Australia, and Russia. Moreover, many countries have carried out agricultural tests and research for the potential cultivation of *Echinacea* (Lin *et al.,* 2011). *Echinacea* consists of nine species, although only three species, namely *E. purpurea, E. pallida,* and *E. angustifolia,* possess medicinal properties. *Echinacea* species contain various bioactive components including alkamides, glycoproteins, polysaccharides, flavonoids, caffeic acid derivatives, and volatile compounds (Pellati *et al.,* 2004; Tsai *et al.,* 2012). Antioxidant (Pellati *et al.,* 2004; Lin *et al*., 2011; Erenler *et al.,* 2015), antimutagenic (Tsai *et al.,* 2012), antibacterial (Stanisavljević *et al.,* 2009), antiviral and immunostimulant activities (Aucoin *et al.,* 2020) of the species were proven in many studies.

Nowadays, there has been an increasing interest in the use of *Echinacea* and its preparations as an immune-modulator in COVID-19 treatment. Some reports have explained that *Echinacea* supplementation may alleviate the severity and interval of infection if taken during the first symptoms (Aucoin *et al.,* 2020). *Echinacea* is also one of the most preferred plants by cancer patients. In addition, some studies have revealed that the methanol extract from the roots of *Echinacea pallida* has antiproliferative activity against various cancer cells (Yaglioglu *et al.,* 2013), and its hexane extract has a higher cytotoxic effect on the tested cancer cells than the other two species (Chicca *et al.,* 2007).

While there are various studies about the utilization of *Echinacea* extracts for medicinal purposes, a recent study revealed that the silver nanoparticles of *E. purpurea* extract could be used as an effective antioxidant in food and pharmacological applications. Synthetic antioxidants are widely used as food supplements to avoid or retard lipid oxidation, which causes the formation of toxic compounds responsible for the bitter odor and taste which decrease food quality and safety. The utilization of synthetic antioxidants such as butylated hydroxyanisole (BHA) in foods raises concerns due to their carcinogenic risks. Thus, the identification of antioxidants from natural and safe sources is becoming increasingly more essential (Gecer *et al.,* 2022).

Echinacea is especially famous for its numerous volatile compounds which exhibit a wide range of beneficial effects. Recently, volatile compounds have attracted enormous attention for replacing synthetic substances in the food and pharmaceutic industries. They are drug candidates for treating various diseases due to their excellent biological effects. They have also gained importance as a non-toxic insecticide which is harmless to health in the fight against insects in stored products and agricultural production (Erenler *et. al.,* 2018; Karan *et.al.,* 2018).

The bioactive compounds in aromatic and medicinal plants largely rely on cultivation area, climate conditions, and harvest time (Thappa *et al.,* 2004). They also differ according to the species and parts of plant. Despite there being studies about the determination of the bioactive properties in *E. purpurea* for the whole plants, the bioactive properties of roots have only been determined for *E. pallida.* Therefore, comprehensive studies are required for assessing the bioactive compounds of the aerial parts of *E. pallida.*

The objectives of this study were: (1) to determine the effects of different harvest times on the bioactive compounds in the aerial parts of *E. pallida* cultivated in Turkey; (2) to assign a suitable solvent for the efficient extraction of phenolics from *E. pallida;* and (3) to determine the antioxidant effect of its extract on vegetable oils.

2. MATERIALS AND METHODS

2.1. Samples

The *E. pallida* plants used in this study were cultivated during the 2008 - 2010 growing seasons in the Experimental Horticulture area of Cumra Agricultural Vocational School in Konya, Turkey. Since Echinacea is a perennial plant, no harvest was done in the first year (2008), which is the planting year. It was harvested in two different developmental stages, at the beginning of flowering and full bloom, in the 2nd and 3rd years (2009-2010). The beginning of flowering is the period when 50% of the petals of the *Echinacea* come out. According to the development of aerial parts, the plants were harvested in June

2009 and 2010, at the beginning of flowering, and in August 2010, at the time of full flowering. The flower, leaf, and stem parts of the plants were brought to the laboratory and dried at room temperature.

2.2. Preparation of the extracts

Plant samples were ground in a laboratory-type grinder (Retsch MM400, Germany). Then the samples were extracted with different solvents, namely ethanol: water (80:20), methanol: water (80:20), and acetone: water mixtures (80:20). A weighed amount of ground samples was extracted with 25 ml of the solvent mixtures for 24 h at ambient temperature in the dark. The extracts were centrifuged at 4100 rpm for 15 min and then separated through a filter (Filter Discs No. 391). After that, the extracts were dried under a vacuum at 40 °C. After determining the extraction yield, the extracts were kept at -22 °C before the analyses of bioactive compounds.

2.3. Determination of total phenolic contents

The total phenolic content of the extracts was determined by the Folin-Ciocalteu colorimetric method (Tulukcu *et al.,* 2009). 40 µL of extract solution (1 mg/mL) were mixed with 2.4 mL distilled water and reacted with a 200-µL Folin-Ciocalteau reagent. After 5 min, 600 µL of a sodium carbonate solution (20% Na_2CO_3) and 760 µL of distilled water were added and the mixture was left for 2 h at ambient temperature in the dark. The absorbance of the samples was measured at 765 nm. The total phenolic content was expressed as mg gallic acid equivalent (GAE)/g of dry extract.

2.4. Determination of antioxidant properties

2.4.1. DPPH assay

The antiradical activity of the extracts was measured according to the 1,1- diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay used by Tulukcu *et al.* (2009) with some modifications. 50 µl of the extract were mixed with 3500 µL of a 0.1 mM methanol solution of DPPH and reacted for 30 min at ambient temperature in the dark. The absorbance of the samples was measured against a methanol blank at 517 nm. The percentage inhibitions of DPPH free radical (I%) was calculated according to the following equation:

$$I\ (\%) = 100 \times (1\text{-Absorbance of sample/ Absorbance of control})$$

As the reference substance, different concentrations of BHA were used (0-1 mg/mL). Then, the radical-scavenging activity of the samples was calculated as mg butylated hydroxyanisole equivalents (BHAE) /g of dry extract (Zulkafli *et al.,* 2014).

2.4.2. Phosphomolybdenum assay

The total antioxidant activity was measured by the phosphomolybdenum assay (Tulukcu *et al.,* 2009). For this purpose, 0.4 mL of extract were mixed with 4 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and then reacted in a water bath at 95 °C for 90 min. The absorbance of the samples was recorded at 695 nm. The antioxidant activity was expressed as mg ascorbic acid equivalents (AAE) /g of dry extract.

2.4.3. Rancimat assay

The antioxidant effect of *E. pallida* extract on the oxidative stability of vegetable oils was determined by a 743 Rancimat device. For this purpose, E. *pallida*'s powdered aerial part (4 g) was extracted by 100 ml of 100% ethanol at ambient temperature for 24 h in the dark. The extract was dried under vacuum at 40 ºC. 500, 1000, and 2000 ppm concentrations of the extracts and BHA (100 ppm) as the positive control were added to refined sunflower and canola oil. The induction period of these concentrations was measured at 120 ºC with 20 L/h air flow in duplicate.

2.5. Volatile compounds analysis

Volatile compounds were analyzed according to the method described by Yalcin *et al.* (2017) with GC-MS coupled to a mass selective detector (Agilent 7890A GC system) and HP-5MS column (60 m x 0.250 mm i.d.; film thickness 0.25 µm). About 3 g homogenized sample were extracted by Headspace (HS)-solid phase microextraction (SPME) at 60 °C for 40 min using a 75-µm carboxen-polydimethylsiloxane fiber (Supelco). GC oven temperature was held at 40 °C for 5 min, heated to 110 °C at 3 °C/min, from 110 °C to 150 °C at 4 °C/min, and from 150 °C to 210 °C at 10 °C/min and held for 15 min. Helium

was used as a carrier gas at 0.5 ml/min. The fiber was desorbed in the injection port for 20 min at 250 °C in the splitless mode. The mass spectrometer was scanned with an ionizing voltage of 70 eV and a scan range of *m*/*z* 35-450. The volatile compounds were identified by comparison with spectra from Flavor 2, NIST 05a, and Wiley7n libraries. The percentage of compounds was calculated from the TIC automated integrator.

2.6. Statistical analysis

The entire experiment was performed in triplicate. Data were analyzed with SAS statistical software. Comparative analyses between significant means were determined by using the analysis of variance and Tukey's multiple range test.

3. RESULTS AND DISCUSSION

The yield of extracts obtained with three different solvents, namely ethanol: water (80:20), methanol: water (80:20), and acetone: water mixtures (80:20), of the flower, leaf and stem parts of *E*. *pallida* harvested in June, 2009, June, 2010 and August 2010 are shown in Table 1. There was a significant difference ($P < 0.05$) in the extract yield of plant parts depending on harvest time. For flower and stem parts of the plant, while decreasing extraction yields were observed with late harvesting, the leaves of the plant did not show any definite tendency. Tulukcu *et al.* (2009) reported the extraction yield of clary sage as between 19.62 - 26.08%, depending on the harvest time. In this study, while the methanol extracts from the leaf parts had the highest extraction yield (15.79%), while the acetone extracts from the stem parts had the lowest extraction yield (4.19%).

The total phenolic content, free radical-scavenging activity, and antioxidant activity of the extracts obtained from different parts of *E. pallida* are provided in Table 2. The differences among the bioactive contents in the leaves, stems and flowers of *E. pallida* in the early and late harvest of the 2010 season were found to be significant ($p < 0.05$). Based on the plant's flowers, which contain the most bioactive substances, no significant difference ($p > 0.05$) was observed in the bioactive properties from June 2009 to June 2010. However, they exhibited higher bioactive properties with the late harvest in 2010. Complying with the present findings, Mistríková and Vaverková (2009) reported that the amount of hydrophilic and lipophilic compounds of *Echinacea*'s flower was higher during the third (ripening) developmental stage compared to the earlier stages, thus the third developmental stage was the best time for harvest. Chen *et al.* (2008) determined that the total phenolic contents in *E. purpurea* harvested in the spring were lower than that of the plants harvested in the autumn. Binns *et al.* (2002) reported increasing chicoric acid contents with age in E. *pallida* wildflowers, accompanied by decreasing values in roots because of developmental transport of this substance from the roots to the other plant parts or spatiotemporal shifts in phenolic pathways. The total phenolic contents in the *E. pallida*'s ethanol ex-

Table 1. Extraction yield of the Echinacea pallida (%)

Plant parts	Harvest Time	Acetone	Methanol	Ethanol
Flower	June 2009	10.29±1.24 ^{aA}	14.33±1.55 ^{aA}	12.61 ±1.86 ^{aA}
	June 2010	9.97±0.54 ^{aB}	14.01±1.00 ^{aA}	12.39±0.09 ^{aB}
	August 2010	7.72±1.05 ^{aB}	10.47±1.15 ^{bA}	7.93±0.79 ^{bB}
Leaf	June 2009	8.16 ±1.15 ^{aB}	15.79±1.82 ^{aA}	11.01 ±0.50 ^{bB}
	June 2010	9.38±0.62 ^{aB}	14.68±0.96 ^{aA}	12.51±0.90 ^{aBA}
	August 2010	9.33 ±1.04 ^{aB}	15.54±2.97 ^{aA}	12.24±0.75 ^{aBA}
Stem	June 2009	5.71±0.83 ^{bA}	7.51±0.85 ^{bA}	6.04 ±0.89 ^{cA}
	June 2010	5.22±1.10 ^{bB}	9.00±0.78 ^{bA}	6.55±0.89 ^{cBA}
	August 2010	4.19±0.41 ^{bB}	6.17±0.91 ^{cA}	4.80±0.77 ^{cB}

Each solvent type consists of a solvent: water mixture (80:20 v: v). Each value is expressed as mean± SD for three different harvest replications (n=3). Different lower-case letters in the same column and different upper-case letters in the same row indicate significant difference ($p < 0.05$), according to 2-way ANOVA with Tukey's test.

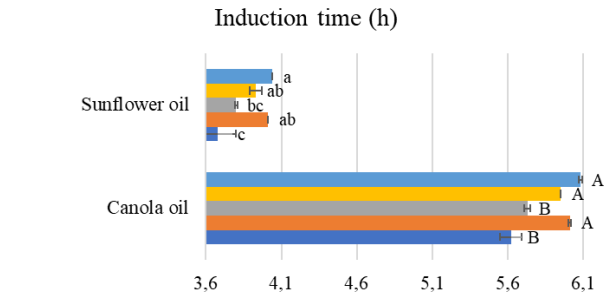
TABLE 2. Total phenolic contents, DPPH radical-scavenging activities and antioxidant activities of the *Echinacea pallida*

Plant parts	Harvest Time	Total phenolic contents (mg GAE/g dry extract)			DPPH radical-scavenging activities (mg BHAE/g dry extract)			Antioxidant activities (mg AAE/g dry extract)		
		Acetone	Methanol	Ethanol	Acetone	Methanol	Ethanol	Acetone	Methanol	Ethanol
Flower	June 2009	173.27±19.2 ^{bA}	131.80±7.1 ^{bB}	106.88±7.6 ^{bB}	317.51±27.23 ^{aA}	236.01±38.13 ^{bBA}	136.59±7.99 ^{bB}	151,23±9.69 ^{bA}	166.78±3.05 ^{bA}	145.00±9.42 ^{bA}
	June 2010	165.46±21.7 ^{bA}	109.37±8.96 ^{bB}	97.23±3.26 ^{bB}	239.08±41.70 ^{bA}	162.58±23.59 ^{cBA}	107.76±4.13 ^{bB}	149.69±3.68 ^{bBA}	156.47±4.06 ^{bA}	139.77±2.93 ^{bB}
	August 2010	225.07±28.74 ^{aA}	168.47±14.1 ^{aBA}	159.23±8.06 ^{aB}	370.33±9.65 ^{aA}	271.6±30.18 ^{aB}	252.67±29.79 ^{aB}	185.09±5.66 ^{aA}	194.99±5.22 ^{aA}	170.85±1.58 ^{aA}
Leaf	June 2009	71.30±7.02 ^{cA}	71.10±2.73 ^{cB}	45,31±7.07 ^{cB}	130.20±12.60 ^{cA}	155.07±4.48 ^{cA}	84.43±15.08 ^{bB}	136.97±14.36 ^{bA}	152.03±4.55 ^{bA}	130.65±6.81 ^{bA}
	June 2010	37.88±1.28 ^{dA}	31.34±0.89 ^{dB}	26.77±0,19 ^{dB}	44.17±4.30 ^{dA}	59.85±2.26 ^{dA}	16.77±1.75 ^{cB}	112.57±2.72 ^{cB}	123.105±0.06 ^{cA}	106.14±0.51 ^{cB}
	August 2010	45.95±1.95 ^{cA}	40.42±4.66 ^{dBA}	31.33±0.21 ^{dB}	63.07±5.28 ^{dA}	70.90±8.47 ^{dA}	38.03±2.87 ^{cB}	120.47±1.87 ^{cB}	131.42±2.08 ^{cA}	114.05±3.36 ^{cB}
Stem	June 2009	66.32±2.24 ^{cA}	55.34±4.22 ^{cB}	43.44±2.05 ^{cC}	95.81±8.20 ^{dA}	84.67±9.16 ^{dA}	47.78±3.16 ^{cB}	112.51±3.10 ^{cBA}	122.1±6.64 ^{cA}	103.06±7.29 ^{cB}
	June 2010	51.29±0.58 ^{cA}	37.10±3.01 ^{dB}	34.40±2.45 ^{dB}	55.04±4.88 ^{dA}	55.43±2.86 ^{dA}	27.25±1.31 ^{cB}	105.28±3.52 ^{cBA}	114.90±2.23 ^{cA}	97.77±5.66 ^{cB}
	August 2010	86.31±5.77 ^{cA}	65.34±4.64 ^{cB}	58.12±1.48 ^{cB}	117.45±6.33 ^{dA}	92.43±5.59 ^{dB}	70.67±3.66 ^{cC}	121.21±2.29 ^{cB}	131.72±2.89 ^{cA}	118.22±2.18 ^{cB}

Each value is expressed as mean± SD from three different harvest replicates x four measurements for each replicate (n=12). Different lower-case letters in the same column and different upper-case letters in the same row indicate significant difference ($p < 0.05$), according to 2-way ANOVA with Tukey's test.

tract were observed as 97.23-159.23, 26.77-31.33, and 34.40-58.12 mg GAE/g for flowers, leaves, and stems in the early and late harvest of the 2010 season, respectively. Stanisavljević *et al.* (2009) reported that the ethanol extract of the whole plant of *E. purpurea* contained 60.2 mg GAE/g dry extract of total phenolics. The highest bioactive contents were obtained from the flowers, complying with the results of Chen *et al.* (2008) and Lin *et al.* (2011). Erenler *et al.* (2015) reported the chicoric acid values, which is the main component of extracts using the LC-MS method, respectively, as 32.39 and 56.15 mg/100 g for flowers and 4.96 and 0.74 mg/100 g for leaves of *E. purpurea* and *E. pallida*. In general, the differences among the bioactive contents in the leaf and stem parts were not significant ($p > 0.05$), which revealed that the plant's stems contained important bioactive components.

Besides harvest times, the extraction solvents also had significant effects on the bioactive contents in the plant parts ($p < 0.05$). The highest total phenolic content was observed in the acetonic extracts, followed by the methanolic extracts; while the lowest total phenolic content was observed in the ethanolic extracts (Table 2). These results are coherent



	Canola oil	Sunflower oil
Echinacea (2000 ppm)	6,08	4,04
Echinacea (1000 ppm)	5,95	3,93
Echinacea (500 ppm)	5,73	3,8
BHA(100 ppm)	6,01	4,01
Control oil	5,62	3,68

FIGURE 1. Each value represents the mean of two replicates (n=2), bar errors show the standard deviations and significant differences ($p < 0.05$), according to one-way ANOVA with Tukey's test.

with those reported by Pellati *et al.* (2004) for the extraction of *E. pallida*. Although the methanol extracts gave the highest extract yields, they had lower total phenolic contents than the acetone extracts. Do *et al.* (2014) reported that proteins and carbohydrates had higher solubility in methanol than in ethanol and acetone. The components (polysaccharide and glycoprotein) of *E. pallida* other than the phenolics may

have increased the extract yield depending on solvent polarity.

The free radical-scavenging and total antioxidant activities of flowers were respectively identified as 185.04 mg BHAE/g and 370.33 mg AAE/g for acetone extracts; as 194.99 mg BHAE/g and 271.60 mg AAE/g for methanol extracts; as 170.85 mg BHAE/g and 252.67 mg AAE/g for ethanol extracts. It is clear from the results that the free radical-scavenging and antioxidant activities are well compatible with the total phenolic content. Tsai *et al.* (2012) observed that the ascorbic acid, BHA, and flower extract of *E. purpurea* exhibited the same highest radical scavenging ability at 100, 200, and 400 μg/ml, respectively. Pellati *et al.* (2004) and Erenler *et al.* (2015) reported that the radical scavenging activity of *Echinacea* species may be attributed to the caffeic acid derivatives, which have a high number of hydroxyl groups in their phenolic rings, which are proven to inhibit free radicals.

While the DPPH assay measures the free radical scavenging ability of the antioxidants found in the extract, the rancimat assay determines the oxidative stability of oils from a technological perspective. Rancimat quantifies the rise in the water conductivity resulting from the volatile degradation products of oil under accelerated oxidation conditions. The induction period represents the time required for the degradation of oxidation products which occurred with oil oxidation (Yalcin *et al.,* 2017).

The induction periods of canola and sunflower oils with or without added extract exposed to accelerated oxidation conditions are schematized in Fig. 1. The BHA (100 ppm) and *E. pallida* extracts (1000 and 2000 ppm) improved the oxidative stability of the oils, with similar induction periods. The *E. pallida* extract (2000 ppm) raised the induction periods of sunflower and canola oils from 3.68 h and 5.62 h to 4.04 h and 6.08 h, respectively ($p < 0.05$). Therefore, *E. pallida* extract exhibited high efficiency in delaying the oxidation of vegetable oils. This promising antioxidant effect for prolonging the shelf life of lipid-containing foods could be attributed to its bioactive components, which exhibit high free radical-scavenging capacity, such as volatile compounds and caffeic acid derivatives, especially cichoric and chlorogenic acid (Lin *et al.,* 2011; Erenler *et al.,* 2015).

Some studies evaluated comparing the antioxidant activity of *E. pallida* with synthetic antioxidants by spectrophotometric methods (Erenler *et al.,* 2015). However, no study was found on the effect of *E. pallida* on the oxidative stability of vegetable oils. Yalcin *et al.* (2017) reported that the IP of corn oil without and with 2000 ppm of grape seed extract changed from 3.18 to 3.31 - 3.41, depending on the seed variety. In another study, saffron extract (1000 ppm) had a similar effect ($p < 0.05$) with BHT (200 ppm) in preventing the oxidation of vegetable oils (Najafi *et al.,* 2022)

The volatile compounds in the flower, leaf, and stem parts of *E. Pallida* harvested in June, 2009, June, 2010 and August, 2010 are shown in Table 3. Bornyl acetate, caryophyllene E, musk ambrette, germacrene D, α-cubebene, α-copaene, α-humulene, α-muurolol, γ-cadinene, and caryophyllene oxide are some of the major volatile compounds which were identified in all the plant parts of *E. pallida.* Terpenes, which have many bioactive properties, exhibited the largest diversity and dominated all plant parts. Previous studies showed considerably higher proportions of terpenes in *Echinacea* species (Mazza and Cottrell, 1999; Thappa *et al.,* 2004; Mirjalili *et al.,* 2006; Lepojević *et al.,* 2017). In this study, bornyl acetate was determined to be one of the most abundant terpenes of all the plant parts. Although it was present in the essential oil of *E. pallida* in the study by Mirjalili *et al.* (2006), it was not identified in the essential oil of *E. purpurea* in some studies by Mazza and Cottrell (1999), Thappa *et al*. (2004), and Mirjalili *et al.* (2006). They reported that Bornyl acetate is the principal component in the above-ground parts of *E. pallida* and *E. angustifolia*, except for *E. purpurea*. There are reports that *E. purpurea* differs from other species due to its high monoterpene content, particularly α-phellandrene and myrcene, not detected for *E. pallida* in this study. The nitrogen-containing heterocycles, such as Imidazo (1,2-a) pyrimidine, 1-Pyrrolidinocyclohexene, 2,3,5,6-tetrahydro-1H-pyrrolizine, pyrazine, and benzenaminium, not detected as a major component in other studies, were present at high rates in the flower parts. They have a promising position in producing new bioactive compounds and in discovering drugs due to their excellent antiproliferative activity (Ivan *et. al.,* 2022). The musk ambrette (6.65-3.42%) was the heterocyclic flavor compound which dominated all the plant parts, but it was not identified in the other studies on *Echinacea* species.

Table 3. Volatile compounds in the flower, leaf, and stem parts of Echinacea pallida (%)

Volatile compounds	Flower			Leaf			Stem		
	June 2009	June 2010	August 2010	June 2009	June 2010	August 2010	June 2009	June 2010	August 2010
Monoterpene hydrocarbons	5.51	2.80	2.47	10.77	7.66	6.62	**9.95**	**12.82**	**9.91**
α -Pinene	0.36±0.00	0.26±0.01	-	0.31±0.03	0.60±0.14	0.62±0.07	0.84±0.06	0.92±0.07	0.45±0.09
β -Pinene	0.17±0.04	-	0.35±0.26	0.99±0.01	0.48±0.10	0.77±0.22	0.97±0.79	0.11±0.11	-
Limonene	1.52±0.02	0.79±0.05	0.33±0.03	4.26±1.11	2.27±0.48	2.00±0.03	1.52±0.23	2.21±0.49	1.13±0.09
Camphene	0.32±0.08	0.14±0.01	-	0.25±0.10	0.14±0.14	0.34±0.02	0.67±0.50	1.35±0.23	1.05±0.08
o- Cymene	0.50±0.03	0.34±0.03	0.32±0.01	2.22±0.38	0.99±0.01	0.93±0.00	0.24±0.01	0.26±0.06	0.18±0.01
Anethole	0.26±0.01	0.27±0.02	0.21±0.01	0.91±0.38	1.65±0.84	0.57±0.00	0.66±0.06	0.96±0.19	1.48±0.10
p -Cymenene	0.16±0.00	-	-	0.37±0.11	0.16±0.16	0.29±0.01	0.35±0.09	0.36±0.03	0.24±0.01
Thuja-2,4-diene	0.34±0.06	-	0.36±0.36	-	-	-	0.76±0.19	0.55±0.01	0.49±0.07
Carvone	1.88±0.01	1.00±0.04	0.90±0.05	1.46±0.16	1.37±0.02	1.10±0.09	2.69±0.10	2.54±0.31	2.50±0.18
Myrcene	-	-	-	-	-	-	1.25±0.04	3.56±0.01	2.39±0.27
Oxygenated Monoterpenes	**6.59**	**5.81**	**4.39**	11.09	10.63	10.51	**20.06**	**26.25**	**19.14**
Myrtenal	0.32±0.02	-	0.11±0.01	0.34±0.02	0.19±0.19	0.35±0.05	0.58±0.02	0.56±0.01	0.46±0.00
Bornyl acetate	3.97±0.56	3.67±0.03	2.51±0.07	6.29±1.22	3.18±0.02	6.11±0.20	16.17±0.61	20.79±0.21	14.35±0.63
Sabinol	-	0.73±0.04	1.06±0.15	0.62±0.12	0.28±0.28	0.54±0.18	-	-	-
Pinocarveol	0.21±0.02	0.11±0.00	0.13±0.02	-	-	0.22±0.04	0.31±0.31	0.46±0.02	0.43±0.02
Carveol	1.15±0.21	0.62±0.06	-	-	-	-	0.89±0.05	0.97±0.00	0.70±0.04
Carvacrol	0.94±0.05	0.68±0.04	0.58±0.00	3.84±0.05	4.63±0.43	3.29±0.50	1.20±0.15	1.18±0.01	1.00±0.08
α- Campholenal	-	-	-	-	2.35±2.35	-	0.53±0.04	0.82±0.15	0.61±0.06
Linalool	-	-	-	-	-	-	-	0. 98±0.09	1.01±0.05
Verbenol	-	-	-	-	-	-	0.38±0.38	0.49±0.00	0.58±0.02
Sesquiterpene hydrocarbons	**28.49**	**31.08**	**28.37**	25.95	22.99	23.29	**11.40**	**12.49**	**16.49**
α-Cubebene	2.09±0.05	1.99±0.19	2.42±0.13	1.15±0.05	1.09±0.06	1.28±0.00	0.97±0.08	1.09±0.12	1.32±0.07
α-Ylangene	0.64±0.05	0.57±0.01	0.59±0.07	0.63±0.02	0.37±0.37	0.57±0.02	0.55±0.00	0.54±0.00	0.53±0.03
α-Copaene	2.92±0.07	3.03±0.09	2.47±0.05	2.48±0.00	2.14±0.76	2.02±0.13	1.61±0.12	1.95±0.15	1.52±0.00
Caryophyllene E	5.54±0.17	6.24±0.13	7.57±0.92	5.11±0.52	5.10±0.62	5.85±0.31	2.48±0.24	2.14±0.11	2.46±0.43
α -Humulene	2.05±0.01	2.96±0.19	2.38±0.04	2.01±0.21	1.42±0.41	1.89±0.16	0.72±0.02	1.06±0.12	1.33±0.11
Germacrene D	3.52±0.04	5.16±0.42	1.58±0.11	3.35±0.12	3.84±3.84	2.27±0.03	0.94±0.94	0.58±0.58	1.05±0.66
γ-Muurolene	1.69±0.21	1.44±0.18	1.45±0.00	1.14±0.06	1.40±0.53	1.21±0.15	0.55±0.22	0.56±0.10	0.61±0.04
β-Cubebene	0.92±0.03	1.21±0.07	0.61±0.11	0.23±0.23	-	0.61±0.61	-	-	-
β-Bourbonene	1.05±0.04	-	1.11±0.04	0.29±0.29	-	1.78±0.54	-	0.93±0.05	1.52±0.25
β-Elemene	1.25±0.03	1.47±0.11	1.50±0.06	1.14±0.18	1.05±0.00	1.10±0.07	0.61±0.07	0.62±0.06	0.61±0.06
β- Copaene	0.88±0.05	0.87±0.01	1.08±0.08	0.71±0.21	1.13±0.87	0.70±0.05	0.43±0.03	0.48±0.04	0.54±0.00
α-Calacorene	-	-	-	0.65±0.02	0.33±0.33	0.24±0.24	-	-	-
β-Longipinene	0.76±0.76	1.08±0.00	1.16±1.16	0.70±0.70	-	-	0.63±0.01	0.72±0.72	1.02±0.46
Muurola-4,5-diene	0.69±0.69	0.75±0.75	0.66±0.03	0.81±0.04	-	-	0.49±0.23	-	0.58±0.11
γ-Cadinene	1.71±0.20	2.25±0.08	1.84±0.09	1.20±0.07	0.54±0.54	0.37±0.08	0.32±0.01	0.55±0.05	0.82±0.09
Calamenene	0.22±0.22	0.26±0.26	0.55±0.07	0.83±0.3	0.30±0.30	0.58±0.05	0.26±0.04	-	0.33±0.01
Murrola-4,5-diene	-	-	0,61±0.11	0.21±0.21	0.42±0.42	0.71±0.06	-	-	-
Cadina-1,4-diene	1.04±0.13	1.11±1.11	0.79±0.04	0.54±0.06	0.72±0.08	0.72±0.06	-	-	-
β-Gurjunene	0.51±0.16	0.69±0.03	-	0.39±0.39	0.47±0.47	0.87±0.14	-	0.25±0.25	0.92±0.19
β- Ylangene	1.01±1.01	-	-	0.62±0.62	0.94±0.94	0.27±0.27	0.34±0.34	0.22±0.22	0.29±0.29
Muurola- 3,5-diene	-	-	-	1.49±0.35	1.28±0.39	0.25±0.25	0.50±0.07	0.80±0.02	0.74±0.74
α- Clovene	-	-	-	0.27±0.27	0.45±0.45	-	-	-	0.30±0.01
Oxygenated Sesquiterpenes	**5.74**	**5.01**	**4.41**	2.20	1.47	3.94	1.86	**2.54**	**3.42**
α -Muurolol	3.47±0.29	3.37±0.16	2.94±0.08	1.66 ±1.66	1.47±1.47	2.68±0.98	-	0.46±0.46	0.86±0.16
Caryophyllene oxide	2.16±0.09	1.41±0.09	1.00±0.07	0.54±0.54	-	1.26 ±0.01	1.18±0.1	1.37±0.11	1.76±0.03
α-Cadinol	0.11±0.11	0.23±0.23	0.47±0.00	-	-	-	-	-	-
Humulene epoxide	-	-	-	-	-	-	0.68±0.09	0.71±0.16	0.80 ±0.05
Aldehyde	0.18	0.15	0.15	7.97	4.6	6.39	1.22	0.58	0.87
Nonanal	0.18±0.01	0.15±0.01	0.15±0.04	0.83±0.00	0.51±0.06	0.58±0.05	1.03±0.10	0.58±0.07	0.55±0.03
Benzaldehyde	-	-	-	2.73±0.77	0.56±0.56	0.99±0.99	-	-	-
2E.4E- hexadienal	-	-	-	0.85±0.11	0.41±0.41	0.74±0.05	-	-	-
2-Pentenal	-	-	-	0.38 ±0.38	0.39 ±0.39	0.75±0.11	-		--
2,4-Heptadienal	-	-	-	0.97±0.33	0.48±0.48	1.14±0.12	-	-	-
2-Hexenal	-	-	-	2.21±0.03	2.25±0.07	2.19±0.08	0.19±0.05	-	0.32±0.02
Ester	2.08	1.64	2.24	6.59	1.36	3.98	1.33	1.36	1.41
Methyl linoleate	2.08±0.76	1.64±0.20	2.24±0.16	2.64 ±0.26	-	-	1.33±0.11	1.36±0.23	1.41±0.03
Diisooctyl sulfate	-	-	-	3.95±1.2	1.36±1.36	3.98±0.53	-	-	-
Alcohol	1.99	1.56	2.64	1.55	1.28	1.77	0.68	0.54	-
n-Pentadecanol	1.52±0.02	1.56±0.04	2.26±0.08	0.88 ±0.88	0.87±0.87	1.41 ±0.01	0.68±0.68	0.54±0.54	-
Benzyl alcohol	0.47±0.02	-	0.38±0.01	0.67±0.06	0.41±0.41	0.36±0.06	-	-	-
Heterocyclic Compounds	**21.25**	**25.19**	**23.07**	12.29	10.98	8.45	**10.11**	**8.41**	**9.53**
Imidazo(1,2-a) pyrimidine	-	3.16±3.16	6.29±1.57	-	-	-	-	-	-
1-Pyrrolidino-1-cyclohexene	4.52±0.51	5.33±0.38	5.95±0.74	0.69±0.25	3.10±3.10	1.59±1.59	2.61±1.48	0.47±0.47	2.12±0.95
2,3,5,6-tetrahydro-1H-pyrrolizine	3.44±0.16	3.25±0.34	1.89±0.33	0.35±0.35	0.38±0.38	0.67±0.08	1.20±0.09	2.00±0.01	1.47±0.10
pyrazine	2.47±0.02	3.51±0.29	2.36±0.51	-	-	-	0.56±0.02	0.93±0.23	1.16±0.29
Musk ambrette	5.06±0.32	4.44±0.06	6.05±0.03	6.65±0.06	3.95±3.22	4.48±0.36	4.66±0.44	3.78±0.23	3.42±0.10

Volatile compounds	Flower			Leaf			Stem		
	June 2009	June 2010	August 2010	June 2009	June 2010	August 2010	June 2009	June 2010	August 2010
Benzenaminium	5.76±0.06	5.50±0.77	0.53±0.53	1.97±1.97	-	0.84±0.84	1.08±1.08	1.23±0.04	1.36±0.29
Benzothiazole	-	-	-	1.66±0.91	2.26±1.39	0.26±0.26	-	-	-
Dihydro actinidolide	-	-	-	0.97±0.29	1.29±0.46	0.61±0.01	-	-	-
Aromatic hydrocarbon	**3.87**	**4.58**	**2.98**	2.14	1.66	2.00	**2.80**	**3.30**	**3.58**
1,4-Dichlorobenzene	-	-	-	0.81±0.06	0.34±0.34	0.68±0.10	0.46±0.03	0.49±0.00	0.64±0.02
bicyclo[5.3.0]decan-2-one	3.87±0.01	4.58±0.31	2.98±0.80	-	-	-	1.39±0.58	0.34±0.34	0.94±0.94
Naphthalene	-	-	-	0.29±0.06	0.16±0.16	0.22±0.01	-	-	-
Tetracosane	-	-	--	0.59±0.19	0.44±0.44	0.56±0.08	-	-	-
Pentadecane	-	-	-	0.22±0.22	0.39±0.39	0.16±0.03	-	-	-
Tridecane	-	-	-	0.23±0.23	0.33±0.33	0.38±0.13	-	-	-
2-Undecene, 6-methyl	-	-	-	-	-	-	0.95±0.95	2.47±0.02	2.00±2.00
Ketone	-	-	-	1.46	2.21	2.00	0.14	0.12	0.14
β- Ionone	-	-	-	0.79±0.03	0.82±0.06	0.93±0.13	0.14±0.00	0.12±0.01	0.14±0.00
3,5-Octadien-2-one	-	-	-	0.67±0.03	1.39±1.39	1.07±0.12	-	-	-
Acid	-	-	-	-	-	-	2.81	1.88	1.12
Acetic acid	-	-	-	-	-	-	2.81±0.13	1.88 ±0.13	1.12±0.11
Total terpene compounds	**46.33**	**44.70**	**39.64**	50.01	42.75	44.36	43.27	54.10	48.96
Total volatile compounds	75.70	77.82	70.72	82.01	64.84	66.95	62.36	70.29	65.61

Each value is expressed as mean± SD for two different harvest replicates (n=2)

The large variations in volatile compounds were generally due to the plant parts rather than harvest time. Caryophyllene E, which is one of the major sesquiterpene compounds in all plant parts, reached its highest value (7.57%) for the late harvest. A sesquiterpene, Germacrene D were detected at 3.52 and 1.58% for flowers and 3.35 and 2.27% for leaves in the early and late harvest, respectively. The proportion of germacrene D was much lower than that reported for the flowerheads of *E. pallida* (Mirjalili *et al.,* 2006), and was like the one reported for *E. pallida* (Mazza and Cottrell, 1999). Thappa *et al.* (2004) observed that the amount of germacrene D, which is one of the most abundant components in Echinacea species, ranged from 7.2% (June) to 33.5% (December) and reported that weather conditions such as temperature and humidity had significant effects on the content and composition of the major terpene hydrocarbons in the flowers of *E. purpurea* during the growing season. Moreover, the percentage of α-copaene, which is one of the major sesquiterpenes compounds, was higher for all the plant parts from the early harvest than from the late harvest. Bornyl acetate, with known antioxidant activity (Karan *et al.,* 2018), showed the most abundant terpenoid component in stem parts and its level decreased from 20.79 to 14.35% with late harvesting. Among the volatile compounds, bornyl acetate is the most promising compound. Karan *et al.* (2018) observed that bornyl acetate showed significant antiproliferative activity against the tested cancer cells. In another study, it was found to have a neuroprotective effect on multiple sclerosis (MS), a neurological autoimmune disease (Lee *et al.,* 2023). Imidazo (1,2-a) pyrimidine compound, which is effective in treating anxiety disorders and ulcers (Goodacre *et al.,* 2006) and preventing unchecked cell growth (Aeluri *et al.,* 2015) was found at high rates in the flower parts of the plant, and its amount increased with late harvesting. Limonene, carveol, carvacrol, and carvone compounds, the major monoterpenes of *E. pallida,* decreased in all plant parts with late harvesting. The total monoterpene and oxygenated monoterpene hydrocarbon contents generally decreased with increasing temperature depending on late harvesting. The monoterpenes (C10) are smaller compounds to sesquiterpenes (C15), and thus they tend to evaporate more easily with the influence of high temperatures (Pirbalouti *et al.,* 2013).

4. CONCLUSIONS

The bioactive properties of *E. pallida* were significantly affected by harvest time. The highest total phenolic content was identified in the flowers of the plant with the late harvest of 2010. Among the solvents, acetone was the most suitable solvent for the efficient extraction of phenolics from *E. pallida*. While terpenes were detected as the dominant volatile compound in all the plant parts, the nitrogen-containing heterocycles were present at high rates in the flower parts. Total terpene content decreased with late harvesting for the flower and stem parts. *E. pallida* extracts exhibited high efficiency in delaying the oxidation of vegetable oils. These findings, which reveal the valuable volatile components and antioxidant activity of *E. pallida* also indicate that it is worth considering for further studies.

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Impact of different treatments on the antioxidant properties of two market types of peanuts grown in Mexico

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SUMMARY: The effect of roasting, frying, microwave heating, and germination on the antioxidant properties, total phenolics and flavonoids content of two types of peanuts (Valencia and Virginia) grown in Mexico was investigated. The thermal treatments affected the phenolic content and the antioxidant capacity of the two varieties of peanuts differently (by ABTS, DPPH, FRAP and iron chelating activity methods). Germination was the best method to increase the antioxidant activity (up to 157% increase in the Virginia variety) and the contents of compounds with nutraceutical potential in the peanuts (up to 59% increase in total phenolics in the Valencia variety and 700% increase in total flavonoids in the Virginia variety). Germinated peanuts could be used as raw material for the production of functional foods.

KEYWORDS: *Antioxidant capacity; Peanut processing; Total flavonoids; Total phenolics*

RESUMEN: ***Efecto del procesamiento sobre las propiedades antioxidantes de dos tipos comerciales de cacahuete cultivados en México.*** Se investigó el efecto del tostado, fritura, tostado en microondas y la germinación, sobre las propiedades antioxidantes, el contenido de compuestos fenólicos totales y flavonoides de dos tipos de cacahuete cultivados en México. Los tratamientos térmicos afectaron de forma diferente al contenido de fenólicos y la capacidad antioxidante de las dos variedades de cacahuete (por los métodos de ABTS, DPPH, FRAP y actividad quelante de hierro). La germinación fue el mejor método para aumentar la actividad antioxidante (hasta en 157% en la variedad Virginia) y el contenido de compuestos con potencial nutracéutico de los cacahuates (hasta en 59% de aumento en los fenólicos totales de la variedad Valencia y 700% de aumento en los flavonoides totales de la variedad Virginia). Los cacahuetes germinados podrían usarse como materia prima para la producción de alimentos funcionales.

PALABRAS CLAVE: *Capacidad antioxidante; Fenólicos totales; Flavonoides totales; Procesamiento del cacahuete*

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1. INTRODUCTION

Currently, food scientists have focused their attention on the development of foods which, in addition to providing the basic nutrients for the maintenance of the organism, supply additional benefits to health, with particular interest in those which provide antioxidant compounds (Serafini and Peluso, 2016). A large number of research works support the role of oxidative stress in the pathogenesis of chronic degenerative diseases such as diabetes, obesity, cardiovascular disease, and cancer, which is why dietary antioxidants have become particularly important, since they counteract the oxidative damage to DNA, lipids and proteins by reactive oxygen species (Phan-Thien *et al*., 2014; Serafini and Peluso, 2016).

The peanut (*Arachis hypogaea* L.) is a legume which is highly consumed worldwide. It is a rich source of valuable nutrients such as dietary fiber, protein, oleic acid, niacin, folate, vitamin E, magnesium, manganese and phosphorous, as well as bioactive compounds, including phytosterols, arginine and phenolic compounds, which produce important beneficial effects on human health. Several studies have associated peanut consumption with a reduced risk of cardiovascular disease, obesity, diabetes, and cancer, among others (Robles-Ramírez *et al*., 2014). An important part of the nutraceutical properties of peanuts is due to their content of phenolic compounds (Chukwumah *et al*., 2007; Phan-Thien *et al*., 2014). These compounds have been shown to be useful in the prevention of diseases related to oxidative stress due to their antioxidant properties and their interaction with cell signaling pathways (Vauzour *et al*., 2010).

Peanuts are consumed as processed foods, either directly in the shell or in the form of peanut butter, peanut oil, snacks (salty, fried, spicy) and confectionery products (Robles-Ramírez *et al*., 2014). Regardless of the type of consumption, a previous heat treatment is applied to peanuts, generally by toasting in a conventional oven, or in oil, in order to decrease their microbial load, facilitate peeling, improve their sensory characteristics and decrease anti physiological factors (Chukwumah *et al*., 2007; Kumar *et al*., 2017). Microwave roasting has also been considered for blanching the peanuts because this method is fast, saves energy and is easy to control (Kumar *et al*., 2017).

On the other hand, there has been a growing tendency in the consumption of sprouts due to their high content of nutrients and nutraceuticals (Geng *et al*., 2021). Germination is an inexpensive and simple procedure during which several biochemical changes occur, improving protein digestibility, decreasing anti-physiological factors and increasing bioactive compounds, including phenolic compounds (Beltrán-Orozco *et al*., 2020). Different studies have demonstrated the increase in the content of phenolic compounds and in the antioxidant activity of different seeds during germination, such as soybeans, broad beans, mung beans, chia, amaranth, broccoli and wheat, among others (Fernandez-Orozco *et al*., 2008; Beltrán-Orozco *et al*., 2020; Geng *et al*., 2021).

The phenolic content and the antioxidant activity of peanuts can change depending on the geographic growth site and the genotype of seeds (Craft *et al*., 2010; Phan-Thien *et al*., 2014; Yang *et al*., 2019). Therefore, the objective of this work was to investigate the effects of dry, oil and microwave roasting, as well as the germination process, on the phenolic content and antioxidant properties of two types of peanuts grown in Mexico.

2. MATERIALS AND METHODS

2.1. Biological material

Peanuts (*Arachis hypogaea* L.) of two market types (Valencia and Virginia), obtained from different locations in Mexico, were used for this research. These varieties were selected based on their high production and preference among consumers. The Valencia peanuts were grown in the municipality of Temoac, Morelos (18° 46′ 20″ N, 98° 46′ 39″ W; 1583 mamsl, annual mean temperature of 19.8 °C, average annual precipitation of 1693 mm). Virginia peanuts were grown in Delicias, Chihuahua (28°11′36″N, 105°28′16″W; 1170 mamsl, annual average temperature of 27.7 °C, mean annual precipitation of 334.2 mm). Whole pods free of microbial contamination were selected and shelled to obtain the grains. Damaged or defective grains were removed and the different treatments were applied to the healthy grains.

2.2. Reagents

Folin-Ciocalteu reagent, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazil), TPTZ (2,4,6-tripyridyl-s-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ferrozine, and sodium persulfate, were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Treatments

Peanuts were subjected to different types of processing (roasting, frying, microwave and germination) to subsequently evaluate their effect on the content of phenolic compounds, flavonoids and antioxidant activity. Untreated dry peanuts were used as a control. A batch of peanuts was dry-roasted in a preheated convection oven at 175 °C for 15 min. Another batch of peanuts was fried for 2.5 min at a ratio of 50 g of seeds in 200 mL of high oleic safflower oil preheated to 175 °C. Then, the excess oil was removed. For the microwave treatment, the peanuts were heated in a microwave oven (Panasonic,

model NN-6462A), at a frequency of 2.45 GHz and 450 W for 3.5 min. After each thermal treatment, the peanuts were cooled, the skin was removed and the skinless seed was ground. For the germination process, the peanuts were washed and disinfected by immersion in a chlorine dioxide solution (0.25 mL of a 10% solution for each L of water) for 10 minutes. Subsequently, they were soaked for 16 h in water at room temperature (23-25 °C), drained and placed in a plastic tray with a perforated lid, on a bed of cotton, covered with filter paper. The sprouts were collected after 3 days of germination, dried in an oven at 50 °C, and ground.

2.4. Proximate analysis

The moisture, protein, fat, ashes and dietary fiber contents of the samples were determined according to 925.10, 923.03, 920.39, 920.87 and 985.9 AOAC methods, respectively (AOAC, 1995).

2.5. Antioxidants extraction

Ground samples (2 g) were extracted by magnetic stirring using 20 mL of 80% methanol for 8 h. Afterwards, the extracts were obtained by filtration through Whatman No. 4 filter paper and stored at -20 °C until analysis.

2.6. Total phenolic content

The total polyphenol content of the samples was assessed using the Singleton *et al*. (1999) methodology. A volume of 20 µL of extract and 1.58 mL of distilled water were combined with 100 µL of Folin-Ciocalteu reagent (previously diluted 1:1 with water). The mixture was kept at rest for 5 min and then 300 µL of 10% sodium carbonate solution were added. After standing for 2 h at room temperature, the absorbance was measured at 765 nm. The results were expressed as mg of gallic acid equivalents per 100 g of dry sample (mg GAE/100 g).

2.7. Total flavonoid content

The method described by Ebrahimzadeh *et al*. (2008) was followed to determine the flavonoid content of the samples. A volume of 0.5 mL of the extract was combined with 1.5 mL of ethanol, 0.1 mL of 1 M potassium acetate, 0.1 mL of 10% aluminum chloride and 2.8 mL of distilled water. The absorbance was read at 415 nm after 30 min of standing at room temperature. A standard curve of quercetin was used to evaluate the flavonoid content of the extracts. The results were expressed in mg quercetin equivalents per 100 g dry weight sample (mg QE/100 g).

2.8. ABTS radical-scavenging activity

The ABTS radical-scavenging activity of peanut extracts was measured according to the method described by Re *et al*. (1999) with slight modifications. A solution containing 7 mM of ABTS and 2.45 mM of potassium persulfate was allowed to stand in the dark at room temperature for 16 h. This stock solution was diluted with ethanol until obtaining an absorbance of 0.7 ± 0.02 at 734 nm. Aliquots of 20 µL of the extracts or Trolox standard solutions were allowed to react with 1980 µL of ABTS•+ solution for 6 min and the absorbance was then measured at 734 nm. Results were expressed in µmol of Trolox equivalents per gram dry weight (µmol TE/g).

2.9. DPPH radical-scavenging activity

The assessment of DPPH radical-scavenging activity was carried out according to Brand-Williams *et al*. (1995). The extracts or Trolox standard solutions (100 µL) were mixed with 2 mL of 0.06 mM DPPH methanol solution and the absorbance measured at 515 nm after 30 min of incubation at room temperature. The antioxidant activity of the samples was expressed in µmol TE/g dry weight.

2.10. Ferric reducing antioxidant potential (FRAP)

The ferric reducing power of peanut samples was measured following the method described by Benzie and Strain (1996) with minor modifications. A working solution was prepared by combining 1 volume of 10 mM TPTZ in 40 mM HCl with 1 volume of 20 mM $FeCl_3 \cdot 4\ H_2O$ and 10 volumes of 300 mM acetate buffer, pH 3.6. The working solution (900 µL) was mixed with 90 µL of distilled water and 30 µL of the sample extract. This mixture was incubated at 37 °C for 30 min and the absorbance was read at 595 nm. The reducing power was calculated from a standard curve prepared by plotting the absorbance against the concentration of Trolox (300-1500 µM).

2.11. Metal chelating activity

To evaluate the ferrous ion chelating activity of the sample extracts, the method described by Ebrahimzadeh *et al*. (2008) was used with slight modifications. The methanolic extracts (0.5 mL) were mixed with 0.05 mL of 2 mM $FeCl_2$ solution. This mixture was left to stand for 5 min and then 0.1 mL of 5 mM ferrozine and 2.35 mL of 80% methanol were added. After 10 min of incubation at room temperature, the absorbance was measured at 562 nm. The iron chelating activity (%) was calculated according to the formula $(A_O - A_1) \times 100/A_O$, where A_O was the absorbance of the control, and A_1 the absorbance of the sample extract.

2.12. Statistical analyses

All determinations were made in triplicate and the results are expressed as the mean ± SD. One-way ANOVA and Tukey multiple comparison tests were used to analyze the differences between means ($p < 0.05$). The relationship between antioxidant compounds and antioxidant capacity measurements was evaluated using Pearson's correlation coefficient. Statistical analyses were carried out in SigmaPlot 13 software (Systat Software Inc., San José, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Proximate analysis

The results of the proximate analysis are shown in Table 1. Both Virginia and Valencia varieties had high contents of protein, lipids and dietary fiber. However, the Virginia type peanuts showed significantly higher content of fat and dietary fiber than the Valencia variety, while the Valencia variety presented higher contents of protein and ashes. In general, the values obtained from the chemical analysis of peanuts were similar to those reported by Mora-Escobedo et al. (2015) in 8 Mexican cultivars. The differences observed in the composition of both varieties were probably due to their different genotype and growing conditions, as several studies have revealed (Craft *et al*., 2010; Phan-Thien *et al*., 2014; Yang *et al*., 2019).

3.2. Total phenolic content

The total phenolic and total flavonoid contents of peanuts preserved with the different treatments are shown in Figure 1A. The contents of total phenolic compounds of the Valencia variety without treatment were lower than those of the corresponding Virginia variety (198.17 and 273.26 mg gallic /100 g, respectively). However, both types of peanuts presented phenolic concentrations within the range of those found in other investigations, which fluctuated between 92 and 1458 mg GAE/100 g in different types and cultivars of peanuts (Craft *et al*., 2010; Win *et al*., 2011; Ferreira *et al*., 2016).

Processing affected the phenolic content of peanuts. In the case of the Virginia variety, the thermal treatments (roasting, frying and microwaving) slightly decreased the contents of phenolic compounds by 16.1%, 7.6% and 18.7%, respectively. In contrast, processing increased the contents of these compounds in Valencia peanuts (from 198.17 to 223.47, 215.36 and 228.84 mg GAE/100 g with roasting, frying and microwaving, respectively), with this difference being significant only for the microwave treatment.

Other studies carried out on peanuts (Ferreira *et al*., 2016; Chukwumah *et al*., 2007) showed that heat treatment significantly increased the amount of soluble phenolic compounds. These are found in the pericarp, the testa and the aleurone layers of the seed, either as free compounds or as esterified compounds which are conjugated to sugars and low-molecular weight components; whereas insoluble phenolic compounds are part of the cell wall of the seed cells, and are covalently bound to high-molecular weight components such as cellulose, hemicellulose, pectins, lignin and structural proteins, which makes their extraction difficult (Ferreira *et al*., 2016). Heat treatment could release the phenolic compounds from the complex structures to which they are attached (Win *et al*., 2011). This would explain the increment of the polyphenolic content of Valencia peanuts after heating. Microwave showed to be better for increasing the polyphenol content of this type of peanuts. Thermal conductivity determines how evenly the temperature is internally dispersed in a material when it is heated conventionally. However, microwave heating generates intense heat throughout the food's structure rather than just at the surface. This causes a greater release and extraction of polyphenols. However, the effect of heat processes on the phenolic and nutrient composition of the grains depends on their size, thickness, form and internal structure, characteristics which may vary with genotype and growth conditions (Ferreira *et al.,* 2016; Kumar *et al.,* 2017). Ali *et al*. (2016) also

Table 1. Proximate analysis of peanut seeds (g/100 g)

Component	Virginia	Valencia
Moisture	3.64 ± 0.06 a	4.31 ± 0.01 b
Protein	27.51 ± 0.32 a	29.64 ± 0.36 b
Lipids	44.79 ± 0.18 b	42.62 ± 0.29 a
Insoluble fiber	13.16 ± 0.90 b	7.09 ± 1.05 a
Soluble fiber	4.25 ± 0.15 b	3.81 ± 0.18 a
Total dietary fiber	17.41 ± 1.04 b	10.91 ± 1.27 a
Ash	2.38 ± 0.01 a	2.85 ± 0.03 b

Values are the mean ± SD (n=3). Different letters in the same row indicate significant differences ($p < 0.05$) according to One-way ANOVA/Tukey's test.

reported an increase in total phenolic compounds in a time-dependent manner, when peanuts were roasted from 0 to 7.5 min in a microwave oven at a frequency of 2450 MHz and 350 W. Craft *et al*. (2010) investigated the effect of roasting and frying on the phenolic content of different varieties and commercial types of peanuts, finding that the type, cultivar and harvest date had an influence on the response to treatment, impacting both the profile and the quantity of phenolic compounds. Rosales-Martínez (2014) observed that during oven roasting of Virginia-type peanuts there was an increase in the phenolic compounds from 370 to 457 mg GAE /100 g sample, while Chukwumah *et al*. (2007) did not obtain any change when frying or roasting this type of peanuts. As can be seen, the behavior of polyphenols after the heat treatments is variable, depending on the peanut variety and the type of thermal process. However, the total content of polyphenols was not highly modified with processing, and the possible benefits to consumers were not affected.

Both varieties of peanuts showed a significant increase in the content of total phenolic compounds during germination, which was consistent with their high biological activity (germination percentages higher than 93%). The Virginia variety showed an increase of 8% and the Valencia variety an increase of 59%. This behavior has also been observed in studies with other seeds. For example, Fernandez-Orozco *et al*. (2008) observed increases between 50 and 300% in different legumes with 4-7 days of germination, while Beltrán-Orozco *et al*. (2020) obtained a 300% increase in the phenolic content of chia seeds after 4 days of germination. The phenolic compounds have been studied as secondary metabolites used by plants in defense against insects, microorganisms and parasitic plants, as well as for their role as signaling molecules to maintain seedling survival (Ndakidemi and Dakora, 2003). Germination possibly triggered the synthesis of phenolic compounds and also caused the release of these compounds from the food matrix of peanut seeds.

3.3. Total flavonoid content

Figure 1B shows the changes in the total flavonoid content observed in both varieties of peanuts subjected to the different treatments. In summary, the Virginia variety showed increases of 70, 74 and 45% in the flavonoid content of peanuts treated with oven roasting, frying and microwave, respectively; while the Valencia variety showed a reduction with roasting, a non-significant change with frying and a 31% increase in the flavonoid content in microwave-treated seeds.

In contrast, Ali *et al*. (2016) found that microwave heating decreased the content of flavonoids in a Bangladeshi peanut cultivar, suggesting their degradation. On the other hand, Chukwumah *et al*. (2007) found that neither oven roasting nor frying increased or modified the total flavonoid content of peanuts; while boiling caused an increase of 20%. This increment was attributed to the presence of proanthocyanidins in the peanut skin. Proanthocyanidins are oligomers of flavan-3-ol, such as catechin, epicatechin and epigallocatechin, which may have migrated from the hull to the cotyledons during thermal treatment. Epicatechin is a flavonoid which has diverse beneficialhealth properties such as antioxidant, anti-inflammatory, antitumor, cardioprotective and neuroprotective activities (Prakash *et al*., 2019). These researchers obtained values of 5 mg QE/100 g in raw Virginia type peanuts with skin and 1 mg QE/100 g in raw peanuts without skin; while in the present work averages of 1.75 and 2.31 mg QE/100 g were found for Virginia and Valencia peanuts, respectively.

It is important to note that germination significantly increased the flavonoid content (approximately 700% in the case of the Virginia variety and 400% in the Valencia variety) probably due to the role that these compounds play in plants to combat predator attack, decrease oxidative stress and as growth regulators, as mentioned above (Ndakidemi and Dakora, 2003). This fact is very important given that flavonoids are the phenolic compounds with the greatest

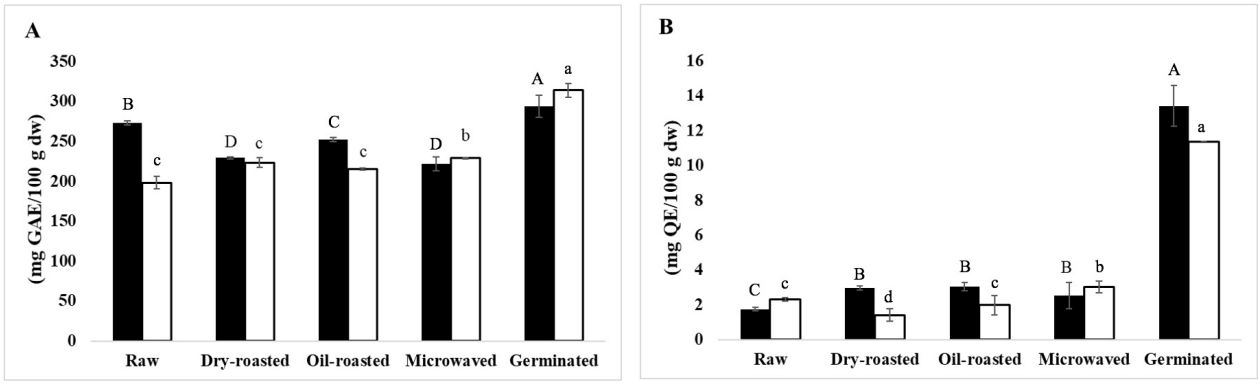

Figure 1. Total phenolic compounds (A) and total flavonoid compounds (B) of Virginia (■) and Valencia (□) peanuts subjected to different treatments. Values are mean ± SD (n=3). Different letters indicate significant differences among treatments according to One-way ANOVA/Tukey's test ($p < 0.05$).

beneficial effects on human health such as antioxidant and anti-inflammatory activities, free radical scavenging capacity, cardiovascular disease prevention, neuroprotective, hepatoprotective, anticancer and antiviral activities, among others (Kumar and Pandey, 2013).

3.4. ABTS radical scavenging activity

Figure 2A shows the antioxidant activity of peanuts as determined by the ABTS method. Results of 12.73, 11.24, 10.93, 11.09, and 14.5 µmol Trolox/g sample were obtained for the Virginia variety, and 11.16, 9.74, 11.11, 11.62, and 12.95 µmol Trolox/g sample for the Valencia variety, in raw, roasted, fried, microwave-treated and germinated samples, respectively. There was no significant difference between the control and the peanuts treated by the different thermal methods, although a tendency to decrease with roasting was shown in the Valencia variety. In the case of the Virginia variety, values of 12.5 to 16.5 µmol Trolox /g of sample without treatment were reported using the ABTS method (Mahatma, 2016), similar to those obtained in this study. On the other hand, Craft *et al.* (2010) obtained values between 3.02 and 11.99 µmol Trolox /g in different types and varieties of peanuts. These researchers found that the antioxidant capacity of peanuts subjected to different heat treatments (dry and oil-roasted) depended on the type, variety and age of the peanut.

A tendency to increase the antioxidant capacity (ABTS method) with germination was also observed in both types of peanuts, although the difference was not significant. Germination has been shown to increase the antioxidant capacity of other legumes such as mungbean (Geng *et al*., 2021), soybeans (Fernandez-Orozco *et al*., 2008), and lupine (Dueñas *et al*., 2009).

There was a positive correlation between total polyphenol content in the raw samples and the antioxidant activity determined by the ABTS method (r = 0.99). However, this correlation decreased in the treated samples (r = 0.82). These results show that polyphenols are mainly responsible for the antioxidant activities of peanuts and that processing generates new compounds that contribute to the antioxidant capacity. For instance, the increased synthesis of the Maillard reaction products during peanut roasting has been related to their higher antioxidant capacity. These comprise phenolic and non-phenolic compounds that could act as free radical scavengers (Kumar *et al*., 2017).

3.5. DPPH radical scavenging activity

Figure 2B shows the antioxidant activity of peanuts following the DPPH method. Results of 3.49, 3.6, 2.98, 3.8, and 8.97 µmol Trolox/g sample were obtained for the Virginia variety, and 5.48, 2.22, 5.69, 4.43, and 8.47 µmol Trolox/g sample for the Valencia variety, in raw, roasted, fried, microwave-treated and germinated samples, respectively. Compared to the unprocessed sample, there was a significant decrease in the antioxidant capacity of the Virginia type peanut when it was fried (14%) and in the Valencia type when it was roasted (60%) or microwave-treated (20%). The roasting conditions (175 °C, 15 min) probably changed the profile of the antioxidant compounds to others with lower antioxidant capacity or there was degradation of the compounds already released or formed.

However, Win *et al*. (2011) reported an increase in the antioxidant activity of peanuts as roasting time increased, at least for the Virginia variety. They found that the samples treated at 160 °C for 20 to 50 minutes showed higher DPPH radical-scavenging activity than untreated and 10 min-treated samples. This was attributed to the release of antioxidant compounds from the cell matrix to which they were attached. This can also be attributed to phenolic and non-phenolic compounds derived from Maillard reactions (Kumar *et al*., 2017).

Germination significantly increased the antioxidant capacity of the two peanut varieties, as determined by the DPPH method. An increase of 257% was obtained for the Virginia variety while in the Valencia variety there was an increase of 154.6%. These values were higher than those obtained by the ABTS method. The DPPH method detects the activity of low-molecular weight antioxidants since the DPPH radical presents a problem of steric inaccessibility (Prior et al., 2005). Therefore, small molecules, which have better access to the radical site, will show an apparent greater antioxidant activity by this method. Low-molecular weight antioxidant compounds were probably released or synthesized during germination.

Khang *et al*. (2016) studied the effect of germination (5 days) on the content of phenolic compounds and the antioxidant activity of six different legumes. Peanuts showed higher antioxidant capacity than soybeans, mung beans, white cowpeas, black beans and Adzuki beans, as determined by the DPPH method. All these legumes showed an increase in the content of total phenolic compounds as germination time increased.

3.6. Ferric reducing antioxidant power (FRAP)

The FRAP method determines the reducing power (electron transfer) of antioxidants, which is related to their degree of hydroxylation and conjugation (Prior *et al*., 2005). In Figure 2C it can be seen that roasting favored the formation of reducing compounds in both varieties of peanuts (increase of 8.3% and 31.8% in the Virginia and Valencia varieties, respectively).

Similarly, Thummakomma *et al*. (2018) obtained an 18% increase in the antioxidant activity (FRAP) of home-roasted peanuts.

The two varieties of peanuts responded differently to frying and microwave heating. Frying increased the reducing capacity of the Virginia variety, but decreased that of the Valencia variety; while microwave heating decreased the reducing power of the Virginia variety and increased that of the Valencia variety. Ali *et al*. (2016) found that microwave heating increased the reducing power of peanuts grown in Bangladesh by up to 7 times, depending on both heating power and time.

Germination increased the reducing power of the Virginia and Valencia varieties by 31.8 and 81.7%, respectively. Therefore, peanut germination generated the production of compounds with reducing power as well as free radical scavengers. In the study of Khang *et al*. (2016) peanuts showed higher reducing power than five other legumes (soybeans, mungo beans, white cowpeas, black beans and Adzuki beans), which demonstrates the high antioxidant potential of this legume.

3.7. Metal chelating activity

Figure 2D shows the metal chelating activity of peanut extracts. The transition metal ion Fe^{2+} has the capacity to transfer individual electrons, thus promoting the generation and spread of numerous radical reactions. The chelation of metal ions is the primary method for preventing the production of reactive oxygen species (ROS) associated with redox active metal catalysis (Ebrahimzadeh *et al*., 2008). The methanolic extracts from the raw samples of both varieties of peanuts had high chelating activity (above 90%) and none of the treatments significantly affected this capacity except in the Valencia variety, in which germination caused a slight decrease in chelating activity. The IC_{50} varied from 0.11 to 0.18 mg/mL.

4. CONCLUSIONS

In this study it was found that the response to the different treatments in the content of total phenolic compounds, flavonoids and antioxidant activity depended on the variety of peanut. However, the treatments commonly used to improve the sensory qualities of this oilseed (roasting and frying) did not greatly affect the antioxidant content in peanuts and, in some cases, even increased it. Germination was the best method to increase the antioxidant activity and the content of compounds with nutraceutical potential in peanuts, thus geminated peanuts could be used as raw material to produce functional foods and nutraceuticals.

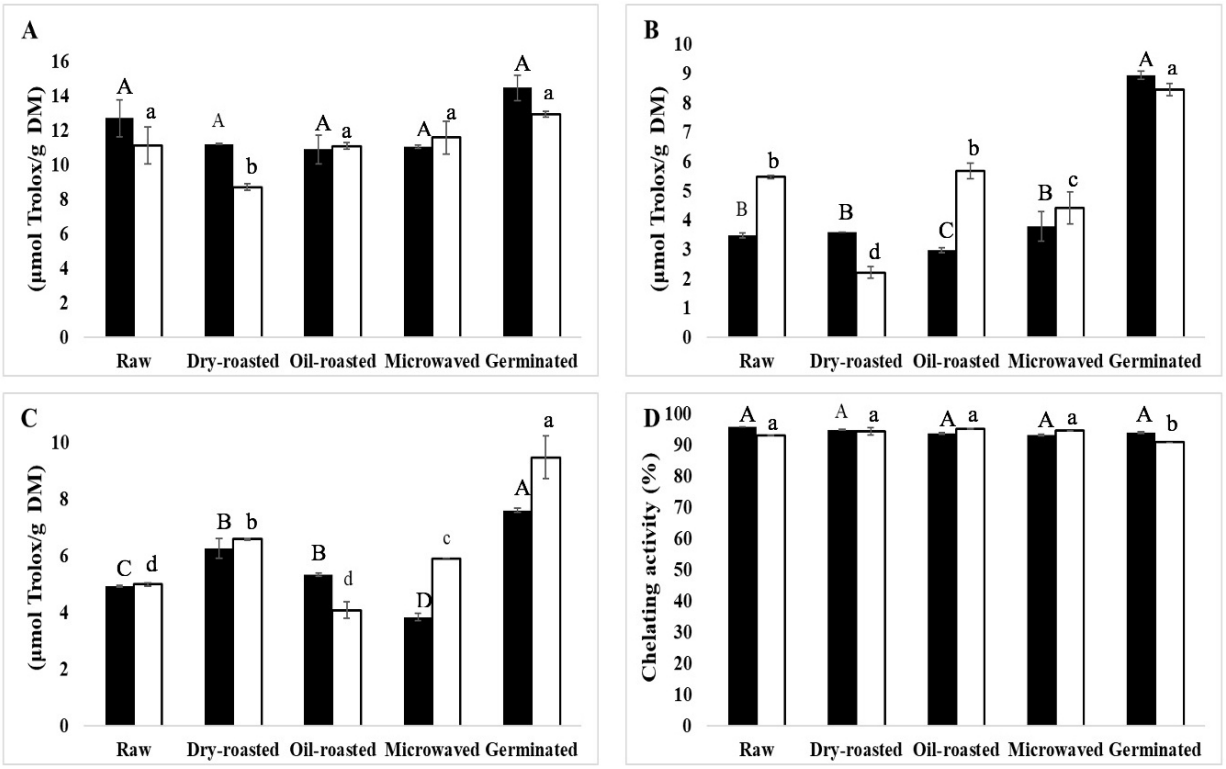

Figure 2. Antioxidant activity as determined by the ABTS method (A), DPPH method (B), reducing power (C) and ferrous chelating activity (D) of Virginia (■) and Valencia (□) peanuts subjected to different treatments. Values are mean ± SD (n=3). Different letters indicate significant differences among treatments according to One-way ANOVA/Tukey's test ($p < 0.05$).

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Optimization of the recovery yield of the enzymatic aqueous extraction of oil from wet açaí decocts using Design of Experiment

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SUMMARY: These last decades, açaí oil has been extensively studied for its biological properties and has gained interest from the health industry. It has thus become necessary to develop eco-friendly extraction techniques. The main objective of this study was the use of experimental designs for the maximization of the recovery yield of the enzymatic aqueous extraction process of açaí oil from wet decocts. A Simplex-Lattice Mixture Design was employed for the optimization of the proportion of three commercial enzymatic preparations. Subsequently, a Central Composite Design was used to identify the optimal values for total enzymatic concentration (0.5–4.5%) and extraction time (2-12h). The "Response Surface Methodology" (RSM) revealed that the maximum yield (60.55 ± 5.98%) was obtained using a 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L and Ultrazym AFP-L at a total enzymatic concentration of 2.85% for 10.9 hours. This study concluded that the enzymatic aqueous extraction of açaí oil is an efficient and sustainable process.

KEYWORDS: *CCD response surface methodology; Euterpe oleracea; Mixture design.*

RESUMEN: ***Optimización del rendimiento de la extracción acuosa enzimática de aceites de açaí mediante cocciones utilizando un diseño experimental***. En las últimas décadas, el aceite de açaí ha sido ampliamente estudiado por sus propiedades biológicas, resultando interesante para las industrias relacionadas con la salud. Por lo tanto, se ha vuelto necesario desarrollar técnicas de extracción ecológicas. El objetivo principal de este estudio es el uso de diseños experimentales para la maximización del rendimiento en la recuperación del proceso de extracción acuosa enzimática del aceite de açaí a partir de cocciones. Se emplea un diseño de mezcla simplex-lattice para la optimización de las proporciones de tres preparaciones enzimáticas comerciales. Posteriormente, se utiliza un Diseño Compuesto Central para identificar los valores óptimos de la concentración enzimática total (0,5–4,5%) y el tiempo de extracción (2–12h). La "Metodología de superficie de respuesta" (RSM) señala que el rendimiento máximo (60,55 ± 5,98%) se obtiene utilizando una mezcla ternaria 0,49:0,25:0,28 de Celluclast 1,5 L, Viscozyme L y Ultrazym AFP-L a una concentración enzimática total de 2,85% durante 10,9 horas. Este estudio indica que la extracción acuosa enzimática del aceite de açaí es un proceso eficiente y sostenible.

PALABRAS CLAVE: *CCD metodología de superficie de respuesta; Diseño de mezclas; Euterpe oleracea.*

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1. INTRODUCTION

The fruit of the species *Euterpe oleracea* is a globous berry, averaging approximately 1 cm in diameter, and 2 g in weight. The pericarp, popularly called pulp, represents only 15% in weight of the fruit, with a thickness of 1-2 mm. During the harvest period, the pulp presents a high concentration of lipids (~ 30-50% on dry basis, d.b.), proteins (~ 5-10%, d.b.), and dietary fibers (~ 20-50%, d.b.) as well as a good source of minerals, phenolic and bioactive compounds (Schauss, 2010; Bichara and Rogez, 2011). The pulp has been widely reported as presenting a high variety of biological activities, mainly anti-inflammatory, antimicrobial, antiprotozoal, antioxidant, antidiabetic and antilipemic (Yamaguchi *et al.*, 2015). More recently, several therapeutic properties have been demonstrated, such as protection against age-related neurodegenerative disorders, cardiovascular diseases, atherosclerosis, control of carcinogenic diseases and global protection of some organs, such as the heart, lung, kidney, and liver (Magalhães *et al.*, 2020).

The presence of the considerable amount of lipids in the pulp of *E. oleracea* fruit, hereafter called açaí oil, its chemical composition and biological activities, seems to play an important role in facilitating the absorption of bioactive compounds by the human organism (Skrovankova *et al.*, 2015).

The chemical composition of açaí oil is highly similar to that of olive and advocado oil, with more than 70% of unsaturated fatty acids, from which ~60-70% are monounsaturated and ~5-15% are polyunsaturated fatty acids. The most abundant fatty acids are oleic acid (~60-70%), followed by palmitic acid (~15-25%) and linoleic acid (~10-15%) (Pacheco-Palencia *et al.*, 2008; Nascimento *et al.*, 2008; Batista *et al.*, 2016). Furthermore, açaí oil contains interesting bioactive compounds, mainly phenolic compounds (Pacheco-Palencia *et al.*, 2008) and tocopherols (Darnet *et al.*, 2011; Lubrano *et al.*, 1994). During the last decade, some works have shown the variety of biological activities and pharmacological applications of açaí oil, including antineoplasic, anti-inflammatory, antilipemic, antimicrobial, antinociceptive, cytotoxic, and genotoxic effects (Magalhães *et al.*, 2020). Due to all these properties, açaí oil has been proven to be very interesting for food, cosmetic and pharmaceutical industries.

Therefore, it has become necessary to develop and optimize extraction techniques for açaí oil. Presently, commercial açaí oil is obtained by conventional extraction methods using organic solvents or hydraulic press. Besides being economical and efficient methods, which provide high yields, such techniques are not healthy or environmentally safe. Recently, some works have focused on alternative and more sustainable extraction methods for açaí oil, such as supercritical CO_2 extraction (Batista *et al.*, 2016; Silva *et al.*, 2019) or enzymatic aqueous extraction (EAE) (Nascimento *et al.*, 2008), with the aim of obtaining not only a high yield but also a good quality oil. The main disadvantage of the conventional and supercritical CO_2 extraction methods is the need to perform the extraction from a pre-processed and dry product, mainly lyophilized aqueous açaí juice, while the EAE process could be performed directly from an "in nature" and wet product (i.e. small pieces of the surrounding pulp of the fruit commonly known as "decocts").

Commonly, the EAE process of oil from fruit or seed is performed through the succession of three steps: the incubation step (considered as the main one during which the extraction takes place), the solid-liquid separation step (during which the solid residue is separated from the liquid phase) and the liquid-liquid separation step (during which lipid and aqueous phases are separated). During the incubation step, enzymes are specifically selected for increasing the permeability of the different structures of the vegetable cell, mainly the primary and secondary walls, and the oleosomes, thus releasing the oil contained inside it into the aqueous medium (Domínguez *et al.,* 1994; Rosenthal *et al.,* 1996; Ricochon and Muniglia, 2010).

Considering the main composition of the cell wall (i.e. mainly lignocellulose and pectin, and, in minor proportions, glycoprotein and glycolipid), the two most interesting classes of enzymes for partially, or completely breaking down the different molecules are the hydrolases (E.C. 3) and lyases (E.C. 4). In particular, enzymes from subclass E.C. 3.1. (e.g. hemicellulase and pectinesterase) acts on ester bonds, E.C. 3.2. (e.g. cellulase, polygalacturonase and glucosidase) acts on glycosidic bonds, E.C. 3.4. (peptidase-class) acts on peptide bonds, and E.C. 4.2. (e.g. pectin and pectate lyases) acts on the carbon-oxygen bonds (Ricochon and Muniglia, 2010).

These different enzymes can be used simultaneously or successively during the EAE process. Commonly, a mixture of carbohydrases with different activities (e.g. E.C. 3.1., 3.2. and 4.2.) are used during the incubation step. The peptidases (e.g. E.C. 3.4.) can be used in combination with carbohydrases during the incubation step (if the objective is to simultaneously extract oil and protein) or can be added in a second phase, during the liquid-liquid separation step (if the purpose is to de-emulsify the protein-lipid emulsion).

The scientific literature is replete with papers studying the effect of these classes of enzymes on oil yield (and eventually interesting bioactive compounds) extracted by the EAE process from a variety of vegetable raw materials (Yusoff *et al.,* 2015). However, to our knowledge, our research group is the only one to specifically study the EAE process for açaí oil for the optimization of its yield.

Numerous unpublished preliminary essays have allowed us to develop a standardized and reproducible EAE process for açaí oil. In particular, they indicate that it is more interesting to extract the oil directly from "in nature" wet açaí decocts than from any other kind of pre-processed and/or dry raw material derived from açaí pulp. This strategy avoids adding water during the juice production, then removing it during drying, as well as significantly limiting the formation of the protein-oil emulsion. Moreover, it has been shown that is it much more efficient to use carbohydrases separately (during the incubation step) and peptidases (during the liquid-liquid separation step). Based on this, our works focused on the maximization of the yield of the oil extracted from açaí wet decocts using only carbohydrases during the entire EAE process.

In a previous work, Ferreira *et al.* (2018) studied four commercial carbohydrase enzymatic preparations (EP). They were studied individually and in combination of two, three and four using iso-enzymatic proportion at an individual enzymatic concentration (i.e. liquid enzymatic preparations weight: wet substrate weight, w:w) of 1%. According to the authors, the 1:1:1 ternary mixture of Celluclast 1.5L, Viscozyme L and Ultrazym AFP-L (i.e. at a total enzymatic concentration of 3%) was identified as the optimal one.

The literature shows that other parameters in the EAE process may have a significant impact on the yield of the extracted oil, such as the proportion of the EP, the total enzymatic concentration, the extraction time, the proportion of water in the mixture, the pH of the medium, the temperature, or the velocity of agitation, amongst others (Yusoff *et al.,* 2015).

In this context, the main objective of our work was the optimization of three parameters – the proportion of the three carbohydrase EP, the total enzymatic concentration and the extraction time – using experimental designs for the maximization of the recovery yield of the enzymatic aqueous extraction process of açaí oil from wet decocts using exclusively carbohydrase EP.

2. MATERIALS AND METHODS

2.1. Raw material

E. oleracea fruits were collected in Abaetetuba (Pará State, Brazil) in the middle of the harvest period (GPS coordinates 1°46'42" S–48°51'07" W). Once collected, the fruits were taken to the laboratory, washed under tap water, and softened in water at 40 ºC for 1 hour. Then, the softened fruits were pulped by manual friction using sieves. Small pieces of the surrounding pulp of the fruits, with an average size of 0.65 cm, commonly called "decocts", were obtained. Lastly, the decocts were stored at -20 °C until the EAE experiments were performed.

2.2. Raw material characterization

The dry matter content of the decocts was determined according to the NFTA Method 2.1.4 (AOAC Official Method 935.29 & 945.15) by drying the manually-milled wet decocts in a drying and sterilizing stove (SOLAB SL-100, São Paulo, Brazil) at 105 °C for 3 hours (or until constant weight). The result was expressed as the ratio between the mass of water and the total mass of the wet decocts (%, w.b.).

The lipid content of the decocts was determined according to the AOAC Official Method 945.16, by submitting milled dry decocts to Soxhlet extraction (SOLAB SL 145/6, São Paulo, Brazil) using petroleum ether at 60 °C for 3 hours. The result was expressed as the ratio between the mass of lipid and the total mass of the dry decocts (%, d.b.).

2.3. Enzymatic preparations

The three commercial EP (LNF Latino Americana, Rio Grande do Sul, Brazil), were selected according to the conclusions of Ferreira *et al.* (2018):

a. Celluclast 1.5 L (C): mixture of three synergistic sub-enzymes, namely exo-β-1,4-glucanase (major component), endo-β-1,4-glucanase and β-1,4-glucosidase, 700 EndoGlucanase Units (EGU)/g;
b. Viscozyme L (V): multi-component carbohydrase containing endo-1,3(4)-β-glucanase (major component), xylanase, cellulase and hemicellulose, 100 Fungal Beta Glucanase Units (FBG)/g;
c. Ultrazym AFP-L (U): mixture of endopolygalacturonase (major components), endo-pectin lyase, and cellulase, 3000 Pectinase Units (PECTU)/ml.

2.4. EAE process of açaí oil

The methodology for the EAE process of açaí oil was based on and adapted from Nascimento *et al.* (2008). Each essay was performed using 100 g of thawed wet açaí decocts. They were thermally pretreated at 70 °C for 1 min using a domestic food steamer (Walita / Philips Jamie Oliver RI9132/01, São Paulo, Brazil) with the aim denaturing the endogenous enzymes and partially cleave the lignin. Then, the decocts and 240 mL of distilled water at ambient temperature (wet decocts: water ≈ 1:2.5 (w:w)) were transferred to a 600 mL beaker sealed with aluminum foil. The three EP were inserted sequentially using a micropipette in the proportion and the total enzymatic concentration according to the experimental designs. A set of triplicate essays was performed without the addition of EP, considered as the negative control.

For the incubation step, the beaker was incubated at 50 °C under orbital agitation of 100 rpm in a shaker (SOLAB SL-222, São Paulo, Brazil) during an extraction time fixed according to the experimental designs. After the incubation, the beaker was cooled in a bath with ice cubes until reaching ambient temperature.

For the solid-liquid separation step, the beaker was left for approximately 24 hours at ambient temperature in order to obtain natural separation of the solid cake (i.e. fibers and high density proteins), the aqueous-proteic phase (i.e. water and low density proteins) and the lipid phase (i.e. oil droplets dispersed on the surface, optionally in protein emulsion). Note that this step may also be realized using a centrifuge lab equipment. The liquid phases (i.e. aqueous-proteic and lipid phases) were then transferred to another smaller beaker.

For the liquid-liquid separation step, about 20 mL of n-hexane (Dinâmica, São Paulo, Brazil) was added onto the surface with a micropipette in order to favor, on the one hand, the separation of the lipid phase from the aqueous-proteic phase, and on the other hand, the agglomeration of all oil droplets. This strategy was selected as a reference standardized technique at laboratory scale with the aim of focusing only on the effect of the carbohydrase EP (i.e. avoid the use of peptidases) and to allow for the most precise analytical quantification of the extracted oil. Then, the supernatant organic-lipid phase was separated from the aqueous-proteic phase using a micropipette and transferred to a 100-mL previously weighed beaker.

This beaker was incubated (at 100 °C for 15 hours) in a drying and sterilizing stove (SOLAB SL-100, São Paulo, Brazil), until constant oil weight (i.e. complete evaporation of the n-hexane), then stored in a desiccator until reaching ambient temperature. Eventually, the mass of the extracted oil was determined by the gravimetric method, using an Analytical balance (SHIMADZU UniBloc AUY 220, São Paulo, Brazil).

The efficiency of the extraction method, i.e. the oil recovery yield, hereafter called yield (expressed in percentage), was calculated by the ratio between the mass of the oil extracted from 100g of wet decocts by the EAE process, and the mass of the total lipid content in 100 g of wet decocts obtained by the reference Soxhlet method (considered as the positive control).

2.5. Experimental designs

The identification of the optimal values for the parameters was made through two experimental designs: a three-component Simplex-Lattice Mixture Design for the proportion of the three EP and a two-factor Central Composite Design for the total enzymatic concentration and the extraction time. The ranges of values were determined according to the literature (using scopus data base) related to the EAE process for vegetable oils.

2.6. Three-component simplex-lattice mixture design

The effect of the proportion of the three EP on the yield was studied using a three-component augmented Simplex-Lattice Mixture Design. The total enzymatic concentration and the extraction time were fixed at 3% and 4 hours, respectively. Our ma-

trix was carried out with the following 5 coded (and uncoded) levels: 0 (0%), 0.33 (33.3%), 0.5 (50%), 6.6 (66.6%) and 1 (100%). The experimental design presents 15 experimental points, in which 3 are single EP essay treatments (essays 1-3), 9 are two-EP mixture essays (i.e. mid-points, essays 4-12) and 3 are three-EP mixture essays (i.e. central point performed in triplicate, essays 13-15) (see Table 1).

2.7. Two-factor central composite design

The effect of the total enzymatic concentration and the extraction time on the yield was studied using a two-factor Central Composite Design 2^2 with $\alpha = 1.41$. Our matrix was carried out with the following 5 coded levels: -1.41, -1, 0, 1, and 1.41. The total enzymatic concentrations investigated were: 0.5, 1.08, 2.5, 3.9, and 4.5% (w:w), and the extraction times studied were: 2, 3.5, 7, 10.5 and 12 hours (see Table 2). The central point (2.5% and 7 hours) was performed in triplicate.

2.8. Statistical analysis

The experimental designs and statistical analysis of the data were performed using the STATISTICA software version 7.0 (Statsoft Inc., Oklahoma, USA). The significance of the models and the coefficient estimates of the factors were evaluated by Analysis of Variance (ANOVA) with a significance level of 5% ($\alpha = 0.05$). The optimization was performed using the Response Surface Methodology.

Table 1. Three-component augmented Simplex-Lattice Mixture Design: matrix of the proportion (expressed in ratio) of the three EP (enzymatic preparations: C = Celluclast 1.5 L, U = Ultrazym AFP and V = Viscozyme L), results of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) and comparison with Ferreira *et al.* (2018).

	Ratio			EAE oil recovery yield (%)	
Essay nº	**C**	**U**	**V**	**Our work**	**Ferreira *et al.* (2018) $**
Negative Control	0	0	0	29.51±5.92 $	34.91±11.81
1	1	0	0	42.04	41.52±4.31 *
2	0	1	0	39.14	47.71±11.72 *
3	0	0	1	37.40	46.19±8.31 *
4	0.5	0.5	0	47.08	55.02±13.73 **
5	0.5	0	0.5	45.64	56.20±9.81 **
6	0	0.5	0.5	28.56	46.81±6.15 **
7	0.33	0.66	0	42.74	
8	0.66	0.33	0	50.43	
9	0.33	0	0.66	42.96	
10	0.66	0	0.33	45.56	
11	0	0.33	0.66	36.05	
12	0	66.6	0.33	26.64	
13	0.33	0.33	0.33	51.25	
14	0.33	0.33	0.33	56.81	63.78±3.01
15	0.33	0.33	0.33	49.93	

The essays were performed using a total enzymatic concentration of 3% and an extraction time of 4 hours. The experimental design presents 15 experimental points, in which 3 are single EP essay treatments (essays 1-3), 9 are two-EP mixture essays (i.e. mid points, essays 4-12) and 3 are three-EP mixture essays (i.e. central point performed in triplicate, essays 13-15).

$The results are presented as the mean value of triplicate ± standard deviation.

* At a total enzymatic concentration of 1%.

**At a total enzymatic concentration of 2%.

Table 2. Two-factor Central Composite Design: matrix of the total enzymatic concentration (expressed in %) and the extraction time (expressed in hours) and results of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %).

Essay nº	Total enzymatic concentration (%)	Extraction time (hours)	EAE oil recovery yield (%)
1	1.08	3.5	45.86
2	1.08	10.5	54.39
3	3.9	3.5	57.61
4	3.9	10.5	59.24
5	0.5	7	48.44
6	4.5	7	48.12
7	2.5	2	50.36
8	2.5	12	60.19
9	2.5	7	58.92
10	2.5	7	56.72
11	2.5	7	57.01

The essays were performed using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L Ultrazym AFP-L, respectively. The experimental design presents 11 experimental points in which the central point (2.5% and 7 hours) was performed in triplicate (i.e. essays 9-11).

3. RESULTS AND DISCUSSION

3.1. Raw material characterization

The decocts presented a dry matter content ranging from 45.14 ± 0.33% to 46.50 ± 0.51% w.b. This result is in agreement with those obtained (but not presented) by Ferreira *et al.* (2018) with an average of 50.98% w.b. at the beginning and 45.5% w.b. at the middle of the harvest. No other comparison was possible with the literature because all the authors work from the pre-processed pulp, mainly aqueous juice.

The decocts used throughout our work showed a high variation in the total lipid content, which ranged from 15.20 ± 0.23% to 24.30 ± 0.61% d.b., with an average value of 18.82 ± 3.6% d.b.. This variation in the total lipid content can be justified by the heterogeneity of the vegetable raw material, which may have presented variations in its composition depending on the harvest and the extraction moment itself (Bichara and Rogez, 2011). Our results are slightly lower than those obtained (but not presented) by Ferreira *et al.* (2018), with an average of 28.33% d.b. and 22.45% d.b., at the beginning and in the middle of the harvest, respectively. These values are significantly lower than those obtained from açaí juice by Nascimento *et al.* (2008) with 42.6% d.b., Batista *et al.* (2016) with 45.4% d.b. and Silva *et al.* (2019) with 49-57%. It is worth mentioning that there are more fiber contents in the decocts than the juice, contributing to the reduction in the total lipid content of the decocts compared to the juice.

3.2. Negative and positive control

Considering the mathematical expression of the yield used in our paper, the yield of the positive control (i.e. using the AOAC Official Method 945.16) was 100%, while the yield obtained experimentally of the negative control (i.e. using the EAE process without EP, with an extraction time of 4 hours) was 29.51 ± 5.92%. This result is similar to the one obtained by Ferreira *et al.* (2018) under the same conditions with 34.91 ± 11.81% (see Table 1). Note that the yields of the negative control are similar to those obtained by other authors who studied the EAE process for oil from different vegetable matrices (see Table 3).

Table 3. Comparison of the recovery yield (%) of the oil extracted by the EAE process from different vegetable matrices, using similar values for the total enzymatic concentration and extraction time to ours, and using several enzymatic preparations, including cellulase, hemicellulase and/or pectinase, individually and in combination (using iso-enzymatic proportion).

Reference	Matrix	Enzymatic preparations			Total enzymatic concentration (%)	Extraction time (hours)	Oil recovery yield (%)						
		Cellulase	Hemicell.	Pectinase			Negative Control	1 EP Cell.	1 EP Hemicell.	1 Ep Pect.	2 EP	3 EP	4 EP
Our work	Açaí decocts	C	V	U	3	4	29	42	37	39	28-47	53	
Ferreira *et al.* 2018	Açaí decocts	C	V	U	1-3	4	34	41	46	47	46-56	63	
Abdulkarim *et al.* 2006	Moringa seed	C	-	other	2	36	35	65		56			74 *
Zhang *et al.* 2007	Rapeseed	other	-	other	2.5	3	48	69		85	75-88	84 **	
Tabtabei and Diosady, 2013	Yellow mustard flour	C	V	other	3	3	55	67	68	65	72	76	
Mai *et al.* 2013	Gac fruit	other	-	other	10	2	5-10	12		20	29	40 #	62 #
Dela Cruz *et al.* 2007 @	Vutalao seed	other			2	4	29	79			97 $	93 $	
Silvamany and Jahim 2015 @	Palm	other	Other	other	0.3	2	53	73	79	71	71-79	90	

Legend: C=Celluclast 1.5L, U=Ultrazym AFP-L, V=Viscozyme L, EP=Enzymatic Preparation. @ use of Simplex-Lattice Mixture Design, * including a protease and a amylase, ** including a glucanase, # including a amylase and/or a protease, $ including a amylase and/or a xylanase

3.3. Three-component augmented Simplex-Lattice Mixture Design

The matrix of the experimental design and the results of the three-component augmented Simplex-Lattice Mixture Design for the maximization of the yield as a function of the proportion of the three EP is presented in Table 1. One can easily observe a significant increase in these yields, whatever the proportion used, compared to the negative control.

Our experimental results can be directly compared with the ones obtained by Ferreira *et al.* (2018), who studied the effect of these three EP individually and in combination using iso-enzymatic proportion at an individual enzymatic concentration of 1%, on the yield of the oil extracted from açaí wet decocts. One can observe that our results, all obtained at an enzymatic concentration of 3%, are slightly lower (see essays 1-3, essays 4-6 and essays 13-15, Table 1). One can observe a variability in our results at the central point (see essays 13-15, Table 1), with an average experimental yield of 52.63 ± 3.66%. Such variability may be explained by the use of biological materials, and the independent repetition of all the essays. Note also that the central point provides the maximum experimental yield.

Model fitting. The fitting of the different order models (i.e. linear, quadratic or cubic) to the experimental results of the yields showed that the cubic was the most appropriate one. In particular, the low global p-value (< 0.0028) indicated that the model was significant, the high R^2 (> 0.90) was satisfactory to validate the significance of the model, the high R^2_{adj} (> 0.83) showed a good relationship between the experimental data and the fitted model and the high lack-of-fit p-value (> 0.6) indicated that the model seemed to accurately fit the experimental data. The significant coefficients (p-value < 0.05) of the factors of the cubic model are those of the linear effects (p-value < 0.0001), followed by the cubic effect (p-value = 0.0026), and the quadratic effect between Ultrazym AFP-L and Viscozyme L (p-value = 0.0415).

Optimal value of the proportion of the three enzymatic preparations. The optimal value of the proportion of the three EP for predicted maximum yield was determined by Response Surface Methodology. In particular, the contoured response surface using the special cubic model clearly showed that the highest yields were achieved with enzymatic mixtures containing more than 25% of Celluclast 1.5L (see red colored region in Figure 1). According to the prediction profiling, the judicious proportion of the three EP which maximized the yield was 45.9% of Celluclast 1.5 L, 25.3% of Viscozyme L and 28.8% of Ultrazym AFP-L (see red vertical line that indicated the optimal value of each EP, in Figure 2). In these conditions, the predicted maximum yield was 54.07 ± 8.91%. Note that this value is comparable to the maximum yield obtained experimentally (see essays 13-15, Table 1).

Understanding of the enzymatic phenomena. Our results demonstrate that Celluclast 1.5L allowed for obtaining higher yield than using Viscozyme L or Ultrazym AFP-L (see essays 1-3 in Table 1). This can be explained by the fact that endo-enzymes randomly cleave internal bonds of the polymer chain, while exo-enzymes act on the end of the polymer chain. In particular, exo-glucanase (e.g. Celluclast 1.5 L) is essential in the breakdown of crystalline cellulose, and is considered an "input enzyme" for the releasing of oil contained in the vegetable cell (Domínguez *et al.* 1994). However, due to the overlapping of the

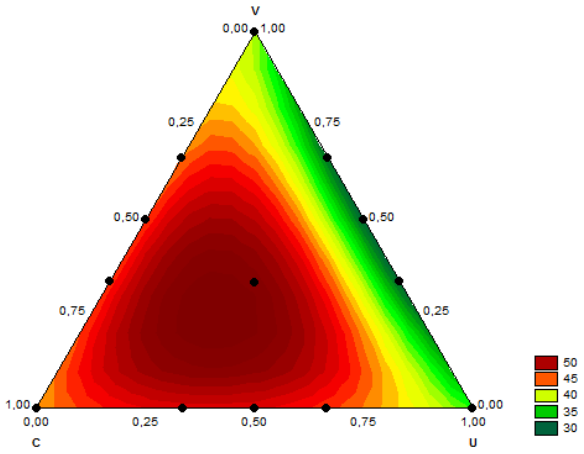

Figure 1. Contoured Response Surface of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the ratio of the three EP (enzymatic preparations: C = Celluclast 1.5 L, U = Ultrazym AFP and V = Viscozyme L) using the cubic model for the three-component augmented Simplex-Lattice Mixture Design.
The assays were performed using a total enzymatic concentration of 3% and an extraction time of 4 hours. The experimental design presents 15 experimental points, in which 3 are single EP essay treatments (i.e. vertex of the triangle), 9 are two-EP mixture essays (i.e. on the edges of the triangle) and 3 are three-EP mixture essays (i.e. central point performed in triplicate).

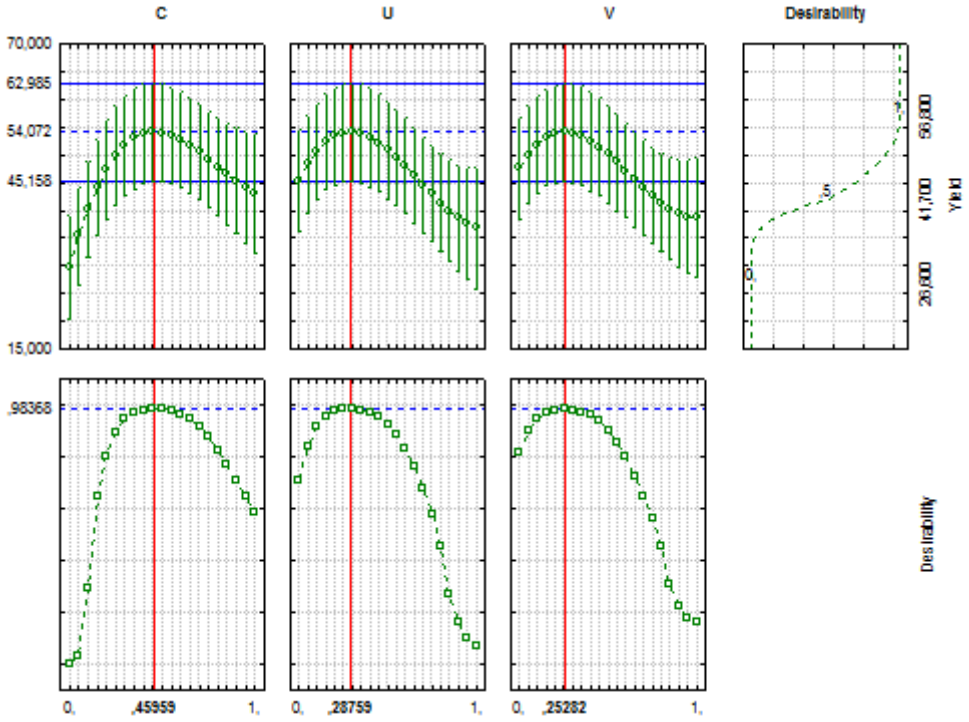

Figure 2. Prediction profiling (with prediction intervals) and desirability function of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the ratio of the three EP (enzymatic preparations: C = Celluclast 1.5 L, U = Ultrazym AFP and V = Viscozyme L) using the cubic model of the three-component augmented Simplex-Lattice Mixture Design. The essays were performed using a total enzymatic concentration of 3% and an extraction time of 4 hours.

different polymers of the vegetable cell wall, the accessibility of Celluclast 1.5 L to its specific substrate is intrinsically low. Therefore, enzymatic mixtures containing other class-type enzymes, in particular endo-enzymes, are more competitive. Our results clearly demonstrate that the enzymatic mixtures of two EP containing Celluclast 1.5L with Viscozyme L or Ultrazym AFP-L show yields higher than those obtained with the use of only one EP (see essays 4-5, 7-10 in Table 1, and red color region in Figure 1). This finding can be explained by the fact that these two endo-enzymes easily and randomly act on different polysaccharides of the vegetable cell wall, and in particular generate ends of cellulose chain for the subsequent action of Celluclast 1.5L (Silvamany and Jahim, 2015). On the other hand, as already pointed out by Ferreira *et al.* (2018), the essays carried out without Celluclast 1.5 L are considered non-advantageous as showing yields lower than those obtained with the use of only one EP (see essays 6, 11-12 in Table 1, and green color region in Figure 1).

Comparison with the literature. Some papers have already studied the effect of several EP, including cellulase, hemicellulase and/or pectinase, individually and in combination (using iso-enzymatic proportion), on the yield of the oil extracted by the EAE process from different vegetable matrices, using similar values for the enzymatic concentration and extraction time to ours (see e.g. Abdulkarim *et al.*, 2006; Zhang *et al.*, 2007; Tabtabei and Diosady, 2013; Mai *et al.*, 2013, and Ferreira *et al.*, 2018, Table 3). As observed, the results obtained by these authors are of the same order as those obtained in our work. Most of authors pointed out that the cellulase and the pectinase are among the main enzymes which contribute to increasing the yield. Moreover, most of the papers concluded that a synergetic action of the different EP on the vegetable cell wall is necessary to maximize the release of oil.

However, to our knowledge, the literature presents very few works related to the optimization of enzymatic mixtures for the aqueous extraction of oil by employing a Simplex-Lattice Mixture Design. One can cite the papers of Dela Cruz *et al.* (2007) and Silvamany and Jahim (2015) (see Table 3). More specifically, Dela Cruz *et al.* (2007) ob-

served that ternary mixtures did not provide significantly higher yields than binary mixtures, and concluded that the maximum yield was achieved using a 1:1 binary mixture of xylanase and cellulose (or amylase). Silvamany and Jahim (2015) showed that maximum experimental yield was acheived using a 1:1:1 ternary mixture of cellulase, hemicelulase and pectinase, an a maximum predicted yield with ratio 0.46:0.34:0.2 (celulase : hemicelulase : pectinase). Such an enzymatic proportion is very similar to that obtained in our work.

3.4. Two-factor Central Composite Design

Table 2 shows the matrix of the experimental design and the results of the two-factor Central Composite Design for the maximization of the yield as a function of the total enzymatic concentration and the extraction time, using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L and Ultrazym AFP-L, respectively. Once more, one can observe a variability in the results at the central point (i.e. total enzymatic concentration: 2.5%, extraction time: 7 hours) (see essays 9-11, Table 2), with an experimental average yield of 57.55 ± 1.19%. One can observe that the results obtained for the essays realized with a total enzymatic concentration of 2.5% and extraction times of 2 and 7 hours (see essays 7 and 9-11, Table 2) are coherent with the one predicted by the previous model for a total enzymatic concentration of 3% and an extraction time of 4 hours. Note that the highest experimental result was obtained for essay 8, with 60.19%, performed using a total enzymatic concentration of 2.5% and an extraction time of 12 hours.

Model fitting. The fitting of the different order models (i.e. including linear, quadratic or interaction effects, and their combination) to the experimental results of the yields showed that the simpler second order models (i.e. that combine the linear & quadratic effects) were the most appropriate ones. In particular, they are characterized by the lowest global p-value (< 0.08), the highest R^2 (> 0.75), the highest R^2_{adj} (= 0.59), and the highest lack-of-fit p-value > 0.08. This is considered a good result in view of the great variability in handling biological raw materials. The most significant coefficients (p-value < 0.05) of the factor of the second order model are those of the quadratic effect (p-value = 0.015) of the total enzymatic concentration and the linear effect (p-value = 0.019) of the extraction time, followed by the linear effect (p-value = 0.041) of the total enzymatic concentration.

Optimization of the total enzymatic concentration and extraction time. The optimal values for the two factors for predicted maximum yield are identified by Response Surface Methodology. One can easily observe the quadratic effect of the total enzymatic concentration and the linear effect of the extraction time on the yield (see contoured response surface in Figure 3). According to the prediction profiling, the values for the total enzymatic concentration and the extraction time which maximized the yield are 2.85% and 10.9 hours, respectively (see red vertical line that indicates optimum value for each factor, in Figure 4). In these conditions, the predicted yield was 60.55 ± 5.98%. Note that this value is similar to the maximum yield obtained experimentally (see essay 8, Table 2).

As the optimized extraction time lies at the upper extremity of the range of values, some more independent essays were performed in triplicate using extraction times of 8, 12 and 16 hours in order to statistically compare the experimental yields obtained. The results (57.55 ± 1.19%, 57.28 ± 2.77% and 56.03 ± 1.43%, respectively) were analyzed by Analysis of Variance (ANOVA) with a significance level of 5% (α = 0.05) and show that there is no statistical difference between the results (p-values > 0.05).

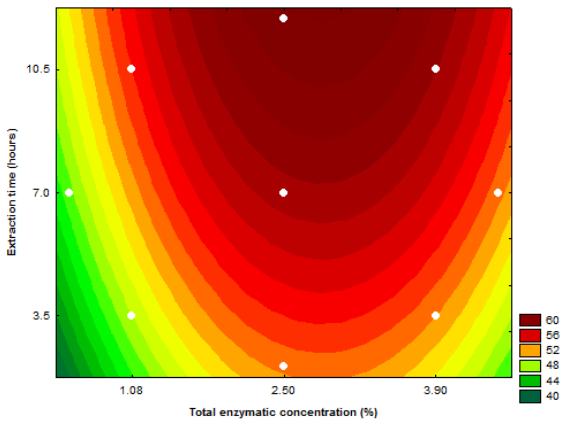

Figure 3. Contoured Response Surface of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the total enzymatic concentration (expressed in %) and the extraction time (expressed in hours) using the second order model for the two-factor Central Composite Design.
The essays were performed using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L Ultrazym AFP-L, respectively. The experimental design presents 11 experimental points in which the central point (2.5% and 7 hours) was performed in triplicate.

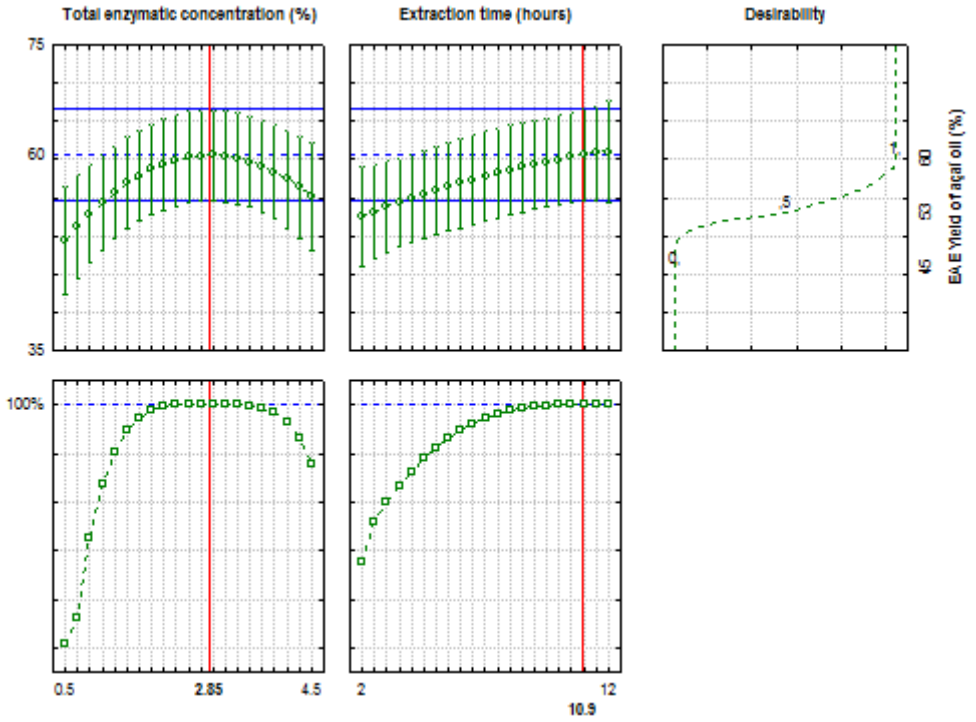

Figure 4. Prediction profiling and desirability function of the EAE (Enzymatic Aqueous Extraction) oil recovery yield (expressed in %) as a function of the total enzymatic concentration (expressed in %) and extraction time (expressed in hours) using the second order model for the two-factor Central Composite Design. The essays were performed using the 0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L Ultrazym AFP-L, re

Understanding of the enzymatic phenomena. As pointed out previously, the yield is significantly influenced by the quadratic effect of the total enzymatic concentration. The yield was enhanced when the total enzymatic concentration increased, because the enzymatic mixture of the three EP hydrolyzed the different polymers of the vegetable cell wall, which enable the release of the oil. However, once the total enzymatic concentration reached the maximum value, the yield slightly decreased with further increase in the total enzymatic concentration. This may be explained by possible enzyme aggregation caused by excess enzymes, which restricted their individual flexibility and activity, by the accumulation of intermediate products which inhibit the enzymatic activity, or by the stabilization of a fat emulsion in water by the enzymes which act as protein surfactants. Regarding the extraction time, the statistical treatment pointed out the significant linear effect of the extraction time, but the prediction profiling, the desirability function and the extra experimental essays showed that an increase in the extraction time up to 12 hours did not provide a significantly ($\alpha = 0.05$) higher yield compared to 8 hours. Some authors explained that a long extraction time might contribute to the depletion of the substrates and/or the product inhibition of enzymes (Zhang *et al.*, 2007; Li *et al.*, 2011), to a partial inactivation of the enzyme and/or to the adsorption of the oil onto the remaining solid fraction, or to the liberation of cellular components including phospholipids, glycolipids, and proteins, which form lipid–protein aggregates and emulsion stabilization (Nguyen *et al.*, 2020), consequently reducing the yield.

Comparison with the literature. Some papers have already used experimental designs for the identification of the optimal values of the total enzymatic concentration and the extraction time (among others) for the maximization of the yield of the oil extracted by the EAE process from different vegetable matrices, using cellulase, hemicellulase and/or pectinase (see Table 4).

Regarding the total enzymatic concentration, Xie *et al.* (2011), Mai *et al.* (2013), Nguyen *et al.* (2020) and Liu *et al.* (2020) also observed the significant quadratic effect of the total enzymatic concentra-

Table 4. Comparison of the ranges and optimal values for the total enzymatic concentration (%) and the extraction time (hours) suited by the Experimental Design for the maximization of the yield (%) of the oil extracted by the EAE process from different vegetable matrices, using cellulase, hemicellulase and/or pectinase.

Reference	Matrix	Enzymatic preparations			Methodology	Total enzymatic concentration (%)				Extraction time (hours)				Max Yield
		Cellulase	Hemicell.	Pectinase		Range	Optimal	L	Q	Range	Optimal	L	Q	%
* Abdulkarim *et al.* 2006	Moringa seed	C	-	other	OFAT	0.5-2.5	>2			0-48	>36			74
* Zhang *et al.* 2007	Rapeseed	other	-	other	OFAT	0-5	>2.5			1-7	>4			90
* Tabtabei and Diosady, 2013	Yellow mustard flour	C	V	other	OFAT	-	3			-	3			76
Zhang *et al.* 2007	Rapeseed	other	-	other	RSM	0.5-1.5	1.5	X		1-3	3	X		75
Womeni *et al.* 2008	Bush mango kernel	-	V	other	RSM	0-2	>1.4	X		6.6-18.4	>14	X	X	83
Xie *et al.* 2011	Wheat germ	-	V	-	RSM	0.1-4	1.1		X	0.5-24	>5.25	X	X	66
* Mai *et al.* 2013	Gac fruit	other	-	other	RSM	5-25	14	X	X	1-3	2	X	X	80
Nguyen *et al.* 2020	Sacha Inchi Seed	other	-	-	RSM	2-6	4.5	X	X	2-6	5	X	X	95
Liu *et al.* 2020	Peanut	other	V	other	RSM	0.5-2	1.5	X	X	0.3-2.3	1.5		X	78

C=Celluclast 1.5L, U=Ultrazym AFP-L, V=Viscozyme L, OFAT=One Factor At a Time, RSM=Response Surface Methodology.
** References also present in Table 3. L. Linear factors. Q. Quadratic factors.*

tion on yield. Other papers (Abdulkarim *et al.* 2006; Zhang *et al.* 2007 and Womeni *et al.*, 2008) showed a stabilization of the yield above a value of total enzymatic concentration (see Total enzymatic concentration (%), Table 4).

Regarding the enzymatic time, Zhang *et al.* 2007, Womeni *et al.* 2008, Xie *et al.* 2011, Mai *et al.* 2013 and Nguyen *et al.* 2020 also pointed out the significant linear effect of the extraction time, even if they showed that an increase in the extraction time up to the predicted optimal value did not provide a significant higher yield. This was also the conclusion of the papers of Abdulkarim *et al.* (2006); Zhang *et al.* (2007) and Liu *et al.* (2020) (see Extraction time (hours), Table 4).

4. CONCLUSIONS

In the present work, an efficient and sustainable enzymatic aqueous extraction process for açaí oil is suggested. Its particularities are to perform the extraction from "in nature" wet açaí decocts and using exclusively carbohydrases. Three enzymatic aqueous extraction parameters (i.e. proportion of three commercial carbohydrase enzymatic preparations, total enzymatic concentration and extraction time) were optimized using two successive Experimental Designs coupled to Response Surface Methodology. Under the optimum conditions identified (0.49:0.25:0.28 ternary mixture of Celluclast 1.5 L, Viscozyme L and Ultrazym AFP-L, total enzymatic concentration: 2.85%, extraction time: 10.9 hours), the highest oil recovery yield of 60.55 ± 5.98% was estimated. This process allows satisfactory yields compared to conventional extraction methods, with the advantage of being carried out under environmentally-friendly conditions. Moreover, this technique is relatively accessible and easily implantable by Amazonian communities, and could help to create new market opportunities.

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Application of an edible coating developed with Andean potato starch and carboxymethyl-cellulose for lipid reduction during frying

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SUMMARY: This work aimed to search for alternative uses for different varieties of Andean potatoes (*Solanum tuberosum ssp andigenum*) which have been reintroduced in north-western Argentina. Specifically, the development of simple and compound films made with hydrocolloids such as carboxymethyl-cellulose (CMC) and starch (S) extracted from Andean potatoes var. Runa, and its application as a cover in the deep frying of Andean potato chips var. Waycha was studied to minimize oil absorption. The effect of prior bleaching of the chips with different media was also evaluated: water, calcium chloride solution, and ascorbic acid. The coatings were applied to potatoes chips before being fried. The results showed that the type of oil used did not affect absorption by the chips. The bleaching treatments with calcium chloride and coating with S/CMC, showed a significant reduction in oil absorption (39.5 % ± 0.7), delayed its oxidation, and decreased the loss of tocopherols during the frying process. It also contributed to the physical and sensory characteristics of the final product, which presented high acceptability by consumers.

KEYWORDS: *Andean potato chips; Andean potato starch; Coating; Frying; Oil absorption*

RESUMEN: ***Aplicación de un recubrimiento comestible desarrollado con almidón de papa andina y carboximetilcelulosa para la reducción de lípidos durante la fritura.*** Este trabajo tuvo como objetivo buscar alternativas de uso para diferentes variedades de papas andinas (*Solanum tuberosum ssp andigenum*) reintroducidas en el noroeste argentino. Específicamente, el desarrollo de películas simples y compuestas elaboradas con hidrocoloides como la carboximetilcelulosa (CMC) y el almidón (S) extraído de papa andina variedad Runa, y su aplicación como cobertura en frituras de papas andinas var. Waycha fue estudiado para minimizar la absorción de aceite. También se evaluó el efecto del blanqueo previo de las hojuelas con diferentes medios: agua, solución de cloruro de calcio y ácido ascórbico. Los recubrimientos se aplicaron a patatas fritas antes de freírlas. Los resultados mostraron que el tipo de aceite utilizado no afectó su absorción por parte de las hojuelas. Los tratamientos de blanqueo con cloruro de calcio y recubrimiento con S/CMC, mostraron una reducción significativa en la absorción de aceite (39,5 % ± 0,7), retrasaron su oxidación y disminuyeron la pérdida de tocoferoles durante el proceso de fritura. También contribuyó a las características físicas y sensoriales del producto final, que presentó alta aceptabilidad por parte de los consumidores.

PALABRAS CLAVE: *Absorción de aceite; Almidón de papa andina; Fritura; Hojuelas de papas andinas; Recubrimiento*

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1. INTRODUCTION

Many strategies have been proposed to reduce oil absorption in food during frying (Crosa *et al.*, 2014). The application of a hydrocolloid coatings has been one of the most promising methods (Naghavi *et al.*, 2018). These modify the food surface, decreasing its permeability. Among the materials used to make coatings are methylcellulose (MC), hydroxypropyl-cellulose (HPC), carboxymethyl-cellulose (CMC) and hydroxypropylmethyl-cellulose (HPMC) (Mai Tran *et al.*, 2007). The use of natural polymers in packaging and food additive applications is gaining popularity, due to the prevention of environmental problems. Edible coatings must contain substances that comply with food standards and must also be economical, easy to apply and respectful of the environment. Cellulose is a very abundant natural polysaccharide, it is the structural component of the cell wall of plants, and therefore, it is obtained from various natural sources, such as food waste, cereal bran and fruit peels. The main reasons for the common use of CMC are its viscosity, flocculant property, excellent oil resistance, transparency, non-toxicity, and low price. It has GRAS status from the FDA (Yaldirim–Yalcin *et al*., 2022). Starch, as one of the most abundant, sustainable, and low-cost commercial biopolymers, has versatile applications in many industries (e.g., food, paper, packaging, etc.). Potato varieties to produce fried products at the industrial level must meet external and internal quality characteristics. Environmental and genetic effects determine external qualities. The internal ones refer to the chemical composition of tubers which includes the content of sugars, dry matter, and starch, among others. Reducing sugars (fructose and glucose) play a critical role in the industrial process, so the legislation establishes contents lower than 0.25% for the elaboration of French fries and less than 0.2% (Colman *et al.*, 2009) for potatoes in flakes. Frying is an operation widely used in the food industry. It is based on the transfer of heat from hot oil to foods, causing water removal and oil absorption. The high consumption of fried products is a risk factor for health due to the high energy density and the possible formation of toxic compounds. The origin and oil composition can influence the absorption of oil into food during the frying process, as well as temperature and frying time, type of food, porosity, and pretreatments applied (Alvis *et al.*, 2015). During frying, reactions responsible for taste, color, and texture occur and unhealthy components are formed such as polar compounds (Jimenez *et al.*, 2017). During repeated use of the frying oil, it degrades and changes its composition, generating a mixture of polar compounds that act as wetting agents which reduce the surface tension between water and oil, causing an increase in oil absorption.

This paper proposed to search technological alternatives for use of Andean potato *(Solanum tuberosum ssp. andigenum*) genotypes which have been reintroduced in northwestern Argentina to revalue these relegated foods. For this purpose, the objective was to study the application of films elaborated with hydrocolloids and starches extracted from Andean potatoes var. Runa and to study their application in the deep frying of chips of the var. Waycha, in order to reduce oil absorption.

2. MATERIALS AND METHODS

2.1. Materials

Starch extracted from the Runa Andean potato variety (30% amylose content) (Calliope *et al.*, 2019), carboxymethyl-cellulose (CMC) (food grade, Rettenmaier & Sohne GMBH + Co), calcium chloride and ascorbic acid (food grade) were used for the formulation of coatings. The Andean variety Waycha was used for frying assays. Commercial potato chips were used as reference to calculate the relative reduction in oil absorption. Sunflower oil and an oil blend (soybean/sunflower, ratio: 94/6) were used for frying.

2.2. Formulation and properties of films

Starch Extraction. Healthy tubers of the Andean potato var. Runa were used to extract starch. They were cut into cubes of 2–3 cm, submerged in sodium bisulfite (1.2 $g \cdot L^{-1}$) and then crushed in an Omni Mixer. The obtained mixture was filtered; and the residue was washed repeatedly with a sodium bisulfite solution. The filtrate was centrifuged and the starch obtained was washed with alcohol, dried at 40 °C, and packaged until analysis (Calliope *et al.*, 2019).

Formulation of films. The concentrations of the components were: S (1%); S/CMC (1:0.5; 1.5%), and CMC (1%), which were dispersed in distilled water, heated (S) at 80 °C and (CMC) at 100 °C for 1 h and cooled to room temperature (25 °C). 13 g of each formulation were poured onto 10 cm diameter

polystyrene plates and allowed to dry for 6 h in a forced-flow oven at 35 °C and then maintained for 15 h at 53% relative humidity in room temperature.

Thickness. The film thickness was measured with an analog micrometer (Digimess, Argentina, sensibility 0.0001mm); the measurement was evaluated with the average of 5 different points of the film.

Water Vapour Permeability (WVP). The WVP was determined gravimetrically according to the standard method ASTM E96 (2000). The films were conditioned for 48 h in a desiccator at 25 °C and 53% relative humidity (RH) using a supersaturated solution of Mg $(NO_3)_2$. The measurements of WPV (quadruplicate) were performed according to Slavutsky and Bertuzzi (2015).

The water vapor transmission rate (WVTR) was calculated from equations 1 and the water vapor permeability (P) was calculated from equation 2

$$WVTR = \frac{G}{A} \quad \text{(Eq. 1)}$$

$$P = cte \cdot \frac{WVTR \cdot l}{P_{w0} - P_{w2}} \quad \text{(Eq. 2)}$$

Where: G: slope of linear regression; A: area of the exposed film; l: film thickness; P_{w0}: partial pressure of water vapor in the air on the surface of distilled water; P_{w2}: partial pressure of water vapor on the surface of the film outside the cup; cte: constant to satisfy unit conversion.

Solubility. Solubility was measured as a percentage of dry matter in the film solubilized in water for 24 h immersion. The samples, previously dried in an oven at 105 °C, were weighed (1 g) and placed in a beaker with 50 mL of distilled water at 30 °C, with constant stirring. The non-solubilized material was then separated by centrifugation (Sigma 4K10, Germany) and dried to determine the weight of the dry matter. The tests were performed in triplicate and the solubility was calculated as follows:

$$solubility = \frac{Initial\ dry\ weight - final\ dry\ weight}{Initial\ dry\ weight} x\ 100 \quad \text{(Eq. 3)}$$

Sorption isotherms. The films were cut into pieces of approximately 2 cm^2 and placed in a desiccator with P_2O_5 for 48 h. After that, they were placed in containers with controlled relative humidity using different supersaturated saline solutions (range of aw: 0.10 to 0.90) (Spiess and Wolf, 1983). The weight of the samples was recorded until the difference between the two consecutive weighings was less than 1 mg. Absorption tests were performed in triplicate at each aw. The data obtained were adjusted by the sorption model of BET (Equation 4).

$$W_e = \frac{W_0 \cdot C \cdot a_w}{(1 - a_w) \cdot (1+(C - 1)a_w)} \quad \text{(Eq. 4)}$$

Where W_e is the equilibrium moisture content (g water/100g dry film), W_0 is the moisture content in monolayer (g water/100g dry film) and C is adsorption constant of the first layer dependent on temperature. The quality of the fit was assessed through R^2.

Color. The color of the films was determined with a colorimeter (Colorquest XE Hunter Lab, USA) versus a standard film (L=94; a=−0.11 and b=3.2). All measurements were performed in triplicate. Total color difference (ΔE), was calculated according to Equation 5:

$$\Delta e = ((L_{standar}-L_{sample})^2 + (a_{standar}-a_{sample})^2 + (b_{standar}-b_{sample})^2)^{1/2} \quad \text{(Eq. 5)}$$

Contact angle. To evaluate the wettability of the oils to the different surfaces, the contact angles were measured using a goniometer (Standard Goniometer with DROP image model 200-00, Ramé-Hart Instrument Co., USA). The oil (10 µL) was dropped onto the surface of the film, using a micro-syringe. The contact angle was measured in 5 points on each film (Zdanowicz and Johansson, 2016). Each analysis was performed in sextuplicate at 25 °C.

2.3. Potato Chips

Analysis of the raw material. Reducing sugar content was determined according to the dinitrosalicylic (DNS) acid method (Miller, 1959). The DNS reagent contained 10 g/L 3,5-dinitrosalicylic acid, 16 g/L NaOH and 300 g/L sodium potassium tartrate (Rochelle salt). 3 mL DNS reagent and 1 mL supernatant sample were mixed in a test tube and heated in a boiling water bath for 5 min. Subsequently, they were placed in a cold water bath for 2 minutes, shaken and left to rest for 10 minutes. The reacted mixture was measured for absorbance at 540 nm in a spectropho-

tometer (Mapada, model UV 6300 PC). Glucose p.a. (Merck) was used for the standard curve.

Blanching treatments. The potatoes were washed and cut without peeling in the form of chips (2.5 mm thick); then three scalding processes were applied: 1) water boiling for 5 min, 2) aqueous solution of calcium chloride (0.5%), and 3) ascorbic acid solution (1%), with the same temperature/time conditions.

Coating application. After blanching, the potatoes were drained on absorbent paper and immediately immersed in the solutions described in section 2.2.2., at 25 °C for 2 min, then the potatoes were drained and the surface moisture was removed in a convection oven at 40 °C for 20 min. An uncoated sample was used as control.

Frying process. The potatoes (150 ± 5 g) were fried in 3 L of oil in a domestic fryer without reposition. The temperature/time conditions were 180 ± 10 °C/3 min. The fried chips were drained for 2 min in the fryer basket and stored for 24 h until analysis. The oil absorption of the chips with different blanching and coatings was studied in the first frying cycle. To determine the behavior of the oils concerning the tested coatings, 40 frying cycles were performed for each type of oil; 10 g of oil was taken in cycles 1, 20, 40, and stored at -20 °C until analysis.

Chip evaluation. In the first frying cycle, the lipid content and moisture/solid matter were determined (AOAC, 2016).

To calculate the reduction in oil absorption (% ROA, Equation 6), a commercial potato chip was taken as a reference.

$$\%ROA = \left(\frac{\%LChA \times 100}{\%LChC}\right) \quad \text{(Eq. 6)}$$

% LChA: percentage of lipids of Andean potato chips, scalded with coatings
% LChC: percentage of lipids of commercial potato chips, label value: 30.4 g/100 g potato.

Color. It was measured by a Colorimeter (Colorquest XE Hunter Lab, USA). The average of 5 readings was calculated. A chip without frying was taken as reference. The measure of color change was evaluated according to Equation 5. Where L*a*b*standard were the values for fresh potatoes and L*a*b*sample were the values for fried chips.

Sensory evaluation. Chip samples from the first frying cycle in the two types of oil were used to carry out the sensory analysis with 48 untrained consumers (Sullivan, 2017). Four different samples were put on a colorless plate and arranged according to the master sheet. The consumers evaluated sensory attributes of color, odor, flavor, acceptability and, texture. The consumers assessed the samples using a 5- point hedonic scale where 5-very pleasant, 4-pleasant, 3-neither like nor dislike, 2-unpleasant, 1-very unpleasant. Four-digit random numbers were used to identify each sample.

2.4. Analysis of fresh and used oils

Fatty acid composition. Fatty acid methyl esters (FAMEs) were prepared according to IUPAC (1987). The FA were quantified in a gas chromatograph model 2014 (Shimadzu, Japan) equipped with column SP 2560 (100 mm x 0.25 mm). A mixture of FAME (Supelco FAME Mix C4-C24 18919) was employed as standard.

Calculated oxidizability (Cox). The Cox value of the oils was calculated by the percentage of unsaturated C18 fatty acids, applying Equation 7, as proposed by Rossi *et al.* (2013):

$$\text{Cox} = [1(\text{oleic acid \%}) + 10.3(\text{linoleic acid \%}) + 21.6\ (\text{linolenic acid \%})]/100 \quad \text{(Eq. 7)}$$

Tocopherols. They were determined by the AOCS Method Ce8-89. A chromatograph (Shimadzu model 20, Japan) was used, with a fluorescence detector, a Phenomenex C18 silica column (250 × 4.6 mm, 5.0 μm); the mobile phase was acetonitrile, methanol, water with phosphoric acid and isopropanol (the flow rate was kept constant at 1.0 mL/min). Tocopherol isomers (α-, β-γ-, δ-) were identified using standards (Sigma Aldrich). Isopropanol (1 mL) was added to the oil sample (30 mL) and then injected into the HPLC equipment.

Polar compounds. They were determined by adsorption chromatography. Stationary phase Silica gel (Merck) particle size 0.063-0.200 mm was used as the mobile phase for non-polar compounds ethyl ether/petroleum ether 10:90 v/v, and diethyl ether for the polar fraction. Polar compounds (PC) were quantified, such as the difference between the initial mass of oil and the eluted non-polar fraction (IUPAC, 1987).

2.5. Statistical analysis

The means were analyzed by analysis of Variance. Differences among samples were analyzed according to Tukey's test. Differences among treatments were considered significant at ($p < 0.05$). To determine the influence of the scalding solution, coating addition, and oil absorption during frying, the 3-way interaction method was applied. Software Infostat 2017 and Graph pad prism version 5.01 were used.

3. RESULTS AND DISCUSSION

3.1. Films

The average results of the measured parameters are shown in Table 1. The water vapor permeability (WVP) of the edible films should be as low as possible in order to control the transfer of moisture between the food and the surrounding atmosphere. S/CMC films showed a significantly lower WVP value than CMC films, which would be related to the thickness of the film and the intrinsic characteristic of each material (Basiak *et al.*, 2017). The permeation phenomenon depends on three stages: adsorption, diffusion, and desorption. The diffusion stage depends on the thickness of the film and the tortuous path which hinder the passage of water molecules through the film matrix, while the other two are independent of it. Starch has a semi-crystalline structure; the amylose is capable of forming a tortuous path that decreases the diffusion of water through the film. The different behavior of starch and CMC films may be due to this. No significant difference was observed between S/CMC and S films. This indicated that in the composite films, permeability was related to the presence of starch. Almasi *et al.* (2010) postulated that starch forms hydrogen bonds with the hydroxyl groups of the CMC, and this strong structure could reduce the diffusion of water into the material. Therefore, the addition of CMC would improve the water-resistance of the starch matrix. The water solubility of the S films was high and higher than that reported by Basiak *et al.* (2017). This would be related to the higher amylose content of the starch in the potato var. Runa used. The combination with CMC produced a decrease in solubility of approximately 50%. This behavior was also observed by Ghanbarzadeh *et al.* (2010).

Table 1. Parameters measured in the developed films

Parameters		S	CMC	S/CMC
Thickness (μm) 10^{-5}		6.42 ± 0.20^{c}	4.86 ± 0.07^{a}	5.63 ± 0.08^{b}
Barrier properties water vapor permeability (10^{-10} g· m^{-1}·s^{-1}·Pa^{-1})		2.12 ± 0.23^{ab}	2.73 ± 0.21^{b}	1.94 ± 0.29^{a}
Solubility in water (%)		26.24 ± 6.66^{b}	9.25 ± 2.23^{a}	13.06 ± 1.34^{a}
BET	w_0	3.09 ± 0.13^{b}	2.00 ± 0.10^{a}	3.23 ± 0.15^{b}
	c	15.87 ± 2.58^{b}	5.01 ± 4.50^{a}	6.15 ± 5.36^{a}
	R^2	0.9292	0.9388	0.9240
Color	L	92.98 ± 0.09^{a}	93.32 ± 0.06^{b}	93.24 ± 0.17^{b}
	a	-0.33 ± 0.03^{c}	-0.57 ± 0.02^{a}	-0.52 ± 0.02^{b}
	b	3.60 ± 0.19^{a}	3.90 ± 0.27^{a}	3.47 ± 0.87^{a}
	AE	1.11 ± 0.15^{b}	1.08 ± 0.22^{ab}	0.91 ± 0.15^{a}
Surface properties	θ (OB)	15.80 ± 1.20^{a}	18.90 ± 3.90^{ab}	20.60 ± 3.90^{b}
	θ (SO)	18.80 ± 4.10^{a}	16.80 ± 1.80^{a}	19.10 ± 3.10^{a}

S: starch; CMC: carboxymethyl-cellulose; S/CMC: combination starch with carboxymethyl-cellulose.
w0: monolayer moisture content, c: constant related to heat sorption for monolayer, R^2: coefficient of determination. θ (OB): contact angle of oil blend; θ (SO): contact angle of sunflower oil; color parameters (L, a, b). Values are the average ± SD of triplicate samples.
Values having the same letter for a parameter within the same row are not significantly different at p level > 0.05 according to the Tukey test.

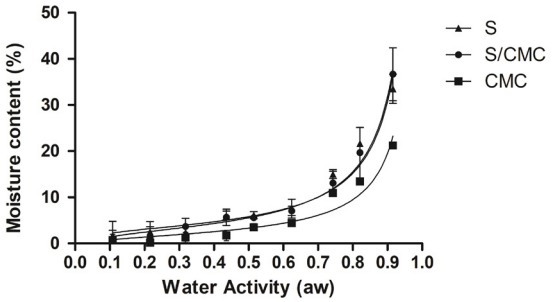

Figure 1. Moisture Absorption Isotherms Of Films. Coating: Starch (S); Carboxymethyl-cellulose (CMC); combination starch with carboxymethyl-cellulose (S/CMC). Continuous line: BET model.

Figure 1 shows the absorption isotherms obtained at 25 °C for the three films formulated. The curves were typical of polymers with affinity for water. The curves showed a slight relative slope at low a_w values, while they were exponential at aw values greater than 0.60. Other authors reported similar behavior in starch-based films (Slavutsky and Bertuzzi, 2015). The experimental data indicated that the CMC film had the lowest water absorption, while the S/CMC film showed similar behavior to the S film. The adjustment parameters obtained with the BET model (Equation 4) of each film showed that the constant c influenced the sigmoidal shape of the isotherms, particularly in the low range of a_w. The values for c would indicate that the moisture absorption of the matrix studied could occur more easily in the upper layers than in the monolayer. These results showed that the stage that controlled the water permeability of the S films was diffusion and not the adsorption/desorption of water. Instead, the stage that controlled the water permeability phenomena for CMC films depended on the absorption/desorption phenomena. This was probably due to the lower water sorption capacity of these films. However, S/CMC films presented a similar value to the S films for the water permeability and sorption isotherm, but the solubility in water was similar to CMC films. This was probably due to the influence of the semi-crystalline structure of starch and the interaction through hydrogen bonds between both polymers.

Starch films showed greater opalescence, which could be explained by their greater thickness, which was probably due to the higher amylose content (Basiak *et al.*, 2017). Regarding color, parameter L showed significant differences which were lower for the S film. In parameter a, all the films had a greenish hue, which was higher for the CMC, and in b the film, hue was yellow with no significant differences between them. In general, the optical values for the films presented good transparency. Ghanbarzadeh *et al.* (2010) observed in a study conducted with S/CMC composite films that the CMC aggregate produced clearer films. These changes can be further described with the ΔE function, which showed a significant decrease ($p < 0.05$) when CMC was added.

The contact angle was used as an indicator of the degree of interaction between the oils and the surface of the films. The three films had surfaces with moisturizing properties which confirmed their hydrophilicity. An increase in the contact angle between the oil and the film indicates a lower affinity between both materials. The highest value (20.6°) was obtained for S/CMC films in the oil blend. This is accordance with the frying experiments (Table 2 and Figure 2), in which the potato chips with the lowest oil content were those covered by S/CMC. The contact angles of the CMC and S films were smaller, indicating surfaces with greater affinity to oils.

3.2. Chips

Colman *et al.* (2009) reported that the minimum values for the dry matter (DM) and reducing sugar (RS) content for a potato to be suitable for frying are 25 and 0.2%, respectively. The Andean potato var. Waycha, contains 26.38 ± 0.31 DM and 0.18 ± 0.06 RS. Therefore, the Waycha potato variety meets both conditions. It is also larger than other varieties, which would indicate that it could be suitable for use in the production of fried potato chips. The DM content decreased with all the bleaching treatments. Significant differences in DM content were found among chips scalded with water (observed decrease from 26.4 to 23.8%), with respect to treatments with ascorbic acid (22.6%) and calcium chloride (22.1%). This could be due to different migrations of soluble potato compounds to the bleaching medium. The oil content was significantly affected ($p > 0.05$) by the kind of scalding and the type of coating used (Table 2). The oils employed did not significantly influence absorption. Figures 2a and 2b, show the percentages of absorbed oil reduction according to the treatments applied, taking as reference a commercial potato chip (Label value 30.4 g oil/100g). Control samples scalded in water and without coating showed

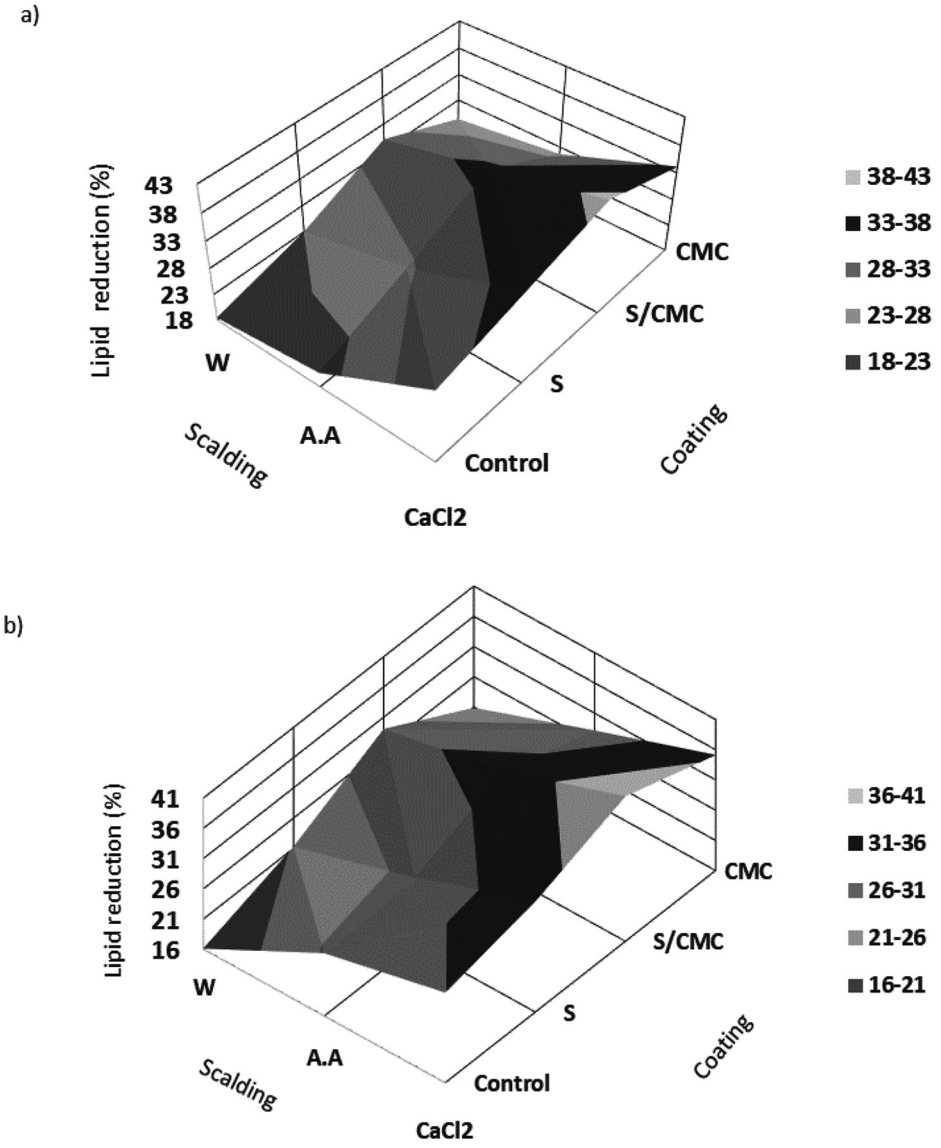

Figure 2. Reduction in fat content in Andean potato chips vs. commercial chips, according to treatments applied. a) Frying oil: sunflower, b) Frying oil: blend (Sunflower/soybean).
Coating: Starch (S); Carboxymethyl-cellulose (CMC); combination starch with carboxymethyl-cellulose (S/CMC). Scalding: Water (W), Ascorbic acid (A.A); Calcium chloride (CaCl2).

a slight reduction in oil content (16.1-18.5%), compared to those scalded in calcium chloride with S/CMC coating (39.1–40.1%). These results indicated that calcium chloride stabilized the structure of the tissue during the frying process. The texture of the potato depends on the presence of pectin substances, which are part of the intercellular material. Pectinolytic enzymes produce free carboxylic groups, which can react with divalent ions such as calcium and magnesium, creating more rigid structures and increasing firmness. The formation of these calcium/pectin complexes causes the reaffirmation of the cell wall and increase the stiffness of the medium of the laminar cell wall (Hernandez *et al.*, 2014), and therefore its structure better resists the frying process. Table 2 and Figures 2a and 2b show that the coatings influenced the decrease in oil absorption. The results showed significant differences ($p > 0.05$) among treatments. The one with the greatest effect was the coating of S/CMC (39.5% ± 0.7) combined with scalding in calcium chloride; while the one with the least effect was the control without coating and

Table 2. Oil content in Waycha potato chips (g/100 g) with different treatments

Treatment	Sunflower oil				Oil Blend				Mean (o*b)
Blanching	Coating				Coating				
	Control	S	S/CMC	CMC	Control	S	S/CMC	CMC	
Water	24.79±0.18^{l}	23.40±0.26^{ij}	21.25±0.09^{def}	23.05±0.09^{ij}	25.50±0.13^{m}	23.93±0.09^{ijk}	21.57±0.10^{efg}	24.06±0.24^{ijk}	23.44±1.47^{b}
AA (1%)	24.08±0.21^{k}	22.07±0.08^{gh}	19.95±0.16^{b}	22.30±0.15^{h}	22.37±0.06^{h}	22.12±0.04^{gh}	20.33±0.17^{bc}	21.85±0.11^{fgh}	21.88±1.28^{b}
CaCl2 (0.5%)	20.83±0.16^{cd}	19.68±0.11^{b}	18.52±0.13^{a}	20.03±0.08^{b}	20.99±0.45^{cde}	19.97±0.65^{b}	18.24±0.07^{a}	19.72±0.17^{b}	19.75±0.97^{a}
Mean (o*f)	23.23±1.80^{c}	21.72±1.61^{b}	19.91±1.17^{a}	21.79±1.34^{b}	22.95±1.98^{c}	22.01±1.72^{b}	20.05±1.44^{a}	21.88±1.85^{b}	

References: Control: Uncoated, AA: ascorbic acid. Mean (o*b): interaction oil*blanching; Mean (o*f): interaction oil*film. Letters inside box are the interaction of (o*b*f). Each value in the table is the mean of three replicates. Means with a common letter are not significantly different ($p > 0.05$) according to the Tukey test.

scalding in water (17.3% ± 1.7). This reduction in oil absorption could be attributed to the fact that the starch undergoes structural changes in which the crystals of amylose and amylopectin are reorganized and promote the formation of a gel that functions as a barrier to the entrance of oil (Hasbún *et al.*, 2009). Varela and Fiszman (2011) and Freitas *et al.* (2009) postulated that CMC increases water retention capacity and, consequently, prevents the replacement of water with oil. In addition, since the polymer is hydrophilic, it forms a thin layer on the surface of the food which acts as a barrier to the incorporation of oil. Ali *et al.* (2012) observed that CMC increased surface tension, which facilitated the draining of surface oil. Likewise, calcium chloride is a cross-linking agent which forms a fine network which prevents the migration of oil to the potato during the frying process (Hasbún *et al.*, 2009).

The results showed that the type of oil had no significant influence on absorption ($p > 0.05$).

Table 3 shows color changes during 40 frying cycles. In the parameters L* a* and b* within the frying cycles with coatings there were significant differences in the L values. It was observed that chips with coating darkened as the cycles continued. For parameter a, the potatoes with coatings in both oils showed significant differences between the first and the last frying, with an increase in redness in the last cycles, possibly due to the effect of the coating. While in parameter b, there was greater variation, with the exception of chips without coating fried in the oil blend. In the other treatments, the intensity of the yellow color varied, and a defined pattern was not found. In all cases, the yellow/gold color, typical of fried products, was characteristic. The parameter ΔE was used to evaluate the color change between the different processes tested. The mean values for color L*a*b (Table 3) show that the potatoes with coating, fried in sunflower oil, presented statistical differences with respect to those without coating. It is also observed that the highest ΔE corresponded to the treatments with edible coating, due to the parameters *b followed by *a. This could be due to the presence of the coating and the type of oil. However, the sensory attributes of acceptability and texture for fried coated potatoes in sunflower oil presented a favorable statistical difference compared to those without coating in the same type of oil. The re-

Table 3. Color parameters and preference scores for sensory attributes of Waycha coated potato chips with S/CMC and scalding with calcium chloride. Tocopherol content in fresh and used oils

Properties	Variable	SO		OB	
		without coating	with coating	without coating	with coating
*Chips Color L*a*b*	L(St)	80.37±0.23	80.37±0.23	80.37±0.23	80.37±0.23
	L(1)´	78.85±1.28^{a}	78.82±1.24^{a}	77.49±0.88^{a}	77.42±1.01^{a}
	L(20)´	74.18±1.50^{a}	75.05±0.49^{ab}	78.52±3.52^{a}	72.48±2.78^{ab}
	L(40)´	70.63±4.72^{a}	69.44±2.07^{c}	73.25±2.39^{a}	63.07±2.59^{b}
	a(St)	1.04±0.15	1.04±0.15	1.04±0.15	1.04±0.15
	a(1)´	1.46±1.12^{a}	1.72±0.58^{b}	4.17±0.61^{a}	4.87±0.78^{b}
	a(20)´	5.75±2.13^{a}	2.27±0.40^{b}	3.92±3.43^{a}	4.96±1.77^{b}
	a(40)´	5.79±4.52^{a}	6.23±0.14^{a}	5.71±0.82^{a}	14.65±1.39^{a}
	b(St)	31.39±0.53	31.39±0.53	31.39±0.53	31.39±0.53
	b(1)´	38.37±3.29^{ab}	41.29±0.77^{a}	36.93±1.14^{a}	37.18±0.63^{bc}
	b(20)´	42.97±2.75^{a}	32.42±1.80^{b}	40.58±2.27^{a}	40.75±1.37^{ab}
	b(40)´	38.10±5.60^{ab}	41.48±1.14^{a}	41.08±2.51^{a}	41.56±1.19^{a}
	ΔE(1)´	7.15	10.04	6.99	7.54
	ΔE(20)´	13.95	5.56	9.81	12.85
	ΔE(40)´	12.74	15.13	12.9	24.25
**Sensory attributes of fried chips 1º cycle with film and blanching (calcium chloride)	Color	4.0±0.4^{a}	4.2±0.4^{a}	3.8±0.8^{a}	4.0±0.6^{a}
	Odor	3.5±0.5^{ab}	4.0±0.4^{b}	3.4±0.7^{ab}	3.3±0.8^{a}
	Flavor	3.7±0.9^{b}	4.3±0.5^{b}	2.7±0.7^{a}	3.6±0.9^{b}
	Acceptability	3.8±0.8^{a}	4.7±0.5^{b}	3.5±0.9^{a}	4.0±1.0^{ab}
	Texture	2.9±0.9^{a}	4.4±0.9^{c}	3.0±0.9^{ab}	3.9±0.8^{bc}
	Mean	3.6±0.4^{a}	4.3±0.3^{b}	3.3±0.4^{a}	3.8±0.3^{ab}
***Tocopherol Content in oils (ppm)	α (fresh)	650.5±1.0^{c}		88.0±1.4^{a}	
	F40	530.5±1.0^{a}	548.9±2.6^{b}	91.5±1.2^{ab}	97.5±0.7^{b}
	β–ϒ (fresh)	121,5±2.5^{c}		2059.2±2.7^{c}	
	F40	106.8±0.9^{a}	114.6±1.1^{b}	1688.4±2.4^{a}	1899.1±0.9^{b}
	δ- (fresh)	22.2±0.3^{a}		647.8±1.6^{b}	
	F40	21.9±1.1^{a}	25.6±1.2^{a}	621.6±1.1^{a}	766.6±0.4^{c}
	Total (fresh)	794.1±1.1^{c}		2795.0±5.7^{c}	
	Total F40	659.1±1.3^{a}	688.2±2.6^{b}	2401.5±0.1^{a}	2763.2±1.1^{b}

*Standard (St): raw potato; SO: Sunflower oil; OB: Oil blend. Number of frying cycles; coating combined starch/ carboxymethyl-cellulose: S/CMC; the number in brackets is the number of frying cycles. * Different letters (a, b, c) indicate significant differences among results in columns ($p < 0.05$). **The ratings are based on a 5-point hedonic scale where 1=very unpleasant, 5=very pleasant; Means with one common letter per row are not significantly different ($p > 0.05$). *** Comparison of means between fresh potatoes and frying cycle 40 (F40) with and without coatings. Each observation is a mean of 10 replicate experiments. Different letters for fraction (α- βϫ- δ) indicate significant differences ($p < 0.05$).

sults of this study show that the coating with S/CMC did not affect the color and provided a better "crunchy" texture to the fries in the two types of oils used; while the attributes described for the product without coating were "bitter taste, burnt and oily". This indicates that the coating contributed to eliminating these perceptions. García *et al*. (2002) reported that CMC edible coatings affected the color of potato chip samples, but did not change the characteristic texture.

The coating not only impacted the reduction in the oil content but also slightly improved the sensory at-

tributes of the fries. During frying, the oil is exposed to high temperatures in the presence of air and humidity, which generates oxidation, hydrolysis and polymerization reactions (Rimac-Brnčić *et al.*, 2004). This is why the oil usage times are reflected in the color and changes in its composition (Navas *et al.*, 2007).

3.3. Analysis of fresh and used oils

Figure 3 shows the fatty acid fractions: saturated (SFA), monounsaturated (MFA), polyunsaturated (PFA), and Trans (TFA) of fresh oils and after being used in 40 frying cycles. The SO had a lower content of polyunsaturated fatty acids (5.6%) than the OB (7.03); so, its calculated oxidability was different as well. There was an increase in the SFA content in OB with increasing frying cycles, and in TFA when potato chips were uncoated. In the SO the MFA fraction increased and TFA were generated when uncoated chips were fried. It is known that TFA has been related to temperature and times the same oil is used. In this study, the temperature was maintained at 180 °C, but the times of frying changed, which explains the increase of TFA in cycle frying 40 in both cases. Also, the increase in TFA has been related to the kind of food and the fatty acid composition of the oil. The food varied due to the presence of the coating, which favored a lower increase of TFA, apparently acting as a protective agent against it. The higher amount of TFA observed in the oil blend was probably due to its composition related to less oxidative stability compared to sunflower oil. Cis to Trans isomerization begins when the frying temperature is higher than 150 °C (Bhardwaj *et al.*, 2016). However, the TFA content in the oils with 40 frying cycles was less than the maximum established (5%) by Argentine legislation.

Tocopherols are natural components of oils with a protective effect against oil oxidation. During the processing, storage, and use of oils there are partial losses of these components. Table 3 shows the content of the different types of tocopherols in fresh SO and OB and after being used in 40 frying cycles. The tocopherol content was significantly higher in the OB than in SO, both fresh. When both were used in 40 frying cycles, the total tocopherol content decreased significantly, which was less noticeable when covered chips were fried. Rossi *et al.* (2017) reported that frying potato chips in eight different types of vegetable oils, including pure and blended sunflower oil, tocopherols decreased rapidly after the third hour of

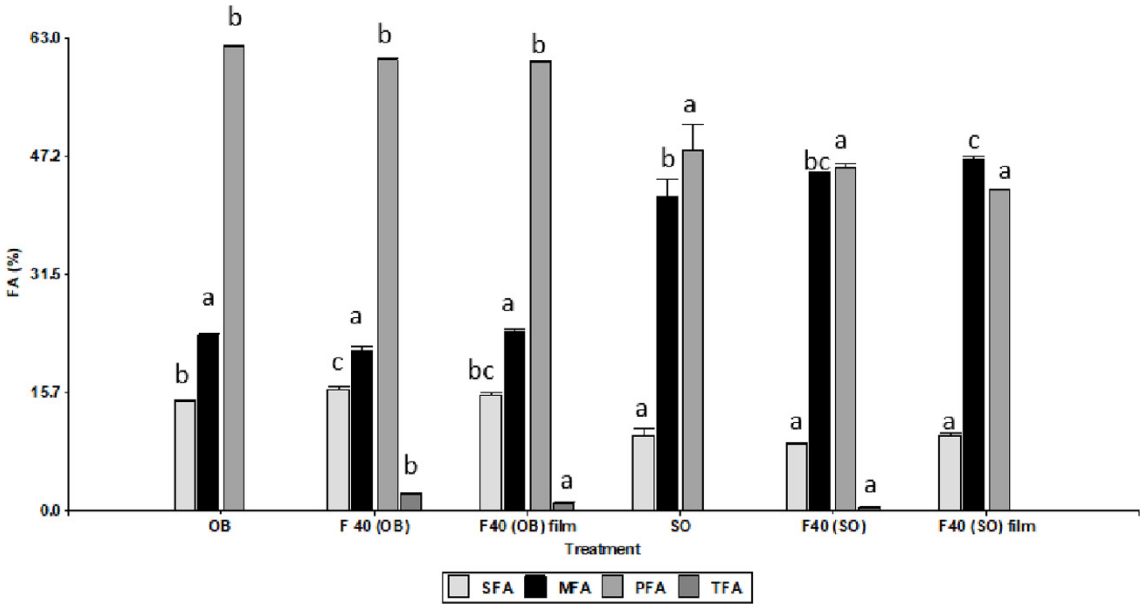

Figure 3. Fatty acid fraction of fresh oils and those used for frying

OB: oil blend (Sunflower/soybean); F40 (OB): Frying cycle 40 in oil blend; F40 (OB) film: Frying cycle 40 in oil blend chips with film; SO: sunflower oil; F40 (SO): Frying cycle 40 in sunflower oil; F40 (SO) film: frying cycle 40 in sunflower oil with film. Fatty acids: saturated (SFA), monounsaturated (MFA), polyunsaturated (PFA), and trans (TFA). Different letters indicate significant differences by the Tukey test ($p < 0.05$) among the same fatty acid fraction with different treatments.

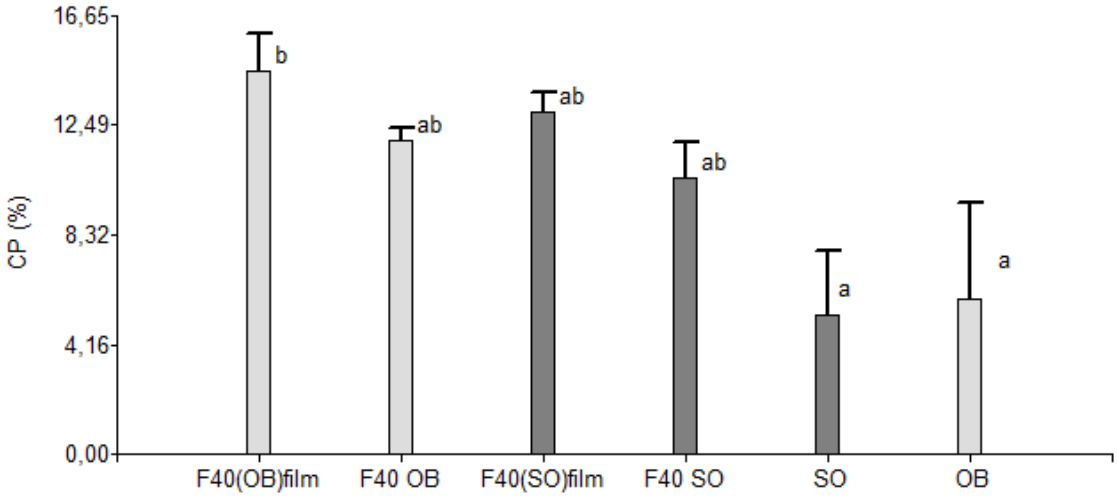

FIGURE 4. Polar compounds in fresh oils (OB and SO) and used in 40 frying cycles (F40) of potatoes with and without coating

Each observation is an average of 6 replicates. The same letter for the same parameter is not significantly different at $p > 0.05$ according to the Tukey test.

continuous use of the oils. In this study, the frying cycles exceeded 3 hours of oil use. The mechanism of the reaction to eliminate radicals of tocopherols requires that they lose their mobile hydrogen atom in the hydroxyl group, forming more stable free radicals than fatty acids. It follows that the rapid oxidation of tocopherols corresponds to greater antioxidant power. However, if all other fractions are taken into account, it could be assumed that, in the case of oils containing higher levels of PFA, the double bond that determines unsaturation competes with tocopherols as substrates for oxidation, resulting in a less rapid decrease in these antioxidants. In contrast, in the case of low polyunsaturated oils, tocopherols would constitute the substrates that react more easily with oxygen. In fact, it was reported in the literature that in the propagation phase of the reaction, peroxy fatty acid-free radicals preferentially react with the phenolic hydrogen of the tocopherol molecule (Rossi *et al.*, 2017). The initial concentrations of PCs in the fresh oils were within the reported values (Ramírez Botero *et al.*, 2012). After 40 frying cycles (Fig. 4), the PC content increased, but there were no statistically significant differences due to the application of the coating. In this study, it can be inferred that the changes in the increase in polar compounds are related to the amount of frying cycles carried out. In addition, the fatty acid profile of the oils is an important factor which contributes to the generation of polar compounds, consequently, the oil blend, with a higher degree of unsaturation, presents more polar compounds than sunflower oil. It was also possible to observe that sunflower oil was more resistant to oxidative deterioration during frying, probably due to containing more monounsaturated fatty acids (Jadhav *et al.*, 2022). These results indicated that under the conditions used in this study, CP content was not generated in concentrations higher than the limits established by countries which have their content legislated. For example, Spain, France, Italy, and Chile accept a maximum value of 25% of polar compound content, while Germany accepts 24% and Austria and Switzerland up to 27% (Suaterna Hurtado, 2009).

CONCLUSIONS

The Waycha variety is suitable for producing potato chips because the contents of dry matter and reducing sugars meet the conditions established for that purpose.

The coating formulated with starch extracted from the Runa variety combined with CMC and applied to chips scalded in calcium chloride contributed to decreasing oil absorption by 39.5% during the frying process. This formulation did not increase the formation of polar compounds. In addition, it contributed to a reduction in the loss of tocopherols during the frying process and improved the physical and sensory characteristics of the final product, which had high acceptability by consumers.

These results confirm two technological applications for Andean potatoes which can be used to contribute to a healthy diet. In addition, both materials used in the preparation of edible coatings are ecological and respectful of the environment since they will contribute to the recycling of waste from food and other industries.

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Effects of cold and hot smoking processes and the addition of natural *Dunaliella salina* polyphenol extract on the biochemical quality and shelf life of *Sander lucioperca* fillets after storage for 90 days

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SUMMARY: The effects of cold and hot smoking and the addition of *Dunaliella salina* polyphenol extract on the biochemical quality and shelf-life of *Sander lucioperca* fillets after storage for 90 days at 0–4 °C were examined. The results showed a significant increase in protein, lipid, free fatty acid, and 1,1-diphenyl-2-picrylhydrazyl contents, and a decrease in peroxide and thiobarbituric acid reactive substances, and volatile base nitrogen levels in cold (CSF) and hot (HSF) smoked fillets covered with or without extract and stored for 1, 20, and 90 days compared to fresh fillets (FF). Saturated and monounsaturated fatty acids exhibited a significant increase in FF and CSF and HSF covered with or without extract. The total polyunsaturated fatty acids revealed a significant decrease in FF and CSF and HSF with or without extract. Therefore, cold and hot smoking and polyphenol extract improved the biochemical quality and storage shelf-life of fillets for 90 days at 0–4 °C.

KEYWORDS: *Antioxidants; Cold and hot smoking; Dunaliella salina microalgae; Fatty acids; Freshwater fish; Polyphenols*

RESUMEN: ***Efecto de procesos de ahumado frío y caliente y la adición de extracto polifenólico natural de* Dunaliella salina *sobre la calidad bioquímica y la vida útil de filetes de* Sander lucioperca *almacenados durante 90 días.*** Se examinaron los efectos del ahumado en frío y en caliente y la adición de extracto de polifenoles de *Dunaliella salina* sobre la calidad bioquímica y la vida útil de filetes de *Sander lucioperca* almacenados durante 90 días a 0–4 °C. Los resultados mostraron un aumento significativo en los contenidos de proteínas, lípidos, ácidos grasos libres y 1,1-difenil-2-picrilhidrazilo, y una disminución en las sustancias reactivas de peróxido y ácido tiobarbitúrico, y los niveles de nitrógeno básico volátil en frío (LCR) y caliente (HSF) de filetes ahumados cubiertos con o sin extracto y almacenados durante 1, 20 y 90 días en comparación con los filetes frescos (FF). Los ácidos grasos saturados y monoinsaturados exhibieron un aumento significativo en FF y LCR y HSF cubiertos con o sin extracto. Los ácidos grasos poliinsaturados totales revelaron una disminución significativa en FF y CSF y HSF con o sin extracto. Por lo tanto, el ahumado en frío y en caliente y el extracto de polifenoles mejoraron la calidad bioquímica y la vida útil durante el almacenamiento de los filetes durante 90 días a 0–4 °C.

PALABRAS CLAVE: *Ácidos grasos; Ahumado en frío y en caliente; Antioxidantes; Microalga Dunaliella salina; Pescado de agua dulce; Polifenoles*

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1. INTRODUCTION

Fish is a highly biodegradable food due to its susceptibility to oxidation, which assists in the growth of pathogenic microorganisms (Chaillou *et al*., 2015) and eventually leads to the formation of off-odor and flavor, and finally to rot (De Souza-Franco *et al*., 2010). Therefore, it must be precisely handled and preserved to retard its spoilage and to assure microbial safety and a marketable shelf-life (Amaral *et al*., 2021). Indeed, some chemical quality indices have so far been developed to assess the level and extension of fish spoilage, such as the total volatile basic-nitrogen (TVB-N) and trimethylamine-nitrogen (TMA-N), thiobarbituric acid (TBA) value, and the presence of biogenic amines (histamine, cadaverine, tyramine, and putrescine) produced by the decarboxylation of specific free amino acids by the action of microorganisms (Silbande *et al*., 2018). TVB-N implies the measurement of volatile basic nitrogenous compounds, such as trimethylamine (TMA), dimethylamine (DMA), and ammonia (NH_3), which are produced by bacteria, from the action of enzymes or the deamination of amino acids (Kostaki *et al*., 2009). The proposed value of TVB-N for spoilage initiation is 30–35 mg N/100 g; however, some studies present lower levels depending on the fish species (Kostaki *et al*., 2009). TMA-N is the main constituent of non-protein nitrogen fraction, produced by the bacterial spoilage, enzymatic activity, and decomposition of TMA-N-oxide, and is responsible for the fishy odor. The upper limit of TMA-N values considered for spoilage acceptance is 10–15 mg TMA-N/100 g, but lower limits are also suggested by other authors (Kostaki *et al*., 2009). Regarding lipid oxidation, the TBA value is used to measure the malondialdehyde (MDA) content. The quality values range between 2–4 mg MDA/kg, but this value might not reflect the actual rate of lipid oxidation because MDA can interact with other components (Kostaki *et al*., 2009). In addition, several methods, including vacuum packaging, modified atmosphere packaging, active packaging, and chemical additives, such as organic acids and natural extracts, combined with freezing systems, have been applied to impede its decomposition (Amaral *et al*., 2021).

Extensive fish farming, as in the case of *Sander lucioperca* (pikeperch or zander), offers the opportunity for fishermen to cost-effectively harvest fish. Although the nutritional quality of *S. lucioperca* is high because it is rich in polyunsaturated fatty acids (PUFA), vitamins, and minerals (Bouriga *et al*., 2020), this species is not overly valued by consumers, mainly due to its undesirable taste and flavor compared to marine fish. The smoking process can offer such a marketing alternative to freshwater fish and result in high-quality and acceptable products (Bouriga *et al*., 2012). As far as known, smoking treatments (hot, cold, liquid, and electrostatic) have been documented as useful processes for preserving the quality of seafood and can be achieved through both traditional and innovative techniques (Karsli and Çağlak, 2021). Overall, some constituents of smoking substances are aldehydes, ketones, alcohols, acids, hydrocarbons, esters, phenols, and ethers. These substances are applied to the surface of the wires and then penetrate the muscle, giving the products their final color and taste. However, some reports have shown that smoking processes have a negative impact on the nutritional value of fish fillets (Bouriga *et al*., 2012). Thus, the addition of antioxidants can be a useful technique for preserving the quality of fillets.

Given that consumers are becoming more health conscious, there has been a strong demand for the use of functional foods and the addition of natural ingredients. For this reason, several authors have focused on microalgae as potential sources of compounds with functional, nutritional, antimicrobial, and antioxidant properties (Cakmak *et al*., 2014). Among the compounds that can be obtained from microalgae are antioxidants, which have been widely used as food conserves in the food industry (Madhavi *et al*., 1996**)**. In addition, natural antioxidants, such as polyphenols, are now more extensively used due to their physiological benefits to human health. In this context, herbs have been the most valuable antioxidants used to protect smoked fillets, especially green algae of the genus *Dunaliella*. Of this genus, *D. salina* is a green, halophilic microalga commonly found in sea salt fields. This microalga is famous for its high commercial, economic, and industrial value due to its persistent capacity to produce large amounts of polyphenols and carotenoids, especially β-carotene, which has been widely used as an important natural antimicrobial and antioxidant for nutrient preservation in food, feed, and the pharmaceutical industry due to its high physiological properties, as well as biodiesel because of its high unsaturated fatty acid content (Cakmak *et al*., 2014). In addition, polyphenols and β-carotene function as scavenger compounds to protect the fillets from the generation of free radicals (Burton and Ingold, 1984).

Despite the immense number of works on smoking processes and their potential effects on fish fillets, the use of *Dunaliella salina* as a natural antioxidant has not been well explored. Hence, the current study was conducted to examine the effect of both cold and hot smoking processes and the addition of two graded concentrations (0.5 and 1% v/w) of natural *Dunaliella salina* polyphenol (pp) antioxidant extract on the biochemical quality and shelf-life and consumption of *Sander lucioperca* fillets during storage for 1, 20, and 90 days, respectively, in a refrigerator at 0–4 °C.

2. MATERIAL AND METHODS

2.1. Polyphenol antioxidant extract

Dunaliella salina samples were collected in May 2019 from Chott El Djerid, an endorheic salt lake, situated in southern Tunisia (33°54′42.21″N, 8°31′7.98″E). The antioxidant extract was prepared following the method described by Messina *et al*. (2015), in which 10 g of dried and pulverized microalgae were extracted with 100 mL of distilled water and then incubated in a shaker for 24 h in the dark. Afterwards, the mixture was filtered and lyophilized. The final solution was prepared by dissolving 10 g of the freeze-dried extract in 1000 mL of distilled water (10 $g \cdot L^{-1}$ of distilled water), with a polyphenol content equal to 500 mg of gallic acid equivalents (GAE)/L (Messina *et al*., 2015).

2.2. Sampling and smoking procedure of *Sander lucioperca*

S. lucioperca samples were collected in May 2019 during 10 fishing operations from Sidi El Barrak Reservoir (Beja Governorate, northwest Tunisia, 37°01'N, 09°39'E). A total of 45 fish samples, ranging from 12.5 to 28.6 cm (mean 24.3 ± 3.2 cm) in total length and 3400 to 1900 g (mean 700 ± 2.20 g) in total weight, were collected using a 30 mm mesh trammel net. The samples were preserved in ice and then transported to the laboratory where they were weighed, measured, de-capitated, and cleaned. These samples were longitudinally cut into fillets measuring 13.6 –18.2 cm (mean 15.8 ± 2.6 cm) in length, 3.2–4.4 cm (mean 3.1 ± 0.23 cm) in thickness, 7.4–9.8 cm (mean 6.78 ± 1.12 cm) in width, and 200.4–420 g (mean 264 ± 0.86 g) in weight. The fillets were divided into three groups (100 g each): the first consisted of fresh fillets without any additives, the second comprised fillets covered with a final concentration of 0.5%, i.e., 50 mL pp (v)/100 g fillets (w), of the polyphenol (pp) antioxidant *D. salina* extract using a micropipette, and the third included fillets that were treated with the polyphenol antioxidant extract as the second group, but with a final concentration of 1% pp, i.e., 100 mL pp (v)/100 g fillets (w), extract. Afterwards, the three groups were smoked in cold and hot conditions. The cold-smoked fillets were dried for 2 h in the smoking chamber at a temperature of 30–35 °C, while the hot-smoked fillets were introduced into the industrial smoking chamber using a peripheral smoke generator. The process of smoking the fillets was accomplished as follows: pre-drying the fish surface at 50–60 °C for 150 min, followed by hot smoking at 65–70 °C for 30 min, and finally steaming at a temperature of 68–72 °C. In both smoking processes, oak wood was used (Bouriga *et al*., 2020). Finally, the fillets were cooled in cold air at 10 °C. Subsequently, the smoked fillets were vacuum-sealed and stored in a refrigerator at 0–4 °C for 90 days. For the biochemical analyses, fresh and vacuum-sealed smoked fillet samples were gradually tested after 1, 6, 20, 50, and 90 days of storage at 0–4 °C. However, the results obtained from the storage periods 6, 20, 50, and 90 showed a similar significant gradual increase or decrease in each fillet type, as the case may be, so we reduced and summarized the data by presenting here only the results for three storage periods, namely 1, 20, and 90. All flesh samples were stored at -80 °C until analysis.

2.3. Biochemical analyses related to shelf-life

2.3.1. Protein determination

The total protein content of *S. lucioperca* fillets was determined by estimating their total nitrogen content using the Kjeldahl method 981.10 of the AOAC. Approximately 1 g of raw material was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing two copper catalyst tablets in a heat block (Kjeltec system 2020 digestor, Tecator Inc., Herndon, VA, USA) at 420 °C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization and titration. The amount of total nitrogen obtained was multiplied with both the traditional conversion factor of 6.25 and species-specific conversion factors (Mariotti *et al*., 2008) in order to determine total protein content. The protein content was expressed as a mean percentage (%) of the wet weight (ww) of three replicates.

2.3.2. Lipid determination

The total lipids were determined according to the protocol described by Bouriga *et al*. (2020). Briefly, 10 grams of fresh or smoked fillet samples were homogenized for 8-10 min at 4 °C in a mixture of chloroform: methanol (1:2) using a Polytron homogenizer (Malaysia). The homogenate was added to 5 mL NaCl saturated solution and 20 mL chloroform with Butylated hydroxytoluene (BHT; 50 ppm), and then homogenized for 7 and 5 min, respectively. Then, 20 mL of distilled water were added and the solution was homogenized again for 1 min. The obtained mixture was incubated in an ultrasound bath for 10 min, and a vacuum cleaner with Buchner funnel and chloroform. The organic fraction was extracted with a separating funnel, dried with sodium sulfate, and evaporated to dryness in the rotary evaporator (Stuart™, UK). The obtained oil was solubilized in a known volume of chloroform with BHT (50 ppm) and stored at -20 °C. The total lipids were expressed as a mean percentage (%) of the wet weight (ww) of six replicates.

2.3.3. Peroxide value (PV)

The peroxide value of the fillet samples was determined according to the IDF standard method, 74A: 1991(9), with the ferric thiocyanate method based on the ability of lipid peroxides to oxidize ferrous ions at a low pH. The resulting ferric ions were reacted to thiocyanate and the concentration of the complex formed was determined by spectrophotometry (Jenway 6315, UK) at 500 nm. The standard sample was determined by the reaction of a series of aliquots of a 10-µg/ml iron (III) chloride standard solution, 10 mM ammonium thiocyanate, and a sufficient amount of chloroform/methanol mixture (7:3). The results were expressed as mequivalent of oxygen per kg of lipid (meq O_2/kg) and the values were presented as a mean percentage (%) of three replicates.

2.3.4. Thiobarbituric acid reactive substances (TBARS)

The production of thiobarbituric acid reactive substances (TBARS) was determined based on the AOAC (1998) method. Oil samples were dissolved in 1-butanol, mixed with 0.2% TBA in 1-butanol, incubated in a water bath for 2 h at 95 °C, then cooled under tap water. The absorbance was determined using a spectrophotometer at 532 nm, and the standard curve was established by the TBARS reaction of a series of aliquots of 0.2 mM TMP (1,1,3,3-tetramethoxypropane) prepared in 1-butanol. The results were expressed in mg MDA (malondialdehyde)/kg of oil and values were presented as a mean percentage (%) of three replicates.

2.3.5. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant activity

To measure the antiradical activity, the synthetic radical DPPH (1,1-diphenyl-2-picrylhydrazyl) was used according to the method of Bersuder (Bersuder *et al*., 1998). Briefly, 10 mg of the fillet sample were suspended in 0.5 mL distilled water. Afterwards, 1.2 mL absolute ethanol and 0.2 mL DPPH solution (50 µM in ethanol) were mixed and incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm using a T70 UV-visible spectrophotometer. The results were expressed as a mean percentage (%) of three replicates of inhibition or trapping activity and were calculated by the following formula:

$$\text{DPPH trapping activity} = ((\text{control OD} - \text{sample OD})/\text{control OD}) \times 100.$$

2.3.6. Total fatty acid (FA) determination

Fatty acid methyl esters (FAME) were determined following the AOAC 963.15 methodology (AOAC, 1990), with slight modification. In brief, the analysis was done in a Varian Agilent 6890 N gas chromatograph (Agilent Technologies, Santa Clara, USA), equipped with an auto-sampler and fitted with a split/splitless injector and a flame ionization detector (FID). Separation was performed in an Innowax 30 × 0.25 capillary column (25 m × 0.25 mm i.d., film thickness) (Agilent Technologies, Santa Clara, USA). The temperature was programmed from 180 to 200 °C at 4 °C/min, held for 10 min at 200 °C, heated to 210 °C at 4° C/min, and held at 210 °C for 14.5 min using an injector and FID at 250 °C. The fatty acid contents in the total lipids of the samples were estimated using nonadecanoic acid methyl ester C19:0 Me (Sigma Chemical Co. Ltd), as an internal standard (10 mg/mL) based on the ratio of the peak area. The fatty acid sequences ranged according to their chromatographic retention times, and the values are given as a mean percentage (%) of total fatty acid methyl esters of six replicates.

2.3.7. Free fatty acids (FFA) determination

The samples (50 mg each) were homogenized with cyclohexane and copper acetate-pyridine reagent and stirred by vortex for 2 min, and then centrifuged at 9000 rpm for 20 min, and detected at 710 nm. The quantitative analysis of each fatty acid was performed for six replicates of each sample using nonadecanoic acid (C19:0, Sigma Chemical Co. Ltd), as an internal standard, and values were presented as a mean percentage (%) of three replicates.

2.3.8. Total volatile base nitrogen (TVB-N)

The total volatile basic nitrogen (TVB-N) was measured by direct distillation of the homogenized samples according to the EU Commission Regulation (EC) No 2074/2005 (EEC, 2005). The sample was ground carefully by a meat grinder. Exactly 10 g ± 0.1 g of the ground sample were weighed in a suitable container, mixed with a 90-mL 6% perchloric acid solution, homogenized for two min with a blender, and then filtered. Steam distillation of 50 mL of the extract after sufficient alkalinization with 20% NaOH (6.5 mL) and the addition of several drops of phenolphthalein (1 g/100 mL 95% ethanol) and a few drops of silicone anti-foaming agent, began immediately. The steam distillation was regulated so that around 100 mL of distillate was produced within 10 min. The distillation outflow tube was submerged in a receiver with a 100-mL 3% boric acid solution, to which three to five drops of the indicator solution, Tashiro Mixed Indicator (2 g methyl-red and 1 g methylene-blue, dissolved in 1000 mL 95% ethanol), were added. After exactly 10 min the distillation was ended. The volatile bases contained in the receiver solution were determined by titration with a standard hydrochloric solution 0.01M till the pH reached 5.0 ± 0.1. The TVB-N (mg/100 g sample) = (V1 - V0) × 0.14 × 2 × 100/ M, where V1 = Volume of 0.01 M hydrochloric acid solution in mL for sample; V0 = Volume of 0.01 M HCL solution in mL for blank (50 mL 6% perchloric acid solution was used instead of the extract); M = sample wet weight (ww) in g. The values were expressed as a mean percentage (%) of the wet weight (ww) of three replicates.

2.4. Statistical analysis

The results are presented as means ± standard deviation (SD). All biochemical indicators were statistically analyzed using SPSS software on triplicate samples, except total lipids and fatty acids, and free fatty acids, which were statistically analyzed on six triplicate samples. Normality and homogeneity of the variance of the results were confirmed by Kolmogorov-Smirnov and Levene's tests, respectively. One-way ANOVA followed by the Tukey test was performed to determine the significant differences between fresh, cold-, and hot-smoked fillets with or without antioxidant extract and was set either at $p < 0.05$, $p < 0.01$, $p < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Biochemical composition of fresh and smoked fillets

The biochemical composition of total proteins and lipids (%) in fresh and smoked *S. lucioperca* fillets are presented in Table 1. The percentage of total protein content remained nearly constant (range = 18.36–18.07%) in the fresh fillets (FF) during the 1, 20, and 90 days of storage, and showed a gradual significant increase ($p < 0.001$) in the levels (range = 41.27–51.31%) in both cold- (CSF) and hot-smoked (HSF) fillets covered with or without the two graded concentrations (0.5 and 1%) of *D. salina* polyphenol extract compared to FF during the three storage periods. However, this significant increase remained nearly constant in both CSF (41.27%) *vs* HSF (41.78%) without the covered extracts and CSF (42.57%) *vs* HSF (42.31%) covered with 0.5% of the polyphenol extract; while it increased in HSF (51.31%) covered with 1% of the polyphenol extract compared to CSF (42.31%) covered with 1% of the polyphenol extract during the three storage periods.

In addition, the total percentage of lipids revealed gradually decreased levels (1.86, 1.29, and 0.98%, respectively) in FF during the three storage periods and differentially significantly increased levels ($p < 0.001$) in both CSF and HSF with or without 0.5 or 1% of the polyphenol covering extracts during the three storage periods, with the highest similar levels in CSF and HSF covered with 0.5% of the extract and the highest

TABLE 1. Variations in the biochemical composition of total proteins and lipids (in %) in fresh (FF), cold-smoked (CSF), hot-smoked (HSF) fillets of *Sander lucioperca*, and cold-smoked (CSF) and hot-smoked (HSF) fillets covered with *Dunaliella salina* polyphenol (pp) antioxidant extract at 0.5 and 1% concentrations during three storage periods.

Storage days	FF		CSF		CSF+0.5% pp		CSF+1% pp		HSF		HSF+0.5% pp		HSF+1% pp	
	Proteins	Lipids	Proteins	Lipids	Proteins	Lipids	Proteins	Lipids	Proteins	Lipids	Proteins	Lipids	Proteins	Lipids
1	18.36±1.13^{a}	1.86±0.12^{a}	41.27±2.57^{a}	6.92±1.27^{a}	42.57±1.13^{a}	7.76±2.23^{a}	42.31±2.57^{a}	6.92±1.12^{a}	41.78±3.27^{a}	7.05±1.92^{a}	42.57±1.13^{a}	7.76±2.23^{a}	51.31±3.22^{a}	9.01±2.07^{a}
20	18.20±1.54^{a}	1.29±0.07^{a}	39.94±2.68^{a}	5.87±0.73^{a}	40.20±1.54^{a}	7.19±1.87^{a}	41.98±1.27^{a}	5.87±1.21^{a}	40.36±2.38^{a}	6.36±1.37^{a}	40.20±1.54^{a}	7.19±1.87^{a}	46.07±2.39^{a}	6.22±1.73^{a}
90	18.07±1.17^{a}	0.98±0.09^{a}	39.07±1.31^{a}	2.02±0.15^{a}	39.01±0.22^{a}	5.98±0.09^{b}	41.04±1.72^{a}	4.31±1.62^{a}	38.27±1.43^{a}	5.01±0.18^{b}	39.01±0.22^{a}	5.98±0.09^{b}	44.69±1.86^{a}	4.56±1.57^{a}
ANOVA	*	***	***	***	*	**	*	**	**	**	*	**	**	***

Values are means ± standard deviation (n = 3 for proteins and n = 6 for lipids); Different superscript letters in the same column represent significant differences($p < 0.05$, one-way ANOVA with Tukey test); Significant differences in total proteins and lipids percentages between FF and CSF and HSF with or without antioxidant extract during the three storage periods are presented ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

level, especially after the day 1 of storage, in HSF covered with 1% of the extract (Table 1).

In the present study, the total proteins remained unaltered in FF over the 1, 20, and 90 days of storage. This stability in total protein content may be due to the retention of water in the fillets during these storage periods. However, its significant increase in both CSF and CSF, as well as in HSF and HSF, covered with 0.5 and 1% of the extracts, compared to FF during the three storage periods can be attributed not only to the decrease in moisture and water content, and the increase of ash associated with evaporation during the smoking process, but also to the effect of polyphenols, including phenolic acids and flavonoids, vitamin E, tocopherols, and tocotrienols, which are powerful antioxidants found in the *D. salina* extract and oil (Cakmak *et al*., 2014), which protect the cell membrane from oxidative damage and consequently prevent protein and lipid oxidation (Garavaglia *et al*., 2016). In addition, the significant decrease ($p < 0.05$) in protein levels in both HSF and HSF covered with 0.5% of the extract after the 20 and 90 days of storage may be due to signs of the enzymatic autolytic activity causing the spoilage (Ayeloja *et al*., 2020), which was discontinued in the HSF covered with 1% of the extract after e 90 days of storage.

On the other hand, the significant decrease ($p < 0.001$) in the lipid content in FF during the three storage periods can be attributed to the progression in reducing the antioxidant properties and total phenolic content, which reversibly increased in CSF and HSF with or without 0.5 and 1% of the extracts during the three storage periods. This significant increase can be explained in terms of the dehydration of the fillets during smoking and the incorporation of *D. salina* oil into the fillets covered with 0.5 and 1% of the extracts, which led to stopping the degradation of lipids by eliminating free radicals. Similar results were found with sardine samples canned in grape seed and olive oils (Bouriga *et al*., 2022). Nevertheless, the significant decrease in lipid levels in FF after the 20 and 90 days of storage suggests the hydrolysis of some lipid fractions associated with the progression of microbial spoilage (Burton and Ingold, 1984).

3.2. Lipid oxidation indices in fresh and smoked fillets

The levels of the peroxide value (PV) and TBARS levels in the *S. lucioperca* FF and CSF and HSF covered with or without 0.5 and 1% of *D. salina* polyphenol antioxidant extracts are given in Table 2. The results showed that PV increased gradually during the storage periods from 1, 20 to 90 days, with the highest significant level ($p < 0.001$) found after 90 days of storage (Table 2). However, the PV in both CSF and CSF covered with 0.5 and 1% of the extract revealed a gradual decrease over the three storage periods compared to its level in FF. In HSF, although the PV indicated a significant increase ($p < 0.05$) after day 1 of storage and a significant decrease ($p < 0.05$) after the 20 days, it revealed a highly significant increase after 90 days compared to CSF. In HSF covered with 0.5 and 1% of the extract, compared with HSF, the PV displayed a slight decrease after 1 day of storage, a constant level at 1% concentration of the extract after the 20 days, and a significantly decreased level after 90 days; but this level was reduced significantly at 0.5% concentration

TABLE 2. Variations in the peroxide (PV, in meq active O2/kg lipid), thiobarbituric acid reactive substances (TBARS, in mg MDA/kg lipid), and 1,1-diphenyl-2-picrylhydrazyl (DPPH, in g/100g lipids) values in fresh (FF), cold-smoked (CSF), hot-smoked (HSF) fillets of *Sander lucioperca*, and cold smoked (CSF), and hot smoked (HSF) fillets covered with *Dunaliella salina* polyphenol (pp) antioxidant extract at 0.5 and 1% concentrations during the three storage periods

Parameter	Storage days	FF	CSF	CSF+0.5% pp	CSF+1% pp	HSF	HSF+0.5% pp	HSF+1% pp	ANOVA
PV	1	6.41±0.23^{a}	4.13±0.27^{a}	3.97±0.12^{a}	3.01±0.57^{b}	5.76±0.22^{b}	4.52±0.42^{c}	4.22±0.10^{c}	***
	20	10.06±1.04^{a}	9.13±1.41^{a}	6.08±1.06^{a}	5.27±1.42^{b}	7.27±0.47^{b}	5.58±0.18^{c}	6.73±0.76^{c}	***
	90	47.86±2.53^{a}	26.21±1.43^{a}	16.48±2.57^{b}	15.78±1.27^{c}	31.38±1.67^{b}	17.98±1.79^{b}	14.72±1.56^{c}	***
TBARS	1	0.52±0.12^{a}	0.96±0.17^{b}	0.67±0.08^{a}	0.55±0.03^{a}	1.38±0.31^{b}	1.17±0.38^{c}	1.03±0.15^{c}	***
	20	4.06±0.13^{a}	2.12±0.31^{c}	2.07±0.08^{b}	1.74±0.41^{b}	3.27±0.38^{a}	1.74±0.54^{b}	2.36±0.42^{c}	***
	90	8.41±1.05^{a}	3.68±0.35^{b}	3.31±0.52^{c}	2.11±0.72^{c}	7.12±1.07^{a}	5.03±0.67^{b}	4.78±1.04^{c}	***
DPPH	1	69.13±0.39^{a}	72.0±0.58^{a}	74.08±0.83^{a}	77.48±0.23^{a}	72.75±0.43^{b}	81.94±0.95^{c}	88.57±0.83^{c}	***
	20	33.72±0.57^{a}	37.12±0.53^{c}	44.12±0.43^{c}	53.96±0.32^{c}	39.52±0.26^{b}	48.98±0.46^{a}	57.14±0.35^{a}	***
	90	18.10±0.12^{a}	21.89±0.45^{b}	26.67±0.36^{c}	28.56±0.13^{c}	21.65±0.34^{a}	27.98±0.27^{b}	31.88±0.23^{b}	***

Values are means ± standard deviation (n = 3); Different superscript letters in the same column represent significant differences ($p < 0.05$, one-way ANOVA with Tukey test); Significant differences between FF and CSF and HSF with or without antioxidant extract during the three storage periods are presented ($^{***}p < 0.001$).

of the extract. This significantly increased the PV level in FF after 90 days of storage and it is higher than that found in *Sardinella gibbosa* after 6 and 9 days of storage (Chaijan *et al*., 2006). In addition, the PV levels in both CSF and HSF with or without 0.5 and 1% of the polyphenol extracts during the three storage periods were higher than those reported for *S. lucioperca* by Bouriga *et al*. (2020), although these levels were significantly decreased by the addition of these polyphenols (Table 2). These higher levels of PV are likely due to the hot temperature of the smoking process and the higher content of PUFAs, which are highly sensitive to primary oxidative reactions induced by molecular oxygen, and fall within the acceptable limits (10–20 mEq/kg) declared by Connell (1995).

In *S. lucioperca* FF, Bouriga *et al*. (2020) reported that the TBARS original level was 0.49 mg MDA/kg oil. This level increased here to 0.52 mg MDA/kg after the 1 day of storage, indicating that lipid oxidation had occurred and progressively significantly increased ($p < 0.001$) after 20 and 90 days of storage (Table 2). This significant increase is consistent with that found in *Trachurus trachurus* during frozen storage (Aubourg *et al*., 2002) In CSF, the level was significantly higher than in FF, constant after 1 day of storage, and decreased significantly ($p < 0.05$) after 20 and 90 days in CSF covered with 0.5 and 1% of the extract. In HSF, the level revealed a gradual significant decline in SHF covered with 0.5 and 1% of the extract after the 20 and 90 days of storage. However, compared to its level in FF, it exhibited a significant elevation after 1 day of storage and a reduction in HSF and HSF covered with 0.5 and 1% of the extract after 20 and 90 days of storage. In comparison with both CFS and CSF covered with 0.5 and 1% of the extract, the TBARS level displayed a significant increase ($p < 0.001$), especially in HSF and HSF covered with 0.5% of the extract, and constant in HSF after 1 day of storage. However, its level was significantly higher in both HSF and HSF covered with 1% of the extract than in both CSF and CSF covered with 0.5 and 1% of the extract, constant in both CSF and HSF covered with 1% of the extract after 20 days of storage, and significantly higher in both HSF and HSF covered with 0.5 and 1% of the extract after 90 days of storage. This significant increase in the TBARS level beyond the acceptable limit (8 mg MDA/kg) in FF was significantly decreased in both CSF and HSF with or without the covering polyphenol antioxidant extracts during the three storage periods. Despite this decrease, the levels were significantly higher than those observed by Bouriga *et al*. (2020) and Bouriga *et al*. (2022) and lower than those found by Chaijan *et al*. (2006). This increase in TBARS reflects the increase in the formation of aldehydes, relatively polar secondary reaction products (Kolakowska, 2002) due to the increase in phospholipids. In addition, it is worth not-

ing that the decrease in TBARS levels was concurrent with the gradual increase in PV during the three storage periods, a condition which is inconsistent with that described by Chaijan *et al*. (2006). This was probably due to an increase in hydroperoxides, especially aldehydes, in the later stages of secondary lipid oxidation as a result of the greater release of free iron and other prooxidants from the muscle which were excessively degraded when storage time was increased. However, this increase in hydroperoxides was reduced in both CSF and HSF by the addition of 0.5 and 1% of the covering polyphenol extracts.

3.3. Free radical (DPPH) activity in fresh and smoked fillets

The DPPH activity in *S. lucioperca* FF, as well as in both CSF and HSF covered with or without 0.5 and 1% of *D. salina* polyphenol antioxidant extracts are shown in Table 2. Overall, the DPPH activity exhibited a significant decline in FF over the 1, 20, and 90 days of storage, respectively, while a progressive elevation in the activity was observed in both CSF and CSF covered with the two concentrations of the polyphenol antioxidant extracts during the three storage periods. Conversely, the activity showed a significant decrease ($p < 0.05$) in HSF compared to HSF covered with 0.5 and 1% of the extract during the three periods of storage. However, in comparison with its activity in FF, as well as in CSF and CSF covered with 0.5 and 1% of the extract, the activity level in HSF was significantly higher than in FF and lower than in CSF covered with 0.5 and 1% of the extract during the three periods of storage. On the other hand, the level in HSF covered with 0.5 and 1% of the extract was significantly higher than that in CSF covered with 0.5 and 1% of the extract, respectively, during the three periods of storage. Indeed, the DPPH free radical assay has been used to assess the antioxidant activity based on the electron transfer that produces a violet solution in ethanol (Huang *et al*., 2005). Overall, the DPPH activity showed a gradual significant decrease ($p < 0.05$) in both FF and CSF, as well as in HSF covered with or without 0.5 and 1% of the polyphenol extract, with an increase in the storage periods from 1 to 90 days. However, the levels of activity exhibited a significant increase in both CSF and HSF, compared to FF, with the addition of the polyphenol extract. Moreover, this increased activity was concurrent with the increase in lipid content. Therefore, we can assume that there was a close correlation between the polyphenol content, the DPPH activity, and the increase in lipid peroxidation.

3.4. Total fatty acid (FA) composition in fresh, cold-, and hot-smoked fillets

The total FA composition in FF differed significantly during the three storage periods (1, 20, and 90), with a significant increase ($p < 0.05$) in both total saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), and a significant decrease ($p < 0.05$) in total polyunsaturated fatty acids (PUFAs) after the three storage periods (Table 3). This significant increase was confirmed by the considerable elevation of all SFAs (C14:0, C16:0, and C18:0) and MUFAs (C16:1n-7, C18:1n-7, and C18:1n-9), while the total decrease in PUFAs was only observed in C20:5n-3, C22:5n-3, C22:6n-3, and PUFA-n-3. Similarly, in both CSF and HSF, all SFAs and MUFAs exhibited a progressively significant increase ($p < 0.05$) in level during the three storage periods (1, 20, and 90). However, the total PUFAs, as well as PUFA–n-3 and –n-6, indicated a significant gradual decrease ($p < 0.05$) during these storage periods. Similarly, when CSF and HSF were covered with both concentrations of 0.5 and 1% of *D. salina* extract, all SFAs and MUFAs showed a remarkable increase ($p < 0.05$) over the 1, 20, and 90 days of storage, while the total PUFAs and PUFA–n-3 and –n-6 displayed a substantial significant decrease ($p < 0.05$) during these storage periods.

Regarding the variation in the FA composition in FF, it was shown that the significant increase in total SAFs was dominated by palmitic acid (C:16), which showed the highest increase (40.49%) after the 90 days of storage. This significant increase was also continued at different rates from day 1 to day 90 of storage in both CSF and HSF covered with or without 0.5 and 1% of the antioxidant extract. In addition, the significant increase in total MUFAs in FF was dominated by oleic acid (C18:1n-9), with the highest increase (17.95%) after 90 days of storage. This increase also persisted in differential proportions from day 1 to day 90 of storage in both CSF and HSF covered with or without 0.5 and 1% of the extract. However, the significant reduction in PUFAs was prevailed by docosahexaenoic acid (C22:6n-3) in FF, with the highest decrease (1.67%) found after

TABLE 3. Total fatty acid (FA) composition (in %) in fresh (FF), cold-smoked (CSF), and hot-smoked (HSF) fillets of *Sander lucioperca*, and CSF and HSF fillets covered with *Dunaliella salina* polyphenol (pp) antioxidant extract at 0.5 and 1% concentrations during the three storage periods

FA	FF			CSF			CSF+0.5% pp			CSF+1% pp		
	1 day	20 days	90 days	1 day	20 days	90 days	1 day	20 days	90 days	1 day	20 days	90 days
C14:0	4.65±0.19a	5.09±1.12a	6.14±1.78b	5.72±0.25a	7.72±1.12b*	8.32±0.67c**	5.89±0.25a	6.41±1.52b	7.92±1.55c	6.72±1.05a*	7.12±1.78b**	8.01±1.23c***
C16:0	16.4±0.89a	20.46±1.52b	22.19±2.5c	17.65±2.14a	18.23±1.95b*	21.29±1.48c	16.5±0.87a	17.57±2.4b**	20.76±2.48c*	18.21±1.56a**	18.31±2.55b	19.7±2.37c
C18:0	2.76 ±0.66a	4.01±1.73b	7.15±3.45c	2.92 ±0.78a	4.57±0.73c	7.89±0.47c	3.19±0.78a	3.73±0.47b	6.37±1.51c*	4.22±0.96a***	4.27±0.36a	6.03±0.58c**
C20:0	0.78 ± 0.03b	0.81 ± 0.05c	1.72 ± 0.18c	0.68 ± 0.04b	0.79 ± 0.07b	1.56 ± 0.11a	0.87 ± 0.02b	0.93 ± 0.01c	1.29 ± 0.06b	0.61 ± 0.03b	0.78 ± 0.02a	1.18 ± 0.21c
C22:0	0.52 ± 0.01b	1.12 ± 0.07b	1.41 ± 0.09b	0.34 ± 0.02c	0.57 ± 0.01b	1.47 ± 0.04b	1.22 ± 0.07b	1.07 ± 0.11b	1.63 ± 0.15b	1.22 ± 0.17c	1.41 ± 0.14b	1.57 ± 0.33b
C24:0	1.33 ± 0.08b	2.48 ± 0.15b	3.29 ± 0.21b	1.69 ± 0.13b	2.54 ± 0.27	3.66 ± 0.31c	1.53 ± 0.26b	1.27 ± 0.17c	2.7 ± 0.31c	1.86 ± 0.47b	2.68 ± 0.48b	2.29 ± 0.53b
Total SFA	26.44±2.03a	33.97±3.12c	40.49±2.30c	29.00±3.41a**	34.42±3.27b	44.19±3.06c***	29.2±2.10a**	30.98±2.92a	40.67±2.94c	32.84±2.10a***	34.57±3.41a	38.78±3.07b
C16:1n-7	6.75 ±1.51a	8.80±1.55a	9.984±2.47c	7.82 ±1.51a*	9.41±1.59b*	12.06±3.02c**	6.40±0.46a	7.37±1.42b	10.12±1.21c	5.92±0.47a*	6.68±1.25b**	9.42±1.36c
C18:1n-7	2.90±0.50a	4.25±0.58b	5.12±2.72c	3.51±0.78a*	4.12±0.68b	6.43 ±0.18c*	3.81±0.27a*	4.21±0.72b	6.87±1.36c*	3.36±0.27a	3.86±0.58a*	4.11±1.27b
C18:1n-9	12.23±2.40a	15.40±2.05a	17.95±3.22b	12.85±2.74a	13.78±2.02b*	16.08±0.87c*	13.53±1.13a	14.31±3.05b	17.31±2.87c	12.68±1.41a	13.57±2.42b*	19.52±2.31c**
C20:1	1.11±0.32a	1.96±0.65b	3.04±0.61c	1.27±0.12a	1.62±0.44a	2.08±0.41b	1.09±0.21a	1.51±0.66b	2.12±0.37a	1.31±0.20b	1.39±0.36a	0.97±0.02b
C22:1	1.02 ± 0.05a	1.68 ± 0.05b	2.74 ± 0.07b	1.08 ± 0.03a	1.31 ± 0.03a	1.55 ± 0.03a	1.01 ± 0.05a	1.32 ± 0.03a	1.83 ± 0.07a	0.98 ± 0.01a	1.09 ± 0.06b	0.65 ± 0.03a
C24:1	0.76 ± 0.01b	1.14 ± 0.06b	1.63 ± 0.04b	1.01 ± 0.02b	1.08 ± 0.06b	1.49 ± 0.02c	0.88 ± 0.03b	0.95 ± 0.04c	1.78 ± 0.06b	0.73 ± 0.01b	1.06 ± 0.02b	0.18 ± 0.01c
Total MUFA	24.77±2.05a	33.23±2.56b	40.47±3.67c	27.54±2.31a**	31.32±3.73b*	39.69±2.73c	26.72±1.58a*	29.70±3.41b*	40.03±3.21c	24.98±1.23a	27.65±2.73b**	34.85±3.14c***
C18:2n-6 (LA)	4.68±0.97a	5.40±1.96b	7.32±1.51b	5.69±0.82a*	3.78±1.25b***	3.02±1.78b***	6.32±2.48a**	4.92±1.63b	3.96±0.69c***	7.01±1.57a***	5.36±0.44b	4.00±0.73c***
C20:2n-6	0.96 ± 0.01a	0.42 ± 0.02b	0.01 ± 0.00a	1.48 ± 0.16a	1.09 ± 0.12b	0.36 ± 0.01c	0.09 ± 0.01b	1.34 ± 0.4c	0.46 ± 0.02b	1.39 ± 0.3c	1.19 ± 0.2b	0.75 ± 0.1c
C20:3n-3	1.06 ± 0.04b	0.51 ± 0.05c	0.03 ± 0.01b	1.29 ± 0.03c	1.12 ± 0.05b	0.49 ± 0.03b	0.03 ± 0.02b	1.2 ± 0.1b	0.57 ± 0.03b	0.99 ± 0.07b	1.2 ± 0.4b	0.46 ± 0.01c
C20:4n-6 (ARA)	11.24±2.35a	12.23±2.65b	13.05±2.29c	12.44±7.78a	11.22±2.42b*	11.23±1.65a*	9.98±1.75a*	7.78±1.57b**	9.52±1.38a***	10.02±1.41a	9.08±1.36b***	7.14±0.68c***
C20:5n-3 (EPA)	7.93 ±1.46a	3.49±0.51b	1.22±0.54c	8.57 ±1.33a*	4.98±1.94b*	1.74±2.31a	5.67±0.36a**	3.98±1.72b	3.21±0.42a***	6.41±0.51a*	5.31±0.87b**	4.12±0.35c***
C22:4n-6	1.00 ± 0.03b	0.59 ± 0.06b	0.01 ± 0.00b	1.25 ± 0.07c	1.04 ± 0.05b	0.5 ± 0.02c	0.21 ± 0.01b	1.43 ± 0.6b	0.13 ± 0.01c	1.69 ± 0.5b	2.08 ± 0.8b	0.88 ± 0.02c
C22:5n-3 (DPA)	1.78±0.23a	1.27±0.72a	0.78±0.06b	2.42±0.27a**	1.63±0.12a	1.02±0,05a	1.31±0.68a	0.97±0.09b	0.98±0.08b	1.76±0.21a	1.36±0,01a	1.02±0.04b
C22:6n-3 (DHA)	13.68±2.78a	8.78±2.86b	1.67±0.57c	12.63±2.56a*	8.46±1.41b	6.98±2.97c***	14.85±2.35a*	10.37±1.52b**	7.21±0.33c***	13.78±2.27a	12.21±1.41b**	9.42±0.84c***
PUFA-n-3	23.39±1.15a	13.54±1.09c	3.67±0.08c	23.62±3.06a	15.07±0.76c**	9.74±0.63c***	21.83±2.58a*	15.32±2.13c*	11.40±1.3c***	21.95±1.61a*	18.88±1.2b***	14.56±2.33c***
PUFA-n-6	15.92±1.22a	17.63±1.11b	20.37±1.98c	18.12±0.97a**	15.00±1.35a*	14.25±1.53b***	16.30±1.00a	12.70±1.18b**	13.48±1.3b***	17.03±1.22a*	14.44±1.37b**	11.14±0.51c***
Total PUFA	42.33±1.32a	32.69±2.78b	24.09±2.09c	45.77±3.02a*	33.32±2.78b	25.34±3.12c	37.8±3.1a***	31.99±3.12b	26.04±3.07c	43.05±4.08a	37.79±2.44b	27.79±3.51b*

the 90 days of storage. This decrease also proceeded to different percentages from day 1 to day 90 of storage in both CSF and HSF covered with or without 0.5 and 1% of the extract. Similar results of the significant reduction in PUFAs at the end of smoking (90 days) due to a decrease in PUFAs of the n-3 series (Tot n-3) and a simultaneous increase in MUFAs have been found in *Argyrosomus regius* fillets cold-smoked in combination with 1% *Halocnemum strobilaceum* antioxidant for 35 days of storage at 4 °C (Messina *et al*., 2021). In comparison with the previous work on *S. lucioperca*, the current increase in total SAFs is consistent with that also found in CSF and HSF by Bouriga *et al*. (2022). However, the increase in total MUFAs recorded here is not in line with that reported by these authors, as total MUFAs showed a significant decrease in CSF and HSF. Nevertheless, there is agreement about the decrease reported here and there in total PUFAs in both CSF and HSF. Therefore, we can assume that the *D. salina* polyphenol antioxidant extract had a significant effect not only on the increase in SAFs and MUFAs but also on their percentages in both CFS and HSF.

3.5. Free fatty acid (FFA) content in fresh, cold-, and hot-smoked fillets

The profile of the FFA content in FF showed a progressive significant increase ($p < 0.05$) over the 1, 20, and 90 days of storage. This significant increase was gradually decreased in CSF covered with or without 0.5 and 1% of *D. salina* polyphenol antioxidant extract and then increased again in HSF during the three periods of storage but with the highest level in HSF (Table 4). Overall, the formation of FFA, as a marker

TABLE 3. Continued

FA	HSF			HSF+0.5% pp			HSF+1% pp		
	1 day	20 days	90 days	1 day	20 days	90 days	1 day	20 days	90 days
C14:0	5.28±0.63a*	6.92±1.51b*	9.01±1.44c***	4.69±0.51a	5.08±1.03b	6.27±1.07c	4.73±0.27a	5.27±1.22b	6.55±1.43b**
C16:0	16.98±3.08a	17.86±2.44b**	22.18±1.55c	15.21±2.45a*	16.12±2.76b***	20.36±1.28c**	15.39±2.64a*	15.68±2.31a***	18.6±1.59b***
C18:0	3.26±0.92a	4.13±0.87b	7.41±0.51c	3.12±0.84a	4.01±0.56a	6.81±0.36c	3.58±0.72a	3.78±0.56a*	5.62±0.36b**
C20:0	0.61 ± 0.01a	0.73 ± 0.03b	1.59 ± 0.03b	0.24 ± 0.00a	0.84 ± 0.02b	1.42 ± 0.16a	0.57 ± 0.02b	0.69 ± 0.03b	1.09 ± 0.07b
C22:0	0.92 ± 0.04b	1.05 ± 0.13b	1.87 ± 0.05c	0.69 ± 0.01b	1.02 ± 0.07c	1.89 ± 0.12b	0.97 ± 0.06b	1.01 ± 0.01a	1.64 ± 0.08b
C24:0	1.61 ± 0.12c	2.21 ± 0.16b	2.55 ± 0.15b	2.1 ± 0.23c	1.9 ± 0.15b	1.91 ± 0.24b	1.7 ± 0.18b	1.86 ± 0.09a	2.08 ± 0.16b
Total SFA	28.66±3.24a**	32.90±3.15b	44.61±4.05c***	26.05±2.54a	28.97±2.69b**	38.66±3.12c**	26.94±3.35a	28.29±2.56b**	35.58±2.86c***
C16:1n-7	8.43±1.26a**	8.78±1.21a	13.76±2.58c**	7.21±0.96a	7.92±1.26a	11.32±2.37c*	7.44±0.82a	7.66±1.41a	9.78±2.57b
C18:1n-9	11.46±2.52a	12.02±2.15b	17.45±2.41c	10.23±1.78a	11.12±2.27b	14.36±2.28c	10.51±1.45a	10.77±2.31b	11.90±2.45b
C18:1n-7	4.36±0.89a***	4.99±0.38b	7.16±0.23c**	3.78±0.27a*	4.97±0.12b	6.46 ±0.47c	3.97±0.68a*	4.37±0.12b	5.86±0.47b
C20:1	1.21±0.57b	1.32±0.61a	1.38±0.72c	1.16±0.21c	1.48±0.22a	1.66±0.63a	1.2±0.18c	1.31±0.09b	1.43±0.12c
C22:1	1.07 ± 0.03a	1.46 ± 0.05a	1.16 ± 0.12b	1.01 ± 0.01b	1.10 ± 0.01b	1.31 ± 0.02b	1.16 ± 0.04b	1.68 ± 0.07b	1.63 ± 0.05b
C24:1	0.96 ± 0.04b	1.16 ± 0.07b	0.84 ± 0.01b	0.95 ± 0.00c	1 ± 0.12b	1.15 ± 0.03b	1.06 ± 0.01b	0.73 ± 0.02c	0.99 ± 0.03b
Total MUFA	27.49±2.72a**	29.73±4.21b	41.75±2.86c	24.34±2.15a	27.59±2.76b**	36.26±3.12c***	25.37±2.68a	26.52±2.39b**	31.59±3.28b***
C18:2n-6 (LA)	6.21±0.74a***	4.21±1.13a	2.27±0.14b***	7.04±0.66a***	6.54±0.76a*	4.31±0.21b***	7.01±0.66a***	6.81±0.15a**	6.01±0.18b**
C20:2n-6	1.22 ± 0.2b	1.51 ± 0.6c	0.89 ± 0.01b	1.96 ± 0.5b	1.79 ± 0.6c	1.44 ± 0.3b	1.65 ± 0.4c	2.48 ± 0.54b	1.22 ± 0.17c
C20:3n-3	1.94 ± 0.5a	1.27 ± 0.1b	0.87 ± 0.01b	1.59 ± 0.2c	1.55 ± 0.36b	0.87 ± 0.01a	1.61 ± 0.7c	1.73 ± 0.31c	1.12 ± 0.09a
C20:4n-6 (ARA)	13.25±2.31a**	12.08±1.31b	8.97±1.13a***	11.78±2.58a	11.12±1.26a	7.33±1.09c***	11.66±2.45a	11.36±1.13a	10.16±1.19c**
C20:5n-3 (EPA)	7.12±1.15a	5.96±1.26b**	1.21±0.27c	7.68±1.08a	6.96±1.37b***	4.95±0.83c***	7.51±1.11a	7.38±1.78b***	6.06±0.27c***
C22:4n-6	2.05 ± 0.02b	1.89 ± 0.03c	0.92 ± 0.02b	2.47 ± 0.54c	2.58 ± 0.33b	2.39 ± 0.12b	3.01 ± 0.8a	3.44 ± 0.92b	1.16 ± 0.53b
C22:5n-3 (DPA)	2.51±0.14a**	2.04±0.17a*	1.02±0.01b	3.07±0.27a**	2.47±0.24a**	1.64±0.03b*	3.05±0.13a**	2.81±0.51a**	2.51±0.15b***
C22:6n-3 (DHA)	12.41±2.22a	11.09±1.28b***	6.14±2.37c***	13.61±2.52a	12.79±1.09b***	9.26±1.99c***	13.87±2.42a	13.16±1.28b***	12.65±1.37c***
PUFA-n-3	22.06±1.14a	19.11±1.67b***	8.37±0.78c***	24.37±2.08a*	22.23±1.53b***	15.86±1.47c***	24.43±2.69a*	23.35±1.31a***	21.23±1.70b
PUFA-n-6	19.46±1.42a**	16.31±1.27b*	11.25±0.99c***	18.83±1.66a**	17.67±2.04a	11.64±1.38c***	18.67±0.76a**	18.17±1.28a*	16.18±1.54b***
Total PUFA	46.71±4.31a**	40.05±3.51b**	22.29±2.05c	49.29±4.57a***	45.80±2.48b***	32.19±2.24c***	49.37±3.34a***	49.17±4.97b***	40.89±2.51c***

Values are means ± standard deviation (n = 6); Different superscript letters in the same column represent significant differences ($p < 0.05$, one-way ANOVA with Tukey test); Significant differences between FF and CSF and HSF with or without antioxidant extract during the three storage periods are presented ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); NS = non-significant, FAs = fatty acids, SFAs = saturated fatty acids, MUFAs = monounsaturated fatty acids, PUFAs = polyunsaturated fatty acids, LA = linoleic acid, ARA = arachidonic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid, PUFA-n-3 = Omega-3 fatty acid, PUFA-n-6 = Omega-6 fatty acid.

of lipolysis in smoked oil fish fillets during storage, has been so far associated with fat content, lipolytic activity, and temperature (Bouriga *et al*., 2020). In *S. lucioperca* FF, FFA was represented by 1.24% of total lipids, a level which is within the range (1–7%) found in crude fish (Bimbo, 1998) and lower than that reported by Aidos *et al*. (2001) in herring oil. This level was significantly increased (7.57%) as the storage periods increased, with the highest increase (7.89%) shown in HSF after 90 days of storage. Similar results have previously been recorded by Bouriga *et al*. (2020). Such an increase, particularly in HSF, can be attributed to the lipolysis generated by lipases or phospholipases (Chaijan *et al*., 2006; Bouriga *et al*., 2020). In addition, the results obtained indicated that both smoking and the addition of 0.5 and 1% of the polyphenol extracts were below the acceptable limit (7 g/100g).

3.6. Total volatile base nitrogen (TVB-N) values in fresh, cold-, and hot-smoked fillets

The TVB-N indicated significantly increased levels ($p < 0.001$) in FF during the three storage periods, with the highest level found after 90 days of storage. In addition, differentially significant decreased levels ($p < 0.05$) were found in CSF and CSF covered with 0.5 and 1% of the extracts, as well as in HSF, and HSF covered with 0.5 and 1% of the extracts, during the three storage periods, with the highest similar level in CSF and HSF, especially after 90 days of storage, compared to CSF and HSF covered with 0.5 and 1% of the extracts (Table 4). This significant decrease ($p < 0.001$) in the level of TVB-N in CSF and HSF covered with or without 0.5 and 1% of the covering extracts, compared to FF during the three storage periods, is inconsistent with the findings of Karsli and Caglak (2021), who report-

TABLE 4. Variations in the free fatty acids (FFAs, in g/100g lipids) and total volatile basic nitrogen (*TVB-N, in* mg MDA/kg lipids) values in fresh (FF), cold-smoked (CSF), hot-smoked (HSF) fillets of *Sander lucioperca*, and cold-smoked (CSF), and hot-smoked (HSF) fillets covered with *Dunaliella salina* polyphenol (pp) antioxidant extract at 0.5 and 1% concentrations during the three storage periods

Parameter	Storage days	FF	CSF	CSF+0.5% pp	CSF+1% pp	HSF	HSF+0.5% pp	HSF+1% pp	ANOVA
FFAs	1	1.24±0.07a	1.78±0.17a	1.52±0.04a	1.38±0.02a	3.42±0.10b	2.14±0.16c	2.04±0.37c	***
	20	3.76±0.49a	2.39±0.71c	2.05±0.13c	1.69±0.39c	4.27±0.38b	3.47±0.44a	3.12±0.37a	***
	90	7.57±1.21a	4.96±0.72b	4.23±0.47c	3.29±0.62c	7.89±1.53a	4.92±0.47b	4.47±1.36b	***
TVB-N	1	5.32±0.47a	3.73±0.06b	3.48±0.10b	2.78±0.10a	2.86±0.12a	2.48±0.09a	2.28±0.17b	***
	20	10.06±0.09a	6.27±0.27b	5.2±0.02b	4.2±0.28a	6.05±1.23a	4,89±0.16a	3.21±1.69b	***
	90	22.21±0.43a	19.42±1.22a	16.8±1.52b	15.7±1.02c	19.42±1.22a	13.62±1.38b	12.7±1.31b	***

Values are means ± standard deviation (n = 3); Different superscript letters in the same column represent significant differences ($p < 0.05$, one-way ANOVA with Tukey test); Significant differences between FF and CSF and HSF with or without antioxidant extract during the three storage periods are presented ($^{***}p < 0.001$).

ed a gradual significant increase in TVB-N during the storage of the smoked fish. In this respect, Arous *et al*. (2014) reported that the increase in TVB-N levels resulted from the production of dimethylamine, trimethylamine, and ammonia associated with the destructive effect of microorganisms on proteins during storage. In addition, Karsli and Caglak (2021) attributed the increase in the TVB-N levels to the loss of water during smoking and the increase in proteolytic activity during salting and smoking. Therefore, in addition to the water loss during smoking, we can assume here that the significant decrease in the TVB-N levels in CSF and HSF with or without 0.5 and 1% of the covering extracts was due to increased deamination of adenosine monophosphate or amino acids, which led to an increase in ammonia release, resulting from the combined effect of smoking and the addition of *D. salina* extract (Bouriga *et al*., 2022).

4. CONCLUSIONS

The results showed a significant increase in proteins, lipids, FFAs, and DDPH contents, and a decrease in PV, TBARS, and TVB-N levels in cold (CSF) and hot (HSF) smoked fillets of *Sander lucioperca* covered with or without 0.5 and 1% of *Dunaliella salina* polyphenol antioxidant extract and stored for 1, 20, and 90 days compared to fresh fillets (FF). Saturated (SFAs) and monounsaturated (MUFAs) fatty acids exhibited a significant increase in FF and CSF and HSF covered with or without polyphenol extract. Total polyunsaturated fatty acids (PUFAs) revealed a significant decrease in FF and CSF and HSF with or without the extract. Therefore, cold and hot smoking processes and the addition of 0.5 and 1% of natural *D. salina* polyphenol antioxidant extract was a valuable and promising method to improve the biochemical quality, shelf-life, and consumption of *S. lucioperca* fillets stored for up to 90 days in a refrigerator at 0-4 °C.

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Variation in seed morphology and selected oil parameters of neem (*Azadirachta indica* A. Juss.) from different agroclimatic zones in Tamil Nadu, India

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SUMMARY: Tamil Nadu, in southern India, has the second-largest number of neem trees in the country. The oil from the seeds has high economic significance for cottage industries in the region. This paper examines 28 Candidate Plus Trees (CPTs) selected from six agroclimatic zones in Tamil Nadu which exhibit exceptional traits such as superior growth and other desirable characteristics. We aimed to understand seed morphology variations and physicochemical properties in the oil across different regions. Significant differences were observed for morphometric traits. Fruit production correlated negatively with rainfall. 100-seed kernel weight and seed length correlated with oil percentage. Rainfall influenced seed breadth and pericarp weight. Clustering using morphological characters did not group genotypes from the same region; while soil type could distinguish them. Correlation helped us determine the prominent features which influence the traits of interest, which can be useful for breeding programs, cultivation practices, and the development of neem-based products in Tamil Nadu and beyond.

KEYWORDS: *Oil quality; Physical traits; Physicochemical properties; Variability.*

RESUMEN: ***Variación en la morfología de semillas y parámetros de aceites seleccionados de lilas india (*Azadirachta indica *A. Juss.) de diferentes zonas agroclimáticas de Tamil Nadu, India.*** Tamil Nadu, en el sur de la India, tiene el segundo mayor número de árboles de lilas india del país. El aceite de las semillas tiene una gran importancia económica para las industrias artesanales de la región. En este trabajo se examinan 28 árboles Candidate Plus (CPT) seleccionados de seis zonas agroclimáticas en Tamil Nadu, que exhiben rasgos excepcionales como un crecimiento superior y otras características deseables. Nuestro objetivo era comprender las variaciones de la morfología de las semillas y las propiedades fisicoquímicas del aceite en diferentes regiones. Se observaron diferencias significativas para los rasgos morfométricos. La producción de frutos se correlacionó negativamente con las precipitaciones. El peso de 100 semillas y granos y la longitud de la semilla se correlacionaron con el porcentaje de aceite. Las lluvias influyeron en el ancho de la semilla y el peso del pericarpio. El agrupamiento utilizando caracteres morfológicos no agrupó genotipos de una misma región, mientras que el tipo de suelo pudo distinguirlos. La correlación nos ayudó a diseccionar las características prominentes que influyen en las características de interés, que pueden informar los programas de reproducción, las prácticas de cultivo y el desarrollo de productos a base de neem en Tamil Nadu y más allá.

PALABRAS CLAVE: *Calidad del aceite; Propiedades fisicoquímicas; Rasgos físicos; Variabilidad.*

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1. INTRODUCTION

Azadirachta indica A. Juss., (Neem; Family: Meliaceae), an evergreen, multipurpose tree which is native to India, grows in the arid, semi-arid, and tropical conditions in Pakistan, Bangladesh, Srilanka, Malaysia, Indonesia, Thailand, the Middle East, Sudan and Niger (Kaushik *et al.*, 2007). The species shows wide adaptability. It is seen as an avenue, ornamental agroforestry or roadside tree. It grows in clay, saline, alkaline, dry, stony, shallow soils, including high calcareous soil (Pattnaik *et al.*, 2006, Atabani *et al.*, 2013), and tolerates high temperatures, low rainfall, long spells of drought and salinity. For centuries, it has been used in traditional medicine. Various parts are used in the Ayurvedic and Unani systems of medicine. It is estimated that ~ 25 million neem trees exist in India, the highest recorded from Uttar Pradesh, followed closely by Tamil Nadu, Madhya Pradesh, Andhra Pradesh, and Karnataka. A fully grown tree produces about 50 kg fruits annually. The productivity of neem oil mainly varies from 2 to 4 t/ha/yr (Kaushik *et al.*, 2007).

Neem Seed Kernel Extract (NSKE) is widely used in agriculture. About 20–30% of the seed weight constitutes oil – the kernels contain 40–50% of an acrid green to brown oil (Atabani *et al.*, 2013). The oil comprises fatty acids, mainly oleic acid (13.50–26.76%), palmitic acid (6.88–11.99%), linoleic acid (7.32–11.17%), stearic acid (4.29–13.08%), and arachidic acid (0.53–1.27%) (Adigwe *et al.*, 2022). Azadirachtin, salannin, and other limonoids are also present in small quantities. Neem oil, being one of the least toxic to humans and beneficial organisms, is very promising in the control of numerous pests.

Kaushik *et al*. (2007) and Jessinta *et al*. (2014), provide information on the variation in tree morphology, vegetative and reproductive phenology, and seed morphology of the neem species across different geographic locations. However, there is a lack of information specifically related to agroclimatic zones. Tamil Nadu is the second in India's highest number of neem trees, so we felt it imperative to understand the variations across agro-climate zones. This study is significant considering the state's high number of neem trees and their relative abundance (12%), as reported by the Trees Outside Forests (TOF) inventory (FSI, 2019).

Hence, this paper discusses variations in seed morphology and seed oil properties in neem from various agroclimatic regions of Tamil Nadu. It would also enable us to predict site-source matched provenances for future planting.

2. MATERIALS AND METHODS

2.1. Collection of fruits

An extensive survey was carried out during 2019-20 in different agroclimatic regions of Tamil Nadu to select superior phenotypes of neem. Tamil Nadu has wide variations in its agro climatic zones (ACZ). The state is divided into seven ACZs. The present study covered six zones, leaving behind the Hilly region (Nilgiris), where neem trees are not present (FSI, 2019). The agroclimatic zones with precipitation and soil details are shown in Table 1. The details of selected Candidate Plus Trees (CPTs) concerning fruit yield, growth superiority, pest and disease incidence are presented in Table 2. The data on height, girth at breast height (GBH), clear bole height (CBH) and crown diameter were recorded and the seeding behavior of the selected CPTs was scored on a scale of 1 to 5 (1- Poor fruiting and 5 - Heavy fruiting). Fully mature, yellowish-green fruits were collected from the CPTs. Within 24 hours of collection, the fruits were de-pulped and their seeds were washed thoroughly with tap water to remove pulp, dirt and impurities. The seeds were shade-dried and stored under ambient conditions. All experiments were conducted in a completely randomized design with four replicates.

2.2. Measurement of seed morphometric characters

Neem seeds (100 in four replicates – 25 in each) were spread on the glass plate of a macro viewer and images were captured. Using Leica Q Win, the length (cm) and breadth (cm) of the seeds were recorded. The 100-seed weight (g), 100-seed kernel weight (g) and pericarp weight (g) of the seeds were estimated using an electronic balance and pericarp percentage was calculated from the recorded values. The moisture content of the neem seeds was estimated as per ASAE (1998).

2.3. Oil extraction and characterization

Twenty grams of seed kernel powder were used for oil extraction. The oil content was extracted using the standardized Soxhlet method. Petroleum ether was used as solvent.

Table 1. Agroclimatic zones (ACZ) with the number of trees selected in parenthesis, along with precipitation and soil details

ACZ No	Agroclimatic zones	Locations	Minimum Annual Rainfall (mm)	Maximum Annual Rainfall (mm)	Total Annual Rainfall (mm)	Soil Type
1	Western (15)	Coimbatore	121.8	673.6	1206.1	Red or Black Loam
		Tiruppur	20.5	146.9	600.3	
		Erode	28.7	212.1	653.4	
2	NorthEastern (1)	Kallakurichi	36.3	278.5	860.1	Red clay loam
3	High Rainfall (3)	Thoothukudi	5.7	61.8	593.1	Saline Coastal
4	NorthWestern (2)	Salem	54.3	365.1	850.8	Non-Calcareous red and brown
5	Cauvery Delta (1)	Trichy	26.3	242.2	715.8	Red Loamy or Alluvium
6	Southern (9)	Ramnathapuram	14.1	113.0	805.3	Red Sandy
		Tirunelveli	26.9	135.6	841.8	

Source: District Statistics, TN (2020)

Table 2. Variation in tree morphometric traits in the selected Candidate Plus Trees (CPTs) of *Azadirachta indica.*

ACZ	Accession code	Tree height (m)	Girth at Breast Height (cm)	Clear Bole Height (m)	Crown diameter (m)	Seeding behavior
ACZ1	IFGTB AI 1	12.16	150	1.3	11.2	5
ACZ1	IFGTB AI 2	13.28	155	0.85	10.56	4
ACZ1	IFGTB AI 3	13.06	148	1.2	12.10	4
ACZ1	IFGTB AI 4	12.46	151	1	13	3
ACZ1	IFGTB AI 6	13.12	136	1.62	10.60	4
ACZ1	IFGTB AI 9	11.10	142	1.30	10.89	4
ACZ1	IFGTB AI 10	14.23	155	1.85	13.5	3
ACZ1	IFGTB AI 12	11.56	126	1.32	12.16	4
ACZ1	IFGTB AI 13	13.65	134	1.25	14.18	4
ACZ1	IFGTB AI 14	14.12	128	1	10.10	4
ACZ1	IFGTB AI 16	12.10	145	1.32	13.36	3
ACZ1	IFGTB AI 17	11.22	123	1.23	14.65	4
ACZ2	IFGTB AI 18	13.08	169	1.65	15.11	4
ACZ3	IFGTB AI 19	10	135	1.11	10.0	5
ACZ3	IFGTB AI 20	9.80	110	1.21	10.48	5
ACZ3	IFGTB AI 21	14.12	130	1.5	16.15	5
ACZ4	IFGTB AI 22	12.28	142	1.33	11.34	4
ACZ4	IFGTB AI 23	13.36	132	1.62	12.25	5
ACZ5	IFGTB AI 28	16.11	186	1.25	18.12	4
ACZ6	IFGTB AI 29	8.98	110	1.36	10	4
ACZ6	IFGTB AI 31	15.50	168	1.22	17.10	4
ACZ6	IFGTB AI 32	17.22	185	1.14	20.42	3
ACZ6	IFGTB AI 33	10.15	125	1.19	10.23	3
ACZ6	IFGTB AI 34	13.15	129	1.23	15.18	4
ACZ6	IFGTB AI 37	12.18	116	1.54	12.13	4
ACZ1	IFGTB AI 38	15.50	128	1.65	16.26	4
ACZ1	IFGTB AI 40	10.48	130	1.10	13.15	4
ACZ1	IFGTB AI 41	11.24	126	1.05	12.22	4

ACZ: Agroclimatic Zone; IFGTB AI: Institute of Forest Genetics and Tree Breeding *Azadirachta indica*

2.3.1. Physical properties

Oil color was determined visually and the odor was determined by the volatilized smell. The pH of the oil samples was analyzed using pH indicator strips (Merck, Germany). The refractive index (RI) of the oil was estimated using the standard method of AOAC (2007) and quantified using a pen Refractometer (Atago, Japan) with a resolution and accuracy value of ± 0.1 and ± 0.2%, respectively at 10-60

°C. The specific viscosity of the oil was measured according to the standard method ASTM (2003) at 30 °C using the Engler Viscometer and expressed in Degrees Engler (°Engler).

2.3.2. Chemical properties

Acid value. The acid value was determined according to the method described by AOAC (2007) and expressed as the KOH (in mg) necessary to neutralize the free fatty acids contained in 1 g of oil.

Saponification value. A measurement of the fatty acid chain length in oils was determined by standard procedures (AOAC, 2007) and expressed in milligrams KOH absorbed per gram of oil.

Iodine value. The iodine value was estimated by Oomah *et al.* (2000).

Peroxide value. The peroxide value was determined by Cox and Pearson (1962) and expressed in meq $O_2 \cdot kg^{-1}$ of oil.

Specific gravity. Specific gravity was measured following the standard method of AOCS (1997).

2.4. Statistical analysis

A statistical analysis was conducted using SPSS (v. 20). Analysis of variance followed by post-hoc (Duncan's Multiple Range Test (DMRT)) was performed at a 5% significance level. Correlation analyses were employed to find the relationship between the meteorological data and seed morphology with the oil contents. The dendrogram was constructed by the Ward method of cluster analysis on the Average distances between zones. To determine the robustness of the dendrogram, the data was bootstrapped with 1000 replicates.

3. RESULTS

3.1. Variations in plus trees and morphometric characteristics of the seeds

The tree height of the individual trees varied from 8.98 m in IFGTB AI 29 to 17.22 m in IFGTB AI 32; whereas the highest GBH was recorded in IFGTB AI 28 (186 cm) and the lowest value in IFGTB AI 29 (110 cm) (Table 2). Variations were also observed among the different ACZs. The GBH and seeding behavior among the ACZs showed significant ($P < 0.05$) variations. The highest GBH was recorded in ACZ 5 (186 cm), while ACZ 3 recorded the lowest (125 cm) values. Seeding behavior was highest in ACZ 3 and lowest in ACZs 1 and 6. A strong (0.531) negative correlation was observed between seeding behavior and total annual rainfall (data not shown), indicating the influence of yearly rainfall on seeding behavior. Crown diameter, however, did not show any relation to seeding behavior.

Significant variations were recorded among the 28 Candidate plus trees for all seed characters (Table 3). The highest seed length was recorded in IFGTB AI-1 (2.11 cm) followed by IFGTB AI 38 (2.07 cm); whereas the lowest values were registered for IFGTB AI 33 (1.05 cm). Seed breadth was the highest in IFGTB AI 41 (1.09 cm) followed by IFGTB AI 38 (0.91cm) and lowest in IFGTB AI 20 (0.56cm). The 100-seed weight varied from 10.14 g in IFGTB-17 to 36.38g in IFGTB AI - 1. The highest hundred-kernel weight of 19.93 g was recorded for IFGTB AI- 1 followed by 15.44 g for IFGTB AI - 38. The pericarp percentage showed significant differences among all the plus trees. Three plus trees viz., IFGTB AI 14 (60%), IFGTB AI 22 (59.46%) and IFGTB AI 23 (59.02%) showed superiority in pericarp percentage. IFGTB AI-41 recorded the highest pericarp weight (17.25 g) and the lowest (5.12 g) was recorded for IFGTB AI 20. The maximum kernel weight was recorded for IFGTB AI 1 (19.93 g) and the minimum was for IFGTB AI 40 (5.18 g). (Table 3). The oil content of neem seed kernels exhibited large variations (25.06 to 45.45%) in different zones (Table 3). The maximum seed oil content was recorded for IFGTB AI 1 (45.45%) followed by IFGTB AI 38 (43.96%) and the minimum percentage was recorded for IFGTB AI 33 (25.06 %).

There were significant ($P < 0.05$) variations among the ACZs as well. ACZ5 recorded high values for seed length, 100-seed and kernel weight and oil percentage. However, ACZ 4 showed high values for breadth, pericarp weight and pericarp percentage (Table 4), suggesting fruits with thicker pericarps.

The correlation matrix of seed characteristics, oil content and rainfall are given in Table 5. Seed morphometric characteristics such as 100-seed weight ($r = 0.959$), 100-kernel weight ($r = 0.848$) and seed length ($r = 0.874$) recorded strong positive correlation with oil percentage of neem seeds. However, the correlation between seed breadth and oil percentage was negligible ($r = 0.08$). Rainfall influenced seed breadth ($r = 0.311$) and pericarp weight ($r = 0.308$). The total minimum and maximum rainfall influenced pericarp percentage (Table 5).

Table 3. Variations in the morphological characteristics of Neem seeds collected from different parts of Tamil Nadu

S.No.	Candidate Plus Trees	Seed Length (cm)	Seed Breadth (cm)	100-Seed weight (g)	100-Seed kernel weight (g)	Pericarp weight (g)	Pericarp (%)
1	IFGTB AI 1	2.11	0.68	36.38	19.93	8.2	46.25
2	IFGTB AI 2	1.56	0.69	20.64	9.47	8.2	45.57
3	IFGTB AI 3	1.69	0.7	26.72	13.95	9.45	55.65
4	IFGTB AI 4	1.45	0.66	21.68	9.76	7.62	47.19
5	IFGTB AI 6	1.37	0.65	17.77	9.55	6.23	52.99
6	IFGTB AI 9	1.5	0.64	16.19	8.97	6.79	39.23
7	IFGTB AI 10	1.46	0.59	17.32	12.95	5.98	47.82
8	IFGTB AI 12	1.59	0.66	25.06	9.6	7.35	42.30
9	IFGTB AI 13	1.53	0.58	17.67	8.97	5.16	50.69
10	IFGTB AI 14	1.32	0.84	13.92	6.48	10.32	59.78
11	IFGTB AI 16	1.54	0.67	22.12	11.96	7.94	56.84
12	IFGTB AI 17	1.23	0.65	10.14	4.98	6.8	44.38
13	IFGTB AI 18	1.52	0.67	16.93	8.47	7.28	54.71
14	IFGTB AI 19	1.45	0.65	16.52	8.6	7.06	42.54
15	IFGTB AI 20	1.55	0.56	25.11	12.95	5.12	14.55
16	IFGTB AI 21	1.6	0.66	21.88	10.95	7.81	43.79
17	IFGTB AI 22	1.38	0.88	15.27	7.47	11.55	59.24
18	IFGTB AI 23	1.5	0.8	18.62	10.5	11.1	58.80
19	IFGTB AI 28	1.85	0.68	26.89	14.95	9.16	48.99
20	IFGTB AI 29	1.35	0.73	19.28	10.46	10.34	44.26
21	IFGTB AI 31	1.65	0.74	17.26	13.95	10.88	48.23
22	IFGTB AI 32	1.54	0.76	22.99	9.96	10.75	58.74
23	IFGTB AI 33	1.05	0.65	11.71	5.48	7.54	32.69
24	IFGTB AI 34	1.32	0.84	13.26	6.75	12.65	47.97
25	IFGTB AI 37	1.29	0.78	12.83	5.98	11.75	47.62
26	IFGTB AI 38	2.07	0.91	26.9	15.44	11.2	52.28
27	IFGTB AI 40	1.28	0.72	12.46.	5.18	12.12	44.68
28	IFGTB AI 41	1.34	1.09	16.09	8.47	17.25	45.95
	Mean	1.50	0.71	19.52	10.07	9.05	47.63
	SEd	0.07	0.03	0.89	0.47	0.43	2.15
	Critical Difference	0.09	0.04	1.26	0.66	0.61	3.04

*Values are means of four replicates. IFGTB AI: Institute of Forest Genetics and Tree Breeding *Azadirachta indica.*

Table 4. Variations in morphometric traits in *Azadirachta indica* seeds from different agro-climatic zones (ACZ). Means compared using Duncan's Multiple Range Test (DMRT).

ACZ	Seed Length (cm)	Seed Breadth (cm)	100-Seed weight (g)	100-Seed kernel weight (g)	Pericarp weight (g)	Pericarp (%)	Oil (%)
ACZ1	1.54 ± 0.26b	0.72 ± 0.14ab	20.07 ± 6.65b	10.38 ± 3.91b	8.71 ± 3.03ab	48.77 ± 6.13b	33.89 ± 6.34b
ACZ2	1.52 ± 0.09b	0.67 ± 0.04b	16.93 ± 1.02b	8.47 ± 0.51b	7.28 ± 0.44b	54.71 ± 3.28ab	30.46 ± 1.83b
ACZ3	1.53 ± 0.1b	0.62 ± 0.06b	21.17 ± 3.92ab	10.83 ± 1.97b	6.66 ± 1.25b	33.63 ± 14.44c	33.99 ± 4.29b
ACZ4	1.44 ± 0.1b	0.84 ± 0.06a	16.95 ± 2.05b	8.99 ± 1.73b	11.33 ± 0.66a	59.02 ± 3.18a	30.57 ± 3.13b
ACZ5	1.85 ± 0.11a	0.68 ± 0.04b	26.89 ± 1.61a	14.95 ± 0.9a	9.16 ± 0.55ab	48.99 ± 2.94b	43.2 ± 2.59a
ACZ6	1.37 ± 0.21b	0.75 ± 0.07ab	16.22 ± 4.22b	8.76 ± 3.12b	10.65 ± 1.72a	46.59 ± 8.23b	29.25 ± 4.01b

*Values are means of four replicates. Means were compared using DMRT. The values denoted by a different letter indicate significant differences among treatments (**$p < 0.05$**).

Table 5. Correlation studies of seed parameters and oil content in *Azadiracta indica* with precipitation

Correlations	Seed Length	Seed Breadth	100-Seed Weight	100-Kernel Weight	Pericarp Weight	Pericarp %	Minimum Annual Rainfall	Maximum Annual Rainfall	Total Annual Rainfall	Oil %
Seed Length	1	0.065	.860**	.873**	-0.051	0.169	0.113	0.154	-0.128	.874**
Seed Breadth		1	-0.106	-0.094	.903**	.422**	0.148	0.141	.311**	-0.08
100-Seed Weight			1	.892**	-0.143	0.019	0.064	0.092	-0.198	.959**
100-Kernel Weight				1	-0.121	0.039	0.032	0.059	-0.164	.848**
Pericarp Weight					1	.368**	0.027	0.015	.308**	-0.144
Pericarp %						1	.418**	.444**	.535**	0.072
Minimum Annual Rainfall							1	.985**	.647**	0.115
Maximum Annual Rainfall								1	.609**	0.153
Total Annual Rainfall									1	-0.176
Oil %										1

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

Table 6. Variations in physical and chemical properties of Neem oil extracted from seeds collected from different parts of Tamil Nadu

S.No.	Candidate Plus Trees	Specific gravity ($g \cdot ml^{-1}$)	Refractive Index	Viscosity ($mm^2 \cdot s^{-1}$)	Moisture (%)	pH	Oil percentage	Peroxide value (meq $O_2 \cdot kg^{-1}$ oil)	Saponification value (mg $KOH \cdot g^{-1}$)	Iodine value ($gI \cdot 100\ g^{-1}$)	Acid value (mg $KOH \cdot g^{-1}$)
1	IFGTB AI 1	0.80	1.66	44.73	14.70	4.19	45.45	12.28	167.67	39.45	20.13
2	IFGTB AI 2	0.83	1.45	45.33	1.70	4.20	33.86	10.20	234.74	52.09	25.71
3	IFGTB AI 3	0.82	1.46	49.02	3.84	4.34	40.9	11.40	190.74	40.96	26.83
4	IFGTB AI 4	0.79	1.67	51.21	0.60	4.79	34.13	10.68	245.91	41.98	22.36
5	IFGTB AI 6	0.59	1.62	54.00	3.45	5.03	32.06	13.23	190.03	27.82	27.95
6	IFGTB AI 9	0.85	1.66	45.23	3.53	4.38	29.4	13.07	190.03	37.93	21.24
7	IFGTB AI 10	0.75	1.46	53.80	1.10	4.82	31	8.37	312.98	42.48	22.36
8	IFGTB AI 12	0.81	1.65	44.54	1.57	4.18	40.8	12.20	122.96	26.8	18.99
9	IFGTB AI 13	0.75	1.65	45.33	0.76	4.35	33.33	12.28	190.03	24.59	21.24
10	IFGTB AI 14	0.81	1.66	53.30	0.92	4.75	27.9	11.56	212.38	63.21	26.83
11	IFGTB AI 16	0.80	1.46	54.10	1.13	4.05	34.53	12.04	245.91	47.03	26.83
12	IFGTB AI 17	0.75	1.65	47.42	3.17	4.32	26	7.81	234.74	60.68	24.59
13	IFGTB AI 18	0.76	1.65	49.71	0.70	4.57	30.46	10.76	299.1	65.23	21.24
14	IFGTB AI 19	0.70	1.65	51.51	0.80	4.20	29.4	7.25	279.45	54.11	25.71
15	IFGTB AI 20	0.77	1.65	54.60	1.51	4.56	38.4	10.04	201.2	64.23	29.06
16	IFGTB AI 21	0.81	1.65	54.10	3.79	4.81	34.17	10.84	223.56	60.68	22.36
17	IFGTB AI 22	0.91	1.64	51.71	0.60	4.51	28.13	11.40	290.63	55.12	25.71
18	IFGTB AI 23	0.83	1.64	53.50	2.58	4.12	33	7.33	223.56	63.21	24.59
19	IFGTB AI 28	0.80	1.64	51.21	2.21	4.90	43.2	8.69	201.20	52.59	24.59
20	IFGTB AI 29	0.79	1.65	55.19	6.48	5.43	31.26	7.01	312.98	66.75	31.3
21	IFGTB AI 31	0.78	1.64	49.02	8.65	5.28	30.23	9.96	268.27	66.25	25.71
22	IFGTB AI 32	0.80	1.64	50.31	6.46	4.07	35.73	7.41	297.91	39.95	29.06
23	IFGTB AI 33	0.79	1.64	51.91	2.10	4.19	25.06	10.60	212.38	50.57	25.71
24	IFGTB AI 34	0.86	1.64	58.18	6.39	5.50	26.8	9.80	279.45	67.77	37.77
25	IFGTB AI 37	0.81	1.65	58.88	1.88	5.40	26.4	3.35	319.54	67.26	33.54
26	IFGTB AI 38	0.83	1.64	57.18	2.50	5.22	43.96	6.94	299.89	64.23	26.83
27	IFGTB AI 40	0.84	1.65	54.60	1.28	4.54	26.26	11.88	245.91	64.23	29.06
28	IFGTB AI 41	0.68	1.65	57.29	1.99	5.45	28.8	5.58	312.98	70.3	38.01
	Mean	0.78	1.62	51.67	3.08	4.64	32.87	9.78	243.07	52.76	26.26
	SEd	0.03	0.07	2.30	0.19	0.20	5.77	0.44	10.90	2.41	1.17
	Critical Difference	0.05	0.10	3.25	0.27	0.29	15.17	0.63	15.42	3.41	1.81

*Values are means of four replicates. IFGTB AI: Institute of Forest Genetics and Tree Breeding *Azadirachta indica.*

3.2. Physical properties

The physical properties of neem seed oil across the plus trees (Table 6) and zone-wise (Table 7) reveal significant differences only in specific gravity. No significant differences were observed among trees within the same zone. The highest specific gravity was observed in oil from ACZ4 (0.87) and the lowest in ACZ 3 (0.72). The viscosity of the neem oil in zones ranged from 44.8 to 53.59 $mm^2 \cdot s^{-1,}$ with values for individual trees ranging between 44.54 $mm^2 \cdot s^{-1}$ and 58.88 $mm^2 \cdot s^{-1}$. The oil's refractive index was the highest in IFGTB AI 4 (1.67) and the lowest in IFGTB AI 2 (1.45). The average pH in the seed oil was 4.64 and the maximum was recorded for IFGTB AI 34 (5.5) and minimum for IFGTB AI 16 (4.05) (Table 6).

3.3. Chemical properties

The chemical properties of neem seed oil are shown in Table 7. Except for peroxide values, all other chemical properties were significantly different ($P < 0.05$) across zones and among chemical properties (Table 7). Zone ACZ 2 recorded high values for both saponification and iodine values. ACZ 6, 5 and 3 recorded high values for saponification, acid and peroxide values, respectively. The highest saponification value in the oil was obtained for IFGTB AI 37 (319.54 mg $KOH \cdot g^{-1}$), followed by IFGTB AI 10, IFGTB AI 29 and IFGTB AI 41 (312.98 mg $KOH \cdot g^{-1}$), and the lowest was detected in IFGTB AI 12 (122.96 mg $KOH \cdot g^{-1}$) (Table 6). The acid value of the oil was found to be at its highest in IFGTB AI 41 (38.01 mg $KOH \cdot g^{-1}$) and its lowest in IFGTB AI 12 (18.99 mg $KOH \cdot g^{-1}$). The highest iodine value in the oil was found for IFGTB AI 41 (70.3 $gI \cdot 100\ g^{-1}$), followed by IFGTB AI 34 (67.77$g \cdot ml^{-1}$), with the minimum value recorded for IFGTB AI 13 (24.59 $gI \cdot 100\ g^{-1}$) (Table 6).

Clustering revealed that neem from ACZ 3 was distinct from the rest of the zones. In the second clade, neem from ACZ 5 was clustered separately. ACZs 1 and 6 were grouped together. Likewise, ACZs 2 and 4 were grouped together.

4. DISCUSSION

Cataloguing seed morphological features from natural populations is considered the first step in understanding the genetic variability of a species. Multiple factors could induce and maintain variation in seed features. Large seeds are favored as they produce large and vigorous seedlings. On the contrary, smaller seeds may have a better selection advantage due to broader and more effective dispersal (Eriksson 1999). Fruit and seed characteristics, namely weight, length, width, diameter, yield and oil content, are reported highly variable both within and among the provenances of neem (Kundu and Tigerstadt, 1998; Jindal *et al.*, 1999). The present study also revealed significant variation among individual trees (CPTs) and among agroclimatic zones. Kundu and Tigerstadt (1997) reported distinct clustering of neem provenances based on rainfall regions. A strong negative correlation was observed in the present study between seeding behavior and total annual rainfall, in line with earlier reports. Thus, it could be concluded that rainfall plays a crucial role in seed-bearing.

Seeds are influenced by various factors, such as geographic area, climate, genetic variability, agronomic conditions, plant morphology and physiology,

Table 7. Physical and chemical properties of neem seed oil from different agro-climatic zones (ACZ). Means compared using DMRT.

ACZ	Specific gravity	Refractive Index*	Viscocity* (mm^2/s)	pH*	Peroxide value* (meq $O_2 \cdot kg^{-1}$ oil)	Saponification value (mg $KOH \cdot g^{-1}$)	Acid value (mg $KOH \cdot g^{-1}$)	Iodine value ($g \cdot ml^{-1}$)
ACZ1	0.79 ± 0.09ab	1.60 ± 0.17	52.67 ± 6.36	4.74 ± 0.62	9.99 ± 2.62	240.71 ± 53.48abc	26.5 ± 4.94b	47.99 ± 14.07b
ACZ2	0.76 ± 0.08ab	1.65 ± 0.18	44.80 ± 4.81	4.58 ± 0.49	10.78 ± 1.16	299.60 ± 32.16a	20.16 ± 2.16c	66.36 ± 7.12a
ACZ3	0.72 ± 0.08b	1.65 ± 0.15	52.03 ± 5.29	4.52 ± 0.5	8.09 ± 3.66	197.81 ± 70.8bc	24.26 ± 4.32bc	59.77 ± 7.13ab
ACZ4	0.87 ± 0.09a	1.64 ± 0.16	52.99 ± 5.25	4.32 ± 0.47	11.86 ± 1.24	257.52 ± 44.45ab	23.51 ± 3.34bc	66.87 ± 7.51a
ACZ5	0.80 ± 0.09ab	1.64 ± 0.18	53.59 ± 5.75	4.91 ± 0.53	8.70 ± 0.93	190.35 ± 20.43c	38.07 ± 4.09a	52.68 ± 5.65ab
ACZ6	0.81 ± 0.08ab	1.65 ± 0.15	49.81 ± 6.13	4.61 ± 0.76	9.53 ± 2.07	268.07 ± 43.33a	26.50 ± 3.37b	54.68 ± 15.99ab

*Values are means of four replicates. Means were compared using DMRT. The values denoted by a different letter indicate significant differences between treatments ($p < 0.05$). *Not Significant

collection and storage of plant material (Fernandes *et al.*, 2019). Though our reports are consistent with reports on variability in seed length in neem seeds collected from five provenances in northern and western India (Kaura *et al.*, 1998), the 100-seed weight ranged from 10.14 to 36.38 g, while they report 0.8 to 3.5 g, indicating smaller seeds. Variation in seed parameters such as seed diameter, seed length, kernel-to-seed ratio, 100-kernel weight and 100-seed weight have also been reported by Gupta *et al.* (2012) in different provenances in Gujarat. Kumaran *et al.* (1993) reported high heritability for seed length, seed oil content, and 100-seed weight. These parameters could be a robust selection index for neem.

We also obtained significant correlations between oil content and 100-seed and kernel weight in accordance with their findings. Neem seeds collected from different locations in Tamil Nadu showed a positive correlation between oil content and the number of hours of sunshine (Sridharan *et al.*, 1998). As presented in Table 4, rainfall influenced seed breadth, pericarp weight and percentage, suggesting that rounder fruits with thicker pericarps could be observed in high rainfall areas. Seed and kernel weight, which positively correlated with oil content, can be considered promising traits for the early selection of seed sources. Similar results have been reported for other tree-borne oil seeds (Kaura *et al.* 1998).

Seed oil content varies among tree-borne oil seeds (Vollmann *et al.* 2007). Oil yield is also affected by tree age, seed extraction method, seed storage, and environmental factors. This variation in the present study and other seed morphological attributes presents us with a viable selection alternative from base seed material at a very early stage. This could be useful for the improvement of programs, especially considering that the neem is commercially important for its oil and azadirachtin contents. We recorded 14 to 60% oil content in neem seed kernels, similar to Kaura *et al.*, (1998) and Tomar *et al.* (2011), who report the influence of agroclimatic zones on oil content.

The present study recorded the highest oil percentage from ACZ 5, comprising alluvial soil, which is rich in minerals, especially potash. This may have contributed to its high oil content, as indicated by Devaranavadagi *et al.* (2003), who reported oil content variation due to climatic and site conditions. Sidhu *et al.* (2003) also reported low oil contents in neem from arid, saline and coastal regions.

The Bureau of Indian Standards (BIS) IS 4765: 1975 Specification for Neem Kernel Oil and Depulped Neem Seed Oil (Reaffirmed in 2018) prescribes a specific gravity range of 0.908-0.934 for neem oils. None of the samples fell within this range. The BIS also defined ranges for saponification value (188-205 mg $KOH \cdot g^{-1}$), iodine value (65-80 $gI \cdot 100\ g^{-1}$) and acid value (15 mg $KOH \cdot g^{-1}$).

Acid value is a relative measure of rancidity as free fatty acids, while iodine value defines the drying quality of the oil. The low iodine values obtained for the samples studied represents the fewer unsaturated bonds, indicating that the low tendency of the oil to undergo oxidative rancidity. Accordingly, the peroxide value, an indication of the rate of rancidity was also low (Table 7). The presence of water or moisture contributes to hydrolysis, thus leading to higher acid values, and reducing the storage capacity of the oil (Do *et al.*, 2022).

The saponification value, a measure of the average molecular weight of all the fatty acids in the sample in triglycerides, varied from 190.35 to 299.6 mg/KOH. Hussein *et al.* (2021) also reported a wide range of variation in neem. A higher acid value increases saponification value (Hussein *et al.*, 2021), thus increasing the possible utilization of the oil in soaps and cosmetics. The proportion of each fatty acid in the oil may vary from tree to tree because of genetic make-up. A high saponification value implies the potential tendency to soap formation, and long-stored degraded oils are good for soaps and toiletry product productions (Hussein *et al.*, 2021). The prevalence of a wide variation in the neem accessions collected from different agroclimatic zones helped us classify the oil's utility prospects from the different agro-climatic zones. Genotypes with higher saponification values could be recommended for the cosmetic industry.

Morphometric techniques are valuable tools for exploring population differentiation, allowing more rigorous comparisons within a genus (Kolawole *et al.* 2016). Morphological characterization reveals diversity between germplasm. The clustering of zones based on seed morphology and oil content revealed that soil played a significant role in grouping the accessions. The saline coastal (ACZ 3) and rich alluvial (ACZ 5) zones remained distinct. However, it is to be noted that distinction based on morphological characteristics may not cluster genotypes from the

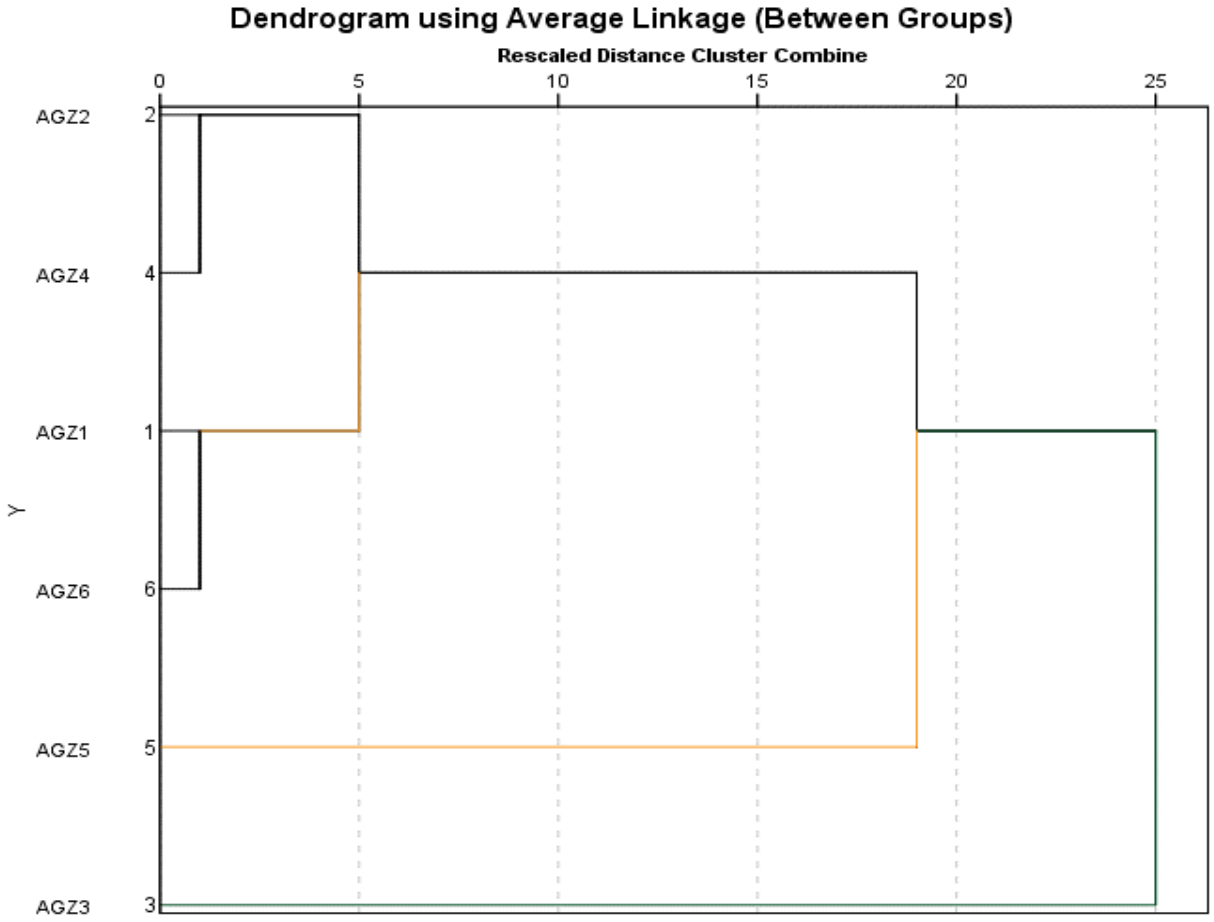

FIGURE 1. Dendrogram of neem from different Agro-climatic Zones (ACZ) studied based on Ward method using Average linkage.

same region within the same group (Figure 1). However, Kolawole *et al.* (2021) suggested phenetic dendrograms as the first step to grouping variables, dissecting the prominent features which influence the traits of interest, followed by a rigorous selection.

Understanding the variations in seed morphology and seed oil properties across agroclimatic zones allows for a comprehensive understanding of the species' adaptability and response to different climatic conditions. This knowledge can aid in selecting appropriate neem provenances for future planting in specific agroclimatic regions. Variations in seed morphology and seed oil properties have implications for various applications of neem products. Neem oil, derived from the seeds, is widely used in agriculture, medicine, cosmetics, and other industries. The quality and composition of neem oil is observed to vary based on geographical factors, which in turn affects its efficacy and suitability for different purposes.

Studying the seed morphology and seed oil properties across various agroclimatic regions in Tamil Nadu provided insights into the geographic variations within the neem species. This information contributes to the development of site-source matched provenances, helping in the selection of neem trees that are well-suited to specific agroclimatic conditions.

5. CONCLUSIONS

Cottage industries in Tamil Nadu extensively utilize neem for the production of various products. Neem oil, extracted from neem seeds, is a primary ingredient in the manufacturing of soaps, shampoos, hair oils, and other personal care items. Neem-based products from Tamil Nadu's cottage industries have the potential for export, contributing to the state's economy. With a wide distribution of the species in the state, assessing the extent of variability becomes pertinent. This paves the way for developing a defined tree program by targeting oil. Soil type and rainfall influenced oil content. Of the twenty-eight superior trees identified from six agro-climatic

zones, the oil content was the highest in those growing in alluvial soil. Fruit yield was also dependent on rainfall. Information on the influence of environmental variables is crucial to identify high oil-yielding populations. This would enable the selection of genotypes for developing improved and adapted cultivars. Superior genotypes from these selections can also be used for establishing large scale plantations.

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Kinetic and thermodynamic parameters of curcumin in edible oils with different degrees of unsaturation

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SUMMARY: The antioxidant activity of curcumin (0.02–0.1%) was evaluated in olive, sesame, and safflower oils at 373, 383, and 393 K. The results were examined in contrast to the effects of tocopherol (0.1%) and BHT (0.02%), so that the inhibitory function of curcumin was evaluated comparatively. The activation energy of oxidation was determined for olive (82.94 kJ·mol^{-1}), sesame (77.39 kJ·mol^{-1}) and safflower oils (74.42 kJ·mol^{-1}). Adding curcumin (0.1%) enhanced the activation energy by 26.26, 26.64, and 38.81% in the case of olive, safflower, and sesame oils, respectively. Based on Gibbs free energy, curcumin functioned more effectively in olive oil at 373 K (growth coefficient: 1.52%), compared to the action of the other two antioxidants, namely tocopherol (1.43%) and BHT (1.39%). The efficiency of curcumin was lower in oils which had a higher degree of polyunsaturation due to the disproportionation of the hydrogen-donating mechanism and the rate of free-radical formation in these oils.

KEYWORDS: *Activated complex theory; Antioxidant activity; Curcumin; Hydrogen donating mechanism; Lipid oxidation; Thermodynamic parameters.*

RESUMEN: ***Parámetros cinéticos y termodinámicos de la curcumina en aceites comestibles con diferentes grados de insaturación.*** La actividad antioxidante de la curcumina (0,02–0,1 %) se evaluó en aceites de oliva, sésamo y cártamo a 373, 383 y 393 K. Los resultados se contrastaron con los efectos del tocoferol (0,1 %) y del BHT (0,02%), por lo que se evaluó comparativamente la función inhibitoria de la curcumina. Se determinó la energía de activación de la oxidación para los aceites de oliva (82,94 kJ·mol^{-1}), sésamo (77,39 kJ·mol^{-1}) y cártamo (74,42 kJ·mol^{-1}). La adición de curcumina (0,1 %) mejoró la energía de activación en un 26,26 %, 26,64 % y 38,81 % en el caso de los aceites de oliva, cártamo y sésamo, respectivamente. Según la energía libre de Gibbs, la curcumina funcionó de manera más eficaz en aceite de oliva a 373 K (coeficiente de crecimiento: 1,52 %), en comparación con la acción de los otros dos antioxidantes; es decir, tocoferol (1,43 %) y BHT (1,39 %). La eficiencia de la curcumina fue menor en los aceites que tenían un mayor grado de poliinsaturación debido a la desproporción del mecanismo de donación de hidrógeno y la tasa de formación de radicales libres en estos aceites.

PALABRAS CLAVE: *Actividad antioxidante; Curcumina; Mecanismo de donación de hidrógeno; Oxidación de lípidos; Parámetros termodinámicos; Teoría del complejo activado*

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1. INTRODUCTION

Antioxidant addition is among the most effective strategies which can assist researchers and producers in preventing the chemical spoilage of vegetable oils. In fact, antioxidants are inhibitors of oxidation chain reactions which have different functions which emanate from their structural nature. In recent research, natural antioxidants have received a great deal of attention because of their safe applicability which entrusts public awareness and serves as reliable alternatives to the possible carcinogenicity of synthetic antioxidants. Curcumin (diferuloylmethane), has two phenolic rings and a beta-diketone part which is known as a natural antioxidant originating from saffron, clove, and turmeric. The antioxidant activity of this compound has been frequently reported by many researchers (Aliabbasi *et al.*, 2021; Barzegar, 2012; Hewlings and Kalman, 2017; Kumar *et al.*, 2016). In addition, curcumin plays a valuable role in the treatment and prevention of various types of diseases such as inflammatory, anti-cancer, anti-blood pressure, anti-fungal, anti-viral, anti-fibrotic, anti-ulcer, and anti-toxic activities. Also, curcumin plays important roles in food technology, including stability improvement, quality enhancement, and decreasing lipid oxidation (Mandal *et al*., 2023; Hewlings and Kalman, 2017). In general, antioxidant activity can be potentially related to the presence of phenolic groups in the chemical structure of antioxidants (Frankel, 2012). Two mechanisms of hydrogen donating and electron transfer are effective in the occurrence of antioxidant activity. Curcumin is assumed to have desirable antioxidant activity which can be attributed to having two phenolic rings. However, the antioxidant activity of curcumin can be affected by the presence of methoxy groups in the ortho position of benzene rings (in the proximity of hydroxyl groups) due to the high electronegativity of these groups (Barzegar, 2012).

Curcumin shows low bioavailability due to its high hydrophobicity. It is very common to use carriers to solve this defect and also to inhibit degradation caused by oxidation, or protection against environmental factors such as pH changes. On the other hand, the amount of its dissolution in vegetable oils is adequate. However, the log p of this antioxidant is 1.945, which is considered a relatively non-polar antioxidant due to the many functional groups in its structure. Different researchers have reported that antioxidants show good antioxidant activity in opposite environments in terms of polarity. In this way, the performance of curcumin in vegetable oils is expected to be favorable (Jokar *et al*., 2022; Malik *et al*., 2016; Malik *et al*., 2020; Waraho, *et al*., 2011).

Lipid oxidation is one of the main causes of quality loss in lipid systems. This reaction is accompanied by the production of harmful products that tend to threaten human health (Shahidi, 2005). The rate of this reaction is affected by temperature at an exponential rate, revealing an interdependence which is well explained by the Arrhenius equation (Farhoosh and Hoseini-Yazdi, 2014; Shim and Lee, 2011). In fact, this equation is part of the collision theory, based on which, effective collisions should take place between reactant molecules before a chemical reaction could occur. An effective collision occurs when the reactant molecules hit each other with sufficient energy and at the right angle. Specifically, it is the number of collisions within a certain timeframe that controls the reaction rate. Therefore, the reaction rate can change by any factor affecting the collision of reactants (e.g. temperature) (Kamal-Eldin and Yanishlieva, 2005). The increase in temperature can change the pathways of oxidation reactions and may cause performative defects in the occurrence of antioxidant activity. It should be noted that antioxidants can reduce effective collisions between oxidation reactants, although an important question is how much the performance of natural antioxidants can be affected by the operating conditions and by high temperatures, even as the antioxidants contain diverse functional groups (such as curcumin).

Hydroperoxides are among the most important precursors of the oxidation reactions of oils. Different oils produce different hydroperoxides, depending on the degree of unsaturation of their constituent fatty acids, which create several diverse secondary products, depending on oxidation reactions. Oils with more oleic acid, such as olive oil, produce more stable hydroperoxides, and oils such as sesame contain more linoleic acid, which generates more unstable hydroperoxides which break down more quickly into secondary oxidation products. Depending on their natural chemical structures, antioxidants indicate different functions against hydroperoxides. Thus, the performance of an antioxidant can be different in various oils (Laguerre *et al.*, 2020, Toorni and Golmakani, 2022).

Nevertheless, the present research aimed to investigate the potential application of a natural, avail-

able and inexpensive antioxidant such as curcumin. This research was planned in several sections. First, the performance of curcumin was investigated in various oils with different degrees of unsaturation. Second, the changes in the antioxidant activity of curcumin were checked by increasing concentration, and the best concentration was also determined. The effectiveness of curcumin was evaluated by increasing temperature, especially at temperatures above 100 °C. Furthermore, the efficacy of curcumin was compared to synthetic antioxidants in order to replace them. These sections have been established in order to develop detailed instructions so that this valuable antioxidant could be used effectively. In practice, different concentrations of curcumin were added to olive, sesame, and safflower oils, and the antioxidant activity of curcumin was measured by kinetic and thermodynamic equations, according to data obtained from the Rancimat device at different temperatures. To evaluate the inhibitory power of curcumin, its effectiveness was compared to natural (tocopherol) and synthetic (BHT) antioxidants.

2. MATERIALS AND METHODS

2.1. Materials

Refined, bleached, and deodorized safflower, sesame and olive oils which did not contain any added synthetic antioxidants were supplied by the Narges and Golbarg-e-Baharan companies. The oils were stored at -18 °C. Curcumin (CAS No. 458-37-7) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade, and standard markers were supplied by Sigma-Aldrich (St. Louis, MO) and Merck (Darmstadt, Germany). The antioxidant capacity of curcumin was measured by the DPPH assay according to the method described by Sanchez *et al*., (1998). The amount of IC_{50} for curcumin was 23.61 ± 3.45 μM.

2.2. Fatty acid composition

Fatty acid composition was determined by gas-liquid chromatography (Hewlett-Packard, 5890, Palo Alto, CA) according to the AOCS official method Ce 2-66 (Firestone, 2009). For this purpose, oils (0.3 g in 7 mL n-hexane) were converted into their corresponding fatty acid methyl esters by mixing with a solution of methanolic potassium hydroxide (2 mL of 7 N) for 15 min at 65 °C. The gas chromatography device was equipped with a flame ionization detector and a CP-FIL 88 column (with 60 m length, 0.22 mm internal diameter, and 0.2 μm film thickness) (Supelco Inc., Bellefonte, PA). The injector, detector, and oven temperatures were set at 225, 265, and 195 °C, respectively. Nitrogen gas was applied as carrier gas at a flow rate of 1 $mL \cdot min^{-1}$.

2.3. Induction period

A Rancimat device (Methrom, model 743 Herisau, Switzerland) was used for determining the induction periods (IPs) of the oils under study. In brief, a batch of 3 ± 0.001 g per oil, containing 0.02, 0.06, or 0.1% (w/w) curcumin, 0.1% (w/w) tocopherol, or 0.02% (w/w) BHT was weighed in separate reaction vessels and placed into the heating channel of the Rancimat. The airflow rate was regulated on 20 $L \cdot h^{-1}$, and the temperature inside the conductivity tube was constantly maintained at 25 °C. The oxidative stability of the oils was automatically determined at 373, 383, and 393 K by Apparatus software.

2.4. Analysis of kinetic and thermodynamic data

Temperature coefficient (T_C, 1/K) was calculated by the slope (parameter a) of the curve which fitted as follows:

$$\text{Log IP} = T_C.T + \log IP_0 \quad \text{Eq. (1)}$$

where T is temperature (K), and IP_0 is IP at a reference temperature. The temperature acceleration factor was embodied in the Q_{10} number, showing the increase in reaction rate due to a 10 °C rise in temperature, as obtained from Eq. (2).

$$Q_{10} = \frac{\text{IP at T °C}}{\text{IP at (T+10) °C}} \rightarrow Q_{10} = 10^{-10T_C} \quad \text{Eq. (2)}$$

According to Eq. (3) (Arrhenius equation), the activation energy (E_a) and frequency factor (A) of the secondary oxidation products were calculated by plotting the natural logarithm of k (1/IP) vs the 1/RT.

$$\ln k = -\left(\frac{E_a}{RT}\right) + \ln A \quad \text{Eq. (3)}$$

where R and T represent the molar gas constant (8.3143 J/mol K) and temperature (K), respectively.

According to Eq. (4) and Eq. (5), the Arrhenius equation parameters were calculated using the obtained slope (a) and intercept (b) from Eq. (3).

$$E_a = -a \quad \text{Eq. (4)}$$

$$A = \exp(b) \quad \text{Eq. (5)}$$

Enthalpy (ΔH^{++}) and entropy (ΔS^{++}) of activation were determined by the Eyring equation (Eq. (6)) via drawing ln (k/T) against 1/RT.

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \left(\frac{\Delta S^{++}}{R}\right) - \left(\frac{\Delta H^{++}}{RT}\right) \quad \text{Eq. (6)}$$

where k_B and h are the Boltzmann constant (1.380658 × 10^{-23} J K^{-1}) and Planck's constant (6.6260755 × 10^{-34} J s), respectively. The values for enthalpy and entropy were calculated using the slope and intercept related to the mentioned relationship, according to Eq. (7) and Eq. (8).

$$\Delta H^{++} = -a \quad \text{Eq. (7)}$$

$$\Delta S^{++} = \left[b - \ln\left(\frac{k_B}{h}\right)\right] \times R \quad \text{Eq. (8)}$$

ΔH^{++} and ΔS^{++} were used to determine the Gibbs free energy equation according to Eq. (9).

$$\Delta G^{++} = \Delta H^{++} - T\Delta S^{++} \quad \text{Eq. (9)}$$

The percentage of changes (C%) in the kinetic parameters, relevant to inhibited oxidation, were compared to non-inhibited and were calculated according to the following equation:

$$C\% = \left(\sum_{i=1}^{3}([AH]_i(X_i - X_c)100/X_c)\right) / \sum_{i=1}^{3}[AH]_i \quad \text{Eq. (10)}$$

where X_i and X_c are kinetic parameters in the presence and absence of the antioxidant, respectively.

The percentage of changes in ΔG^{++} due to temperature variations was determined using linear regression between the natural logarithm of changes (%) in ΔG^{++} and temperature. A decrease in the effectiveness of antioxidants (Ef_d) due to an increase in temperature of 10 °C was measured by the slope (a) of said relationship according to Eq. (11).

$$Ef_d = \exp(10a) \quad \text{Eq. (11)}$$

2.5. Statistical analysis

All evaluations were carried out in triplicate, and the analysis of variance (ANOVA) of the results was performed by SPSS Software. Significant differences among means were compared by Duncan's statistical test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Oxidative potential of oils

The fatty acid composition of each oil showed a significant difference from other oils in terms of linoleic (C18:2), oleic (C18:1), and palmitic (C16:0) acids (Table 1). The highest amount of saturated fatty acids was found in olive oil, followed by sesame and safflower oils. The highest value for monounsaturated fatty acids, an important symbol of oxidative stability, was observed in olive oil; whereas no significant difference was observed between safflower and sesame oils in this respect. As expected, the highest

Table 1. Fatty acids composition (%) of different vegetable oils.

Fatty acid	Safflower	Sesame	Olive
C12:0	0.04 ± 0.00^{b}*	0.12 ± 0.01^{a}	0.08 ± 0.01^{a}
C14:0	0.07 ± 0.01^{a}	0.07 ± 0.00^{a}	0.03 ± 0.00^{b}
C16:0	5.74 ± 0.11^{c}	8.28 ± 0.16^{b}	13.43 ± 0.06^{a}
C16:1	0.12 ± 0.13^{b}	0.17 ± 0.01^{b}	0.91 ± 0.02^{a}
C18:0	2.55 ± 0.19^{b}	4.56 ± 0.18^{a}	2.49 ± 0.09^{b}
C18:1	32.91 ± 0.37^{b}	32.67 ± 0.13^{c}	69.33 ± 0.13^{a}
C18:2	58.06 ± 0.27^{a}	53.36 ± 0.11^{b}	12.44 ± 0.13^{c}
C18:3 (n-3)	0.06 ± 0.01^{b}	0.20 ± 0.14^{b}	0.62 ± 0.02^{a}
C18:3 (n-6)	0.17 ± 0.02^{b}	0.17 ± 0.01^{b}	0.27 ± 0.01^{a}
C20:1	0.28 ± 0.02^{b}	0.41 ± 0.01^{a}	0.41 ± 0.00^{a}
SFA **	8.40 ±0.20^{c}	13.03 ± 0.04^{b}	16.02 ±0.12^{a}
MUFA	33.30 ±0.40^{b}	33.25 ± 0.13^{b}	70.66 ±0.15^{a}
PUFA	58.30 ±0.55^{a}	53.72 ± 0.16^{b}	13.32 ±0.13^{c}

* All experiments were performed in triplicate. The results were checked using ANOVA and were compared by Duncan's test ($P < 0.05$). In each row, averages (± standard deviation) with different lowercase letters are statistically different.
** Saturated fatty acids; Monounsaturated fatty acids; Polyunsaturated fatty acids.

and lowest polyunsaturation degrees (presented as the nutritional index of the oils) belonged to safflower and olive oils, respectively. Since the average relative rates of oxidation, pertaining to linolenic, linoleic, oleic, and stearic fatty acids were 2500, 1200, 100, and 1, respectively (Hsieh and Kinsella, 1989). The results provided information on the antioxidant activity of curcumin in the diverse lipid systems and in terms of degree of unsaturation.

3.2. Curcumin and oxidative stability period

The absolute values for the oxidation IPs are shown in Table 2. As predicted, the control samples exhibited different levels of oxidative stability. Even though the oxidative stabilities of the oils were not doubled compared to each other, this trend was somewhat maintained as temperatures rose from 373 to 393 K. The results showed that the addition of curcumin increased the oxidative stability of the oils. Despite the fact that increasing the concentration of curcumin improved the oxidative stability of the oils, the growth slope of the IP was unique for each oil (Figure 1a). Such contradictory behavior of curcumin can be attributed to the occurrence of complex chemical reactions. These reactions are most likely due to the residual radical of curcumin after releasing $[H]^+$ and by the excitation of oxidation chain reactions, whereby lipid substrates are attacked (Fennema, 1996). Thus, the best and most economical concentration of curcumin was 0.02% in the bulk oils of this study. Also, the growth slope of IPs, due to increasing temperature eventuated in a significant decrease which appeared more noticeable in the sesame oil. By comparing the performance of the antioxidants in sesame oil, the presence of curcumin at a concentration of 0.02% could be seen as a competitive approach in comparison with the function of tocopherol and BHT. Nonetheless, the behavioral pattern of curcumin requires further discussion on specific measurements and detail in comparison with other antioxidants so as to elucidate the inhibitory mechanism of this valuable natural antioxidant.

TABLE 2. Induction periods (IPs) and thermal kinetic parameters of olive, sesame, and safflower oils in the presence of different concentrations of curcumin, tocopherol, and BHT and at different temperatures.

Oil	AO	*C* (%)	IP (h)			T_C (×10-2)	Q_{10}
			373 K	383 K	393 K		
Safflower	Con	-	4.02 ±0.08^{o}*	2.15 ±0.04^{l}	1.19 ±0.02^{m}	-2.65 ±0.07^{j}	1.84 ±0.03^{j}
	CUR	0.02	6.93 ±0.03^{n}	3.52 ±0.10^{k}	1.65 ±0.03^{k}	-3.12 ±0.05^{f}	2.05 ±0.02^{f}
	CUR	0.06	7.42 ±0.03^{m}	3.66 ±0.03^{k}	1.59 ±0.04^{k}	-3.35 ±0.06^{de}	2.16 ±0.03^{de}
	CUR	0.10	7.96 ±0.06^{k}	3.64 ±0.03^{k}	1.70 ±0.05^{j}	-3.36 ±0.05^{d}	2.17 ±0.02^{d}
	TCP	0.10	9.07 ±0.03^{i}	3.94 ±0.03^{j}	1.72 ±0.02^{j}	-3.61 ±0.01^{c}	2.30 ±0.01^{c}
	BHT	0.02	8.44 ±0.06^{j}	4.15 ±0.03^{i}	1.45 ±0.05^{l}	-3.82 ±0.06^{a}	2.41 ±0.03^{a}
Sesame	Con	-	7.81 ±0.03^{l}	3.81 ±0.10^{j}	2.20 ±0.03^{i}	-2.75 ±0.02^{i}	1.89 ±0.01^{i}
	CUR	0.02	15.36 ±0.04^{h}	7.16 ±0.04^{g}	2.83 ±0.03^{h}	-3.67 ±0.02^{b}	2.33 ±0.01^{b}
	CUR	0.06	16.50 ±0.08^{g}	7.31 ±0.03^{f}	2.98 ±0.07^{fg}	-3.72 ±0.06^{b}	2.35 ±0.03^{b}
	CUR	0.10	16.64 ±0.24^{g}	6.63 ±0.09^{h}	2.86 ±0.06^{g}	-3.83 ±0.07^{a}	2.41 ±0.04^{a}
	TCP	0.10	13.93 ±0.03^{b}	7.09 ±0.02^{g}	3.10 ±0.03^{f}	-3.26 ±0.03^{e}	2.12 ±0.01^{e}
	BHT	0.02	16.99 ±0.07^{f}	7.39 ±0.09^{f}	3.51 ±0.04^{e}	-3.42 ±0.03^{d}	2.20 ±0.03^{d}
Olive	Con	-	15.41 ±0.08^{h}	7.31 ±0.04^{f}	3.96 ±0.04^{d}	-2.95 ±0.02^{h}	1.97 ±0.01^{h}
	CUR	0.02	22.38 ±0.08^{e}	9.84 ±0.05^{e}	4.23 ±0.03^{c}	-3.62 ±0.02^{c}	2.30 ±0.01^{c}
	CUR	0.06	25.98 ±0.02^{d}	11.07 ±0.03^{d}	4.92 ±0.04^{b}	-3.61 ±0.2^{c}	2.30 ±0.01^{c}
	CUR	0.10	27.86 ±0.06^{a}	11.89 ±0.07^{b}	5.00 ±0.06^{b}	-3.73 ±0.02^{b}	2.36 ±0.01^{b}
	TCP	0.10	27.16 ±0.05^{b}	13.21 ±0.04^{a}	6.72 ±0.03^{a}	-3.03 ±0.01^{g}	2.01 ±0.01^{g}
	BHT	0.02	26.52 ±0.04^{c}	11.49 ±0.08^{c}	4.99 ±0.02^{b}	-3.63 ±0.00^{e}	2.31 ±0.00^{e}

* All experiments were performed in triplicate. The results were checked using ANOVA and were compared by Duncan's test ($P < 0.05$). Means ± SD (standard deviation) within a column with the same lowercase letters are not significantly different.
AO: antioxidant; *C*: antioxidant concentration; Con: control sample; CUR: curcumin; TCP: tocopherol; BHT: butylated hydroxytoluene; T_C: temperature coefficient (K^{-1}); Q_{10}: temperature acceleration factor.

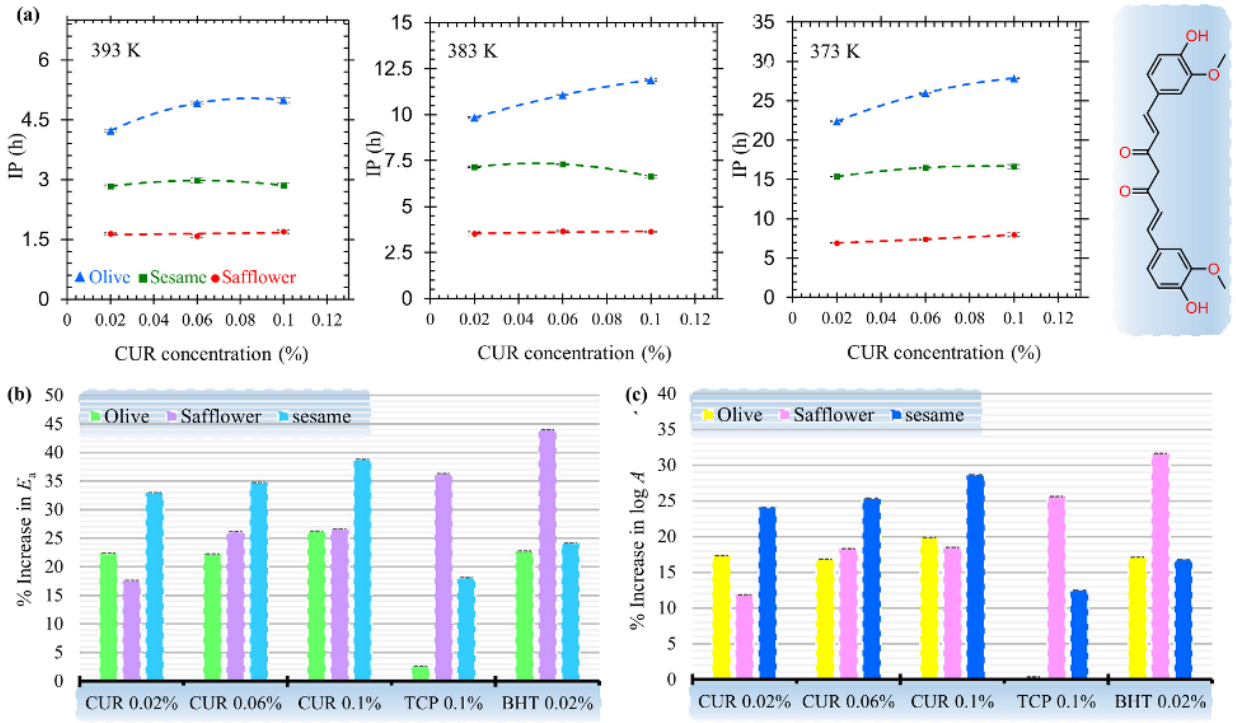

Figure 1. (a) Relationship between curcumin (CUR) concentration and induction period (IP) for the oxidation of different vegetable oils at 373, 383, and 393 K. **(b,c)** Percentage of increase in activation energy (E_a) or logarithm of frequency factor (Log A) compared to the control samples for the oxidation of different vegetable oils in the presence of various concentrations of curcumin (CUR), tocopherol (TCP), and butylated hydroxytoluene (BHT) at 373, 383, and 393 K. All experiments were performed in triplicate. The results were checked using ANOVA and were compared by Duncan's test ($P < 0.05$).

3.3. Temperature and curcumin function

It is clear that the rise in temperature is a critical parameter in consideration when monitoring the performance of antioxidants. The role of heat is important in two aspects. First, there are the potential effects on the mechanistic pathway of the oxidation process. It has been confirmed that the general trend of the oxidation process tends to change at temperatures higher than 60 °C (Zheng *et al.*, 2020). Although the reaction rate also increases, the effect of the pathway change is much higher than the reaction rate (Keramat *et al.*, 2021). Second, the effects of temperature on antioxidant molecules which can cause decomposition or evaporation. Meanwhile, heat can convert the antioxidants into per-oxidant compounds. The T_c and Q_{10} indices are considered very important tools for evaluating the performance of antioxidants against temperature rise. The percentage of changes in these parameters was lower in olive oil, with the highest amount of oleic acid, compared to the sesame and safflower oils (Table 2). The occurrence of such changes is likely related to the higher resistance of oleic acid at high temperatures.

In general, according to the data in Table 2, it was revealed that the shelf-life of oils in the presence of curcumin has no significant differences with the samples containing synthetic antioxidants. The observation of such effects reveals the favorable inhibitory power of curcumin.

3.4. Parameters of Arrhenius model

Oxidation reactions of the unsaturated oils tend to occur under any circumstances, while heat accelerates the rate of these reactions like a catalyst. The parameters of the Arrhenius equation actually represent the relationship between temperature and the occurrence of chemical reactions. This relationship has been frequently used in predicting the behavior of reactants and in estimating how many reaction products can be produced (Golmakani *et al.*, 2020a; Toorani and Golmakani, 2022; Zheng *et al.*, 2020). Since the amount of secondary oxidation products was a criterion for estimating Arrhenius parameters such as E_a, the measured parameter was introduced as the "activation energy of curcumin". In fact, the term refers to a rise in the required energy, created by

Table 3. Thermal kinetic parameters of olive, sesame, and safflower oils in the presence of different concentrations of curcumin, tocopherol, and BHT at different temperatures (373, 383, and 393 K).

Oil	AO*	C (%)	Arrhenius equation parameters		Eyring equation parameters		Gibbs free energy at 373–393 K		
			E_a	log A	$\Delta H^{\neq}$	$\Delta S^{\neq}$	$\Delta G^{\neq}_{373}$	$\Delta G^{\neq}_{383}$	$\Delta G^{\neq}_{393}$
Safflower	Con	-	74.4±.0^{i}†	13.4±.3^{j}	71.2±2.0^{j}	-135.5±0.0^{j}	121.8±0.0^{o}	123.1±0.0^{m}	124.5±0.1^{n}
	CUR	0.02	87.5±.4^{f}	15.0±.2^{f}	84.3±.4^{f}	-105.1±.7^{f}	123.5±0.0^{n}	124.6±0.0^{l}	125.6±0.1^{kl}
	CUR	0.06	93.9±.6de	15.8±0.2^{de}	90.7±.6de	-88.6±.2de	123.7±0.0^{m}	124.6±0.0^{l}	125.5±0.1^{l}
	CUR	0.10	94.2±.3^{d}	15.8±.2^{d}	91.0±.3^{d}	-88.1±.6^{d}	123.9±0.0^{l}	124.8±0.0^{k}	125.6±0.1^{kl}
	TCP	0.10	101.4±.4^{c}	16.8±.1^{c}	98.2±.4^{c}	-69.9±.1^{c}	124.3±0.0^{j}	125.0±0.0^{j}	125.7±0.0^{k}
	BHT	0.02	107.1±.6^{a}	17.6±.2^{a}	103.9±.6^{a}	-54.4±.3^{a}	124.2±0.0^{k}	124.6±0.0^{k}	125.3±0.0^{m}
Sesame	Con	-	77.4±.5^{i}	13.5±.1^{i}	74.2±.2^{i}	-132.9±.2^{i}	123.7±0.0^{m}	125.1±0.0^{i}	126.4±0.0^{j}
	CUR	0.02	102.9±.6^{b}	16.8±.1^{b}	99.7±.6^{b}	-70.5±.6^{b}	126.0±0.0^{g}	126.7±0.0^{h}	127.4±0.0^{i}
	CUR	0.06	104.3±.6^{b}	16.9±.2^{b}	101.1±.6^{b}	-67.3±.3^{b}	126.2±0.0^{f}	126.9±0.0^{g}	127.6±0.1^{h}
	CUR	0.10	107.4±.0^{a}	17.4±.3^{a}	104.2±.0^{a}	-58.8±.4^{a}	126.1±0.0^{f}	126.7±0.0^{h}	127.3±0.1^{i}
	TCP	0.10	91.4±.8^{e}	15.2±.1^{e}	88.2±.8^{e}	-100.5±.1^{e}	125.7±0.0^{i}	126.7±0.0^{h}	127.7±0.0^{g}
	BHT	0.02	96.1±.9^{d}	15.8±.13^{d}	92.9±.9^{d}	-89.3±.5^{d}	126.2±0.0^{f}	127.1±0.0^{f}	128.0±0.0^{f}
Olive	Con	-	82.9±.6^{h}	14.0±.1^{h}	79.7±.6^{h}	-123.7±1.5^{h}	125.9±0.0^{h}	127.1±0.0^{f}	128.3±0.0^{e}
	CUR	0.02	101.5±.5^{c}	16.4±.1^{c}	98.3±.5^{c}	-77.2±.3^{c}	127.1±0.0^{e}	127.9±0.0^{e}	128.7±0.0^{d}
	CUR	0.06	101.4±.5^{c}	16.3±.1^{c}	98.2±.5^{c}	-78.6±.2^{c}	127.5±0.0^{d}	128.3±0.0^{d}	129.1±0.0^{c}
	CUR	0.10	104.7±.6^{b}	16.8±.6^{b}	101.5±.6^{b}	-70.4±.5^{b}	127.8±0.0^{a}	128.5±0.0^{b}	129.2±0.0^{b}
	TCP	0.10	85.1±.3^{g}	14.0±.0^{g}	81.9±.3^{g}	-122.7±.9^{g}	127.7±0.0^{b}	128.9±0.0^{a}	130.1±0.0^{a}
	BHT	0.02	101.8±.1^{c}	16.4±.0^{c}	98.6±.1^{c}	-77.8±.2^{c}	127.6±0.0^{c}	128.4±0.0^{c}	129.2±0.0^{b}

† All experiments were performed in triplicate. The results were checked using ANOVA and were compared by Duncan's test ($P < 0.05$). Means ± SD (standard deviation) within a column with the same lowercase letters are not significantly different.
AO: antioxidant; *C*: antioxidant concentration; Con: control sample; CUR: curcumin; TCP: tocopherol; BHT: butylated hydroxytoluene; E_a: activation energy (kJ·mol^{-1}); log *A*: logarithm of frequency factor (h^{-1}); $\Delta H^{\neq}$: activation enthalpy (kJ·mol^{-1}); $\Delta S^{\neq}$: activation entropy (J mol^{-1}); $\Delta G^{\neq}$: Gibbs free energy (kJ·mol^{-1}).

curcumin or other antioxidants, for lipid oxidation to occur less easily. The results of E_a showed that curcumin desirably increased the oxidative resistance of all oils even at 393 K (Table 3). One of the concerns before starting the research was the thermal degradation of curcumin and the destruction of its inhibitory function at high temperatures due to the presence of many functional groups in the chemical structure of curcumin. However, the results showed that this antioxidant is highly capable of resisting 393 K. The highest value for E_a was observed in sesame oil when using 0.1% curcumin, which was higher than in samples with any other antioxidant.

Compared to the control samples, the percentage of increase in E_a by curcumin ultimately led to finding the optimal performance of this antioxidant in the lipid systems under study (Figure 1b). The highest amount of this parameter was found in sesame oil – in response to different concentrations of curcumin. It has been proven that this factor cannot be considered exclusively as an appropriate criterion for predicting the intensity of antioxidant activity (Toorani *et al.*, 2021). It is clear that the function of an antioxidant is affected by both factors of the Arrhenius equation, namely E_a and *A*. The results of the *A* parameter (log *A*) showed that this factor increased significantly in the presence of curcumin. A powerful antioxidant is characterized by a high E_a and a low *A* value. Since the trend of changes in these two parameters was incremental, it became difficult to judge the optimal performance of curcumin. The percentage of increase in *A* showed a noticeable growth in all samples (Figure 1c). However, the lowest growth percent was observed in the safflower oil with a maximum amount of PUFA. It is clear that the variations in *A* values per antioxidant were proportional to the changes in E_a, so that a decrease in *A* significantly reduced the E_a value. The *A* value was mathematically defined as serving a reac-

tion rate whereby all reactants have enough energy for a chemical reaction to occur (i.e. $E_a = 0$) or in a case where the temperature inclines to infinity ($T \rightarrow \infty$) (Toorani and Golmakani, 2022). In fact, *A* is a criterion of reaction rate, showing the reactivity of lipid systems against oxidation. Therefore, considering the two Arrhenius parameters together is the most reliable way to understand how fast a reaction proceeds.

3.5. Thermodynamic indices

According to the activation complex theory, the reactants should form an intermediate compound by interconnection and structural rearrangement to facilitate the production of the final product. As an activated complex, it has more energy than the reactants and the products, thereby defining the overall rate of chemical reactions. The stored energy (ΔH^{++}) in this structure is consumed, while changing the length and angles of reactant bonds, thereby causing structural integrity and molecular rearrangement (Atkins *et al.*, 2014). ΔS^{++} is a symbol of environmental disorder and indicates an associative mechanism in which the reactants create an intermediate complex (Espenson, 1995). Higher values for ΔS^{++} show an increase in the possibility of creating activated complexes. In fact, the reaction is likely to occur by participating the fewer molecules of reactants. A lower ΔS^{++} value (more negative) and higher ΔH^{++} value are indicative of the high stability of lipid systems against temperature changes. According to Table 3, the highest and the lowest amount of ΔH^{++} among lipid systems in the non-inhibited condition was observed in olive and safflower oils, (79.7 vs. 71.2 kJ mol^{-1}), respectively. In the presence of curcumin (0.1%), however, maximum ΔH^{++} was observed in sesame oil (104.2 kJ mol^{-1}). The highest and lowest ΔH^{++}, considering all concentrations of curcumin, were observed in sesame and safflower oils, respectively. This can be attributed to a mismatch between the produced hydroperoxides and the function of inhibitory mechanisms of antioxidants (Toorani and Golmakani, 2021). As the precursor of oxidation products, the generated hydroperoxides in safflower oil were very unstable due to noticeable amounts of linoleic acid and the presence of allylic and bis-allylic carbons in its acyl chain. On the other hand, the presence of a methoxy group in the vicinity of the antioxidant agent (-OH), due to high electronegativity, decreased the rate of hydrogen release into the medium, thereby preventing quench free radicals (Barzegar, 2012). Thus, the tendency of curcumin to trap extremely active hydroperoxides is practically negligible.

The results indicated that adding curcumin to the oils increased ΔS^{++} in all samples. The highest and lowest values for ΔS^{++} were observed in sesame oil with 0.1% curcumin and in safflower oil with 0.02% curcumin, respectively. However, none of the parameters of the Eyring equation alone could predict the optimal performance of curcumin or any other antioxidant.

An analysis of the results showed that variations in the Eyring and the Arrhenius parameters were interdependent. Two appropriate linear models were obtained between the parameters of the Eyring and Arrhenius equations (Figure 2a and Figure 2b). These relationships were identical in the case of each oil with different unsaturation degrees. The obtained experimental models were quite similar to the equations reported by Farhoosh and Hoseini-yazdi (2014). Specifically, the slopes were identical, whereas the intercepts were slightly different. According to the transition state theory, the Eq.s (12) and (13) show the involved variables for converting the parameters of Eyring and Arrhenius models to each other.

$$A = \left(\frac{Tk_b}{h}\right) + exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \quad \text{Eq. (12)}$$

$$E_a \sim \Delta H^{++} \quad \text{Eq. (13)}$$

3.6. Curcumin performance based on Gibbs free energy

It is proven that ΔG^{++} is a suitable parameter for the purpose of comparing the oxidative stability of oils (Golmakani *et al.*, 2020b; Veloso *et al.*, 2020). Higher values for ΔG^{++} indicate higher stability of the lipid systems against oxidation reactions. In olive and sesame oils, the ΔG^{++} values showed that curcumin performance was better than tocopherol and BHT at 373 K (Table 3). However, higher temperatures slightly reduced the effectiveness of curcumin, since the ΔG^{++} was more affected by ΔS^{++} values than by ΔH^{++}. Accordingly, more negative ΔS^{++} values further increased the ΔG^{++}, which means an increase in oxidative stability. In safflower oil, curcumin was less effective than tocopherol and BHT in enhancing oxidative stability at 373 K.

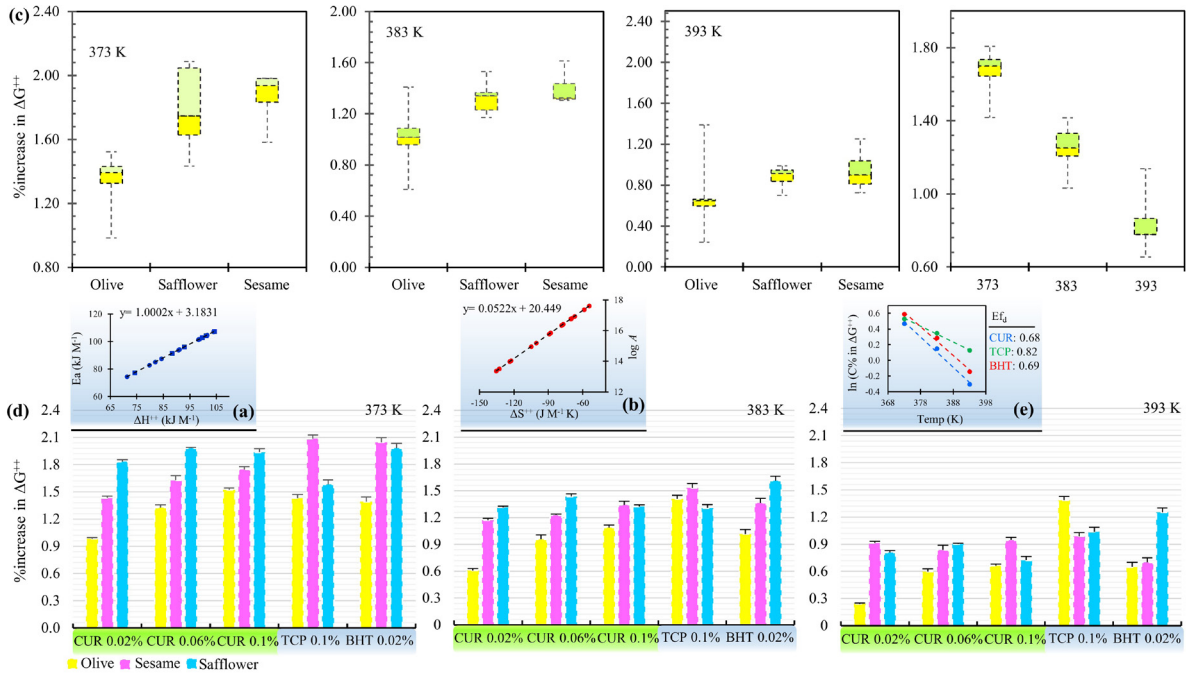

Figure 2. (a,b) Linear relationships between the changes in activation energy (E_a) and activation enthalpy (ΔH^{++}) or logarithm of frequency factor (log A) and activation entropy (ΔS^{++}) for the oxidation of olive, sesame, and safflower oils in the presence of various concentrations of curcumin, tocopherol, and butylated hydroxytoluene (BHT) at 373, 383, and 393 K. **(c)** Scattering curve of increase in Gibbs free energy (ΔG^{++}) for the oxidation of different vegetable oils in the presence of various concentrations of curcumin at 373, 383, and 393 K, and scattering curve of increase in Gibbs free energy (ΔG^{++}) for all oils at 373, 383, and 393 K. **(d)** Percentage of increase in ΔG^{++} compared to the control samples for the oxidation of different vegetable oils in the presence of various concentrations of curcumin (CUR), tocopherol (TCP), and butylated hydroxytoluene (BHT) at 373, 383, and 393 K. **(e)** A decrease in the effectiveness of antioxidants (Ef_d) under study due to temperature increase. All experiments were performed in triplicate. The results were checked using ANOVA and were compared by Duncan's test ($P < 0.05$).

Nonetheless, this trend changed at higher temperatures (383 and 393 K) and made curcumin show no significant difference in function compared to the other two antioxidants. While increasing the temperature enhances the effect of entropy coefficient in the equation of ΔG^{++} (Eq. (9)), changes in this index are further revealed at higher temperatures. In safflower oil, ΔH^{++} was much lower than in the other two oils, and the curcumin effect was more apparent in ΔS^{++}. It should be noted that the increase in ΔG^{++}, due to rising temperatures, arose from the endothermic nature of the active complex taking form during the oxidation reactions (Toorani and Golmakani, 2022).

The changes in ΔG^{++} at different temperatures indicated a lower dispersion of data pertaining to various lipid systems (Figure 2c). A lack of outlier data reflected the accuracy of the calculations and normality of the data. Also, the size of the box plots of the lipid systems was different, meaning that the growth coefficient in curcumin efficiency differed per oil when the curcumin concentration increased. Furthermore, the distribution of data indicated an increase in their dispersion, parallel to the increase in temperature. This can be attributed to a high degree of antioxidant susceptibility to heat, the decomposition caused thereof, and/or loss via volatilization, which is known as the carry-through property (Dugan and Kraybill, 1956).

Based on the percentage of increase in ΔG^{++}, the best performance of curcumin was observed in sesame oil, where its efficiency was higher than tocopherol but not significantly different from BHT (Figure 2d). Surprisingly, curcumin (0.02%) showed optimal performance, although the antioxidant activity of curcumin was less than that of the other two antioxidants in olive and safflower oils. The difference in the function of curcumin in various oils is likely due to a difference in the types of active radicals which were produced and of the hydroperoxides in these oils, which were not well adapted to the inhibitory mechanisms of this antioxidant. In all samples containing curcumin, the results showed that the percentage of growth in ΔG^{++} decreased in a consistent manner when the temperature increased. Also, a similar trend was observed in the case of tocopherol, but the decline in growth was lower in the presence of

BHT, possibly because of the high resistance of BHT to evaporation and thermal degradation. It is clear that an increase in temperature reduced the effectiveness of all antioxidants. The Ef_d of curcumin was 0.68 by an increase of 10 K (regardless of oil type and antioxidant concentration) (Figure 2e).

3.7. Relationship between IP and ΔG^{++}

As can be seen in Figure 3, power equations with high correlations ($R^2 > 0.99$) can properly describe the relationship between the values for IP and ΔG^{++}. The obtained nonlinear relationships indicated that the inhibitory power of phenolic antioxidants did not increase linearly by an increase in their concentration. This approach did not change fundamentally by increasing the temperature. On the other hand, the obtained exponential equations enable predictions of ΔG^{++} using simple data such as IP. It is clear that the estimation of ΔG^{++} at various temperatures can enable suitable assessments of the efficiency of antioxidants.

4. CONCLUSIONS

This research revealed that curcumin is substantially capable of antioxidant activity and can compete with synthetic antioxidants such as BHT. Also, curcumin showed different abilities to inhibit the oxidative reactions in different lipid systems. While curcumin showed appropriate antioxidant activity in lipid systems with higher unsaturation degrees, a maximum achievable efficiency was not observed. This result was attributed to a mismatch between the hydrogen donating mechanism of curcumin and the rate of hydroperoxide production by polyunsaturated systems, which were likely caused by the presence of methoxy groups in the benzene rings of the chemical structure of curcumin. Since the hydroxyl group in the phenolic ring is directly responsible for showing antioxidant activity, the presence of the methoxy group in its vicinity increases the energy required for separating hydrogen from the hydroxyl group. This phenomenon can be related to the high electronegativity of the methoxy group. As a result, the hydrogen donating mechanism was impaired in its function of quenching more reactive radicals produced by polyunsaturated fatty acids. Therefore, it is suggested that this natural antioxidant can be used in bulk oils with lower unsaturation degrees like olive or sesame oils in a concentration of 0.1% to achieve maximum antioxidant capacity. Furthermore, the results of the present study proved that curcumin is not thermally decomposed in the temperature range of 373-393 K, and that its antioxidant activity does not undergo fundamental changes. The results of this project do not necessarily suggest that synthetic antioxidants such as BHT can be generally replaced with curcumin, but that replacing a portion of synthetic antioxidants with this valuable herbal compound can be useful.

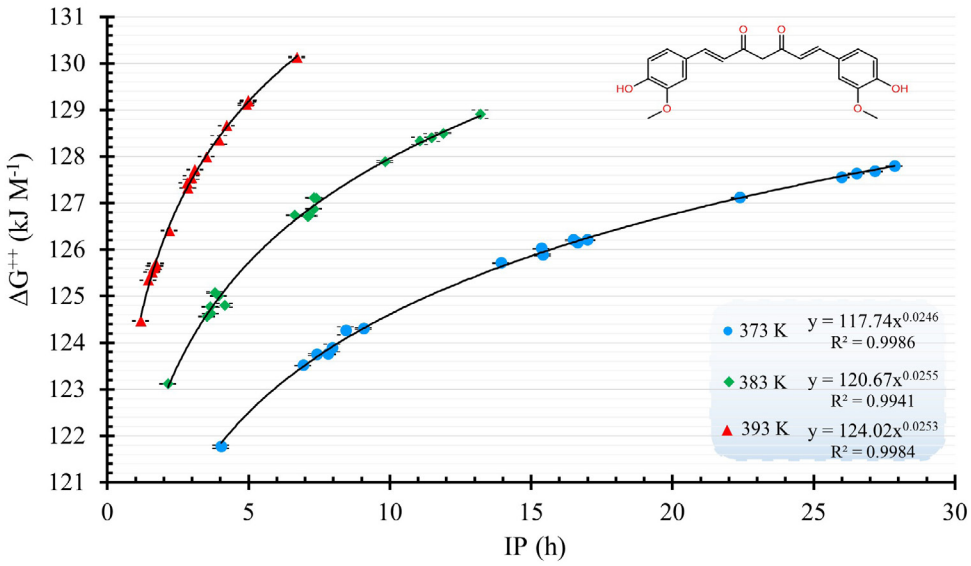

Figure 3. Relationship between Gibbs free energy (ΔG^{++}) of the activated complex formation and induction period (IP) for the oxidation of different vegetable oils in the presence of different concentrations of curcumin at 373, 383, and 393 K. All experiments were performed in triplicate. The results were checked using ANOVA and were compared by Duncan's test ($P < 0.05$).

5. ACKNOWLEDGMENTS

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Influence of the impurity content on the density and viscosity of olive oily fluids

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SUMMARY: In this work, as a case study, the measurement of the density (ρ) and the dynamic viscosity (μ) of 12 different fluids (taken from a conventional oil mill) has been carried out. The variability of the samples processed shows that their impurity contents c (between 0.5–5.87%), together with the temperature t (which varied between 20–30 ºC), can affect the values of ρ and μ. However, this variation has been shown to be different depending on the case, being of the order of 1% for density or even more than 50% for dynamic viscosity. The fact that μ can be sensitive to the presence of impurities opens up a line of study that could be used to estimate such impuritiy content, in real time, by means of relatively simple methods.

KEYWORDS: *Impurities; Mill; Olive Oil; Process; Temperature; Water*

RESUMEN: ***Influencia del contenido de impurezas sobre la densidad y viscosidad de fluidos oleosos.*** En este trabajo, como caso de estudio, se ha llevado a cabo la medida de la densidad (ρ) y de la viscosidad dinámica (μ) de 12 fluidos diferentes tomados de una almazara convencional. La variabilidad de las muestras procesadas indica que el contenido de impurezas c (entre el 0,5%–5,87 %), junto con la temperatura t (que varió nominalmente entre 20 ºC–30 ºC), pueden afectar a los valores de ρ y μ. Sin embargo, esta variación se ha mostrado diferente dependiendo del caso, siendo del orden del 1 % para la densidad o incluso mayor del 50% para la viscosidad dinámica. El hecho de que μ pueda ser sensible a la presencia de impurezas abre una línea de estudio que podría ser aprovechada para estimar tal contenido de impurezas, en tiempo real, por medio de métodos relativamente sencillos.

PALABRAS CLAVE: *Aceite de Oliva; Agua; Almazara; Impurezas; Proceso; Temperatura*

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1. INTRODUCTION

The production of olive oil requires a series of unit operations which, applied to the incoming raw olives, serve to obtain the final product. Focusing our attention on the intermediate and final stages of this process, it should be noted that the production fluids treated are usually accompanied by a series of undesirable impurities (Dammak *et al*., 2015; Gila *et al*., 2020) which must be separated from the main juice in order to obtain the desired EVOO (extra virgin olive oil). In this context, it is well-known (Hermoso *et al*., 1996; Uceda *et al*., 2006) that currently, after milling and malaxation, a first centrifugation is often applied (with a horizontal centrifuge, usually called 'decanter'). The result of this operation is an oily phase which still contains remnants of vegetation water and other substances such as pulp, olive stones and even yeasts. In order to eliminate these substances, it is also common to carry out other subsequent operations which finally allow the production of the EVOO. These operations include sieving, a second centrifugation (with a vertical or "plate" separator), filtering and natural decantation (these last two operations are not always performed in the order indicated).

Taking into account the set of unit operations mentioned above, it is clear that, for their management, it may be of interest to know beforehand, among many other factors, the physical properties of the oily fluids treated in the final stages of the process, since this knowledge could help to improve the yield and quality of the final product (Hermoso *et al*., 1996; Alba, 2008). In this sense, it is noteworthy that there are several works that have addressed the study of properties such as the density and the viscosity of olive oil and its fatty acids (Cedeño *et al*., 1999; Bonnet *et al*., 2011; Nierat *et al*., 2013; Gila, 2017), paying special attention to the variations that these properties can undergo with temperature changes. In other works, the focus has been mainly on the search for robust models which could allow the estimation of such properties with different types of oils, including olive oil (Herschel, 1922; Fasina *et al*., 2006; Giap, 2010; Esteban *et al*., 2012; Sahasrabudhe *et al*., 2017). However, the presence of impurities and their influence on these parameters is an area of study that perhaps has not yet received enough attention (Gila, 2017), despite the fact that these substances can affect the quality and preservation of the EVOO, which may ultimately have a negative impact on its price.

In the above context, this work is presented to treat (as a case study) the determination of the density (ρ) and the dynamic viscosity (μ), at different temperatures, of various oily fluids taken at different production stages, and campaign dates, from a conventional oil mill. To this end, the content of impurities (c) present in these production fluids (non-final products) will be evaluated, and an attempt will be made to relate this content to the density and viscosity variations observed. Likewise, the Newtonian nature of these intermediary products will also be analyzed to verify whether the impurities present in them could cause any change in this respect. This analysis will be made by means of inexpensive devices that were designed and built in a laboratory. In addition, the effort developed in this work will try to elucidate whether the content of impurities, present in any oily fluid, could be estimated by simpler and faster methods than those currently used, which could be of interest for future applications.

2. MATERIALS AND METHODS

2.1. Test samples

In this work, 12 oily samples (approximately 10 l each) were collected between December 2019 and February 2020 from an olive oil mill ('*almazara*') located near Priego de Córdoba (Córdoba, Spain). Each of these samples had different characteristics and appearance (see Figure 1), mainly due to the date of collection and the point chosen for sampling. In summary, Table 1 shows some data of interest related to these samples. At this point, it should be noted that the production process carried out by the aforementioned '*almazara*' was based on a 'continuous conventional system'. In this system, each processing line uses (after cleaning, washing, milling and kneading) a two-way centrifugal decanter ('horizontal centrifuge') for solid-liquid separation, and a disc stack centrifuge ('vertical centrifuge') for liquid-liquid separation (Uceda *et al*., 2006). In addition, the oil must is not filtered, but it can be sieved after the decanter step and can be clarified (with dynamic settling tanks) after the vertical centrifuge.

2.2. Lab equipment

In order to conduct the necessary experiments, it was decided to build several tube viscometers (Steffe, 1996), whose geometry is described in Figure 2.

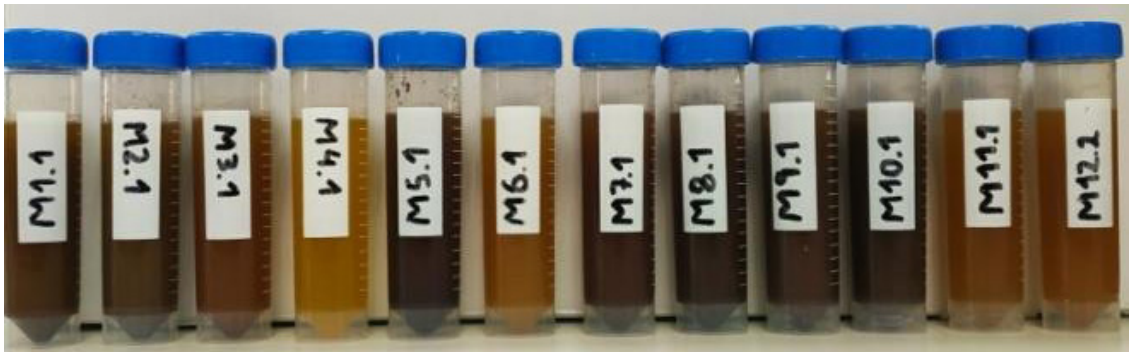

FIGURE 1. Appearance of some sub-samples taken from the oily fluids available.

TABLE 1. Sampling date, sampling point and origin (source) of the test samples

Sample	Sampling Date	Sampling Point - Source
M1	Dec 10th 2019	Sieve after Decanter – tree olives
M2	Dec 10th 2019	Sieve after Decanter – tree olives
M3	Jan 14th 2020	Sieve after Decanter – tree olives
M4	Jan 14th 2020	Vertical Centrifuge – tree olives
M5	Jan 30th 2020	Decanter directly – tree olives
M6	Jan 30th 2020	Vertical Centrifuge – tree olives
M7	Jan 30th 2020	Sieve after Decanter – tree and ground olives
M8	Jan 30th 2020	Sieve after Decanter – tree olives
M9	Feb 11th 2020	Sieve after Decanter – tree olives
M10	Feb 11th 2020	Sieve after Decanter – ground olives
M11	Feb 11th 2020	Sieve after Decanter – tree olives
M12	Feb 11th 2020	Vertical Centrifuge – tree olives

For manufacturing each viscometer, a calibrated plastic vessel was mainly required (to contain the fluid to be tested), as well as a transparent polyethylene tube (whose length and diameter were fixed by design to facilitate the development of laminar flow through the duct). The vessel utilized in each device had a capacity of one liter and it was marked with different reference lines to measure the fluid heads (h) reached inside it. The tube length (L) and the diameter (D), selected to construct the viscometers, followed these combinations to modulate different flow rates, shear rates or shear stresses throughout the experiments: $L = 0.2$ m, $D = 4.07$ mm; $L = 0.4$ m, $D = 4.07$ mm; $L = 1$ m, $D = 7.63$ mm; $L = 1{,}20$ m, $D = 3.87$ mm and $L = 1.60$ m, $D = 4.10$ mm. The tube insertion length (δ) inside the vessel (see Figure 2) was 0.01 m in all cases.

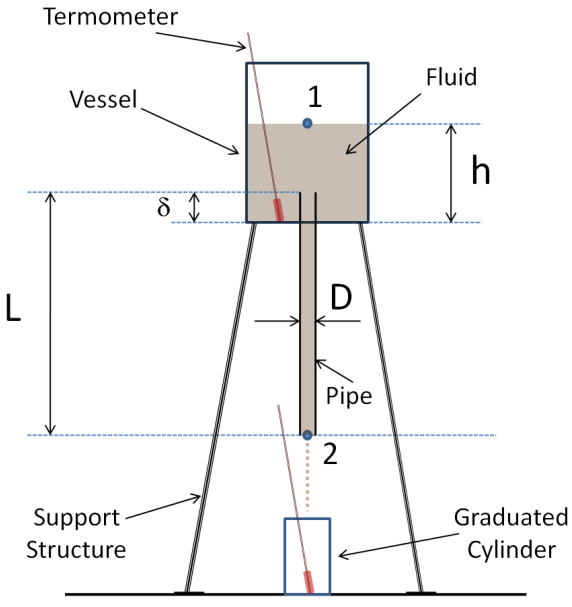

FIGURE 2. Scheme of a tube viscometer used in this study.

In addition to the above, other auxiliary materials were needed to run the tests: two glass thermometers (to determine the fluid temperature at the viscometer pipe ends) with a measuring range of -10 ºC to +60 ºC and with an accuracy of ±1 ºC; six laboratory graduated cylinders of 100 ml (with an accuracy of ±1 ml), and five more of 250 ml (with an accuracy of ± 2 ml), to collect the volumes that would be use-

ful for determining densities and flow rates; two laboratory scales with accuracies of ±0.01 g and ±0.001 g, respectively; a digital chronometer (with a resolution of 0.01 s); A centrifuge with adjustable temperature and speed controls (suitable for 50 ml test tubes from -9 ºC to +40 ºC, and from 200 rpm to 14000 rpm, respectively); two refrigerating chambers (at 4.5 ºC and 12 ºC) and a freezing chamber (at -18 ºC) for storing samples during testing; ice blocks, hot water and 10-l buckets for rapid cooling (or heating) of oily samples by indirect contact; auxiliary bottles of 1.5 l to contain samples if required; calibrated 50-ml tubes for centrifugal separation; pipettes and syringes for handling or transferring samples; a measuring tape (calibrated in millimetres) and various office supplies (scissors, adhesive tape, markers, etc.).

2.3. Viscosity and density tests of oily samples

The experiments in this section were performed using a viscometer with a tube length (L) of 0.4 m and a diameter (D) of 4.07 mm. Having made the above clarification, it should be noted that the original samples (see section 2.1 and Table 1) were first homogenized by manual shaking and then divided into different 1.5-l auxiliary bottles to facilitate the tasks involved in the tests. The bottles were kept cold (at 4.5 ºC or -18 ºC, depending on the expected time before use) to avoid any microbiological activity (such as fermentation due to the presence of yeasts).

When it was convenient, one of the refrigerated bottles (at 4.5 ºC) was taken and tempered in the laboratory (at 23 ºC) for (1 – 2) h. Bearing in mind that the fluid temperature in the tests had to be similar to that present in the industrial production of olive oil (of the order of 20 ºC – 30 ºC), each viscosity experiment was started when the sample temperature was approaching 20 ºC. At that moment, the fluid was poured into the viscometer vessel (which was exposed to the atmosphere) and, after its stabilization and its flow through the tube, it was collected for a given time in a lab cylinder (placed in the lower part of the experimental setup, see Figure 2). Fluid volume (V), stored in the graduated cylinder, was also weighed with a laboratory scale to estimate its density ρ, (Eq. 1)

$$\rho = \frac{m}{V} \quad \text{(Eq. 1)}$$

where m was the mass linked to V.

In addition, flow rate Q was estimated from V taking into account the time τ needed to collect such fluid volume (Eq. 2)

$$Q = \frac{V}{\tau} \quad \text{(Eq. 2)}$$

On the other hand, the fluid temperature was measured at two points (at the input and output ends of the viscometer pipe), and adjusted by indirect contact using hot or cold water before the fluid was poured into the viscometer vessel. The fluid height h inside the vessel (see Figure 2) was observed at the beginning and at the end of each test, and the mean of these measurements was taken as a representative value (usually in the range of 4 −6 cm).

From the procedure described above, which was applied 144 times (12 tests, in the range of 20 ºC−30 ºC, for each of the 12 types of fluid treated), and taking into account what it is described in section 2.2, the values for dynamic viscosity μ were estimated from the following expression (Eq. 3), based on the Hagen-Poiseuille equation and on the energy equation (Singh and Heldman, 2009).

$$\mu = \frac{\Delta p \pi D^4}{128 L Q} = \frac{\rho g (h + L - \delta) \pi D^4}{128 L Q} \quad \text{(Eq. 3)}$$

where $\Delta p = \rho\, g\, (h + L - \delta)$ is the pressure loss between 1 and 2 (see Figure 2), g is the gravitational acceleration, and the other variables are those just indicated in this section and in paragraph 2.2. The use of Eq. 3, to estimate μ requires, in principle, the assumptions that the fluid tested is Newtonian, the kinetic heads are negligible and the flow is stationary and perfectly laminar throughout the entire length L. This last assumption implies that the effect of the inlet length and the local head losses at the pipe inlet can also be neglected. The accuracy of the previous approximations was analyzed for flows such as those treated here in Tirado (2020), where it was verified that for oily fluids at very low Reynolds numbers (of the order or less than 10) such premises could be accepted. To minimise possible errors, it should also be noted that the values of ρ employed in Eq. 3, were obtained, in each case, from polynomial interpolation taking 12 pair of values (t, ρ) available as reference for each type of fluid from the experiments.

2.4. Impurity content tests

Considering as impurities, as a whole, all those remains of vegetation water, pulp, olive stone (and even yeasts) present in the EVOO, in this work this matter content was determined according to an alternative method (Vallesquino and Tirado, 2023), different from the official one (IOC, 2019), in order to obtain sufficiently accurate results while simplifying the experimental procedure. Therefore, the impurity content c, expressed as a percentage of the volume of impurities (V_{imp}) and the sample volume (V_{sp}) (Eq. 4):

$$c\ (\%) = \frac{V_{imp}}{V_{sp}} 100 \quad \text{(Eq. 4)}$$

could be estimated as follows: firstly, two samples (of around 40 ml) were taken from every type of fluid previously treated in the 'viscosity and density tests of oily samples' (see section 2.3), and they were poured into two centrifuge tubes like those shown in Figure 1. These tubes were allowed to decant naturally in the laboratory, between (24 – 48) h at 23 ºC, and then centrifuged at 3900 rpm for 12 min at 27 ºC. After that, the volume of impurities present in each sample was clearly visible (see the lower part of each tube in Figure 3, as an example), and this volume (in many cases very small) could be determined by applying the appropriate operations (Vallesquino and Tirado, 2023).

The bulk density of the impurities was determined separately, with the aim of estimating a more representative value than that which could be obtained from sub-samples like those shown in Figure 3. With this in mind, and depending on the content of non-oily remains, among 2 − 4 samples of each type of fluid were randomly selected (from those collected in 1.5-l bottles, as described in section 2.3), and the impurities present in their bottoms were extracted. Such sediments were formed by gravitational decantation until an effective separation between the substances could be visually verified. This task could take no less than (2–3) wk per sample, applying temperatures between 4.5 − 23 ºC to avoid unwanted fermentations. Next, the bottom content extracted from these samples was homogenized and 50 ml were poured into one or two centrifuge tubes (depending on the amount of impurities available) and centrifuged at 3900 rpm for 12 min at 27 ºC. Once this operation had been carried out, the appearance of the samples obtained was similar to that shown in Figure 3, but with a more abundant impurity phase (of the order of 30 ml), in which it was generally possible to observe vegetation water (about 55%) and insoluble solids (about 45%). The bulk density of each impurity sample could then be estimated by a simple fraction between the mass and the volume of the impurities (after carefully removing the residual oil that could be present in each tube).

2.5. Rheological tests of oily samples

These experiments were designed to verify whether the dynamic behavior of the fluids studied in this work was in accordance with Newton's law of viscosity. Therefore, the relationship between σ_w (wall shear stress of the flow) and $\left(\frac{du}{dr}\right)_w$ (wall shear rate) was analyzed. According to Steffe (1996), σ_w can be expressed as follows (Eq. 5) for the laminar flow of a time-independent fluid through a cylindrical tube (Figure 2):

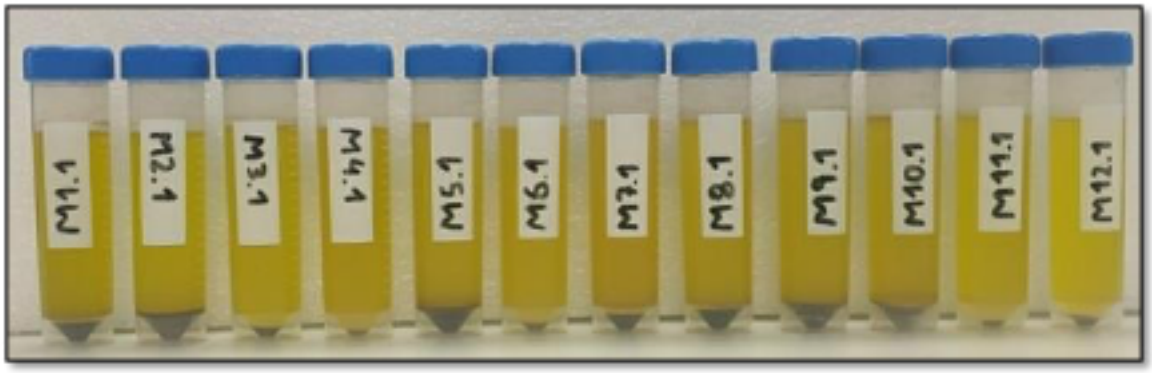

FIGURE 3. Appearance of the sub-samples shown in Figure 1 after 24 h of natural decantation and further centrifugation at 3900 rpm for 12 min at 27 ºC.

$$\sigma_w = \frac{\rho g(h + L - \delta)D}{4L} \quad \text{(Eq. 5)}$$

For its part, the shear rate $\left(\frac{du}{dr}\right)_w$ depends on the fluid treated. However, this variable could be expressed as a function 'f[ ]' of this elemental ratio (γ_e) (Eq. 6):

$$\left(\frac{du}{dr}\right)_w = f\left[\frac{32Q}{\pi D^3}\right] = f[\gamma_e] \quad \text{(Eq. 6)}$$

which can be used in further analysis.

Considering the above premises (together with the available resources), and trying to obtain an adequate set of pairs (γ_e, σ_w), it was considered appropriate to use four of the viscometers already mentioned in section 2.2 in these tests. The dimensions of these devices were: L = 0.2 m, D = 4.07 mm; L = 1 m, D = 7.63 mm; L= 1.20 m, D = 3.87 mm and L= 1.60 m, D = 4.10 mm. In addition, four samples were tested with each of these viscometers: M1, M4, M7 and M10, chosen because they showed a representative variability in impurity content. In order to avoid unwanted fermentations (due to the racking, aeration and duration of these tests), about 3 l of each of these samples were pasteurized (at 80 ºC for 45 min). They were then cooled (at a temperature close to 23 ºC) and analyzed 5 times with each of the viscometers cited above, following a procedure analogous to that described in section 2.3. Furthermore, it should be noted that the data previously obtained from the unpasteurized samples (according to section 2.3) were used as control data for the corresponding comparisons.

3. RESULTS AND DISCUSSION

3.1. Impurity density and content

Table 2 shows the data related to the impurity content c, as well as the bulk density values (ρ_{ap}) linked to them. From these data, it is worth noting that the minimum and maximum c values are of the order of 0.5% (sample M4) and 5.87% (sample M2), respectively. These data can be considered normal when compared to those obtained by other authors (Hermoso *et al*. 1996; Uceda *et al*. 2006; Bejaoui *et al*., 2013; Dammak *et al*., 2015; Gila, 2017), who presented c results which varied from 1 to 15% for fluids taken from the decanter, and from 0.2 − 0.6%

Table 2. Impurity content c (%) and impurity bulk density (ρ_{ap}) of the samples treated.

Sample and Date	c (%)	ρ_{ap} (kg / m^3)
M1 - Dec 10th 2019	3.61 ± 0.11	1240 ± 8.2
M2 - Dec 10th 2019	5.87 ± 0.33	1167 ± 10.3
M3 - Jan 14th 2020	1.13 ± 0.13	1196 ± 7.5
M4 - Jan 14th 2020	0.50 ± 0.12	1108
M5 - Jan 30th 2020	5.00 ± 0.18	1171 ± 12.5
M6 - Jan 30th 2020	1.14 ± 0.14	1089 ± 5.7
M7 - Jan 30th 2020	2.00 ± 0.20	1146 ± 9.1
M8 - Jan 30th 2020	4.05 ± 0.20	1168 ± 6.5
M9 - Feb 11th 2020	4.50 ± 0.24	1135 ± 9.7
M10 - Feb 11th 2020	5.38 ± 0.50	1080 ± 11.0
M11 - Feb 11th 2020	0.89 ± 0.14	1218
M12 - Feb 11th 2020	1.27 ± 0.05	1126 ± 5.2

The values shown are the mean ± SD of two replicates (for samples M4 and M11 ρ_{ap} a single test was done in due to the impurity content available).

(Bejaoui *et al*., 2013; Dammak *et al*., 2015) for samples collected from the vertical centrifuge (note that samples M4, M6 and M12 came from the vertical centrifuge, and the other ones from the horizontal decanter; see Table 1). The factors causing this variability (in this study and in those cited) can be diverse and they could have their roots in agronomic and technological aspects (Hermoso *et al*., 1996, Uceda *et al*., 2006; Vallesquino and Tirado, 2023). Most likely, in the absence of more data, the lower c results (for the samples taken from the decanter in this study) could be related to longer residence times, higher processing temperatures and smaller outlet radii for the oily phase. In this context, sample M11 could be a representative example of these operating conditions, as it appears to have been taken from a vertical centrifuge, but was actually collected from a decanter.

Regarding the bulk density values (ρ_{ap}) presented in Table 2, it is noteworthy that they all fell within a range of 1080 kg/m^3 − 1240 kg/m^3. Attempting to compare these data with those of other studies, it is notorious that Gila (2017) points out that there are few references on this issue in the literature, placing ρ_{ap} (through simulations) in a range of 1025 kg/m^3 to 1225 kg/m^3. In Alba (2008), the density of oily wastewaters (*alpechines*) is set around 1050 kg/m^3, and that of the pomace at 1200 kg/m^3 (values compatible with those presented here).

It is also remarkable that ρ_{ap} seems to be smaller for the samples taken from the vertical centrifuge

(M4, M6 and M12) compared to those obtained from the decanter. This could make sense if one considers that, according to Stokes's law (Mafart and Béliard, 1994), the densest and largest particles are easier to separate from the liquid phase in the first centrifugation (with the decanter). Similarly, although the number of data available is not large, the values for ρ_{ap} in Table 2 seem to show a decreasing trend as the production campaign progresses. This could be related to the changing nature of the raw material (maturity stage) and could be analyzed from a greater number of samples in future works.

3.2. Rheological tests results

Figure 4 shows the data of the shear stress σ_w (see Eq. 5), as a function of the elemental ratio $\gamma_e = \frac{32Q}{\pi D^3}$, obtained from the rheological tests developed according to section 2.5 (at laboratory temperature of 23 ºC). The fluids treated in these experiments were: M1, M4, M7 and M10. It can be seen in Figure 4 that the relationship between σ_w and γ_e is linear, with zero ordinate at the origin. According to Steffe (1996), the data presented do not agree well with the Bingham or power law models, but they do with that expected for a Newtonian fluid. In such a case (Newtonian fluid), it could be stated directly (Eq. 7):

$$\left(\frac{du}{dr}\right)_w = \gamma_e = \frac{32\,Q}{\pi\,D^3} \qquad \text{(Eq. 7)}$$

$$\sigma_w = \mu\left(\frac{du}{dr}\right)_w \qquad \text{(Eq. 8)}$$

which implies that the shear rate $\left(\frac{du}{dr}\right)_w$ is directly equal to $\gamma_e = \frac{32Q}{\pi D^3}$, and that the slope of the function $\left(\frac{du}{dr}\right)_w = f[\gamma_e]$ (see also Eq. (6)) corresponds to the dynamic viscosity (μ) of the fluids tested. In this scenario, it so happens that these fluids would have a μ value (at 23 ºC) that could be of the order of 0.07 Pa·s (sample M4) or 0.074 Pa·s (sample M10).

Other authors, like Fasina *et al*. (2006), Bonnet *et al*. (2011) or Gila (2017), have also found that olive oil (without impurities) shows a Newtonian behavior, reporting values for μ which are similar to those indicated here. This circumstance confirms that the moderate presence of impurities does not seem to affect the Newtonian nature of olive oil. On the other hand, it is worth noting that the pasteurization

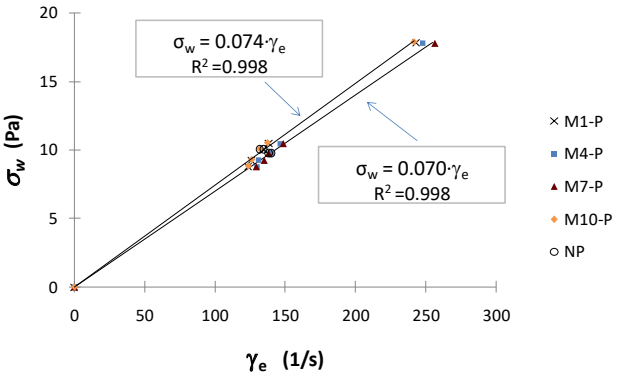

Figure 4. Relationship between γ_e (shear rate) and σ_w (shear stress) for some samples (M1, M4, M7 and M10) after pasteurization (-P) and without pasteurization ('O' symbol).
Each pair of values (γ_e, σ_w) was obtained for t = 23 ºC by polynomial interpolation from 5 replicates made at temperatures between (20 – 25) ºC. The experimental error expected is of the order of 4.2%.

treatment does not seem to have any effect on the rheological behavior of the fluids analyzed, since the control samples (without heat treatment, see section 2.3) have values for μ that are equivalent to those of the pasteurized samples. This aspect can be observed in Figure 4, where the values for M1, M4, M7 and M10 (marked with 'O' symbol and taken from the control samples) are perfectly in line with the rest of the values (obtained from pasteurized samples).

Besides the former, the data presented in Figure 4 are not evenly distributed. This effect is due to the design of the viscometers used, which conditions the values for γ_e that they can present. For instance, in those viscometers with $D \approx 4$ mm, γ_e was in the range of 120 – 150 s^{-1}, and the remaining γ_e values were related to the device with D = 7.63 mm (including the singular point (0,0), explained by the fact that no fluid could be retained at the bottom end of the viscometer tube when it was finally emptied). Considering the results obtained in this study, it could be recommended for future works to follow an experimental design slightly different from the one treated here. In this regard, a better approach could be implemented by using a set of tube viscometers with a greater variety of tube diameters and fewer length options. Nevertheless, the results shown in Figure 4 could be useful references for the olive oil industry, since γ_e (see Eq. 7) is usually less than 250 s^{-1} for oily flows of (1000 – 2000) l/h flowing through (3–5) cm diameter tubes (common operating values associated with decanters or vertical centrifuges).

3.3. Density and viscosity of the oily samples

Figure 5 presents the density values (ρ) for all the samples tested. On the left side of this Figure, ρ is plotted as a function of the impurity content c (see Eq. 4), taken as reference, as a first approximation, only three temperature values (t = 20 ºC, t = 25 ºC and t = 30 ºC) to carry out a simpler analysis. On the right side of Figure 5, ρ is shown according to the model results which will be presented later.

Observing the relationship between ρ, c, and t in the cited Figure, it is clear (despite the scatter) that the density showed a linear increasing trend as the impurity content increased. However, ρ tended to decrease as temperature t increased (at 30 ºC the values for ρ were, in general, smaller than at 25 or 20 ºC). Likewise, the gap (separation) between the functions $\rho = f(c)$ maintained a certain linearity (the ordinate at the origin and the slope of such functions grew linearly with t), which suggests that the general relationship between ρ, c and t could be of the form:

$$\rho = (a_1 c + a_2)(a_3 t + a_4) \quad \text{(Eq. 9)}$$

where a_1, a_2, a_3 and a_4 are fitting parameters. In the scientific literature, the density of different types of oils, including olive oil, is usually adjusted by means of linear functions that depend on temperature (Esteban *et al*., 2012; Gila, 2017). In addition, their resulting values are similar to those presented here, taking into account that the oils treated in this study contained impurities. Considering the above, ρ data were fitted to Eq. (9) as follows:

$$\rho = (0.957\,c + 1.423 \cdot 10^{3})(-4.030 \cdot 10^{-4}\,t + 0.644) \quad \text{(Eq. 10)}$$

where the determination coefficient (R^2) was equivalent to 0.402 and the residual mean square error (RMS) was of the order of 11.070 $(kg/m^3)^2$.

Figure 5 (right side) presents the fluid density values ρ_{est}, estimated from Eq. (10), in comparison to those measured in the experiments (ρ_{ms}). Except for the scale factor of this Figure, which in this case tends to exaggerate the magnitude of the deviations, the main trend established by Eq. (10) is in agreement with the experimental data. Moreover, Figure 5 shows that, in order to accurately take into account the effect of the impurity content on ρ, it is necessary to measure the fluid density with a lower tolerance (of the order of 0.1%), since the density variations are not very sensitive to changes in c values. In this respect, it should be noted that the data scatter shown in this Figure is the result of possible errors associated with the measurement of volumes using the materials mentioned in section 2.2. These errors are unlikely to be greater than ± 1.5%, but they are sufficient to produce a maximum deviation of ± 14 kg/m^3, which is compatible with the RMS value just indicated.

Regarding the dynamic viscosity (μ), Figure 6 presents the values of μ in a similar way to that already performed with ρ. On the left side of such a Figure, three reference t values have been taken to analyze the variation in μ with respect to the impurity content c. It is observed, as in the case of ρ, that

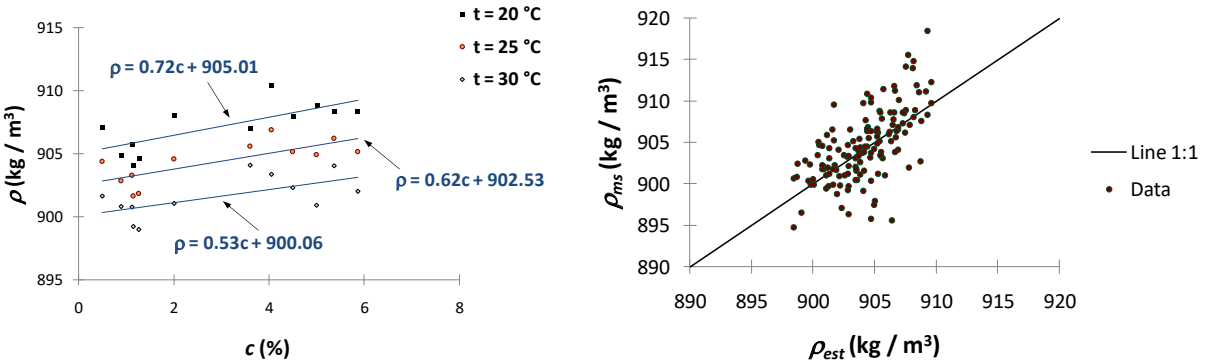

Figure 5. Relationship between the impurity content (c) and the density of oily fluids (ρ), at t temperatures of 20, 25 and 30 ºC (left side), and comparison between the estimated density (ρ_{est}) and the measured density (ρ_{ms}) from the experiments (right side). Each ρ value, on the left side, was obtained by polynomial interpolation (at constant impurity content c) from 12 replicates made at different temperatures between (20 – 30) ºC. On the right side, each value for ρ was obtained from a single test, with R^2 = 0.402 and $(RMS)^{0.5}$ = 3.327 kg/m^3.

μ follows a linearly increasing trend with c, and a decreasing one (but not linear) with respect to t. On this occasion, the intercept and the slope of the fitting functions $\mu = f(c)$ do not vary linearly with t, which leads to the assumption that the possible relationship between μ, c and t could be of the type:

$$\mu = (b_1 c + b_2)\left(\frac{b_3}{t} + b_4\right) \quad \text{(Eq. 11)}$$

$$\mu = (b_1 c + b_2)\left(\frac{b_3}{t^2} + \frac{b_4}{t} + b_5\right) \quad \text{(Eq. 12)}$$

where b_1, b_2, b_3, b_4, and b_5 are fitting parameters.

According to several authors (Fasina *et al*. 2006; Esteban *et al*., 2012; Gila, 2017; Sahasrabudhe *et al*., 2017), the viscosity of diverse oils (including olive oil) could be estimated from different models which, essentially, are based on the Arrhenius equation:

$$\mu = A e^{\left(\frac{E_a}{RT}\right)} \quad \text{(Eq. 13)}$$

where A is an empirical parameter, E_a is the activation energy, R is the universal gas constant, and T is the absolute temperature. Taking into account the exponential nature of the previous model, and the development of Taylor's series for any exponential function (Spiegel and Ribero, 1970):

$$e^x = 1 + x + \frac{x^2}{2!} + \frac{x^3}{3!} + \cdots \quad \text{(Eq. 14)}$$

it is obvious that Eq. (13) could be reformulated as a function such as:

$$\mu = d_1 + \frac{d_2}{T} + \frac{d_3}{T^2} + \cdots \quad \text{(Eq. 15)}$$

which is directly related to the Slotte's equation (Herschel, 1922). Note that, again, d_1, d_2, d_3 … are fitting parameters. For short temperature ranges (as occur in this study), where the slope of the function $\mu = f(T)$ does not change excessively with temperature, it may be feasible to apply an expression like Eq. (15) by only using, as an approximation, two or three equation terms. Furthermore, if T is well above 273 K, Eq. (15) could even be adapted for Celsius degrees, achieving quite reasonable results (Vallesquino and Sánchez, 2023). Bearing in mind the above, μ data were adjusted for two models, such as those shown in Eqs. (11) and (12), in which the Celsius scale was used in the first one (Eq. 16) and the Kelvin scale was applied in the second one (Eq. 17):

$$\mu = \left(-1.437\cdot10^{-3}\, c - 0.094\right)\left(\frac{-18.403}{t} + 0.073\right) \quad \text{(Eq. 16)}$$
$$R^2 = 0.982; \quad RMS = 3.252\cdot10^{-6}\ (Pa\cdot s)^2$$

$$\mu = (4.672\cdot10^{-2} c + 3.087)\left(\frac{1.794\cdot10^{5}}{T^2} - \frac{1.118\cdot10^{3}}{T} + 1.751\right)$$
$$R^2 = 0.987; \quad RMS = 2.351\cdot10^{-6}\ (Pa\cdot s)^2 \quad \text{(Eq. 17)}$$

On the right side of Figure 6, the values for dynamic viscosity estimated by means of Eq. (17)

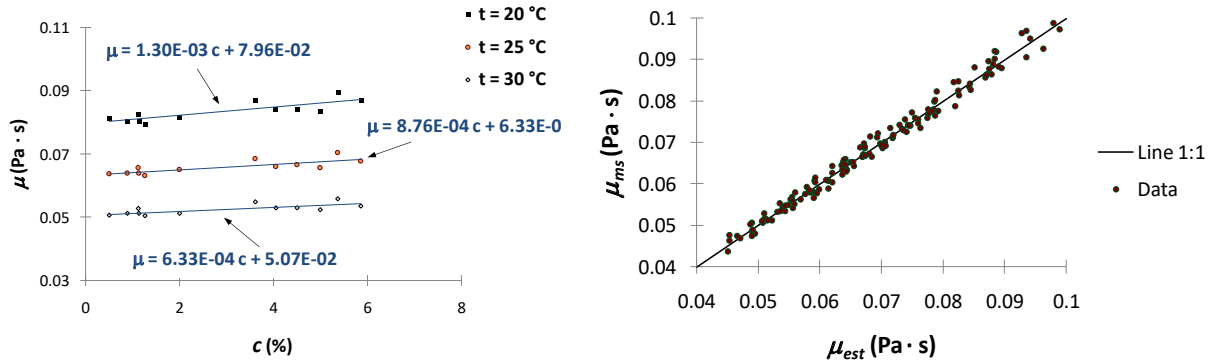

Figure 6. Relationship between the impurity content (c) and the dynamic viscosity (μ), at t temperatures of 20, 25 and 30 (left side), and comparison between the estimated viscosity (μ_{est}) and the measured viscosity (μ_{ms}) from the experiments (right side).
Each μ value, on the left side, was obtained by polynomial interpolation (at constant impurity content c) from 12 replicates made at different temperatures between (20 – 30) ºC. On the right side, each value of μ was obtained from a single test, with $R^2 = 0.987$ and $(RMS)^{0.5} = 1.533\cdot10^{-3}$ Pa·s.

(μ_{est}) are plotted against those obtained from the experiments (μ_{ms}). It should be noted that, for the sake of clarity, the data related to Eq. 16 are not displayed in the Figure because they would overlap with those estimated from Eq. 17, (as can be inferred from R^2 and RMS values associated to these equations).

On the other hand, it is also remarkable that the fitting accuracy of Eqs. 16 and 17 is higher than that of Eq. 10, (see also the right side of Figures 5 and 6). This fact reveals that the dynamic viscosity of olive oil is a variable that is sensitive to variations in temperature (this is well-known), but it is sensitive, as well, to variations in the impurity content. This aspect is relevant because, similar to the approach presented by Vallesquino and Sánchez (2023), by combining expressions such as those established in Eqs. 3 and 16 (or in Eqs. 3 and 17) it could be possible to calibrate empirical functions (ψ) of the form:

$$c = \psi(\Delta p, Q, t, L, D) \quad \text{(Eq. 18.a)}$$

$$\text{or} \quad c = \psi(\Delta p, Q, T, L, D) \quad \text{(Eq. 18.b)}$$

These ψ functions could, in the end, make it possible to estimate the impurity content c in real time (present in any oily must) if some simple sensors were installed on the production line. As an example, if Eqs. 3 and 16 were considered in this case, Eq. 18.a could be reformulated as:

$$c\ (\%) = \frac{\frac{\Delta P \pi D^4}{128 L Q}}{-1.437 \cdot 10^{-3}\left(-\frac{18.403}{t} + 0.073\right) - \frac{0.094}{1.437 \cdot 10^{-3}}} \quad \text{(Eq. 19)}$$

where c is obtained, as a percentage, from two plant design parameters (L and D, in m) and three process line variables like Q (flow rate, in m^3/s), t (temperature, in ºC) and Δp (pressure drop, in Pa). Although Eq. (19) is only valid, as a rough approximation, for certain operating conditions (related to some samples taken from a determinate conventional olive oil mill), this approach opens the possibility of studying similar models that could be applied in any '*almazara*', which could be of interest for the improvement of processes and products in the olive oil sector. In this regard, it should be pointed out that, in real manufacturing processes, knowledge of the impurity content in oily fluids could help to make appropriate decisions on factors such as the residence time of the processed fluids in decanters (or in vertical centrifuges) and the choice of the most suitable type of clarification to be applied after the second centrifugation.

4. CONCLUSIONS

As a case study, the density (ρ) and the dynamic viscosity (μ) of 12 different oily fluids (taken from a conventional mill) have been measured, verifying the variability that these physical properties may present as a function of temperature (t) and the impurity content (c) of the samples treated.

In the case of ρ, its relationship with t and c has shown a linear trend, but this dependence is weak since large increases in temperature or impurity content are required for the density to vary significantly. In this work, it could serve as a reference that the density has experienced variations, of the order of less than 1%, when the temperature varied between 20−30 ºC and the impurity content between 0.5−5.87 %.

Regarding dynamic viscosity, its dependence on t and c is of a different nature, as it can be quadratic for t and simply linear for c, in a context of Newtonian fluid behavior. The fact that the viscosity varies of the order of 50% or more, between 20 −30 ºC for a given value of c, allows one to confirm (as is usual for fluids) that μ strongly depends on t. In the case of the relationship between μ and c (for temperatures between 20 −30 ºC), it is notable that the dependence of μ on c is not so strong, but it is appreciable: when the impurity content varies from 0.5 to 5.87%, the dynamic viscosity of the oily fluids can reach variations of the order of 7 − 10% (much higher than that reported in the case of ρ). In this scenario, the relationship between μ and c could be used to determine the impurity content of a given oily fluid on line, (requiring only the implementation of some simple sensors on the process line), which could be used, ultimately, to improve the quality of the olive oil and its industrial process.

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Comprehensive characterization of physicochemical, thermal, compositional, and sensory properties of cold-pressed rosehip seed oil

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SUMMARY: In this study, cold-pressed rosehip seed oil was fully characterized. Acidity and oxidation levels were near the limit values or slightly exceeded them and improvement in the storage conditions was suggested. The oil started to crystallize at -45.25 °C, and melt at -25.56 °C. Linoleic acid (51.1%), β-sitosterol (84.6%), γ-tocopherol (773.76 µg/g) and rosmarinic acid (31.38 µg/g) were determined as major fatty acid, sterol, tocopherol and phenolic compound, respectively. For the first time, aromatic volatile compounds and sensory descriptive terms were determined for cold-pressed rosehip seed oil. Sixty-seven volatile compounds were detected and L-limonene was found to be a major volatile compound. According to the sensory analysis, timber/kindling and raw vegetable tastes/aromas were found to be relatively dominant. Consequently, it is thought that rosehip seeds can be used as a raw material for edible and nutritionally-rich cold-pressed oil production and/or as source oil for functional food preparations.

KEYWORDS: *Cold-Press; Composition; Physicochemical Property; Rosehip Seed; Sensorial Description; Volatile Compound*

RESUMEN: ***Caracterización integral de las propiedades fisicoquímicas, térmicas, composicionales y sensoriales del aceite de semilla de rosa mosqueta prensado en frío.*** En este estudio se caracterizó completamente el aceite de semilla de rosa mosqueta prensado en frío. Los niveles de acidez y oxidación estaban cerca de los valores límite o los excedían ligeramente y se sugirió mejorar las condiciones de almacenamiento. El aceite comenzó a cristalizar a -45,25°C y a fundirse a -25,56°C. Se determinó el ácido linoleico (51,1%), β-sitosterol (84,6%), γ-tocoferol (773,76 µg/g) y ácido rosmarínico (31,38 µg/g) como principal ácido graso, esterol, tocoferol y compuesto fenólico, respectivamente. Por primera vez, se determinaron compuestos aromáticos volátiles y términos descriptivos sensoriales para el aceite de semilla de rosa mosqueta prensado en frío. Se detectaron sesenta y siete compuestos volátiles y se descubrió que el L-limoneno era un compuesto volátil importante. Según el análisis sensorial, se encontró que los sabores/aromas de madera/astillas y vegetales crudos eran relativamente dominantes. En consecuencia, se cree que las semillas de rosa mosqueta pueden usarse como materia prima para la producción de aceite prensado en frío comestible y nutritivo y/o como aceite fuente para preparaciones de alimentos funcionales.

PALABRAS CLAVE: *Composición; Compuestos volátiles; Descripción Sensorial; Prensado en frío; Propiedades fisicoquímicas; Semilla de rosa mosqueta*

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1. INTRODUCTION

Rosehip (*Rosa canina* L.) is a plant from the Rosaceae family with more than 200 species and approximately 18000 varieties in the world. Rosehip plant is rarely affected by environmental conditions, and can grow on infertile soils with a harsh climate. It spreads naturally in Asia, Caucasia, Europe, and Africa. It is a 2-3-meter tall plant in the form of a bush. Its flowers can be of different colors, from white to pink. Rosehip plant has 3-15 leaves, which are mostly green and sometimes slightly bluish in color with a drop-shaped and hairless structure. Rosehip fruits are round, egg-shaped or elliptical and their colors vary from orange to red. There are about 20-30 hairy and hard-shelled seeds inside the fruit. Although it varies according to the rosehip type, generally, the harvest begins in July and continues until mid-November (Türkben, 2003; BUGEM, 2020).

According to the Ministry of Agriculture and Forestry of Turkey, the annual rosehip production was 195 tons in 2014, and 3783 tons in 2018. In 2019, cultivated production was 998 tons and 113.4 tons were collected from nature. The farmers were promoted by the ministry for more production (BUGEM, 2020). The global rosehip oil market value was predicted as 141 million dollars in 2021, and projected to reach 275.6 million dollars in 2028. Further, the oil was estimated to be segmented mostly in skin and hair care products, and predicted to extend into other functional preparations (Anonymous, 2023).

Worldwide, rosehip fruit is generally consumed as jam, marmalade, and tea. During the processing of rosehip fruit into these products, generally, rind and seeds are removed as wastes. Rosehip seeds, which are between 30 and 40% by weight in fresh fruit and removed a waste during the processing of rosehips, are not adequately utilized. The seeds have been used in the cosmetic and pharmaceutical industries in recent years (Göknur, 2013; Çağlar and Demirci, 2017). The composition of rosehip seeds was reported as 1.94–2.09% ash, 6.89–8.64% protein, and 6.92– 8.60% oil (Kadakal, 2002). In a recent review (Mannozzi *et al*., 2020), different studies in the literature showed that rosehip seeds contain around 1.2–3.9% ash, 3.0–11.5% protein, 6.3–17.8% oil, and 40.0–89.0% carbohydrates, including fiber.

Since significant amounts of seed are obtained as a waste during rosehip processing, concern about valorizing them has been raised. In particular, the oil contained in the seeds received attention from researchers and producers (Barros *et al*., 2011; Güneş *et al*., 1027; Dabrowska *et al*., 2019; Mannozzi *et al*., 2020). The above-cited literature indicated that rosehip oil contains approximately 3.0-8.0% palmitic, 1.5-3.5% stearic, 13.0-23.0% oleic, 35.0-56.0% linoleic, and 14.0-35.0% linoleic acid as major fatty acids. The variation seems to be due to different varieties, regions and climates, but generally the oil seems to be unsaturated and essential fatty acid rich. Further, the oil was shown to contain 5.29 mg/100 g total tocopherol, of which 3.47 mg was γ-tocopherol (Barros *et al*., 2011). Also, the main sterol in rosehip oil was β-sitosterol, and the presence of brassicasterol, campesterol, stigmasterol, Δ-5- and Δ-7-avenasterol was reported (Mannozzi *et al*., 2020). Therefore, rosehip oil is rich in bio-active substances such as essential fatty acids, phytosterols, tocopherols, and carotenoids, and it might have functional uses as anti-inflammatory, anti-obesity, anti-oxidant, anti-diabetic, skin care, and others in culinary applications, cosmetics, and pharmaceutical industries (Barros *et al*., 2011; Göknur, 2013; Mannozzi *et al*., 2020).

Considering that rosehip seed oil is rich in bioactive compounds, it is thought that rosehip seeds can be used in the production of gourmet oil. Cold pressing is a very suitable technique for gourmet oil production like rosehip seed oil. The cold-press technique yields clean, sensorially acceptable, and high-quality oils and by this technique all the naturally present bioactive compounds are preserved (Aydeniz *et al*., 2014).

In this study, the aim was to characterize cold-pressed rosehip seed oil. Physicochemical, thermal, compositional, and sensorial properties of the oil were determined. Some data (composition of aromatic volatiles and sensory properties) were presented for the first time for those interested in utilizing this special oil. Hence, possible uses for this new oil would be foreseen.

2. MATERIALS AND METHODS

2.1. Materials

The cold-pressed rosehip seed oil used in this study was purchased from Bade Natural (Istanbul, Turkey). The company informed us that the rosehip seeds used in the oil production were obtained from rosehip fruit harvested in the Gümüşhane region of Turkey in November 2020, and the cold-pressed oil was generated

in June, 2021. The oil analyses started in our laboratory in December, 2021. Therefore, the oil was stored for almost 6 months under premium conditions before the analyses took place. All chemicals, solvents, and standards used in the analyses were of analytical or chromatographic grade and purchased from Sigma (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany). Cold-pressed rosehip seed oil is shown in Figure 1.

FIGURE 1. Cold-pressed rosehip seed oil

2.2. Physicochemical properties of the oil

Specific gravity was determined according to the AOCS Cc 10b-25 method (AOCS, 2017), specific extinction values were measured with a spectrophotometer (Shimadzu UV-1800, Shimadzu Co., Kyoto, Japan) according to the AOCS Ch 5-91 method (AOCS, 2017), and refractive index was determined by an Abbe 5 refractometer (Bellingham and Stanley, Tunbridge Wells, UK). Apparent viscosity was measured by a Brookfield DV II + Pro Viscometer (Brookfield Eng. Lab., Inc., Middleborough, MA, USA) with LV-SC4-18 spindle and 50-rpm rotation speed at room temperature. Color values (L, a*, and b*) were measured with a Minolta colorimeter CR-400 (Konica, Minolta Sensing, Osaka, Japan).

Free fatty acidity, peroxide value, *p*-anisidine value, iodine number, saponification number and unsaponifiable matter contents were measured according to AOCS Ca 5a-40, AOCS Cd 8b-90, AOCS Cd 18-90, AOCS Cd 1b-87, AOCS Cd 3-25, and TSE 894, respectively (TSE, 1970; AOCS, 2017). The total phenolic content and antioxidant capacity of the oil were determined according to Aydeniz *et al*. (2014) with an Agilent 8453 UV–Vis Spectrophotometer (Waldbronn, Germany). The total carotenoid content of the oil was measured according to Franke *et al.* (2010) by using an Agilent 8453 UV–Vis Spectrophotometer (Waldbronn, Germany).

2.3. Thermal analyses of the oil

The thermal properties of the oil were measured with a differential scanning calorimeter (DSC, Perkin-Elmer DSC 4000, Waltham, MA). Melting and crystallization parameters of the oil were determined according to Aydeniz *et al.* (2014). A 5–10 mg sample was placed into an aluminum pan, sealed hermetically, and analyzed against an empty pan. The thermal program was as follows: heating from 20 to 110 °C at a rate of 10 °C/min; cooling from 110 to −40 °C at a rate of 10 °C/min; holding at that temperature for 3 min and then heating from −40 to 50 °C at a rate of 5 °C/min. Nitrogen (99.99%) was used during analysis. The thermal parameters were calculated with Pyris 1 Manager Software.

The oxidative induction time (OIT) of the oil was determined according to Aydeniz *et al.* (2014). A 5-10 mg sample was placed into an aluminum pan and analyzed against an empty pan. The thermal program was as follows: heating from 30 to 170 °C at a rate of 50 °C/min with nitrogen (99.99%) and holding at that temperature for 30 min with oxygen (99.99%). The OIT of the oil was calculated with Pyris 1 Manager Software.

2.4. Determination of the fatty acid, sterol, and tocopherol compositions

The fatty acid composition of the oil was determined according to TGK 2017/26 (2017). First, fatty acid methyl esters (FAME) were prepared. 100 mg of the oil were dissolved into 2 ml heptane. Then, 0.2 ml of 2 M methanolic KOH were added and the mixture was vortexed for 30 s. Finally, the mixture was centrifuged at 6461 xg for 5 min (Sigma 2-16K, Sartorius, Germany) and the clear phase was taken into a vial for injection. The fatty acid composition was determined by a Gas Chromatograph-FID (Agilent Technologies 7890B, Palo Alto, CA, US) with a HP 88 capillary column (100 m × 0.25 mm ID × 0.2 μm

film thickness, J&W Scientific Co., CA, USA). The injection volume was 0.2 µl and the injector split ratio was 1:10. Hydrogen was used as carrier gas at a flow rate of 1.7 ml/min. Hydrogen (40 ml/min) and dry air (450 ml/min) were detector gases. The inlet temperature was 250 °C and detector temperature was 280 °C. The oven temperature program was as follows: holding at 130 °C for 1 min, heating to 170 °C at a rate of 6.5 °C/min, heating to 215 °C at a rate of 2.75 °C/min and holding at that temperature for 12 min, then heating to 230 °C at a rate of 40 °C/min, holding at that temperature for 5 min. Fatty acids were identified by using a FAME standard mixture (37-components, 4-24, Supelco, Bellefonte, PA, USA).

The sterol composition was determined according to the TSE EN ISO 12228 method (TSE, 1999). First, the unsaponifiable matters were obtained, and then the sterol fractions were separated with Thin Layer Chromatography (TLC). The sterol composition was determined by Gas Chromatograph-FID (Agilent Technologies 7890B) with a DB5 capillary column (30 m × 0.25 mm ID × 0.1 µm film thickness, J&W Scientific Co). Injection volume was 1 µl and the injector split ratio was 1:100. Hydrogen was used as carrier gas at a flow rate of 0.7 ml/min. Hydrogen (30 ml/min) and dry air (400 ml/min) were detector gases. The inlet temperature was 290 °C and detector temperature was 300 °C. The oven temperature program was as follows: holding at 60 °C for 2 min, heating to 220 °C at a rate of 40 °C/min and holding at that temperature for 1 min, then heating to 310 °C at a rate of 5 °C/min, then holding at that temperature for 30 min. The sterols were identified using commercial standards.

Tocopherol composition was determined according to Grilo *et al*. (2014) with minor modifications. 0.5 ml oil were diluted to 5 ml with dichloromethane and analyzed by a HPLC (Shimadzu Co., Kyoto, Japan) with Inertsil SIL 100A column (250 mm× 4.6 mm× 5 µm, GL Sciences Inc., Japan) and a RF-20A fluorescent detector. A methanol:water (97:3, v/v) mixture was used as the mobile phase. Isocratic elution was used with 0.8 ml/min flow rate. Detector wavelengths were 290 and 330 nm for excitation and emission, respectively. Commercial standards were used for the identification and quantification of the tocopherols.

2.5. Determination of phenolic compounds in the oil

Approximately 3 g of oil were dissolved into 3 ml of hexane, and then 3 ml of methanol were added. After that, the mixture was centrifuged at 6461 xg for 3 minutes (Sigma 2-16K, Sartorius, Germany) and the supernatant was collected. This process was repeated three times. Then, the methanolic phase was evaporated, the residue was dissolved in 1 ml of methanol and placed into a vial for injection. The phenolic composition of the oil was analyzed by a HPLC (Shimadzu Co., Kyoto, Japan) equipped with an Agilent Eclipse XDB-C18 column (250 mm × 4.6 mm × 5 µm) and a SPD-M20A diode array detector (λmax=278nm). Acetic acid:water (3:97, v:v) (A) and methanol (B) were used as the mobile phases and flow gradients used in this analysis were as follows: 0. min 0% A, 0.1–18 min 80% A, 18–24 min 70% A, 24–30 min 67.5% A, 30–36 min 45% A, 36–40 min 0% A, 40–45 min 60% A, and 45–47 min 80% A. Column temperature was 30 °C, flow rate was 0.8 ml/min and the injection volume was 20µl. Commercial standards (Sigma-Aldrich and Fluka Chem Co., St. Louis, MO, USA) were used for the identification and quantification of the phenolic compounds.

2.6. Determination of aromatic volatile compounds in the oil

The aromatic volatile compounds in the oil were determined according to Aydeniz *et al*. (2014). First, 2 g of oil were placed into an amber-colored vial and the vial was kept in a water bath at 60 °C for 30 min. Then, the volatile compounds collected at the headspace of the vial were taken with the SPME fiber coated with 75 µm carboxen/polydimethylsiloxane coating. GC-MS (Shimadzu 2010 SE) with a Restek Rtx-5Sil MS column (30 m x 0.25 mm, 0.25 µm, Fisher Scientific International, Inc., USA) was used to determine the volatile compounds. Helium was used as carrier gas with 1.61 ml/min flow rate. Injector and detector temperatures were set to 250 °C, and 70 eV ionization energy was used. The temperature program was as follows: holding at 40 °C for 2 min and heating to 250 °C at a rate of 4 °C/min. For the identification of compounds, The National Institute of Standards and Technology and Wiley Registry of Mass Spectral Data were used.

2.7. Sensory descriptive analysis of the oil

A sensory descriptive analysis of the oil was carried out through Quantitative Descriptive Analysis (QDA) (Meilgaard *et al*., 1991). There were six female and six male trained panelists aged between 21 and 52. These panelists were trained for at least 10

Table 1. Descriptive terms and references used in the sensory descriptive analysis

Descriptor	Definition	Reference
Spicy	Aroma perceived from spice blends	Aqueous solution of red pepper-black pepper-thyme
Earthy	The odor detected from moist soil	Wet soil
Timber/Kindling	The odor perceived from the dry wood	Pieces of wood and kindling, toothpick
Raw Vegetable	The flavor of raw vegetables	Fresh green beans
Bitter	Basic taste of caffeine and quinone	0.05% caffeine solution (full bitter)

hours on different days and in different sessions. Under the management of the panel leader, five different sensory terms were developed to describe cold-pressed rosehip seed oil. The descriptive terms and references used in this analysis are presented in Table 1. Samples were served in glasses at room temperature. A 10 cm line scale from 1 at minimum intensity to 10 at maximum intensity was used to quantify the sensory characteristics. Water, unsalted cracker, dry coffee, and expectoration cups were provided to panelists in addition to the samples.

2.8. Statistical analysis

Two different cold-pressed rosehip seed oil samples from the same production year were obtained. Each analysis for each sample was made at least in duplicate, or in triplicate. The results of the sensory analysis are presented as means with standard deviation. All other data are presented as means with standard errors.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties of the oil

The physiochemical properties of the cold-pressed rosehip seed oil are presented in Table 2. Specific gravity and refractive index values depend on the fatty acid compositions of oils and vary according to the oil type. In a study about rosehip seed oil, the specific gravity value was found to be 0.927 at 20 °C (Concha *et al.,* 2006). The refractive index value of rosehip seed oil was measured as 1.481 and 1.478 in the studies of Concha *et al.* (2006) and Nino *et al.* (2020), respectively. Our results concur with the literature.

The viscosity of the sample at 25 °C was determined as 49.5 cP. In a study (Milic *et al.,* 2020), the viscosity of cold-pressed rosehip seed oil was measured as 89.4 cP at 20 °C. The viscosity value of the oil sample did not exactly match with the literature,

Table 2. Physicochemical properties of the oil

Property	Value
Specific gravity (25 °C)	0.92 ± 0.01
Specific extinctions	
E232	3.76 ± 0.15
E270	2.84 ± 0.01
Refractive index (25 °C)	1.48 ± 0.00
Viscosity (25 °C, cP)	49.50 ± 0.30
Color	
L	22.96 ± 0.01
a*	0.93 ± 0.02
b*	2.26 ± 0.01
Free fatty acids (linoleic acid %)	1.61 ± 0.07
Acid value (mg KOH/g oil)	3.22 ± 0.14
Peroxide value (meqO_2/kg oil)	14.54 ± 0.91
p-Anisidine value	3.35 ± 0.10
Iodine value (g I_2/100 g oil)	170.04 ± 2.54
Saponification value (mg KOH/g oil)	193.47 ± 2.22
Unsaponifiable matter (%)	1.37 ± 0.04
Total phenolic content (mg GA/100 g)	20.33 ± 1.07
Total carotenoid (mg/kg)	46.50 ± 0.90
Antioxidant capacity (μmol TE/g)	1.43 ± 0.02

Results are expressed as mean ± SE. Each analysis was done at least in duplicate, or in triplicate

probably due to differences in the measurement temperature and genotype of the rosehip seeds. The L, a*, and b* color values are presented in Table 2. There is no data in the literature for comparison. As seen in Figure 1, the oil is dark orange in color which matches with the measured color values.

The free fatty acidity and acid values are shown in Table 2. According to the codex, the acid value should be a maximum of 4.0 mg KOH/g oil for cold-pressed oils (Codex, 2012). Therefore, it can be said that the acid value of the sample was near the limit value but within acceptable limits. Peroxide and

p-anisidine values are used to determine primary and secondary oxidation products, respectively. According to codex, the limit for the peroxide value for cold-pressed oils is 15 meqO_2/kg oil (Codex, 2012). The peroxide value of the oil was near the limit value but within acceptable limits. The peroxide value for cold-pressed rosehip seed oil was measured as 1.2 and 2.1 meqO_2/kg oil in the studies of Grajzer *et al*. (2015) and Tenekeci (2017), respectively. These peroxide values are much lower than the value in our study. In our study, the *p*-anisidine value was measured as 3.35. In a study by (Grajzer *et al*., 2015), *p*-anisidine values for cold-pressed rosehip oils from two different manufacturers were found to be 2.5 and 7.7. Specific extinction values at 232 and 270 nm are the indicators of primary and secondary oxidation products. According to codex, E232 and E270 values for extra virgin olive oil should be a maximum of 2.50 and 0.22, respectively (Codex, 2017). Oil types are different, but production techniques are similar; therefore, these limit values could be suitable for comparison. The oil sample in this study exceeded the limit values given in codex. Oxidation probably occurred during the storage of the seeds (before cold pressing) and during the cold-pressing process. Therefore, storage conditions should be improved.

Iodine number and saponification values are parameters which vary according to oil type. The iodine number and the saponification value for the cold-pressed rosehip oil were determined as 170.04 g I_2/100 g oil and 193.47 mg KOH/g oil, respectively. In some studies (Grajzer *et al*., 2015; Milic *et al.,* 2020), the iodine number of the cold-pressed rosehip oil was measured as 160 and 157.8 g I_2/100 g oil. The saponification value was determined as 187.4 mg KOH/g oil in the study by Concha *et al.* (2006) and 184.2 mg KOH/g oil in the study by Milic *et al.* (2020). Although there are some slight differences, our results generally concur with the literature.

In this study, the unsaponifiable matter content in the cold-pressed rosehip seed oil was determined as 1.37%. In one study (Concha *et al.,* 2006), the unsaponifiable matter content in the rosehip seed oil was found to be 1.4%. Our result concurs with the literature. Total phenolic content and total carotenoid content are shown in Table 2. It is known that phenolic compounds have various health effects and affect the sensory properties of oils (Gioxari *et al.,* 2016). The total phenolic content in rosehip seed oil was measured as 21.54 mg GA/100 g in one study (İlyasoğlu, 2014) and 31.08 mg GA/100 g in another study (Demir *et al.,* 2014). The results from this study generally concur with the literature. In this study, the total carotenoid content in the cold-pressed rosehip seed oil was measured as 46.5 mg/kg. In one study (Fromm *et al.,* 2012), the total carotenoid content in rosehip seed oil was found to be 39.15 mg/kg; while in another study (Grajzer *et al.,* 2015), it was determined as 36.4 mg/kg. It was observed that the carotenoid content in the sample was slightly higher, possibly due to variety or region.

The antioxidant capacity of the oil is shown in Table 2. In some studies, the antioxidant capacity of rosehip seed oil was determined as 1.77 µmol TE/g (İlyasoğlu, 2014), 2.53 µmol TE/g (Grajzer *et al.,* 2015), and 1.69 µmol TE/g (Güney, 2020). Compared to these studies, the antioxidant capacity of the oil sample was slightly lower.

3.2. Thermal properties of the oil

The melting and crystallization temperatures, enthalpies, and OIT of the oil are presented in Table 3. Greater amounts of saturated fatty acids are known to cause higher melting temperatures (Mayfield *et al.,* 2015). Unsaturated fatty acids were dominant in the oil, so, a lower melting point was an expected result. There is no data in the literature for direct comparison. In the study by Ramos *et al.* (2016), the thermal properties of seeds, pulp, leaves and seed oil of *Rosa rubiginosa* were investigated. In the thermal analysis of the seeds, a peak at around -37 ºC was observed

Table 3. Thermal properties of the oil

Property	Value
Melting	
$Onset_m$ (°C)	-25.56 ± 0.17
T_m (°C)	-22.13 ± 0.15
ΔH_m (J/g)	16.68 ± 1.41
Crystallization	
$Onset_c$ (°C)	-45.25 ± 1.15
T_c (°C)	-47.00 ± 1.42
ΔH_c (J/g)	-29.92 ± 1.76
OIT (170°C, min)	3.85 ± 0.04

Results are expressed as mean ± SE.
Each analysis was done at least in duplicate, or in triplicate.
OIT: Oxidative induction time.

and this peak was associated with the crystallization of α-linolenic acid and linoleic acid, which are major fatty acids in the seed oil. The results of this study are similar to this study.

OIT is the time required for the onset of oil oxidation at a given temperature and it is used as an indicator of the oxidative stability of oils. The OIT of our oil sample was determined as 3.85 min at 170 °C. It is thought that the oxidative stability of the oil was low because it was rich in polyunsaturated fatty acids (Table 4). In one study, rosehip seed oils from two different manufacturers were analyzed at 140 °C and the OIT values were found as 26 min and 23 min (Grajzer *et al.,* 2015). The OIT value for our sample is much lower, probably, due to differences in the analysis temperatures. Clearly, this oil has low oxidative stability near frying temperatures.

3.3. Fatty acid, sterol, and tocopherol compositions of the oil

Thirteen fatty acids were determined in rosehip seed oil and the results are presented in Table 4. It was observed that rosehip seed oil contained higher amounts of unsaturated fatty acids (92.5%). Linoleic acid was determined as the major fatty acid with 51.1% and it was followed by α-linolenic acid and oleic acid with 21.4 and 19.3%, respectively. In one study (İlyasoğlu, 2014), linoleic acid, oleic acid, and α-linolenic acid were measured as major fatty acids with 54.05, 19.50, and 19.37%, respectively. In another study (Grajzer *et al.,* 2015), cold-pressed rosehip seed oils from two different manufacturers were analyzed and linoleic acid, oleic acid, and α-linolenic acid were found as major fatty acids with 51.7-44.4, 16.3-14.7 and 21.5-31.8%, respectively. In general, the results concur with the literature.

The sterol composition of the cold-pressed rosehip seed oil is presented in Table 4. β-Sitosterol was determined as the major sterol with 84.6%. It was followed by Δ-5-avenasterol (3.8%), campesterol (3.7%), Δ-7-stigmastenol (2.7%), and stigmasterol (1.6%). In a study (İlyasoğlu, 2014), β-sitosterol, Δ-5-avenasterol, campesterol and Δ-7-stigmastenol contents were measured as 544, 31.6, 23.3 and 41.4 mg/100g, respectively. In the study of Grajzer *et al.* (2015), the β-sitosterol, Δ-5-avenasterol, campesterol and stigmasterol contents of the cold-pressed rosehip seed oils were found to be 5297.3-4753.3, 242.4-379.1, 192.3-205.4, and 77.9-60.2 mg/kg, respectively. The data seems to be within the ranges reported in the literature.

Table 4. Fatty acid, sterol, and tocopherol compositions of the oil

Compound	Value
Fatty acids (%)	
Palmitic	3.70 ± 0.01
Palmitoleic	0.10 ± 0.05
Margaric	0.10 ± 0.00
Heptadecenoic	0.10 ± 0.00
Stearic	2.20 ± 0.00
Oleic	19.30 ± 0.05
Linoleic	51.10 ± 0.05
α-Linolenic	21.40 ± 0.05
Arachidic	1.10 ± 0.00
Eicosanoic	0.40 ± 0.00
Arachidonic	0.10 ± 0.00
Behenic	0.20 ± 0.05
Lignoceric	0.10 ± 0.00
ΣSFA	7.50
ΣUFA	92.50
Sterols (%)	
β-Sitosterol	84.60 ± 3.60
Δ-5-Avenasterol	3.80 ± 0.01
Campesterol	3.70 ± 0.00
Δ-7-Stigmastenol	2.70 ± 0.02
Stigmasterol	1.60 ± 0.00
Δ-7-Avenasterol	1.00 ± 0.00
Δ-5.24- Stigmastadienol	0.60 ± 0.00
Clerosterol	0.60 ± 0.00
Cholesterol	0.60 ± 0.00
Brassicasterol	0.20 ± 0.00
Erythrodiol+Uvaol	0.20 ± 0.01
Tocopherols (µg/g oil)	
γ-Tocopherol	773.76 ± 161.21
α-Tocopherol	266.08 ± 39.6
δ-Tocopherol	35.83 ± 0.75
β-Tocopherol	6.33 ± 1.49
Total	1082

Results are expressed as mean ± SE.
Each analysis was done at least in duplicate, or in triplicate.
SFA: Saturated fatty acid; UFA: Unsaturated fatty acid.

Tocopherols are known as oil-soluble antioxidant and sources of vitamin E. As seen in Table 4, the total tocopherol content in the oil was measured as 1082 µg/g oil. γ-Tocopherol (773.76 µg/g

oil) was determined as the major tocopherol and it was followed by α-tocopherol (266.08 µg/g oil). In one study (Fromm *et al.,* 2012), the total tocopherol content in the rosehip seed oil was measured as 1099.9 mg/kg oil and γ-tocopherol was determined as the major tocopherol. In another study (Grajzer *et al.,* 2015), the tocopherol compositions of cold-pressed rosehip seed oils from two different manufacturers were analyzed. The total tocopherol contents in the rosehip seed oils were determined as 1124.7 and 1037.6 mg/kg oil and, γ-tocopherol was found as the major tocopherol in both oils. The tocopherol composition of the oil is similar to those reported in the literature.

3.4. Phenolic composition of the oil

The phenolic composition of cold-pressed rosehip seed oil is presented in Table 5. Fifteen types of phenolic compounds were quantified in the oil. Rosmarinic acid was the major phenolic compound with 31.38 µg/g oil. It was followed by benzoic acid, campherol, vanillin, caffeic acid, catechin, and quercetin. In one study (Grajzer *et al.,* 2015), the phenolic compositions of cold-pressed rosehip seed oils from two different manufacturers were analyzed. In one of the samples, *p*-coumaric acid was determined as the major phenolic compound, followed by vanillic acid, ferulic acid, and vanillin. In the other sample, again, *p*-coumaric acid was found to be the major phenolic compound, followed by vanillin, vanillic acid, and sinapinic acid. Our results are quite different from these studies, probably because of the differences in genotype of the seeds and agricultural conditions.

Table 5. Phenolic composition of the oil

Phenolic compound	Value (µg/g oil)
Protocatechic acid	0.08 ± 0.00
Catechin	0.96 ± 0.08
p-Hydroxybenzoic acid	0.46 ± 0.02
Caffeic acid	0.93 ± 0.03
Syringic acid	0.23 ± 0.01
Vanillin	1.10 ± 0.01
p-Coumaric acid	0.13 ± 0.00
Ferulic acid	0.45 ± 0.00
Benzoic acid	2.01 ± 0.12
o-Coumaric acid	0.05 ± 0.01
Hesperidin	0.27 ± 0.08
Rosmarinic acid	31.38 ± 1.09
Cinnamic acid	0.42 ± 0.01
Quercetin	0.82 ± 0.01
Kaempferol	1.30 ± 0.07

Results are expressed as mean ± SE. Analysis was done in duplicate.

3.5. Aromatic volatile compounds in the oil

The aromatic volatile compounds detected in the oil are presented in Table 6. Sixty-seven aromatic volatile compounds were quantified in cold-pressed rosehip seed oil. L-Limonene, which has aroma descriptions such as orange, terpene, and pine, was detected as a major aromatic volatile compound with a ratio of 24%. It was followed by 2,4-heptadienal, (E,E) (7.58%) and hexanal (7.02%), which have aroma descriptions such as fresh, green, vegetable. Linalyl acetate (sweet, green, bergamot, lavender), beta-myrcene (peppery, terpene, spicy), and *trans*-2-nonenal (oily, green, cucumber) were also detected in the oil at a ratio of approximately 4%. Dominant volatile compounds in the oil concur with the descriptive terms determined with the sensory descriptive analysis.

To the best of our knowledge, there is no study in the literature about the aromatic volatile compounds in the cold-pressed rosehip seed oil. In the study of Murathan *et al.* (2016), oil was extracted from rosehip fruit with soxhlet equipment and aromatic volatile compounds were analyzed. Butanoic acid, 1,2-propanediol, α-caryophyllene and naphthalene were determined predominantly. The data on volatile aromatics would greatly contribute to the literature referring to cold-pressed rosehip oil.

3.6. Sensory descriptions of the cold pressed rosehip seed oil

The sensory descriptive terms developed for the cold-pressed rosehip seed oil are shown in Table 7. Five sensory attributes were determined for description of the cold-pressed rosehip seed oil, namely spicy, earthy, timber/kindling, raw vegetable and bitter. Timber/kindling and raw vegetable tastes/aromas were found to be relatively dominant. Generally, these results concur with the results from the aromatic volatile compounds analysis. To the best

TABLE 6. **Aromatic volatile compounds in the oil**

No.	RI[a]	Volatile compound	Aroma definition[b]	Area %
1	445	Ethanol	Strong alcoholic, ethereal, medical	1.15 ± 0.1
2	500	2-Propanone	Solvent, ether, apple	1.58 ± 0.0
3	602	Acetic acid	Sour, burning, cheesy	4.04 ± 0.8
4	623	2-Butenal	Flower	0.95 ± 0.1
5	678	1-Penten-3-one	Pepperoni, onion	0.15 ± 0.0
6	690	Propionic acid	Acid, cheese, vinegar	0.13 ± 0.0
7	698	Pentanal	Fermented bread	0.48 ± 0.1
8	723	3-Methyl-1-butanol	Fusel oil, whiskey, fruity	1.96 ± 0.3
9	750	2-Pentenal, (E)-	Green, tomato, fruit	0.45 ± 0.0
10	773	Toluene	Sweet	0.25 ± 0.0
11	790	1-Octene	Kerosene	0.08 ± 0.0
12	799	Hexanal	Fresh, green, leaf	7.02 ± 0.1
13	851	(E)-2-Hexenal	Green, banana, cheese	1.86 ± 0.1
14	892	Styrene	Sweet balm, flower	0.61 ± 0.0
15	908	o-Xylene	Geranium	0.16 ± 0.0
16	911	2,4-Hexadienal	Oily, sweet, green, spice	0.07 ± 0.0
17	915	2-Acetylfuran	Sweet balm, almond, caramel	0.07 ± 0.0
18	924	α-Thujene	Woody, green grass	2.47 ± 0.5
19	928	Pentanoic acid	Acidic, sharp, cheese	0.22 ± 0.0
20	948	α-Pinene	Fresh camphor, pine, woody	0.46 ± 0.1
21	952	*trans*-2-Heptenal	Green, vegetables, oily	2.27 ± 0.2
22	954	Benzaldehyde	Sharp, almond, bitter	0.52 ± 0.0
23	958	Heptenal	Fruity, green	0.24 ± 0.0
24	972	1-Octen-3-ol	Fungus, soil, mold, green	0.31 ± 0.0
25	973	β-Pinene	Dry wood, pine, green	0.25 ± 0.0
26	978	1-Octen-3-one	Herbaceous, mushroom, soil	0.08 ± 0.0
27	983	β-Myrcene	Peppery, terpene, spicy	4.16 ± 0.7
28	986	6-Methyl-5-hepten-2-one	Citrus, green, apple	0.89 ± 0.0
29	1000	*trans, trans*-2,4-Heptadienal	Oily, green, vegetable	2.26 ± 0.1
30	1003	Hexanoic acid, ethyl ester	Sweet fruit, pineapple, banana	0.84 ± 0.1
31	1007	Octanal	Aldehyde, waxy, orange peel, oily	0.43 ± 0.0
32	1009	δ-3-Carene	Citrus, herbaceous, pine	0.47 ± 0.0
33	1012	l-Phellandrene	Mint, menthol	0.35 ± 0.0
34	1013	2,4-Heptadienal	Oily, green, vegetable	7.59 ± 0.1
35	1023	Cymene	Fresh citrus, terpene, spice	3.18 ± 0.1
36	1027	l-Limonene	Orange, terpene, pine	24.8 ± 0.1
37	1030	Eucalyptol (1,8-cineole)	Eucalyptus, camphor, medicine, herb	1.02 ± 0.1
38	1036	*cis*- Ocimene	Citrus, tropical, green, terpene	0.21 ± 0.0
39	1042	Oct-3(E)-en-2-one	Earthy, spicy, sweet, mushroom	0.16 ± 0.0
40	1044	β-Ocimene	Citrus, tropical, green, woody	0.58 ± 0.0
41	1059	2-Octenal	Oily, sweet, green	0.63 ± 0.2
42	1080	Heptanoic acid	Rancid, sour cheese, sweat	0.07 ± 0.0
43	1081	3,5-Octadiene-2-one	Fruity, oily, mushroom	0.70 ± 0.0
44	1083	α-Terpinolene	Fresh, woody, pine, sweet	0.07 ± 0.0
45	1086	1-Methyl-4-isopropenylbenzene	Phenolic, spicy, clove	0.13 ± 0.0
46	1093	2-Nonanone	Fresh, green, herbaceous	0.14 ± 0.1
47	1095	Ethyl heptanoate	Fruity, pineapple, wine	0.09 ± 0.0
48	1102	Linalool	Citrus, flower, sweet	1.90 ± 0.0
49	1103	Nonanal	Waxy, aldehydeic, citrus, lemon	1.42 ± 0.3
50	1120	*cis*-Methyl-4-octenoate	Green, fruity, waxy	0.29 ± 0.0
51	1125	Methyl octanoate	Waxy, green, orange, vegetable	0.22 ± 0.0
52	1128	Sabinene	Woody, terpene, pine	0.14 ± 0.1
53	1135	Camphor	Mint, herbaceous	0.24 ± 0.0
54	1155	2,6-Nonadienal, (E,Z)-	Green, oily, cucumber	0.51 ± 0.0
55	1161	*trans*-2-Nonenal	Oily, green, cucumber	4.15 ± 0.1
56	1184	4-Octenoic acid, ethyl ether	Fruity, pear, citrus	0.23 ± 0.0
57	1185	Decanal	Sweet, waxy, orange peel, flower	0.16 ± 0.0
58	1200	Dodecane	Alkane	0.67 ± 0.0
59	1202	Benzaldehyde, 2-hydroxy-6-methyl	Almonds, cherries	0.36 ± 0.0
60	1215	2,4-*trans, trans*-Nonadienal	Oily, melon, waxy, green	0.07 ± 0.0
61	1261	Linalyl acetate	Sweet, green, bergamot, lavender	4.42 ± 0.5
62	1263	*trans*-2-Decenal	Waxy, oily, earthy, mushroom	0.12 ± 0.0
63	1265	*trans*-Anethole	Sweet anise, licorice, mimosa	0.53 ± 0.0
64	1331	2,4-Decadienal	Orange, sweet, fresh, citrus,green	1.50 ± 0.2
65	1342	Neryl acetate	Floral, rose, soap	0.14 ± 0.0
66	1457	Alloaromadendren	Woody	0.10 ± 0.0
67	1469	1-Dodecanol	Soil, soap, waxy, coconut	0.18 ± 0.0

Results are expressed as mean of Area %. Analysis was done in duplicate.

[a] RI (Kovat Index) on Rtx-5 MS column

[b] Aroma definitions of the volatile compounds are found from the web pages of http://www.thegoodscentscompany.com and http://www.flavornet.org

TABLE 7. **Sensory descriptive properties of the oil**

Property	Value
Spicy	1.2 ± 0.3
Earthy	1.0 ± 0.5
Timber/Kindling	5.8 ± 0.7
Raw Vegetable	3.5 ± 1.2
Bitter	0.5 ± 0.1

Results are expressed as mean ± SD. Analysis was done in duplicate.

of our knowledge, for comparison, there is no study about the sensory properties of rosehip seed oil in the literature. Therefore, this study provides very important data for those who would have interest in using this oil.

CONCLUSIONS

In this study, rosehip seed oil produced with the cold-press technique was characterized completely. It was observed that cold-pressed rosehip seed oil was rich in unsaturated fatty acids, essential fatty acids, sterols, tocopherols and phenolic compounds. For cold-pressed rosehip seed oil, for the first time, aromatic volatile compounds were determined and sensory descriptive terms were provided. The aroma descriptions of the dominant volatile compounds in the oil were found to agree with the panel and the determined sensory descriptive terms. Acidity and oxidation levels in the oil were found to be slightly high and it was suggested to improve the storage conditions of both the seeds and pressed oils. In conclusion, rosehip seeds can be utilized for edible and nutritionally-rich cold-pressed oil production for various food applications, functional foods and cosmetics.

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Impact of different harvest times on fatty acid profile, sterol, tocopherol and bioactive properties of hazelnut oil

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SUMMARY: This study was carried out to determine the effects of different harvest times of hazelnuts on their lipid profiles and bioactive compound accumulations. Tombul hazelnut cultivar was harvested at four different harvest stages, namely in PH (pre-harvest time), EH (early harvest time), NH (normal harvest time) and LH (late harvest time). According to the results of the study, oil accumulation continued up to NH but did not further increase into LH (from 49.58 to 58.54 mg/100 g). Oxidative stability indices changed positively due to decreased poly-unsaturated fatty acids (PUFA) from 9.87 to 7.70% in LH. The highest total sterol amount was reached in LH (122.32 mg/100 g). Although the change in the tocopherol content in the oil was irregular with the progression of the harvest time, its amount in the nuts increased continuously. Total carotenoid, phenolic, flavonoid, and antioxidant activity (DPPH and ABTS) peaked in EH and decreased to a minimum in LH.

KEYWORDS: *Antoxidant activity; Fatty acid profile; Hazelnut oil; Oxidative stability; Sterol; Tocopherol.*

RESUMEN: ***Impacto de diferentes épocas de cosecha en el perfil de ácidos grasos, esteroles, tocoferoles y propiedades bioactivas del aceite de avellana.*** Este estudio se llevó a cabo para determinar los efectos de diferentes épocas de cosecha de avellanas sobre los perfiles de lípidos y las acumulaciones de compuestos bioactivos. El cultivar de avellana Tombul se cosechó en cuatro épocas diferentes en el orden: PH (época previa a la cosecha), EH (época de cosecha temprana), NH (época de cosecha normal) y LH (época de cosecha tardía) con referencia al NH. Según los resultados del estudio, la acumulación de aceite continuó hasta NH, pero no aumentó más en LH (de 49,58 a 58,54 mg/100 g). Los índices de estabilidad oxidativa cambiaron positivamente debido a la disminución de los ácidos grasos poliinsaturados (AGPI) del 9,87 % al 7,70 % en la LH. La cantidad total de esteroles más alta se alcanzó en la LH (122,32 mg/100 g). Aunque el cambio en el contenido de tocoferoles en el aceite fue irregular con el avance del tiempo de cosecha, su cantidad en la nuez aumentó continuamente. La actividad total de carotenoides, fenólicos, flavonoides y antioxidantes (DPPH y ABTS) alcanzó su punto máximo en EH y disminuyó a un mínimo en LH.

PALABRAS CLAVE: *Aceite de avellana; Actividad antioxidante; Estabilidad oxidativa; Esteroles; Perfil de ácidos grasos; Tocoferoles.*

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1. INTRODUCTION

In recent years, nuts and their beneficial effects on human health have attracted great attention by researchers. Their special oil profiles and their polyphenolic compounds underlie the beneficial impacts of nut consumption as determined from epidemiological research and nutrition trials (Ciemniewska-Żytkiewicz *et al*., 2015a). Together with walnuts, hazelnuts constitute approximately 60% of the dried nut production in Europe (Pycia *et al*., 2019). The hazelnut is a member of the *Betulaceae* family and is one of the most popular nuts worldwide due to its pleasant taste, nutrients, mono and polyunsaturated fatty acids, fat-soluble bioactive substances such as phytosterol, tocopherol, and polyphenols (Karaosmanoğlu and Üstün, 2019).

The chemical compositions of nuts, which are important for human nutrition, change during nut development, as reported in the literature (Cristofori *et al*., 2015; Ilyasoglu 2015; Gama *et al*., 2020). For example, Seyhan *et al.* (2007) reported that the oil ratio increased from 48.10 to 59.83% in the Tombul hazelnut variety between the early harvest and normal harvest time, the protein and ash ratios decreased, and the oleic acid ratio increased from 74.60 to 80.64, while the linoleic and palmitic acid ratios decreased. Ilyasoğlu (2015) noted that there were significant changes in sterol ratios with the progression of harvest time, and the total amount of sterols, which was 293 mg/100 g in EH, decreased to 147 mg/100 g in NH. Ciemniewska-Zytkiewicz *et al*. (2015a) reported that MUFA increased (22.02-79.17%), PUFA decreased (30.09-10.28%) and SFA remained unchanged with maturation in Polish hazelnuts. In the same study, it was stated that total tocopherol increased from 22.48 mg/100 g to 38.24 mg/100 g and alpha-tocopherol was dominant at all stages. Again, in Polish hazelnut cultivars, Pycia *et al*. (2019) reported a decrease in polyphenol and antioxidant activity with the progression of harvest time.

In Turkey, hazelnuts reach harvest maturity in August. However, in very large and sloping lands, harvesting is usually done by hand. For this reason, a large number of hazelnut workers are needed during the harvest season and the inability to meet the need causes early or late harvest. In addition, producers dealing with other works other than hazelnut and climatic conditions are other reasons for early or late harvest. The progression of harvest time can cause various chemical changes in nuts. For example, Kazantzis *et al*. (2003) reported that more lipid oxidation occurred and oil quality deteriorated in late harvested almonds compared to early harvested almonds. In another study conducted on Macadamia nuts, it was reported that late harvest shortened the shelf-life of the nuts (Gama *et al*., 2020). On the other hand, in *Torreya granndis* nuts, late harvest was reported to increase dry matter content, oil content and nutritional quality (Wang *et al*., 2021). In addition, the effects of early and normal harvest time on fatty acid composition in Italian (Cristofori *et al*., 2015), Polish (Ciemniewska-Zytkiewicz *et al*. 2015a) and Turkish (İlyasoğlu, 2016) hazelnuts, tocopherol content in Polish hazelnuts (Ciemniewska-Zytkiewicz *et al*. 2015a; Pycia *et al*., 2020), sterol composition in Turkish hazelnuts (Ilyasoglu, 2015) and antioxidant activity in Polish hazelnut cultivars (Pycia *et al*., 2020) were investigated. Despite these data reported on hazelnuts and other nuts, so far no study has been found in the literature on the effect of late harvest time of hazelnuts on oil composition and bioactive properties. The main purpose of our study is to reveal the effect of different harvest times on fatty acids, sterol, tocopherol composition, total carotenoid, total phenolic, total flavonoid and antioxidant activities (DPPH and ABTS). In addition, no study has been found in the literature examining the changes in tocopherol content, composition and carotenoid amount during nut development in Turkish hazelnut cultivars. Another aim of our study is to eliminate this deficiency.

2. MATERIALS AND METHODS

2.1. Nut samples

The Tombul hazelnut, which is the most important Turkish hazelnut cultivar, was chosen as the research material. Hazelnut samples were harvested by hand from three orchards in the Batlama valley, Akköy (40°51’38.52”N, 38°18’58.69”E), Seyitköy (40°51’40.40”N, 38°19’09.72”E) and Alınca (40°51’59.55”N, 38°19’00.26”E) villages of Giresun (Turkey). Sampling was harvested from each orchard according to the “Z” pattern for homogeneous and random sample selection. Sample trees were marked and nuts were collected from the same trees in determined harvest times. Sampling was carried out in four stages with reference to the reguler harvest

time in Turkey (second half of August): PH, 14 July; EH, 1 August; NH, 18 August and LH, 6 September. Harvested hazelnuts were separated from their green husks, placed in plastic bags and kept at -18 °C. The drying process was carried out for 3 days between 09:00 a.m. and 08:00 p.m. in ambient conditions (average temperature 24.7 °C). The samples were laid on a 5x5 m jute cover on the concrete ground and mixed 5 times a day during the drying period. After 8:00 p.m. in the evening, each group was gathered in the middle, and they were covered with a nylon cover to prevent moisture transfer from outside. At the end of the drying period, the humidity in all samples decreased below 6% and the samples were kept at -18 °C until the day of analysis.

2.2. Reagents and standards

Unless otherwise stated, all chemicals used were from Sigma-Aldrich-Fluka Co. Ltd. (Prolab, Turkey). Potassium hydroxide (KOH) and anhydrous sodium sulfate (Na_2SO_4) from Carlo Erba (Italy), N,O-bis (trimethylsilyl) trifluoroacetamide, trimethyl chlorosilane, 5α-cholasten-3-β-ol, tocopherol from Merck (Germany) and the standard blend of methyl esters of fatty acids were obtained from Supelco (USA).

2.3. Oil extraction

The shelled hazelnuts used in the analysis were hand-cracked (3 kg) and separated from their shells. Oil extraction was performed with a test-scale screw-press device with a 3-kW regulatable speed electric engine. The screw turning speed was adjusted to 60 rpm to extract 80% of the total oil (Demirtas *et al*., 2013).

2.4. Determination of fatty acids

The fatty acid profile of the nuts was defined according to the method reported by Demirtas *et al*. (2013). The fatty acids were determined on a gas chromatography-flame ionization detector (GC-FID) (Perkin Elmer, Autosystem GLX, USA) equipped with a SP®-2560 (100 m x 0.25 mm x 0.2 µm, Supelco, USA) column. GC-FID operating conditions: carrier gas, helium; flow rate 0.5 mL/min; injection temperature, 280 °C; detector temperature, 260 °C; the oven temperature program was adjusted to keep the initial temperature at 120 °C for 2 minutes, increase it to 220 °C at 5 °C/min and hold it at this temperature for 10 minutes. Data were evaluated with Total Chrom Navigator and explained as % fatty acid.

2.5. Oxidative stability and health indices

The oleic acid/linoleic acid ratio (O/L), iodine value (IV) (Belviso *et al*., 2017), atherogenic (AI) and thrombogenic (TI) indices (Ulbricht and Southgate 1991), hypocholesterolemic-to-Hypercholesterolemic ratio (h/H) and the peroxide index (PI) (Hanczakowska *et al*., 2015) values were calculated according to the equations below:

$$\text{O/L} = \text{oleic acid/linoleic acid} \quad (1)$$

$$\text{IV} = (\text{palmitoleic acid} \times 1.901) + (\text{oleic acid} \times 0.899) + (\text{linoleic acid} \times 1.814) + (\text{linolenic acid} \times 2.737) \quad (2)$$

$$\text{PI} = (\%\ \text{monoenoic} \times 0.025) + (\%\ \text{dienoic} \times 1) + (\%\ \text{trienoic} \times 2) + (\%\ \text{tetraenoic} \times 4) + (\%\ \text{pentaenoic} \times 6) + (\%\ \text{hexaenoic} \times 8) \quad (3)$$

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

$$\text{TI} = (C14{:}0 + C16{:}0 + C18{:}0)/((0.5 \times \sum \text{MUFA}) + (0.5 \times \sum \text{FA}\omega 6) + (3 \times \text{FA}\omega 3) \quad (5)$$

(MUFA: mono-unsaturated fatty acids, FAω6: omega-6 fatty acids, FAω3: omega-3 fatty acids)

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

2.6. Determination of sterol composition

The sterol profile of the nut oils was determined according to the procedure reported by Demirtas *et al*. (2013). Sterols were determined with a GC-FID (Perkin Elmer, Autosystem GLX, USA) and SE-54 (5%-phenyl-1%-vinylmethylpolysiloxane), 30 m x 0.32 mm x 0.25 µm (Agilent, USA) column. GC-FID operating conditions: carrier gas, helium; flow rate 0.8 mL/min; injection temperature, 280 °C; detector temperature, 300 °C; the oven temperature program was adjusted so that the initial temperature was held at 60 °C for 2 minutes, increased to 220 °C at 40 °C/min, held for 1 minute, increased to 310 °C at 5 °C/min and remained at this temperature for 30 minutes. Individual sterols without standards were identified using relative retention time (RRT) of 5α-cholestan-3β-ol. Datas were evaluated with TotalChrom Navigator and expressed as mg/100 g oil.

2.7. Determination of tocol composition

Tocol isomers of the oil samples extracted from kernels with pellicle were determined according the method reported by Demirtas *et al*. (2013). The contents in tocopherols were calculated using external standards, and measured by a HPLC (Agilent Series 1100, Germany) with fluorescence detector and normal phase column (5µm LiCrosorb Si60 25 cm x 4.6 mm i.d., HiChrom, UK). Chromatographic separation was performed with an isocratic tetrahydrofuran/n-heptane (3.8%, v/v) carrier at a flow rate of 1 mL/min. The column temperature was held at 40 °C. The wavelength of the detector was set at 270 nm for excitation and 310 nm for emission. The results with Chemstation were expressed as mean values ± standard deviation as mg/100 g oil.

2.8. Spectrophotometric analyses

2.8.1. Extract preparation of phenols, flavonoids and antioxidant activity assays

Phenolic compounds were extracted according to the method reported by Karaosmanoğlu (2022). 250 g hazelnut sample and a methanol/water (75/10) solution was used for extraction. The extract obtained as a result of the process was used in the total phenolic and flavenoid contents, and antioxidant capacity experiments.

2.8.2. Determination of total phenolic compounds, total flavonoids and antioxidant activities (DPPH and ABTS methods)

Biochemical properties were measured in nut kernels. Total phenolic compounds, total flavonoids and antioxidant activity were determined as biochemical properties and these analyses were performed according to the method described by Karaosmanoğlu (2022). All measurements were performed using a spectrophotometer (Shimadzu, Japan). Total phenolic compounds were determined using the Folin-Ciocalteu reagent. Total phenols were expressed as gallic acid equivalents (mg GAE/100 g) and total flavonoids were expressed as mg catechin equivalents (mg CE/100 g). Antioxidant activity was measured using the DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS [2,2-azinobis (3-ethylbenzoth iazoline-6-sulfonic acid) diammonium salt] tests and stated as Trolox equivalents per 100 g (mgTE/100 g).

2.8.3. Extract preparation and determination of total carotenoid content

For the carotenoid analysis, extraction was done with the help of methyl alcohol and ultrasonic homogenizer (Bandelin MS72, Germany). The absorbance of the extracts was read by the spectrophotometer at 663 nm, 645 nm and 440.5 nm. Firstly, the amounts of chlorophyll a and chlorophyll b, and then the total amount of carotenoids were calculated using the formulas below (Amira, 2011).

$$Chlorophyll\ a = (12.7xA_{663} - 2.69xA_{645}) \; x\frac{v}{1000xw} \quad (7)$$

$$Chlorophyll\ b = (22.9xA_{645} - 4.68xA_{663}) \; x\frac{v}{1000xw} \quad (8)$$

$$Total\ carotenoid = 46.95x(A_{440.5} - 0.268 \; x\ \text{chlorophyll}\ a + b) \quad (9)$$

W: weight by grams for extracted; v: final size of extracted; A: absorbance.

2.9. Statistical analysis

Analyses were conducted using JMP (pro-16) statistical software. One-way ANOVA followed by Tukey's post-hoc test was used to compare the means of the study sets. A p-value of less than 0.05 was considered statistically significant. Results were expressed as means ± standart deviation (n=3) for each determination. Principal component analysis (PCA) was also performed using JMP (pro-16) software.

3. RESULTS AND DISCUSSION

3.1. Variations in oil content at different harvest times

The amount of oil in hazelnuts collected at four different harvest times are summarized in Table 1. Oil in the kernel increased from PH to NH, from 49.58 mg/100 g to 58.54 mg/100 g ($P < 0.01$), but did not change in LH. The most substantial increase was between PH and EH, with a variation of about 15%. Seyhan *et al*. (2007) reported the oil content in Tombul hazelnut as 48.10, 59.83 mg/100 g in EH and NH, respectively, in line with our results. Ciemniewska-Zytkiewicz *et al*. (2015a) studied Polish hazelnuts, and Cristofori *et al*. (2015) studied Italian hazelnuts and reported that the oil content in ha-

Table 1. Oil content (mg/100g), fatty acid composition (%), oxidative stability and healthy indices of hazelnuts at different harvest times

	PH	EH	NH	LH	
Oil	49.58±0.01 c	56.67±0.01 b	58.54±0.17 a	58.19±0.15 a	**
Palmitic	4.71±0.01 c	4.63±0.01 d	4.80±0.01 b	4.99±0.01 a	**
Palmitoleic	0.11±0.00 b	0.10±0.00 c	0.11±0.00 b	0.12±0.00 a	*
Heptadecanoic	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00	ns
Stearic	2.44±0.01 c	2.92±0.01 b	3.04±0.01 a	3.05±0.01 a	**
Oleic	82.41±0.01 c	82.99±0.01 b	82.68±0.02 b	83.55±0.11 a	**
Elaidic	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	ns
Linoleic	9.80±0.01 a	8.85±0.01 b	8.88±0.01 b	7.65±0.01 d	**
α- linolenic	0.07±0.00 a	0.06±0.00 b	0.05±0.00 b	0.05±0.00 b	**
Arachidic	0.12±0.00 a	0.10±0.00 c	0.11±0.00 bc	0.12±0.00 ab	**
Eicosenoic	0.14±0.00	0.15±0.01	0.15±0.01	0.15±0.01	ns
Behenic	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	ns
Miristic	0.02±0.00	0.02±0.00	0.03±0.00	0.03±0.00	ns
SFA	7.34±0.01 d	7.72±0.01 c	8.03±0.01 b	8.24±0.01 a	**
MUFA	82.68±0.01 c	83.26±0.01 b	82.81±0.02 c	83.84±0.10 a	**
PUFA	9.87± 0.01 a	8.91±0.01 b	8.93±0.01 b	7.70±0.01 c	**
UFA	92.56±0.01 a	92.17±0.01 b	91.74±0.01 c	91.54±0.10 c	**
Oxidative stability indices					
UFA/SFA	12.60±0.01 a	11.93±0.02 b	11.42±0.01 c	11.10±0.02 d	**
O/L	8.40±0.01 d	9.37±0.01 b	9.30±0.01 c	10.91±0.01 a	**
IV	92.27±0.01 a	91.02±0.01 b	90.78±0.01 c	89.36±0.09 d	**
PI	12.01±0.01 a	11.05±0.01 b	11.05±0.01 b	9.85±0.01 c	**
Healthy indices					
AI	0.05±0.00 b	0.05±0.00 b	0.05±0.00 b	0.06±0.00 a	**
TI	0.15±0.00 d	0.16±0.00 c	0.17±0.00 b	0.18±0.00 a	**
h/H	19.50±0.03 b	19.75±0.03 a	18.97±0.01 c	18.18±0.05 d	**

All values are presented as means ± standard deviation (n= 3). The different superscript letters (a-d) in the same row indicate statistically significant difference according to the Tukey test. * significant at $P < 0.05$, ** significant at $P < 0.01$, ns: not significant. Nd: not detected. SFA-saturated fatty acids, MUFA- mono unsaturated fatty acids, PUFA- poly unsaturated fatty acids, UFA- unsaturated fatty acids, O/L- oleic acid/linoleic acid, IV- iodine value, PI- peroxidisability index, AI- atherogenic index, TI- thrombogenicity index, h/H- hypocholesterolemic to Hypercholesterolemic ratio. PH- pre-harvest time, EH- early harvest time, NH- normal harvest time, LH- late harvest time

zelnuts increased as nut ripening progressed. In our study, it was observed that late harvest stage did not increase oil accumulation in the nuts. Similarly, Kazantzis *et al*. (2003) found that late harvest did not change the oil content in the nuts in a study conducted on almonds. This can be explained by the cessation of the activity of the biosynthesis pathway that forms triglycerides with maturation.

3.2. Variations in fatty acid composition at different harvest times

The variations in the fatty acid profile of nuts harvested in different stages are presented in Table 1. The predominant fatty acid was oleic acid at all harvest times, followed by linoleic, palmitic and stearic acids. Reporting the same fatty acid order, Cristofori *et al*. (2015) reported the major fatty acid, oleic acid, at the level of 80-83%, with stearic and palmitic saturated fatty acids below 10%. Although there is no knowledge on the impact of LH on the fatty acid profile of hazelnuts, Seyhan *et al*. (2007) and İlyasoğlu (2016) reported a similar ranking between early harvest and normal harvest periods. Minor fatty acids ranged from PH to LH at 0.54 to 0.42%.

It has been observed that the amount of oleic acid increased as maturation progressed, and the

ratio, which was 82.41% in PH, reached the highest level at 83.55% in LH ($P < 0.01$). In contrast to oleic acid, the ratio of linoleic acid decreased from 9.80 to 7.65% as maturation progressed ($P < 0.01$). They showed an opposite pattern of accumulation of oleic and linoleic acids during nut development ($R = -0.948$), which is in close agreement with data reported by Ciemniewska-Zytkiewicz *et al*. (2015a) and İlyasoğlu (2016). In addition, with the progression of maturation, on average, a slight decrease in linoleic acid was emphasized by Cristofori *et al*. (2015), despite the increase in oleic acid in Italian hazelnut cultivars. The negative correlation between oleic and linoleic acid accumulation can be explained by the decrease in linoleic acid synthesis due to the slowdown in the activity of the Δ12-desaturase enzyme, which catalyzes the synthesis of linoleic acid from oleic acid, because of seasonal temperature increase (Bouali *et al*., 2013). In addition, lipoxygenase activity may have caused a decrease in the content of linoleic acid (İlyasoğlu, 2016). The slowdown in linoleic acid synthesis was accompanied by α-linoleic acid, which may be associated with the slowing of the Δ6-desaturase enzyme activity due to temperature increase. Unlike α-linoleic acid, palmitoleic acid increased slightly, but other minor fatty acids were not affected by harvest time ($P > 0.05$). The palmitic acid ratio fluctuated between harvest times, while stearic acid increased from PH (2.44%) to NH (3.04%) but remained the same in LH.

The highest amount of SFA (saturated fatty acids) was detected in LH (8.24%) among harvest times, associated with a rise in the amount of stearic and palmitic fatty acids ($P < 0.01$). Although the MUFA ratio fluctuated over time, it increased from 82.68 to 83.84 between PH and LH in relation to oleic acid. Unlike MUFA, PUFA (poly-unsaturated fatty acids) was highest in PH (9.87%) and lowest in LH (7.70%). This decrease is largely associated with a drop in the content in linoleic acid with nut ripening. It has been observed that as the maturation progressed, the amount of UFA (unsaturated fatty acids) decreased up to NH but did not change in LH. In general, it has been determined that LH caused more SFA and MUFA and less PUFA accumulation than NH. Kazantzis *et al*. (2003) reported that late harvested nuts contained more SFA in a study conducted on almonds.

3.3. Variations in oxidative stability and healthy indices at different harvest times

Depending on the changes in the fatty acid profile of hazelnuts harvested at different harvest times, significant differences emerged in the indices of oxidation resistance and health effects (Table 1). The effect of harvest times on these indices is evaluated below. A lower UFA/SFA rate is considered to have a longer shelf-life (Alasalvar *et al*., 2003). With the delay in harvest time, this ratio decreased continuously and reached the lowest level at 11.10 in LH ($P < 0.01$). A greater O/L value means better oxidative stability (Karaosmanoğlu 2022). Although the O/L value fluctuated between harvest times, the lowest value was found in PH (8.40), the highest value in LH (10.91), and the difference was statistically significant. The increase between NH (9.30) and LH (10.91) was slightly significant. Consistent with our work, Seyhan *et al*. (2007) reported that the O/L ratio increased with maturation. Low IV is an indication that oils are less reactive, more stable, more resistant to rancidity and oxidation (Belviso *et al*., 2017). The IV, which was 92.27 in PH, showed a continuous decrease and reached the lowest value in LH (89.36). The lowest IV in LH may be related to the decrease in linoleic acid along with the delay in harvest time because the lowest linoleic acid ratio was detected in LH. We previously (Karaosmanoğlu and Üstün, 2019) reported the IV of NH hazelnuts in the range of 89.81-94.40, which is comparable to to the present study. The PI value reflects the grade of unsaturation of dietary oils and is used as an indicator of PUFA peroxidation (Hanczakowska *et al*., 2015). In this work, the highest PI value was detected in PH and decreased slightly with maturation.

The oxidation ratios of fatty acids are approximately 1:10:100:200 for stearic, oleic, linoleic and linolenic acids, respectively (Alasalvar *et al*., 2003). Therefore, LH samples with higher stearic acid levels and lower linoleic acid levels are more stable against oxidative changes. As a result, it can be said that LH hazelnuts may be more durable against lipid oxidation and have a longer shelf-life because of lower UFA/SFA, and IV, and higher O/L values. The resistance of LH to oxidation is largely associated with high SFA and low PUFA. It should not be forgotten that this situation may reduce the positive effects in terms of health, despite the potential to extend the shelf-life of hazelnuts.

AI, TI and h/H values are indices used to estimate the lipid character of foods and are calculated based on their fatty acid composition. The values for these indices indicate the quality of fats for the risk of pro-atherogenic, pro-thrombogenic and cardiovascular disease. (Hanczakowska *et al*., 2015). In order to protect cardiovascular health, AI and TI are expected to be low and h/H to be high. With the late harvest, AI and TI values slightly increased compared to NH, while h/H decreased (0.06, 0.18, 18.18, respectively) ($P < 0.01$). Even if the late harvest negatively changed the AI and TI values, it was 2.3 times lower for AI and 1.8 times lower for TI than the values reported by Ulbricht and Southgate (1991) for olives (0.14, 0.32, respectively).

3.4. Variations in sterol composition at different harvest times

Seven different sterols detected and quantified in hazelnut samples which were harvested at different harvest times are listed in Table 2. The prominent sterols β-sitosterol, campesterol and Δ5-avenasterol accounted for more than 93% of total sterols, while other sterols (stigmasterol, Δ7-stigmastenol, Δ7-avenasterol, Δ7-campesterol) were in small amounts. β-sitosterol was the most plentiful sterol, accounting for more than 85% of the all sterols. Similar to our results, Alasalvar *et al*. (2009) reported in Turkish hazelnuts and Amaral *et al*. (2006) in Portuguese hazelnuts, that major sterols constituted more than 90% of total sterols and β-sitosterol was the predominant sterol. The amount of total sterol (116.14 mg/100g in NH) was quite consistent with the results of Alasalvar *et al*. (2003) (113.52 mg/100g) but were higher than those reported by Ciemniewska-Zytkiewicz *et al*. (2015b) (130.32-152.22 mg/100g). This may be due to variety, geographical location or environmental factors (Alasalvar *et al*., 2003).

In our study β-sitosterol was detected to be the predominant sterol at all harvest times and the same sterols were determined (Δ7-campesterol could not be detected in LH). β-sitosterol was found to be increased and Δ5-avenasterol was found to be decreased in hazelnuts with LH time ($P < 0.01$). Since Δ5-avenasterol is a precursor in the biosynthesis of β-sitosterol (Fernandez-Cuesta *et al*., 2013), the different enzymatic efficiency in LH may explain the negative relationship between both sterol accumulations. Similar to β-sitosterol, total sterol increased significantly ($P < 0.01$) in LH (122.32 mg/100 g) compared to NH (116.14 mg/100 g), but PH, EH and NH were in the same group. Harvest times did not affect the accumulation of campesterol and Δ7-avenasterol, while the changes in stigmasterol and Δ7-campesterol were irregular. There is no data in the literature on the effect of late harvest on sterol synthesis. Consistent with our results, the same major sterols were detected in a single study examining the sterol change during maturation stages, and it was reported that β-sitosterol was dominant in all maturation stages, but contrary to our results, the amount of sterols decreased as time progressed (Ilyasoglu 2015). This difference may be due to the fact that the samples were collected much earlier than the harvest maturity in the study, because the values reported ripe nuts were comparable to our results, and abiotic factors may also have affected sterol biosynthesis (Misina *et al*., 2020). Similar to our results, it

Table 2. Sterol composition (mg/100 g oil) of hazelnut oil at different harvest times

	PH	EH	NH	LH	
Campesterol	6.69±0.01	5.95±0.29	6.04±0.1	6.54±0.31	ns
Stigmasterol	1.31±0.03 ab	1.27±0.01 b	0.97±0.01 c	1.50±0.08 a	**
Δ7-stigmastenol	1.78±0.16 b	1.52±0.19 b	1.71±0.01 b	2.78±0.13 a	**
β-sitosterol	100.80±0.32 b	98.19±0.88 b	100.39±0.46 b	105.45±1.33 a	**
Δ5-avenasterol	4.22±0.11 b	3.66±0.31 b	5.11±0.01 a	2.80±0.07 c	**
Δ7-avenasterol	0.90±0.15	1.17±0.05	0.82±0.01	1.14±0.15	ns
Δ7-campesterol	0.83±0.01 b	1.55±0.11 a	0.86±0.01 b	nd	**
Total sterol	116.41±0.47 b	112.67±1.08 b	116.14±0.40 b	122.32±1.34 a	**

All values are presented as means ± standard deviation (n= 3). The different superscript letters (a-d) in the same row indicate statistically significant difference according tothe Tukey test, * significant at $P < 0.05$, ** significant at $P < 0.01$, ns: not significant, Nd: not detected, PH: pre harvest time, EH: early harvest time, NH: normal harvest time, LH: late harvest time

has been reported that the total sterol (Yorulmaz and Konuşkan 2017) in olives increased as harvest time prolongated. The results have shown that LH led to an increase in the sterol content of the samples.

3.5. Variations in tocopherol composition and carotenoid amount at different harvest times

The compositions and amounts of tocopherol in hazelnuts harvested at varied harvest times are summarized in Table 3. In samples collected in NH, α-tocopherol (47.17 mg/100 g) was the predominant isomer, followed by γ-tocopherol (6.26 mg/100 g) and β-tocopherol (1.22 mg/100 g). α-tocopherol constituted 86% of the total tocopherol. A similar profile of tocopherols was reported by Ciemniewska-Zytkiewicz *et al*. (2015a). Alasalvar *et al*. (2009) reported the α-tocopherol (34.5 mg/100 g) and total theocopherol (46.9 mg/100 g) contents of Turkish Tombul hazelnuts, which are quite consistent with our research data.

In this research, it has been observed that α-tocopherol was predominantly homologous at harvest times, its concentration ranged from 53.59 mg/100 g (PH) to 47.17 mg/100 g (NH), and decreased as the harvest time progressed except for LH ($P < 0.01$). A parallel increase in the amount of total tocopherol was observed with α-tocopherol. γ-tocopherol accumulation was inversely correlated with α-tocopherol ($R= -0.765$). This may be due to the alteration of gamma tocopherol methyltransferase enzyme activity, which converts γ-tocopherol to α-tocopherol, depending on abiotic stress factors (Jin and Daniell 2014). While β-tocopherol decreased slightly between PH and EH and remained stable at other harvest times, δ-tocopherol was detected only in a low quantity at PH. Unlike our study, Ciemniewska-Zytkiewicz *et al*. (2015a) reported that the amount of tocopherol increased as maturation progressed. Considering that the most intense period in tocopherol biosynthesis is the first period of nut development (Bouali *et al*., 2013), the reason for the difference with the literature may be that the sampling started close to ripening, since the primary objective of the work was to determine the effects of late harvest. On the other hand, it is thought that the main biochemical functions of tocopherols are to protect PUFA against peroxidation (Bouali *et al*., 2013; Bouali *et al*., 2022). While the ratio of PUFA in total fatty acids was 9.87% in PH, it decreased to 7.70% in LH. Therefore, the decrease in tocopherol levels as the ripening progressed can also be attributed to the decrease in the degree of unsaturation of fatty acids. Although the amount of tocopherol in the oil decreased, the total amount of tocopherol in the nut increased due to the increase in the amount of oil over time (PH, EH, NH, LH; 30.25 mg/100 g, 31.86 mg/100 g, 31.99 mg/100 g, 32.30 mg/100 g, respectively) ($R= 0.979$). A similar increase was reported in Polish hazelnuts (Pycia *et al*., 2020).

As seen in Table 3, harvest time had a significant effect on carotenoid concentration ($P < 0.01$). The amount of carotenoids, which peaked in EH (3.96 mg/g nut) showed a continuous decrease in NH (2.04 mg/g nut) and LH (1.60 mg/g nut) with the progression of harvest time. This decrease can be explained by the blockage of carotenoid production as

Table 3. Tocopherol composition (mg/100 g oil) and total carotenoid content (mg/g nut) of hazelnut oil at different harvest times

	PH	EH	NH	LH	
α- tocopherol	53.59±0.07 a	49.72±0.01 b	47.17±0.01 c	49.76±0.03 b	**
β- tocopherol	2.77±0.12 a	1.49±0.01 b	1.22±0.01 b	1.29±0.01 b	**
γ- tocopherol	4.59±0.01 c	5.22±0.01 b	6.26±0.01 a	4.45±0.01 d	**
δ- tocopherol	0.02±0.00	nd	nd	nd	ns
Total tocopherol	61.02±0.05 a	56.41±0.03 b	54.65±0.02 d	55.51±0.04 c	**
Vitamin E	55.16±0.04 a	50.84±0.01 b	48.28±0.01 c	50.72±0.03 b	**
Carotenoid	1.53±0.01 d	3.96±0.01 a	2.04±0.01 b	1.60±0.01 c	**

All values are presented as means ± standard deviation (n= 3). The different superscript letters (a-d) in the same row indicate statistically significant difference according to the Tukey test. * significant at $P < 0.05$, ** significant at $P < 0.01$, ns: not significant, Nd: not detected, PH: pre-harvest time, EH: early harvest time, NH: normal harvest time, LH: late harvest time, Vitamin E (expressed as α-tocopherol equivalents). The conversion factors for vitamin E activity were as follows: α-Tocopherol x 1.00, β-tocopherol x 0.40, γ-tocopherol x 0.10, δ-tocopherol x 0.01 (Alasalvar *et al*., 2009).

a result of the inhibition of enzymes which catalyze the oxidation steps for carotenoid synthesis with nut ripening (Bouali *et al*., 2013). Similar to our results, in some studies conducted on pecan nuts and olives, researchers reported carotenoid loss with the progression of ripening (Bouali et al., 2013; Yorulmaz and Konuskan 2017; Baccouri *et al*., 2008).

3.6. Variations in total phenolic compounds, flavonoids and antioxidant activities at different harvest times

The changes in the amounts of total phenolic compounds (TPC) in hazelnuts among harvest times are shown in Figure 1. TPC was determined as 251.18 mgGAE/100 g in hazelnuts collected in NH, consistent with some previous studies (Arcan and Yemenicioğlu 2009). Significant differences were detected in the TPCs of the samples among harvest times and the periods were listed as EH > NH > PH > LH ($P < 0.01$). The TPC in LH (174.84 mgGAE/100 g) was observed to be 62% lower than that in NH, and 30% less than that in NH (450.71 mgGAE/100 g), where the greatest amount was detected. It is known that there is a difference between the enzymatic activities of polyphenol oxidase (POD) and peroxidase enzymes in hazelnuts at different harvest times (Seyhan *et al*., 2007). The decrease in TPC as harvest time progressed can be explained by altered POD and peroxidase enzyme activity. A similar downward trend was reported by Pycia et al. (2020) for Polish hazelnuts and Pycia *et al*. (2019) for walnuts. In agreement with our results, Baccouri et al. (2008) associated the decreasing oleuropein concentration with progressive harvest time with the increased hydrolytic enzyme activity of olives with ripening. On the other hand, similar to TPC, the highest TFC was detected in EH (277.85 mgCE/100 g) and the lowest

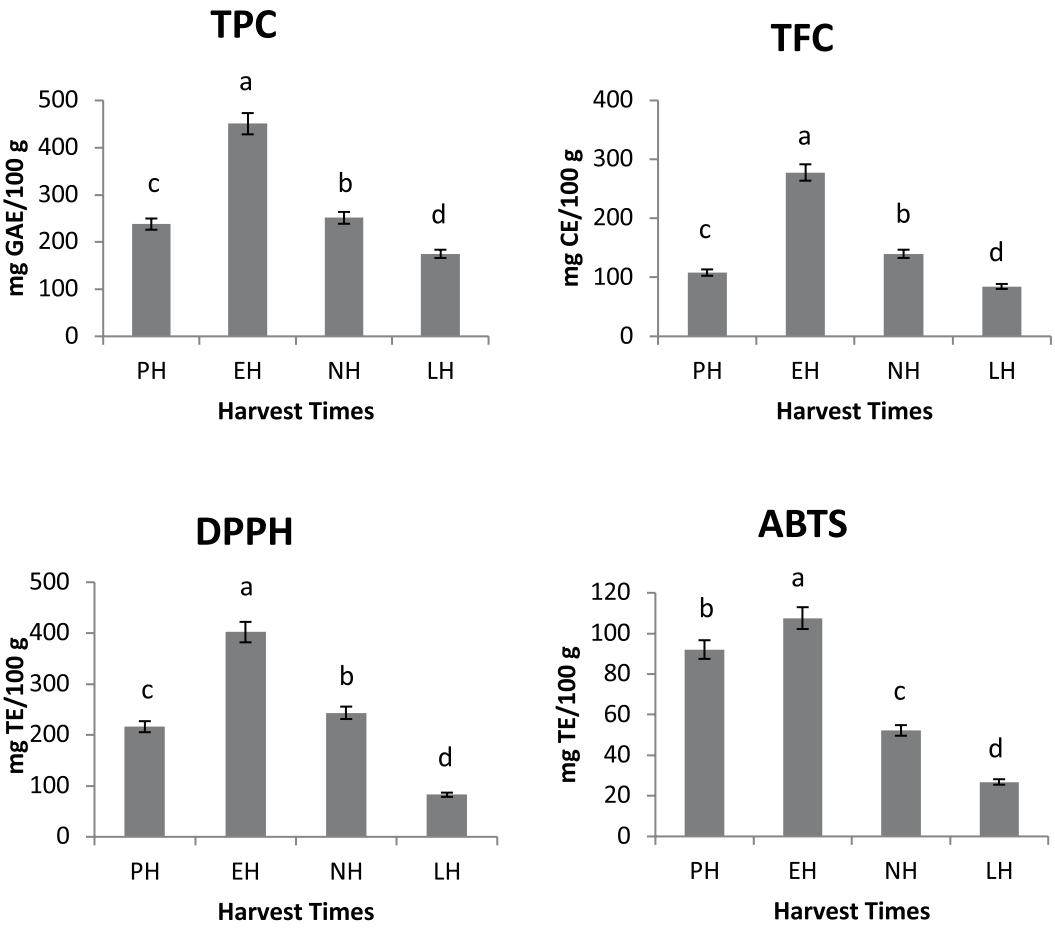

Figure 1. Changes in total phenolic compounds (TPC), total flavonoids (TFC) and antioxidant activities (DPPH and ABTS) of hazelnuts at different harvest times (n=3). Different lowercase letters in the same parameter indicate the statistical difference of harvest times according to the Tukey test ($P < 0.01$). PH: pre-harvest time, EH: early harvest time, NH: normal harvest time, LH: late harvest time.

in LH (84.61 mgCE/100 g). With the late harvest, an approximately 40% decrease was observed in TFC compared to NH. Persic *et al*. (2018) reported that TFC first peaked and then decreased in Slovenian hazelnuts as the harvest time was delayed. The ratios of TFC in TPC varied from 45 to 61% among harvest times. There is a decrease in phenolic compounds as the biological processes ends with ripening and the fruit no longer needs protection from herbivores (Persic *et al*., 2018; Pycia *et al*., 2020). In this study, this information was confirmed for hazelnuts.

In the present study the antioxidant capacities of hazelnut samples were determined by two different methods (DPPH and ABTS) and were found to be significantly affected by the harvest times ($P < 0.01$) (Figure 1). According to the results, EH was characterized by the highest antioxidant capacity (402.03 mgTE/100 g, 107.54 mgTE/100 g; DPPH, ABTS respectively). In both methods, antioxidant activity peaked in EH and then decreased continuously. Similarly, it has been reported that antioxidant activity decreased with ripening in Polish hazelnuts (Pycia *et al*., 2020) and walnuts (Pycia *et al*., 2019). Wang *et al*. (2021) have found that the antioxidant activity in *Torreya granndis* nuts decreased with ripening after the peak point. In LH compared to NH, 66 and 51% decrements were found for DPPH and ABTS, respectively. When the correlation between antioxidant capacity and TPC was evaluated, the lower antioxidant activity of LH can be explained by the decrease in

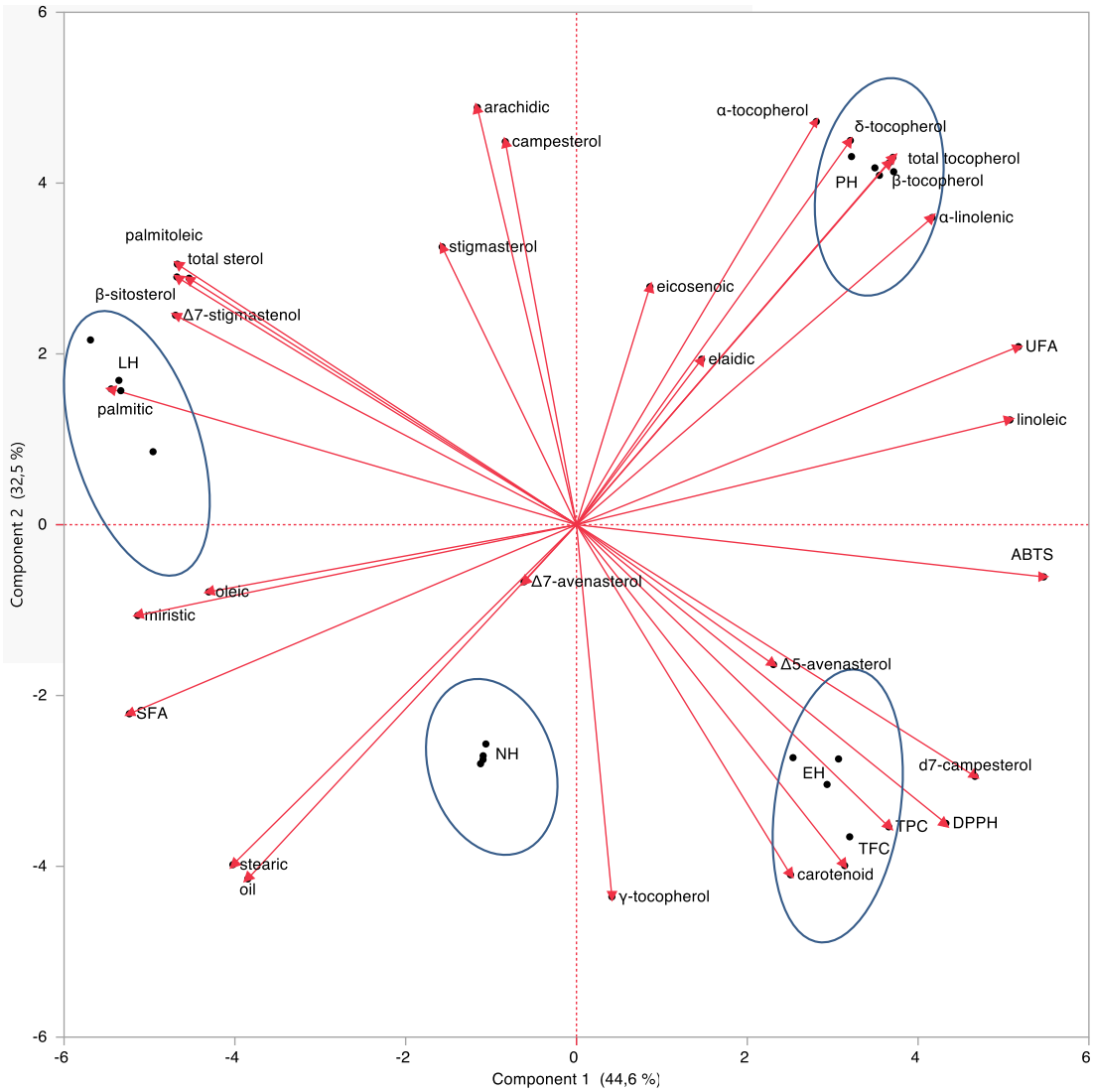

Figure 2. Biplot of the relationship of bioactive compounds in hazelnuts with different harvest times (n=3). PH- pre-harvest time, EH- early harvest time, NH- normal harvest time, LH- late harvest time.

total phenol content due to the progression of harvest times (r=0.960, r=0.797, DPPH and ABTS, respectively). This result is consistent with previous studies reporting that the antioxidant activity of hazelnuts is correlated with TPC (Arcan and Yemenicioğlu 2009).

3.7. Principal component analysis

Principal component analysis (PCA) was applied to get an overview of the sample variations and to reduce the initial variables to a small number of principal components. PC1 (Component 1) and PC2 (Component 2) components explained 44.6 and 32.5% of the total variability, respectively (Figure 2**)**. The lower right quadrant contains variables that correlate quite well with each other. Here, it can be seen that TPC and TFC and two methods used to determine antioxidant capacity (DPPH and ABTS) are correlated. There is also a strong positive correlation between carotenoid and antioxidant activity. This group includes EH samples. In the left upper quadrant, there were, among others, β-sitosterol, total sterol, and palmitic acid, whose accumulation increased with LH. The positive effect of LH on sterol accumulation is clearly seen in this quadrant. In the upper right corner where the pH samples are located, all tocopherols except γ-tocopherol are included in addition to UFA. However, it should not be forgotten that this appearance is related to the accumulation of tocopherol in the oil because the amount of tocopherol in the nuts was higher in the LH samples. As a result, four different harvest times were clearly separated by PCA and ANOVA was largely confirmed.

4. CONCLUSIONS

According to the results, although there was no difference in oil accumulation between the NH and LH periods, SFA and MUFA increased while PUFA decreased. It can be thought that LH hazelnuts may be more resistant to oxidation due to lower UFA/SFA, IV, and PI, and higher O/L values related to the changes in the fatty acid profile. The highest total sterol and β-sterol levels were reached in LH, but there was no difference among the other periods. Although the amount of tocopherols in the oil showed an irregular change between harvest times, it showed a continuous increase in the nuts due to an increase in oil. On the other hand, carotenoids, TPC, TFC and antioxidant activities peaked in EH and then decreased to the lowest level in LH with a continuous decrease. Although LH caused a loss of polyphenols, it can be thought that it can increase the resistance to lipid oxidation and extend storage stability and shelf-life. As a result, late harvesting can be recommended to preserve the kernel quality of hazelnuts, which must be stored for certain periods until they are consumed or processed. In addition, hazelnuts to be used for special food designs and pharmaceutical purposes are recommended to be harvested early due to the increase in polyphenol content and antioxidant capacity.

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