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Computational studies on physico-chemical properties in the quality analysis of corn and peanut oil

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SUMMARY: Oils are commonly used in cooking as a frying medium which has been constantly subjected to different levels of heating. In this work, we have considered the most commonly used oils namely peanut oil and corn oil. Quality analyses of corn and peanut oils were made by relating macroscopic properties (ultrasonic velocity, viscosity, and density) to microscopic parameters (intermolecular free length, adiabatic compressibility etc.,) by subjecting them to six cycles of heating (190 ˚C). Variation in the mentioned property indexes, the degree of degradation and reusability for the next heating cycle that could be used in the food industry and processing were monitored. Using Newton-Laplace and Wood's equation, the adiabatic compressibility, acoustic impedance, and intermolecular free length of the oil were estimated from the experimental data. Ultrasonic velocity was observed linearly as related to viscosity with the dependency factor ($R^2 = 0.932$). With the aid of experiential data, the physical thermodynamic parameters, particularly particle size, packing factor, chemical potential, and L-J potential were computed. A high correlation factor was observed by fitting ultrasonic velocity, viscosity, and density to Parthasarathy and Bakshi, and Rodenbush equations. In the study, ultrasonic velocity, a macroscopic parameter, could be decoded to determine the microscopic variations in oil subjected to different temperatures in an industrial application.

KEYWORDS**:** *Corn oil; LJ potential modeling; Peanut oil; Ultrasonic velocity; Viscosity*

RESUMEN: ***Estudios computacionales de propiedades físico-químicas en análisis de calidad de aceites de maíz y maní.*** Los aceites se utilizan comúnmente en la cocina como un medio para freír y se someten de forma continua a diferentes niveles de calentamiento. En este trabajo, hemos considerado dos de los aceites comunmente utilizados, como los de maní y maíz. Los análisis de calidad de los aceites de maíz y maní se han realizado relacionando propiedades macroscópicas (velocidad ultrasónica, viscosidad y densidad) con parámetros microscópicos (longitud libre intermolecular, compresibilidad adiabática, etc.) sometiéndolo a seis ciclos de calentamiento (190 ºC). La variación en las propiedades mencionadas indica el grado de degradación y su reutilización para el siguiente ciclo de calentamiento que podría ser lo usado en la industria y procesamiento de alimentos. Se utiliza la ecuación de Newton-Laplace y Wood, y a partir de los datos experimentales se estimaron la compresibilidad adiabática, la impedancia acústica y la longitud libre intermolecular de los aceites. La velocidad ultrasónica se observó estar linealmente relacionada con la viscosidad con el factor de dependencia ($R^2 = 0{,}932$). Con la ayuda de los datos experimentales, se calcularon los parámetros termodinámicos físicos como el tamaño de partícula, factor de empaquetamiento, potencial químico y potencial L-J. Se observó un factor de correlación alto ajustando la velocidad, viscosidad y densidad ultrasónicas a las ecuaciones de Parthasarathy y Bakshi y Rodenbush. En el estudio, la velocidad ultrasónica, un parámetro macroscópico, podría decodificarse para determinar las variaciones microscópicas en el aceite sometido a diferentes temperaturas en una solicitud industrial.

PALABRAS CLAVE: *Aceite de cacahuete; Aceite de maíz; Modelado potencial de LJ; Velocidad ultrasónica; Viscosidad*

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1. INTRODUCTION

The ultrasonic technique is a non-destructive method used to estimate the quality of food products such as vegetables, milk, fruits, egg, fish, meat products, etc. An ultrasonic wave with a frequency greater than 1MHz is called a diagnostic wave which can interact with each particle of food items (Benedito *et al.,* 2007). Hence the estimation of the composition of any food material can be done using ultrasonic waves. Low-intensity ultrasonic waves can be used to evaluate the quality, texture, and compositional changes in fats and oils (Benedito *et al.*, 2002). Ultrasonic waves interact with short and long-chain fatty acids and can reveal the structure and degree of saturated fatty acids in the oil. Ultrasonic velocity through vegetable oil decreases with the increase in temperature and can be illustrated in three different regions (Benedito *et al.,* 2002; Izbaim *et al*., 2010). A decrease in the ultrasonic velocity in region I (temperature range 0 to 22 °C) exhibits a negative temperature coefficient due to the presence of the polymers monomer, dimer, trimer, and oligomer in the oil. When the molecular dimension of the polymer is high, it attenuates the ultrasonic wave which decreases its velocity. In region II (temperature range 24 to 37 ˚C approximately) a lowering of velocity is due to the slight melting of the above polymer. A decrease in the velocity in region III (temperature range 38 to 50 °C approximately) has been observed due to saturated bonds in fatty acids (McClements and Gunasekaran).

Frying is one of the most important cooking processes in the food industry. Oil is the sole base product in the preparation of fried foods. During the deep-frying process in snack industries, the oil undergoes heat and mass transfer when it is exposed to temperatures from 180 °C to its smoke point temperature, which in turn accelerates chemical reactions like oxidation, polymerization, hydrolysis, etc. (Katharina *et al.,* 2012; Adolfo *et al.,* 2006; Pushan *et al.,* 2014). In general, fatty acids in the oil are made up of an even number of carbon atoms arranged in zig-zag fashion containing one or more carbon -to- carbon with one or more double bond, and a single carboxyl group (Reidoon, 2005). Oil is a mixture of a large number of fatty acids such as saturated fatty acids (SFA)- without any double bond; monounsaturated fatty acids (MUFA) - with a single double bond; and polyunsaturated fatty acids (PUFA) - with more than one double bond (Benedito *et al.,* 2007; Benedito *et al.,* 2002; Reidoon, 2005; Alireza and Abdolabbas, 2019). Karolina *et al*. (2020) investigated the quality of olive oil for cooking vegetables under domestic condition using its physical and chemical properties.

In the present study, fatty acid molecules were considered as a combination of hard spheres (atoms) to determine their chemical potential and particle size. The oil samples considered for the study were corn oil and peanut oil, which are highly used for frying. Corn oil has more than 85% unsaturated fatty acids and 13-14% saturated fatty acids (Reidoon, 2005). Peanut oil has 84.4% unsaturated fatty acids and 18-19% saturated fatty acids (David William, 2008; Fasino *et al.,* 2006).

Dilapidation of oil has a negative effect on the aroma, color, texture, excellence, and safety of fried foodstuffs (Adolfo *et al.,* 2006; Reidoon, 2005). In the evaluation of the quality of vegetable oil, researchers have developed analytical methods to estimate the degree of degradation of oil with heating cycles (Benedito *et al.,* 2007; Adolfo *et al.,* 2006, Reidoon, 2005). Though analytical analyses remain precise, they are quite laborious and costly. The consumption of degraded oil leads to atherosclerosis, coronary artery diseases, and increases LDL cholesterol, blood pressure, colon cancer, etc. (David, 2008; Fasino *et al.,* 2006). Hence, in this study to analyze its quality, physical and chemical properties were used as an effective tool. The non-invasive ultrasonic method was used to characterize the oil by the interaction of the wave with a molecule in the oil that produces chemical changes (Benedito *et al.,* 2007, Katharina *et al.,* 2012). Viscosity is a conventional factor still used as an important index in the quality analysis of oil in some countries. The viscosity of oil varies with the contents in saturated and unsaturated fatty acids in the oil. Hence castoff as a quality index estimates the degradation level in oil (Fasina *et al.,* 2006; Mohammad and Seyyed, 2020). The formation of free fatty acids and polar compounds due to chemical reactions upon heating decreases the reusability of oil.

In this study, corn and peanut oils exposed to six different cycles of heating to a frying temperature were taken as samples. Physical properties such as ultrasonic velocity, viscosity, density, acoustic impedance, adiabatic compressibility, and intermolecular free length were studied to determine the thermal degradation of the oils at every cycle of heating. Since oil is a dense and viscous liquid made up of short and long-chain fatty acids to take it as freely sliding molecules, hard-sphere models such as Percus-Yevick (PY), Scaled Particle Theory (SPT) and Mansoori, Carnahan, Starling, and Leland (MCSL) model were adopted in the determination of chemical potential, interaction potential, particle size and the packing factor of atoms in fatty acids in oil (Everett, 1963). These parameters were estimated with experimentally determined ultrasound velocity as the main input. The study related the distribution of molecules in oil to degradation and also to the forces existing between molecules in corn oil and peanut oil.

2. MATERIALS AND METHODS

2.1. Sample Preparation

The branded samples of corn (*Zea mays)* and peanut (*Arachis hypogaea)* oil needed for the study were bought from a supermarket in Thanjavur. They were separated as unheated and heated (190 ˚C) samples at different cycles and stored at ambient temperature. Heated samples were prepared by repeated heating cycles in a copper beaker surrounded with an oil bath to 190 °C six times (Rubalya *et al.,* 2016). Each unheated sample was stored as a set of three 100 ml samples (Total of 42 samples) in dark bottles.

2.2. Experimental Measurement

2.2.1. Ultrasonic Measurement

A of velocity measurement was taken for all 42 samples by taking 20 ml of the sample in the liquid column of Ultrasonic interferometer (F-80, Mittal Enterprises, Delhi, India) (Rubalya *et al.,* 2013). The device was provided with an electric signal connected to a flat plate mounted on a piezo-electric crystal with capacitive reactance that produces 2 MHz frequency. The parameters associated with ultrasonic velocity (U), (i) Acoustic impedance (Z) relate the velocity of sound and density of the medium as given by the equation:

$$Z = \rho U \text{ kg /m}^2\text{s} \qquad (1)$$

(ii) Newton- Laplace equation links the elastic bulk modulus (K) of liquid with velocity and density of oil by:

$$K = \rho U^2 \text{ N/m}^2 \qquad (2)$$

(iii) Wood’s equation gives the relation between adiabatic compressibility with velocity and density of sound in liquid:

$$\beta = \frac{1}{\rho U^2} \text{ m}^2\text{/N} \qquad (3)$$

(iv) Molecular free length L_f, it is the distance covered by the propagating acoustic waves between the surfaces of two neighboring molecules in a liquid. Intermolecular free length is the ratio of available volume to the surface area of the molecule. Jacobson (1953) and Pandey *et al.* (1979) have shown an empirical relation between ultrasonic velocity U, the density ρ and the intermolecular free length L_f of a liquid as:

$$\begin{aligned} &L_f U \rho^{1/2} = K, \\ &\text{and } L^2{}_f = K^2 \beta \quad N^{-1} m^2 \qquad (4) \\ &\textit{where} \quad L_f = K \surd \beta \textit{ i.e. } L_f = K \surd 1/\left(U^2 \rho\right) \end{aligned}$$

where, K is Jacobson constant at room temperatures and β is the adiabatic compressibility of liquid.

2.2.2. Density Measurement

The density measurement of density was determined using Pycnometer, following the ASTM standard method D891-09 (Rubalya *et al.,* 2016).

2.2.3. Viscosity Measurement

The kinematic viscosity (η) of the oil was measured using a Redwood viscometer (Associated Instrument Manufacturers India Private Limited, New Delhi, India) (Rubalya *et al.,* 2016).

2.3. Statistical analysis

All data pertaining to viscosity, density, and ultrasonic velocity were recorded as mean ± SD and analyzed using SPSS (version 15). One-way analysis of variance was performed by ANOVA procedures. Significant differences among the parameters were determined by Duncan’s multiple range tests. Time of heating was taken as an independent parameter; whereas viscosity, density, and ultrasonic velocity were dependent parameters. Significance and variances (p) are illustrated in the footnotes of Tables 1 and 2.

2.4. Theory

2.4.1. Study of Empirical equations

The packing fractions of molecules in corn and peanut oil were analyzed using PY, SPT, and MCSL models (Heying and Corti, 2004; Lebowitz, 1964; Mandell and Reiss, 1975; Mansoori *et al.,* 1971). The analyses were carried out by employing the experimentally measured ultrasound velocity as they are directly related to the thermodynamic behaviour of the system. The PY model proposes an equation of state for hard-sphere fluids, which was analyzed in terms of the viral co-efficient (Mandell and Reiss, 1975). The PY equation mainly involves the radial distribution function of a fluid, which can be generalized to an m-component mixture (Lebowitz, 1964; Percus and Yevick, 1958). SPT explains the feasibility of cavity formation in hard-sphere mixture fluids (Mansoori *et al.,* 1971; Reiss *et al.,* 1959). In addition, the glycerol in the oil leaves a space for the fatty acids to occupy and forms a cavity. A system under study is an m-component mixture and this is possible only because the probability of cavity formation is large (Percus and Yevick, 1958; Reiss *et al.,* 1959; Ravi *et al.,* 2008). An equation relating ultrasonic velocity (U) with the change in the molecular weight using the MCSL model incorporating particle interaction in the PY equation is given as (Wertheim, 1963; Yarnell *et al.,* 1973):

$$U = X^{-1}\sqrt{\frac{RT}{\gamma M}} \tag{5}$$

where, R- gas constant, T- temperature, γ- specific heat ratio, M – molecular weight, X^{-1} is the expression that depends on three different theories that define the state of the system (Wertheim, 1963; Yarnell *et al.,* 1973; Yu *et al.,* 2002). The three theories that define these systems are the Percus-Yevick (PY) approach, Scaled Particle Theory (SPT) and Mansoori Carnahan Starling Leland (MCSL) model (Wertheim, 1963; Yu *et al.,* 2002). Based on these models, the equation for X takes the form:

$$X_{PY}^{-1} = \frac{1+2y}{(1-y)^2} \tag{6}$$

$$X_{SPT}^{-1} = \frac{(1+2y)^2}{(1-y)^3} \tag{7}$$

$$X_{MCSL}^{-1} = \frac{y^4 - 4y^3 + 4y^2 + 4y + 1}{(1-y)^4} \tag{8}$$

Using equations 6, 7 and 8 packing fraction and hence particle diameter (y) were deduced using an expression with ρ_n which is the number density of the uniform fluid and σ the particle size:

$$y = \frac{\pi}{\ } \rho_n \sigma^3 \tag{9}$$

2.4.2. Estimation of chemical potential (µ/kT)

Chemical potential is the energy that can be emitted or absorbed when a particle is added to a system. It is also known as partial molar free energy. In heterogeneous systems, it is the change in internal energy of the system when new particles are added or when the condition of the system is altered like the variation in temperature. The density of a uniform hard-sphere fluid is related to its chemical potential through the Carnahan – Starling equation of state and is given in equation 1, respectively (Wertheim, 1963; Yarnell *et al.,* 1973; Yu *et al.,* 2002; Lennard, 1924):

$$\left[\frac{\mu}{kT}\right] = \ln(\rho_n) + \left\{ y\left(\frac{8+9y+3y^2}{(1-y)^2}\right)\right\} \tag{10}$$

where, ρ_n is the number density of the uniform fluid.

2.4.3. Estimation of Lennard – Jones potential (LJ Potential)

The Lennard – Jones potential (5- 8 equations) defines the interaction between a pair of neutral atoms or molecules. The potential description includes i) repulsive term r^{12} and ii) long-range attractive term r^{6} and the potential is defined as (Yarnell *et al.,* 1973; Lennard, 1924):

$$V_{LJ}(r) = \frac{4\varepsilon}{k}\left[\left(\frac{\sigma}{r}\right)^{12} - \left(\frac{\sigma}{r}\right)^{6}\right] \tag{11}$$

where, ε/k defines inter particle- potential and can be estimated using the following equation (Lennard, 1924):

$$\frac{\varepsilon}{k} = \frac{2T}{n_s}\left[-\frac{\mu}{kT} + \mu_o - 3\ln\Delta\right] \tag{12}$$

where, n_s – number of nearest neighboring atoms.

3. RESULTS AND DISCUSSION

3.1. Variation in physical property of oil

Tables 1 and 2 show the variations in viscosity, density, ultrasonic velocity and ultrasonic parameters such as velocity, acoustic impedance and intermolecular free length of corn and peanut oil exposed to different heating times.

3.1.1. Ultrasonic velocity

Ultrasound velocity (U) is subjected to intermolecular interfaces and molecular connotations, which enhance the significance of ultrasound velocity measurements for the molecular state, structure, composition and various processes (Izbaim *et al.,* 2010). From the Tables 1 and 2, it can be observed that ultrasonic velocity decreases with cycles of heating and is due to less transmission of ultrasonic wave and scattering produced by molecular clusters in the oil (Rubalya *et al.,* 2013).

Figures 1 (a), (b) and (c) illustrate the disparity in ultrasonic velocity, adiabatic compressibility, and viscosity with heating time, which is observed to be greater in peanut oil compared to corn oil. The formation of polymers and saturated fatty acids due to hydrogenation, oxidation, and hydrolysis accelerate the clustering of molecules that increase density and viscosity in the oil, and in turn, reduce the ultrasonic velocity (Benedito *et al.,* 2007; Rubalya *et al.,* 2013; Izbaim *et al.,* 2010). The reduction in ultrasonic velocity reflects a decrease in intermolecular free length due to the conversion of a double bond in fatty acids to a single bond by the addition of a hydrogen atom that forms saturated fatty acids by which the packing density of molecule increases (Rubalya *et al.,* 2017).

TABLE 1. Variations in parameters (ρ), (η), (U), (Z), (L) and (β) of corn oil upon heating

Time of heating in h	Density (ρ) 10^3 kg/m^3	Viscosity (η) 10^{-6}m^2/s	Ultrasonic velocity (U) 10^3 m/s	Acoustic impedance (Z) 10^6kg/m^{2}s	Intermolecular free length (L) 10^{-10} m	Adiabatic Compressibility (β) 10^{-10} N^{-1} m^2
0	0.904 ± 0.008^a	61.74 ± 0.15^b	1.48 ± 0.035^a	1.539	0.329	4.390
0.5	0.909 ± 0.005^a	76.41 ± 0.23^b	1.45 ± 0.028^a	1.581	0.338	4.364
1	0.911 ± 0.012^a	89.22 ± 0.14^a	1.44 ± 0.019^a	1.598	0.342	4.345
1.5	0.919 ± 0.003^a	93.90 ± 0.27^a	1.41 ± 0.027^b	1.678	0.359	4.227
2	0.923 ± 0.017^a	109.51 ± 0.13^a	1.38 ± 0.030^b	1.697	0.363	4.269
3	0.926 ± 0.015^a	112.42 ± 0.08^a	1.35 ± 0.023^b	1.701	0.364	4.355
5	0.928 ± 0.011^b	118.33 ± 0.04^a	1.31 ± 0.029^a	1.677	0.360	4.553

One-way ANOVA analysis was determined (n=3) by Duncan's multiple range tests. a p-value < 0.001and b p-value < 0.05 are significant.

TABLE 2. Variations in parameters (ρ), (η), (U), (Z), (L) and (β) of peanut oil upon heating

Time of heating in h	Density (ρ)103 kg/m^3	Viscosity(η) 10^{-6}m^2/s	Ultrasonic velocity (U)10^3 m/s	Acoustic impedance (Z) 10^6 kg/m^{2}s	Intermolecular free length (L) x10^{-10} m	Adiabatic Compressibility (β) 10^{-10} N^{-1} m^2
0	0.932 ± 0.044^b	68.51 ± 0.06^b	1.46 ± 0.039^c	1.415	0.303	4.853
0.5	0.944 ± 0.072^a	76.12 ± 0.53^c	1.44 ± 0.046^b	1.405	0.301	4.929
1	0.949 ± 0.065^a	105.39 ± 0.28^a	1.43 ± 0.012^b	1.396	0.299	5.002
1.5	0.956 ± 0.029^b	132.26 ± 0.17^a	1.41 ± 0.080^a	1.415	0.303	5.019
2	0.967 ± 0.087^c	151.24 ± 0.42^c	1.32 ± 0.028^b	1.449	0.310	5.227
3	0.971 ± 0.024^b	176.73 ± 0.61^c	1.19 ± 0.035^b	1.354	0.290	6.205
5	0.983 ± 0.031^b	183.45 ± 0.34^b	0.94 ± 0.004^c	1.085	0.232	9.807

One-way ANOVA analysis was determined (n=3) by Duncan's multiple range tests. ^{a}p-value < 0.001, ^{b}p-value < 0.05, and ^{c}p-value < 0.005 are significant.

Adiabatic compressibility, Bulk modulus, and molecular free length determine the arrangement of atoms and molecules that influence the inter-atomic forces like London dispersion force, and Van der Waals force, which are seen between molecules in liquid systems (Izbaim *et al.,* 2010; Rubalya *et al.,* 2017; Sankarappa *et al.,* 2005). The nature of forces and their strength between molecules can be understood by determining their internal pressure (Lei *et al.,* 2016). Figure 1 (b) shows an increase in adiabatic compressibility with heating time for peanut oil. However, it remains almost constant for corn oil as the specific molecular interaction between molecules is almost the same.

Tables 1 and 2 elucidate the variation in acoustic impedance with heating time for peanut and corn oil. Acoustic impedance (Z) resists the flow of the ultrasonic wave through the liquid. When bonding between atoms becomes strong, the density of the oil sample will increase with the proliferation in packing density. Unsaturated fatty acids like oleic and linoleic acids get saturated to palmitic and stearic SFA that reduces the flow of sound in oil (Benedito *et al.,* 2007; Benedito *et al.,* 2002). Corn oil shows an experiential rise of Z up to 10.5% after six cycles of heating but in peanut oil, it varies by 33.45%. The Z value of peanut is found to be greater , as the density of the oil increases due to an increase in SFA formed by degradation. Jose Benedito (2007) studied the quality of fried oils using the ultrasonic parameter and correlated it with polar and polymer compounds that are formed by the degradation in oil. Lei Zang *et al.* (2016) have stated that oil exposed to an ultrasonic oscillatory wave produce cavitation bubbles as it passes through the sample, inducing pressure variation. This interaction of the ultrasonic wave with the oil and the breaking of cavitation bubbles will induce the oxidation effect and accelerate thermal degradation. Hence, from the measured ultrasonic velocity, the quality of oil and its further usage can be evaluated.

3.1.2. Viscosity

Variation in viscosity with respect to heating time for corn and peanut oils is shown in Figure 1 (c). The viscosity

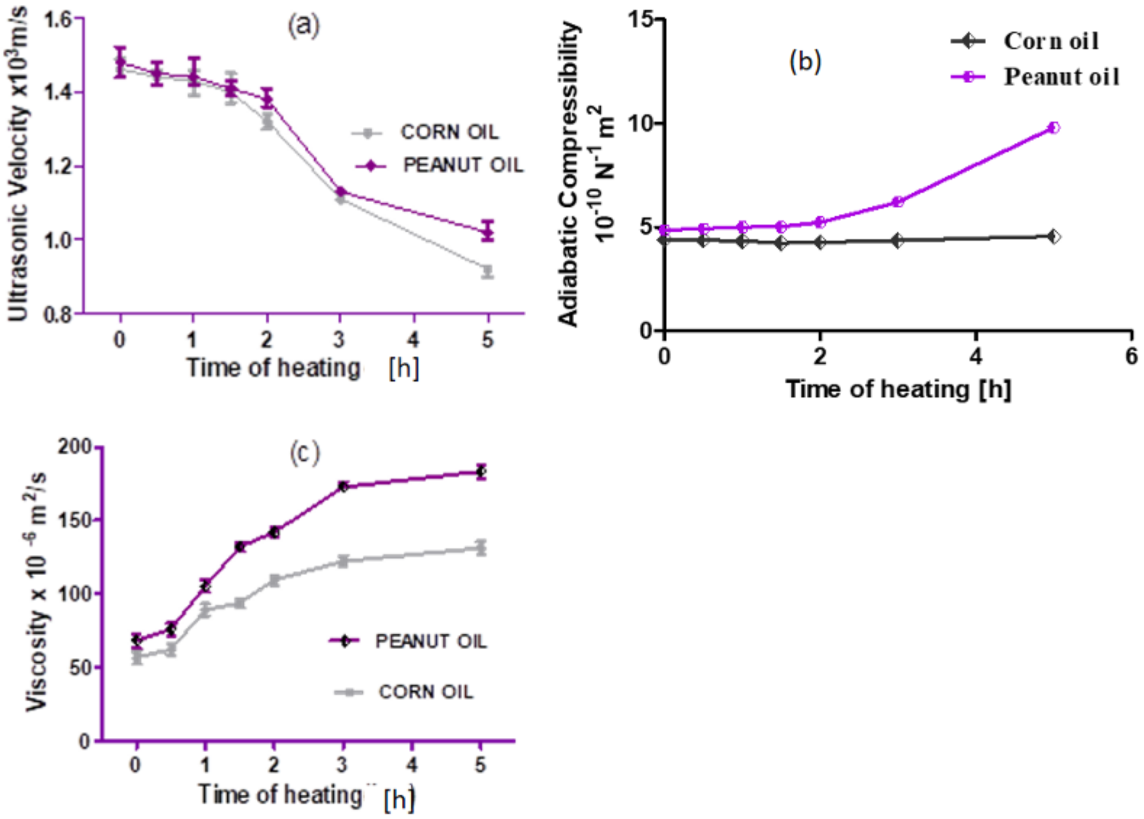

Figure 1. Variation of (a) Ultrasonic velocity for three trials (n=3) (b) Adiabatic compressibility (for n=1) (c) Viscosity for three trials (n=3) of peanut and corn oil with time of heating. The observed experimental deviation has been marked in (a) and (c).

of the oil is less when it is composed of a large amount of unsaturated fatty acids and becomes high with an increase in long-chain saturated fatty acids (Adolfo *et al.,* 2006; Fasina *et al.,* 2006; Rubalya *et al.,* 2016). Thus, the viscosity of oil increases with an increase in heating cycles as the quantity of SFA becomes greater. This variation in kinematic viscosity exhibits a steeper increase in peanut oil compared to corn oil, which is due to the molecular structural change in composition and decrease in the degree of unsaturated fatty acids (David, 2008; Everett, 1963). Viscosity ranges from 56.7 to 131.3 $\times10^{-6}$ m^2/s for corn oil; whereas it varies for peanut oil from 68.51 to 183.5 $\times10^{-6}$ m^2/s in six cycles of heating. Viscosity increases by 47.6% in corn oil after five hours of heating and is observed to be nearer to twice the times of its unheated value. This is due to the increase in altered double bonds to a single bond in fatty acids. The reason for variation in viscosity is due to the existence of long-chain saturated fatty acids (Rubalya *et al.,* 2016, Rubalya *et al.,* 2017). Similarly, from the rheological study, peanut oil shows a viscosity value 62.7% greater than the first time of heating. This is due to the increase in saturated bonds formed by the degradation of oil and also to the presence of arachidic and behenic acid with C22 long-chain saturated fatty acids (Reidoon, 2005).

3.2. Correlation between ultrasonic velocity and viscosity

Figure 2 exemplifies the correlation of ultrasonic velocity with viscosity and dependency factor $R^2 = 0.932$. A scattered graph is drawn between the two parameters and the trend line shows a decrease in velocity with an in-

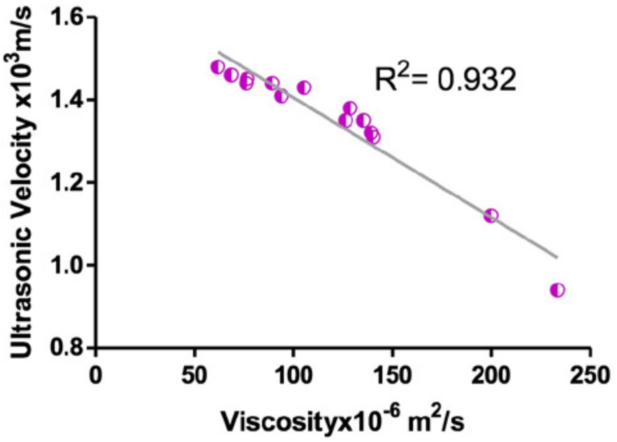

Figure 2. Correlation between average data (n=1) of viscosity and ultrasonic velocity of peanut and corn oil.

crease in viscosity due to the formation of large amounts of polymers, and polar compounds which increase molecular clustering (Benedito *et al.,* 2007; Benedito *et al.,* 2002; Rubalya *et al.,* 2016). It can be inferred from the Figure 2 that ultrasonic velocity and viscosity are negatively correlated because of an increase in chain length of triglycerides, saturated fatty acids and the molecular weight of chemical species. Variation in ultrasonic velocity with viscosity has been correlated for both peanut and corn oil samples and the corresponding equation is given in eq. 13, respectively:

$$Ultrasonic\,velocity = -0.0023\left(viscosity\right) + 1.65 \quad (13)$$

3.2.1. Density

Tables 1 and 2 show that the density of corn oil and peanut oil increases with heating cycles. After 6 cycles of heating, it is observed that the density of corn oil increases by 10.9% whereas in peanut oil it increases by 18%. The experimental density of oil was calculated after each cycle of heating at room temperature. The density of oils greatly depends on the molecular weight of its chemical compounds. In general, the molecular weight of oil rises due to the formation of mono, di and triglyceride oligomers and also with an increase in saturated fatty acids. From the measured physical properties, it is pragmatic that changes in molecular structure in the composition of oil influence the changes in the physical property.

Ultrasonic velocity (U) with a viscosity (η) and density (ρ) are related to the empirical equation given by:

$$\frac{(U)^{1/3}}{\rho} = A + \frac{B}{\sqrt{\eta}} \quad (14)$$

The values are fitted to the above equation and the dependency factor (R^2) was experiential between 0.922 and 0.966. Constants A= -0.387 and B = 0.403 are correlation coefficients in linear fitting of the above equation (15). The variations are due to the change in the molecular weight of unsaturated fatty acids and other unsaturated chemical composites in oil even after repeated cycles of frying. The oil contains 90% of short and long-chain fatty acids and triglycerides that increase the number of molecules per unit volume, which highly influences the density and viscosity of the oil. Rodenbush *et al.* (1999) had related density and viscosity of oil as both factors are temperature-dependent by the equation:

$$\rho = D + \frac{E}{\sqrt{\eta}} \quad (15)$$

Density and viscosity are important quality analysis indices in the oil industry for the pumping, pipelining, designing of the processing system, etc. If the density is known the viscosity of the oil can be determined using equation (15). The dependency of density with viscosity using the Rodenbush equation varies from 0.902 to 913 and was computed using least square fitting, where D = 1.308 and E = -1.302 are the correlation coefficients. The variation in accuracy is due to the heavy molecules polymer: monomer, dimer, and triacylglycerol, which are formed upon heating the oil at the frying temperature in different time cycles.

3.3. Hard sphere model for liquid system

The density of oil increases with heating time and so the packing density of oil also increases. The estimated values for the packing fraction of corn and peanut oil samples at different heating conditions are illustrated in Tables 3 and 4 using the four different models. The packing fraction determined by the PY model is very high compared to the SPT and MCSL models. The investigation illustrates that the number of atoms in a given volume increases with heating time and the system becomes dense by increasing the duration of heating because of this high packing fraction.

To compute PY, SPT and MCSL hard-sphere models, the molecular weight of the fatty acids in oil was determined by Tadao (1985). In his study, a linear variation between the molecular weight and viscosity was estimated for the oil and modified oil. He also observed that the molecular weight of th base oil was disseminated in a narrow range; whereas for blended oil there was a wide range of distribution. When the oil was heated to a smoke point temperature, it underwent chemical breakdown and this complicated behavior of molecules in the oil was explained using the Marangoni effect (Tadao, 1985). The related molecular weight (M) of oil with kinematic viscosity in a heat transfer analysis was estimated by taking the measured viscosity and relative approximate molecular weight variations between up to 588 g/mol of monomer, dimer and trimer forms of fatty acids using the linear fitting relation (Tadao, 1985).

3.3.1. Packing fraction

The backscattering of the ultrasonic wave per unit volume by the ensemble of the molecule in a plane can be theoretically estimated using the PY model (Percus and Yevick, 1958; Wertheim, 1963). The packing fraction of molecules in oil upon degradation with irregular shape in a given volume can be predicted using the PY model. Tables 3 and 4 exemplify that up to sample S4 the packing fraction increases and decreases slightly to 0.002% towards S7 due to the spatially random distribution of molecules in a given volume of oil. The estimated packing fraction by the SPT model envisages the compressi-

Table 3. Oil System: Computed chemical parameters of Corn oil heated to 190 °C by considering the average of ultrasound velocity (n=1)

Model: PY (Percus-Yevick)				
Time of heating (h)	**Packing fraction (y)**	**Particle Size (r) [µ m]**	**Chemical Potential**	**Inter-particle potential**
0	0.9329	0.1137	6,888	-13,050
0.5	0.9331	0.1157	6,948	-13,088
1	0.9341	0.1187	7,264	-13,280
1.5	0.9337	0.1188	7,126	-13,196
2	0.9333	0.1197	7,016	-13,129
3	0.9331	0.1208	6,936	13,080
5	0.9323	0.1214	6,712	-12,940
Model: SPT (Scaled Particle Theory)				
0	0.7822	0.1072	260.2	-5,135
0.5	0.7826	0.1091	261.3	-5,137
1	0.7846	0.1121	267.09	-5,153
1.5	0.7837	0.1121	264.4	-5,145
2	0.7831	0.1129	262.5	-5,139
3	0.7825	0.1140	260.94	-5,134
5	0.7810	0.1144	256.70	-5,121
Model: MCSL (Mansoori Carnahan Starling Leland)				
0	0.7074	0.1037	137.06	-4,973
0.5	0.7078	0.1055	137.37	-4,969
1	0.7099	0.1084	139.22	-4,960
1.5	0.70895	0.1084	138.34	-4,963
2	0.7083	0.1092	137.72	-4,964
3	0.70773	0.1102	137.18	-4,964
5	0.70618	0.1107	135.78	-4,969

bility of hard-sphere in an oil sample which is of medium density. This confirms the cavity formation. As the major constituents of oils are fatty acids, they combine to form a system if their potentials coincide. Tables 3 and 4 illustrate the packing fraction increase with an increase in the duration of heating to up to 1.5 hours. As heating cycles are increased, unsaturated fatty acids in *'cis'* form are converted to *'trans'* form of saturated fatty acids (Pushan *et al.,* 2009; Reidoon, 2005; David, 2008; Fasina *et al.,* 2006). Because of this number of particles within the reference area and its size, its packing fraction in a given volume is greater. When heating cycles are increased the heavy molecules may disintegrate to form molecules of lower dimension. Using the MCSL model the bulk phase behavior in a complex liquid like oil under pressure variation of the propagation of the ultrasonic wave will exhibit the density functional theory. The packing fraction of atoms/molecules that are calculated using the models increases till 90 minutes of heating time and then decreases by 0.005% at 5 hours of heating. In this bulk medium liquid the calculated packing fraction varies in the order PY > SPT> MCSL. By comparing the corn and peanut oil using three models, it has been observed that peanut oil has greater packing fraction.

3.3.2. Particle size

Figure 3 shows the variation in particle diameter/size of hard-sphere with heating time by comparing the behavior of corn and peanut oil. The calculated particle size of peanut oil is greater than corn oil using all three models (PY, SPT, MCSL) due to the presence of a large amount of long-chain saturated fatty acids. These hard-sphere models exemplify the microscopic description of the distribution of molecules in a given volume according to its dimension. The particle size using the PY model for peanut oil starts increasing by 0.008% for 0.5 hours heated sample and 0.001% for 5 hours compared to corn oil in a micrometer range. Using the SPT model, a systematic analytical method, the particle size varies from 0.02% to 0.09% from 1.5 to 5 hours of heating. The changes in the particle size using the fundamental measure theory MCSL model are very significant compared to the other two models.

3.3.3. Chemical potential (CP)

In colloidal science and liquid state theory, the three models (PY, SPT, MCSL) are very important in the study of the distribution of heavy and low weight molecules that are formed when exposed to different temperatures (Reiss

Table 4. Oil System: Computed chemical parameters of Peanut oil heated to 190 °C by considering the average of ultrasound velocity (n=1)

Time of heating (h)	Packing fraction (y)	Particle Size (r) [μ m]	Chemical Potential	Inter-particle potential
Model: PY (Percus-Yevick)				
0	0.93265	0.1137	6,813	-13,003
0.5	0.93280	0.1151	6,857	-13,031
1	0.93442	0.1196	7,369	-13,342
1.5	0.93447	0.1209	7,385	-13,352
2	0.93286	0.1221	6,875	-13,042
3	0.92979	0.1230	6,027	-12,492
5	0.9215	0.1232	4,342	-11,226
Model: SPT (Scaled Particle Theory)				
0	0.78172	0.1072	258.84	-5,131
0.5	0.7820	0.1086	259.60	-5,134
1	0.78524	0.1129	268.99	-5,159
1.5	0.78534	0.1141	269.264	-5,159
2	0.78211	0.1152	259.73	-5,129
3	0.77607	0.1158	243.36	-5,081
5	0.7603	0.1155	207.91	-4,988
Model: MCSL (Mansoori Carnahan Starling Leland)				
0	0.70689	0.1037	136.61	-4,975
0.5	0.70719	0.1050	136.84	-4,972
1	0.71055	0.1092	139.80	-4,958
1.5	0.71067	0.1104	139.88	-4,956
2	0.70730	0.1114	136.76	-4,964
3	0.70104	0.1119	131.35	-4,984
5	0.69485	0.1116	119.25	-5,059

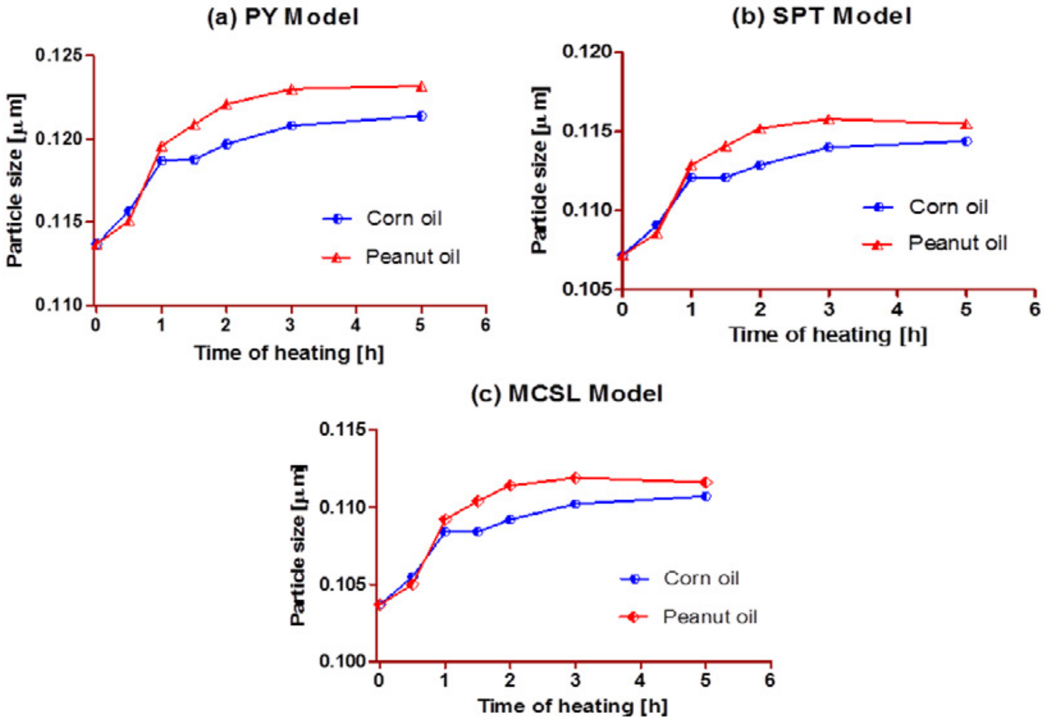

Figure 3. Variation of Particle size with time of heating to 190°C for corn and peanut oil using (a) PY model (b) SPT and (c) MCSL model by considering the average value of ultrasound data as the input with n=1.

et al., 1959; Ravi *et al.,* 2008). Fatty acid molecules in glycerol get distributed in oil depending on the change in the chemical potential (μ/kT), and also in inter-particle potential. Tables 3 and 4 elucidate the discrepancy of chemical potential (CP) with heating time. It can be observed that CP increases till 1 hour heated sample as more RCOO- and H+ ions are formed by oxidation reaction. When the oil is further exposed to repetitive cycles of heating, Peroxides RCOO- undergo a hydrolysis chemical reaction with glycerol to form monomer, dimer, trimer, etc. Hence the CP value decreases for the sample S7 due to the energy released in the formation of polymers. Using the PY model, the CP value of corn oil is observed to increase by 35.2% compared to peanut oil as it has a smaller quantity of saturated fatty acids and also highly potent antioxidants. Moreover, peanut oil has a fractional amount of C22 long-chain fatty acid. In the SPT model, the CP value for corn oil is 19% greater than peanut oil, whereas it is greater than 12% using the MCSL model. By comparing the computed CP value for the three models PY> SPT>MCSL the interatomic distribution in molecules with the time of heating can be explained.

Corn oil has comparatively greater stability and can withstand even more heating cycles as more external energy is needed to alter the chemical species in oil samples (chemically inert) and this is justified by the observed high values of chemical potential and inter-particle potential. The packing fraction of the corn oil increases from 0.7822 to 0.7846 with an increased duration of heating to up to 1.5 hours and slowly dissociates thereafter and reaches a lower value of 0.7810 after 5 hours of heating. The same trend is observed for peanut oil but with a faster dissociation rate. The packing fraction of peanut oil increases from 0.7817 to 0.7853 and dissociates down to 0.7603. As the packing fractions were estimated from the experimentally obtained ultrasound that traveled through the samples it gives insight about the oil's status. This stability shows that the formation of the polar products like triglycerides; polymer compounds, free fatty acids, and carbonyl compounds are less in corn oil than in peanut oil. The particle size of corn oil is less than peanut oil as peanut oil contains C22 Arachidic acid; Behenic acid saturated long-chain fatty acid (Reidoon, 2005). Hence the degradation degree of corn oil is comparatively lower than peanut oil after 6 cycles of heating.

3.3.4. Inter particle potential L-J

The Lennard-Jones (L-J) potential defines the interactive energy, which may be attractive or repulsive between the degraded polar molecules formed with heating cycles (Yarnell *et al.,* 1973, Lennar, 1924). Incorporating the data

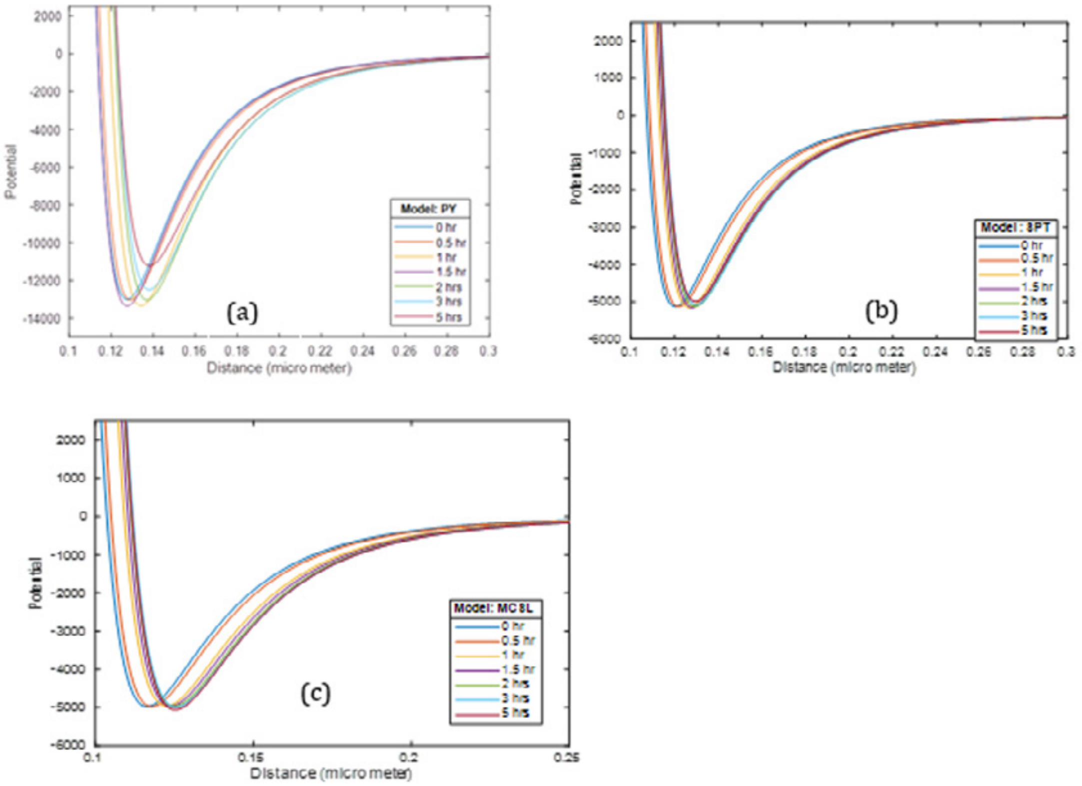

FIGURE 4. L-J potential plot for peanut oil at different duration of heating time to 190°C using (a) PY model (b) SPT model (c) MCSL model with n=1. The duration of heating has been given as inset. The interaction potential changes with heating duration.

from Tables 3 and 4, inter-particle potential L-J has been plotted and is shown in Figures 4 and 5 for corn and peanut oil. Figure 4 (a) presents the way in which the minimum potential energy of corn oil upsurge by the increases in inter-particle distance by 8.57% from 0 to 5 hours of heating using the PY hard-sphere model; whereas using the SPT model it increases by 13%. From the graph, the closest possible distance g(r) (also termed radial distribution function) has been observed around 0.12. A shift in internal energy is observed from the comparison plot which varies with heating cycles. The plot is drawn for 1.5-hour heating and 5 hours of heating and a shift was observed in peanut samples due to the breaking of bonds. Changes in the dynamic properties with the propagation of an ultrasonic wave through the sample is due to the presence of irregular long-chain molecules that produce more pressure than short-chain fatty acid in the oil. Using the MCSL hard-sphere model, the lowest potential energy increases by 13.6% from 0.5 to 5 hours of heating with respect to the interatomic distance of atoms in corn oil. Results obtained from the MCSL model almost match the SPT results.

Figure 5 (a) illustrates the variation in potential with a 7.4% increase in the interatomic distance using the PY model in peanut oil. The variation in interatomic distance is 7.69% using SPT and 16.4% using the MCSL model. The results calculated using the PY and SPT models are almost the same; whereas the MCSL model estimation is nearly twice the predicted PY and SPT. Hence to conclude, using this demonstrated L-J potential one can estimate the distribution of molecules using the hard-sphere models with an increase in heating duration.

Particle fraction, particle size, chemical potential, and L-J potential were computed using MATLAB. The low packing fraction and particle size of peanut oil were exemplified and compared to corn oil as it possesses a large amount of saturated and monounsaturated fatty acids.

Simulated output exhibits the distribution of molecules in oil samples that are made up of long and short flexible fatty acid chains and also additive mixtures of glycerol, antioxidants, etc., and agrees with existing data and the standard equations of state. The three hard-sphere models help to understand the chemical reaction taking place in oil exposed to different heating cycles. The CP and L-J potential also states remarkable computational advantages in the simulation to understand the different chemical composition in the oil and their potential variations after each cycle of heating (Stanciu, 2019).

4. CONCLUSION

Variations in the ultrasonic velocity, density, and viscosity of peanut oil and corn oil samples exposed to different

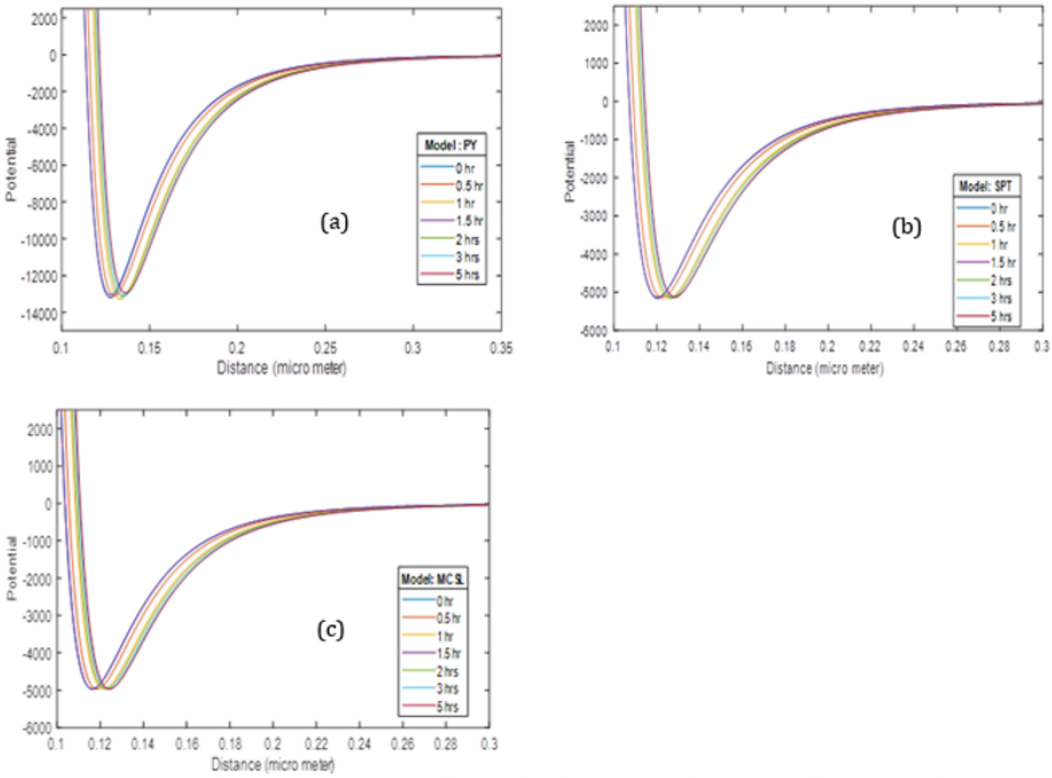

FIGURE 5. L-J potential plot for peanut oil at different duration of heating time to 190°C using (a) PY model (b) SPT model (c) MCSL model with n=1. The duration of heating has been given as inset. The interaction potential changes with heating duration.

heating cycles at 190 ºC were studied. From the physical parameter variations, it is observed that compared to peanut oil, corn oil can be used in more frying cycles. In corn oil, inter-particle potential, chemical potential, and particle diameter exhibit higher oxidative stability than peanut oil. Molecular simulation (PY, SPT, MCSL) can be used to study the distribution of molecules in oil upon degradation and also the forces existing between the atoms or molecules. From a practical perspective, these theories can be used to estimate the equation of state for long and short-chain polymer molecules in oil. The L-J potential graph shows the maximum closest distance between particles from the depth of the graph that is plotted, which exhibits the required external energy to break the bonding in long-chain fatty acids. L-J has been drawn based on the fact that SPT and MCSL matches well than PY, which illustrates that corn oil can be used for more heating cycles compared to peanut oil with less degradation. From the measured physical properties and using the computed particle distance against the potential of corn and peanut oil, the quality of the oil can be decoded.

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Effect of selenium and zinc foliar application to increase the quantitative and qualitative yields of rapeseed at different sowing dates

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SUMMARY: The sowing date is an important factor for expanding the cultivated area of rapeseed and affects seed yield, oil content, and fatty acid compounds. Micronutrient elements play an important role in improving the vegetative and reproductive growth of the plant, especially under conditions of biological and environmental stresses. A two-year experiment (2014-2016) was performed to study the response of rapeseed genotypes to foliar application of micronutrients on different sowing dates. The treatments were arranged as a factorial-split plot in a randomized complete block design with three replicates. Three sowing dates of 7 (well-timed sowing date), 17, and 27 (delayed sowing dates) October and two levels of foliar application with pure water (control), selenium (1.5%), zinc (1.5%), and selenium+zinc (1.5%) were factorial in the main plots and five genotypes of SW102, Ahmadi, GKH2624, GK-Gabriella, and Okapi were randomized in the subplots (a total of 30 treatments). Seed yield, oil yield and content, oleic acid, and linoleic acid were reduced when rapeseeds were cultivated on 17 and 27 October, while the contents in palmitic, linolenic, and erucic acids, and glucosinolate increased ($p < 0.01$). a selenium+zinc treatment improved seed yield, oil content and yield ($p < 0.01$). The oil quality increased due to increased contents of oleic and linoleic acids under the selenium+zinc treatment ($p < 0.01$). The GK-Gabriella and GKH2624 genotypes are recommended to be sown on well-timed (7 October) and delayed sowing dates (17 and 27 October) and treated with selenium+zinc due to the higher oil yield, linoleic and oleic acids.

KEYWORDS: *Delayed sowing; Fatty acid composition; Foliar application*

RESUMEN: ***Efecto de la aplicación foliar de selenio y zinc para aumentar los rendimientos cuantitativos y cualitativos de colza en diferentes fechas de siembra.*** La fecha de siembra es un factor importante para expandir el área cultivada de colza que afecta el rendimiento de la semilla, el contenido de aceite y la composición en ácidos grasos. Los micronutrientes juegan un papel importante en la mejora del crecimiento vegetativo y reproductivo de la planta, especialmente en condiciones de estrés biológico y ambiental. Se realizó un experimento de dos años (2014-2016) para estudiar la respuesta de los genotipos de colza a la aplicación foliar de micronutrientes en diferentes fechas de siembra. Los tratamientos se organizaron como una parcela dividida factorial en un diseño de bloques completos al azar con tres repeticiones. Tres fechas de siembra del 7 (fecha de siembra en el momento oportuno), 17 y 27 (fechas de siembra retrasadas) de octubre y dos niveles de aplicación foliar con agua pura (control), selenio (1,5%), zinc (1,5%) y selenio + zinc (1.5%) fueron factoriales en las parcelas principales y cinco genotipos de SW102, Ahmadi, GKH2624, GK-Gabriella y Okapi fueron aleatorizados en las subparcelas (un total de 30 tratamientos). El rendimiento de semilla, el contenido y rendimiento de aceite, los ácidos grasos oleico y linoleico se redujeron cuando se cultivaron semillas de colza los días 17 y 27 de octubre, mientras que los contenidos de los ácidos grasos palmítico, linolénico y erúcico y glucosinolato aumentaron ($p <0,01$). El tratamiento con selenio + zinc mejoró el rendimiento de semillas, el contenido de aceite y el rendimiento ($p <0,01$). La calidad del aceite aumentó debido al mayor contenido de ácidos oleico y linoleico bajo tratamiento con selenio + zinc ($p <0.01$). Se recomiendan los genotipos GK-Gabriella y GKH2624 sembrados en fechas oportunas (7 de octubre) y tardía (17 y 27 de octubre) y tratados con selenio + zinc, respectivamente, debido al mayor rendimiento de aceite y contenido de los ácidos linoleico y oleico.

PALABRAS CLAVE: *Aplicación foliar; Composición en ácidos grasos; Siembra retrasada*

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1. INTRODUCTION

Rapeseed with the scientific name of *Brassica napus* L. is considered to be one of the most important oilseed plants in the world and it is the third oilseed crop after oil palm and soybean.

According to the latest Food and Agriculture Organization (FAO) report, total rapeseed production in the world and Iran were 72.2 and 0.128 million tons, respectively. Due to its suitable agricultural properties, this oilseed crop can be used as an alternative to cereal-based crop rotations, especially in arid and semi-arid climates (Hamzei and Soltani, 2012). Rapeseed is mainly considered by farmers due to its high oil content (40-45%), and one of the healthiest edible oils due to its low content in saturated fatty acids (7%), high content in monounsaturated fatty acids (60%) and sufficient content in polyunsaturated fatty acids (12%) (Starner *et al.,* 1999).

In order to produce high-quality oil as well as acceptable oil yield in different environmental conditions, it is necessary to manage agronomic practices such as identifying high-yielding genotypes, setting of well-timed sowing dates, and nutritional capacity. Selecting the appropriate genotype in each region can lead to achieving optimal quantitative and qualitative yields. The genotype is considered as the most important determinant factor in fatty acid composition (Knowles, 1988). However, the interaction of genotype with environmental conditions affects the quality of rapeseed fatty acid compounds (Gunasekera *et al.,* 2006). On the other hand, in rapeseed cultivable areas, harvesting crops grown in spring or summer may lead to a delay in rapeseed cultivation, increasing frost risk, and crop losses. Under these circumstances, farmers are less inclined to expand rapeseed cultivation. In fact, the sowing date is an important factor for expanding the cultivated area of rapeseed and affects seed yield, oil content, and fatty acid compounds (Koutroubas and Papadoska, 2005). Accordingly, the evaluation of rapeseed genotypes' response to different sowing dates as well as a selection of high-yielding genotypes under these conditions can have significant positive effects on the expanding area under the cultivation of this crop (Moradi Aghdam *et al.,* 2019). If suitable genotypes for delayed sowing are identified and introduced, it is also possible to reduce the severity of early-season cold damage. In a study by Nazeri *et al.* (2018), the quantitative and qualitative yields of different rapeseed cultivars were evaluated on conventional (7 October) and delayed (27 October) sowing dates in Iran and it was reported that a 20-day delay in sowing reduced rapeseed seed yield by 60%. In another study, Moradi Aghdam *et al.* (2019) investigated the quantitative and qualitative traits of five rapeseed genotypes on two sowing dates of 7 October and 1 November (delayed and optimum sowing dates, respectively) in Iran and indicated that SW102 and HW1 genotypes produced the highest seed yield (3877 and 3801 kg·ha^{-1}) and had a high-quality fatty acid composition, making them suitable for delayed sowing.

Supplying micronutrient elements could improve plant growth and development under different environmental conditions. While micronutrients are not directly involved in plant metabolism and life cycle completion, they play an important role in improving the vegetative and reproductive growth of the plant, especially under conditions of biological and environmental stress. Zinc is a micronutrient that plays an important role in many biological processes in plants and is essential for plant growth and reproduction as well as animal and human health, although it may pollute soil, water, and nutrient chains in excessive amounts (Lebourg *et al.,* 1998). From an agricultural point of view, increasing zinc content in the seeds is a desired qualitative factor and the quantitative and qualitative yields of crops may be reduced under zinc deficiency conditions (Noulas *et al.,* 2018). This element is a factor in reducing the adverse effects of stress on a plant system, and plant growth and metabolism will be affected by any factor affecting its efficiency (Noulas *et al.,* 2018). In a study, Shahsavari *et al.* (2014) concluded that zinc application as a micronutrient element significantly increased oil yield and content in rapeseed. Selenium as another micronutrient element at low concentrations can increase resistance to oxidative stress through increasing plant antioxidant properties and reducing lipid peroxidation (Seppanen *et al.,* 2003). Selenium application increases carbohydrate metabolism (Zhu *et al.,* 2004) and prevents the plant's chlorophyll degradation under environmental stress (Seppanen *et al.,* 2003). Furthermore, glutathione as an important component in chlorophyll which is affected by selenium and this process probably increases photosynthesis and yield through production mechanisms (Valladares *et al.,* 2008). The study results of Bybordi (2016) showed that selenium application increased plant height, pod number, seed number, biological yield, harvest index, and rapeseed oil content while respiration rate, proline, and malondialdehyde contents were decreased.

Regarding the importance of rapeseed as an important oilseed crop, the present study was conducted and aimed to improve the quantitative and qualitative yield of rapeseed genotypes sown on conventional and delayed sowing dates by applying selenium and zinc elements.

2. MATERIALS AND METHODS

2.1. Experimental description and treatments

A two-year experiment (2014-2015 and 2015-2016) was performed at the Research Field of Seed and Plant Improvement Institute (SPII), Karaj, Iran, to study the response of rapeseed genotypes to foliar application of zinc and selenium at different sowing dates. The experiment site (Karaj) is located at an altitude of 1321 meters above

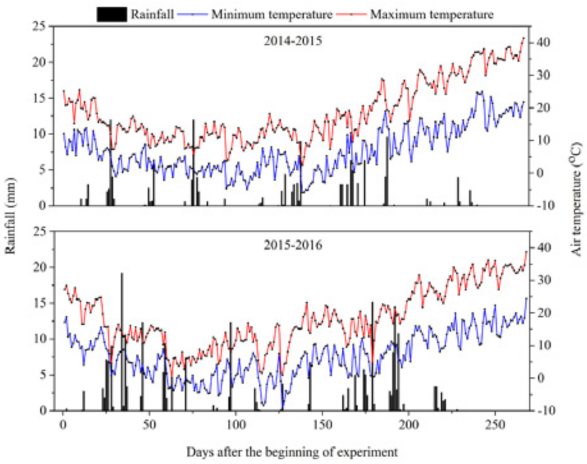

Figure 1. Daily minimum and maximum air temperatures (˚C), and rainfall (mm) recorded over the growing season in 2014-2015 and 2015-2016.

sea level with 35˚ 59´ N latitude and 50˚ 75´ E longitude. The daily climatic data of the experiment location during the rapeseed growth period are presented in Figure 1.

The experiment was conducted as a factorial-split plot in a randomized complete block design (RCBD) with three replicates. In the present study, three sowing dates of 7 (well-timed sowing), 17, and 27 October (delayed sowing dates) and two levels of foliar application with pure water (control), selenium (1.5%), zinc (1.5%), and selenium+zinc (1.5%) were factorial in the main plots and five genotypes of SW102 (early-maturity line), GKH2624 and Okapi (mid-maturity cultivar), and Ahmadi and GK-Gabriella (late-maturity cultivar) were randomized in the subplots (a total of 30 treatments). Sodium selenate (as selenium source) and chelated zinc (as zinc source) were applied in two stages of (i) before rosette (ii) and stem elongation for each sowing date and genotype, respectively.

2.2. Field practices

In this study, experimental plots consisted of six 6 m lines with 30 cm spacing between the lines, with two lateral lines considered as margins. The inter-plant spacing in each line was also 5 cm. It should be noted that the distance between the blocks and main plots in each block were 7 and 2.4 m, respectively.

In order to determine physical and chemical properties of the soil at the experiment site, soil samples were randomly taken at 0-30 and 30-60 cm depths. The experiment field soil was clay loam (Table 1). Fertilization was performed according to the results of soil analysis and rapeseed fertilizer recommendations. Accordingly, 50 kg·ha^{-1} nitrogen and 70 kg·ha^{-1} P_2O_5 from two sources of urea and ammonium phosphate and 10 kg·ha^{-1} K_2O (as pre-plant) and 46 kg·ha^{-1} nitrogen (equivalent to 100 kg·ha^{-1} urea) were used at the beginning of the stem elongation stage. The weeds were controlled by applying 2.5 liters per hectare of trifluralin before sowing as well as hand weeding during the rapeseed growth period. Finally, the seed of rapeseed genotypes was sown on the mentioned sowing dates. Irrigation was carried out throughout the growing period based on 80 mm evaporation from a Class A evaporation pan.

2.3. Qualitative and quantitative traits

Rapeseed seed yield was determined by hand-harvesting 3.6 m^2 in the center of each plot and weighed using a precise scale. It should be noted that the final harvest for each plot over two years was performed when 50% of the seeds in the main siliques and primary branches turned brown. In order to measure and determine the oil content in the rapeseed, three grams of seeds were selected from each experimental plot and the oil content was measured using a Nuclear Magnetic Resonance (NMR) German Broker Brand minispec mq20 model according to the international standard ISON.5511. For this purpose, the calibration of the device was performed with a reference sample and the product calibration with pre-prepared standard samples, and then three grams of rapeseed seeds were transferred to the device-specific cell. The cell containing the specimen was placed at a specific location and the amount of oil was monitored in less than 1 minute. After determining the oil content in the rapeseed seed, oil yield was obtained by multiplying the seed yield by oil content.

In the present study, rapeseed seed oil samples were extracted according to the method proposed by Azadmard-Damirchi *et al.* (2005) and Fathi-Achachlouei and

Table 1. Physicochemical properties of soil collected from the study site.

Year	Depth (cm)	EC (ds·m^{-1})	pH	Organic carbon (%)	Total nitrogen (%)	Available phosphorus (mg·kg^{-1})	Available potassium (mg·kg^{-1})	Soil texture
2014-2015	0-30	1.45	7.9	0.91	0.08	14.7	197	clay loam
	30-60	1.24	7.2	0.99	0.07	15.8	155	
2015-2016	0-30	1.51	8.0	0.89	0.09	14.5	200	clay loam
	30-60	1.25	7.1	0.98	0.07	16.1	152	

Azadmard-Damirchi (2009). In summary, rapeseed seed samples (approximately 10 g) with 30 ml hexane/isopropanol (3:2, v/v) were kept at room temperature for one hour in metal tubes containing four metal balloons to facilitate homogeneity. In order to extract oil from 100 g of rapeseed seed, 10 tubes were used, each tube containing 10 g of rapeseed seed. After one hour of shaking the samples, the extract was filtered through filter paper with a Buchner funnel under vacuum. The remained defatted cake was washed twice with the same solution to extract the entire potential oil content. Then, 35 ml of sodium sulfate was added to the solvent containing the oil and thoroughly mixed. Subsequently, the organic solvent layers containing the oil were separated and evaporated at 35 °C under reduced pressure. Finally, the extracted oil was stored at -20 °C for subsequent analyses.

Fatty acid methyl esters were prepared from oil samples according to the method proposed by Savage *et al.* (1997). Briefly, 2 ml of 0.01 M NaOH solution were added to a tube containing the oil sample (about 10 mg) dissolved in 0.5 M hexane and then placed in a water bath at 60 °C for 10 minutes. Thereafter, a boron trifluoride solution in methanol (20% of boron trifluoride in methanol) was added, and the samples were kept in a 60 °C water bath for 10 minutes. The sample was cooled under running water, and 2 ml of sodium chloride 20% and 1 ml of hexane were added. After complete mixing, the hexane layer containing fatty acid methyl esters was separated by centrifugation.

Fatty acid methyl esters were analyzed using gas chromatography (GC) based on the method proposed by Azadmard-Damirchi and Dutta (2006). The GC instrument was equipped with a flame ionization detector and a Split/Splitless injector. The injector and detector temperatures were 230 and 250 °C, respectively. The oven conditions were such that from 158 to 220 °C, the temperature rose to 2 °C per minute and was maintained at each temperature for five minutes. Helium was used as carrier gas and nitrogen was used as an auxiliary gas at a flow rate of 30 ml per minute. Fatty acid methyl esters were identified by comparing their shelf-life with standard fatty acid methyl esters, and peak areas were reported as the percentage of total fatty acids. In this study, palmitic, stearic, oleic, erucic, linoleic and linolenic fatty acids were measured.

Rapeseed seed oil glucosinolate content was measured using a Varian Spectrophotometer Cary 100 equipped with a 50-m-long CP-Sil 88 capillary, 0.25 mm inner diameter, and 0.2 µm static phase thickness (Harinder *et al.,* 2007).

2.4. Data analysis

A combined analysis of variance was performed using SAS software (version 9.2). The mean comparison was performed by the least significant difference (LSD) at 5% probability level, and interactions among treatments were determined with by-processing.

3. RESULTS AND DISSCUSION

The results indicated that the simple effect of year was significant for all studied traits except for linolenic acid and glucosinolate (Table 2). The mean comparison results

Table 2. Analysis of variance (mean squares) on quantitative and qualitative traits as affected by sowing date, foliar application, and genotype.

S.O.V	df	Seed yield	Oil yield	Oil content	Palmitic acid	Linoleic acid	Linolenic acid	Oleic acid	Erucic acid	Glucosinolate content
Y	1	**	**	**	*	*	ns	**	*	ns
SD	2	**	**	**	**	**	**	**	**	**
Y*SD	2	ns	*	ns	ns	**	**	**	**	**
FA	3	**	**	**	**	**	**	**	**	**
Y×FA	3	ns	ns	ns	ns	ns	ns	ns	ns	ns
SD×FA	6	ns	ns	**	**	**	**	**	**	**
Y×SD×FA	6	ns	ns	ns	ns	ns	ns	ns	ns	ns
G	4	**	**	**	**	**	**	**	**	**
Y×C	4	ns	ns	ns	ns	ns	ns	ns	ns	ns
SD×G	8	**	**	**	**	**	**	**	**	**
Y×SD×G	8	ns	ns	ns	ns	ns	ns	ns	ns	ns
FA×G	12	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×FA×G	12	ns	ns	ns	ns	ns	ns	ns	ns	ns
SD×FA×G	24	ns	ns	ns	ns	ns	ns	ns	ns	ns
Y×SD×FA×G	24	ns	ns	ns	ns	ns	ns	ns	ns	ns
CV (%)		11.02	10.94	7.54	5.69	3.12	3.74	0.97	8.93	4.76

ns: not significant, * and ** Significant at the 5% and 1% levels of probability, respectively.
Y: Year, SD: Sowing date, FA: Foliar application, G: Genotype

TABLE 3. Mean comparison (± standard deviation) of main effect of year on qualitative and quantitative traits of rapeseed.

Year	Seed yield	Oil yield	Palmitic acid	Oleic acid	Linoleic acid	Linolenic acid	Erucic acid
	Kg·ha^{-1}		%				
2014-2015	3056±1222b	1216±590b	38.9±2.76b	4.68±0.70b	16.6±2.53a	57.3±4.17b	0.31±0.12b
2015-2016	3656±1326a	1416±616a	39.3±2.74a	5.06±0.68a	15.5±1.73b	59.9±3.91a	0.33±0.10a

Means followed by similar letters in columns are not significantly different at 5% probability level by the LSD test. All treatments were performed in three replicates.

showed that seed yield, oil yield, oil content, palmitic acid, oleic acid, and erucic acid were higher in the first year (2015-2016) than the second year (2014-2015); while the linoleic acid in the first year was higher than the second year (Table 3). The difference between studied traits in the two experimental years can be attributed to differences in the weather. (Figure 1). As shown in Figure 1, total rainfall during the rapeseed growing period was 140.7 mm in the first year of the experiment; while in the second year of the experiment 238 mm of rainfall were recorded. On the one hand, in the last months of the rapeseed growing period (April, May, and June), which is important for the quantitative and qualitative rapeseed yields, the amount of rainfall in the first year was 67% lower than the second year (Figure 1). In addition, the mean temperatures in April, May, and June in the first year were 1.75 °C lower than in the second year (Figure 1).

The results of the combined analysis of variance showed that the oil and palmitic acid contents were significantly affected by the simple effects of sowing date, foliar application, and genotype as well as two-way interactions between sowing date × foliar application, and sowing date × genotype (Table 2). The seed yield was affected by the simple effects of sowing date, foliar application, genotype, and interaction of sowing date × genotype. The oil yield was affected by the simple effects of sowing date, foliar application, and genotype as well as two-way interactions between year × sowing date, and sowing date × genotype at a 1% probability level. As shown in Table 2, the simple effects of sowing date, foliar application, and genotype as well as interactions of sowing date × year, sowing date × foliar application, and sowing date × genotype were significant for linoleic, linolenic, oleic, erucic fatty acids, and glucosinolate contents.

3.1. Seed yield

The results of mean comparison showed that the foliar application of selenium + zinc produced the highest seed yield with an average of 3692 kg·ha^{-1} and had 17.7, 7.6, and 11.2% higher seed yield compared to the control, selenium, and zinc treatments, respectively (Figure 2).

The foliar application of selenium, zinc, and selenium + zinc increased the seed yield of rapeseed on three sowing dates, indicating their desired effect on increasing the photosynthesis rate of rapeseed and seed yield (Shoja *et al.,* 2018). Sieprawska *et al.* (2015) reported that the selenium element is important due to activating the antioxidant system in plant cells against environmental stresses such as drought, ultraviolet rays, and high/low temperatures. The formation of reactive oxygen species (ROS) is intensified when zinc deficiency has occurred; while the application of zinc provides protection against cold stress in plants (Cakmak, 2006). It is worth noting that the protective range of micronutrients such as selenium and zinc varies depending on the stress and genotype. Fang *et al.* (2008) in a study investigated the effect of foliar application of selenium, zinc, and iron and reported that rice seed yield increased significantly compared to the none-foliar application of micronutrients.

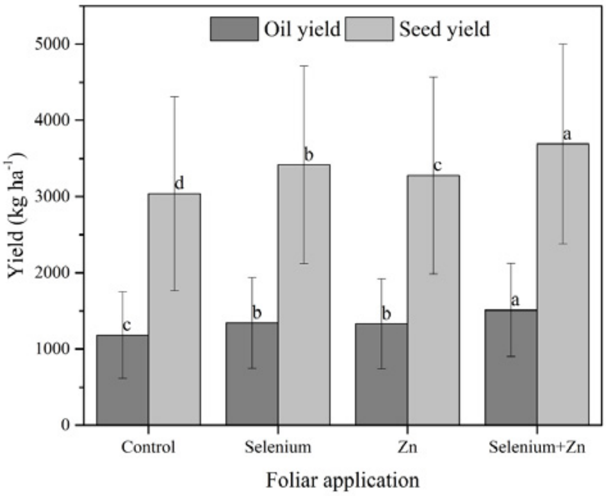

FIGURE 2. Seed and oil yields of rapeseed as affected by main effect of foliar application. Means followed by similar letters in bars are not significantly different at 5% probability level by the LSD test. The vertical bars are standard deviation. The LSD values for seed and oil yields are 129.4 and 54.75 kg·ha^{-1}, respectively. All treatments were performed in three replicates

The interaction between sowing date and genotype showed that the rapeseed seed yield (on average across genotypes) was reduced by 45.7 and 52.5% on the sowing dates of 17 and 27 October (delayed sowing dates) compared to 7 October (well-timed sowing date), respec-

Table 4. Mean comparison (± standard deviation) of interaction effect of sowing date and foliar application on qualitative and quantitative traits of rapeseed.

Sowing date	Foliar application	Oil content (%)	Palmitic acid	Oleic acid	Linoleic acid	Linolenic acid	Erucic acid	Glucosinolate (µmol·g^{-1})
			(%)					
7 October	Control	41.06±0.46d	4.30±0.36a	61.08±1.15d	18.21±1.38b	4.36±0.39ab	0.190±0.04a	9.13±1.16ab
	Selenium	41.57±0.46c	3.87±0.33b	61.89±1.19c	18.60±1.45ab	4.10±0.35c	0.156±0.03b	8.29±0.96c
	zinc	42.36±0.48b	4.49±0.37a	63.01±1.18b	18.80±1.42ab	4.50±0.41a	0.196±0.04a	9.52±1.21a
	Selenium+zinc	42.89±0.47a	4.03±0.34b	63.84±1.23a	19.21±1.23a	4.23±0.36bc	0.161±0.04b	8.56±1.00bc
17 October	Control	38.57±0.51d	5.63±0.41a	58.35±1.66c	15.17±0.58c	6.51±0.48ab	0.429±0.04ab	15.22±1.02b
	Selenium	39.00±0.55c	5.13±0.36d	59.22±1.69c	15.49±0.65b	6.24±0.46c	0.399±0.05c	14.49±1.18c
	zinc	39.78±0.52b	5.87±0.43a	60.19±1.71b	15.66±0.60b	6.72±0.50a	0.443±0.04a	15.86±1.06a
	Selenium+zinc	40.23±0.57a	5.35±0.38c	61.09±1.75a	16.00±0.67a	6.44±0.48bc	0.411±0.05bc	15.11±1.23b
27 October	Control	34.10±0.45d	4.91±0.36b	50.98±1.45c	13.02±0.50d	5.68±0.42c	0.375±0.03b	13.11±0.88c
	Selenium	34.49±0.49c	4.48±0.32c	51.74±1.48c	13.30±0.56c	5.44±0.40d	0.348±0.04c	12.49±1.02d
	zinc	37.35±0.49b	5.45±0.40a	55.89±1.59b	14.67±0.56b	6.30±0.47a	0.416±0.03a	14.41±0.97a
	Selenium+zinc	37.77±0.53a	4.97±0.35b	56.72±1.62a	14.99±0.63a	6.04±0.45b	0.386±0.04b	13.72±1.12b

Means followed by similar letters for each treatment in columns are not significantly different at 5% probability level by the LSD test. All treatments were performed in three replicates.

tively (Table 5). GK-Gabriella and Okapi on 7 October with the averages of 5355 and 5273 kg·ha^{-1}, GKH2624 and GK-Gabriella on 17 October with the averages of 3068 and 2936 kg·ha^{-1}, and GKH2624, GK-Gabriella, and Okapi on 27 October with the averages of 2682, 2567, and 2504 kg·ha^{-1} were known as the superior genotypes, respectively (Table 5).

As previously mentioned, delayed sowing (on average across genotype and foliar application treatments) reduced rapeseed seed yield. Delayed sowing led to a more rapid development of the crop, a reduction in the number of days from emergence to flowering and maturity, and the duration of flowering and seed filling, which has a negative impact on final performance and reduces seed yield (Faraji *et al.,* 2009). The higher seed yield due to optimum sowing date can be attributed to better plant growth conditions which help the plant grow faster and reach the rosette stage before the winter frost. On the other hand, timely sowing reduces drought and heat stresses at the end of the season and increases seed yield (Turner 2004; Gunasekera *et al.,* 2006). Obviously, seed yield losses are greater when the delay in sowing is longer. Moradi Aghdam *et al.* (2019) reported that delayed sowing of rapeseed cultivars on 1 November compared to the well-timed sowing date on 7 October reduced seed yield by 38.4%. Faraji *et al.* (2009), in a study evaluating different sowing dates including 9 November (optimum sowing date), 6 December, 5 January, 4 February, and 6 March and concluded that the sowing of rapeseed genotypes after optimum sowing date reduced rapeseed seed yield by 18, 19.6, 31.3, and 77.5%, respectively.

3.2. Oil content

As shown in Table 4, the oil content in rapeseed (averaged across foliar application treatments) was reduced by delayed sowing dates (17 and 27 October) compared to well-timed sowing date (7 October). The average across foliar application treatments, with rapeseed plants sown on 7 October had the highest oil content with an average of 41.97%; while the oil contents in rapeseed plants sown on 17 and 27 October were 39.39 and 35.92%, respectively (Table 4). The slicing interaction of sowing date × foliar application showed that selenium + zinc treatment on three sowing dates 7, 17, and 27 October produced the highest oil content with averages of 42.89, 40.23, and 37.77%, respectively.

The oil contents in the studied genotypes were reduced when rapeseed plants sown on 17 and 27 October were compared to 7 October. The highest oil content (42.4%) on 7 October was observed in the GK-Gabriella genotype and the lowest oil content (35.45%) on 27 October was observed in SW102 genotype (Table 5). The average seed oil content of rapeseed across genotypes on 7 October (41.94%) was higher than those of 17 and 27 October (39.34 and 35.92%, respectively) (Table 5).

Obviously, quality and quantity yields are determined by genetic and environmental factors as well as management practices (Ashrafi and Razmjoo, 2010; Eyni-Nargeseh *et al.,* 2020). In general, genetic factors are important and effective parameters for crop oil content and environmental factors have little effect on this trait (Robertson and Holland, 2004).

In the present study, the sowing dates and foliar application of micronutrients as different environmental and management factors and genotypes as a genetic factor were investigated and a significant difference was observed between the levels of each treatment in terms of oil content. In this regard, the results of Eyni-Nargeseh *et al.* (2020) showed a statistically significant difference among 17 rapeseed genotypes in terms of oil content. Nazeri *et al.* (2018) evaluated the response of rapeseed genotypes to a delay in sowing date and reported that the oil content in genotypes was significantly different and the delayed sowing of rapeseed reduced the oil contents in rapeseed genotypes. The application of zinc can increase oil content and yield due to an increase in auxin biosynthesis, chlorophyll content, nitrogen, and phosphorus uptake as well as a reduction in sodium concentration in plant tissues (Moinuddin and Imas, 2008). The results of Shoja *et al.* (2018) showed that the seed oil content in rapeseed was increased under the foliar application of zinc compared to the control treatment (non-foliar application). The combined application of micronutrients can lead to more useful results. Eskandari Zanjani *et al.* (2012) concluded that the combined use of selenium and zeolite resulted in increased oil content and yield in rapeseed.

3.3. Oil yield

The selenium+zinc treatment had the highest seed oil yield with an average of 1512 kg·ha^{-1} and produced 21.8, 12, and 11.2% more seed oil compared to the control, zinc, and selenium treatments, respectively (Figure 2). Increased seed oil yield under the foliar application of micronutrients can be the result of further assimilation during the seed filling period, which is associated with an increased photosynthesis of siliques (Shahbaz *et al.,* 2018). Zaman Fashami *et al.* (2018) investigated the response of different rapeseed cultivars to a foliar application of selenium and reported that the oil yield of rapeseed cultivars under selenium foliar application conditions was 5.8% greater than the control treatment (no foliar application). Shahsavari (2019) studied the effects of zeolite and zinc on the quantitative and qualitative yields of rapeseed and showed that although the foliar application of zinc increased the seed oil yield of rapeseed, the combined use of zeolite and zinc produced even greater seed oil yield.

With respect to the average across sowing dates, the highest seed oil yield was related to 7 October with the averages of 1942.75 and 2257.43 kg·ha^{-1} in 2014-2015 and 2015-2016 growing seasons, respectively, and the sowing

TABLE 5. Mean comparison (± standard deviation) of interaction effect of planting date and genotype on qualitative and quantitative traits of rapeseed.

Sowing date	**Genotype**	**Seed yield (kg·ha^{-1})**	**Oil content (%)**	**Oil yield (kg·ha^{-1})**	**Palmitic acid**	**Oleic acid**	**Linoleic acid**	**Linolenic acid**	**Erucic acid**	**Glucosinolate Content (µmolg^{-1})**
					%					
7 October	Ahmadi	4897±722b	41.8±0.80b	2051±323b	3.92±0.30b	18.5±1.38ab	4.39±0.27b	62.3±1.60ab	0.189±0.03b	9.1±0.85b
	SW102	4831±586b	41.7±0.74b	2018±262c	3.85±0.26b	18.4±1.46ab	4.48±0.37b	62.1±1.38b	0.196±0.03b	9.4±0.79b
	Okapi	5273±523a	42.3±0.80a	2223±243a	4.29±0.38a	19.0±1.53a	4.02±0.24c	62.6±1.53ab	0.144±0.02c	8.0±0.77c
	GKH2624	4620±541b	41.5±0.77c	1922±248c	4.43±0.42a	18.2±1.49b	4.68±0.32a	62.1±1.78b	0.219±0.03a	9.9±0.92a
	GK-Gabriella	5355±594a	42.4±0.79a	2275±278a	4.36±0.38a	19.1±1.41a	3.92±0.22c	62.9±1.55a	0.130±0.02c	7.8±0.95c
17 October	Ahmadi	2386±458b	38.9±0.75c	932±188b	5.4±0.43ab	15.2±0.54b	6.7±0.47a	59.2±1.99a	0.458±0.02a	15.9±0.92a
	SW102	2280±465b	38.8±0.80c	889±195b	5.4±0.39ab	15.0±0.49b	6.8±0.43a	59.1±2.05a	0.476±0.02a	16.1±1.08a
	Okapi	2864±568b	39.5±0.79b	1135±235a	5.6±0.51a	15.7±0.62a	6.3±0.37b	59.8±1.95a	0.405±0.03b	14.8±0.90b
	GKH2624	3068±493a	39.8±0.71a	1224±209a	5.3±0.44b	16.0±0.63a	6.1±0.37b	60.1±1.92a	0.380±0.04c	14.2±0.90c
	GK-Gabriella	2936±490a	39.7±0.72ab	1168±204a	5.6±0.59a	15.8±0.64a	6.2±0.43b	60.0±1.92a	0.393±0.04bc	14.6±1.10bc
27 October	Ahmadi	2086±428b	35.55±1.69c	745±174b	4.9±0.46ab	13.6±0.93b	6.1±0.53a	53.4±2.96a	0.415±0.02a	14.1±1.01a
	SW102	1994±432b	35.45±1.71c	711±177b	4.9±0.43ab	13.5±0.90b	6.2±0.50a	53.3±3.00a	0.424±0.03a	14.2±1.14a
	Okapi	2504±530a	36.07±1.73b	907±216a	5.0±0.52a	14.1±1.00a	5.7±0.44b	53.9±2.96a	0.367±0.03b	13.1±0.98b
	GKH2624	2682±472a	36.34±1.71a	979±203a	4.8±0.46b	14.3±1.01a	5.5±0.44b	54.2±2.95a	0.344±0.04c	12.6±0.97c
	GK-Gabriella	2567±467a	36.22±1.71ab	933±196a	5.0±0.58a	14.2±1.01a	5.6±0.48b	54.1±2.95a	0.356±0.04bc	12.9±1.12bc

Means followed by similar letters for each treatment in columns are not significantly different at 5% probability level by the LSD test. All treatments were performed in three replicates.

dates of 17 and 27 October showed a significant yield reduction compared to 7 October in terms of oil yield (Table 6). The results of the slicing interaction between sowing date and genotype showed that the seed oil yield varied from 711 kg·ha^{-1} for the SW102 genotype on 27 October to 2275 kg ha^{-1} for the GK-Gabriella genotype on 7 October. When averaged by genotypes, the sowing of rapeseed plants on 17 and 27 October compared to 7 October resulted in 49 and 52% reductions in seed oil yield (Table 5). The difference in the oil yield by genotype is due to the unique characteristics of each genotype which give different yields (Naseri *et al.,* 2012; Eyni-Nargeseh *et al.,* 2020). Oil yield differences between genotypes have been reported for rapeseed by Safavi Fard *et al.* (2018). The reduction in seed oil yield under delayed sowing dates is probably due to higher temperatures during the seed filling period, which can reduce seed size and consequently oil yield (Nazeri *et al.,* 2018). Of course, the seed oil yield is directly affected by seed yield and oil content; the reasons for which were earlier discussed. The reduction in rapeseed oil yield due to delayed sowing dates has been reported by Adamsen and Coffelt (2005) and Moradi Aghdam *et al.* (2019).

3.4. Palmitic acid

After foliar application treatments, the average palmitic acid contents were 4.17, 5.49, and 4.95% on 7, 17, and 27 October, respectively (Table 4). The results showed that the foliar application of zinc resulted in the highest palmitic acid contents on three sowing dates of 7, 17, and 27 October with averages of 4.49, 5.87, and 5.45%, respectively (Table 4); whereas the foliar application of selenium produced the lowest palmitic acid contents with averages of 3.87, 5.13, and 4.48% under such conditions.

Across genotypes, the average palmitic acid contents were 3.97, 5.46, and 4.92% on 7, 17, and 27 October, respectively (Table 5). The results showed that the highest palmitic acid content on 7 October belonged to the GKH2624 genotype (4.43%) followed by GK-Gabriella (4.36%) and Okapi (4.29%) genotypes (Table 5). On the sowing date of 17 October, the GK-Gabriella and Okapi genotypes had the highest palmitic acid content with an average of 5.6%. The highest content in palmitic acid on 27 October belonged to the GK-Gabriella and Okapi genotypes with an average of 5%.

The contents in saturated and unsaturated fatty acids depend on different factors such as genotype, management, and environmental conditions and these compounds may be increased or reduced under different conditions (Eyni-Nargeseh *et al.,* 2020; Safavi Fard *et al.,* 2018). The results of Eyni-Nargeseh *et al.* (2020) showed that the palmitic acid content in 17 rapeseed genotypes was significantly different due to genetic differences among genotypes. As one of the management practices, sowing date can have different effects on fatty acid composition (Maradi Aghdam *et al.,* 2019; Nazeri *et al.,* 2018). Long periods of high temperatures during the seed filling stage can produce seeds with low oil content and quality (Omidi *et al.,* 2010). In one study, Turhan *et al.* (2011) investigated the effect of sowing dates (10, 20, and 30 October, and 10 November) on rapeseed fatty acid compositions and reported that delayed sowing dates reduced palmitic acid content. Also, the composition of rapeseed fatty acids is significantly affected by fertilizer management such as micronutrients (Shoja *et al.,* 2018). The study results of Bybordi and Mamedov (2010) showed that the application of micronutrients (zinc and iron) increased the content in saturated fatty acids. Zaman Fashami *et al.* (2018) analysed the effect of foliar application of selenium on the quantitative and qualitative yields of different rapeseed cultivars and concluded that palmitic acid content showed an increasing trend under foliar application treatments.

3.5. Linoleic acid

As shown in Table 4, linoleic acid varied from 19.21% on 7 October when treated with selenium + zinc to 13.02% on 27 October in the control treatment. After foliar application treatments, the linoleic acid content was 18.70% on the sowing date of 7 October and reduced by 16.7 and 25.2% on 17 and 27 October, respectively (Table 4). Shoja *et al.* (2018) investigated the effect of the foliar application of micronutrients on the quantitative and qualitative properties of rapeseed seed oil and showed that the lowest linoleic acid content was related to a non-foliar application treatment (41.41 mg·g^{-1}); while the highest linoleic acid content was detected in the foliar application of zinc + boron (55.55 mg·g^{-1}).

The highest content in linoleic acid among the studied sowing dates in both years of the experiment was detected on 7 October (Table 6). When compared to 17 and 27 October, the linoleic acid content was 20 and 28.1% higher on 7 October in the 2014-2015 growing season, and 13 and 21.8% higher in the 2015-2016 growing season, respectively. The results showed that the linoleic acid content of the studied genotypes was reduced with delay in sowing so that the amounts of this trait on 17 and 27 October were 16.6 and 25.2% less than 7 October (Table 5).

Linoleic acid content ranged from 19.1% for the GK-Gabriella genotype on 7 October to 13.5% for the SW102 genotype on 27 October (Table 5). On the sowing dates of 17 and 27 October, the highest linoleic acid content belonged to the GKH2624 genotype with the averages of 16 and 14.3%, respectively. In separate studies, Eyni-Nargeseh *et al.* (2020) and Nazeri *et al.* (2018) stated that different genotypes showed statistically significant differences in terms of linoleic acid content. The results of Turhan *et al.* (2011) showed that delay in sowing significantly reduced the linoleic acid content. Moradi Aghdam

Table 6. Mean comparison (± standard deviation) of interaction effect of year and sowing date on qualitative and quantitative traits of rapeseed.

Year	Sowing date	Oil yield Kg·ha^{-1}	Linoleic acid	Linolenic acid	Oleic acid	Erucic acid	Glucosinolate content µmol·g^{-1}
			(%)				
2014-2015	7 October	1942.75±254a	19.86±1.10a	4.16±0.39c	61.52±1.36a	0.158±0.04c	9.36±1.04c
	17 October	948.82±184b	15.89±0.67b	6.22±0.45a	58.17±1.24b	0.413±0.05a	15.23±1.35a
	27 October	758.73±171c	14.28±1.02c	5.64±0.50b	52.44±2.54c	0.375±0.05c	13.48±1.33b
2015-2016	7 October	2257.43±257a	17.54±0.62a	4.43±0.37c	63.39±1.18a	0.193±0.04c	8.43±1.12c
	17 October	1190.70±237b	15.26±0.55b	6.73±0.43a	61.25±1.22b	0.428±0.03a	15.11±1.07a
	27 October	952.22±219c	13.71±0.93c	6.09±0.49b	55.22±2.64c	0.388±0.03b	13.38±1.11b

Means followed by similar letters for each treatment in columns are not significantly different at 5% probability level by the LSD test. All treatments were performed in three replicates.

et al. (2019) evaluated the response of different rapeseed cultivars to delayed sowing and concluded that linoleic acid content was reduced by 15.2% due to delayed sowing date (1 November) compared to the well-timed sowing date (7 October).

3.6. Linolenic acid

After the foliar application treatments, the average linolenic acid contents were 4.3, 6.8, and 5.87% on 7, 17, and 27 October, respectively (Table 4). It should be noted that the foliar application of zinc on the three sowing dates (7, 17, and 27 October) produced the highest linolenic acid contents (4.5, 6.72, and 6.30%, respectively) (Table 4). Zaman Fashami *et al.* (2018) concluded that the foliar application of selenium significantly increased the linolenic acid contents in different rapeseed cultivars. Shoja *et al.* (2018) reported that the amount of linolenic acid in rapeseed was significantly increased when zinc, boron, and sulphur were applied compared to the non-foliar application treatment.

The results showed that in the 2014-2015 and 2015-2016 growing seasons, the sowing date of 17 October showed the highest linolenic acid contents with the averages of 6.22 and 6.73%, respectively (Table 6). The average linolenic acid contents across genotypes were 4.3, 6.42, and 5.82% on 7, 17, and 27 October, respectively (Table 5). The difference between genotypes in terms of linolenic acid content has been reported by Safavi Fard *et al.* (2018) and Eyni-Nargeseh *et al.* (2020). Omidi *et al.* (2010) studied rapeseed fatty acid compounds as affected by environmental, genotype, and agronomic parameters and found that the linolenic acid content of the studied genotypes showed an increasing trend due to delayed sowing. The results of Moradi Aghdam *et al.* (2019) showed a 28.8% increase in linolenic acid content as a result of delayed sowing dates. Nazeri *et al.* (2018) also investigated the response of rapeseed genotypes to two sowing dates including 7 October (well-timed sowing date) and 17 October (delayed sowing date) and showed that linolenic acid content increased by 30.7% with delayed sowing date.

3.7. Oleic acid

The oleic acid content varied from 63.84% on 7 October and treated with selenium + zinc treatment to 50.98% on 27 October for the non-foliar application treatment (control) (Table 4). the average oleic acid content across foliar application treatments on 7 October was 62.45% and was reduced by 4.4 and 13.8% on 17 and 27 October, respectively (Table 4). In a study, Hashem *et al.* (2013) showed the protective role of selenium foliar application on rapeseed and reported that oleic acid content increased when selenium was applied compared to non-foliar application. The study results of Shoja *et al.* (2018) showed that the foliar application of micronutrients including boron, zinc, and sulphur resulted in increased oleic acid content. Their results also showed that the combined application of these micronutrients had more beneficial effects.

The results illustrated that the rapeseed plants sown on 7 October had the highest amount of oleic acid with the averages of 61.52 and 63.39% in the 2014-2015 and 2015-2016 growing seasons, respectively (Table 6). Averaged by genotypes, the highest oleic acid content was detected on 7 October with an average of 62.4%, and was reduced by 4.4 and 13.8% on 17 and 27 October, respectively (Table 5). The difference in the studied genotypes in terms of oleic acid content is related to genetic differences. Farahani *et al.* (2019) and Eyni-Nargeseh *et al.* (2020) reported differences among genotypes in terms of oleic acid content in separate studies. When compared to 17 and 27 October, higher oleic acid content on 7 October could be attributed to cooler temperatures at the seed filling stage (Fayyaz-UI-Hassan *et al.,* 2005). Nazeri *et al.* (2018) investigated the response of different rapeseed cultivars to well-timed (7 October) and delayed (27 October) sowing dates and found that oleic acid content on 7 October was 2.1% more than 27 October. Moradi Aghdam *et al.* (2019) showed in another study that the oleic acid content of different rapeseed genotypes was significantly reduced with a delay in sowing.

3.8. Erucic acid

Average erucic acid contents across foliar application treatments were 0.175, 0.418, and 0.381% on 7, 17, and 27 October, respectively (Table 4). Erucic acid is one of the most important fatty acids for rapeseed oil and its edible use, which is needed in small quantities (Gecgel *et al.,* 2007), and its content should not exceed 2% (Moradi Aghdam *et al.,* 2019). The results revealed that the foliar application of selenium provided the minimum erucic acid contents on 7, 17, and 27 October with averages of 0.156, 0.399, and 0.348%, respectively (Table 4). Shahsavari *et al.* (2014) investigated the effect of the foliar application of zinc and zeolite on the qualitative yield and composition of the fatty acids in rapeseed and found that the erucic acid content was significantly affected by foliar application and increased when zinc and zeolite were applied compared to a non-foliar application treatment. Shoja *et al.* (2018) investigated the response of rapeseed fatty acid compounds to foliar application of micronutrients (sulfur, boron, and zinc) and concluded that it led to a decrease in erucic acid content compared the non-foliar application treatment.

The results showed that the highest erucic acid content was obtained on 17 October with averages of 0.413 and 0.428% in 2014-2015 and 2015-2016 growing seasons, respectively (Table 6). Averaged by genotypes, rapeseed plants sown on 7 October had the minimum erucic acid content with an average of 0.175%, while erucic acid contents were 0.422 and 0.383% on sowing dates of 17 and 27 October, respectively (Table 5). Plant growth and development as well as oil quality and quantity in rapeseed are affected if maximum daily temperatures are greater than 27 ˚C (Morrison and Stewart, 2002). When rapeseed is sown late in the fall, long periods of high temperatures during the seed filling stage can produce seeds with low oil content and quality (Omidi *et al.,* 2010; Nazeri *et al.,* 2018). The study results of Samadzadeh Ghale Joughi *et al.* (2018) showed that the delayed sowing of rapeseed increased erucic acid contents in all studied genotypes. The difference in erucic acid content was due to genetic differences between genotypes, and in agreement with the current study results, Moradi Aghdam *et al.* (2019) reported that studied cultivars were significantly different in terms of erucic acid content.

3.9. Glucosinolate

After foliar application treatments, the average glucosinolate content was 8.87 μmol·g^{-1} on 7 October; while the glucosinolate content for rapeseed plants sown on 17 and 27 October were 15.17 and 13.43 μmol g^{-1}, respectively (Table 4). It should be noted that for all treatments the glucosinolate content did not exceed the standard level for edible consumption (30 μmol·g^{-1}). Increasing glucosinolate content resulted in a reduction in the quality and nutritional value of rapeseed oil (Sulisbury *et al.,* 1987), which is affected by hereditary and environmental factors (Fieldsend *et al.,* 1991). The results of Shahsavari *et al.* (2014) in evaluating the quantitative and qualitative responses of rapeseed to foliar application of zeolite and zinc showed that glucosinolate content was significantly reduced when zeolite and zinc were applied compared to non-foliar application.

The highest glucosinolate content was observed on 17 October with averages of 15.23 and 15.11 μmol·g^{-1} in the 2014-2015 and 2015-2016 growing seasons, respectively (Table 6). Averaged across genotypes, glucosinolate content was 8.84 μmol g^{-1} on 7 October, and increased by 71 and 51.4% on 17 and 27 October, respectively (Table 5). Glucosinolate content is dependent on environmental and genetic factors (Fieldsend *et al.,* 1991) and its content might vary between genotypes (Farahani *et al.,* 2019). The difference in glucosinolate content due to genetic differences between cultivars has been reported by Li *et al.* (2019) and Safavi Fard *et al.* (2018). A delay in sowing date also has a significant effect on glucosinolate content in rapeseed as an environmental factor. In this regard, Samadzadeh Ghale Joughi *et al.* (2018) investigated the effect of sowing date on the qualitative yield of different rapeseed cultivars and found that a 10-day delay in sowing resulted in a 69% increase in the seed glucosinolate content in rapeseed. Increased glucosinolate content in rapeseed cultivars with delayed sowing dates has also been reported by Moradi Aghdam *et al.* (2019).

4. CONCLUSIONS

Our study illustrated that rapeseed genotypes sown on delayed sowing dates produced lower seed yield in comparison to the well-timed sowing date mainly due to a reduction in the number of days from emergence to flowering and maturity, and the duration of flowering and seed filling. Owing to the importance of oleic and linoleic fatty acids in improving the oil quality regarding edible uses, the delay in sowing date of rapeseed caused a reduction in oil quality by dropping these traits as well as increasing erucic acid and glucosinolate contents. The combined application of selenium + zinc had a more positive effect on oil qualitative and quantitative yield when rapeseeds were planted on well-timed and delayed sowing dates. The improvement in the oil quality of rapeseed genotypes was due to enhanced oleic and linoleic acid contents. As a general result, The GK-Gabriella and GKH2624 genotypes sown on well-timed (7 October) and delayed sowing dates (17 and 27 October) and treated with selenium+zinc are recommended due to higher oil yield, and linoleic and oleic acid contents.

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Oil quality characterization of the Arauco variety from the main olive growing areas of Mendoza (Argentina)

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SUMMARY: 'Arauco' forms part of the over two thousand olives (*Olea europaea* L.) varieties identified worldwide with the peculiarity of being the only recognized variety from Argentina. In this work, the fruit and oil characteristics from the 'Arauco' variety cultivated in the three main olive growing zones of Mendoza province (Argentina) were evaluated over two harvests (2016 and 2017). The characteristics assessed were oil and water concentrations, pulp/pit ratio, industrial yield, oxidative stability, fatty acid profile, total phenols and total flavonoids, among others. The results showed relatively high oleic acid and phenolic contents, together with low acidity and extinction coefficients (K_{232} and K_{270}), as well as a well-balanced fatty acid profile. In addition, three interesting relationships among oxidative stability and ratios of monounsaturated fatty acids/polyunsaturated fatty acids (R^2=0.96), oleic/linoleic (R^2=0.96) and stearic acid (R^2=0.93) were observed. These results showed that the 'Arauco' variety cultivated in Mendoza provides excellent oil quality.

KEYWORDS: *Fatty acid profile; Olea europaea L.; Olive oil; Phenolic content*

RESUMEN: ***Caracterización de la calidad del aceite de la variedad Arauco en las principales*** áreas ***productoras de Mendoza (Argentina).*** 'Arauco' forma parte de las más de dos mil variedades de olivo reconocidas mundialmente, con la particularidad de ser la única variedad reconocida de Argentina. En este trabajo, se evaluaron las características de frutos y aceite de la variedad 'Arauco' cultivada en las tres principales áreas productoras de la provincia de Mendoza (Argentina), durante dos cosechas (2016 y 2017). Las características estudiadas fueron concentración de agua y aceite, relación pulpa/hueso, rendimiento industrial, estabilidad oxidativa, perfil de ácidos grasos, fenoles y flavonoides totales entre otras. Los resultados mostraron un contenido relativamente alto de fenoles y de ácido oleico, sumado a bajos coeficientes de extinción (K_{232} y K_{270}) y acidez, así como un perfil de ácidos grasos bien equilibrado. Además, se observaron tres interesantes relaciones entre estabilidad oxidativa y las relaciones de ácidos grasos monoinsaturados/poliinsaturados (R^2=0.96), oleico/linoleico (R^2=0.96) y el ácido esteárico (R^2=0.93). Los resultados mostraron que la variedad 'Arauco' cultivada en Mendoza da aceite de excelente calidad.

PALABRAS CLAVE: *Aceite de oliva; Contenido fenólico; Perfil de ácidos grasos; Olea europaea L.*

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1. INTRODUCTION

The olive tree is an ancient species from the Mediterranean region. Evidence of its use dates back to 3000 years before Christ, when Crete already exported olive oil to the Egyptians (Riley, 2002). In the American continent, it was first introduced by colonizers in the fifteenth century, and it was thanks to its plasticity that this crop spread to new environments. Nowadays, only four varieties are recognized in America: 'Arauco' in Argentina, 'Azapa' in Chile, 'Mission' in the United States (Bartolini *et al.,* 1998), and 'Criolla' in Peru (Castillo-Llanque *et al.,* 2008).

In Argentina, the commercial expansion of the olive crop took place after 1850 with the arrival of European immigrants. In 1992, tax exemption laws benefited the northwestern provinces (i.e. Catamarca, La Rioja and San Juan) located between latitude 28°S and 31°S, which widely expanded their olive cultivation surface area (SAGPYA, 2010). Before that law, Mendoza (a southern province located at 32°S latitude) was the main olive growing area due to its environmental conditions (i.e. low daily temperatures) (Gómez Del Campo *et al.,* 2010). This, added to other environmental conditions such as low nightly temperatures, high irradiance during fruit maturation and the quality of irrigation water, favored the production of oil of excellent quality. Cornejo *et al.* (2014), evaluating oil quality of 'Arbequina' from three different regions in the San Juan province (31°S), observed that high temperatures (above 35 ºC) affected oleic and linoleic acid composition, and affected the olive oil's shelf-life. Similar results were obtained by Rondanini *et al.* (2011) and Rondanini *et al.* (2014), who evaluated seventeen varieties from warm valleys of La Rioja province (29°S). This particular situation entails difficulties in international marketing, because an oil can be suspected of fraud or adulteration for this reason.

In recent years, many studies have been conducted in Argentina to describe the morphologic, agronomic and management characteristics of foreign varieties (Rondanini *et al.,* 2011; Torres and Maestri, 2006; Torres *et al.,* 2017), although little is yet known about the local 'Arauco' variety. For example, some studies describe it as a table olive variety due to its considerable size and high pulp/pit ratio (Fernández *et al.,* 2014; Fernandez *et al.,* 2018). Other studies highlight olive oil from 'Arauco' variety for its well-balanced fatty acid composition (Bodoira *et al.,* 2015) and high phenolic content (Bodoira *et al.,* 2015; Ceci and Carelli, 2007; Monasterio *et al.*, 2017). High phenolic content oils are increasingly interesting due to their nutraceutical quality (Aguilera *et al.,* 2004; Visioli and Galli, 1998) and sensory attributes like bitterness and pungency (Inarejos-García *et al.,* 2013). In addition, among olive oil phenolic compounds, flavonoids are an important group with a key role due to their antioxidant effects (Bouaziz *et al.*, 2005).

Ceci and Carelli (2010) studied olive oils from a wide range of Argentinian zones that showed an increase in the concentration of some fatty acids (mainly oleic acid) and phenol compounds with an increase in latitude. Similar results were found by Rondanini *et al.* (2014) and Trentacoste *et al.* (2020). Ceci and Carelli (2010) also detected an increase in the oleic/(linoleic + linolenic) ratio and oxidative stability toward higher southern latitudes (i.e. colder environments). In Mendoza, 'Arauco' has been one of the most cultivated varieties (20% of the total provincial olive crop surface area) mainly grown in the North (Maipú and Lavalle) and East (Junín, Rivadavia and San Martín) of the province. 'Arauco' predominates in olive orchards with trees over fifty years old with traditional management (low density, surface irrigation and manual harvest) (Baroni *et al.,* 2010). The new trend in olive farming toward growing just a few varieties with demonstrated adaptation to high plant density, drip irrigation and mechanization, increases the risk of loss in genetic variability. The 'Arauco' variety has not been selected for new orchards; it has even been superseded by more easily adapted varieties. Precisely for this reason, knowing the characteristics of this variety is becoming progressively more important as its excellent nutraceutical attributes are increasingly appreciated. In this sense, the aims of this work were to: (i) evaluate the fruit characteristics and oil composition of the 'Arauco' variety in the three main olive growing areas of Mendoza (Junín, Rivadavia and Maipú), and (ii) explore the relationship between oil and fruit traits affected by environmental (regional) conditions.

2. MATERIALS AND METHODS

2.1. Zones and plant material

Assays were carried out over two harvest years (2016 and 2017) on the 'Arauco' variety grown in orchards with similar management and ages, located in three important olive growing areas of the Mendoza province (see maps in Figure 1).

Zone 1 (Junín) is included in the Olive Germplasm Collection of Mendoza, which is situated in the experimental farm of INTA in Junín (33º06´S, 68º29´W, 653 m.a.s.l). The plantation was established in 1956. The region has a historical average temperature of 17.3 ºC, a rainfall of 275 mm (mainly in summer and far below olive crop water requirements) and a frost-free period from November to April. Trees from the olive collection are spaced 12 m x 12 m (traditional system) and fortnightly flood irrigated to replenish the soil water content over the growing seasons.

Zone 2 (Rivadavia) is located within a commercial olive orchard in Los Campamentos, Rivadavia (33º15´S, 68º26´W, 660 m.a.s.l). The plantation was established in 1980. The region has a historical average temperature of 18.5 ºC, a rain-

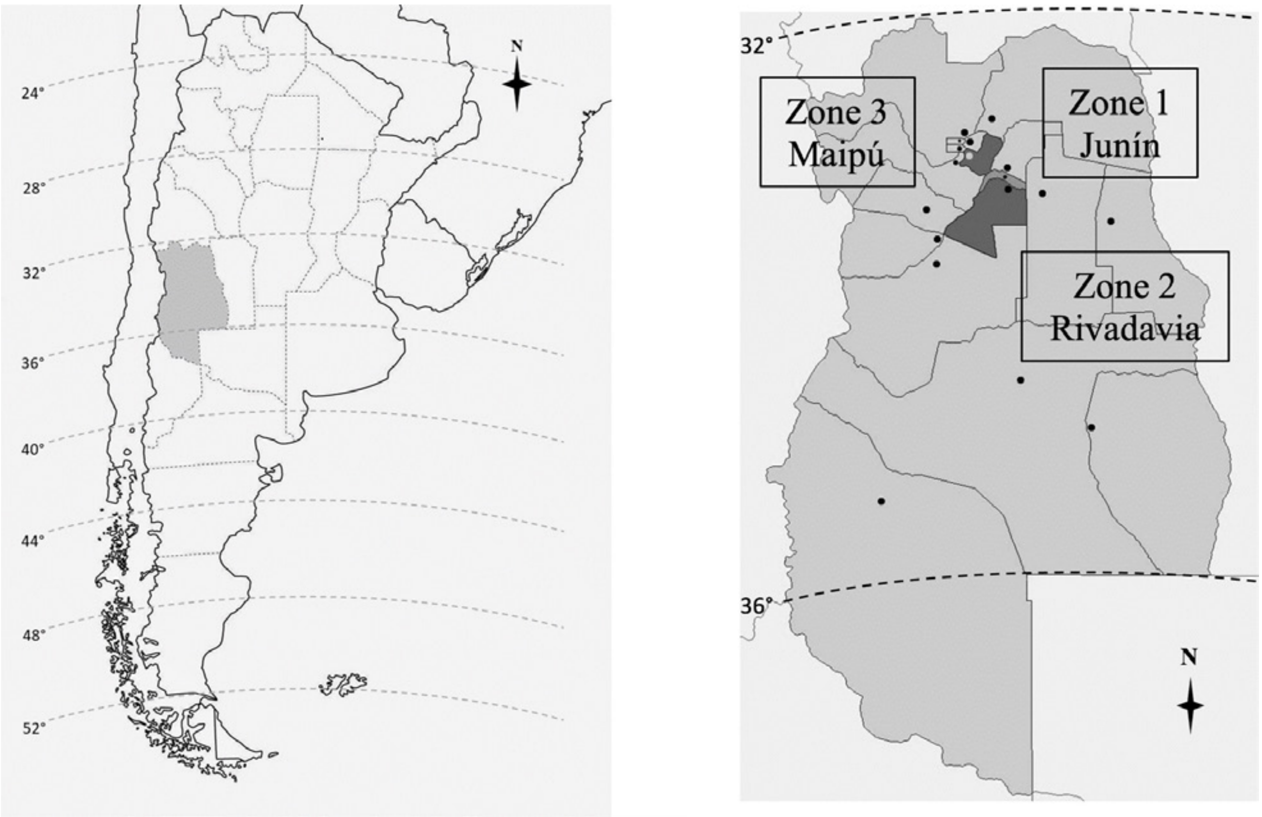

Figure 1. Map of Argentina highlighting Mendoza province and map of Mendoza highlighting the main three olive growing zones evaluated.

fall of 195 mm (mainly in summer) and a frost-free period from November to March. Trees are spaced 10 m x 10 m (traditional system) and drip irrigated to replenish the soil water content and thus avoid water deficit.

Zone 3 (Maipú) is situated within a commercial olive orchard in Russell, Maipú (33°0’S, 68°44’W, and 850 m.a.s.l). The plantation was established in 1986. The region has a historical average temperature of 19.5 ºC, a rainfall of 120.5 mm (mainly in summer) and a frost-free period from October to April. Trees are spaced 10 m x 10 m (traditional system) and flood irrigated to replenish the soil water content and avoid water deficit.

From each orchard, four trees were randomly selected before collecting olives with similar crown volume and high fruit load. Manual harvest was conducted during the first half of May, before frost events. A fruit sample of 20 kg per tree (replicate) was taken. One replicate was used for machine conditioning and the oil obtained was not considered for the study. From the other three replicates, a fruit subsample of 2 kg was randomly selected to perform fruit analysis (pulp oil concentration in fresh and dry basis, fruit weight, fruit humidity, pulp/pit ratio and maturity index). The rest of the fruits (18 kg) were milled to extract olive oil. Briefly, olive fruits were crushed with a hammer mill and the olive paste obtained was malaxated for 40 min until passing through the decanter (Spremolive New 20 kg·h^{-1}, Toscana Enologica Mori) without the addition of water. Afterwards, the olive oil obtained was filtered and stored in a cold and dark place until its analytical parameters were determined.

2.2. Morphological and chemical characteristics of the fruit

Fruit dry weight and water concentration were determined in a sample of 100 fruits which were fresh-weighed and oven-dried (60 °C) to a constant weight. Fruit dry weight was also measured. Next, water concentration was calculated from the difference between fresh and dry weight divided by fresh weight. Pulp/pit ratio was determined from a sample of 50 randomly selected and weighed fruits; then, their pits were manually separated and weighed. Another sample of 100 fruits was randomly selected to determine maturity index, according to Uceda and Frías (1975). Industrial yield was calculated by dividing kilograms of olive oil obtained by kilograms of milled fruit and then multiplied by 100 to express it as percentage.

Pulp oil concentration was determined in triplicate according to Avidan *et al*. (1999). In brief, 5 g of pulp were weighed and oven-dried (60 ºC) to constant weight. Dried-pulp was weighed and crushed in a mortar and transferred to a test tube containing 10 mL of petroleum ether. After 12 h of shaking, the samples were filtered, transferred to a new, previously-weighed test tube and air-evaporated. Later, samples were oven-dried (60 °C) to constant weight. Pulp oil concentration was calculated on fresh basis (POCfb) and dry basis (POCdb) from weight difference.

2.3. Analytical methods

Oil quality was determined through acidity (expressed as oleic acid) and extinction coefficients (K_{232} and K_{270}) according to the International Olive Council (IOC, 2015a, 2015b). In addition, oil oxidative stability was calculated by measuring oxidation induction time using Rancimat equipment (Metrohm Ltd., Herisau, Switzerland). In summary, a sample of oil (3 g) was force-oxidized by means of air flow (10 $L.h^{-1}$) and heat (110 ºC). As a result, the necessary time to reach the curve inflection point was obtained.

Total phenolic content was determined according to the International Olive Council method (IOC, 2017). Briefly, a portion of olive oil (2 g) was weighed and mixed together with the internal standard syringic acid. Next, it was extracted by an ultrasound bath at room temperature with a mix of methanol/water (80/20 v/v). Afterwards, it was centrifuged and the supernatant was filtered and injected into a liquid chromatograph Dionex Ultimate 3000 HPLC system (CA, USA) with a UV detector at 280 nm. A C_{18} reverse-phase column, Roc® Restek (250 x 4.6 mm; 5 µm) (Pennsylvania, USA) was used, employing a gradient of water 0.2% phosphoric acid ((v/v)/methanol/acetonitrile) as indicated by the official methodology.

Total flavonoids were determined by the modified method of Zhishen *et al.* (1999). A sample of olive oil (6 g) was added to 10 mL of methanol/water solution (4:1 v/v), the mixture was shaken for 10 min at 4000 rpm, and the supernatant was extracted and put in a flask. This process was repeated three times. In a 10 mL volumetric flask, 1 mL of sample was added to 4 mL of distilled H_2O. Next, 0.3 mL of sodium nitrite (5% p/v) were added. After 5 min, 0.3 mL of aluminum chloride (10% p/v) were added, and 6 min later, 2 mL of sodium hydroxide (1M) were added. The volumetric flask was filled to the mark with distilled water. Flavonoids were measured by spectrophotometry at 510 nm of absorbance against a blank.

Olive oil fatty acid composition was determined by the IOC method (2001). An oil sample was weighed and dissolved with heptane. Afterwards, it was derivatized with a methanolic potassium hydroxide solution. The solution was left to decant and the supernatant was analyzed with a Shimadzu GC 2010 Plus gas chromatography system (Shimadzu, Japan), using a hydrogen flame ionization detector and a ZB-FAME capillary column (60 m x 0.25 mm; 0.2 um) (Zebron, Phenomenex, USA). Detection and injection were set at 260 ºC and 240 ºC, respectively, using hydrogen as carrier gas. The amounts of fatty acids were expressed as relative area percentages and identified by comparing their retention times with those of standard solutions. The fatty acids detected were palmitic (C16:0), palmitoleic (C16:1), margaric (C17:0), margaroleic (C17:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), arachidic (C20:0), eicosenoic (C20:1) and behenic (C22:0).

2.4. Statistical analysis

Data of zones and harvest years were submitted for variance analysis and means separated using the Tukey-test (α=0.05) and InfoStat statistical software version 1.5 (Di Rienzo *et al.,* 2002). A regression analysis was applied to study the relationships among all the parameters analyzed in both fruits and oil.

3. RESULTS AND DISCUSSION

Absolute maximum, absolute minimum and mean temperatures, relative humidity and accumulated rainfall from August to May (i.e. from bud-break to harvest) for the two seasons studied (2015-2016 and 2016-2017), and the three main olive growing zones in Mendoza are shown in Figure 2 and Table 1. Mean temperatures in Zones 1, 2 and 3 were 16.6 ºC, 17.1 ºC and 17.3 ºC, respectively, for the 2015-2016 season, and 17.9 ºC, 18.3 ºC and 18.1 ºC, respectively, for the 2016-2017 season. The highest temperatures during the fruit development period (i.e. January-May), were 39.6 ºC and 42.0 ºC, recorded in January, 2016 and January, 2017 in Zone 2, respectively. While the lowest temperatures recorded were -2.8 ºC in April, 2016 and -2.3 ºC in May, 2017, both in Zone 2. Thus, the main difference among zones was thermal amplitude, mainly during the January-May period, which was higher in Zone 2 (average 2015 and 2016 = 27.9 ºC), followed by Zones 1 (26.5 ºC) and 3 (24.5 ºC) in an east-west gradient. Accumulated rainfall ranged from the lowest values recorded in Zone 3 (195 and 168 mm in 2016 and 2017, respectively), intermediate in Zone 1 (425 and 239) to the highest values in Zone 2 (425 and 239, respectively). The 2015-2016 season was wetter than the 2016-2017 season. Orchards were frequently irrigated because the amount of rainfall was far below crop water requirements.

3.1. Fruit characteristics

The fruit characteristics of the ‘Arauco’ variety from the three main olive growing zones of Mendoza for the two seasons are shown in Table 2.

Taking into account zones and crop seasons, fruits from the ‘Arauco’ variety showed fresh and dry weight of 5.1 g and 1.8 g, respectively, pulp/pit ratio of 7.8, water concentration of 64.4% and oil concentration of 15.8% and 50.1% on fresh and dry weight basis, respectively, and a maturity index of 1.5. ‘Arauco’ has a large size, pulp/pit ratio higher than 5, and slow change in skin color,

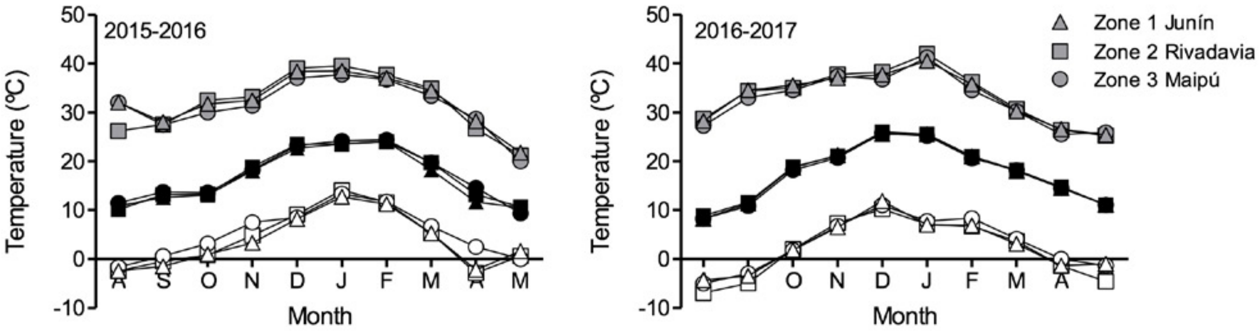

Figure 2. Absolute maximum (gray symbols), mean (black symbols) and absolute minimum (white symbols) temperatures from August to May during 2015-2016 and 2016-2017 growing seasons in three olive growing zones of Mendoza, Argentina.

characteristics which are highly appreciated for table olive elaboration (Bodoira *et al.*, 2015; Kailis and Harris, 2007), which constitutes the main industrial use of this variety in Argentina.

Fruit characteristics were significantly different among zones with the exception of pulp oil concentration. Across seasons, the fruits collected from Zone 2 showed higher fresh weight (6.4 g) and dry weight (2.2 g), pulp/pit ratio (8.9) and were more mature (2.3) than the fruits collected from Zones 1 and 3 (average 4.4 g, 1.6 g, 7.3, 1.1, following the same sequence as Zone 2). The fruit water concentration from Zones 1 and 2 was on average 65.4% higher than Zone 3 (62.6%). During oil processing, the oil extraction yield showed a similar pattern, with the highest value in Zone 2, intermediate in Zone 1, and the lowest in Zone 3. The earlier maturation and higher fruit size and pulp/stone ratio observed in Zone 2 seem to be more associated with fruit load and orchard management than with environmental conditions.

In all environments, except Zone 2 (MI = 2.3), fruits were harvested with a maturity index lower than 1.5 prior to the occurrence of severe early frost, which served to ensure high-quality oil. Bodoira *et al.* (2015) evaluated the olive variety 'Arauco' in the San Juan province and determined maximum oil concentration at a low maturity index (below 1) and maturity index between 1 and 2 to achieve maximum oil quality. In a previous study carried out in Mendoza on the 'Arauco' variety, it was observed that harvest before mid-May led to obtaining maximum oil quality by reducing olive exposure to severe frost events (Trentacoste *et al.,* 2020). Similarly, Morelló *et al.* (2003) evaluated the effects of frost on the Arbequina variety oil and determined a significant decrease in phenol content and pigments due to frost.

3.2. Oil characteristics

Basic oil quality parameters (phenolic content, oxidative stability, acidity, extinction coefficients) and total

Table 1. Total rainfall, relative humidity (RH) and average monthly maximum, mean and minimum temperatures from November to May during 2015-2016 and 2016-2017 growing seasons in the three principal olive zones of Mendoza, Argentina

Average date*		**Max temp (ºC)**	**Mean temp (ºC)**	**Min temp (ºC)**	**Rainfall (mm)**	**RH (%)**
2016	Zone 1	33.0	18.4	5.8	425.0	69.8
	Zone 2	33.2	19.1	6.1	514.4	60.4
	Zone 3	32.2	19.1	7.2	194.6	61.7
2017	Zone 1	34.6	19.7	6.2	239.2	59.5
	Zone 2	35.9	20.1	5.1	382.6	50.5
	Zone 3	33.9	20.1	7.1	168.4	51.1

*Nov/May average dates (2015-2016 and 2016-2017).

Table 2. Fruit characteristics (mean ± standard deviation, n=6) from 'Arauco' variety evaluated during two harvests (2016 and 2017) in the three main olive zones of Mendoza, Argentina

Variable	**Zone 1**	**Zone 2**	**Zone 3**
POCfb (%)	14.3±1.3	14.7±1.0	18.4±5.1
POCdb (%)	50.5±1.7	49.7±1.2	50.3±4.9
MI	1.1±0.4b	2.3±0.6a	1.2±0.2b
FFW (g·100 fruits^{-1})	474.8±87.0b	645.5±51.1a	407.7±28.4b
FDW (g·100 fruits^{-1})	164.7±27.9b	222.7±25.7a	152.3±7.3b
P/P	7.9±1.1b	8.9±0.4a	6.6±0.5c
IY (%)	4.9±1.1b	6.6±1.8a	3.4±0.7c
WC (%)	65.2±2.4a	65.6±1.4a	62.6±1.8b

POCfb: pulp oil concentration on fresh basis, POCdb: pulp oil concentration in dry basis, MI: maturity index, FFW: fruit fresh weight, FDW: fruit dry weight, P/P: pulp/pit ratio, IY: industrial yield, WC: water concentration. Significant differences in the same row are shown by different letters ($p < 0.05$). Tukey test ($p < 0.05$) was used for comparison of means

Table 3. Oil characteristics (mean ± standard deviation, n=6) from 'Arauco' variety evaluated during two harvests (2016 and 2017) in the three main olive zones of Mendoza, Argentina

Variable	Zone 1	Zone 2	Zone 3
Acidity ≤ 0.8% oleic acid*	0.19±0.1	0.17±0.0	0.20±0.1
K232 ≤ 2.50*	1.05±0.6b	1.15±0.7a	0.91±0.8c
K270 ≤ 0.22*	0.09±0.0	0.09±0.0	0.10±0.0
Oxidative stability (h)	8.5±1.4b	7.8±0.3b	10.4±0.8a
Total phenol content (mg·kg^{-1})	401.7±63.1	438.8±59.4	462.2±70.3
Total flavonoid content (mg·kg^{-1})	50.2±25.3	61.5±17.1	49.9±35.3
Fatty acids			
Palmitic acid (C16:0) 7.50 - 20.0%*	16.2±2.7b	18.1±0.9a	14.6±1.1c
Palmitoleic acid (C16:1) 0.3 - 3.5%*	1.5±0.4b	2.2±0.2a	1.1±0.2c
Margaric acid (C17:0) ≤ 0.4%*	0.1±0.1ab	0.1±0.0a	nd
Margaroleic acid (C17:1) ≤ 0.6%*	0.1±0.1	0.1±0.0	0.1±0.0
Stearic acid (C18:0) 0.5 - 5.0%*	2.3±0.1b	2.3±0.1b	2.7±0.1a
Oleic acid (C18:1) 55.0 - 83.0%*	65.6±5.7b	59.1±1.3c	71.7±0.5a
Linoleic acid (C18:2) 2,5 - 21,0%*	12.2±2.7b	16.2±0.8a	8.0±0.4c
Arachidonic acid (C20:0) ≤ 0.6%*	0.5±0.1	0.5±0.1	0.4±0.2
Linolenic acid (C18:3) ≤ 1.0%*	1.0±0.1	1.0±0.3	0.9±0.1
Eicosenoic acid (C20:1) ≤ 0.5%*	0.3±0.1	0.3±0.0	0.3±0.1
Behenic acid (C22:0) ≤ 0.2%*	0.2±0.1	0.2±0.0	0.2±0.0
MUFA / PUFA	5.4±1.5b	3.6±0.2c	8.2±0.3a
Oleic / linoleic	5.7±1.7b	3.7±0.3c	9.0±0.4a
UFA / SFA	4.3±0.7a	3.7±0.2b	4.6±0.2a

*Limit according to the IOC standards for extra virgin olive oil. Significant differences in the same row are shown by different letters ($p < 0.05$). Tukey test ($p < 0.05$) was used for comparison of means. K232 and K270: Extinction coefficients measured at 232 and 270 nm. MUFA/PUFA: monounsaturated fatty acids/polyunsaturated fatty acids. UFA/SFA: unsaturated fatty acids/saturated fatty acids. nd: not detected

flavonoids are shown in Table 3. All measured samples were classified as extra virgin olive oil according to IOC, 2015a; IOC, 2015b; IOC, 2017. On average, the 'Arauco' variety presented 0.18% acidity, 0.09 extinction coefficients K_{270}, 434 mg·kg^{-1} phenolic content and 54 mg·kg^{-1} total flavonoids with non-significant differences among zones, in contrast to oxidative stability (8.9 h) and extinction coefficient K_{232} (1.04). The oil from Zone 3 showed the highest oxidative stability (10.4 h), and the oil from Zone 2 the highest extinction coefficient (K_{232}=1.2).

It is worth noting that the phenol content in 'Arauco' oil was above 400 mg·kg^{-1}, regardless of zone. According to Montedoro *et al.* (1992), 'Arauco' oil could be classified as a variety within the "medium" category (i.e. 200-500 mg·kg^{-1}) in relation to phenolic content. The phenolic content range observed here coincides with Monasterio *et al.* (2017), who evaluated oil from eight olive varieties from Mendoza province, including 'Arauco'. The authors determined the phenolic content in 'Arauco' oil to be within the range of 233 to 404 mg·kg^{-1}. In addition, the phenolic content in the oil from 'Arauco' olives grown in Mendoza was similar to that of 'Arauco' oil from San Juan (433 mg·kg^{-1}) (33ºS, Bodoira *et al.,* 2015) and higher than that from La Rioja province (166 mg·kg^{-1}) (28ºS, Ceci and Carelli, 2010). These results reveal a possible latitudinal gradient where phenols increase with increasing latitude in the Southern hemisphere in relation to a decrease in temperature (Mousa *et al.,* 1996), which is greater than the thermal gradient observed among the zones studied.

The fatty acid composition of olive oils from the three main growing zones of Mendoza during the two seasons is shown in Table 3. All samples were within IOC legal limits. On average across zones and seasons, the 'Arauco' variety presented the following fatty acid profile: 16.29±2.25% palmitic acid, 1.59±0.54% palmitoleic acid, 0.04±0.01% margaric acid, 0.09±0.01% margaroleic acid, 2.42±0.22% stearic acid, 65.47±6.15% oleic acid, 12.13±3.74% linoleic acid, 0.49±0.11% arachidonic acid, 0.98±0.18% linolenic acid, 0.3±0.05% eicosenoic acid, and 0.18±0.02% behenic acid. The contents in palmitic, palmitoleic, margaric, stearic, oleic and linoleic acids were significantly different among zones. Conversely, the contents in margaroleic acid, arachidonic acid, linolenic acid, eicosenoic acid and behenic acid were not significantly different among zones.

The oil from Zone 2 presented the highest contents in palmitic, palmitoleic, margaric and linoleic acids, and the lowest oleic and stearic acids contents. (the latter not significantly different from Zone 1) with respect to oils from Zones 1 and 3. Maximum temperature during fruit development would partially explain the lower oleic acid content in Zone 2 and a reverse trend for palmitic and linoleic acids. García-Inza *et al. (*2014) carried out a manipulation experiment over the fruit-growth period in which fruits were exposed to increments of 5 ºC and 10 ºC above ambient temperature. The authors observed that the contents in palmitic and linoleic acids in oil increased linearly with fruit temperature, while oleic acid content decreased. In addition, greater fruit maturity from Zone 2 could contribute to explaining the fatty acid pattern. Gómez del Campo and García (2012) found that more mature fruits produced oil with lower oleic acid content; while the polyunsaturated fatty acid percentage was greater than in less mature fruits.

In the 'Arauco' oils studied, the oleic acid content was higher than 57%, above the IOC limit (55%). The oleic acid content in oil from 'Arauco' olives grown in Mendoza was similar to the oleic acid range observed in 'Arauco' oil from San Juan (33ºS, Bodoira *et al.,* 2015) and higher than the 53.7-54.3% obtained in La Rioja and Catamarca provinces (28ºS, Ceci and Carelli, 2007; Rondanini *et al.,* 2011). For the same variety, pooled results reveal a possible latitudinal gradient where oleic acid content increases with increasing latitude in the Southern hemisphere in relation to a decrease in temperature, as previously described by Rondanini *et al. (*2011).

The main monounsaturated fatty acid was oleic, which ranged from 59.1 to 71.7%; the main saturated fatty acid was palmitic, ranging from 14.6 to 18.1%; and the main polyunsaturated fatty acid was linoleic, which ranged from 8.0 to 16.2%. Together, they accounted for 93.4 to 94.3% of the fatty acid content. Monounsaturated fatty acid/polyunsaturated fatty acid (MUFA/PUFA), oleic/linoleic acid, and unsaturated/saturated fatty acid (UFA/SFA) ratios were calculated (Table 3). On average, the 'Arauco' variety showed the following values for the three zones and two seasons: MUFA/PUFA = 5.6, oleic/linoleic = 5.9, and UFA/SFA = 4.2. These values were lower than those around 4.4 which were observed for the same ratios estimated in previous studies on the Arbequina olive variety in Junín (Zone 1) Mendoza, (Lémole *et al.*, 2018). In addition, Zone 3 presented the highest MUFA/PUFA and oleic/linoleic ratios, which were significantly different from the other zones; while the UFA/SFA ratio was similar in both Zones 1 and 3 and significantly higher than in Zone 2.

In addition, linear regressions were studied among all the oil traits analyzed. Some interesting relations were identified between oxidative stability (OS) and fatty acids, or their ratios. Thus, a positive and closer relationship was found between the OS and MUFA/PUFA ratio (R^2=0.96), than between the OS and UFA/SFA ratio (R^2=0.66). Bhatnagar *et al. (*2009) determined that OS is negatively related to PUFA. Our results showed that the induction time to oxidation in 'Arauco' oils was more related to monounsaturated fatty acids than to total phenolic and total flavonoid compounds. This could be explained by the fact that all the samples showed similarly high phenolic contents. Given that the highest content in monounsaturated and polyunsaturated acids in olive oils is due to oleic and linoleic acids, respectively, OS was positively related to the oleic/linoleic ratio (R^2=0.96). Nevertheless, not every monounsaturated acid plays such an important role given that palmitoleic acid (C16:1) displayed negative relationships with OS (R^2=0.79). Other negative relationships were found between OS and linoleic and palmitic acids (R^2=0.90 and R^2=0.72, respectively). Martínez *et al. (*2014) determined that linoleic acid is highly susceptible to oxidation due to its key role in the lipoxygenase pathway as the precursor of many volatile compounds. In addition, stearic acid (C18:0) showed a positive relationship with OS (R^2=0.93). Bhatnagar *et al. (*2009) evaluated blends with coconut oil, and observed that an increase in saturated fatty acids improved their oxidative stability.

4. CONCLUSION

The results of the present study highlight the excellent characteristics of the 'Arauco' variety grown in Mendoza from a nutritional viewpoint in terms of oil quality. The data obtained were not affected by abnormal weather conditions. Environmental variability was slight; therefore, other factors such as fruit load could have contributed to the differences obtained in fruits and oils. However, the total variability in the parameters evaluated was within IOC legal limits. 'Arauco' olive oil from Mendoza presented high oleic acid contents, which was positively correlated with oxidative stability rather than total phenolic and flavonoid contents. MUFA/PUFA and oleic/linoleic ratios and stearic acid were also positively correlated with oxidative stability. In addition, 'Arauco' showed high fruit fresh weight and high pulp/pit ratio, which explains its extensive use as a double-purpose variety.

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Fatty acid composition, phytochemicals and antioxidant potential of *Capparis spinosa* seeds

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SUMMARY: The present study evaluates the contents in bioactive compounds, antioxidant activity, oil content and fatty acid composition of *Capparis spinosa* seeds. Samples were collected from 5 different habitats (AH: Ahar; KU: Kurdistan; U1, U2 and U3: Urmia) in Iran. The oil content in the seeds ranged from 16 to 27%. The predominant fatty acid was linoleic acid (45–50%) followed by oleic acid (30–39%), palmitic acid (2–8%) and stearic acid (2–3%). Total phenolic content (TPC) varied from 16.3 to 24.2 mg GAE/ g DW; total flavonoid content (TFC) ranged from 1.48 to 3.05 mg QE/g DW; and the antioxidant activity (DPPH assay) of the seeds was between 35 and 63%. The compounds obtained from different genotypes of *C. spinosa* seeds had different compositions, great antioxidant capacity and unsaturated fatty acids, and therefore could be a prospective source of natural bioactive molecules for the food and health industry.

KEYWORDS: *Antioxidant activity; Bioactive compounds; Fatty acids; Flavonoids; Phenolic compounds*

RESUMEN: ***Composición de ácidos grasos, fitoquímicos y potencial antioxidante de las semillas de* Capparis spinosa.** El presente estudio evaluó el contenido de compuestos bioactivos, actividad antioxidante, contenido de aceite y composición de ácidos grasos de las semillas de *C. spinosa*. Se recolectaron muestras de cinco hábitats diferentes (AH: Ahar; KU: Kurdistán; U1, U2 y U3: Urmia) en Irán. El contenido de aceite de las semillas osciló entre el 15,66 y el 27,50%. El ácido graso predominante fue el ácido linoleico (45–50%) seguido por el oleico (30–39%), el palmítico (2–8%) y el esteárico (2–3%). El contenido fenólico total varió de 16,3 a 24,2 mg GAE/g DW; el contenido total de flavonoides entre 1,48 y 3,05 mg QE/g DW; la actividad antioxidante de las semillas estuvo entre 34,68 y 62,74%. Los compuestos obtenidos de semillas de *C. spinosa* tienen gran capacidad antioxidante y ácidos grasos insaturados, por lo que podrían ser una fuente de moléculas bioactivas naturales en la industria alimentaria y de la salud.

PALABRAS CLAVE: *Ácidos grasos; Actividad antioxidante; Compuestos bioactivos; Compuestos fenólicos; Flavonoides*

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1. INTRODUCTION

The caper is a perennial shrub plant and is the common name of the genus *Capparis*, in the family *Capparidaceae*. It is distributed in the subtropical and tropical regions with 250 species (Wojdyło *et al*., 2019). The taxonomy of the genus in Iran has been the subject of much debate. Boissier (1867), combined *Capparis parviflora* and *Capparis mucronifolia* species in the *Capparis* spinosa as varieties. However, Zohary (1960) introduced five species with several varieties in Iran. *Capparis spinosa,* with three varieties, var. spinosa, var. parviflora and var. Mucronifolia, has been cited in In Flora Iranica (Hedge and Lamond, 1970). Capparis species are mostly distributed in the south of Iran, however, *Capparis spinosa* is widely distributed in all regions of Iran.

Different organs of caper-like fruits and flower buds are commonly used by the local population for their anti-inflammatory, antidiabetic, antihyperlipidemic, antihypertensive, antihepatotoxic and anticarcinogenic activities since the caper and its derivatives, such as pickled caper buds are rich in vitamins, essential minerals, fatty acids and proteins (Özcan *et al*., 2004; Arslan and Özcan, 2007; Özcan, 2008; Bakr and El Bishbishy, 2016). *C. spinosa* seeds are rich in lipids, containing unsaturated fatty acids (Oleic acid, vaccenic acid, linoleic acid), bioactive (phenolic compounds) and nutraceutical (flavonoids and sterols) compounds (Matthäus and Özcan, 2005). However, Duman and Özcan (2014) reported that capers had high levels of Ca, K, Mg, Na, and S minerals but caper seeds involved more mineral matter than oils. During the last decade, several natural compounds such as synthetic additive replacers (Domínguez *et al*., 2020; Lorenzo *et al*., 2018) have been investigated in the food industry. *C. spinosa* is a possible source of these compounds. *C. spinosa* seeds contain a relatively high amount of oil (23–33%) (El-Waseif and Badr, 2018), rich in phospholipids (phosphatidylinositol), with a high content in tocopherols (γ-tocopherols and δ-tocopherols), sterols (sitosterol, campesterol and stigmasterol), carotenoids (α-tocopherol) and glucosinolates (Glucocapperin) (Matthäus and Özcan, 2005; Tlili *et al*., 2009; Zhang and Ma, 2018).

It is well known that the genotype and region could exert a great impact on the oil composition and functional and nutritional properties (Alirezalu *et al*., 2016). However, there are limited data relating to the effects of genotype and location on *C. spinosa* oil composition. In addition to environmental conditions that have considerable effects on the biosynthesis of secondary metabolites, genetic factors can also contribute to the biosynthesis and accumulation of these compounds. The interaction of environmental factors and the genetic background of plants play a substantial role in the accumulation of metabolites (Alirezalu *et al*., 2018; Shaghaghi *et al*., 2019; Alirezalu *et al*., 2020). Some genes contribute to the biosynthesis of fatty acids and phenolics in medicinal plants. The expression of these genes varies in various species. Moreover, cytochromes-P450 isoforms contribute to the phenylpropanoid metabolism and by effecting several reactions, they regulate phenolic synthesis (Ayabe and Akashi, 2006).

The purpose of the present study was to determine, for the first time, oil content, fatty acid composition, phytochemicals and antioxidant activity of the oil from caper seeds collected from 5 different regions.

2. MATERIALS AND METHODS

2.1. Sample preparation

The ripe fruits of *C. spinosa* genotypes (accession) from 5 region of West Iran were collected from 27 June until 10 July 2016 (Table 1). Details of collection sites are presented in Table 1. The seeds were obtained from ripened fruit (5 plants from every region). The freeze-dried fruits were squashed in a mortar and sifted using a 60-mesh screen to separate the seeds from the pulp. After air-drying at 30 °C for 72 h, seeds were removed, mixed and used for additional analysis (Fazio *et al*., 2013).

2.2. Oil extraction

For this study, 5 g of a powdered sample from each plant seed were extracted by hexane solvent (300 mL) using a Soxhlet extractor for 5 h at 80 °C in order to prevent damage to minor compounds. The extract was filtered through filter papers containing sodium sulphate and vacuum evaporated (around 150 mbar) in a rotary evaporator until the n-Hexane portions were removed at 40 °C and then stored -20 °C for further analysis. The oil content in the seeds was expressed on a percent basis, based on whole samples. Oil content was then measured using Equation 1.

$$(\%)\ \text{Oil content} = (W_1 - W_2)/W_3 \times 100 \qquad \text{Eq. 1}$$

W_1 = Weight of dried sample befor extraction + filter bag
W_2 = Weight of dried sample after extraction + filter bag
W_3 = Original weight of simple

2.3. Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared from the oil samples according to the method described by Savage *et al*. (1997). FAME) and were derived from the esterification of 20 mg extracted oil with 2 mL 0.01 M NaOH in dry methanol at 60 °C for 30 min under continuous shaking and transesterification of glycerolipids with boron trichloride/methanol. The FAMEs were determined by GC (Agilent technologies, 6890N, USA) according to the method reported by Azadmard-Damirchi and Dutta

TABLE 1. Sampling locations of the different caper genotypes.

Code	Species	Collection sites	Geographical location		
			Longitude (N)	**Latitude (E)**	**Altitude (m)**
AH	*C. spinosa*	East Azerbaijan /Ahar (Qareh daq)	38°47´32.72”	47°11´54.84 “	2172
KU	*C. spinosa*	Kurdistan / Kurdistan	34°16´58.41”	47°01´07.86”	1553
U1	*C. spinosa*	West Azerbaijan/Urmia(1)	37°45´17.82”	44°42´31.68”	1735
U2	*C. spinosa*	West Azerbaijan/Urmia(2)	37°20´31.37”	45°08´57.87”	1350
U3	*C. spinosa*	West Azerbaijan/Urmia(3)	37°27´39.13”	44°57´05.86”	1624

(2006). The GC instrument was equipped with a capillary injection valve and DB-Wax capillary column (30 m x 0.25 mm i.d and 0.25 µm of thickness), which was treated with polyethylene glycol and a flame ionization detector (FID). The FAMEs were analyzed by comparison of their retention times with standard FAMEs and the peak areas are reported as a percentage of the total fatty acids.

2.4. Preparation of seed extract

One g of caper seed was powdered by liquid nitrogen and extracted with 20 mL of 80% methanol, then mixed gradually for 1 h using a magnetic stirrer. The extract was filtered using filter paper (Whatman No.1). The resulting extract was kept at −70 °C for corresponding analysis.

2.5. Total phenolic content (TPC)

The TPC in the seed extracts was determined by Folin-Ciocalteau method Singleton *et al.* (1999) with some modification. Briefly, 100 µL of methanolic extracts were shaken for 1 min with 1 mL of diluited (1:10) Folin–Ciocalteu reagent and held at 25 °C for 5 min. Then 800 µL of sodium carbonate (10%) were added and the final volume was made up to 5.0 mL with distilled water. After that, the mixture was left at room temperature for 2 h. Finally, the absorbance at 760 nm was measured by a spectrophotometer (UNICO, China). Gallic acid was expressed as a standard solution and TPC was used as mg GAE/g DW.

2.6. Total flavonoid content (TFC)

The TFC was measured by an adapted colorimetric method using aluminium chloride ($AlCl_3$) (Ordoñez *et al.*, 2006). Briefly, 400 µL of seed extract were added to 0.3 mL distilled water followed by 0.03 mL $NaNO_2$ (5%). The mixture was held for 5 min at 25 °C, and then 0.03 mL of $AlCl_3$ (10%) were added. After 5 min, the mixture was treated with 0.2 mL of 1 mM NaOH. Finally, the reaction was diluted to 100 mL with distilled water. The absorbance versus prepared blank was measured at 420 nm. TFC was reported as mg of quercetin equivalents per g of sample dry weight (mg of QE/g DW).

2.7. Antioxidant activity

To measure the antioxidant activity by the DPPH (2, 2’-diphenyl-1-picrylhydrazyl) free radical scavenging method, 500 µL of methanolic extracts were mixed with 1 mL of the DPPH solution. The mixture was held in the dark at 20 °C for 30 min. Absorbance was determined at 517 nm using UV-Vis spectrophotometer (UNICO, China). The percentage of inhibition (I%) of free radical DPPH was calculated using the formula: RSA% = [(Ablank - Asample)/Ablank] × 100 (Nakajima *et al.*, 2004).

2.8. Statistical analysis

All of the analyses (5 regions × 5 sampling point × 3 experiments) were carried out in a completely randomized design. SAS 9.1.3 software package (SAS Institute, US) was used for statistical analysis of the data. Normal distribution and variance homogeneity had been previously tested (Shapiro-Wilk). The data were submitted to one-way analysis of variance (ANOVA). The different parameters studied in the present research were included in the model as dependent variables, while genotype was included as a fixed effect. Duncan’s test was carried out when the ANOVA was significant ($P < 0.05$), in order to determine differences between means. The principal component analysis (PCA) and hierarchical cluster analysis (HCA) based on Ward’s method and correlation analysis based on Pearson’s method were performed among the variables (fatty acid composition) by MINITAB 13.2.

3. RESULTS AND DISCUSSION

3.1. Oil content

The oil contents in the seeds from wild fruits of the caper genotypes are reported in Figure 1. There were significant differences ($P < 0.05$) in oil content among the

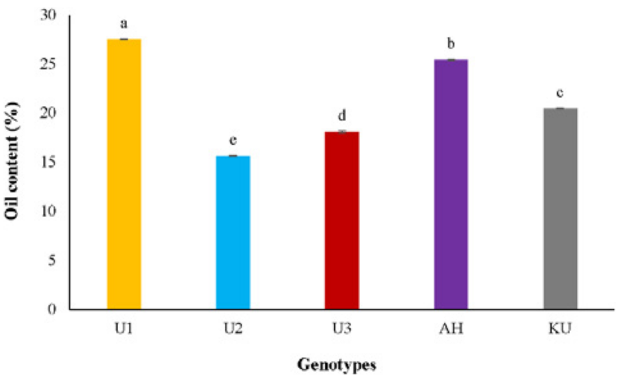

Figure 1. Oil content (g/100 g) of caper seeds in different genotypes. a-e Means (Three experiments) with different letters within treatments were significantly different at the level of $P < 0.05$. Mean comparisons of genotypes were carried out by Duncan's Multiple Range Test (DMRT) at α=0.05.

genotypes. Oil contents in samples ranged from 16 to 27%. Two genotypes (U1 (Urmia) and AH (Ahar)) had significantly higher oil contents than the other genotypes. The highest and the lowest oil contents were found in the U1 (27.50%) and U2 (16.66%) (Urmia) genotypes, respectively. El Amri *et al.* (2019) revealed that the oil content in *C. spinosa* was not only affected by location, but also the highest oil content was observed in big seeds.

These results agree with Tlili *et al.* (2009), who revealed that oil content in caper seeds ranged from 23 to 34% in Tunisia. Zia-Ul-Haq *et al.* (2011) obtained a similar oil content (29.1%) in *Capparis decidua*. Matthäus and Özcan (2005) found that habitat had a high impact on the oil content of Turkish *C. spinosa* seeds. The range of oil content in the present study was higher than that reported in Italian *C. spinosa* subsp. *Rupestris* (Argentieri *et al.*, 2012), which can be related to agro-ecological growing conditions (Čolić *et al.*, 2017). These differences could be due to geographic distribution, soil quality, climatic conditions of an area, and size of seeds and/or using different analysis methods. Caper seeds with high oil content are considered to have potential for food and pharmaceutical applications.

3.2. Fatty acids composition

The fatty acid composition of the oil samples is reported in Table 2. Eleven fatty acids were detected in the extracted oils from *C. spinosa* seeds and ranged from C14:0 to C20:0. The results showed significant differences ($P < 0.01$) in the fatty acid composition of the oil. Among the fatty acids, the predominant constituent was linoleic acid (C18:2n-6) followed by oleic acid (C18:1n-9), palmitic acid (C16:0) and stearic acid (C18:0). The presence of these fatty acids was also reported in other studies (Akgül and Özcan, 1999; Matthäus and Özcan, 2005; Saadaoui *et al.*, 2015; Tlili *et al.*, 2009; Yuldasheva *et al.*, 2008). The monounsaturated fatty acids in the caper oil were

Table 2. Fatty acid composition (g/100 g) in seed oil genotypes of capers.

Fatty acid composition	**Genotypes**				
	U1	**U2**	**U3**	**AH**	**KU**
Myristic acid; C14:0	0.34 ± 0.01^{b}	0.25 ± 0.00^{d}	0.40 ± 0.01^{a}	0.27 ± 0.00^{c}	0.27 ± 0.01^{c}
Palmitic acid; C16:0	1.87 ± 0.02^{e}	6.46 ± 0.05^{c}	7.05 ± 0.04^{b}	6.39 ± 0.04^{d}	7.88 ± 0.02^{a}
Palmitoleic acid; C16:1n-7	0.59 ± 0.09^{c}	1.68 ± 0.055^{a}	1.49 ± 0.07^{ab}	1.29 ± 0.03^{b}	1.35 ± 0.04^{ab}
Stearic acid; C18:0	2.75 ± 0.05^{a}	2.29 ± 0.01^{c}	2.37 ± 0.0^{b}	2.23 ± 0.02^{c}	2.71 ± 0.02^{a}
Oleic acid; C18:1n-9	38.88 ± 0.03^{a}	34.78 ± 0.03^{b}	34.18 ± 0.02^{c}	34.73 ± 0.03^{b}	30.52 ± 0.05^{d}
Linoleic acid; C18:2n-6	47.30 ± 0.02^{c}	45.59 ± 0.02^{d}	45.62 ± 0.02^{d}	47.71 ± 0.02^{b}	49.41 ± 0.05^{a}
α-Linolenic acid; C18:3n-3	1.33 ± 0.01^{a}	1.39 ± 0.1^{a}	1.19 ± 0.02^{ab}	0.69 ± 0.01^{c}	1.07 ± 0.0^{b}
γ-Linolenic acid; C18:3n-6	0.46 ± 0.00^{c}	0.49 ± 0.01^{b}	0.56 ± 0.00^{a}	0.43 ± 0.00^{d}	0.47 ± 0.00^{c}
Stearidonic acid; C18:4n-3	0.72 ± 0.03^{bc}	0.80 ± 0.03^{a}	0.78 ± 0.04^{ab}	0.71 ± 0.01^{c}	0.73 ± 0.02^{bc}
Arachidic acid; C20:0	0.59 ± 0.02^{b}	0.74 ± 0.02^{a}	0.69 ± 0.05^{a}	0.53 ± 0.04^{b}	0.51 ± 0.03^{b}
UFA	89.28 ± 1.32^{a}	84.73 ± 1.75^{bc}	83.82 ± 1.01^{c}	85.56 ± 0.71^{b}	83.55 ± 0.91^{c}
SFA	5.55 ± 0.45^{d}	9.74 ± 0.71^{bc}	10.51 ± 0.54^{ab}	9.42 ± 0.09^{c}	11.37 ± 0.42^{a}

UFA: Unsaturated fatty acid; SFA: Saturated fatty acid; Values are expressed as means ± standard deviation (n=3). $^{a-e}$ Means (Three experiments) with different letters within the same row were significantly different at the level of $P < 0.05$. Mean comparisons of genotypes were carried out by Duncan's Multiple Range Test (DMRT) at α=0.05.

palmitoleic and oleic acid. Our results were in agreement than those reported by Matthäus and Özcan (2005), who determined that linoleic acid (24.6-50.5%) was the major fatty acid in *C. spinosa* genotypes. In contrast, other authors found oleic acid as the major fatty acid in caper seed oil (Akgül and Özcan, 1999; Saadaoui *et al*., 2015; Tlili *et al*., 2009). Zia-Ul-Haq *et al*. (2011) reported that the major fatty acids were linoleic acid (47.3%) followed by oleic acid (33.2%) in *Capparis decidua,* which agree with the values reported in the present study. The KU (Kurdistan) genotype showed the highest content in linoleic acid, while U2 and U3 (Urmia) the lowest ones. As mentioned above, the second most abundant fatty acid was oleic acid, ranging from 30.52 (KU) to 38.88% (U1). Myristic acid (C14:0), γ-linolenic acid (C18:3n-6), stearidonic acid (C18:4n-3) and arashidic acid (C20:0) were present below 1% in all genotypes. The presence of these minor fatty acids in *C. spinosa* seed oil was also reported in different studies (Givianrad *et al*., 2011; Saadaoui *et al*., 2015; Tlili *et al*., 2009). The palmitoleic acid (C16:1n-7) in caper genotypes ranged from 0.59% in U1 to 1.68% in U2. The linolenic acid content varied from 0.69 to 1.33% in AH and U2. Stearidonic acid (C18:4n-3) oscillated from 0.71 (AH) to 0.8% in the (U2) genotype. The lowest amount of γ-linolenic acid (C18:3n-6) was present (> 0.56%) in most of the caper genotypes investigated.

The predominant saturated fatty acids in the caper seed oil were palmitic acid (1.87-7.88%), followed by stearic acid (2.23-2.71%), arachidic acid (0.51-0.69%) and myristic acid (0.25-0.40%). The highest levels of palmitic and stearic acid were observed in the KU genotype and U3 genotype, which contained the highest contents in myristic acid and arashidic acids. A similar fatty acid profile has also been reported for Tunisian *C. spinosa*. Tunisian genotypes showed higher myristic, palmitic, palmitoleic, and oleic acid contents and the lowest amount of linoleic acid compared to our genotypes. There was a large variation in palmitic, oleic, linoleic and palmitoleic acids in comparison with stearic and stearidonic acids. The U1 genotype showed the highest content in unsaturated fatty acid. According to some reports, ecological conditions, genetic variation, seed maturity, seed production environment and geographical origin can affect the fatty acid composition of seeds (Acar *et al*., 2008; Saadaoui *et al*., 2015; Saxena *et al*., 2017), which could explain the differences found in the present research.

Several investigations were conducted to demonstrate the effect of different fatty acids on human health. To this regard, it is well known that saturated fatty acids have negative effects on health and promote the appearance of various diseases. A recent study published by The Word Health Organization about the effects of SFA on serum lipids and lipoproteins concluded that each 1% of dietary energy as SFA replaced with an equivalent amount of cis–PUFA or cis-MUFA resulted in a significant reduction in total, LDL and HDL cholesterol and in triglyceride contents (Mensink, 2016). In a similar way, a study reviewed the effect of fatty acids on health found that the intake of SFA increased LDL-cholesterol, favored inflammation processes and was associated with an increased incidence of type 2 diabetes (Calder, 2015). However, this evidence has been questioned by recent systematic reviews and meta-analyses of data on mortality in relation to exposure to SFA either through the diet or in the bloodstream.

On the other hand, the replacement of SFA with MUFA, particularly oleic acid, may improve glucose control and insulin sensitivity and would be expected to lower the risk of cardiovascular disease (Calder, 2015). In the same way, the consumption of PUFA, especially n-3 fatty acids, has a high impact on health and plays an important role in the prevention of cardiovascular disease. (Nagy and Tiuca, 2017).

Therefore, with the aforementioned, the caper could be an excellent source for obtaining healthy oil with potential use in the food industry. In fact, caper seeds presented higher amounts of oil content and unsaturated (linoleic and oleic acids) fatty acids. In addition, the U1 and AH genotypes showed the higher oil and unsaturated fatty contents and the lowest amounts of saturated fatty acids. Therefore, these two genotypes could be the best options for food application.

3.3. Correlation analysis

The results of the simple correlation coefficients of oil content and fatty acids are presented in Table 3. Studies have shown fatty acid levels do not affect oil content (Johansson *et al*., 2000; Onemli, 2012). A significant positive correlation was observed between myristic acid and α-linolenic acid (r=0.646**).

Palmitic acid was positively correlated (r= 0.833**) with palmitoleic acid and negatively correlated (r= -0.920**) with oleic acid. The negative correlation was revealed when palmitic acid increased and oleic acid decreased. The results agreed with previous reports by multiple authors (Lamaisri *et al*., 2015; Raheja *et al*., 1987). Stearidonic acid showed to correlate positively with palmitoleic acid, γ-linolenic acid and arachidic acid and negatively with linoleic acid.

A positive significant correlation was observed between stearic acid and linoleic acid (r=0.566*). However, observed differences between *C. spinosa* seeds from different habitats were similar to the data published by Onemli (Onemli, 2012). Negative correlations existing between linoleic and γ-linolenic acid can be shown by α- and γ-linolenic acids synthesized by the desaturation of linoleic acid in plants. This is in agreement with results found by other reports by Johansson et al (Johansson *et*

Table 3. Correlation coefficients among fatty acid composition of the caper genotypes

Fatty acids	C14:0	C16:0	C16:1n-7	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	C18:3n-6	C18:4n-3	C20:0
C14:0	1									
C16:0	-0.253	1								
C16:1n-7	-0.236	0.833**	1							
C18:0	0.112	-0.414	-0.606*	1						
C18:1n-9	0.342	-0.920**	-0.655**	0.053	1					
C18:2n-6	-0.423	0.107	-0.256	0.566*	-0.439	1				
C18:3n-3	0.232	-0.327	-0.073	0.321	0.300	-0.460	1			
C18:3n-6	0.646**	0.345	0.422	-0.144	-0.186	-0.598*	0.457	1		
C18:4n-3	0.062	0.381	0.698**	-0.373	-0.177	-0.614*	0.368	0.625*	1	
C20:0	0.232	0.082	0.388	-0.390	0.160	-0.852**	0.654**	0.666**	0.658**	1

* Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level. C14:0; Myristic acid, C16:0; Palmitic acid, C16:1n-7; Palmitoleic acid, C18:0; Stearic acid, C18:1n-9; Oleic acid, C18:2n-6; Linoleic acid, C18:3n-3; α-Linolenic acid, C18:3n-6; γ-Linolenic acid, C18:4n-3; Stearidonic acid, C20:0; Arachidic acid

al., 2000). Arachidic acid in caper oil was positively correlated with α-linolenic acid and γ-linolenic acid.

3.4. Phytochemicals and antioxidant activity in seeds

The TPC in the caper seed extracts of the 5 studied genotypes are shown in Figure 2. The amount of TPC varied from 16.3 to 24.2 mg GAE/g DW in the different genotypes. In a recent study, some authors found values between 3 and 10 mg of polyphenols/g DW of *C. spinosa* flowers (Wojdyło *et al*., 2019). In contrast, other authors reported that the TPC of the aerial part of *C. spinosa* ranged between 14.8 and 87.5 mg GAE/g in roots and 4.49-58.6 mg GAE/g in roots (Baghiani *et al*., 2012). Baghiani *et al*. (2012) reported that the extraction solvent exerted a great influence on TPC contents. They found that the chloroform extract showed the highest levels of TPC followed by ethyl acetate extract and crud extract. This fact could explain the differences reported by different researchers (Alirezalu *et al*., 2020).

The highest TPC was observed in the KU and U1 genotypes and the lowest content was observed in the U2 seeds. Indeed, climatic factors influenced oxidative stress and the generation of reactive oxygen species in perennial plants and led to excessive phenolic compound production in aggressive environments (Lamien-Meda *et al*., 2010). Phenolic compounds in plant extracts are considered as minor components for eliciting nutraceuticals, antimicrobial activity and antioxidant properties (Alirezalu *et al*., 2019). In the present research, the TPC variation was lower than those reported in a previous study (Wojdyło *et al*., 2019). This authors also reported that cultivar had a significant influence on polyphenol content, which agreed with our results (Wojdyło *et al*., 2019).

As shown in Figure 3, the concentration of flavonoids in seed extracts varied from 1.48 to 3.05 mg QE/g DW. All the extracts analyzed showed high TFC. The data showed that the highest TFC was measured in the KU genotype and the lowest content was observed in U2 genotype. According with Mamati *et al*. (2006), different biosynthetic pathway of phenolic compounds and related enzyme expression in the growth stages can be effected by the phenolic content in plants. Baghiani *et al*. (2012) reported values of TFC which range between 23.5 and 298 mg QE/g in aerial parts and between 0.25 and 2.12 mg QE/g in roots. These authors found that ethyl acetate was the best solvent to extract flavonoids from plant material. This aspect could explain, in part, the differences found between this research and our data. Health benefits, especially the antioxidant potential of food, depend on the type and amount of flavonoids (Wojdyło *et al*., 2019). As mentioned for TPC, the cultivar, genotypes and different caper organs (flowers, berries, leaves, seeds) also had a significant influence on the TFC content (Baghiani *et al*., 2012b; Wojdyło *et al*., 2019), which agree with the variations observed in the present study.

The antioxidant activity of extracts from the caper seeds of different genotypes is presented in Figure 4. The values for DPPH radical scavenging activity were directly dependent on the level of total phenolic compounds present in the methanolic extracts. The results of DPPH radical scavenging activity of seed extracts ranged between 35 and 63%. The highest free radical scavenging properties were obtained in the seed extract from the KU genotype followed by the extracts from AH>U1>U3>U2. The phenolic compounds and related antioxidant activity of different seeds were analyzed, and it was observed that there was a significant ($P < 0.05$) linear correlation among TPC, TFC and antioxidant capacities (Katalinic *et al*., 2006).

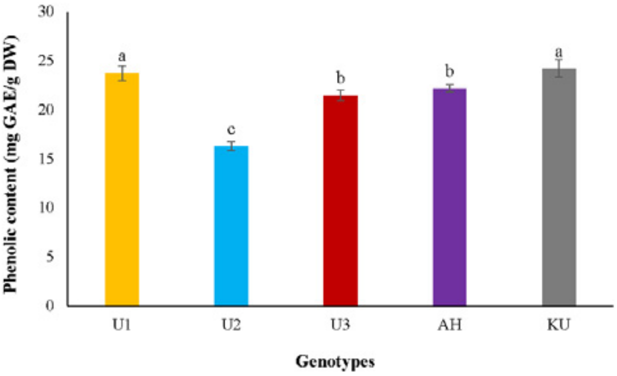

FIGURE 2. Phenolic contents (mg GAE/g DW) of methanolic extracts from caper seeds of different genotypes. $^{a-c}$ Means (Three experiments) with different letters within treatments were significantly different at the level of $P < 0.05$. Mean comparisons of genotypes were carried out by Duncan's Multiple Range Test (DMRT) at α=0.05.

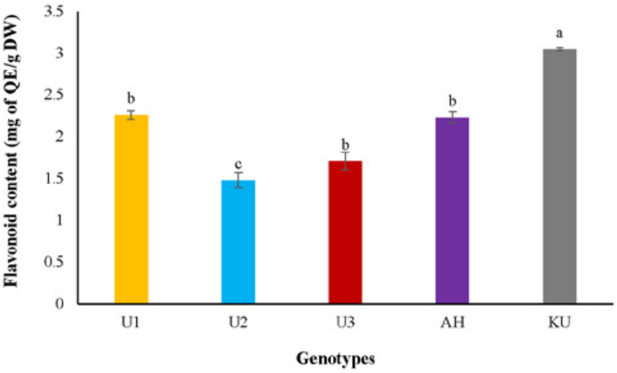

FIGURE 3. Flavonoid contents (mg QE/g DW) of methanolic extracts from caper seeds in different genotypes. $^{a-c}$ Means (Three experiments) with different letters within treatments were significantly different at the level of $P < 0.05$. Mean comparisons of genotypes were carried out by Duncan's Multiple Range Test (DMRT) at α=0.05.

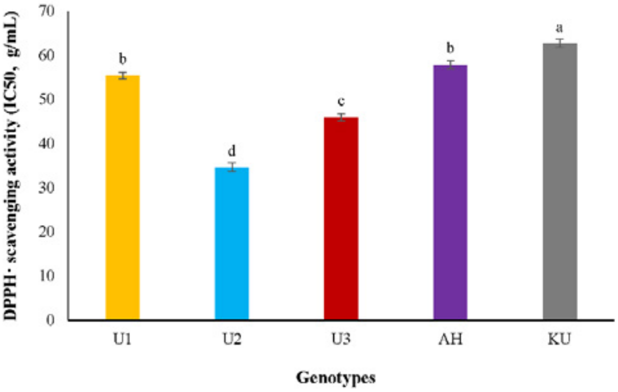

FIGURE 4. Antioxidant activity (%) of methanolic extracts from caper seeds in different genotypes. $^{a-d}$ Means (Three experiments) with different letters within treatments were significantly different at the level of $P < 0.05$. Mean comparisons of genotypes were carried out by Duncan's Multiple Range Test (DMRT) at α=0.05.

Wojdyło *et al*. (2019) found 34 flavonols, 10 hydroxycinnamic acids, and 5 flavan-3-ols in *C. spinosa* flowers. From flavonols, these authors found derivatives of quercetin, kaempferol, isorhamnetin, myricetin and rutin. They also found (+)-catechin and (-)-epicatechin and dimers and trimmers from procyanidins (flavan-3-ols) and ferulic, sinapic, quinic, coumaroylquinic and caffeoylquinic acids and their derivatives (hydroxycinnamic acid derivatives). It is well known that these compounds present high antioxidant activity. El-Ghorab *et al*. (2007) found that the dichloromethane and methanol extracts from caper buds and leaves exhibited higher antioxidant activities than those of their essential oils in testing systems.

The KU genotype contained high amounts of TPC and TFC and showed high antioxidant activity. Several studies have reported that the phenolic compound in plants through their scavenging or chelating activity are associated with their antioxidant activities, their free radical scavenging activity is attributed to free hydroxyl groups (Chang *et al*., 2001; Ghafar *et al*., 2017; Ghimire *et al*., 2011; Ibrahim and El-Masry, 2016). In addition, flavonoids, in fact are a large group of plant phytochemicals as powerful antioxidants with potent free radical scavenging properties and whose utilization as regulators of redox-sensitive signalling pathways is of great interest (Izzi *et al*., 2012). Thus, the variation in antioxidant activity between genotypes was directly related with the phenolic contents. Some studies on the phytochemical composition of various extracts from *C. spinosa* have proven the presence of several bioactive compounds such as vitamins, polyphenols and flavonoids, which are known for antioxidant properties which extend the shelf-life of foods (Matsuyama *et al*., 2009). Tlili *et al*. (2010) reported that *C. spinosa* extract is rich in phenolic compounds and vitamin antioxidants and can be used as a promising constituent for increasing the nutritional and medicinal properties of foods.

3.5. Principal component (PCA) and hierarchical cluster analysis (HCA)

The scores for the principal component analysis of caper genotypes are presented in Figure 5A. The first two principal components accounted for 78% (PC1 = 25% and PC2 = 53%, respectively) of the total variation. The characteristics that contributed positively to PC1 were oil content, linoleic acid, stearic acid, antioxidant activity, TPC, and TFC; whereas negative contributions were observed for stearidonic acid, arachidic acid, γ-linolenic acid, palmitoleic acid, α-linolenic acid and palmitic acid. PC2 was mainly correlated positively with palmitic acid and palmitoleic acid.

The biplot showed three distinct groups. The first group, comprised of two genotypes (U2 and U3) and was collated from the Urmia province. It was characterized by high amounts of stearidonic acid, γ-linolenic acid and arachidic acid along with the lowest amount of total phenols.

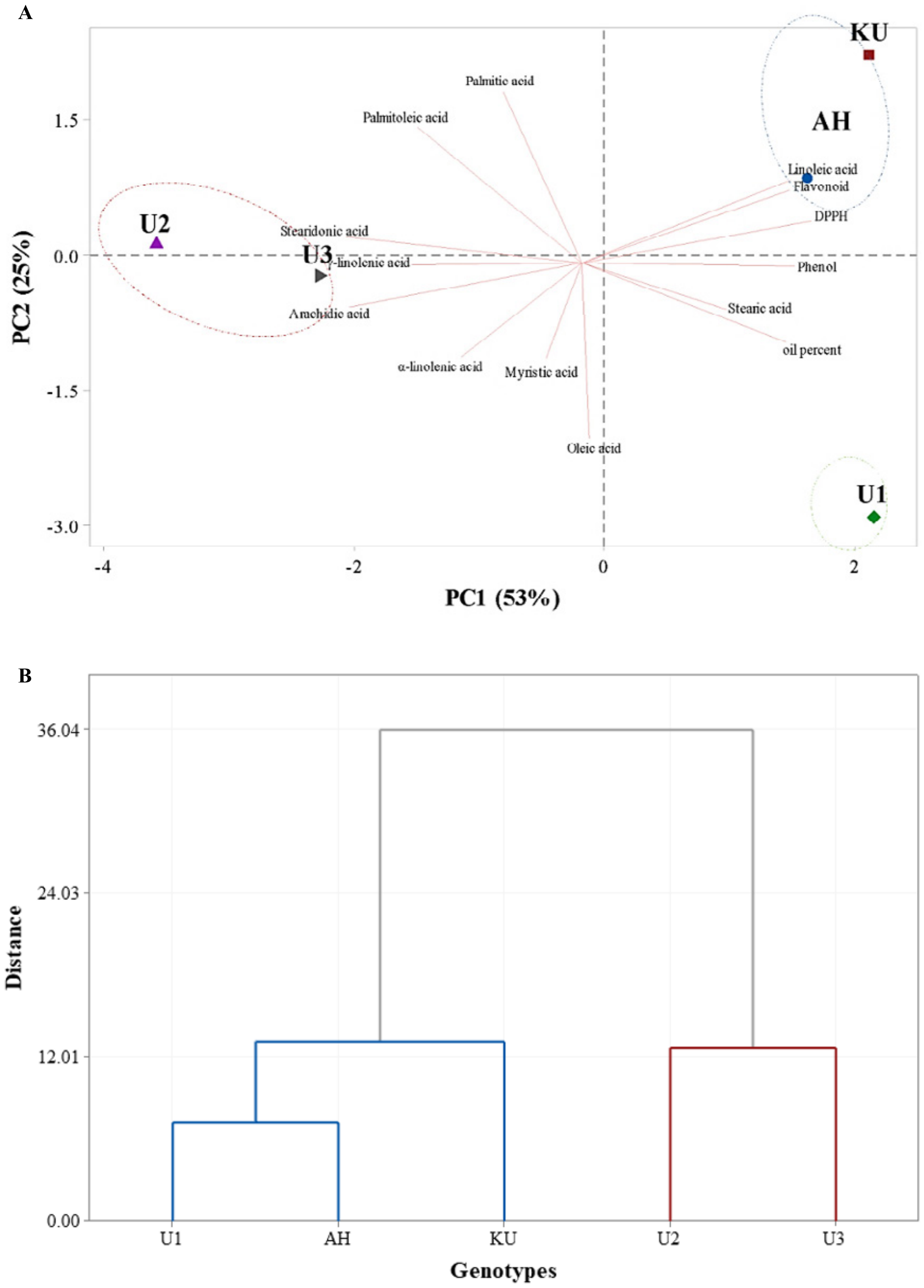

FIGURE 5. A. Principal component analysis (PCA) of caper genotypes; B. Hierarchical cluster analysis (HCA) caper genotypes

The second group included AH and KU genotypes and was characterized by the highest contents in linoleic acid, TFC and antioxidant activity by DPPH assay confirmed that antioxidant activity was mainly due to the presence of flavonoid compounds in caper seeds. The third group (U1 genotype) had the highest amount of oil content, stearic acid and phenols.

A cluster analysis (HCA) of 5 genotypes of capers is shown in Figure 5B. The cluster analysis was performed using antioxidant activity (by DPPH assay), total phenol, total flavonoid, oil content and fatty acid composition. In this case, 5 genotypes were divided into two main clusters. The U2 and U3 genotypes were grouped into the first cluster. They had high amounts of stearidonic acid, γ-linolenic acid and arachidic acid and the lowest amounts of total phenols, total flavonoids, antioxidant activity and oil content. The second cluster was comprised of three genotypes including U1, AH and KU with high amounts of linoleic acid, stearic acid, total phenols, total flavonoids, oil content and antioxidant activity.

Generally, variation in fatty acid composition, TPC, TFC and antioxidant activity was detected among the genotypes grown in West Iran. Genetic variability in capers may be utilized in trait-specific breeding programs. These results showed that the seeds of capers are promising sources of natural antioxidants, unsaturated fatty acids and bioactive compounds which are beneficial to the food and pharmaceutical industries. Based on the obtained results, U1 and KU genotypes are the most suitable to be used in breeding programs due to their unsaturated fatty acids, phenolic compounds and antioxidant activity.

4. CONCLUSIONS

To the best of our knowledge, this report is the first study on oil content and fatty acid composition combined with phenolic compounds and antioxidant activity in *C. spinosa* seeds collected from different genotypes from Iran. The oils obtained from *C. spinosa* seeds of different genotype had different chemical compositions and antioxidant activity. The extracted oil from U1 constituted high levels of oil content and unsaturated fatty acids. The extract from KU was rich in TPC, TFC and showed the highest free radical scavenging activity. In conclusion, the *C. spinosa* seeds showed efficient antioxidant potential and can be an alternative for synthetic antioxidants in food and pharmaceutical products. In addition, the high oil content and special unsaturated composition make *C. spinosa* seeds a potential healthy oil source which can be used in the reformulation of several foods.

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Development and characterization of ethanol-free spearmint essential oil nanoemulsion for food applications using the low energy technique

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SUMMARY: Different emulsifiable concentrates containing spearmint essential oil (SEO) were made and evaluated for their potential for giving ethanol-free nanoemulsion spontaneously upon dilution into water. Each one of these formulas had its specific composition regarding the type of excipients, surfactants, surfactant/SEO ratio and surfactant concentration. The results of this evaluation indicated that the chemical composition of SEO has a profound effect on the formation and physical stability of the nanoemulsion. The incorporation of excipients such as long chain triglyceride and propylene glycol into the emulsifiable concentrates at only 1.0% can lead to a stable nanoemulsion that resists Ostwald ripening. A particle size measurement showed that the diameter of SEO in the nanoemulsion was 28.2 nm and its nanostructure was maintained for 3 months. The application of a mixture of binary nonionic food-permitted surfactants enhanced the thermal stability of the nanoemulsion at up to 50 °C. The developed ethanol-free SEO nanoemulsion has promising industrial applications in food and beverage flavoring.

***KEYWORDS*:** *Essential oil; Food applications; Low energy; Nanoemulsion; Solvent-free*

RESUMEN: ***Desarrollo y caracterización de nanoemulsiones de aceite esencial de menta verde sin etanol para aplicaciones alimentarias mediante una técnica de baja energía.*** Se elaboraron y evaluaron diferentes concentrados emulsionables que contenían aceite esencial de menta verde (AMV) para determinar su potencial para dar nanoemulsión libre de etanol de forma espontánea tras la dilución en agua. Cada una de estas fórmulas tenía su composición específica en cuanto al tipo de excipientes, tensioactivos, relación tensioactivo/AMV y concentración de tensioactivo. Los resultados de esta evaluación indicaron que la composición química del AMV tiene un marcado efecto en la formación y estabilidad física de la nanoemulsión. La incorporación de excipientes como triglicéridos de cadena larga y propilenglicol en los concentrados emulsionables a solo 1,0% puede conducir a una nanoemulsión estable que resiste la maduración de Ostwald. La medida del tamaño de partícula mostró que el diámetro del AMV en la nanoemulsión era de 28,2 nm y su nanoestructura se mantuvo durante 3 meses. La aplicación de una mezcla de tensioactivos no iónicos binarios alimentariamente permitidos mejoró la estabilidad térmica de la nanoemulsión hasta 50 °C. La nanoemulsión AMV sin etanol desarrollada tiene una aplicación industrial prometedora en el sabor de alimentos y bebidas.

PALABRAS CLAVE: *Aceite esencial; Aplicaciones alimentarias; Energía baja; Libre de disolventes; Nanoemulsión*

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1. INTRODUCTION

Essential oils (EOs) are natural volatile extracts obtained from aromatic plants. They are frequently used as flavoring agents in food and beverages. They are also used in some cosmetic products such as fragrances, mouth washes and tooth paste due to their fragrant and antiseptic properties. Generally, EOs are highly concentrated extracts so they must be fairly diluted before application. Water, which is the most appreciated and economically feasible solvent, is inappropriate for the dilution or delivery of EOs due to their hydrophobic nature. Therefore, EOs are usually diluted and delivered using large amounts of organic solvents like ethanol and triacetin. Nowadays, the application of such solvents in food and beverages may not be appreciated by different sectors of consumers. Therefore, the emulsification of EOs in water to form solvent-free flavor delivery systems has become a promising trend for the food and beverage industry (Given, 2009). In such emulsions, EOs exist as tiny microscopic particles dispersed homogenously in water using the appropriate surfactant. However, such EO emulsions are kinetically stable which means that they can stay physically stable only for a limited period of time. Afterwards the oil droplets will flocculate, coalesce and finally separate from the system.

One of the trends that can be adopted to prolong the period of the physical stability of EO emulsions is by reducing the particle size of the dispersed EO droplets from the micrometer (1-5 µm) to the nanometer (20 nm-200 nm) size range (McClements and Jafari, 2018). The light mass of EO nanoparticles makes them more respondent to the bombardment from all directions by the molecules of the aqueous continuous phase in phenomena known as the Brownian motion (Mason *et al*., 2006). As a result, EO nanoparticles are kept in a state of continuous internal agitation which prevents their flocculation and subsequent coalescence and separation. That in return can increase the period of physical stability of the EO nanoemulsions compared to the same formula fabricated in the form of traditional macroemulsion with larger particle size (1-5 µm). Based on that principle, EO nanoemulsions were formulated for applications in food and beverage flavoring (Given, 2009; Barzegar *et al*., 2018) and for food preservation (Mazarei, and Rafati, 2019; Chu *et al*., 2020; Liew *et al*., 2020).

Two major approaches can be applied for the fabrication of EO nanoemultions, including the high energy and the low energy methods (McClements and Jafari, 2018). In the high energy method, a shear or cavitation forces are introduced into the system via high energy equipment to disrupt the EO phase into ultra-fine droplets in a continuous aqueous phase. Examples include sonicators (Rostamia *et al*., 2018), high pressure homogenizers (Martin-Piñero *et al*., 2019) and microfluidizers (Llinares *et al*., 2018). On the other hand, the low energy methods depend on the handling of the interfacial properties of the system by managing the appropriate type and ratio of excipients and surfactants used in the formulation process (Komaiko and McClements, 2016). Therefore, the low energy method does not rely on high shear equipment for the fabrication of nanoemulsions. The low energy method can possibly be carried out by three different approaches, namely spontaneous emulsification (Yildirim *et al*., 2017), phase inversion temperature (Chuesiang *et al*., 2018), and phase inversion composition (Chu *et al*., 2020). Each of these methods has its own preparation steps, advantages and liability which were conclusively reviewed elsewhere (Komaiko and McClements, 2016).

In the current investigation, the author aimed to develop ethanol-free and physically stable spearmint essential oil (SEO) nanoemulsion. This essential oil was chosen due to its promising potentials as a natural flavoring agent in innovative beverages like soft drinks and flavored water. The oil also has antimicrobial activity against some food related fungi (Ji *et al*., 2019) and pathogenic bacteria (Snoussi *et al*., 2015). All of the previously mentioned potentials of SEO in beverages and food necessitate the development of a water-based colloidal system like nanoemulsion for the delivery of SEO in these applications.

It is important to point out that there were previous investigators who reported the formulation of SEO nanoemulsion. However, they used high temperature (65 °C) along with high energy equipment (Tubtimsri *et al*., 2018) or large amounts of ethanol in their formulations (Wangjit *et al*., 2016).

Based on that, the current investigation was devoted to the development and characterization of ethanol-free SEO nanoemulsion fabricated at room temperature. The low energy spontaneous emulsification method is adopted for that purpose due to its simplicity, non-destructive property on the structure of EO (as it takes place at room temperature), and economic feasibility to scale up to industrial level. The appropriate measurements which are necessary to characterize the development of nanoemulsion were carried out. Critical issues that can radically influence the formulation and thermal stability of ethanol-free SEO nanoemulsions were also investigated and discussed in detail.

2. MATERIALS AND METHODS

2.1. Materials

Spearmint essential oil (SEO) was obtained from a freshly distilled batch produced at the Horticultural Research Institute, Medicinal and Aromatic Plant Research Section, Kanater, Egypt. The oil was obtained from the aerial parts (leaf and stacks) of *Mentha spicata*, Family La-

Table 1. Composition of the different sets of spearmint essential oil emulsifiable concentrates.

Sets of SEO	Surfactant(s) and their weight ratios	Composition of the final nanoemulsion (weight %)[a]					
		Sufactant(s)	Essential oils (SEO)	Ripening inhibitor		Co-surfactant (PG)	S/O ratio
				(LCT)	(MCT)		
Set (I)							
I(a)	T80	6	6	---	---	---	1
I(b)	T80	6	5	1	---	---	1
I(c)	T80	6	5	---	1	---	1
I(d)	T80	6	5	1	---	1	1
I(e)	T80	3	5	1	---	1	0.5
Set (II)							
II(a)	T80-T20 (2:1)	6	5	1	---	1	1
II(b)	T80-T20 (1:1)	6	5	1	---	1	1
II(c)	T80-T20 (1:2)	6	5	1	---	1	1
Set (III)							
III (a)	T80-Cr (1:1)	6	5	1	---	1	1
III (b)	T80-Cr (2:1)	6	5	1	---	1	1
III (c)	T80-Cr (1:2)	6	5	1	---	1	1

[a]Each emulsifiable concentrate of the three sets is added to the amount of water sufficient to give 100% final SEO nanoemulsion.
SEO: spearmint essential oil; T80: Tween 80; T20: Tween 20; Cr: Cremophor RH40.
LCT: Long-chain triglyceride; MCT: médium-chain triglyceride.
S/O ratio: surfactant / oil ratio; PG: propylene glycol.
Samples are made in duplicate.

miaceae, after steam distillation, as indicated by the supplier. After distillation, SEO was kept at – 4 °C in dark glass bottles with no headspace till analysis and formulation. Tween 80, Cremophor (Kolliphor) RH40®, propylene glycol, *l*-carvone, *d*-limonene, 1,8-cineol and pulegone were purchased from Sigma-Aldrich (St. Louis Missouri, USA). Sunflower oil, which was used as a source of long chain triglyceride (LCT), was purchased from the local market. Labrafac™ lipophile WL 1349, which is a medium-chain triglyceride (MCT) composed of C_8 and C_{10} fatty acids, was donated from Gattefosse, France.

2.2. Determination of the chemical composition of SEO

SEO was subjected to gas chromatographic (GC) analysis to reveal the structure of its major volatile constituents. For that purpose, SEO (20 µL) was diluted in 1 mL diethyl ether in a glass vial. Then, 2.0 µL of this mixture were injected (at a split ratio 10:1) into Agilent GC equipped with a flame ionization detector (FID). A fused silica capillary column DB5 (30 m × 0.32 mm × 0.25 µm) was used to separate the different volatile components. The oven temperature was programmed from 50 °C to 220 °C at a rate of 3 °C/min. The injector and detector temperatures were 220 °C and 230 °C, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min. Standard samples of *l*-carvone and *d*-limonene, 1,8-cineol and pulegone were used to identify their corresponding equivalents in SEO. The volatile oil constituents were reported as percent of the total eluted peak areas after FID. All values were means of two injections ±S.D.

2.3. Calculation of the HLB values of the binary surfactant systems

The HLB (the hydrophilic lipophylic balance) of a binary surfactant mixture made of (Tween 80/Tween 20) at different ratios was mathematically calculated using the original formula (Strianse and Lanzet, 1960):

$$HLB_{mix} = (\Phi_A \text{ x } HLB_A) + (1\text{- } \Phi_A \text{ x } HLB_B)$$

Where, Φ_A: Mass fraction of the first surfactant; HLB_A: HLB of the first surfactant; (1- Φ_A): Mass fraction of the second surfactant; HLB_B: HLB of the second surfactant

2.4. Formulation of SEO nanoemulsions

Three principle sets of SEO emulsifiable concentrates which included 11 different formulas were made at specific composition and weight ratios as described in Table 1. The desired SEO nanoemulsions were fabricated from the emulsifiable concentrates using the low-energy spontaneous emulsification method as previously described (El-Sayed *et al.*, 2017), from the original work of Anton and Vandamme (Anton and Vandamme, 2009). In more detail, the formulation process was initiated by the preparation of three SEO emulsifiable concentrates which were made as follows: SEO was first mixed with MCT or LCT to form a clear isotropic mixed oil phase; then, the later was added to the single surfactant (Set I) or the binary surfactant mix (Set II & III) in the presence or absence of PG as a cosurfactant (Table 1); the whole mixture was then vortexed to end up with different SEO emulsifiable concentrates. It was possible to heat the concentrates up to 40 °C using water bath in order to decrease the viscosity of the sets to intimate the mixing of ingredients during a vortex application.

Finally, after 30 minutes of equilibration at room temperature, each set of SEO emulsifiable concentrates was titrated drop-wise to a calculated amount of distilled water which was sufficient to produce nanoemulsions containing 6.0% of total oil phase, as illustrated in Table 1. During titration, water was subjected to continuous agitation using a magnetic bar till the whole load of each SEO concentrate was titrated. The samples were then transferred to transparent glass vials and stored at room temperature (~ 20 °C) for further analysis. All formulations were made in duplicate.

2.5. Characterization of SEO nanoemulsions

2.5.1. Appearance

Glass vials containing the different SEO nanoemulsions were examined visually against bright light to check their opacity or translucency. The UV-Vis absorption values (A) for all samples were also evaluated spectrophotometrically using Shimadzu (UV-160 1PC spectrophotometer, Japan) at wave length = 600 nm to establish a quantitative measure for the visual examination of transparency of the samples. The absorption values (A) are an average of two measurements from two different formulations.

2.5.2. Particle size

The particle size of the samples was measured using the dynamic light scattering instrument Zetasizer (Nano-ZS model ZEN3600, Nanoseries, Malvern Instruments, UK). Measurements were taken at 20 ºC unless otherwise specified, with a fixed angle of 173º. Before measurement, all samples were filtered through 0.20 µm single-use syringe filter unit (Minisart®, Sartoius Stedium Biotech GmbH Germany) to remove impurities. Each sample was diluted before measurement with distilled water to only 0.05% to prevent multiple scattering. The measurements are based on the Brownian motion of the hydrated particles thus; it provides information on the hydrodynamic diameter (nm) of the SEO particles. The sizes quoted are the *z*-average mean of the SEO droplet's hydrodynamic diameter (nm) obtained from 6 measurements for each sample (2 duplicate x 3 measurements each) ±SD, as shown in Table (2). The particle size distribution curves are plotted from the dynamic laser scattering results from duplicate samples.

2.6. Evaluation of storage stability

The different formulas of SEO nanoemulsions were subjected to a storage stability test by being left to stand undisturbed on the bench at room temperature (20 ºC ±2) for 3 months. In addition, another storage stability test at

Table 2. HLB values and particle size analysis of the different SEO nanoemulsions formulated and stored at different temperatures.

Sets of SEO	HLB of surfactant(s)	Particle size (nm) and polydispersibility index (PDI)			
		Zero time at 20 ºC	3-month storage at 20 ºC	5-days storage at 40 ºC	5-days storage at 50 ºC
Set (I)					
I(a)	15	121.3±1.7 (PDI 0.42)	Creamy layer after 24h	Cloudy after 24h	NM
I(b)	15	38.0 ±0.5 (PDI 0.30)	35.0±0.2 (PDI 0.2)	Cloudy after 24h	NM
I(c)	15	96.3 ±0.8 (PDI 0.41)	Creamy layer after 24h	Cloudy after 24h	NM
I(d)	15	28.2 ±0.1 (PDI 0.19)	25.9±0.1 (PDI 0.1)	Cloudy after 24h	NM
I(e)	15	62.4 ±0.2 (PDI 0.18)	71.9±0.7 (PDI 0.3)	Cloudy after 24h	NM
Set (II)					
II(a)	15.55	29.5±0.2 (PDI 0.2)	NM	Cloudy after 24h	NM
II(b)	15.85	69.2±2.0 (PDI 0.5)	NM	Cloudy after 24h	NM
II(c)	16.11	106.9±0.6 (PDI 0.2)	NM	Cloudy after 24h	NM
Set (III)					
III (a)	NC	25.4±0.1 (PDI 0.1)	NM	26.7±0.2 (PDI 0.1)	49.1±0.1 (PDI 0.2)
III (b)	NC	24.4±0.1 (PDI 0.1)	NM	44.7±1.4 (PDI 0.2)	75.5±0.2 (PDI 0.1)
III (c)	NC	32.0±0.3 (PDI 0.2)	NM	NM	NM

HLB: hydrophilic lipophilic balance; NC: Not calculated because there is no specific HLB value for Cremophor RH 40.
NM: Not measured because of the physical instability manifested by the cloudy appearance; Samples were made in duplicate.

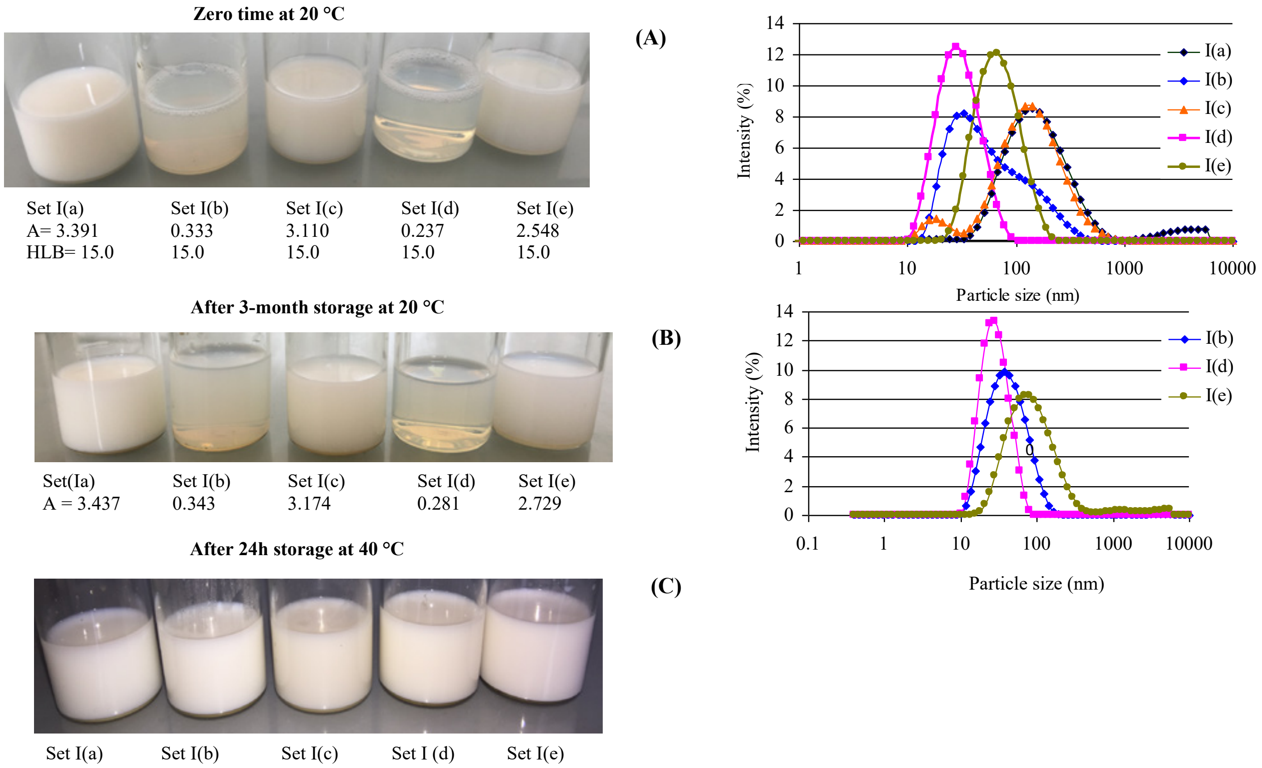


Figure 1. Appearance, UV-Vis absorption "A" at 600 nm, (left column) and their corresponding particle size analysis (right column) of the different spearmint essential oil nanoemulsions fabricated from set (I) using single surfactant at different storage periods and temperatures. The absorption values (A) are an average of two measurements from two different formulations, SDs are too small (≤0.005) to be indicated. The particle size distributions are an average of two measurements from two different formulations, plotted from dynamic laser scattering results.

higher temperatures was performed by incubating the formulas (undisturbed) at 40 ºC and 50 ºC ±1 for 5 consecutive days. At the end of the storage period, the formulas were visually inspected to detect any change in appearance, which was also quantified as absorbance values (A) using UV-Viz examination at 600 nm. Finally, the particle size of the stored samples was re-analyzed for potential alteration during storage as previously described in section (2.5.2).

2.7. Statistical analyses

Data reported after the gas chromatographic analysis of SEO and also after the particle size measurements of the nanoemulsions were the means of at least 2 replicates ± S.D.

3. RESULTS

3.1. Chemical composition of spearmint essential oil (SEO)

The composition of SEO was revealed using the gas chromatographic analysis (GC). The results indicated the presence of high contents in *l*-carvone (63.3±0.9%), and *d*-limonene (12.4±0.3%) besides much lower contents in 1,8-cineol (5.6±0.1%) and pulegone (3.6±0.1%). All together, these components accounted for about 85.0% of the oil composition, which came within the average percentages that were previously reported for each one of these components in SEO (Edris *et al*., 2003; Bouyahya *et al*., 2017; Snoussi *et al*., 2015). No attempts were made to identify the rest of the components because the characterized components were sufficient for the purpose of the GC analysis.

3.2. Formulation and characterization of SEO nanoemulsion of Set (I)

3.2.1. The effect of triglyceride

In a trial to produce ethanol-free nanoemulsion upon dilution into water, a basic tertiary emulsifiable concentrate (Ia) composed of SEO (6.0%), single surfactant (6.0%), water (88.0%) was made (Table 1). No traces of triglyceride oil were used in this formula to see if the desired nanoemulsion could be developed using SEO as the sole oil phase. The results showed that upon dilution of (Ia) emulsifiable concentrate into water, a traditional cloudy emulsion which had high UV-Vis absorbance (A= 3.39, Figure 1A) was obtained. That indicated a failure in the formulation of the desired nanoemulsion. Although the average particle size of that emulsion was 121.3 nm (Table 2), its size distribution analysis showed a second population of particles with an average size of about 5.0 µm (Figure 1A, chart). That makes the emulsion of (Ia) appear cloudier due to enhanced light scattering by the large particle population which also caused a relatively high polydispersibility index (Table 2).

Regarding physical stability, emulsion (Ia) demonstrated poor stability as evident from the appearance of a heavy creamy layer on the walls of the glass vial upon tilting to 45º within only 24h of storage at room temperature (20 °C). These results are likely to be due to the phenomena of Ostwald ripening, (OR).

The incorporation of 1.0 wt% long-chain triglyceride (LCT), such as sunflower oil (set Ib, Table 1) led to the inhibition of OR and positively influenced the appearance, particle size, and shelf-life stability of the obtained SEO nanoemulsion, (Figure 1A and Table 2). This was manifested by a change in UV-Vis absorption of the nanoemulsion of (Ib) to become 0.33, which was in accordance with its less cloudy appearance compared to (Ia), as shown in Figure 1A. In addition, the nanoemulsion of set (Ib) was characterized by a much smaller particle size (38.0±0.5 nm) with better size distribution and lower PDI, as evident from the data in Table 2 and Figure 1A (chart). Unlike the emulsion of set (Ia), which creamed after 24h, the new nanoemulsion of (Ib) which contained LCT overcame OR and showed much higher physical stability. It maintained the same appearance with almost the same UV-Vis absorption even after the 3-month storage period at 20 ºC (Figure 1B). The particle size of nanoemulsion (Ib) after the end of the 3-month storage was 35.0±0.2 nm (Table 2) and its size distribution was monomodal (Figure 1B, chart). The particle size of (Ib) and its distribution pattern were even better after storage than at time zero. The reason behind this result is that storing (Ib) for 3months provided enough time for SEO nanoparticles to equilibrate and attain their final size. That chance was not offered to the same formula at time zero because particle sizing took place shortly after fabrication.

The reader may notice that the particle sizing of emulsions (Ia) and (Ic) are excluded from analysis after the 3-month storage period and seemed absent from Figure 1B (chart). This is because they initially showed early creaming after 24h (Ia), and 1 week (Ic), respectively, as will be shown in the results regarding nanoemulsion of set (Ic).

Despite the high physical stability of the nanoemulsion of (Ib) at 20 ºC, it showed temperature sensitivity when stored at 40 ºC for only 24h. That was manifested by the conversion of its appearance into milky white (Figure 1C), indicating an increase in particle size and loss of the nanostructure.

The incorporation of 1.0 wt% of another triglyceride oil with medium-chain fatty acids (MCT) such as C_8 and C_{10} (set Ic, Table 1) also led to the inhibition of OR. However, the quality of the obtained emulsion of (Ic) was inferior compared to the nanoemulsion of (Ib) which was formulated using LCT. For instance, Figure 1A indicates that the appearance of nanoemulsion (Ic) was cloudy with a much higher UV-Vis absorbance value compared to (Ib). The particle size of (Ic) was 96.3±0.8 nm which represents more than double the size of nanoemulsion (Ib), as shown in Table 2. The particle size distribution of (Ic) also showed a bimodal pattern which justified its relatively high PDI (0.41, Table 2). The shelf-life stability of emulsion (Ic) was also inferior compared to (Ib). After only 1 week of storage at room temperature, the nanoemulsion of (Ic) showed a thin creamy layer on the side of the glass vials upon tilting to 45º. Regarding thermal stability, the emulsion of (Ic) became even cloudier after storage for 24h at 40 ºC (Figure 1C).

3.2.2. The effect of cosurfactant

An additional emulsifiable concentrate (Id) was formulated with the incorporation of 1.0 wt% propylene glycol (PG) as a cosurfactant (Table 1). Results of the effect of PG on the formation and stability of SEO nanoemulsion showed that PG increased the transparency of the nanoemulsion (Id) to become the clearest among those of set (I), (Figure 1A). The average particle size of that nanoemulsion was 28.2±0.1 nm which is considered to be the smallest among all nanoemulsions of set (I) as shown in (Table 2). This result demonstrates the potential of PG at only 1.0 wt% of the formula to act as an efficient cosurfactant that can replace ethanol for enhancing the formulation of SEO nanoemulsion with an ultra-fine particle size.

The helf-life stability study indicated that the nanoemulsion which contained PG was physically stable for 3(+) months as long as the temperature was ≤ 20 ºC. No noticeable change in transparency or in the average particle size after storage (25.9±0.1 nm) was detected as shown in Table 2, and Figure (1B, chart). It is worth noting that these results cannot be attained with PG concentration < 1.0%, as revealed in a preliminary experiment in the current study (data not shown).

Unfortunately, despite the distinctive effect of PG on the formulation and stability of SEO nanoemulsion of (Id), the formula showed temperature sensitivity when stored at 40 ºC for only 24h. That was manifested by a radical change in appearance from the most translucent nanoemulsion into milky white macroemulsion (Figure 1C). That indicates a dramatic increase in particle size and loss of nanostructure.

3.2.3. The effect of surfactant/oil ratio (S/O ratio)

A fifth emulsifiable concentrate (Ie) was made among the formulas of set (I) using the ingredients of formula (Id) after reducing its S/O ratio from 1.0 to 0.5 (Table 1).

The effect of surfactant reduction on the stability of the ethanol-free SEO nanoemulsion was studies. The results in Figure 1A show that the obtained nanoemulsion of (Ie) had an opaque appearance with a monomodal particle size distribution. The average particle size was 62.4±0.2 nm (Table 2), which is about double the size of the nanoemulsion of (Id) in which the S/O ratio was higher (equals 1.0). After a 3-month storage period at 20 °C the particle size of (Ie) slightly changed from 62.4 nm to 71.9±0.7 nm (Table 2), with monomodal size distribution as shown in Figure 1B, **(**chart).

There was also no detected separation or formation of creamy layer after the 3-month storage period. This result is considered to be satisfactory for a stable nanoemulsion because the particle size remained < 100 nm after storage, with no signs of creaming. The reader can also take into consideration that this result was obtained at 50.0% less surfactant with respect to the most ideal physically stable nanoemulsion of (Id).

Subjecting the nanoemulsion of (Ie) thermal stress by storage at 40 ºC led to the conversion of the nanoemulsion into a milky white macroemulsion after only 24h (Figure 1C).

3.3. The effect of HLB on the formation and thermal stability of SEO nanoemulsions

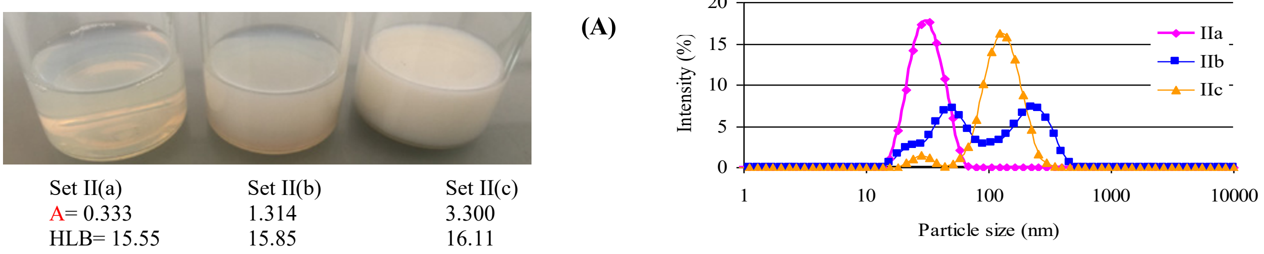
A mixture of binary surfactants composed of Tween 80 and Tween 20 at three different weight ratios was used to formulate a second set of SEO nanoemulsions (set II, Table 1). This mixture of surfactants offers different HLB (hydrophilic lipophylic balance) values, which can affect the formation and thermal stability of SEO nanoemulsion. The results illustrated in Figure 2A and Table 2 reveal that the nanoemulsion of set (IIa) had a translucent appearance and monomodal size distribution pattern accompanied by ultra-fine particle size (29.5±0.2 nm). On the other hand, nanoemulsions of (IIb and IIc) had much larger particle size (69.2±2.0 nm and 106.9±0.6 nm, respectively). This means that the HLB value for formula IIa (15.55, Table 2), which corresponds to the surfactant mixture composed of (T80-T20), at 2:1, is more suitable for the formation of SEO naoemulsion than the HLB values for the other surfactant ratios of II(b) and II(c), (Table 2).

The three nanoemulsions (IIa-c) were subjected to a mild thermal stress by storing at 40 °C. The results showed that this treatment led to destabilization, as evident from the change in the appearance of all nanoemulsions of set (II) to milky white after only 24h (Figure 2B).

Based on the previously obtained results, a second trial was performed to enhance the thermal stability of the SEO nanoemulsion, by formulating a third set of emulsifiable concentrate (set III, Table 1). This set was made using a different surfactant mixture made of Tween 80 and Cremophor RH40, which is another food-permitted surfactant.

The results from that approach indicated that the nanoemulsions of (IIIa) and (IIIb) had excellent transparent/

Zero time at 20 °C



After 24h storage at 40 °C

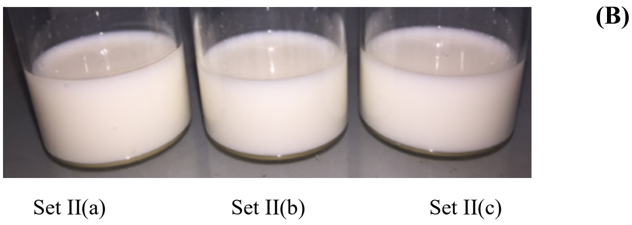
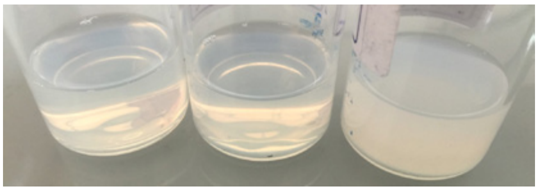


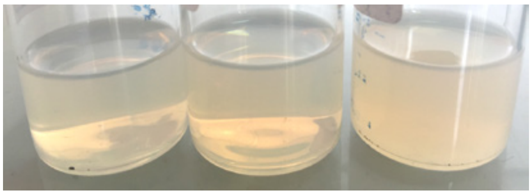
Figure 2. Appearance, UV-Vis absorption “A” at 600 nm, HLB values, (left column) and their corresponding particle size analysis (right column) of the different spearmint essential oil nanoemulsions fabricated from set (II) using family binary surfactant mix at different storage periods and temperatures.
HLB: hydrophilic lipophilic balance
The absorption values (A) are an average of two measurements from two different formulations, SDs are too small (≤0.005) to be indicated.
The particle size distributions are an average of two measurements from two different formulations, plotted from dynamic laser scattering results.

Zero time at 20 °C



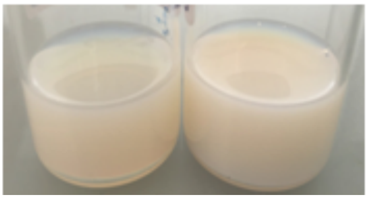
Set III(a) A= 0.182
Set III(b) 0.166
Set III(c) 1.134

Stored for 5 days at 40 °C



Set III(a) A= 0.217
Set III(b) 0.268
Set III(c) 1.256

Stored for 5 days at 50 °C



Set III(a) A= 1.131
Set III(b) 3.02

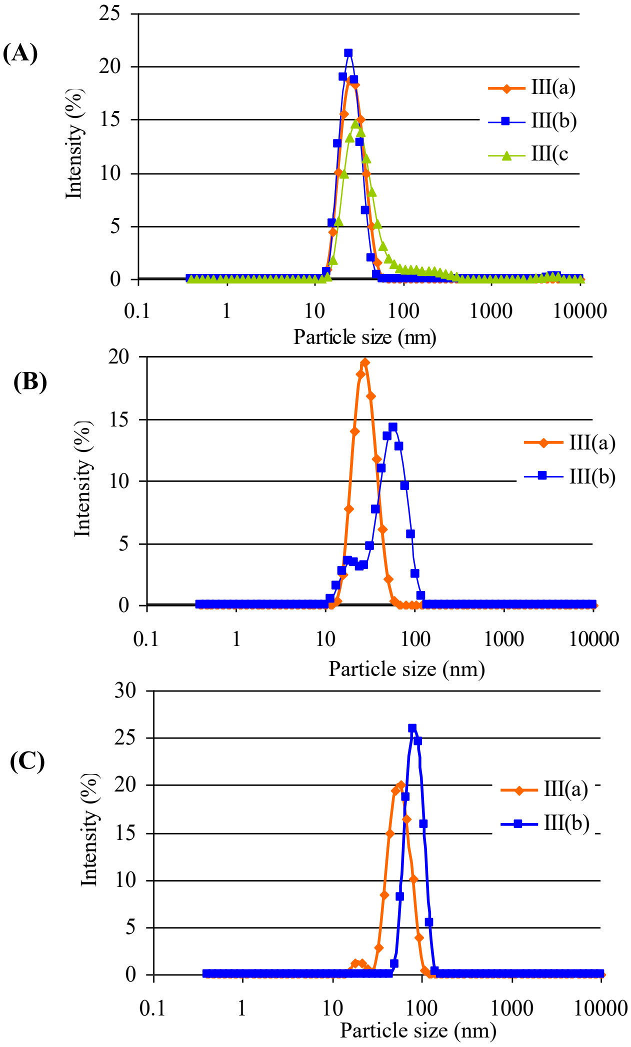


FIGURE 3. Appearance, UV-Vis absorption "A" at 600 nm, (left column) and their corresponding particle size analysis (right column) of the different spearmint essential oil nanoemulsions fabricated from set (III) using hetero binary surfactant mix at different storage periods and temperatures. The absorption values (A) are an average of two measurements from two different formulations, SDs are too small (≤0.005) to be indicated. The particle size distributions are an average of two measurements from two different formulations, plotted from dynamic laser scattering results.

translucent appearance (Figure 3A) with very small particle size (25.4±0.1 nm and 24.4±0.1 nm, respectively), as shown in Figure (3A, chart), and Table (2). On the other hand, the nanoemulsion of set (IIIc) was more opaque with higher absorbance in UV-Vis. Despite the small average particle size of this nanoemulsion (32.0±0.3 nm), its size distribution showed a second small population of larger particles with average particle size 114.0 nm (Figure 3A, chart), Which may have been responsible for the opaque appearance of (IIIc).

Subjecting all nanoemulsions of set (III) to a thermal stability evaluation by storing at 40 °C for 5 days indicated that only slight changes were observed in visual appearance and in the change in the UV-Vis absorbance of the three nanoemulsions, (Figure 3B). Interestingly, none of these samples turned milky white as was the case with nanoemulsions of sets (I and II), even after 5 days of storage at 40 ºC. The particle size of SEO nanoemulsion of set (IIIa) after the thermal treatment was 26.7±0.2 nm, which was almost the same as before the thermal treatment (25.4±0.0 nm, Table 2). On the other hand, the size of (IIIb) increased by about double after the thermal storage to become 44.7±1.4 nm. The nanoemulsion of set (IIIc) was excluded from particle sizing due to its cloudier appearance after the thermal treatment at 40 ºC compared to (IIIa,b), indicating less thermal tolerance (Figure 3B).

From these promising results it can be concluded that SEO nanoemulsions of set (IIIa) and (IIIb) are more thermally stable and temperature tolerant up to 40 °C compared to all nanoemulsions produced using sets I and II.

Therefore, the same nanoemulsions were further challenged with a second round of higher temperature by storing at 50 °C for another 5 days. The results from this treatment showed that the transparency of the nanoemulsions of set (IIIa) and (IIIb) decreased but did not become totally milky white (Figure 3C). The particle size after the second thermal treatment (at 50 °C) was 49.1±0.1 and 75.5±0.2 nm, respectively, (Table 2). In addition, their PDI was 0.149 and 0.114, respectively, indicating a uniform and narrow particle size distribution (Figure 3C**,** chart).

4. DISCUSSION

4.1. Chemical composition of SEO

A GC analysis of SEO was established for two main reasons: first, to confirm the authenticity of the supplied oil through the characterization of its major volatile constituents; and second, to justify the stability behavior of the developed ethanol-free SEO nanoemulsions based on examination of its main constituents.

As shown in the results section, the major constituents of SEO were *l*-carvone, *d*-limonene with some other lower contents of 1,8-cineol and pulegone. This result can authenticate the oil sample in the current study as *Mentha spicata,* which is precisely the targeted oil. It is important to note that there are other species of mint which may also be used in the food industry, such as *Mentha piperita* and *Mentha pulegium*. These species are characterized by different chemical compositions which are rich in menthol and pulegone, respectively.

Therefore, they can show different behavior in the formation of nanoemulsion compared to *Mentha spicata,* which was used in the current study.

4.2. Formulation and characterization of SEO nanoemulsion of Set (I)

Set (I, Table 1) is a group of five emulsifiable concentrates which were formulated to examine their potential to give ethanol-free SEO nanoemulsion spontaneously upon dilution into water. This set was formulated using Tween 80 as a sole surfactant in the absence or presence of other food-grade excipients. These excipients include long-chain triglycerides (LCT) as in formula (Ib) and médium-chain triglycerides (MCT) as in (Ic). Both triglycerides are known as Ostwald ripening inhibitors (ORI) which are commonly used to stabilize EO nanoemulsions. Beside the triglycerides, a third excipient, namely propylene glycol (PG) was also incorporated along with the ORI to replace ethanol as another food-permitted cosurfactant (formula Id). The surfactant to oil ratio (S/O ratio) in the previous four formulas (Ia - Id) was kept at 1.0, which means that the mass of surfactant equals that of SEO, (Table 1). This relatively high ratio (i.e. high amount of surfactant) was used because it is usually applied in the low energy emulsification method to compensate for the absence of high shear energy equipment (Anton, and Vandamme, 2009). Beside formulas (Ia-Id), a fifth formula (Ie) was fabricated using the same excipients as in (Id) after reducing the amount of surfactant by 50.0% so that the S/O ratio became only 0.5 (Table 1).

The choice of the constituents for the previously mentioned five emulsifiable concentrates (Ia to Ie) of set (I) was meant to be a pattern for evaluating the effect of surfactant type, excipients, and the S/O ratio on the formulation and physical stability of the ethanol-free nanoemulsions at room temperature and also under thermal challenging conditions. Therefore, parameters like appearance, UV-Vis absorption at 600 nm, particle size measurement, and the change of these parameters after storage at higher temperatures were assessed and taken as indicators in this evaluation. The effect of each one of these parameters on nanoemulsion formation and stability is discussed in details in the following sections.

4.2.1. The effect of triglyceride

Formula (Ia), which contained no traces of triglycerides (Table 1), failed to produce SEO nanoemulsion and showed poor physical stability in less than 24h. One should bear in mind that the S/O ratio of emulsion (Ia) equals 1.0. That means the presence of a high amount of surfactant, enough to support the formation of a stable emulsion even without using high energy shear equipment in the emulsification process. Therefore, there should be a factor which caused such rapid creaming in this formula which is likely to be the phenomena of Ostwald ripening (OR), (McClements *et al.*, 2012). OR occurs in nanoemulsions (or emulsions) when the oil phase is slightly soluble in water, such as the case with essential oils. OR takes place as a result of the growth of large oil droplets at the expense of tiny ones. That happens due to the continuous migration (through water) of oil from the tiny droplet to the larger droplets under the effect of the high curvature pressure "Laplace pressure" which is exerted on the tiny droplets. Therefore, large droplets become larger and finally float to the surface causing a creamy layer in a short period of time, just a few hours after formulation (McClements *et al*., 2012).

EO emulsions and nanoemulsions are a typical example of the manifestation of OR due to the slight water solubility of their ingredients (McClements *et al*., 2012). This was clearly proven in the current study in the case of formula (Ia) due to the slight water solubility of the major volatile constituents of SEO. For instance, the water solubility of *l*-carvone, *d*-limonene and 1,8-cineol, which are the three major constituents of SEO, was 27.0 $mg \cdot l^{-1}$, 13.8 $mg \cdot l^{-1}$ and 552.0 $mg \cdot l^{-1}$, respectively, as indicated by the official database (PPDB & BPDB, web sites). These three constituents together represented about 81.2% of the total composition of SEO, as was revealed in the current study by GC analysis. Therefore, the author concludes that OR contributes significantly to the observed early creaming of formula (Ia).

In a trial to inhibit OR and to develop more stable SEO nanoemulsion, we incorporated equal amounts (1.0wt %) of two different triglyceride oils into formula (Ib) and (Ic). The first was sunflower oil, which is considered to have long-chain triglycerides (LCT, set Ib), while the second was synthetic medium-chain triglycerides (MCT) made of C_8-C_{10} (set Ic), as shown in Table 1.

The entropy of mixing of SEO with these triglyceride oils provided chemical potential against OR and counterbalanced the driving force for EO migration from tiny particles to large ones (McClements *et al*., 2012).

This treatment showed a noticeable improvement in the appearance, particle size and storage stability of the SEO nanoemulsion, as shown in the results section (Table 2) and (Figure 1A, B). However, it was clear from the results that LCT was a much better excipient than MCT in the formulation of stable SEO nanoemulsion with respect to two aspects: first, better inhibition of OR; and second, greater influence on the reduction of SEO particle size. Detailed justifications of these two findings are discussed in the next passage.

First, the ability of LCT to inhibit OR to a better degree compared to MCT is due to its strong hydrophobic nature (HLB 4.0) compared to that of MCT (HLB 8.0), (Wooster *et al*., 2008). The strong hydrophobicity of LCT originates from its long chain fatty acids which include linoleic (C18:2), oleic (C18:1) and stearic (C18:0) acids. These fatty acids constitute the composition of the sunflower oil which was used as LCT. Therefore, the spiking of SEO with such a small amount (1.0 wt%) of LCT, (as in nanoemulsion of set Ib) can reduce the water solubility of its volatile ingredients to a great extent compared to MCT, leading to better inhibition of OR. That conclusion came in accordance with another study (McClements *et al*., 2012) which further concluded that the hydrocarbon chain length of the triglyceride can affect the interfacial tension between EO droplets and water, and consequently, affect the overall nanoemulsion stability. It is worth indicating that MCT is composed of medium-chain caprylic (C_8) and capric (C_{10}) acids which are less hydrophobic than the other long-chain fatty acids.

On the other hand, our second finding concerning the greater influence of LCT on the reduction of the particle size of SEO nanoemulsion of set (Ib) compared to (Ic) can be justified based on the physical affinity between the oil phase mixture (SEO/triglyceride) and the utilized surfactant. The high affinity between these ingredients leads to a high tendency toward the production of nanoemulsion with ultra-fine particle size (Anton and Vandamme, 2009). Our results indicated that the affinity between (SEO/LCT) and Tween 80, which is used as a surfactant, is higher than that of (SEO/MCT) and the same surfactant. This conclusion is based on the ability of (SEO/LCT) to produce nanoemulsions (Ib) with smaller particle size (38.0 nm) compared to (Ic, 96.0 nm) which was made of (SEO/MCT), as shown in Table 2.

4.2.2. The effect of cosurfactant

Ethanol is usually used as cosurfactant in a relatively high amount to facilitate the formulation of EO nanoemulsions. It can be left in the nanomemulsion after fabrication (Zhang *et al*., 2017), or removed by evaporation in a process known as solvent displacement (Katata *et al*., 2017). However, the presence of ethanol in food and beverages is met with objection from some consumers who seek solvent-free products.

Therefore, out of the desire to eliminate ethanol from food or beverages, we replaced ethanol with only 1.0 wt% PG as cosurfactant. PG is a food-permitted ingredient which has a wide safety margin that can reach up to 25mg/Kg body wt./day (Joint FAO/WHO, 2002). Therefore, another emulsifiable concentrate (set Id, Table 1) was developed based on the ingredients of the promising formula (Ib) with the incorporation of 1.0 wt% PG as cosurfactant (Table 1). As a result, a new SEO nanoemulsion (Id) with better transparent appearance (Figure 1A), smallest particle size (28.2 nm, Table 2) and high stability was obtained.

The mechanism of the action of PG as cosurfactant which is able to facilitate the formation of nanoparticles and to enhance its physical stability is similar to ethanol and other short chain alcohols (Garti *et al*., 2001). PG can arrange itself along with the main surfactant at the interfacial layer between SEO and water, leading to the formation of a mixed surfactant film. This behavior lends flexibility to the film around SEO droplets, leading to an ease in attaining the maximum curvature required for ultra-fine oil particles to be developed. In addition, PG can modify the hydrophobicity of the main surfactant, making it more compatible with the SEO phase.

It is important to note that the role of PG in the formation of ethanol-free nanoemulsion cannot be generalized to include all other types of essential oils. This statement was concluded by the author himself who found that PG does not make any improvement in the formulation of clove or cinnamon EO nanoemulsions (data not published). The particle size of these nanoemulsions increased in the presence of PG rather than decreased as expected. At this point, the real reasons behind that peculiar behavior are not known. However, it can be speculated that the chemical composition of the EO as well as its interaction with the chosen surfactant must have an effect. Further investigations are required in this respect to unravel the scientific basis behind the effect of PG on the formation of various types of EO nanoemulsions.

4.2.3. The effect of surfactant/oil ratio (S/O ratio)

The S/O ratio is considered to be a key factor in the fabrication of EO nanoemulsions using the low energy spontaneous emulsification method. This ratio represents the product of dividing the mass of surfactant by that of the oil phase. The common ratio which is usually used in the art to induce the formation of nanoemulsion is 1.0 (i.e. equal masses of surfactant and oil). However, this ratio can be increased in the case of other types of formulations to become 2.0 (Komaiko and McClements, 2015). This means doubling the amount of surfactant relative to EO.

In the current study the S/O ratio equal to 1.0 was used in the four emulsifiable concentrates of set (I) from (Ia-Id). However, a reduction in the amount of surfactant is generally appreciated in order to fulfill the requirements of legislation in food and beverages and also to proceed economically. However, that trend is considered to be a challenge for formulators due to the crucial role of the amount of surfactant in the stability of nanoemulsions formulated according to the low energy method.

This issue was addressed in the current study by the fabrication of a fifth emulsifiable concentrate (Ie) which was made using ingredients from formula (Id) after the reduction in its S/O ratio from 1.0 to 0.5 (Table 1). That means a 50.0% reduction in the amount of surfactant relative to the four other emulsifiable concentrates of set (I). The results showed that this nanoemulsion was physically stable and had particle size < 100 nm even after a 3-month storage period at 20 °C. It is important to note that this result is unattainable in the absence of PG as cosurfactant. This affirms the importance of this compound as a cosurfactant in the formation of physically stable and ethanol-free SEO nanoemulsion, especially at a reduced S/O ratio. On the other hand, at 40 °C, the appearance of this nanoemulsion changed to milky white within 24h, indicating a loss in the nanostructure (Figure 1C). This elicits the temperature sensitivity of (Ie) nanoemulsion as is the case with all nanoemulsions of set (I).

4.3. The effect of HLB on the formation and thermal stability of SEO nanoemulsions

From the above-mentioned results, it can generally be concluded that the common feature among the SEO nanoemulsions of set (I) is their temperature sensitivity under moderate thermal stress (40 °C, Figure 1C). This thermal intolerance is a common phenomenon among nanoemulsions formulated using nonionic surfactants due to their phase inversion which is induced by temperature. The temperature at which the phase inversion of nanoemulsion takes place is called the phase inversion temperature.

At this temperature, the hydrophilic head groups of the nonionic surfactants become dehydrated due to the breaking down of their hydrogen bonds with water. This in return decreases the HLB (hydrophilic lipophilic balance) of the surfactant, making it more hydrophobic. This causes the nanoemulsion to become highly unstable and turn into a cloudy macroemulsion (Kunieda and Shinoda, 1982).

Previous investigations dealt with this issue by incorporating a second surfactant besides the main one in order to modify the HLB to make it appropriate for improving the thermal stability of the nanoemulsion (Guttoff *et al*., 2015). For instance, the previous reference used sodium dodecyl sulfate to act as a second surfactant to improve the thermal stability of vitamin D nanoemulsion. However, that kind of anionic surfactant is not permitted in food and beverage applications.

Therefore, to render SEO nanoemulsions edible, the author of the current study substituted the anionic surfactant with two nonionic food-permitted surfactants which were mixed, separately, with the principle surfactant (Tween 80), to form a food-permitted binary surfactant mixture. Accordingly, two more new sets of SEO emulsifiable concentrates namely, set (II) and set (III), were fabricated as shown in Table (1).

SEO nanoemulsions of set (II) were formulated using a mixture of surfactants composed of Tween 80 and Tween 20 at 3 different weight ratios (Table 1). This surfactant mixture is called family binary because it belongs to the same family of polysorbate surfactants but differs only in the length of the fatty acid moiety. Results showed that the surfactants of set (IIa) with an HLB value of 15.55 gave the nanoemulsion with the smallest particles size (29.5±0.2 nm) compare to IIb and IIc, indicating the suitability of that HLB value for SEO.

Regarding storage stability, all nanoemulsions of set (II) were not subjected to the 3-month storage period at 20 °C as was the case with set (I). This is because set (II) nanoemulsions were designed mainly for investigating the effect of surfactant mixtures and their HLB on the thermal stability of SEO nanoemulsions.

In this regard, none of the 3 nanoemulsions of set (II) was thermally stable at 40 °C, as shown in Figure 2B. This indicates the inability of all ratios of the surfactant mixtures of set (II) to provide the appropriate HLB value necessary for extending the range of phase inversion temperature of the nanoemulsion to provide the required thermal stability (Shinoda and Saito, 1969).

As a result, a second trial was performed to enhance the thermal stability of SEO nanoemulsions by formulating a third set of SEO emulsifiable concentrate (set III) using different a surfactant mixture made of Tween 80 and Cremophor RH40 (Table 1). This mixture is called the hetero binary system because it belongs to different families of nonionic surfactants. The purpose was to achieve an appropriate HLB which could improve the thermal stability of SEO nanoemulsions.

It is important to indicate that the different HLB values which result from mixing the surfactants of set (III) cannot be calculated precisely. That is because the HLB value of Cremophor RH40 is not univocal, which means that the surfactant has a wide range of HLB values ranging from 14-16, as indicated by the manufacturer. This makes it difficult to calculate an exact HLB value for the binary mixture of (Tween 80-Cremophor) at the three different ratios (refer to Table 2).

The results from the fabrication of set (III) showed that nanoemulsions (IIIa) and (IIIb) showed the typical characteristics of nanoemulsion regarding their transparent appearance and ultra-fine particle size (Figure 3A and Table 2). Moreover, these nanoemulsions were more thermally stable and temperature tolerant up to 50 °C compared to all nanoemulsions produced using set (I) & (II), as showند in the results section. The author justified that thermal stability is based on hitting the right mixture of surfactants (Tween80-Cremophor) in the right ratio (1:1, formula IIIa and 2:1, formula IIIb, Table 1). As a result, an appropriate HLB value was reached which was able to extend the thermal stability of the nanoemulsion (Shinoda and Saito, 1969).

5. CONCLUSIONS

This article investigated in detail the different aspects that can lead to the development of a stable ethanol-free SEO nanoemulsion using the low energy technique. The results reported in this article could have a promising application in developing water-based SEO nanoemulsions for application as flavoring and aromatizing agent in food and beverages. It also draws the attention of relevant researchers to the deferential effect of PG as a cosurfactant in the formation of water-based essential oil nanoemulsions.

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Enzymatic pre-treatment in cold pressing: Influence on flaxseed, apricot kernel and grape seed oils

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SUMMARY: A commercial enzyme preparation consisting of pectolytic, cellulotic and hemicellulotic enzymes was applied to the oil extraction by cold pressing from apricot kernel, flaxseed and grape seed. The effects of enzyme pre-treatment varied depending on the different oil seed used as raw material. Although the increase in free fatty acidity can be considered as a negative effect (from 0.37 to 0.52), the decrease in peroxide number and *p*-anisidine values, increase in oil yield (22.75%), higher levels of total carotenoids and tocopherols, as well as a remarkable increase in phenolic content (x1.68) and radical scavenging effect (including hydrophilic and lipophilic-induced and total antioxidant capacity) showed that the use of enzyme application in the cold pressing of apricot kernel oil would be beneficial. Many of these positive results could not be achieved in the pressing of flaxseed or grape seed oils under the same conditions. A high negative correlation (r=-92.2) was found between *p*-anisidine value and δ-tocopherol for grapeseed oil. *Hydrophilic* and *lipophilic* antioxidant capacity, total phenolics, and total carotenoids negatively correlated well (r values above 80) with peroxide values for apricot seed oil. Correlation results showed that carotenoids play an important role in the oxidative stability of the oils, where it was much more evident for apricot seed oil (r=-97.5).

KEYWORDS: *Apricot kernel; Bioactive compounds; Cold pressing; Enzymes; Flaxseed; Grapeseed*

RESUMEN: ***Pretratamiento enzimático en el prensado en frío: Influencia en los aceites de linaza, hueso de albaricoque y semilla de uva.*** Se aplicó un preparado enzimático comercial que consta de enzimas pectolíticas, celulóticas y hemicelulóticas en la extracción de aceite de prensado en frío de albaricoque, linaza y semilla de uva. Los efectos del pretratamiento enzimático variaron dependiendo de las diferentes semillas oleaginosas como materia prima. Aunque el aumento de la acidez libre puede considerarse como un efecto negativo (de 0,37 a 0,52), la disminución del valor de peróxidos y de *p*-anisidina, el aumento del rendimiento de aceite (22,75%), niveles más altos de carotenoides totales y tocoferoles, además de un aumento notable en el contenido de fenoles (x1.68) y el efecto captador de radicales (incluida la capacidad antioxidante total e inducida por hidrófilos y lipófilos) mostraron que la aplicación de enzimas en el prensado en frío del aceite de hueso de albaricoque resulta beneficioso. Muchos de estos resultados positivos no se pudieron lograr al prensar los aceites de semillas de linaza y uva, en las mismas condiciones. Se encontró una alta correlación negativa (r = -92.2) entre el valor de *p*-anisidina y el δ-tocoferol para el aceite de semilla de uva. La capacidad antioxidante hidrófila y lipófila, los fenoles totales y carotenoides totales se correlacionaron negativamente bien (valores de r por encima de 80) con los valores de peróxido para el aceite de semilla de albaricoque. Los resultados de correlación mostraron que los carotenoides juegan un papel importante en la estabilidad oxidativa de los aceites, mucho más evidente para el aceite de hueso de albaricoque (r = -97,5).

PALABRAS CLAVE: *Compuestos bioactivos; Enzimas; Hueso de albaricoque; Prensado en frío; Semilla de lino; Semilla de uva*

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1. INTRODUCTION

The cold pressing of oils can be carried out in a screw press or hydraulic press and any organic solvent or external heat is not implied. Today, oil production is mostly done by solvent extraction; however, for some oils such as virgin olive oil, sesame oil or rapeseed oil, pressing is preferred due to their specific properties.

Mechanical pressing has the advantage of safety and simplicity throughout the process, it is less harmful, requires a short time period, and only a small amount of raw materials is necessary for extraction from different oilseeds (Oyinlola *et al*., 2004). In addition, the press cake obtained is rich in protein (Singh and Bargale, 2000). The pressed materials have better natural properties; end products are free of chemicals such as free fatty acids, trans fatty acids and oxidative products (Khan and Hanna, 1983). The higher degree of naturalness of the product obtained and the fact that it is rich in bioactive components explains why these products are increasingly found in markets which sell less processed foods.

Pressing is inefficient compared to solvent extraction. In some cases over 10% of the oil can remain in the press cake (Singh and Bargale, 2000). Enzymes might be used in a pre-treatment stage in order to overcome the low yield problem. The mechanism action of enzymes is to facilitate the release of fat from cells by breaking down cell wall components such as cellulose and pectin.

Enzymatic pre-treatments lead to an increase in oil yield because they tend to either soften and/or destroy cellular structures, thus aiding in extraction. In order to achieve higher oil recovery, enzyme preparations (pectolytic, cellulolytic and hemi cellulolytic) used in pre-treatments have a broad spectrum of activity to disrupt the cell wall structure. Enzymatic hydrolysis, allows for easier oil removal. There is also considered to be a synergistic effect between several enzyme types (Amos and Mohnen, 2019; Neeharika *et al*., 2020).

Apricot kernels are produced as by-products from the food canning industry (Sharma *et al*., 2011). Apricot kernels are rich in essential amino acids, oil, high concentrations of minerals and B group vitamins; its oil is high in unsaturated fatty acids, with oleic acid being dominant (Gupta *et al*., 2012).

Grape seeds (*Vitis vinifera* L.) are the main by-products of winery and molasses. Its oil contains high levels of unsaturated fatty acids, linoleic (58–78%) and oleic (10–20%) (Crews *et al*., 2006) and antioxidant-rich compounds (Kadri *et al*., 2019). Grape seeds constitute about 20% of the fruit weight, and this percentage corresponds to about 40–60% on a dry matter basis. This situation reveals the importance of the complete utilization of grape seeds (Wang *et al*., 2009; Brasky *et al*., 2011).

Flax seeds (*Linum usitatissimum* L.) contain a relatively high amount of polyunsaturated fatty acids (PUFA), lignans, secoisolariciresinol diglucoside (Hasiewicz-Derkacz *et al*., 2015), phenolic acids and flavonoids. Flax oil is valuable due to its high quantity of essential PUFA (73%): α-linolenic acid and linoleic acid (Singh *et al*., 2011; Mridula *et al*., 2011; Mridula *et al*., 2013).

In studies previously conducted on various oilseeds, it has been reported that enzyme application provides an increase in oil yield by up to 12% (Kashyap *et al*., 1997; Shankar *et al*., 1997; Sarkar *et al*., 2004). Enzyme assisted pressing, because of its nontoxic and inflammable features, stands out as an ideal alternative for oilseed extraction (Singh and Singh, 2013). With this in mind, in this study we aimed to investigate the impact of enzymatic pre-treatment prior to oil extraction by mechanical pressing on the recovery of three specialty oils; grape seed oil, apricot seed oil and flaxseed oil. In this study, the effects of enzyme pre-treatment on yield, some quality parameters and some physical and chemical properties of various oilseeds were revealed. In this context, the degree of exposure of minor bioactive components was also presented. Here, the antioxidant activity was examined in detail, and it was deepened by analyzing both lipophilic and hydrophilic fractions. Flaxseed, apricot seeds and grape seeds were selected, as these oilseeds enjoy widespread use due to preferred fatty acid composition (flaxseed and apricot seed) and high phenolic contents (grapeseed and flaxseed).

2. MATERIALS AND METHODS

2.1. Oil samples and chemicals

Oil seeds (flaxseed, grape seed, apricot kernel) were obtained from a local supplier in Konya, Turky. Reagents for measurements were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Water was of ultrapure milli-Q grade. The commercial enzyme was a complex enzyme preparation of cellulases, pectinases and hemicellulolytic enzymes produced by *Aspergillus aculeatus* (SEBMax Olive, Advanced Enzyme Technologies Ltd., India). All the remaining chemicals used for experiments were at least analytical grade. The hydrolyzed or conventionally treated samples were directly subjected to pressing without any roasting or moisture adjustment. The control sample was considered as the sample produced by the conventional method. Neither buffer solution nor enzyme was applied.

Oil was obtained by pressing 1000 g of seeds with a screw press (Karaerler NF 500, Turky) (nozzle diameter of 5 mm, shaft screw diameter of 33 mm, rotation speed of 50 rpm) at oil flowing temperatures below 50 °C (cold pressing).

The oil seeds were ground in a laboratory scale grinder (220 V, 1-30 kg seeds/hour capacity, 1.5 kW, 9000 rpm) to a particule size of 1-2 mm ground. Enzymes were applied by using a buffer solution (0.1 M of aqueous NaH_2PO_4, pH adjusted to 6 with 0.5 M NaOH or phosphoric acid) containing 10 g commercial enzyme mixture. 100 mL of this solution was sprayed onto 1 kg of ground seeds. Then the seeds were incubated at 60 ^{o}C for 3 hours. Enzyme solution was applied at a final enzyme concentration of 1 g enzyme per 100 g seeds. Pressing time was approximately 5 min. The oil extracted was stored in amber glass bottles. The pressing procedure was carried out in three replicates and the average values were reported. The percentage oil yield was obtained by calculating the proportional amount of oil obtained from 100 g of seed (Ezeh *et al.*, 2016).

2.2. Oil analysis

The determination of free fatty acids (FFA), peroxide value, and *p*-Anisidine values (*p*AV) of the extracted oil was carried out according to the standard AOCS official methods Cd 3d-63 and Cd 8b-90, respectively. Instrumental color was measured by a colorimeter [Minolta Chroma meter CR 400 (Osaka, Japan)]. The chromaticity coordinates a^* and b^* measures red-green and yellow-blue, respectively, depending on the negativity and positivity of the values (Criado *et al.*, 2004).

2.3. Fatty acid composition

The fatty acid composition was determined following the ISO standard (ISO, 1990; ISO, 2000). One miligram of oil was dissolved in 1 mL of *n*-heptane and 50 µg of sodium methylate, and the tube was shaken vigorously for 1 min at room temperature. 100 µL of water were added and the tube was centrifuged at 4500 g for 10 min and the lower aqueous phase was removed. Then the solution was mixed with 50 µL of HCl (1 mol with methyl orange) and the lower aqueous phase was rejected. About 20 mg of sodium hydrogen sulphate (monohydrate, extra pure) were added, and after centrifugation at 4500 g for 10 min, the top *n*-heptane phase was used. A Shimadzu GC-2010 Plus/FID/HS-20, with a capillary column, SP 2-4111 was also used (100 m long, 0.25 mm ID, film thickness 0.2 µm). The temperature program was as follows: from 140 °C (for 5 min.); heated to 240 °C (4° C/min) and held for 20 min; injector 250 °C, detector 260 °C; carrier gas 36 cm/s hydrogen; split ratio 1:50; detector gas 30 mL/min hydrogen; 300 mL/min air and 30 mL/min nitrogen; manual injection volume was 1 µL. The peak areas were computed by the integration software, and the results were calculated as weight percent by direct internal normalization.

2.4. Determination of tocopherols

The contents of tocopherol (α-, β-, γ- and δ-forms) in the oil were determined by Shimadzu LC-20A HPLC system equipped with a fluorescence detector and Lichrosorb Si 60 column (250 × 4.0 mm, 5 µm) (Supelco, USA). The emission and excitation wavelengths were set at 330 and 290 nm, respectively. The oil samples were dissolved in n-hexane at 50 mg/mL. A 20 µL volume of the loaded sample on the column was eluted with *n*-hexane/2-propanol (99.5/0.5, v/v) isocratically at 0.8 mL/min. The tocopherol contents were determined by comparing to calibrated standard curves. For the identification of tocopherols, the peak retention times were compared against absolute/ pure tocopherol compounds (α-, β-, γ- and δ –tocopherols) (IUPAC, 1987).

2.5. Carotenoids and chlorophylls in olive oils

7.5 g oil were weighed and dissolved in cyclohexane up to a final volume of 25 mL. Carotenoid contents were calculated from the absorption spectra of the oils by using the specific extinction values. Absorption at 470 nm and 670 nm were recorded for carotenoid (mg lutein / kg oil) and chlorophyll (mg pheophytin *a* / kg oil) fractions, respectively (Minguez-Mosquera *et al*., 1991).

2.6. Total antioxidant capacity measurement and determination of total phenolics

The antioxidant activity of the oil (total fraction) and the hydrophilic and lipophilic fractions were assessed by the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Tuberoso *et al*., 2007; Teixeira *et al*., 2013). 1 g of oil was dissolved in 2 mL of 80% methanol, the solution was agitated for 30 min at room temperature and centrifuged at 700 g for 10 min to separate the methanol phase as hydrophilic fraction, while ethyl acetate was mixed with the remaining solution as lipophilic fraction.

The oil was diluted in ethyl acetate alone, without fractionation as total fraction. Antiradical activity was expressed as percent inhibition of DPPH. For the antiradical activity measurement, a methanolic solution (100 µL) of the extracts from different fractions of the oil was placed in a cuvette and 0.5 mL of a methanolic solution of DPPH (50 mg DPPH/100 mL MeOH) were added. After 20 min incubation in the dark and at ambient temperature (27 °C), the resultant absorbance was recorded at 517 nm (Shimadzu Co., Ltd., Kyoto, Japan). Antiradical activity was expressed as percent inhibition of DPPH (Roginsky and Lissi, 2005).

The methanolic extracts prepared for the antioxidant analysis were used for the determination of total phenolics. The method described by Rigane *et al*., (2011) was performed. The results were expressed as mg of gallic acid per kg of oil.

2.7. Statistical analysis

All parameters analyzed were determined in triplicate. Statistical analysis was performed with the SPSS v. 16 statistical software (SPSS Inc., Chicago, IL, USA). "General linear model multivariate analysis" was used to evaluate oil seed and pretreatment depended differences regarding the parameters analyzed. Significant differences between mean values were evaluated by using the Duncan's new multiple range test (P<0.05).

3. RESULTS AND DISCUSSION

3.1. Influence of enzymatic pre-treatment on oil yield

The yield values forof flaxseed, apricot kernel and grape seed oils are presented in Table 1. Enzyme application showed the most significant increase in oil yield (22.75% increase) in the apricot seed sample. The pre-treatment of mixed enzyme formulation on apricot kernel resulted in 47.33% oil recovery which was 14.22% more than that obtained without enzymes as reported in the study of Bisht *et al.* (2015). Although the enzyme application provided a small increase in oil recovery from grape seed, it was not statistically significant. In a previous study, an enzymatic treatment for 9 hours at 50% moisture along with pre-heating, grape seed oil extraction yield was raised by 59.4% in comparison to the yield obtained from the control without enzymes (Tobar *et al*., 2005). The lower increase in oil yield achieved in the present study compared to that of Tobar *et al.* (2005) can be attributed to the longer incubation time (9 hours) and moisture level applied in their study. We tried to keep these conditional parameters as low as we could in order to suppress the formation of free acidity and oxidation. Soto, Chamy and Zúñiga (2004) also reported that with the use of an enzyme mixture, borage oil yield was 84% in comparison to 77.7% from control samples.

Contrary to expectations, this application significantly reduced oil yield from flaxseed. It is thought that the applied enzyme mixture is not suitable for the flaxseed cell membrane structure and also created a negative result by affecting the moisture ratio. Because the enzymes were applied in a buffer solution which increased the moisture ratio of ground seeds to a level that could not be ignored even 60 °C, 3 hours incubation led to a loss in the moisture level to some extent. This is a well-known effect of moisture which gives rise to the differences between the components of hydrophobic (van der Waals forces) and hydrophilic (hydrogen and ionic bonding) matrixes (Mustafa and Turner, 2011). In flaxseed, enzyme pre-treatment probably reduced the difference between these two fractions and, on the contrary, showed an effect in favor of binding (emulsion). Enzymes affect the release of oil depending on the seed composition. The oil found inside the plant cells (oil bodies, 0.6–2 µm) is a triglyceride matrix which

Table 1. Analysis results of cold pressed seed oils obtained by conventional and enzyme pre-treatment methods.

	Flaxseed oil		**Apricot seed oil**		**Grape seed oil**	
Analysis	**Conventional**	**Enzyme**	**Conventional**	**Enzyme**	**Conventional**	**Enzyme**
Oil yield (%)	37.82±1.09[*]a	30.85±1.94b[†]	31.16±4.13b	38.25±1.76a	10.43±0.92	11.05±0.65
Free fatty acids (%)	1.91±0.09b	2.04±0.47a	0.37±0.03b	0.52±0.02a	0.94±0.05b	0.99±0.07a
Peroxide value (meq O_2/kg_{oil})	2.05±0.15a	1.73±0.17b	1.89±0.17a	1.34±0.40b	18.93±1.56	20.59±1.18
p-Anisidine value	1.40±0.59	1.31±0.15	2.28±0.75a	1.97±0.23b	5.30±1.14	4.46±1.08
Total carotenoids (mg/kg)	0.297±0.03	0.295±	0.376±0.026b	0.731±0.08a	1.237±0.168a	0.719±0.037b
Total phenolics (mg/kg)	62.32±12.94	56.27±7.15	18.44±2.67b	31.08±4.41a	154.13±23.29	127.92±15.03
Antioxidant capacity %						
hydrophilic	29.72±1.14a	16.34±3.65b	17.81±3.90b	33.55±4.24a	40.46±4.51	33.68±1.48
lipophilic	40.37±1.26a	35.61±5.22b	20.59±4.02b	42.93±3.44a	42.12±6.84	31.30±2.48
total	31.85±2.73	28.89±3.17	34.10±4.18	38.31±5.21	42.13±2.17	38.54±3.05
Tocopherols (mg/L)						
α-tocopherol	112.43±24.8	111.60±12.5	119.09±17.6	119.65±9.53	181.49±18.72a	156.59±17.5b
β-tocopherol	186.22±21.17a	154.29±25.9b	0.157±0.05a	0.147±0.04b	95.09±12.4b	117.89±14.6a
γ-tocopherol	416.62±26.8	444.09±31.17	638.84±30.86	619.04±22.46	418.11±46.65	413.00±29.34
δ-tocopherol	146.21±13.06	147.12±10.66	154.12±12.38	150.97±26.67	0.140±0.03	0.147±0.05
Oil color indices						
L*	31.13±3.7	31.88±4.0	31.15±2.9	30.88±1.67	27.44±4.5	27.57±1.53
a*	-1.95±0.7	-1.97±0.31	-1.66±0.8	-1.52±0.6	1.07±0.4a	0.76±0.21b
b*	7.15±0.48	6.82±0.62	3.98±0.25	3.63±0.21	-1.94±0.09b	-0.87±0.06a

[*] mean± standard deviation; results of three replicates (n=3).

[†] small case letters show significant differences between between values and belong to oils of conventional and enzyme-treated samples of the same oilseed (P ≤0.05).

Statistical results obtained from General Linear Model-Multivariate, and Duncan methods.

is compromised of a monolayer of phospholipids linked together with proteins. Proteins give stability to this matrix due to the steric hindrance and electronegative repulsion of hydrophobic proteins on the surface of the oil bodies. Due to the structural properties of protein in the flaxseed, it is thought to be more stable with the addition of enzymes, unlike apricot kernel and grape seed.

On the other hand, in order to achieve a higher amount of oil extraction, enzyme preparations should have a broad spectrum of activity to disrupt the cell wall structure. The commercial enzyme preparate used in this study did not contain protease, which seemed inevitable for flaxseed pressing. Long *et al.* (2011) reported that when a mixture of cellulose, pectinase, and hemicellulose enzymes were applied, the flaxseed oil yield was 73.9%, higher than the oil yield from the application of each enzyme individually. These higher oil yield values reported by Long *et al.* (2011) are of course due to the application of a method completely different from our study (ultrasound-assisted aqueous enzymatic method) to extract the oil. In addition, it is known that the dissolution of proteins, at a pH far from the isoelectric point, disrupts the matrix and enables oil release (Oyinlola *et al.*, 2004). This triggers the idea that a commercial mixture of enzymes along with buffer solution carrying it might have formed a pH close to the isoelectric point in ground flaxseed. A physical effect can also be considered in this case, because products from the degraded materials can negatively affect enzymes to act on their substrates (Ezeh *et al.*, 2016). Particle size is another factor thought to be effective on the release of oil. The physical effect of enzymatic treatment is also involved, which was demonstrated by smaller particle size with a degraded surface of cotyledon cells of enzymatically treated soybeans (Sineiro *et al.*, 1998). Hence, the disruption of walls and cytoplasm leads to a slow and gradual lose in the cellular and sub-cellular structure of cell tissues.

The enzyme pre-treatment duration of hemp seeds was up to 6 hours in study by Latif and Anwar (2009). Nosenko *et al.* (2019) demonstrated that using a different kind of proteolytic enzyme for pumpkin seed pre-treatment resulted in an increase in destroyed cell quantity from 3 to 10.4%. In particular, the acid proteases were the most effective enzymes in increasing destroyed cell content in oily material. The increase in oil extractability by means of enzyme pre-treatment was attributed to the breakdown of the protein network surrounding the lipid bodies and also supports the degradation of the complex lipoprotein molecules into simpler lipid and protein molecules, thereafter enhancing the release of oil (Murphy, 1993; Tzen and Huang, 1992).

Regarding the low oil content in grape seeds (determined between 10-11% in the present study), in previous studies, oil yields between 7-20% were reported and the authors pointed out that high amounts of oil remained in the cake from cold pressing (Matthäus, 2008; Özcan *et al.*, 2017; Özcan and Aljuhaimi, 2017).

3.2. Pre-treatment effects on some quality indices and fatty acid composition of seed oils

The enzyme application was shown to increase free fatty acidity (%) values (Table 1). Although it showed the lowest free fatty acidity value, the highest increase was in apricot oil. Moisture had a big effect on this because the carrier buffer solution which enables the enzyme to be added homogeneously increased the moisture content in the ground seed. Similarly, it has been reported that the acidity of oil increases with increasing moisture content in canola seed (Patil and Singh, 2017). Enzyme treatment also led to an increase in free fatty acids in borage oil from 5.56 to 6.55% compared to the control (Soto *et al.*, 2007). An increase in the acid value (mg KOH/g) of apricot seed oil proportionate to the increase in enzyme concentration was reported by Bisht *et al.* (2015).

Free fatty acids in oil are formed as a result of hydrolysis in the presence of moisture but the non-enzymatic reaction only occurs at high temperatures. Lipase in the seeds may also have increased the hydrolysis reaction which leads to the occurence of free fatty acid during grinding and extraction. The main reason for a high acid value was probably the prolonged activity of native seed enzymes, especially lipases, since the optimum temperature and pH for the lipases of various origin ranges between 30 and 80 °C, and between 4.5 and 11, respectively (Barros *et al.*, 2010).

Peroxide values were found between 0.80-3.00 meq O_2/kg oil. The peroxide values for enzyme-treated samples were lower for flaxseed and apricot oils than that of the control, but it caused a slight increase in grape seed oil which was not statistically significant. In addition, the high peroxide value for grape seed oil was remarkable. Patil and Sing (2017) reported that the moisture content in the seeds was responsible for the high peroxide values of the oil and that the high moisture content facilitated the oxidation of oil, which resulted in rancidity. Akinoso *et al.* (2010) also explained the high peroxide values for sesame oil with the same arguments. The more free fatty acids and primary oxidation products in aqueously extracted oil than cold-pressed oil reported by Konopka *et al.* (2016) might also show the influence of paste moisture on the oxidative stability of pressed oil. Grape seeds are obtained as waste from wine or molasses production, and grape seeds are naturally in contact with water during processing. With the application of enzymes on flaxseed and grape seeds, *p*-anisidine values decreased insignificantly, while the decrease was significant in the case of apricot kernel.

Grape seed oil showed important changes in color. It is understood that enzyme pre-treatment caused a significant decrease in the a* value and increase in b* value (lower redness and blueness) compared to the control.

There was a slight but significant increase in the stearic acid percentage of flaxseed and apricot kernel oils (Table 2). With respect to enzyme pre-treatment, generally there was no significant change in the concentration of fatty acids among the studied oil samples. In addition, a significant increase in the amount of grapeseed omega-3 content can be mentioned. Increases in the sum of SFA and PUFA were determined in the oils of enzyme pre-treated seeds. However, there were negligible decreases in the U/S ratios.

3.3. Minor bioactive compounds and DPPH radical scavenging activity

Carotenoid contents showed ambiguous results with regards to enzyme pre-treatment. Enzyme pre-treatment did not reveal a significant change in carotenoid content of flax seed. However, it

Table 2. Distribution of fatty acids in seed oils obtained by cold pressing with and without enzyme pre-treatment.

	Flaxseed oil		**Apricot seed oil**		**Grape seed oil**	
Fatty acids (relative %)	**Conventional**	**Enzyme**	**Conventional**	**Enzyme**	**Conventional**	**Enzyme**
C16:0	5.12±0.04*	5.03±0.05	4.77±0.05	4.80±0.06	7.52±0.08	7.58±0.04
C18:0	4.12±0.05b	4.23±0.03a	4.12±0.02b	4.22±0.03a	4.32±0.02	4.33±0.03
C18:1 (n-9)	18.02±0.15	18.10±0.17	69.33±0.36	69.28±0.46	18.61±0.12a	18.51±0.11b
C18:2 (n-6)	14.10±0.19b	14.32±0.04a	23.37±0.08	23.44±0.15	68.40±0.17	68.38±0.23
C18:3 (n-3)	57.65±0.76	57.49±0.18	0.32±0.06	0.08±0.02	0.43±0.06b	0.71±0.02a
SFA	9.24	9.26	8.89	9.02	11.84	11.91
PUFA	71.74	71.81	92.70	92.72	87.01	86.89
U/S	9.72	9.71	10.46	10.29	7.39	7.36

*mean± standard deviation; results of three replicates (n=3).

†small case letters show significant differences between values and belong to oils of conventional and enzyme-treated samples of the same oilseed (P ≤0.05).

Statistical results obtained from t-test. U/S: unsaturated/saturated fatty acids; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids.

caused a significant increase in apricot kernel and a significant decrease in grape seed in terms of carotenoid content.

The ratio between the individual representatives of tocopherols in the cold-pressed seed oils practically did not differ. When tocopherols were examined, the most prominent effect of enzyme treatment was on β-tocopherol. The contents of β-tocopherol in flax and apricot seed oils were lower as a result of enzyme application. However, while the β-tocopherol content increased in grape seed oil, at the same time, the α-tocopherol content decreased, although a significant change occurred only in grape seed oil in terms of this tocopherol homologue.

Total phenolics in flaxseed and grapeseed oil exhibited similar changes with enzyme treatment. The phenolic contents in these oil samples showed non-significant decreasing results, but the total phenolic content in apricot oil was almost doubled. Oilseed extracts generally contain high levels of phenolics and have shown excellent antioxidant activities in both *vivo* and *vitro* (Schmidt *et al.*, 2003). However, depending on whether the phenolics are free or bound, only a small proportion can pass into the oil, and most phenols remain in the meal. In recent years, many studies have been conducted in order to increase the phenol content in seed oils (Schmidt and Pokorný, 2005).

Enzyme application did not show a significant effect in terms of antioxidant activity in grape seed oil. In fact, the effect of enzyme application in terms of antioxidant activity from the total fraction was not found statistically significant for all oil seed samples. In this regard, we can say that the effect of enzyme application varied according to the oilseed. That is, hydrophilic and lipophilic-induced antioxidant activity decreased in flaxseed oil. On the contrary, antioxidant activities from these fractions increased by up to 100% in the case of apricot seed oil. Considering the seed grain structure, there is a hard exocarp in flax and grape seed compared to apricot. Therefore, we are of the opinion that the enzymes can penetrate the tissues of apricot kernel more effectively. Here, we see that the DPPH-RSA analysis carried out where the oil is directly used as the sample does not reflect antioxidant capacity sufficiently. Because, according to the DPPH-RSA analysis results we performed directly in oil, enzyme application resulted in a slight increase, but this increase level was not even statistically significant. Phenolic compounds were also affected by enzyme pre-treatment in a similar manner to the DPPH-RSA results. The phenolic content of apricot kernel oil obtained by enzyme application is almost twice (x1.68) that of the control. In contrast to this situation, there was only a small decrease in the contents in phenolic substances in flax and grape seed oils, which were statistically insignificant. On the other hand, the results also revealed the direct relationship between total phenolic content and antioxidant capacity from hydrophilic and lipophilic fractions but not with the total antioxidant capacity determined in the oil. Symoniuk, Ratusz, Ostrowska-

Ligęza, and Krygier (2018) reported that none of the quality characteristics affected the oxidative stability of cold-pressed oils, as determined by the Rancimat test. They associated the induction times of oils with their fatty acid compositions.

3.4. Correlations

Pearson correlation coefficients among the concentrations of bioactive components, antioxidative activitiy percentages as variables and oxidation parameters (*p*-anisidine value and peroxide value), are given in Table 3. The results showed that correlation relationships changed according to the oilseed source. In terms of peroxide value, a negative correlation was observed only with γ-tocopherol in flaxseed oil (r = -59.2); whereas apricot and grape seed oils showed negative correlations between peroxide number and hydrophilic/lipophilic antioxidant capacities, total phenolics and total carotenoid contents. In addition, a negative correlation was found for grape seed oil with α-tocopherol (r = -69.2). These variables correlated with much higher ratios [r values between (-80.4) – (-97.5)] in apricot kernel oil compared to grape seed and flaxseed oils.

p-Anisidine value was negatively correlated with total carotenoids for flaxseed oil (r = -70.3), to *total* antioxidant capacity for grapeseed oil (r = -79.7). A high negative correlation (r = -92.2) could be seen between *p*-anisidine value and δ-tocopherol for grapeseed oil. For apricot seed oil, there were negative poor correlations between *total* antioxidant capacity and total carotenoids with *p*-anisidine value.

Table 3. Correlations (r) ($P < 0.01$) between oxidation parameters and some bioactive components of oil samples

	Variables	***p*-Anisidine value**	**Peroxide value (meq O_2/kg $_{oil}$)**
Flaxseed	*hydrophilic* antioxidant capacity %	-	-
	lipophilic antioxidant capacity %	-	-
	total antioxidant capacity %	-	-
	total carotenoids (mg/kg)	-70.3	-
	α-tocopherol (mg/L)	-	-
	β-tocopherol	-	-
	γ-tocopherol	-	-59.2
	δ-tocopherol	-	-
	total phenolics (mg/kg)	-	-
Apricot seed	*hydrophilic* antioxidant capacity %	-	-80.4
	lipophilic antioxidant capacity %	-	-80.9
	total antioxidant capacity %	-56.5	-
	total carotenoids (mg/kg)	-40.2	-97.5
	α-tocopherol (mg/L)	-	-
	β-tocopherol	-	-
	γ-tocopherol	-	-
	δ-tocopherol	-	-
	total phenolics (mg/kg)	-	-95.0
Grapeseed	*hydrophilic* antioxidant capacity %		-47.7
	lipophilic antioxidant capacity %	-	-39.1
	total antioxidant capacity %	-79.7	-
	total carotenoids (mg/kg)	-	-76.2
	α-tocopherol (mg/L)	-	-69.2
	β-tocopherol	-42.5	-
	γ-tocopherol	-	-
	δ-tocopherol	-92.2	-
	total phenolics (mg/kg)	-	-52.1

Statistical results were obtained by using the Correlation method.

4. CONCLUSIONS

Enzyme pre-treatment increased the free acidity of the oils. It was understood from the decreasing peroxide and *p*-anisidine values that this process had a positive effect on oxidative stability. The results confirmed that the effects of a commercial mixture of several enzymes varied according to the oil seed. As the homologous compound most affected by the enzyme application, *β*-tocopherol content decreased in flaxseed and apricot kernel oils and increased in grape seed oil. Total phenolic content was doubled in apricot kernel oil. Accordingly, antioxidant activity also increased. However, regarding flaxseed and grape seed oils, there were reductions in total phenolics and with a greater level in hydrophilic fraction-induced antioxidant activity. Enzyme pre-treatment, which shows positive results in terms of total carotenoids, tocopherols, oil yield, phenolics, antioxidant activity and oxidative stability, can be recommended for the cold pressing of apricot kernels. The maximum correlations were determined for apricot seed oil between the *hydrophilic* and *lipophilic* antioxidant capacity, total phenolics and total carotenoids with peroxide value which showed r values above 80. Correlation results showed that carotenoids play an important role in the oxidative stability of the seed oils. This effect was much more evident in apricot seed oil and consistent with a very high correlation ratio (r

= -97.5). In addition, regarding apricot oil, a negative high correlation was found between total phenolics and peroxide value which was not seen in the other oils examined. Unlike other oils, the high negative correlation between δ-tocopherol and *p*-anisidine value in grape seed oil was also remarkable.

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Bioactivity of betulinic acid nanoemulsions on skin carcinogenesis in transgenic mice K14E6

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SUMMARY: Alternative therapies for cancer treatment have been developed using bioactive compounds such as betulinic acid (BA). The objective of this study was to investigate the bioactivity of BA in its free form and compare it with its nano-encapsulated form under a skin carcinogenesis protocol in a genetically modified murine model. K14E6 and FVB mice were divided into four groups to be treated with free BA and with betulinic acid nanoemulsion (BANE). Lecithin enriched with medium chain fatty acids (MCFAs) was employed as an emulsifier to prepare the nanoemulsions with a mean droplet size of 40 nm. Skin tumors were induced by exposure to DMBA and TPA directly to the transgenic mice. Tumor development was completely inhibited by BANE and by 70% with free BA. This was validated by histological sections and the gene expression of the Cdk4 and Casp8 genes.

KEYWORDS: *Betulinic acid; Medium chain fatty acids; Mice; Modified lecithin; Nanoemulsion; Skin tumors*

RESUMEN: ***Bioactividad de nanoemulsiones de ácido betulínico en la carcinogénesis de la piel en ratones transgénicos K14E6.*** Terapias alternativas para el tratamiento del cáncer han sido desarrolladas usando compuestos bioactivos como el ácido betulínico (AB). El objetivo de este estudio fue investigar la bioactividad del AB en su forma libre, y compararlo con su forma nano-encapsulada, bajo un protocolo de carcinogénesis cutánea en un modelo murino modificado genéticamente. Los ratones K14E6 y FVB fueron asignados a cuatro grupos tratados con AB libre y con nanoemulsión de ácido betulínico (NEAB). La lecitina enriquecida con ácidos grasos de cadena media (AGCM) se empleó como emulsionante para preparar las nanoemulsiones con un tamaño medio de gota de 40 nm. Los tumores de piel fueron inducidos por la exposición a DMBA y TPA directamente a los ratones transgénicos. El desarrollo del tumor fue completamente inhibido por las NEAB y en 70% por el AB libre. Esto fue validado por las secciones histológicas y la expresión de los genes Cdk4 y Casp8.

PALABRAS CLAVE: *Ácido betulínico; Ácidos grasos de cadena media; Lecitina modificada; Nanoemulsión; Ratones; Tumores de la piel*

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1. INTRODUCTION

Cancer is one of the main causes of death worldwide. It is characterized by a deregulation of cell growth and proliferation (Diaz-Cano, 2015). It is estimated that by 2025 the incidence per year will reach 19.3 million (Bishayee and Block, 2015) and it has been documented that melanoma-type skin cancer is associated with higher morbidity in Latino populations (Rodríguez *et al*., 2018). In addition, the increase in the incidence of skin cancer is mainly attributable to prolonged periods of sun exposure. Firsthand treatments continue to be mainly radiotherapy, chemotherapy, surgery, hormone therapy and targeted therapies. However, these strategies have proven to show adverse secondary side-effects such as damage to adjacent tissues. Because of its incidence, finding alternative therapies to treat it is of utmost importance (Pejin *et al*., 2014). The use of natural compounds as a complementary therapy has attracted great interest in the prevention and treatment of cancer and since several biochemical and genetic mechanisms are involved in the development of cancer, it is necessary to investigate the different pathways in order to propose novel treatments (Palumbo *et al*., 2013; Pejin *et al*., 2013). Betulinic acid (3β, LUP-hydroxy-20 (29) -en-28-oic) is a triterpene isolated mainly from the bark of the white birch tree (*Betula alba*), to which several therapeutic properties have been attributed, with the most promising being its antitumoral effect. It has been reported to induce apoptosis in melanoma cells since 1995 (Fulda and Kroemer, 2009) by the loss of mitochondrial membrane potential through the release of cytochrome C, smac/DIABLO, the activation of caspases 3 and 9 by APAF1 binding among other proteins and, by causing DNA damage (Ali-Seyed *et al*., 2016). However, there are insufficient studies to explain the interaction between BA and cell cycle progression (Chintharlapalli *et al*., 2011). It has also been reported that BA induces cell cycle arrest in the G2/M phase by regulation of the Hiwi protein and Cyclin B (Yang *et al*., 2010). BA has very little pharmacokinetic value due to its low aqueous solubility (~21 µg/mL) (Saneja *et al*., 2018). Nanotechnology-based drug delivery systems in cancer therapy have been demonstrated to be suitable for the administration of lipophilic compounds by improving distribution capacity (Jabir *et al*., 2012). Nanoemulsions are characterized by their droplet size ranging between 20-200 nm (Kabri *et al*., 2011). The main focus of this research is based on assessing the effects of free and nanoencapsulated BA on a skin cancer mouse model.

2. MATERIALS AND METHODS

Phospholipase PLA_1 Lecitase® Ultra was provided by NOVO (Salem, VA); Duolite A568 support was a gift from Rohm & Haas (Barcelona, Spain). Phosphatidylcholine (PC) (95% PC soy) was purchased from Shenyang Tianfeng Bioengineering Technology Co. (Shenyang, Liaoning, China). Free medium-chain fatty acids were obtained from the saponification of commercial Original Thin Oil® (Dover, Idaho). Betulinic acid was purchased from Indofine Chemicals (Hillsborough, NJ). Fifteen K14E6 transgenic mice, descendent from the original mice provided by Prof. Patricio Gariglio, and five control FVB mice were provided by the animal facility of the Universidad Cristóbal Colón in Veracruz. The iTaqTM Universal SYBR Green One-Step (BIO RAD, Hercules, CA) Kit was employed for the RT-PCR assay.

2.1. Obtaining free medium-chain fatty acids (MCFAs)

100 g of Original Thin Oil® were added to 40 g of NaOH dissolved in 100 mL of distilled water and 300 mL of ethanol. The mixture was heated and stirred for 30 minutes before it was transferred to a 2 L separatory funnel. 200 mL of distilled water were added and the unsaponifiable matter was extracted with 300 mL of hexane. The aqueous phase was transferred to another separatory funnel, to which concentrated HCl was added until a pH value of 1.0 was reached. The lower layer was discarded; then 400 mL of hexane and 200 mL of distilled water were added. The mixture was shaken, and the lower layer was discarded. The organic phase was filtered through a bed of anhydrous sodium sulfate and passed through a silica gel column. Hexane was then removed under vacuum in a rotary evaporator at 40 °C.

2.2. Obtaining the emulsifier

An acidolysis reaction was performed using phospholipase PLA_1 immobilized on Duolite A568 (Ochoa-Flores *et al*., 2017). Briefly, the reaction mixture consisted of PC and free MCFAs in a 1:15 molar ratio. The immobilized enzyme was added at 12% of the total substrate weight. The reaction mixture was incubated in an orbital shaker at 50 °C and 300 rpm for 12 hours. Incorporation of MCFAs into PC was determined by alkaline methylation using 1 M sodium methoxide. Fatty acid methyl esters were extracted with hexane and 1 µL of the extract was injected into a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (FID), and a HP-INNOWAX (60 m X 0.25 mm X 0.25 mm) capillary column. The method consisted of 50 °C as initial temperature for 1 minute, followed by a ramp of 7 °C per minute to 200 °C then held for 16 minutes. Total running time was 35 minutes. The injection port was set at 200 °C and the detector at 230 °C. Yield was determined by HPLC analysis. Aliquots were taken from samples and diluted in ethanol to a 10 mg/mL final concentration; 50 µL were injected into the HPLC system consisting of a binary pump (Waters 1525), an automatic injector (Waters 717plus) and a UV-Visible detector (Waters 2487). An Alltech normal phase Econosil Silica (5 µm, 250 x 4.6 mm) was used at 40 °C. The mobile phase consisted of acetonitrile/methanol/phosphoric acid (130:5:1.5 v/v/v) at an isocratic flow rate of 1.8 mL/min.

2.3. Purification of the emulsifier

A 460 mm x 57 mm glass chromatographic column packed with 600 g of silica gel with a 60-200-mesh size and a pore diameter of 150 Å was used. The mobile phase consisted of chloroform to remove the fatty acids, and chloroform/methanol (65:35) to separate the modified PC (Vikbjerg *et al.*, 2006).

2.4. Preparation and characterization of nanoemulsions (NE)

The methodology of Cavazos-Garduño *et al.* (2015) was followed with slight modifications: 1 g of PC was dissolved in 6.0 g of water and 2.5 g of glycerol, by stirring for 5 min; subsequently 0.5 g of medium-chain oil containing 25 mg of BA were added and shaken for 3 min; the two phases were combined and ultrasonicated for 5 min in an Aquawave 9376 ultrasonic bath (Barnstead/Labline). To produce a coarse emulsion, the mixture was homogenized for 3 min at 20,000 rpm using an Ultraturrax T25 homogenizer, subsequently submitted to ultrasonication at a 20% duty cycle amplitude in a Branson Digital Sonicator S-450D for 4 minutes to obtain the O/W NE. For the characterization of nanoemulsions, a Nano-ZS90 dynamic light scattering device (Malvern Instruments Inc., Worcestershire, UK) was employed.

2.5. Betulinic acid nanoemulsions (BANE) under skin carcinogenesis protocol

15 K14E6 (genetically modified) and 5 FVB mice, at 6 weeks of age (25-30 g) were used and maintained in a controlled environment at 24-26 °C, with a relative humidity of 60% and 12/12 h light/dark cycles. The mice were divided into four groups. Group 1 was the healthy control (FVB), group 2 was the sick control, group 3 were mice treated with free betulinic acid (Free BA) and group 4 were mice treated with BANE. Every treatment was orally administered twice a week at a rate of 50 mg/kg body weight (Das *et al.*, 2016). Skin tumors were induced in the previously shaven dorsal area. During the first week a single topical dose of DMBA (7,12-dimethylbenzanthracene) was applied at a concentration of 25 nmol in 0.2 mL acetone. The second week the animals rested. From the third to the eighth week, two doses of TPA (12 o-tetradecanoyl-phorbol-13-acetate) were applied at a concentration of 6.8 nmol in 0.2 mL acetone. Growths were considered as tumors when they were at least 1 mm diameter; their surface area was calculated by the following formula: $A = (\pi)(R1)(R2)$.

2.6. Ethics statement

All animal studies were carried out with the approval of the Instituto Tecnológico de Veracruz (protocol CEI-ITVER/023/2015) and conducted under the National Research Council Guide for the Care and Use of Laboratory Animals, 8th Edition (2011).

2.7. Histological evaluation

Cross-sections of the samples with 5 µm thickness were obtained by means of a sliding microtome (Microm International GmbH, Walldorf, Germany) for further staining with hematoxylin and eosin dye.

2.8. qRT-PCR

RNA extraction was performed according to the Trizol reagent protocol (Sigma-Aldrich, Mexico City). RNA was quantified by spectrometry in a NanoDrop™ set at 260 and 280 nm. RT-PCR primer sequences used were for Cyclin-dependent kinase (CdK4) F: 5’-TTT CTA AGC GGC CTG GAT TTT-3’ R: 5’-CCA GCT TGA CGG TCC CAT TA-3’, Caspase 8

(Casp8) F: 5’-GGC AGG CTT CGA GCA ACA-3’ R: 5’-CGT AGC CAT TCC CAG CAG AA-3’ and glyceraldehyde phosphate dehydrogenase (GAPDH) as the housekeeping gene F: 5’-ATG TGT CCG TCG TGG ATC TGA-3’ R: 5’-TTG AAG TCG CAG GAG ACA ACC T-3’ (Mendoza-Villanueva *et al.*, 2008) to be performed on a RT-PCR using the StepOne™ software and applying the $2^{-\Delta\Delta CT}$ method to calculate the relative quantitation. The iTaqTM Universal SYBR® Green One-Step kit (BIO RAD) was employed.

2.9. Statistical analyses

The statistical analysis of the data was made by analysis of variance (ANOVA) and Tukey’s means comparison test, using the statistical package Minitab v. 18; a value of $p < 0.05$ was considered significant. The student *t*-test was used to calculate the levels of significance in the gene expression analysis. Values of p less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Incorporation of free medium-chain fatty acids (MCFAs) to PC

Free fatty acids were obtained as a product of the saponified medium-chain oil with a composition of 68.93% caprylic acid (C8:0) and 30.77% capric acid (C10:0). Adsorption of the enzyme onto the support was quantified by Bradford obtaining an immobilization of 64%, with 46 mg of protein adsorbed/g of support from an initial suspension of 75 mg of protein/g. Figure 1 shows the kinetics of the incorporation of MCFAs to PC (59%), the yield of PC (47%) and modified PC (86%) during 12 h of reaction.

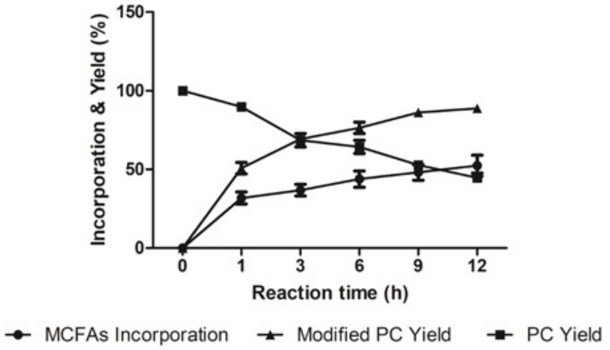

Figure 1. Incorporation of MCFAs, yield of PC and modified PC by acidolysis with PLA_1; Molar ratio of substrates (PC/MCFAs) of 1:15 and 12% enzyme concentration at 50 °C. PC= phosphatidylcholine, MCFAs= medium-chain fatty acids. Tukey’s test was used to compare averages in the incorporation of MCFAs, PC Yield and modified PC Yield. Each data point represents the mean of three replicates and their SD.

3.2. Separation of modified phospholipids by solid phase extraction

120 g of free fatty acids and 22 g of modified PC were recovered. The composition of fatty acids from modified PC used as emulsifier for BANE can be observed in Table 1.

Table 1. Fatty acid composition (molar %) of native and modified PC obtained from column chromatography separation of the PLA_1-catalyzed acidolysis reaction products.

Fatty Acid	Native PC (%)	Modified PC (%)	P
C8:0	ND	42.66 ± 0.62	
C10:0	ND	16.42 ± 1.59	
C12:0	ND	ND	
C14:0	1.18 ± 1.07	ND	
C16:0	12.16 ± 1.56	2.19 ± 0.23	0.008
C16:1	2.45 ± 0.59	ND	
C18:0	3.83 ± 0.50	0.09 ± 0.05	0.006
C18:1 (9)	12.12 ± 0.55	0.65 ± 0.20	0.001
C18:1 (7)	ND	4.62 ± 0.55	
C18:2 (6)	60.83 ± 2.70	27.73 ± 2.39	0.001
C18:3 (3)	6.36 ± 0.25	3.20 ± 0.31	0.001
C20:0	0.67 ± 0.18*	ND	
Total MCFAs		59.08	

PC was modified under the following conditions: PC and MCFAs in a molar ratio 1:15, 12% of enzyme immobilized PLA_1 on Duolite A568 under 12 hours reaction. PC-Phosphatidylcholine, PLA1-Phospholipase A1, MCFAs-Medium chain fatty acids. Values are reported as means ± SD of the triplicate determinations ($P < 0.05$) by two-sample T-test. ND = not detected.

3.3. Preparation and characterization of nanoemulsions (NE) stabilized with modified PC

To compare the droplet size, nanoemulsions were structured with either native PC or modified PC; both systems contained BA and produced nanometer droplet sizes (Table 2).

3.4. Effect of BANE on skin carcinogenesis

In the sick control group, the number of tumors were 7.33 ± 5.8. In the group with the carcinogens and free BA, the mean number of tumors was 2.25 ± 1.25, which represents a 70% reduction, as shown in Figure 2. Finally, in the group treated with the

Table 2. Characterization of NE with native and modified PC as emulsifier.

PC	Droplet size (nm)	PDI	ζ Potential (mv)
Unmodified	88 ± 0.97 ^{a}	0.10 ± 0.036 ^{a}	-3.8 ± 0.5 ^{a}
Modified	40 ± 1.65 ^{b}	0.14 ± 0.018 ^{a}	-15 ± 1.7 ^{b}

Preparations made with 10% phosphatidylcholine or modified phosphatidylcholine, 25% glycerol in the aqueous phase and 25 mg of BA/g of NE and 5% of MCT in the oil phase. NE- Nanoemulsion, PC- Phosphatidylcholine, PDI- Polydispersity Index. Tukey test was used to evaluate mean difference (n=3). Means that do not share a letter are significantly different P=0.0001.

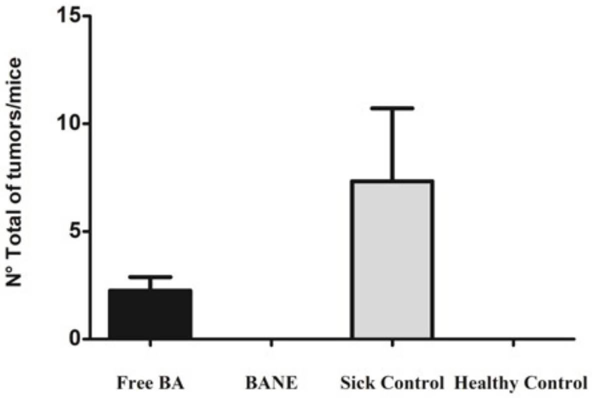

Figure 2. Effect of free BA and BANE on skin tumorigenesis of K14E6 mice with DMBA (25 nmol/0.2 mL acetone) and TPA (6.8 nmol/0.2 mL acetone) for eight weeks. One-way ANOVA, P=0.007. Data points are mean values from five replicates; errors bars represent the SD.

carcinogens and BANE, no skin tumorigenesis was observed. In Table 3, the average tumor surface areas by groups are depicted compared with the healthy control group (FVB).

Table 3. Effect of BA on tumor surface area of all groups (mm^2)

Healthy control (FVB)	Sick Control N/T	Free BA group	BANE group
0.00	15.62 ± 0.78A	14.92±0.97A	0.00±0.00B

Data represent the mean ± SD on each treated group (n= 5), One-way ANOVA/Tuckey *P*=0.002. Free BA-Free betulinic acid, BANE-betulinic acid nanoemulsión.

3.5. Histological evaluation

Figure 3 illustrates the histological results of all 4 treatment groups: (A) healthy control group (FVB), a normal cell architecture is observed in the epidermis, dermis, subcutaneous tissue and appendages. The tissue of the sick control group (only treated with DMBA/TPA) (D), had moderately differentiated squamous keratinizing carcinoma development, composed entirely of atypical squamous epithelial cells with intense keratin production, neoplastic cells with irregular nuclei and abundant mitosis. In the group treated with free BA (G), the mice developed a keratinizing epidermoid carcinoma. The group treated with BANE (J), showed normal skin architecture without tumor lesions of any type and regular morphology was observed. The epidermal structure of the BANE-treated group was similar to the healthy control group.

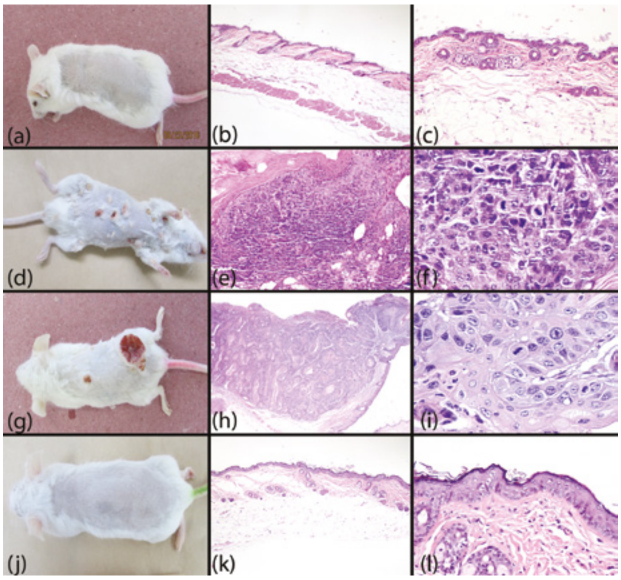

Figure 3. (a) Macroscopic appearance of healthy control; (b) histological dissection of skin without histological alterations (H&E, 2x); (c) histological dissection without alterations in hair follicles and sebaceous glands (H&E, 5x); (d) sick control macroscopic appearance of mouse with multiple epidermoid carcinomas; (e) infiltrating epidermoid carcinoma with areas of surface keratinization (H&E, 5x); (f) neoplastic cells with moderate cytoplasm, pleomorphic nuclei of granular chromatin and abundant mitoses (H&E, 40x); (g) macroscopic aspect of mice treated with free BA, shows two neoplastic lesions in the dorsal region, the largest of which is ulcerated; (h) neoplastic lesion corresponds to infiltrating epidermoid carcinoma with differentiation regions (H&E, 2x); (i) morphological appearance of neoplastic cells with broad keratinizing cytoplasm, large nuclei with dispersed granular chromatin, note also mitosis; (j) macroscopic aspect of mice treated with BANE, does not show neoplasia; (k) histological cut of skin without histological alterations (H&E, 2x); (l) histological dissection with slight chronic inflammatory infiltrate in the dermis (H&E, 10x).

3.6. Gene expresión

The gene expression analysis (Figure 4) showed similar statistical RQ values for each treatment group: Cdk4 and Casp8. In the free BA and BANE

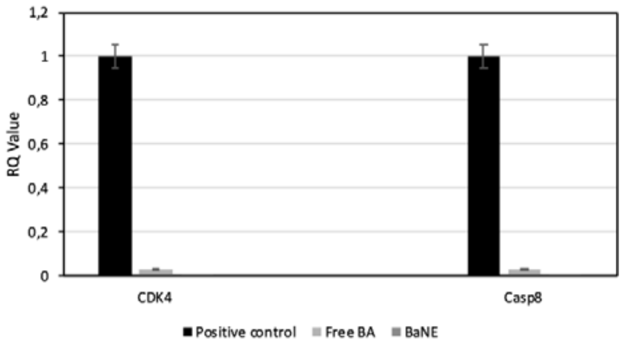

Figure 4. Relative Quantitation values for Cdk4 and Casp8 gene expression. Student *t*-test was employed to calculate the levels of significance, and $P < 0.05$ was considered statistically significant. The data points represent the mean of five replicates and the error bar is the SD.

groups, differences were evident when compared to the healthy and sick control groups; however, there was no difference between their signaling pathway level. RQ values were 0.03 and 0.0002 for Cdk4 and Casp8, respectively; the values for free BA and BANE were 0.04 and 0.0005, respectively. Cdk4 and Casp8 expression levels were reduced when free BA and BANE were supplied, suggesting that the mechanism of action of this compound may influence the cyclin-dependent pathway and repressed Rb1.

4. DISCUSSION

This investigation describes the bioactivity of free BA compared to its nanoemulsified form as well as the advantage of the enzymatic modification that was carried out through the use of phospholipase PLA_1 (Ochoa-Flores *et al.*, 2017). Our results showed an increase in the modification of PC with MCFAs under the optimal conditions mentioned in the results section. Recent research showed that the enzymatic modification of phospholipids by PLA_1 reached a significant level after 24 h of reaction (Li *et al.*, 2014). The choice of oil is an important variable, since it directly affects the NE droplet size, along with its chemical and optical properties (Cavazos-Garduño *et al.*, 2015). Stability increases with small sizes by avoiding gravitational segregation, flocculation by Brown's movement and greater system transparency; their bio-accessibility has been increased in *in vitro* systems when using O/W NE (Salvia-Trujillo *et al.*, 2013). It has been reported that using polylactide-co-glycolide-monomethoxy polyethylene glycol nanoparticles containing BA, where 147 nm globule sizes were obtained, increased its antitumor activity by up to 7 times in an Erlich carcinoma model (Saneja *et al.*, 2017). The hepatoprotective activity of BA in nanoemulsions with sizes of 150.3 nm, a PDI of 0.192, a Ȥ-potential of -10.2 mV has been reported, together with an increase in its solubility when dispersed in olive oil in Wistar rats with CCl_4 as a toxic agent (Harwansh *et al.*, 2017). In the same way, using chicken embryos, the anti-angiogenic effect of nanoemulsions with a globule size of 145 nm and a Ȥ-potential of -39.1 mV has been documented (Dehelean *et al.*, 2011). In this study, particle sizes of 40 nm, a PDI of 0.14, and a Ȥ-potential of -15 mV were attained. The reduction in particle size increased the superficial area, enhancing the absorption capacity of the bioactive by biological membranes such as the intestinal lining and/or skin; i.e., it improved the transport, exposure, and activity of the encapsulated compound, evidencing that a minimum amount can be employed to exert its therapeutic activity. The use of carrier systems has opened a field to investigate the different routes of the carcinogenesis process in murine models. This has been envisioned for the development of drugs that use natural compounds with low bioavailability, such as in the case of BA. In a research regarding skin damage monitoring, the anti-angiogenic process and the damage caused to blood vessels by TPA and DMBA exposure to BALB/c mice were described; betulin was used for 12 weeks and produced a reduction in blood vessels; and no tumor development was observed (Dehelean *et al.*, 2011). When evaluating BA (both topic and oral NE) in C57BL/6J mice, with a liver implanted with B6 melanoma cells, a decrease in metastasis was found to be significant compared to the non-treated group (Ciurlea *et al.*, 2010). When a complex of cyclodextrin with BA (100 mg/kg) was intraperitoneally administered in an *in vivo* melanoma model (C57BL/6J) for 21 days, the tumor volume decreased from 300 to 150 mm^3. However, the administration method, even though it was effective, caused significant pain and distress to the animal. Because of the BA structure, it can be metabolized and degraded, thereby releasing the compound before the target cells could be reached (Soica *et al.*, 2014). In this research, we prepared a nanoemulsion system to be administered orally, without causing harm to the animal. Moreover, it was formulated with nutraceutical materials that provide additional health benefits, such as MCFAs and lecithin. These are important components in the architecture of cell membranes which increase the bioavailability of the bioactive compound and enhance its intestinal absorption by enterocytes, or it is absorbed directly through the lymphatic system. One of the advantages of our nanoemulsified system is the globule size of < 200 nm, allowing better absorption of the compound through cellular membranes. Also, an additional benefit of using MCFAs is the increased bio-accessibility of lipophilic compounds; unlike long-chain fatty acids, MCFAs are more easily dispersed in aqueous phases and confer better protection to the nanoemulsion against lipolytic enzymes; they can also form micelles in the intestine for the solubilization of hydrophobic bioactive compounds. Furthermore, it is hypothesized that the chemical structure of BA resembles cholesterol in terms of its insertion into the plasmatic membrane. It was estimated that BA changed cell membrane fluency, and thereby modulated the signaling pathway associated with membrane microdomains, translocated certain receptors, and inhibited cell proliferation and migration (Chen *et al.*, 2016). It has also been proposed that its antitumor effect is caused by the inhibition of tumor angiogenesis, promoting a decrease in the supply of nutrients and oxygen to potential tumor formations; therefore, a decrease in growth rate was probably caused by low rates of cell necrosis (Aisha *et al.*, 2012), but also increased cellular apoptosis, loss of membrane potential and cell cycle arrest (Saneja *et al.*, 2017). The specific cytotoxicity of BA has been previously found in neuroblastoma and glioblastoma cell lines (Thurnher *et al.*, 2003); however, the mechanism is not yet fully understood. Implicated genes in skin and cervical cancer have been outlined to clarify these conditions and determine whether BA can be effective to counter it. In that study, Ccne2 and Cdk4 were upregulated in skin cancer and showed no changes in cervical cancer. This suggested that the above genes were involved in these particular cancer types (Mendoza-Villanueva *et al.*, 2008). It is important to note that the transgenic mouse model used in our project, K14E6, was designed to express E6 and E7 genes from Human Papilloma Virus (HPV) in skin, promoting the development of skin cancer; these genes were implanted in the embryo's ectoderm for their future expression. It has been reported that E6 inhibits p53, which is a tumor suppressor gene that regulates cell cycle by mediating p21. Additionally, E7 inhibits the expression of the retinoblastoma (RB) gene, another tumor suppressor gene that also regulates cell cycle. Under normal circumstances both p53 and RB cease cell division at the G_1 phase by compromising CDK binding to Cyclins or by restricting DNA replication, respectively. This prevents the progression to the S phase when there is a defect in cell division; conversely, in this case both genes were under-expressed, producing defective and uncontrolled proliferation of skin cancer cells. Casp8 expression levels were also found to be down-regulated in skin cancer, producing a favorable environment for the progression of this type of cancer. Attenuation of apoptosis attributed to Caspase inhibition has been suggested as one of the mechanisms for tumorigenesis onset in several types of cancer. In this manner, alteration in the Caspase expression might be a diagnostic biomarker for skin carcinoma. In our research, subjects treated with BA showed a significant decrease in Cdk4 when compared to our control untreated mice. This biomarker has been attributed as a target for the BA potential therapeutic effect by arresting the cell cycle at the G_1 phase as expected, as well as inducing apoptosis in melanoma cell lines. In this type of cancer Cdk4 is upregulated (Rieber and Rieber, 2006). It has been proposed that BA selectively activates the intrinsic apoptotic pathway in cancer cells by releasing cytochrome C, activating caspase 3 but not caspase 8; BA also regulates members of the Bcl-2 family and induces apoptosis in HeLa cells directly through this intrinsic pathway (Fulda, 2009; Gali-Muhtasib *et al.*, 2015). BA bearing dephosphorylates the 3-kinase (PI3K)/Akt phosphatidylinositol pathway by promoting cell viability in cancer cells, thereby repressing Cdk inhibitors p27 and the p21 cell cycle progression modulator. BA has proven to cease cell cycle progression in cell lines such as HeLa, at the G0/G1 phase through the inhibition of the PI3K/Akt pathway by incubating 30 μmol/L BA after 12 hours; in addition, it was found to contribute to mitochondrial apoptosis by membrane depolarization and increased Caspase activity (Xu *et al.*, 2017).

The results described in this research suggest the enhanced beneficial effect of BA in its nanoemulsified form as a protector against skin carcinogenesis development and progression, as it can mediate multiple pathways as well as intervene in cell cycle regulation. It is still necessary to better clarify the interaction of BA in both its free and nanoencapsulated forms directly on the cell cycle, in order to explain the favorable response that was observed in this work, and thus be able to propose its use as a complementary therapy.

5. CONCLUSIONS

A complete inhibition of tumor development was observed in mice treated with BANE, confirming its antitumor activity *in vivo*, which is consistent with previous studies performed by several authors. Our data suggest a clear advantage of nanoencapsulating BA over its application in its free form. However, further studies remain to be carried out in order to precisely elucidate the molecular mechanisms, gene regulation, and cell cycle interference achieved by the BANE.

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Enzymatic pre-treatment of grape seeds for an oil with higher antioxidant activity

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SUMMARY: The paper investigates the effect of the enzymatic pre-treatment of grape seeds from six Romanian cultivars on the oil extracted. The grape seeds of some white and red Romanian grape varieties were separated from winery waste, washed, dried and ground, with the oil then obtained by extraction with petroleum ether. The extraction was performed directly or after a preliminary treatment with a commercial *pectin lyase*. The enzymatic procedure applied was more cost effective compared to other treatments previously described in which a cocktail of enzymes was used. The quantity of the extracted oil was measured in both types of processing, with an increase being observed for pre-treated samples. The fatty acid profiles (FAPs) of the oils resulted for the treated and untreated seeds were determined. No change in the composition was noticed. The reductive power of these oils was also investigated. Compared to the untreated samples for the same variety, the enzyme pre-treatment resulted in a superior antioxidant capacity.

KEYWORDS: *Antioxidants content; Fatty acid profile; Grape seeds; Pectin lyase treatment; Statistical analysis*

RESUMEN: ***Pretratamiento enzimático de semillas de uva para un aceite con alta actividad antioxidante***. En este artículo se investigó el efecto del tratamiento enzimático de semillas de uva de algunos cultivares rumanos sobre el aceite extraído. Las semillas de uva de variedades seleccionadas de uva rumana blanca y roja se separaron de los residuos de la bodega, se lavaron, secaron y molieron, a continuación el aceite se obtuvo mediante extracción con éter de petróleo. La extracción se realizó directamente o después de un tratamiento preliminar con una liasa de pectina comercial. El procedimiento enzimático aplicado es más rentable en comparación con otros tratamientos descritos anteriormente en los que se utilizó un cóctel de enzimas. La cantidad de aceite extraído se midió en ambos tipos de procesamiento y se observó un aumento para las muestras pretratadas. Se determinaron los perfiles de ácidos grasos (PAG) de los aceites resultantes de las semillas tratadas y no tratadas. No se notó ningún cambio en la composición. También se investigó el poder reductor de estos aceites. En comparación con las muestras no tratadas para la misma variedad, el pre-tratamiento enzimático dio lugar a una capacidad antioxidante superior.

PALABRAS CLAVE: *Análisis estadístico; Contenido antioxidante; Pectina liasa tratamiento; Perfiles de ácidos grasos*

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1. INTRODUCTION

The evaluation of any economic activity may be performed based on the economic value added (EVA) model developed in 1982 by G.B. Stewart, which is still used successfully (Stewart, 2013). In such context the valorization of the biomass waste from the industrial production of wine is important, as obtaining added value is a priority for any sustainable economy.

Romania is a known wine producer, being the 11th producer in the world and the 5th in Europe (European Commission, 2019). A sustainable development for winery implies the adequate management of resources in the production chain and reduction of waste (Maicas and Mateo, 2020). Waste minimization through technological innovation will generate an EVA equity indicator (Machová and Vrbka, 2018). A waste reduction policy is recommended for achieving a sustainable wine making process (Devesa-Rey *et al.*, 2011) and the implementation of the “zero waste” concept (Donner *et al.*, 2020).

Winery wastes are an important part of wine production. Grape pomace is a waste which results as a by-product from the must production; it is composed of around 30% stems, 30% seeds and 40% skins and pulp**.** It is considered an agro-industrial waste, representing about 25% (w/w) of the weight of grapes processed and more than 9 million tons annually (Sirohia *et al.*, 2020, Cvejic Hogervorst *et al.*, 2017).

There are several proposals for solving the problem of pomace. It can be used in different applications such as functional foods and supplements, pharmaceutical and cosmetic products (Galanakis, 2020). The dispersal of it into landfill seems ecologically inappropriate (Dwyer *et al.*, 2014). Viable solutions for winery waste valorization are: oil production (Al-Juhaimi and Ozcan, 2018), manufacture of composite materials (Barbieri *et al.*, 2013), extraction of the antioxidants for food supplements (Nowshehri *et al.*, 2015), bioconversion to valuable chemicals or biofuels (Rani *et al.,* 2020; Zacharof, 2017).

A better biomass valorization implies the separation of the pomace components (Toscano *et al.*, 2013). The seeds represent a great part of these wastes, approximately 47% on dry base (Zhang *et al.*, 2017), making their valorization vital.

The paper investigates the valorization of grape seeds by oil extraction. Residual grape seeds from six Romanian cultivars were processed. In order to increase the outcome, a pre-treatment of the seeds with a commercial *pectin lyase* was experimented. The enzymatic treatment seems appropriate due to the progress in the industrial production of enzymes as well as their numerous industrial applications derived from their properties, namely: low toxicity, energy saving due to mild work conditions, biodegradability, etc. (Choi *et al.*, 2015). An improvement in oil quantity and/or quality was predicted.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Sample preparation

The grape seeds were isolated from the pomace resulting from the wine making process, washed, dried for 24 hours (h) at room temperature, and ground. Seeds from the grape varieties of the 2015 harvest were investigated, namely four red brands, including Cabernet Sauvignon, Feteasca Neagra, Merlot, Pinot Noir and two white brands Columna and Riesling Italian. The material was supplied by the winery of Murfatlar, situated in Dobrogea, a south-eastern region of Romania.

2.1.2. Commercial enzyme

The pectin lyase solution (Pectinex XXL, activity 10.000 U mL^{-1} according to the supplier) was purchased from Novozymes A/S (Bagsvaerd, Denmark).

2.1.3. Chemicals

The 10-14% BF_3 solution in methanol was supplied by Merck (Darmstadt, Germany). Petroleum ether (b.p. 40-60 ºC), analytical grade methylene chloride, methanol, and 96% ethanol solvents were purchased from Sigma Aldrich and were used as delivered. The citric acid and disodium phosphate dihydrate for the buffer solution were supplied by Sigma Aldrich.

2.2. Equipment and procedures

UV-Vis spectra (200-800 nm) were acquired on a Helios Beta apparatus with Vision software (Thermo Electron Corporation, Waltham, MA, United States).

The ^{1}H-NMR spectra were obtained on a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany).

The GC analyses were performed on an Agilent Technologies 7890A instrument (2850 Centerville Road Wilmington, DE 19808-1610 USA), provided with a flame ionization detector.

2.2.1. Enzymatic treatment

Portions of 10 g ground grape seeds were kept for certain amounts of time in a solution of 3 mL commercial enzyme and 37 mL buffer solution of pH 3.5 (0.1M citric acid and 0.2M Na_2HPO_4, 2.3/1 volume ratio), in 100 mL capped glass amber flasks, at room temperature (20 ºC), with gentle intermittent stirring. The optimal time for treatment was established based on the oil yield (see Figure 1). For each grape seed variety the experiments were conducted in triplicate. After treatment, the seeds were separated by filtration, washed and dried before extraction.

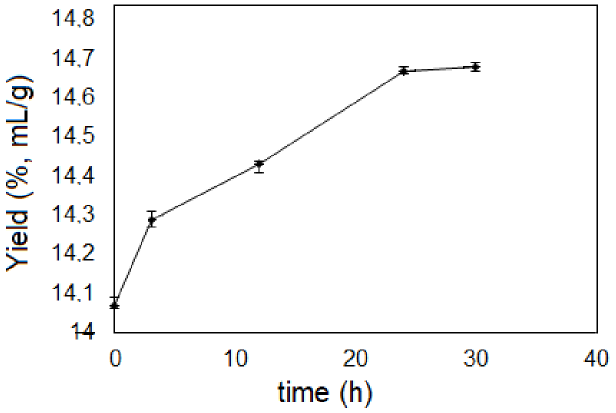

Figure 1. Variation in the extracted oil (mL oil/100 g seeds) with the pre-treatment time for the Pinot Noir variety (Standard deviation for n = 3)

The residual solution was concentrated in a Heidolph rotary evaporator, in a water bath (50 ºC), and the residue was studied by NMR spectroscopy.

For checking the buffer effect on the grape seed a number of experiments were performed by keeping the seeds in the buffer solution (ratio w/v = 1/4) for 24 hours, at room temperature. The quantity of the oil from these samples was in the same range as the corresponding untreated oil.

2.2.2. Oil extraction

The oil samples were obtained from the dried ground seeds (pre-treated or not with enzyme) by the Soxhlet extraction method with petroleum ether (b.p. 40-60 ºC), following the protocol ISO 659 (2009). The solvent was partially recovered by evaporation under atmospheric pressure, heated on a water bath at 65 ºC, using a Heidolph rotary evaporator. The weight of the oil was measured with an analytical balance (accuracy of ± 0.0001 g) and the corresponding volume was calculated using the density value for grape seed oil of 0.92 g/mL (Ceriani *et al.*, 2008). The value of the density was confirmed by checking on a number of experimental samples. The experiments were performed in triplicate, the average volumes of oil being presented in Figure 2. The initial quantity of seeds was the same for both types of experiments (with or without pre-treatment) as measured before pre-treatment.

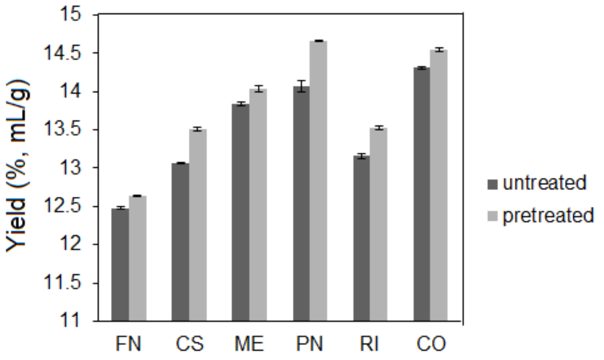

Figure 2. The oil amount (mL/100 g seeds) obtained by extraction from untreated and enzyme pre-treated seeds. (Standard deviation for n = 3; FN – Feteasca Neagra, CS - Cabernet Sauvignon, ME - Merlot, PN - Pinot Noir, RI - Riesling Italian, CO – Columna)

2.2.3. GC analysis of the grape seed oil composition (FAP)

The grape seed oil FAPs were determined following the ISO 12966 (2015) protocol. The free fatty acids were obtained by treatment of 0.5 mg oil with 0.9 mL NaOH 0.5 M solution in methanol, at reflux, for 10 min. Then, they were esterified by treatment with 0.1 mL BF_3 solution and 0.8 mL methanol, at reflux, for another 10 min. After dilution with 4 mL distilled water, the fatty acid methyl esters (FAMEs) were extracted with methylene chloride and were submitted to a standard gas chromatographic (GC) analysis. GC analysis was performed on a highly polar capillary column Supelco SP™ 2560 (100 m × 0.25 mm, 0.20 µm film) with a biscyanopropyl stationary phase. A certified reference material (Supelco™ 37 Component FAME Mix) was used for the identification of the FAPs (see table 1). For each oil sample, three GC analyses were performed. The composition was expressed as a percentage based on the ratio between each peak area and the total area.

2.2.4. Determination of the reductive power of the oil samples

The CUPRAC assay (Apak *et al.*, 2008) was applied to assess the total antioxidative capacity (TAC) of the oil. Samples of oil extracted by sonication in ethanol (1/10, v/v) were treated with 1 mL $CuSO_4$ 10^{-2} M aqueous solution, 1 mL of 7.5 × 10^{-3} M Neocuproine ethanolic solution, 1 mL CH_3COONH_4 buffer (pH 7) and 0.4 mL distilled water. After stirring for 30 min, at room temperature, the absorbance (Abs) at 450 nm was measured. TAC was expressed as a trolox equivalent (TE, μmol per 100 g oil) based on a previously drawn calibration curve ($y = 0.058x$; $R^2 = 0.99$). The average values obtained from three experiments for each oil sample are presented in Table 1.

2.2.5. Determination of the total polyphenol content (TPPC) of the grape seeds

The polyphenols (PPs) were quantified using the known Folin-Ciocalteu (FC) method (Ballus *et al.*, 2015). Dried seeds (2 g), before and after the oil extraction, were extracted with 9 mL ethanol/water (2/1, v/v). Extract samples (0.1 mL) were mixed with the FC reagent (0.5 mL), distilled water (1.5 mL) and, after 5 min, with 20% Na_2CO_3 solution (1.5 mL). The mixture was incubated for 2 h, at room temperature, in the dark. The Abs at 750 nm was measured vs. a blank. The analyses were performed in triplicate, for seeds with or without enzymatic pre-treatment, before and after oil extraction. The TPPC was expressed as gallic acid equivalents (GAE, mg per 100 g dried seeds) derived from a previously obtained calibration curve ($y = 0.061x$; $R^2 = 0.98$) (Table 2).

2.3. Statistical analysis

To identify sample clusters the principal component analysis (PCA) was applied to the grape seed oil compositions. The independent variables were selected by PCA. The ANOVA program was used for the comparison of the mean values. Data analysis was performed using the XLSTAT 2015 software (Addinsoft).

3. RESULTS AND DISCUSSION

3.1. Effects of the pre-treatment with pectin lyase on the grape seed oil yields

The research was focused on the valorization of grape seeds from the waste of a Romanian winery

Table 1. FAPs and antioxidant content in the extracted grape seed oil

Cultivar	FN	FNE	CS	CSE	ME	MEE	PN	PNE	RI	RIE	CO	COE
Fatty acid						**FA Content (%)**						
Palmitic and Stearic	10.58±0.02	10.71±0.01	11.11±0.02	11.29±0.01	9.02±0.02	9.55±0.03	10.34±0.01	10.42±0.01	9.88±0.02	9.99±0.03	8.78±0.01	8.83±0.02
Oleic (18:1 cis-9)	15.47±0.01	15.84±0.03	12.14±0.02	12.36±0.03	12.18±0.02	12.62±0.02	14.39±0.01	14.53±0.03	18.10±0.01	18.18±0.01	17.85±0.01	17.90±0.01
Linoleic (18:2 cis,cis-9,12)	73.76±0.02	73.28±0.01	76.48±0.02	76.12±0.01	78.34±0.01	77.60±0.01	75.05±0.02	74.76±0.01	71.79±0.02	71.54±0.03	73.13±0.01	73.00± 0.02
Linolenic (18:3 cis,cis,cis 9,12,15)	0.19±0.01	0.22±0.01	0.27±0.01	0.22±0.02	0.28±0.02	0.24±0.01	0.23±0.01	0.27±0.01	0.23±0.01	0.28±0.01	0.24±0.01	0.27±0.01
TAC (μmol TE /100 g oil)	72.84±1.14	176.75±1.31	62.43±1.49	131.64±1.45	109.26±1.32	216.18±1.19	134.19±1.28	234.48±1.54	33.45±1.31	58.73±1.40	50.14±1.33	85.12±1.49

Standard deviation for n = 3

FAPs - fatty acid profiles, FA - fatty acids, TAC - total antioxidative capacity, TE - trolox equivalent, FN – Feteasca Neagra, FNE – Feteasca Neagra pre-treated with enzyme, CS - Cabernet Sauvignon, CSE - Cabernet Sauvignon pre-treated with enzyme, ME -Merlot, MEE –Merlot pre-treated with enzyme, PN - Pinot Noir, PNE - Pinot Noir pre-treated with enzyme, CO - Columna, COE - Columna pre-treated with enzyme, RI - Riesling Italian, RIE - Riesling Italian pre-treated with enzyme.

Table 2. Polyphenol content in grape seeds

Sample Variety	**PN**	**ME**	**FN**	**CS**	**CO**	**RI**
Analyzed sample	**Polyphenols as *GAE* (mg/100 g seeds)**					
Initial	132.27±1.33	148.52±1.25	133.74±1.33	149.26±1.35	133.74±1.39	134.48±1.34
After extraction	127.09±1.53	128.57±1.34	130.05±1.44	144.09±1.59	128.57±1.35	127.09±1.33
After pre-treatment and extraction	124.14±1.49	125.62±1.43	115.27±1.48	135.22±1.54	127.83±1.58	124.14±1.34

Standard deviation for n = 3
GAE - gallic acid equivalents, PN - Pinot Noir, ME -Merlot, FN – Feteasca Neagra,
CS - Cabernet Sauvignon, CO - Columna, RI - Riesling Italian.

Murfatlar starting with the production of the grape oil. The oil obtained from grape seeds is a valuable material (Shinagawa *et al.*, 2015). Recent clinical trials showed the beneficial effects of this oil on human health (Kaseb and Biregani 2016; Ismail *et al.*, 2016).

The solvent extraction standardized method used for the oil separation is simple and less energy demanding than other procedures (Castro-Lopez *et al.*, 2016). The yield is relatively high due to the permanent contact of ground seeds with clean solvent, which favors the extraction. The grinding of the seeds helps due to the generation of a larger contact area for seed-solvent. The solvent used, petroleum ether, is industrially accessible and not so toxic because of degrading rapidly in soil and water and having a half-life of 3-8 days in the air (Nalliah, 2014).

Vegetable oils may be produced also by cold pressing the seeds but the yield is usually lower and it is difficult to obtain a constant quality of the product (Ustun–Argon *et al.,* 2020). The yield may be improved by using shockwaves but the process is complex and not always economically viable (Marousek, 2015a).

By comparison with other methods the solvent extraction of the grape seed oil has a low processing cost and is easy to handle (Galankis, 2020).

For improving the extraction process of bioactive compounds a number of procedures have been experimented, among which can be found supercritical fluid extraction, pressurized liquid extraction, microwave and ultrasound assisted extraction, etc. (Azmir *et al.*, 2013; Kumar *et al.*, 2017). Some of these procedures require investments which are suitable only for a large scale production.

Improvements of the extraction process may be done by pre-treatment of seeds before extraction. The literature has reported the following procedures: heating (Ustun–Argon *et al.,* 2020), shockwave treatment (Marousek, 2015a), enzymatic hydrolysis (Passos *et al.*, 2009) or a combination of these methods (Marousek *et al.,* 2015b).

According to the literature (González-Centeno *et al.*, 2010), pectins are polymer constituents of the cell walls in fresh grapes or in grape pomace, which bind other constituents. The study of oil distribution inside the seed showed its presence near the external tegument (Pope *et al.*, 1993). There is a relatively strong interaction between the oil and the seed walls leading to supramolecular structures (Scollary *et al.*, 2012) which block oil removal. Thus, to improve oil extraction, an enzymatic pre-treatment was carried out, with an enzyme specific for pectin breaking. The enzymatic treatment (see paragraph 2.2.1) was performed in batch mode, by maintaining the seeds in a buffered solution of Pectinex XXL, at room temperature and pH 3.5, parameters recommended for this type of enzymes (Najafian *et al.*, 2009). The *pectin lyase* cleaves the pectin (Yadav *et al.*, 2009) giving water soluble compounds (saturated and unsaturated pectic-oligosaccharides). The fragmentation process is improved by the presence of citric acid, which acts both as buffer and ligand for calcium ions (Stanescu *et al.*, 2010). Thus, these ions, which reinforce the pectin structure by making bridge bonds between chains (Ochoa-Villarreal *et al.*, 2012), are removed, aiding in the elimination of pectins. The fracture of the cell walls facilitates the access of solvent and improves the oil extraction yield. The chosen pH was the optimal one for the commercial enzyme used. Room temperature was suitable for the enzyme and did not require added cost for heating. The only parameter to be established was the treatment time. An optimal time of 24 hours was established for the enzymatic treatment based on the volume of the resulted oils for different time ranges (see Figure 1).

The enzymatic pre-treatment of seeds, for 24 h with *Pectinex XXL*, led to more oil, the quantities obtained being 101.3-104.3% compared to untreated seeds (see Figure 2). Comparable results for the enzymatic pre-treatment of seeds have been found by other researchers (Passos *et al.*, 2009). The performance of the described procedure consisted of lower additional costs than those with enzyme cocktails (Passos *et al.*, 2009; Marousek *et al.,* 2015). A preliminary calculation (Tociu, 2019a) indicated a reduced cost (of over 100 times) for the pre-treatment with only Pectinex XXL compared to the cost of the treatment with a mixture of *cellulase*, *xylanase* and *pectinase* performed by Passos *et al.,* (2009), the differences in oil yield being insignificant.

Unfortunately, there are limitations to the application of enzymatic treatments such as the reproducibility of enzyme biosynthesis and the possible negative effects of the stabilizers on commercial products. These are impediments to the application of enzymatic treatments at large scale in processes where the enzyme properties (content, activity) are of great importance.

3.2. Effects of pre-treatment with pectin lyase on the oil properties

3.2.1. FAPs of the grape seed oils

One of the most important features of lipids is their fatty acid profile (FAP). The previous research involving enzymatic pre-treatment (Passos *et al.*, 2009; Marousek *et al.,* 2015b) did not check this aspect for the oil applications. Thus, the extracted oils were analyzed by a GC standard method (see paragraph 2.2.3). The FAPs of the oil samples are presented in Table 1.

The main component of these oils was linoleic acid, a representative polyunsaturated fatty acid (PUFA). The saturated fatty acid (SFA) content was around 10%. Similar FAPs were revealed for Portuguese grape varieties (Fernandes *et al.*, 2013) and Spanish wines with protected denomination of origin (Bada *et al.*, 2015). Due to their low SFAs and high linoleic acid contents these oils present good nutritional qualities (Alsharari *et al.*, 2017). According to the experimental results the pre-treatment changed the FAPs only slightly (≤ 0.4%).

As expected, the statistical analysis revealed a close correlation between PUFA and linoleic acid ($y = 1.004x+0.1003$; $R^2 = 0.99$). The grape variety showed an impact on the content in fatty acids in oil. Principal component analysis (PCA) applied to FAPs disclosed the differences or similarities of the analyzed oils (Figure 3). The PCA analysis revealed the following eigenvalues: F1: 2.067, F2: 1.375, F3: 0.558, F4: 0.000. Thus, the variance values of the principal components (PCs) were 51.66% (F1), 34.39% (F2), 13.95% (F3) and 0.003% (F4). The cumulative percentage for PC1 and PC2 coordinates was over 86.00% so only these 2 PCs had to be considered.

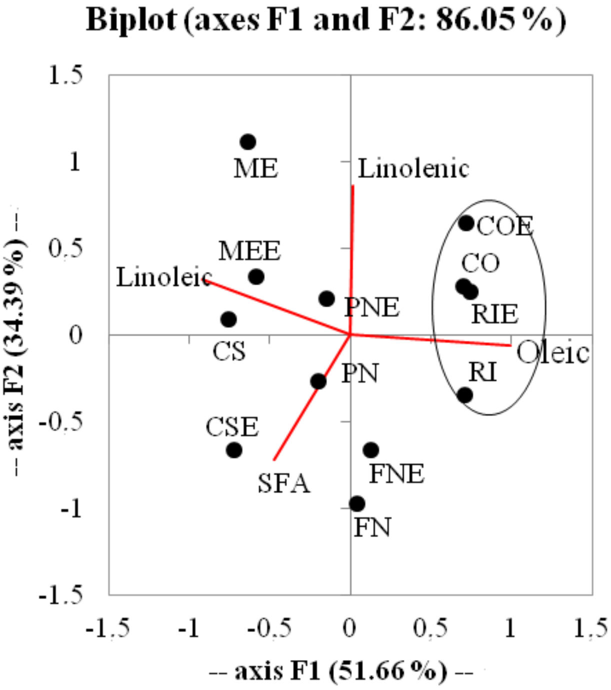

FIGURE 3. Principal component analysis based on FAPs. (CS - Cabernet Sauvignon, FN – Feteasca Neagra, ME -Merlot, PN - Pinot Noir, CO - Columna, RI - Riesling Italian, CSE - Cabernet Sauvignon pre-treated with enzyme, FNE – Feteasca Neagra pre-treated with enzyme, MEE -Merlot pre-treated with enzyme, PNE - Pinot Noir pre-treated with enzyme, COE - Columna pre-treated with enzyme, RIE - Riesling Italian pre-treated with enzyme, SFA - saturated fatty acids)

The oils from Columna and Riesling (with a higher content in oleic acid) as well as Merlot and Cabernet Sauvignon (with higher content in linoleic acid) showed a good discrimination on the F1 (PC1) direction; whereas Feteasca Neagra and Cabernet Sauvignon (with high content in SFA) exhibited good discrimination on the F2 (PC2) direction.

A comparison with oils from other seeds seemed of interest. According to the literature the average values for PUFAs (%) (Chira *et al.*, 2011) for oils obtained from sunflower (59.77 ± 4.80), soybean (55.02 ± 2.88) and rapeseed (25.95 ± 1.70) were by far lower than those for the grape seed oil (75.00 ±

2.40%). Thus, this oil stands out for its high content in healthy PUFAs.

3.2.2. Total antioxidant capacity (TAC) of the grape seed oils

Besides FAP, the antioxidant capacity of vegetable oils is essential for their applications (Chambre *et al.*, 2019). The method used to establish the TAC was the CUPRAC assay. This assay is recommended due to its simplicity, reduced cost and reduced reaction time (Apak *et al.*, 2008). It gives an accurate estimation of the TAC of the analyzed sample. The experimental results obtained for the studied oils are presented in Table 1. The oils obtained after the enzymatic treatments of seeds are richer in antioxidants, with the TAC values being 1.7-2.4 times higher compared to those of oils from untreated seeds (see Table 1). The enzymatic treatment destroyed not only the interactions of oil with the cell walls but also that of the antioxidants (Chamorro *et al.*, 2012), increasing the quantities of extracted antioxidants. As expected, the oils obtained from the red grape varieties had higher antioxidant contents and improved health benefits.

A comparison of the TAC of grape seed oils (see Table 1) with other vegetable oil TACs is of interest. Thus, from the literature data the TAC values, expressed as TE (mmol kg^{-1}) are as follows: 1.79 for extra virgin olive oil, 2.20 (soybean), 1.29 (corn), 1.17 (sunflower) (Pellegrini *et al.*, 2003). Due to the enzymatic treatment the related grape seed oils are ranked in better positions by their TE values.

3.3. Complementary possible valorization of other wastes

The processing of grape seeds for oil extraction generates new wastes. Solutions have to be found for further valorization.

An investigation by ^{1}H-NMR (see Figure 4b) of the residue resulting from the concentration of the solutions obtained after enzymatic treatment using a rotavap showed the presence of valuable products.

Occurrence of pectic-oligosaccharides was suggested by the gel aspect of the residue, as well as the specific ^{1}H-NMR peaks at 4.2-4.5 ppm and around the 5.1 ppm (Winning *et al.*, 2007). Other specific peaks are covered by the concentrated commercial enzyme signal (see Figure 4a) and the signal of the citric acid (2.5-2.8 ppm) from the buffer. Small peaks at 6.6-7.5 ppm evidenced the presence of aromatic compounds which were most likely traces of polyphenols (PPs) (Franz *et al.*, 2014). There was no peak at 5.29 ppm in the residue spectrum (Figure 4b), signal characteristic for –CH=CH- of fatty acid (Chambre *et al.*, 2019) as one may see in Figure 5, proving that no oil was lost during pre-treatment.

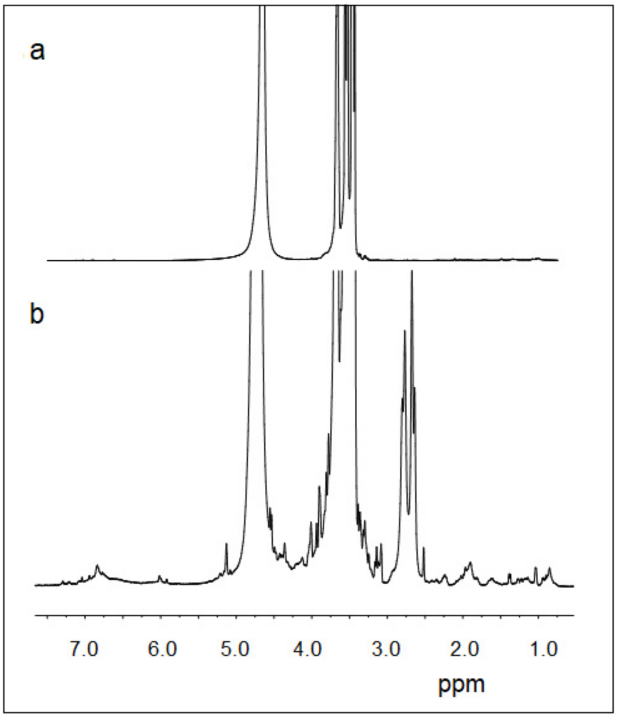

FIGURE 4. ^{1}H-NMR spectra (400 MHz, D_2O, internal standard TSP) for (a) concentrated commercial enzyme; (b) residue obtained by concentrating the solution from the enzymatic pre-treatment

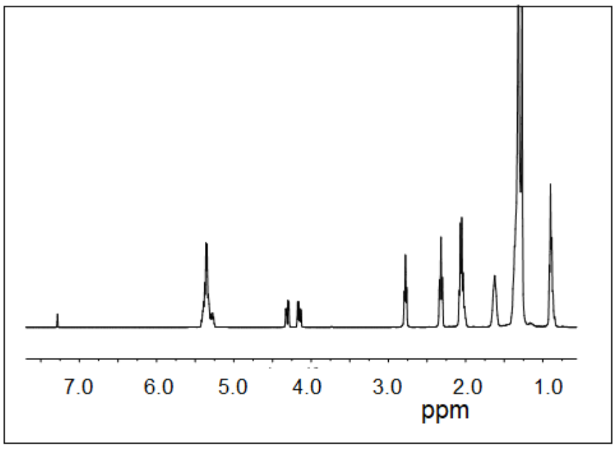

FIGURE 5. ^{1}H-NMR spectrum (400 MHz, D_2O, internal standard TSP) of the oil extracted from the Cabernet Sauvignon seeds

The residual grape seeds from the extraction of oil may be another source for PPs (Maier *et al.*, 2009) as proven by the performed Folin-Ciocalteu assay presented in Table 2.

After the extraction of the oil, the seeds hold over 86% of the initial quantity of PPs, most likely due to the reduced solubility of these compounds in petroleum ether. The literature data claim that vitamin E (tocopherols and tocotrienols) is the main antioxidant in grape seed oils (Wen *et al.*, 2016). This fact was also confirmed by the thermal behavior of grape oils (Chambre *et al.*, 2019).

Further investigation into the valorization of residual grape seed after extraction were also performed by isolating the PPs and using the lignocellulosic material for dye adsorption (Tociu, 2019a; Tociu *et al.*, 2019b).

The economic impact of the enzymatic pre-treatment has to be analyzed. Therefore, an accurate EVA analysis should be performed, considering all the by-products from the grape seeds (oil, PPs, adsorbents, char) not only the oil. Thus, new investigations must be carried out.

4. CONCLUSIONS

The paper presents the effect of a *pectin lyase* pre-treatment on grape seeds from six Romanian grape cultivars, raw materials for oil extraction. The following assertions resulted from the experimental work:

The effect of treatment on oil quantity is comparable to processes using cocktails of enzymes.

The statistical analysis indicates the influence of the seed origin on the FAP.

The enzymatic treatment does not affect the FAP of the extracted oils, which contain a high percentage of healthy unsaturated fatty acids.

The enzymatic pre-treatment increased significantly the antioxidant capacity of the extracted grape seed oils.

The antioxidant capacity of the oils is based mostly on vitamin E components as experimentally proven. Most of PPs remain in seeds.

The pre-treatment is performed in a batch system and in mild conditions not needing a complex production unit.

Further investigation into the residual solutions resulting from the enzymatic treatment to recover valuable products, e.g. oligopectins and PPs, are required.

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Seasonal effects of the fatty acid composition of phospholipid and triacylglycerol in the muscle and liver of male *Salmo trutta macrostigma*

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SUMMARY: The seasonal effects on the fatty acid composition of triacylglycerol (TAG) and phospholipid (PL) in the muscle and liver of male *Salmo trutta macrostigma* were determined using the gas chromatographic (GC) method. The fatty acid (FA) compositions of total lipid, PL and TAG fractions were determined in muscle and liver tissues of *S. trutta macrostigma.* The phospholipids contained a higher proportion of 16:0 compared to the TAG in the muscle tissue of *S. trutta macrostigma.* Docosahexaenoic acid (22:6 ω-3) and eicosapentaenoic acid (20:5 ω-3) contents were high in both muscle and liver tissues. The total lipid contents in the muscle and liver were 1.07-2.45 and 3.00-4.64%, respectively. *S. trutta macrostigma* is a rich source of ω-3 and ω-6, polyunsaturated fatty acids (PUFA) with numerous benefits to human health.

KEYWORDS: *Fatty acid; Phospholipid; Triacylglycerol;* Salmo trutta macrostigma; *Seasonal changes.*

RESUMEN: ***Efecto estacional de la composición de ácidos grasos de fosfolípidos y triacilgliceroles en el músculo e hígado de* Salmo trutta macrostigma *macho.*** El efecto estacional sobre la composición de ácidos grasos de los triacilgliceroles (TAG) y fosfolípidos (PL) en el músculo e hígado de *Salmo trutta macrostigma* macho se determinaron mediante cromatografía de gases (GC). Se han determinado las composiciones de ácidos grasos (FA) de lípidos totales, fracciones de PL y TAG en tejidos musculares y hepáticos de *S. trutta macrostigma*. Los fosfolípidos contenían una mayor proporción de 16:0 en comparación con los TAG en el tejido muscular de *S. trutta macrostigma*. El contenido de ácido docosahexaenoico (22:6 ω-3) y ácido eicosapentaenoico (20:5 ω-3) es alto en el tejido muscular y hepático. El contenido total de lípidos de los músculos e hígado fue de 1,07-2,45% y 3,00-4,64%, respectivamente. *S. trutta macrostigma* es una fuente rica de ω-3 y ω-6 que son ácidos grasos poliinsaturados (PUFA) con numerosos beneficios para la salud humana.

PALABRAS CLAVE: *Ácidos grasos; Cambios estacionales; Fosfolípidos;* Salmo trutta macrostigma; *Triacilgliceroles.*

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1. INTRODUCTION

S. trutta macrostigma inhabiting the River Munzur shows distribution in an area of 80 km, starting 1-2 km south of Munzur Gozeleri on the east of Ovacik, Tunceli, Turkey, up to Tunceli, Turkey, especially in the streams of Munzur and Mercan. This endemic species not only creates an economic value for its taste but also creates an important potential for tourism. They prefer to live in cool (12-19 °C) and oxygen-rich trout zones of a gravel-bed stream with high velocity and upriver areas (Aras *et al.,* 1997). Their spawning season occurs in the period of December-February.

Fish meat, especially trout, is a delicious nutritional source with rich nutritional components that play an important role in meeting the animal protein needs of people (Justi *et al.,* 2003). The deliciousness of fish meat is due to the fats and fatty acids in its structure (Kinsella, 1987). These are not just high energy sources, but they are also very important in that they contain fat-soluble vitamins, combine with proteins to form lipoproteins, and play a role in blood lipid levels.

Fish muscle is the main part of the fish used as human food. Liver tissue is important for fat metabolism and it undertakes important functions, such as the intake, oxidation and transformation of FAs and the provision of long-chain polyunsaturated fatty acids (PUFA) to other tissues (Rincon-Sanchez *et al.,* 1992).

Fish meat is the only animal source of ω-3 group FAs, eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acids (DHA, 22:6 ω-3). The fatty acids in fish meat, especially EPA and DHA, have numerous benefits for human health thanks to their biochemical, metabolic, nutritional properties, and pharmacological effects (Sushchik *et al.,* 2007). These fatty acids play an important role in the membrane biochemistry of cells, such as the fluidity and permeability of the cell membrane; while they also have a direct effect on intermembrane processes such as osmoregulation, absorption and transport of nutrients (Christiansen *et al.,* 1989).

The lipid contents in fish generally depend on seasonal changes and accordingly, nutrient availability, temperature, pH, and salinity of the water where they were caught, spawning cycle, size and physiological status of fish. The FA composition of living things comes from their diets (Kaushik *et al.,* 2006).

It was reported that the FA composition in fish can vary depending on various environmental factors, as well as the species, which are significantly influenced by their bio-cycle capacities (Sargent, 1995), and there may be significant differences between the tissues of a fish due to lipid metabolism (Haliloğlu, 2001). Another important environmental factor, salinity, on the other hand, is known to be effective in the digestion of proteins, fats, and some dietary FAs, especially in some trout species (Borlongan and Benitez, 1992).

The FA content in the food of the fish directly affects the FA content of the fish tissues (Bell *et al.,* 2003). For example, herbivorous fish feeding on algae contain high levels of 18-carbon polyunsaturated fatty acids, with fewer rates of 20 and 22-carbon PUFAs (Henderson and Tocher, 1987). Like trout, carnivorous (flesh-eating) fish can complete the elongation (chain extension) and desaturation (increased degree of unsaturation) process since they feed on other fish and aquatic organisms. These fish, therefore, contain long-chain ω-3 PUFAs at a high rate and linoleic acid at a low rate.

Phospholipids and TAGs play different roles in fish metabolism. Triacylglycerols, also known as neutral oils, are the main component of the fats in our body that are taken from nutrients and make up more than 95% of pure fats. TAGs, mainly stored in adipose tissue, function as energy reserves (Sargent *et al.,* 1995). PLs, which form a small portion of total fats, are the major component of the cell membrane and structure, and 20-carbon polyunsaturated fatty acids that serve as precursors to eicosanoids.

Several studies have been carried out about the FA composition of total lipids of *S. trutta macrostigma* (Aras *et al.,* 2003; Akpınar *et al.,* 2009; Kayım *et al.,* 2011; Ateş *et al.,* 2013) and there are two published reports on the FA composition of the PL and TAG of this fish living in Erzurum (Bayır *et al.,* 2010) and living in the Munzur River (Kayhan *et al.,* 2015). However, the FA composition of PL and TAG of muscle and liver tissue of this species has not been reported previously. In this framework, the objective of this study was to determine the seasonal changes in lipid content and FA composition in the muscle and liver tissue of the brown trout *S. trutta macrostigma* in the Munzur stream, Tunceli, Turkey.

2. MATERIALS AND METHODS

Brown trout (22.93 cm; 137.33 g) were collected by electrofishing at site (39º 21¢ 67² N, 39º 13¢ 55²) in the

Munzur stream, Ovacık, Tunceli. Fish samples were collected every two months for a year (November, January, April, June, July, October). Muscle samples were taken for analyses from the fish body above the lateral line. Fish sex was determined by their gonads. The captured samples varied between 18.35 (in July) and 27 (in April) cm, with weights between 88 and 191 g.

Total lipids were extracted from 1 g of liver and 2 g of muscle tissue. The muscle and liver extracted were homogenized in a chloroform-methanol mixture (Folch, 1957). The thin-layer chromatography technique was used to fractionate the total lipids in the samples. Total lipid extracts of the samples were spotted on plates in a straight line. Total lipids were run in a mixture of petroleum ether-diethyl ether-acetic acid. The bands of the PL and TAG fractions determined by the standards were scraped and transferred to the reaction test-tubes. 3 ml of methanol and 3-5 drops of sulfuric acid were added to each fraction separately and they were heated at 85 °C under refrigerant for 2 hours. Thus, the transformation of fatty acids to fatty acid methyl esters was carried out. Methyl esters were extracted using hexane after the solution was cooled down. A gas chromatography device with Flame Ionization Detector (FID) was used for the analysis of fatty acid methyl esters.

Esterified samples were diagnosed on gas chromatography devices according to the study of Kayhan *et al.* (2015). A SPSS 16 computer program was used to compare fatty acid percentage rates. All data obtained from our study were obtained from the average of three replicates. In the gas chromatographic analysis of fatty acid methyl esters, three samples from each period were injected separately and the three values of the same fatty acid were averaged. A comparison of fatty acid percentages was made by one-way analysis of variance. Differences were determined by the Tukey HSD test. As a result of the statistics, it was accepted that the differences were significant when the data were $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. FA profile of the total lipid of muscle and liver tissue

The ΣSFA of the fish examined was between 31.19-33.72% in muscle tissue. Although there was an irregular increase and decrease in the amount of ΣSFA during the year, it was determined that the amount of ΣSFA did not change significantly during the year, and that it was more stable than ΣMUFA and ΣPUFA and its amount was at the highest level in November, which was also the pre-spawning season and the water temperature was low. It was found that the amounts of ΣSFA and 16:0 and 18:0, which form the majority of ΣSFAs, were not significantly affected by the spawning season or water temperature. The majority of ΣSFA were formed by 16:0 fatty acids (21.98-24.19%) and it was seen that the amount of ΣSFA was affected by the changes in the amount of 16:0.

There were no significant seasonal differences in the amount of 16:0. This indicates that 16:0 fatty acids were key metabolites in fish and their quantity was not affected by food (Ackman *et al.,* 1975).

The most abundant fatty acid in SFAs after 16:0 was 18:0 (4.45-6.32%), and it was found that its amount did not show significant variations during the year.

The amount of ΣMUFA in trout was determined to be 22.01-35.30%. 18:1 ω-9 and accordingly, the lowest ΣMUFA amounts were found in the month of April (post-spawning season) and the highest ΣMUFA was in January (spawning season). 18:1 ω-9 and 16:1 ω-7 FAs were fatty acids with the highest percentages among the MUFAs. The excessive increase in the amount of ΣMUFA was due to the 16:1 ω-7 and 18:1 ω-9 increase in the month of January.

In our study, it was determined that the amount of 16:1 ω-7 and 18:1 ω-9 in ΣMUFA was high. Similar data were obtained in studies carried out on *Oncorhynchus mykiss* (Haliloğlu *et al.,* 2004, Görgün and Akpınar 2007).

The amount of ΣPUFA in trout was determined to be 33.35-46.52%. 22:6 ω-3 and 20:5 ω-3 and accordingly, the highest ΣPUFA amount was found in April. 18:2 ω-6 and 20:4 ω-6 from ω-6 FAs and 18:3 ω-3, 20:5 ω-3, 22:5 ω-3 and 22:6 ω-3 from ω-3 FAs were found to form the vast majority of ΣPUFAs, and showed fluctuations during the year.

There were DHA ω-3 FAs in the muscle tissues of fish at the highest levels. It was determined that the amount of DHA varied during the year, and the amount was the lowest in January and reached the highest level in April. Besides, it was found that the amount of DHA was higher than the amount of EPA in each season. The amount of EPA and DHA was found between 7.16-9.75% and 7.63-22.00%.

Table 1. Fatty acid composition in total lipids of muscle from male *S. trutta macrostigma* (% of total FA)*

Fatty acids	November (2009)	January (2010)	April (2010)	June (2010)	July (2010)	October (2010)
C10:0	-	-	-	-	-	-
C12:0	0.64±0.01a**	0.78±0.03b	-	0.30±0.01c	0.36±0.01c	-
C13:0	-	0.02±0.05a	-	0.62±0.07b	-	1.06±0.01b
C14:0	2.04±0.23a	2.54±0.26b	1.68±0.21a	2.34±0.11ab	1.90±0.10a	1.79±0.21a
C15:0	0.34±0.07a	0.26±0.01b	0.33±0.05a	0.17±0.05c	0.22±0.04bc	0.28±0.06b
C16:0	23.80±0.98a	21.98±0.76b	23.18±0.56a	23.49±0.68a	24.19±0.84c	23.08±0.53a
C17:0	0.58±0.12a	1.17±0.04b	0.46±0.06a	0.53±0.05b	0.80±0.07ab	0.65±0.06ab
C18:0	6.32±0.53a	4.45±0.41b	5.54±0.38ab	5.68±0.51ab	5.39±0.49ab	4.69±0.31b
ΣS.F.A***	33.72±1.01a	31.20±0.96b	31.19±0.86b	33.13±0.87a	32.86±0.72ab	31.55±0.67b
C16:1 ω-7	3.28±0.28a	11.70±0.81b	4.63±0.46a	6.04±0.28c	7.43±0.63bc	7.90±0.72bc
C18:1 ω-9	20.79±0.91a	21.19±0.75a	16.17±056b	18.90±0.48c	19.80±0.59ac	18.28±0.92c
C20:1 ω-9	0.72±0.03a	2.41±0.16b	1.21±0.08c	2.97±0.07b	2.98±0.13b	1.27±0.06c
ΣM.U.F.A.	24.79±0.68a	35.30±1.06b	22.01±0.59c	27.91±0.72d	30.21±0.84bd	27.45±0.67d
C18:2 ω-6	4.62±0.44a	4.05±0.31b	3.90±0.25b	3.63±0.32b	4.54±0.38a	4.81±0.34a
C18:3 ω-3	5.18±0.30a	11.08±0.17b	4.20±0.21c	5.92±0.92a	6.98±0.24d	9.90±0.36db
C20:2 ω-6	0.28±0.06a	0.13±0.01b	0.39±0.03c	0.12±0.03b	0.41±0.04c	0.26±0.07a
C20:3 ω-6	0.47±0.09a	0.27±0.06b	0.33±0.02ab	0.28±0.24b	0.31±0.11ab	0.46±0.07a
C20:4 ω-6	2.02±0.15a	0.80±0.08b	2.45±0.36c	1.68±0.62ab	1.99±0.29ab	1.12±0.36ab
C20:5 ω-3	7.53±0.23a	7.16±0.12a	9.66±0.32b	8.52±0.45ab	9.11±0.37b	9.75±0.42b
C22:5 ω-3	2.90±0.08a	2.23±0.12a	3.59±0.19b	3.03±0.22ab	2.68±0.17a	2.86±0.11a
C22:6 ω-3	18.50±0.57a	7.63±0.51b	22.00±0.64c	15.64±0.73d	10.77±0.56bd	13.46±0.38db
ΣP.U.F.A.	41.50±1.03a	33.35±0.96b	46.52±1.13c	38.82±0.92ab	36.79±0.76ab	42.62±1.04a
ω3	34.11±0.98a	28.10±0.68b	39.45±1.02c	33.11±0.89a	29.54±0.68b	35.97±1.01a
ω6	7.39±0.56a	5.25±0.43b	7.07±0.49a	5.71±0.53b	7.25±0.66a	6.65±.61ab
ω3/ω6	4.61	5.35	5.57	5.79	4.07	5.40

* Means are the averages of 3 replicates

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

*** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

In the month of January, the spawning period, the amount of 18:3 ω-3 was found to be quite high compared to the other months, and the amount of DHA was significantly lower.

18:2 ω-6, 20:2 ω-6, 18:3 ω-3, 20:5 ω-3, 22:5 ω-3 and 22:6 ω-3 were found to form the vast majority of ΣPUFAs in trout. As found in studies performed on other trout, the highest percentage of fatty acids among ω-6 FAs was 18:2 ω-6 (Aras *et al.,* 2003a; Aras *et al.,* 2003b; Akpınar *et al.,* 2009; Kalyoncu *et al.,* 2010).

Due to the high amounts of ω-3 FAs such as EPA and DHA in our study, it can be said that *S. trutta macrostigma* has an important place in human nutrition. Various types of trout, including *S. trutta macrostigma*, were studied previously and it was seen that the most abundant ω-3 FAs in PUFAs were DHA and EPA (Haliloğlu *et al.,* 2002; Aras *et al.,* 2003a; Aras *et al.,* 2003b; Görgün and Akpınar 2007; Kalyoncu *et al.,* 2010).

The fact that the amount of DHA was found to be significantly lower in the month of January, the spawning period, it is believed to have arisen from the fact that DHA in the muscles may have moved to the gonads with the onset of the gonad maturation stage (Jeong *et al.,* 2002).

In our study, the percentage of total unsaturated FAs in all seasons was higher than the percentage of total SFAs.

Fish are poikilothermic living things, in other words, their body temperature changes according to the ambient conditions. Studies have found that temperature is directly effective on fatty acid metabolism. A decrease in the water temperature in the environment where the fish inhabit causes an increase in the carbon numbers of FAs in their structural lipids, and unsaturation (Williams and Hazel 1992). It was stated that the percentage of total SFAs in fish can never exceed the percentage of total unsaturated FAs since they are poikilothermic living things (Akpınar, 1987).

It was found that in the muscle total FA composition of the fish, ω-3 FAs changed more than ω-6 FAs during the year and the amount of ω-3 PUFAs was higher than ω-6 PUFAs in each period. This was effective in determining the ω-3/ ω-6 ratios. The ω-3/ ω-6 ratio was found as 4.07-5.79.

The high ω-3/ω-6 ratio was believed to happen due to the ω-3PUFA content that plays a role in adaptation to high altitude and long winter conditions in trout caught from the River Munzur. The ω-3/ω-6 ratio in the muscle tissue of *S. trutta macrostigma* was found to be higher than many freshwater fish (Haliloğlu *et al.,* 2002; Güler *et al.,* 2007).

Table 2 shows the variations in the liver total FA composition of *S. trutta macrostigma* by months.

The amount of ΣSFA in the liver of trout was determined as 29.41-33.64%. The liver ΣSFA amount showed fluctuations during the year and was found to be higher in the month of January (spawning season) compared to other months. It was found that the 16:0 level was the highest in January and that this finding directly affected the amount of ΣSFA. In many fish species examined, especially trout, it was found that the most abundant component in SFAs in

Table 2. Fatty acid composition in total lipids of liver from male *S. trutta macrostigma* (% of total FA)*

Fatty acids	**November (2009)**	**January (2010)**	**April (2010)**	**June (2010)**	**July (2010)**	**October (2010)**
C12:0	0.10±0.02a**	0.41±0.11b	-	-	-	-
C13:0	-	-	-	-	-	0.03±0.01
C14:0	2.11±0.23a	2.57±0.13a	1.59±0.30b	1.26±0.24c	1.89±0.21b	1.09±0.24c
C15:0	0.49±0.16a	0.46±0.20a	0.29±0.14b	0.19±0.07b	0.25±0.11b	0.14±0.05c
C16:0	20.88±0.44a	23.02±0.34b	20.20±0.38a	21.01±0.34c	21.33±0.52c	21.94±0.45c
C17:0	0.61±0.13a	0.78±0.18b	0.50±0.12a	0.35±0.08c	0.23±0.11c	0.39±0.17c
C18:0	6.78±0.19a	6.40±0.33a	6.83±0.35a	8.09±0.41b	7.54±0.42ab	6.50±0.41a
ΣS.F.A***	30.97±1.03a	33.64±0.65b	29.41±0.54a	30.90±0.73a	31.24±0.65ab	30.09±1.01a
C16:1 ω-7	3.08±0.05a	8.08±0.10b	3.56±0.29c	3.28±0.63a	3.46±0.24ac	3.14±0.18a
C18:1 ω-9	20.88±0.52a	19.98±0.47b	14.54±0.53c	19.65±0.61b	18.45±0.51ab	13.87±0.48c
C20:1 ω-9	0.37±0.11a	1.42±0.10b	0.54±0.21c	0.83±0.20d	0.88±0.22d	0.41±0.30a
ΣM.U.F.A.	24.33±0.95a	29.48±0.76b	18.64±0.65c	23.76±0.68a	22.79±0.44d	17.42±0.25c
C18:2 ω-6	6.19±0.21a	4.21±0.33b	2.66±0.12bc	3.73±0.34c	3.11±0.30c	3.74±0.51c
C18:3 ω-3	2.82±0.10a	6.18±0.12b	2.38±0.20a	3.39±0.33c	2.76±0.21a	3.62±0.19c
C20:2 ω-6	0.50±0.14a	0.30±0.06ab	0.38±0.06ab	0.41±0.03b	0.27±0.03c	0.27±0.12c
C20:3 ω-6	0.41±0.02a	0.27±0.13b	0.28±0.01b	0.39±0.02c	0.28±0.06b	0.40±0.05a
C20:4 ω-6	4.85±0.11a	2.42±0.15b	5.79±0.33c	5.83±0.20c	5.21±0.21ac	4.57±0.22a
C20:5 ω-3	8.42±0.45a	10.87±0.54ab	11.85±0.23b	10.00±0.34ab	11.91±0.10b	12.43±0.31c
C22:5 ω-3	2.27±0.11a	2.26±0.12a	4.24±0.15b	3.28±0.23ab	2.66±0.10a	2.96±0.11a
C22:6 ω-3	19.17±0.10a	10.32±0.19b	24.18±0.38c	18.15±0.71d	19.70±0.41a	24.35±0.19c
ΣP.U.F.A	44.63±1.01a	36.83±1.18b	51.76±1.13c	45.18±1.24a	45.90±1.08a	52.34±1.05c
ω3	32.68±0.99a	29.63±0.99b	42.65±0.99c	34.82±1.05a	37.03±0.99d	43.36±0.71c
ω6	11.95±0.90a	7.20±0.65b	9.11±0.91c	10.36±0.65a	8.87±0.65b	8.98±0.90b
ω3/ω6	2.73	4.11	4.68	3.36	4.17	4.82

* Means are the averages of 3 replicates

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

*** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

the liver, as in the muscles, was 16:0 (Haliloğlu *et al.,* 2002, Aras *et al.,* 2003a, b, Akpınar *et al.,* 2009).

The amount of ΣMUFA in trout was determined as 17.42-29.48%. Like the ΣSFAs, the amount of ΣMUFA was found to show fluctuations during the year and it was higher in the spawning season compared to other periods. The FAs with the highest percentage in the liver ΣMUFAs were 18:1 ω-9 and 16:1 ω-7. 18:1 ω-9 and 16:1 ω-7 are characteristic components for freshwater fish (Osman *et al.,* 2001).

The increase in the amount of ΣMUFA was due to the increase in the amount of 16:1 ω-7. The fact that the amount of ΣMUFA was at its lowest level in the month of October was due to 18:1 ω-9, which was at a minimum level during this period.

The amount of ΣPUFA in the liver was determined as 36.83-52.34%. The highest ΣPUFA amount was found in the month of October and the lowest ΣPUFA was in the month of January, the spawning season. Just like in muscle tissue, it was found that the amount of ΣPUFA in the liver was affected by the spawning season; whereas the amount decreased in this period and increased in the month of April, the post-spawning season. Just like in the muscles, the most common ω-3 FAs in the liver ΣPUFAs of the fish were 20:5 ω-3 and 22:6 ω-3 FAs. It was determined that the amount of ΣPUFA was significantly affected by changes in the amount of 20:5 ω-3 and 22:6ω-3. For example, the reason for the high number of PUFAs in October compared to other periods was due to the increase in the 22:6 ω-3 rate in this period. ω-6 FAs, on the other hand, were found to be more abundant than ΣPUFAs 18:2ω-6 and 20:4 ω-6. It was seen that both the amount of DHA, which is very important for the structure and functions of cells, and AA, the primer substance of the biologi-

TABLE 3. Fatty acid composition in the phospholipid fraction of muscle from male *S. trutta macrostigma* (% of total FA)*

Fatty acids	November (2009)	January (2010)	April (2010)	June (2010)	July (2010)	October (2010)
C14:0	1.15±0.22a**	0.67±0.13b	0.91±0.34ab	1.08±0.24a	1.01±0.21a	0.82±0.21ab
C15:0	0.36±0.16a	0.18±0.10b	0.26±0.22ab	0.21±0.07ab	0.10±0.01c	0.21±0.15ab
C16:0	32.81±0.52a	27.58±0.65b	25.50±0.44c	36.07±0.49d	27.41±0.32b	26.40±0.35bc
C17:0	0.56±0.13a	0.43±0.28b	0.52±0.41a	0.39±0.17b	0.24±0.14c	0.26±0.12bc
C18:0	6.68±0.42a	4.83±0.51b	6.75±0.38a	8.91±0.42c	5.43±0.28ab	3.64±0.44d
ΣS.F.A***	41.56±1.02a	33.69±0.76b	33.94±0.54b	46.66±1.24c	34.19±0.92b	31.33±0.64d
C16:1 ω-7	1.74±0.52a	1.66±0.31a	2.25±0.10b	1.29±0.33c	1.50±0.21ac	1.39±0.41c
C18:1 ω-9	12.65±0.36a	8.44±0.55b	11.52±0.45a	14.16±0.47c	13.50±0.56ac	12.67±0.48a
C20:1 ω-9	0.46±0.12a	0.66±0.24b	0.53±0.21a	0.76±0.20b	0.95±0.22c	0.34±0.30d
ΣM.U.F.A.	14.85±0.39a	10.76±0.42b	14.30±0.48a	16.21±0.28c	15.95±0.5ac	14.40±0.67a
C18:2 ω-6	2.43±0.41a	1.39±0.23b	2.67±0.21a	2.44±0.34a	2.86±0.36c	18.65±0.61d
C18:3 ω-3	4.08±0.25a	4.48±0.32a	2.74±0.29b	3.32±0.33ab	3.79±0.22ab	3.07±0.19ab
C20:2 ω-6	-	0.16±0.06a	0.30±0.04b	0.25±0.10ab	0.37±0.13b	0.16±0.08a
C20:3 ω-6	2.51±0.12a	0.23±0.06b	0.35±0.11c	0.35±0.02c	0.40±0.05c	0.18±0.07b
C20:4 ω-6	-	1.98±0.15a	2.94±0.32b	2.15±0.24ab	2.82±0.27b	1.46±0.22c
C20:5 ω-3	9.60±0.42a	15.01±0.51b	10.93±0.33a	7.96±0.44c	13.28±0.23ab	8.97±0.35c
C22:5 ω-3	2.80±0.13a	4.65±0.18b	4.31±0.15b	2.89±0.22a	4.06±0.16b	2.65±0.12a
C22:6 ω-3	22.09±0.54a	27.58±0.39b	27.27±0.48b	17.62±0.51c	22.27±0.44a	19.07±0.49d
ΣP.U.F.A.	43.51±0.84a	55.48±1.18b	51.51±1.03c	36.98±0.71d	49.85±0.65c	54.21±1.05b
ω3	38.57±0.68a	51.78±0.97b	45.25±0.59ab	31.79±0.53c	43.40±0.49ab	33.76±0.70a
ω6	4.94±0.61a	3.76±0.56b	6.26±0.73c	5.19±0.55ac	6.45±0.46c	20.45±0.31d
ω3/ω6	7.80	13.77	7.22	6.12	6.72	1.65

* Means are the averages of 3 replicates

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

*** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

TABLE 4. Fatty acid composition in the triacylglycerol fraction of muscle from male *S. trutta macrostigma* (% of total FA)*

Fatty acids	**November (2009)**	**January (2010)**	**April (2010)**	**June (2010)**	**July (2010)**	**October (2010)**
C10:0	-	-	-	0.06±0.01**	-	-
C12:0	1.36±0.23a	0.79±0.31b	0.92±0.40c	0.65±0.28d	0.77±0.29b	-
C13:0	1.84±0.34a	-	0.43±0.17b	-	0.21±0.14c	1.49±0.25d
C14:0	5.18±0.28a	3.03±0.19b	3.29±0.41b	4.49±0.35ab	4.01±0.38ab	2.22±0.33c
C15:0	4.60±0.27a	0.39±0.21b	0.99±0.32c	0.34±0.13b	0.27±0.09d	0.26±0.12d
C16:0	16.49±0.69a	22.77±0.41b	25.84±0.61c	26.59±0.55c	23.89±0.52bc	19.70±44d
C17:0	6.63±0.53a	1.34±0.18b	0.98±0.25c	0.57±0.21d	0.38±0.14d	0.79±0.18dc
C18:0	9.20±0.56a	4.36±0.62b	7.32±0.39c	6.21±0.38c	3.69±0.33b	3.29±0.23b
ΣS.F.A***	45.30±0.71a	32.68±0.55b	39.77±0.63c	38.91±0.42c	33.22±0.42b	27.75±0.63d
C16:1 ω-7	4.31±0.34a	13.64±0.63b	6.24±0.69c	7.85±0.43d	10.68±0.52db	9.25±0.32db
C18:1 ω-9	19.16±0.24a	24.30±0.044b	18.76±0.29a	33.70±081c	23.94±0.62b	21.54±0.62ab
C20:1 ω-9	0.79±0.53a	2.10±0.47b	1.45±0.42c	2.31±0.35b	3.94±0.42d	1.45±0.26c
ΣM.U.F.A.	24.26±0.43a	40.04±1.21b	26.45±0.41a	43.86±0.31c	38.56±0.74b	32.24±0.048ab
C18:2 ω-6	11.57±0.73a	4.86±0.73b	4.51±0.52b	5.46±0.93c	5.94±0.87c	19.11±0.72d
C18:3 ω-3	5.00±0.39a	11.80±0.36b	4.59±0.25a	5.34±0.49a	8.35±0.38c	10.94±0.54b
C20:2 ω-6	3.82±0.32a	0.24±0.14b	0.54±0.24b	0.25±0.15b	0.16±0.06b	0.33±0.13b
C20:3 ω-6	-	0.24±0.11a	0.59±0.23b	0.28±0.11a	0.32±0.04ab	0.34±0.14ab
C20:4 ω-6	-	0.73±0.23a	2.05±0.31b	0.58±0.18a	1.05±0.35c	0.63±0.29a
C20:5 ω-3	3.80±0.33a	4.92±0.46b	8.53±0.22c	2.77±0.43d	6.56±0.34bc	4.20±0.25b
C22:5 ω-3	1.51±0.23a	1.42±0.17a	2.32±0.36b	0.73±0.13c	1.45±0.27a	1.53±0.21a
C22:6 ω-3	4.66±0.34a	2.44±0.22b	10.60±0.55c	1.61±0.25d	3.72±0.33ab	2.79±0.24b
ΣP.U.F.A	30.36±0.41a	26.65±0.58b	33.73±0.65a	17.02±0.41c	27.55±0.28b	39.87±0.43d
ω3	14.97±0.72a	20.58±0.83b	26.04±0.68c	10.45±.05d	20.08±1.05b	19.46±1.06b
ω6	15.39±0.90a	6.07±0.91b	7.69±0.96b	6.57±1.48b	7.47±0.46b	20.41±0.63c
ω3/ω6	0.97	3.39	3.38	1.59	2.68	0.95

* Means are the averages of 3 replicates

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

*** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

cally active substances eicosanoids, decreased significantly in January and decreased to the lowest level observed during the year.

It was found that the amounts of ω-3 FAs in liver total lipids were in the muscle tissue and that they was considerably higher than the amounts of ω-6 FAs in every period and that the amount reaches its maximum level in October. The ω-3/ω-6 ratio in this period was determined to be at its highest level due to the increase in the amount of ω-3. This ratio was between 2.73-4.82.

It was determined that the amount of liver ΣPUFA was higher than the amounts of ΣMUFA and ΣSFA in every season. Our results were consistent with the results obtained in previous studies which examined various freshwater fish, including *S. trutta macrostigma* (Akpınar *et al.,* 2009, Aras *et al.,* 2003b, Görgün and Akpınar, 2007). In some studies, on the other hand, it was found that the amount of liver ΣSFA was higher than the amounts of ΣMUFA and ΣPUFA (Aras *et al.,* 2003a, Bayır *et al.,* 2010). This difference is believed to have arisen from several factors such as species of the fish, living environment, the temperature of the water where it was caught, season, differences in the food chain, and the physiological status of the fish, such as gonad maturation, and reproduction during the year (Ackman, 1967).

In our study, we found that long-chain PUFAs varied more than SFAs. It was suggested that gonad maturation and spawning seasons directly affected these variations (Akpınar, 1986).

3.2. FA profile of the PL and TAG fraction of muscle and liver tissue

Tables 3 and 4 show the changes in the FA compositions of the muscle PL and TAG fractions of S. *trutta macrostigma* from the River Munzur by months.

The amount of ΣSFA in the muscle PL fraction of the trout examined in this study was found between 31.33-46.66%. Although it is a PL fraction, the amount of 16:0 with the highest percentage in ΣSFA ratio and SFAs was found to be quite high.

Similar findings were reported in all three types of trout, including *S. trutta macrostigma* from our study (Bayır *et al.,* 2010). Bayır *et al.* (2010), reported that the high 16:0 ratio in the PL fraction may have been caused by increased water temperature in summer and reproductive activity in autumn.

The amount of ΣMUFA in the PL fraction was between 10.76-16.21%. Just like ΣSFAs, the highest amount of ΣMUFA was found in the month of June. The lowest ΣMUFA amount was found in the month of January. ΣMUFAs, which were much lower than ΣPUFA and ΣSFA, and 18:1 ω-9 and 16:1 ω-7, did not vary much during the year.

The amount of ΣPUFA in the PL fraction was found to be 36.98-55.48%. The most common components in the ΣPUFAs were 20:5 ω-3 and 22:6 ω-3. The amount of ΣPUFA due to the decrease in the amount of these components was also low in June, when the air temperature was high compared to other periods.

ω-3 FAs in the PL fraction were significantly higher than ω-6 FAs in each season and were found to be the highest in the month January (spawning season). The ω-3/ω-6 ratio was between 1.65-13.77. The reason for the low rate of ω-3/ω-6 in October was that the amount of 18:2 ω-6 of ω-6 FAs in this period was quite high. The increase in the amount of ω-3/ω-6 in January was due to the increase in the amount of 20:5 ω-3 and 22:6 ω-3.

In the PL fraction, the highest amount was found in ΣPUFA and then in ΣSFA in all periods except June. The lowest amount was in ΣMUFA. Similar findings were obtained in studies examining other freshwater fish (Ackman *et al.,* 2002; Kayhan *et al.,* 2015). Another interesting finding was that the amount of 18:2 ω-6 was quite high in October compared to other periods.

Bayır *et al.* (2010) found that ΣMUFAs were present in the neutral lipid fraction in *S. trutta caspius*. However, the same researchers found the amount of ΣSFA in the PL fraction to be higher than the amount of ΣPUFA. This difference is thought to have arisen from the fact that the fish were caught from different water resources and accordingly the temperature values of their inhabiting areas changed, along with the fatty acid contents of the living things constituting their food. The high amount of ΣPUFA in our study is thought to have arisen from the fact that the water temperature of the River Munzur, where the fish were caught, was lower. It is known that the rate of unsaturated FAs increases especially in fish living in cold water because cells can change the lipid composition of their membranes to adapt to changing temperatures. For example, fish living in cold environments increase the unsaturated fatty acids in their phospholipids as an adaptation to prevent the cell membranes from solidifying in winter. Thus, when the degree of unsaturation of FAs increases, the melting point decreases (Çelik *et al.,* 2008).

The amount of ΣSFA in the muscle TAG fraction of the trout examined was found to be 27.75-45.30%. The lowest ΣSFA amount in the TAG fraction, as in the PL fraction, was found in the month of October. The highest amount of ΣSFA in TAGs was found in the month of November, which is pre-spawning season. This shows that the amount of ΣSFA in TAGs was not affected by the spawning season.

It was determined that the increase in the amount of ΣSFA in the muscle TAG fraction in November resulted from the increase in the amount of 14:0, 15:0, 17:0 and 18:0, rather than the amount of 16:0.

The amount of ΣMUFA in the muscle tissue TAGs of the examined trout was determined as 24.26-43.86%. 18:1 ω-9 and accordingly, the amount of ΣMUFA, was found at their peak in the month of June. The amount of 18:1 ω-9, which has the highest percentage among ΣMUFAs, showed irregular increases and decreases.

The TAG fraction ΣPUFA amount of *S. trutta macrostigma* was found as 17.02-39.87%. The most important reason for the increase in ΣPUFAs in October was that 18:2 ω-6 was found in this period, especially as in PL, in a very high amount. The change in this main fatty acid is thought likely to have arisen from food.

The amount of 18:2 ω-6, which was quite high in TAG in October, and accordingly, the amount of ω-6 FAs, were found to be quite high. This situation affected the ω-3/ω-6 ratio of the TAG fraction in the muscle tissue and the ω-3/ω-6 ratio was found to be lower than 1 in these periods.

The amounts of ΣSFA and ΣMUFA were higher than ΣPUFAs, except in October. This was due to the fact that the fish mostly store saturated and monounsaturated FAs (Kozlova and Khotimchenko, 2000).

In the TAG fraction of muscle tissue, the ω-3/ω-6 ratio was found at values between 0.95-3.39.

When the PL and TAG fractions of the muscle lipids of *S. trutta macrostigma* were compared, the ΣSFA percentages were expected to be higher in the TAG fraction than the PL. However, due to the fact that 16:0, which is the most important component of SFAs in the PL fraction of brown trout, was higher than expected, ΣSFA ratios in both fractions were found close to each other. This result was also found in the study carried out by Bayır *et al.* (2010), on three types of trout, including *S. trutta macrostigma*. From these results, we can affirm that the fact that the amount of 16:0 in the PL fraction was higher than TAG is characteristic of trout (Ackman *et al.,* 2002).

In freshwater fish, on the other hand, this component was found in greater amounts in the TAG fraction.

In our study, the amount of ΣMUFA and the fatty acids forming the ΣMUFA, 16:1 ω-7 and 18:1 ω-9, were higher in the TAG fraction than the PL fraction. The amounts of ΣPUFA and the 20:5 ω-3 and 22:6 ω-3 ratios of ω-3 FAs forming the ΣPUFA were found to be higher in the PL fraction. The increase in the ratios of these components caused an increase in the ω-3/ω-6 ratio in the PL. 18:2 ω-6 and 18:3 ω-3 rates, among other important PUFAs, were found to be higher in the TAG fraction compared to PL.

The fatty acids 20:5 ω-3, 22:5 ω-3 and 22:6 ω-3 abounds in PL, while 16:1 ω-7, 18:1 ω-9, 18:2 ω-6 and 18:3 ω-3 in the TAG fraction. The effect of nutritional lipids on the FA composition of body lipids differs between triacylglycerol and phospholipids. Studies have shown that the FA composition of phospholipids was more greatly affected than triacylglycerols. In freshwater fish, linoleic and linolenic acid, which are taken with food, are exposed to chain extension and their degree of unsaturation is increased. In this way, these fatty acids are transformed to AA, docosapentaenoic, and docosahexaenoic acids. Moreover, it was found that these FAs are involved in the structure of PLs and these components taken with nutrients are stored in triacylglycerols without being changed. Similar data have been previously reported for different types of trout and freshwater fish (Aras *et al.,* 2003; Aras *et al.,* 2003b; Bayır *et al.,* 2010).

The ΣSFA ratio in the liver PL fraction of the trout examined increased in April, June and July, when the temperature was relatively high. In liver PLs, as in muscle tissue, the most common FAs in SFA are 16:0 and 18:0. The changes in the amount of ΣSFA during the year were directly affected by changes in the amount of these FAs. ΣMUFA amounts were found between 16.87-25.42%. The highest amounts of 18:1 ω-9 and ΣMUFA were found in June and the lowest amounts of 18:1 ω-9 and ΣMUFA in October (Table 5).

The highest amount of ΣPUFA was found in October (51.05%) and the lowest amount of ΣPUFA was found in April (32.85%). Most of the fatty acids found in the PUFAs were 20:5 ω-3 and 22:6 ω-3 in PL, just like in the muscle tissue. The amounts of EPA, DHA, and ΣPUFA decreased in April, June, and July, which are warmer compared to other periods, and the amount of these components increased in the pre-spawning seasons, October and November when the temperature began to drop.

As in the muscle PL fraction, in October, the 18:2 ω-6 ratio of ω-6 FAs was found to be significantly higher in liver PL fraction than in other periods. It is thought that this increase was due to the FA contents of the aquatic organisms that the fish feed on during this period. In the PL fraction, ΣPUFA rates were significantly higher than ΣSFA and ΣMUFA in November, January and October; while the ΣMUFA rate was lower than ΣPUFA and ΣSFA.

ω-3/ω-6 ratio was found between 2.25-4.75. The decrease in the rate of ω-3/ω-6 in October was due to the fact that the amounts of ω-6 FAs 18:2 ω-6 were quite high in this period.

The amounts of EPA, DHA, and ΣPUFA decreased in April, June, and July, which are warmer than other periods; while the amount of these components increased in October and November, pre-spawning season, when the temperature began to decrease. Such a relationship between the temperature change and the

TABLE 5. Fatty acid composition in the phospholipid fraction of liver from male *S. trutta macrostigma* (% of total FA)*

Fatty acids	November (2009)	January (2010)	April (2010)	June (2010)	July (2010)	October (2010)
C14:0	1.38±0.32a**	1.77±0.21b	1.71±0.30b	1.39±0.27a	1.98±0.24c	0.68±0.14d
C15:0	0.41±0.06a	1.07±0.13b	0.40±0.21a	0.25±0.17c	0.32±0.08ac	0.24±0.03c
C16:0	21.37±0.96a	22.08±0.51a	29.06±1.04b	26.26±0.76c	30.07±1.11b	24.11±0.65a
C17:0	0.65±0.23a	0.26±0.09b	0.82±0.14c	0.50±0.11d	0.48±0.15d	0.49±0.06d
C18:0	7.46±0.32a	8.59±0.41b	10.72±0.53c	10.31±0.37c	8.48±0.42b	6.41±0.44ab
ΣS.F.A***	31.27±1.20a	33.77±0.98b	42.71±1.14c	38.71±1.24d	41.33±1.28c	31.93±1.20a
C16:1 ω-7	2.03±0255a	4.17±0.31b	2.80±0.39a	2.31±0.23a	3.75±0.29ab	1.93±0.19a
C18:1 ω-9	15.43±0.33a	17.63±0.51b	21.16±0.69c	22.17±0.76c	21.44±0.66c	14.66±0.65a
C20:1 ω-9	0.30±0.07a	0.68±0.12b	0.35±0.06a	0.55±0.13b	0.23±0.03c	0.28±0.09ac
ΣM.U.F.A.	17.76±0.96a	22.48±1.03b	24.31±1.09c	25.03±0.45d	25.42±0.54d	16.87±0.67a
C18:2 ω-6	2.68±0.51a	6.28±0.43b	3.43±0.33c	3.58±0.34c	1.82±0.29a	11.17±0.63d
C18:3 ω-3	2.31±0.10a	2.64±0.16b	1.91±0.21a	2.47±0.34ab	1.10±0.20c	2.50±0.19ab
C20:2 ω-6	0.41±0.14a	0.37±0.06ab	0.42±0.12a	0.34±0.05b	0.36±0.06ab	0.25±0.11c
C20:3 ω-6	0.25±0.10a	0.32±0.06a	0.41±0.11b	0.42±0.05b	0.57±0.06c	0.50±0.07c
C20:4 ω-6	5.51±0.16a	3.67±0.15b	4.82±0.33ab	4.83±0.20ab	3.53±0.21b	3.78±0.26b
C20:5 ω-3	10.43±0.45a	10.60±0.52a	7.45±0.32b	7.63±0.33b	7.49±0.43b	9.60±0.41a
C22:5 ω-3	3.06±0.13a	3.28±0.12a	2.46±0.15b	3.23±0.23a	3.25±0.10a	2.72±0.14b
C22:6 ω-3	26.24±0.64a	16.45±0.49b	11.95±0.38c	13.68±0.71c	15.02±0.44b	20.53±0.49d
ΣP.U.F.A.	50.89±1.23a	43.61±1.18b	32.85±0.98c	36.18±0.68d	33.14±054c	51.05±1.05a
ω3	42.04±0.99a	32.97±0.42b	23.77±0.38c	27.01±.65bc	26.86±0.72bc	35.35±0.59d
ω6	8.85±0.40a	10.64±0.63b	9.08±0.41ab	9.17±0.64ab	6.28±0.49c	15.70±0.93d
ω3/ω6	4.75	3.09	2.61	2.94	4.27	2.25

* Means are the average of 3 replicates

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

*** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

amount of ΣPUFA is a natural consequence. Farkas and Csenger (1976), stated that fish liver has the ability to adjust fatty acid biosynthesis very quickly to the appropriate temperature. Previous studies on various freshwater fish have reported that the amount of ΣPUFA in liver PL and TAG fractions was affected by temperature changes and increased with decreasing temperature (Bayır *et al.,* 2010). 18:2 ω-6 was found to be quite high in October compared to other periods. This increase is thought to have arisen from the FA contents of the aquatic organisms that the fish feed on during this period because 18:2 ω-6 is an essential ingredient in many other invertebrates, and in vertebrates, including fish. This is an ingredient that must be taken externally with nutrients.

The amount of ΣSFA in the TAG fraction of trout liver varied between periods depending on the increase in 16:0, which was between 28.94 and 54.65% (Table 6). The lowest amount of ΣSFA was detected in October; while the highest ΣSFA amount was detected in the month of November, pre-spawning season. It was determined that the amount of ΣSFA during the year was due to the increase in 16:0. However, the excessive increase in the amount of ΣSFA in November was caused by the excessive increase in 14:0 rather than 16:0. ΣMUFA amounts were found between 14.64-34.82%. In November, the pre-spawning period, the rate of 18:1 ω-9, which was the most abundant component in the percentage distribution, decreased by half compared to other periods. The rate of 16:1 ω-7, another MUFA component, decreased significantly in this period compared to other periods.

The amount of ΣPUFA was determined as 25.75-45.99%. As in the PL fraction, the highest amount of ΣPUFA was found in October. The most common fatty acids in the PUFAs were 18:2 ω-6, 18:3 ω-3, 20:5 ω-3 and 22:6 ω-3. FAs other than these formed a small proportion of the PUFAs. Just like in PL, the rate of 18:2 ω-6 in the TAG fraction was found to be significantly higher in October than in other periods.

The ratio of ω-3/ω-6 in the TAG fraction was determined as 0.49-2.98. Since the 18:2 ω-6 percentage of ω-6 FAs in October was higher in this period compared to other periods, it was found that the ratio of ω-3/ω-6 was at its lowest value during the year.

In the TAG fraction, the 18:2 ω-6 in October was significantly higher than in other periods. Hazel (1979) stated that the FAs in the liver triacylglycerol of *Oncorhynchus mykiss* increased due to the decrease in temperature of the FAs that form the ω-3 and ω-6 PUFAs. However, in our study, since 18:2 ω-6, one of the most important ω-6 FA components of PUFAs, was very high in October, PUFAs were found to be high in this period, and not in January when the temperature was the lowest.

CONCLUSION

As a result of the study, it was concluded that, compared to many other freshwater fish, the brown trout *S. trutta macrostigma* is a rich source of ω-3 and ω-6 polyunsaturated fatty acids with numerous benefits to human health, and accordingly the ω-3/ω-6 ratio. It can be said that consuming these fish as nutrients will have important effects on human health.

TABLE 6. Fatty acid composition in the triacylglycerol fraction of liver from male *S. trutta macrostigma* (% of total FA)*

Fatty acids	November (2009)	January (2010)	April (2010)	June (2010)	July (2010)	October (2010)
C10:0	-	-	-	0.38±0.05**	-	-
C12:0	-	0.43±0.12a	1.44±0.18b	0.41±0.15a	-	-
C13:0	-	0.36±0.12a	0.63±0.10b	-	-	0.77±0.08b
C14:0	14.38±0.34a	4.78±0.42b	4.37±0.31b	2.38±0.25c	3.06±0.42bc	1.39±0.24d
C15:0	2.48±0.26a	2.23±0.17a	1.05±0.35c	0.71±0.17c	1.05±0.11c	0.21±0.04d
C16:0	29.61±0.85a	24.40±0.25bc	24.80±0.36bc	26.77±0.42b	22.27±054c	21.80±0.42c
C17:0	1.68±0.23a	0.87±0.17b	1.10±0.21c	0.57±0.20d	0.52±0.14d	0.41±0.07d
C18:0	6.50±0.12a	7.47±0.31ab	6.00±0.43a	8.63±0.45b	5.37±0.30c	4.36±0.34c
ΣS.F.A***	54.65±1.20a	40.54±1.02b	39.39±1.05b	39.85±0.84b	32.27±0.68c	28.94±0.55d
C16:1 ω-7	2.38±034a	9.61±0.75b	8.15±0.49c	4.11±0.63d	8.20±0.54c	4.29±0.24d
C18:1 ω-9	11.46±0.51a	22.49±0.43b	24.28±0.39bc	26.27±0.45c	24.98±0.61bc	20.21±0.48b
C20:1 ω-9	0.80±0.10a	1.49±0.21bc	1.77±0.23b	1.11±0.08c	1.64±0.12b	0.61±0.33a
ΣM.U.F.A.	14.64±0.56a	33.59±0.46bc	34.20±0.49b	31.49±0.65c	34.82±0.54b	25.11±0.57d
C18:2 ω-6	4.95±0.48a	5.49±0.40b	4.93±0.31a	6.73±0.34c	5.10±0.30b	29.10±0.51d
C18:3 ω-3	3.19±0.20a	7.38±0.32c	4.71±0.14b	4.37±0.34b	3.60±0.32a	4.51±0.29b
C20:2 ω-6	0.72±0.14a	0.20±0.06b	0.34±0.04bc	0.41±0.05c	0.17±0.03b	0.28±0.12bc
C20:3 ω-6	2.25±0.22a	0.46±0.13b	0.24±0.07c	0.38±0.02bc	0.20±0.03c	0.23±0.05c
C20:4 ω-6	6.61±0.21a	0.99±0.25b	2.16±0.30c	2.69±0.24c	2.72±0.23c	1.25±0.12d
C20:5 ω-3	4.06±0.35a	6.05±0.50ab	7.31±0.22b	5.92±0.33ab	9.45±0.28c	4.48±0.36a
C22:5 ω-3	1.16±0.12a	1.60±0.10bc	1.50±0.25b	1.64±0.14bc	1.77±0.10c	1.24±0.13a
C22:6 ω-3	7.04±0.25ab	3.58±0.11d	5.15±0.35c	6.36±0.41a	9.74±0.46b	4.90±0.29c
ΣP.U.F.A	29.98±0.68a	25.75±0.57b	26.34±0.73b	28.50±0.52a	32.75±1.02c	45.99±1.05d
ω3	15.45±0.39a	18.61±0.65b	18.67±0.71b	18.29±0.49b	24.56±0.84c	15.13±0.51a
ω6	14.53±0.50a	7.14±0.35b	7.67±0.43b	10.21±0.35c	8.19±0.45b	30.86±0.40d
ω3/ω6	1.06	2.60	2.43	1.79	2.98	0.49

* Means are the averages of 3 replicates

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

*** **SFA:** saturated fatty acids, **MUFA:** monounsaturated fatty acids, **PUFA:** polyunsaturated fatty acids

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Effects of an integrated harvest system on the quality of olive fruit for small producers

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SUMMARY: Small producers confront specific challenges when they opt to produce high-quality olive fruit. Limited resources for investing in harvest machinery and manpower are the main reasons for continuing a traditional harvest method that puts the final product and its economic value at risk. This paper discusses the efficiency of an integrated harvest system as a possible solution to these specific challenges. The system is formed by a newly designed manual harvesting device and the use of a cooling room near the olive grove. Both systems were evaluated to assess their feasibility for optimum conditions before processing. The combined effect of the harvesting method and cold storage on the fruit characteristics (incidence of decay, skin color, weight loss, firmness, respiration, and ethylene production) was evaluated on three different varieties ('Arbequina', 'Picual' and 'Verdial') and four different storage times (0, 4, 8, and 14 days). The results indicate that the proposed harvesting method in combination with an appropriate cooling system offers an affordable alternative for obtaining fruit with the best physiological characteristics.

KEYWORDS: *Branch-shaker; Cold-storage; Inverted umbrella; Olea europaea; Fruit quality*

RESUMEN: ***Efectos de un sistema integrado de recolección para pequeños productores en la calidad de la aceituna recogida***. Los pequeños productores se enfrentan a retos específicos, cuando optan por producir aceitunas de alta calidad. La imposibilidad económica de invertir, tanto en maquinaria de cosecha, como en mano de obra es la razón principal que obliga a continuar una recolección tradicional que pone en riesgo el producto final y su valor económico. Este estudio analiza la eficiencia de un sistema de recolección integrado como una posible solución a estos desafíos específicos. El sistema está formado por un dispositivo de recogida manual de nuevo diseño y el uso de una cámara de frío en la finca. El efecto combinado del método de recolección presentado y el almacenamiento en frío sobre las características de la fruta (incidencia de podredumbre, color de piel, pérdida de peso, firmeza, respiración y producción de etileno) se evaluó en tres variedades diferentes ('Arbequina', 'Picual' y 'Verdial') y diferentes tiempos de almacenamiento (0, 4, 8, 14 días). Los resultados indican que la combinación de un método de recolección mecánica y un sistema de enfriamiento adecuado ofrece una alternativa económicamente asequible para obtener frutos con las mejores características fisiológicas.

PALABRAS CLAVE: *Calidad del fruto; Conservación en frío; Olea europaea; Paraguas invertido; Vibrador de rama*

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1. INTRODUCTION

While it is well-know that Spain stands out as the major producer of olive oil and table olives in the world, it is scarcely mentioned that more than three-quarters of this cultivated area of 2,5 x 10^6 ha consists of plots of less than 10 ha (Cátedra Caja Rural de Jaén, 2020; Junta de Andalucía. Consejería de Agricultura, Pesca y Desarrollo Rural, 2015). This high degree of fragmentation also occurs in Andalusia, where 60% of the national production is concentrated (Colombo and Villanueva, 2018). The majority of these producers are members of cooperative mills to which they sell their fruit. Few of them produce their own oil. A shift towards producing more quality olive oil instead of aiming to maximize the quantity is becoming eminent in Spain, and as a consequence, the availability of fresh undamaged fruit has come to the fore as a critical factor for many mills (Rallo *et al.*, 2018). Evaluating the quality of the fruit is rapidly becoming standard procedure and experiments with automated assessment tools are under development (Puerto *et al.*, 2015; Navarro Soto *et al.*, 2018; Aguilera Puerto *et al.*, 2019). The impact of this shift on the producer's side is straightforward: a better quality means a better price. Meanwhile, a growing number of independent small producers aim to produce their own 'high end' or 'Premium' oil. For them, the necessity to optimize the harvest conditions are even more important regardless of whether they produce their own oil or, as is more often the case, they bring their olives to a private mill where they are processed as a custom-made batch.

Despite the differences between both types of small producers, they are confronted with peculiar constraints. First of all, they are limited in resources when it comes to investing in specialized harvesting machinery or to contract expensive harvesting services. The absence of adequate technology makes the use of manual collection methods (manual beating, shaker combs, or branch shakers) with nets placed under the trees still widespread (Serrano *et al.*, 2012). This traditional method entails the risks of damaging the fruit, be it by the dragging of the nets over the ground or by stepping on the fallen fruit during harvesting (Famiani *et al.*, 2020). Secondly, the labor capacity consists very often of family members. This limited work capacity on the farm determines how long it will take to obtain enough fruit to carry out the transport. As a consequence, storage may be delayed by several days before this amount is reached. Besides, the Andalusian olive farmer who opts to produce his own oil in a private mill has to deal with the requirement of delivering a minimal quantity of various tons before the fruit will be processed as a separate batch.

These factors threaten the production of high-quality olive fruit. Fruit damaged during harvesting directly jeopardizes the quality of the processed oil, while prolonged storage in trailers means rapid deterioration due to the proliferation of fungi and bacteria. As a consequence, after being processed in the mill, only virgin olive oils which are not of optimal quality are produced, which irrevocably reduces their profitability (García and Yousfi, 2006).

Recently, a new type of harvesting device has come to the foreground as a possible solution for small producers to optimize their recollection. It basically consists of a manually displaceable structure with an inverted umbrella that can be placed around the trunk and a system to collect the fallen fruit into a box. Instead of trunk shakers, more economic branch shakers are used to detach to fruit. Various models already exist in the market (Bosco, Olitree, Crendon Machinery), of which some even have a motorized part to facilitate moving the device. Nevertheless, several problems in the handling of the device persist (access to the tree, excess of weight, handling of the boxes), while at the same time its efficiency has not been studied until recently. Plasquy *et al.* (2019) compared the efficiency of a prototype with the traditional recollection method with nets, contrasting different work crews, and the number of branch shakers used. The results showed the importance of the composition of the harvest crew and clarified that when the harvest was done by four operators, of which two used branch shakers, the efficiency of working with a MIU as compared to the use of nets was not significantly different.

To tackle the second major challenge, namely the avoidance of the detrimental process, cold storage of the fruit has been proposed as a possible solution, allowing for reasonable maintenance of its physiological integrity, without harming the quality of the extracted oils because it depends directly on the quality of the fruit from which it comes (Canet and García, 1999). However, besides a large-scale experiment, the technique has not been implemented on an industrial level due to the logistic and infrastructural difficulties that arise when handling large quantities of fruit daily (García *et al.*, 1996). The use of cold storage at the farm level has not been documented nor evaluated thus far. This work presents such a cooling room, designed to store up to 5000 kg at 5 °C at the farm and evaluates its effects on the quality of the fruit, taking into account the aforementioned harvesting methods.

2. MATERIALS AND METHODS

2.1. Location and design

The fruit for the experiment was harvested on a farm in Bollullos Par del Condado in 2017 (Huelva, Andalusia, Spain). The olive grove covers 8 ha and includes 1200 trees, primarily of the cultivars 'Arbequina', 'Picual', and 'Verdial'. The trees were planted between 2005 and 2007 at distances of 6 x 7 m and irrigated according to a deficient regimen.

The 'Arbequina' fruit was collected in the second week of October (mean color index (CI): 34.5); the 'Picual' fruit in the first week of November (CI: 28.5); and the 'Verdial' fruit in the last week of November (CI: 20.81). To simultaneously evaluate the effects of the collection and conservation methods on fruit quality, 40 kg of olives were harvested with each collection method, namely a Manual Inverted Umbrella (MIU), referred to as Recollection Method 1 (R1), and the traditional method with nets, Recollection Method 2 (R2). From this 40 kg set, half was randomly taken and placed in a refrigerated room at 5 ºC, referred to as Storage Method 1 (S1); while the other half was stored indoors, but outside the refrigerator at room temperature, Storage Method 2 (S2), as is usually done with olives before processing. The physiological characteristics of the olives were evaluated at times: 0, 4, 8, and 14 days of storage (ST).

The MIU was designed to catch and collect the olives into boxes, preventing them from touching the ground (Figure 1a). A detailed description of the device can be found in Plasquy *et al.* (2019). The boxes where the picked olives were collected have a capacity of 20 kg, placed under the umbrella, and removed when full using a system of pulls and ropes. The harvesting team consisted of two operators who detached the fruit with branch shakers and two men who handled the MIU and the boxes. The traditional way of harvesting (R2) to which the R1 was compared, used nets that were placed under the tree (Figure 1b). Two operators with branch shakers stepped on the nets and moved around the tree to detach the fruit. Once picked, the nets with the fallen fruit were dragged to the next tree and spread open by two other men after which the operators continued to detach the fruit with the branch shakers. This continued until the nets became too heavy to displace them to the next tree. At that moment the collected olives were transferred into the same type of boxes used in R1.

The cooling room was installed in an existing outhouse on-site at the farm (Figure 1c). The existing construction measured 4.3 x 4.4 m on the ground floor. The inclination

Figure 1. a. Harvesting with the use of the Manual Inverted Umbrella and branch shakers (R1); b. Conventional harvest with nets placed on the ground and using branch shakers (R2); c. Cooling room installation in a barn at the farm.

of the roof served to install a ceiling at 2.5 m, after which two separate spaces were created. Behind the existing barn gate of 3.0 m width, a separation wall was mounted with 10-cm thick extruded polystyrene sandwich insulating panels and a cooling door of 1,2 m width. The inside walls and the ceiling were isolated with insulating panels of 10 cm. The floor was covered with the same panels and finished with wooden boards of 2.5 cm (OSB) and a rubber protector. On the upper level a cold group was placed, formed by a compressor (EMBRACO, UNJ 9232) and the evaporator (LU-VE, SHDN 25-80) was mounted on the ceiling of the cooling room. A thermostat was mounted on the outside to have visual control of the inside temperature. The capacity of the cooling room amounted to 6.000 kg. The system was able to keep the chamber at 5 ºC (± 1 °C). To maintain this temperature, the compressor started every 5 to 10 min and ran for approximately 4 min. The cost to install the cooling room amounted to 2.500 €, including the purchase and installation of the refrigerating group by a technician, the isolation panels, the iron structure, and the finishing of the floor. The energy consumption amounted to 16 kWh, taking into account a consumption of 2 kW for the different motors present, working one-third of the time.

2.2. Fruit characteristics

2.2.1. Incidence of decay

The incidence of decayed fruit was quantified (bruised fruit or with visible fungal infection). Triplicate samples of 100 olives were randomly selected at the beginning of the experiment (Time 0) for each collection method and were kept in S1 or S2 throughout the storage period, with the same samples being used to quantify this parameter on each sampling date.

2.2.2. Weight loss

Tetraplicate samples of 100 olives were randomly selected at the beginning of the experiment (Time 0) for each recollection method and placed in four plastic baskets which were individually weighed on each sampling date with a precision of 0.1 mg and kept in S1 or S2 throughout the storage period.

2.2.3. Skin color and firmness

The color of the fruit was determined on the equatorial zone of the same 100 olives of each variety and each one of the 4 treatments, on each sampling date, using a Minolta CR400 (Minolta Camera Co., Osaka, Japan) chroma-meter with a measuring area of 8 mm in diameter, diffuse illumination and a viewing angle of 0º. The CIE L*a*b* color notation system was applied to determine the parameters L*, a* and b*; where L* indicates lightness, a* means the color axis from green to red, and b* the blue-yellow tone. Using these parameters, a color index was calculated according to the formula: $CI= L^*(b^*-a^*)/100$, which is strongly correlated to olive de-greening during cold storage (Castellano *et al.*, 1993).

Initially, 100 olives were randomly selected for each of the 4 treatments and kept in S1 or S2 throughout the storage period in plastic baskets, using the same replicate of 100 olives in each sample. Firmness measurements were taken randomly in the equatorial zone of each olive, using a Zwick 3300 non-destructive densimeter (Zwick GmbH & Co., Ulm, Germany) with the pressure of a 5 mm diameter disk. The results were expressed in N.

2.2.4. Respiration rate and ethylene production

Triplicates of 30 g olives were randomly taken from the 20 kg box of each treatment and placed in 125-mL open glass jars at 20 ºC for 2 h. Subsequently, they were hermetically sealed for 3 h at the same temperature. The CO_2 contents of the headspace of these jars were determined with a G100 portable gas analyzer (Geotechnical Instrument Ltd., Leamington Spa, UK) and the ethylene content was subsequently evaluated using an ICA portable ethylene analyzer (International Controlled Atmosphere Ltd., Paddock Wood, UK). CO_2 and ethylene concentrations were expressed in mL/kg h and μL/kg h, respectively.

2.3. Statistical analysis

For each cultivar, decay incidence, weight loss, firmness, respiration rate, and ethylene production were analyzed by one-way ANOVA to determine the effect of the four treatments at each storage time (ST). The same variables were analyzed by two-way ANOVA to determine the effect of the treatments and the ST, and by three-way ANOVA to detect the effect of the three independent factors (four ST, two R, and two S). When a significant ($P < 0.05$) effect due to treatment or ST was detected, the Tuckey test was applied to distinguish mean values ($P < 0.05$).

The parameters of skin color did not show normal distributions and were not studied using parametric tests. Each value was identified by the mean value of 100 replicates ± standard deviation (SD). The nonlinear curve fit of the histograms of the IC (OriginPro 9.1.0, OriginLab Corp. Northampton, USA) are presented for 0 and 14 storage days in the four treatments tested (two R and two S) in each cultivar.

3. RESULTS

3.1. Incidence of decay

In all the studied cultivars both the ST and the use of different treatments affected decay incidence in one way at least, which was very significant (P <0.01), considering all the ST and each sampling date independently for this period (Figure 1). During the progress of the ST, the values for this variable increased differently according to the treatment applied. The recollection method turned out to be decisive for the changes in decay incidence in the stored fruit. Thus, the treatments using the R1 showed significantly lower decay than the harvest using nets, regardless of the storage temperature or the cultivar tested. The cold storage also showed an effect on 'Arbequina' and 'Picual' cultivars for this parameter. 'Verdial' olives presented no significant differences due to the different storage temperatures. However, systematically, the treatments with cold storage obtained lower decay values than their counterparts. The interaction between the R and the S only caused an effect on 'Arbequina' olives for this variable. During the ST decay incidence significantly increased in all the treatments and cultivars. However, each variety showed different behavior in this period. Whereas in 'Arbequina' fruit the differences between the treatments R2S2 and the rest increased throughout this period. The differences among the rest of combined treatments decreased and were not significant from the third sampling date (day 8). Similarly, the differences in decay incidence in 'Picual' olives between R2S2 and the other treatments increased over the examined storage time. The three lasting treatments stayed akin with respect to the decay incidence until R1S1 exhibited the significantly lowest values for this parameter at day 14. In contrast, 'Verdial' fruit was affected by R throughout the entire storage period, except for the last sampling date (day 14), when, as for 'Picual' olives, the treatment R1S1 showed the significantly lowest values for this parameter.

3.2. Weight loss

The effect on this parameter was extremely significant for the three varieties (P < 0.001) according to the treatments applied, the progress of storage time, and the interactions of both factors (Table 1). As in the case of decay incidence, the values for this variable increased differently

TABLE 1. Weight loss (%) of olive fruit under four different treatments (T) with two different collection methods (R1: Manual Inverted Umbrella; R2: nets), and two storage methods (S1: 5 °C; S2: room temperature), during storage time (ST): 0, 4, 8, 14 days for 'Arbequina', 'Picual' and 'Verdial' cultivars. Each value indicates the mean value of 4 replicates ± standard deviation.

DAY	R	S	Weight Loss (%)		
			ARBEQUINA	**PICUAL**	**VERDIAL**
0	1	1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	2	1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4	1	1	0.4 ± 0.1 b*	0.6 ± 0.1 b	0.3 ± 0.1 b
		2	0.9 ± 0.0 a	2.6 ± 0.4 a	1.8 ± 0.7 a
	2	1	0.4 ± 0.1 b	0.5 ± 0.1 b	0.5 ± 0.1 b
		2	0.9 ± 0.0 a	2.2 ± 0.1 a	1.3 ± 0.2 a
8	1	1	0.8 ± 0.2 bc	1.2 ± 0.3 b	0.6 ± 0.1 c
		2	1.5 ± 0.4 a	4.6 ± 0.9 a	3.1 ± 1.3 a
	2	1	0.7 ± 0.1 c	1.0 ± 0.2 b	1.0 ± 0.2 c
		2	1.3 ± 0.2 ab	4.1 ± 0.3 a	2.2 ± 0.3 b
14	1	1	1.5 ± 0.4 b	2.0 ± 0.5 b	1.0 ± 0.1 c
		2	3.1 ± 0.8 a	7.3 ± 1.4 a	5.0 ± 2.2 a
	2	1	1.3 ± 0.2 b	1.6 ± 0.2 b	1.7 ± 0.3 c
		2	2.6 ± 0.3 a	6.4 ± 0.7 a	3.5 ± 0.6 b
effect of ST		0.000	0.000	0.000	
effect of T		0.000	0.000	0.000	
effect of ST x T		0.000	0.000	0.000	
effect of R		0.076	0.081	0.268	
effect of S		0.000	0.000	0.000	
effect of ST x R		0.361	0.352	0.876	
effect of ST x S		0.000	0.000	0.000	
effect of R x S		0.579	0.254	0.066	
effect of ST x R x S		0.876	0.903	0.170	

* For each storage day, the values for different combinations of treatments followed by different lowercase letters are significantly different according to the Tukey test (P < 0.05). Absence of letter means no significant effect due to treatment according to one-way ANOVA (P < 0.05).

Table 2. Skin color (L*, a*, b*, IC values) for ‘Arbequina’, ‘Picual’ and ‘Verdial’ cultivars, collected with two different collection methods (R1: Manual Inverted Umbrella; R2: nets) and two storage methods (S1: 5 °C; S2: room temperature) during storage time (0, 4, 8, 14 days). Each value indicates mean value (N = 100) ± SD.

CULTIVAR	DAY	R	S	L	a	b	IC
ARBEQUINA	0	1	1	65 ± 8	-15 ± 8	36 ± 10	34 ± 12
			2	64 ± 8	-14 ± 9	35 ± 10	33 ± 13
		2	1	65 ± 6	-16 ±7	38 ± 8	36 ± 10
			2	64 ± 8	-15 ± 9	38 ± 10	35 ± 13
	4	1	1	64 ±9	-14 ± 9	37 ± 10	34 ± 13
			2	62 ± 9	-13 ± 11	34 ± 11	31 ± 14
		2	1	64 ± 7	-15 ± 8	38 ± 8	35 ± 10
			2	64 ± 7	-16 ± 8	39 ± 9	36 ± 12
	8	1	1	63 ± 9	-13 ± 9	36 ± 10	32 ± 12
			2	60 ± 11	-10 ± 13	32 ± 13	28 ± 16
		2	1	64 ± 7	-15 ± 8	39 ± 8	36 ± 11
			2	62 ± 9	-15 ± 9	38 ± 10	34 ± 13
	14	1	1	63 ± 9	-14 ± 9	37 ± 11	34 ± 13
			2	49 ± 11	1 ± 12	22 ± 13	13 ± 15
		2	1	63 ± 8	-15 ± 9	39 ± 9	35 ± 12
			2	52 ± 10	-2 ± 11	27 ± 11	17 ± 13
PICUAL	0	1	1	62 ±11	-9 ± 12	33 ± 11	28 ± 15
			2	62 ± 11	-9 ± 12	33 ± 11	28 ± 15
		2	1	60 ± 11	-10 ± 13	34 ± 12	29 ± 15
			2	60 ± 11	-10 ± 13	34 ± 12	29 ± 15
	4	1	1	59 ±13	-7 ± 15	31 ± 14	26 ± 17
			2	56 ± 15	-5 ± 16	27 ± 15	22 ± 19
		2	1	59 ± 13	-9 ± 15	33 ± 13	28 ± 17
			2	57 ± 14	-7 ± 15	29 ± 14	24 ± 18
	8	1	1	59 ± 14	-8 ± 15	31 ± 14	26 ± 18
			2	51 ± 16	-2 ± 16	23 ± 16	18 ± 19
		2	1	56 ± 14	-6 ± 16	31 ± 15	25 ± 19
			2	52 ± 16	-3 ± 16	24 ± 15	19 ± 19
	14	1	1	56 ± 15	-5 ± 16	29 ± 15	23 ± 19
			2	44 ± 12	2 ± 10	18 ± 11	10 ± 13
		2	1	55 ± 15	-5 ± 17	30 ± 16	23 ± 20
			2	49 ± 16	-1 ± 15	22 ± 16	16 ± 18
VERDIAL	0	1	1	60 ± 11	-4 ± 18	28 ± 15	21 ± 19
			2	60 ± 11	-4 ± 17	28 ± 15	21 ± 7
		2	1	60 ± 11	'-4 ± 17	28 ± 15	21 ± 19
			2	59 ± 11	-4 ± 17	11 ± 0	21 ± 19
	4	1	1	59 ± 12	-4 ± 18	28 ± 15	21 ± 19
			2	54 ± 14	-3 ± 13	24 ± 13	15 ± 13
		2	1	58 ± 12	-4 ± 16	28 ± 15	21 ± 19
			2	57 ± 13	-4 ± 16	26 ± 14	18 ± 16
	8	1	1	59 ± 12	-6 ± 14	27 ± 15	20 ± 15
			2	50 ± 15	-3 ± 12	15 ± 8	9 ± 8
		2	1	57 ± 13	-6 ± 16	30 ± 15	20 ± 13
			2	53 ± 14	-6 ± 15	18 ± 10	14 ± 13
	14	1	1	58 ± 12	-4 ± 18	31 ± 11	21 ± 16
			2	44 ± 13	8 ± 14	14 ± 13	5 ± 14
		2	1	56 ± 14	-6 ± 14	28 ± 15	20 ± 17
			2	47 ± 14	6 ± 12	16 ± 13	7 ± 13

during storage according to the combination of treatments applied. However, for this parameter, the storage temperature was the only factor that exerted a significant effect on the weight loss of the fruit, regardless of the cultivar tested. Cold storage significantly delayed the increase in this parameter during storage and this effect was increased significantly as the storage time became greater.

3.3. Skin color

According to chromatic values (L*, a* and b*) cold storage delayed fruit ripening. This effect was most evident in the olives, 'Picual' and 'Verdial', which exhibited similar variations in these chromatic parameters from the second sampling date after 4 storage days, and then the differences increased compared to the cold-stored ones, which only showed slight differences from the initial values (Table 2). The 'Arbequina' olives maintained similar color parameters over 8 days of storage, regardless of the harvest or storage methods. However, after 14 days of storage, the olives stored under ambient conditions presented obvious changes in these parameters. While L* and b* values decreased, a* increased. This means that the skin of this fruit was darker, red and blue and less shiny, green and yellow. Consequently, these olives exhibited a darker purple (red + blue) skin color than those stored at 5 °C.

The non-linear curve fits of the CI histogram values obtained from the different treatments in the three cultivars tested after 0 and 14 storage days showed that during the storage period the fruit was separated into three groups (Figures 2, 3, 4). This three-modal profile evidenced that these distributions cannot be statistically evaluated by parametrical tests to know whether the effect of the different factors is significant. However, these histograms showed clear evidence that after 14 days of storage, the treatments kept at ambient temperature, regardless of the method used for harvesting, exhibited a higher frequency in the group with the lowest values for this parameter; whereas the cold-stored ones maintained higher frequency values in the group with the highest values for this chromatic formula. As CI diminished according to the progression of fruit ripening, it seems clear that the use of cold storage delayed this process.

3.4. Firmness

Similarly, as in the cases of weight loss and incidence of decay, the fruit of the 'Picual' and 'Verdial' cultivars tested showed similar behavior in changes in firmness, and were affected in an extremely significant way by the treatments applied, storage time, and interactions between these two factors. In the case of 'Arbequina' the effect of the treatment was only significant at day 14. (Table 3). All the fruit exhibited a decrease in the firmness parameter during storage time, but it was more evident in the fruit stored at ambient temperature, regardless of the method

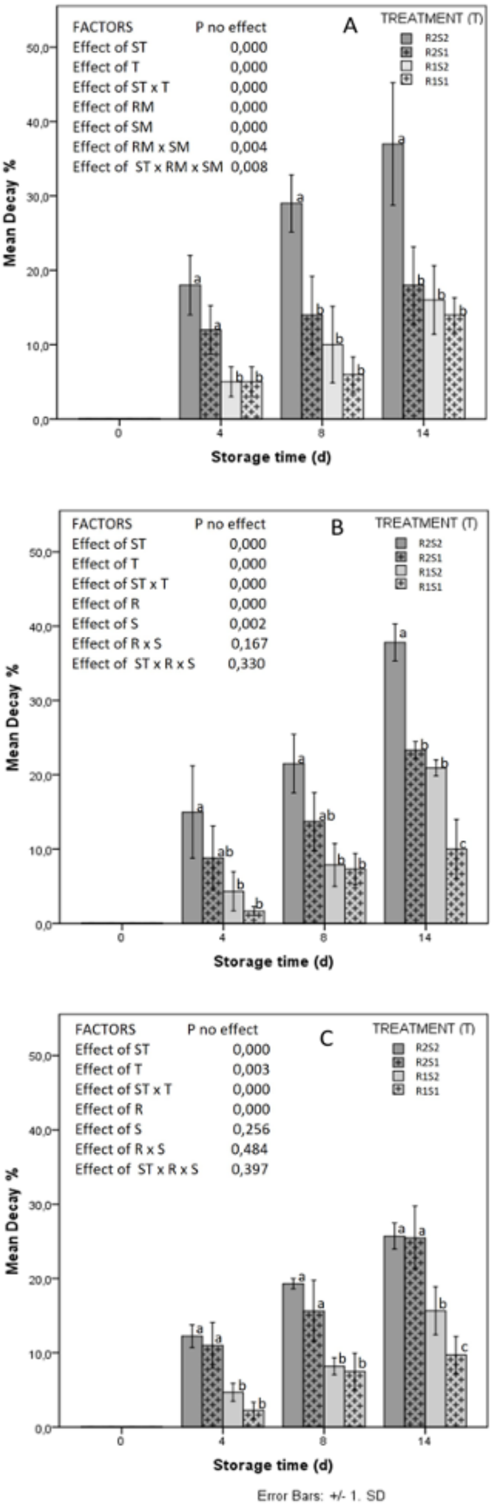

Figure 2. Decay incidence (%) of olive fruit under four different treatments (T) with two different collection methods (R1: Manual Inverted Umbrella; R2: nets) and two storage methods (S1: 5 °C; S2: room temperature) during storage time (ST: 0, 4, 8, 14 days) for 'Arbequina' (A), 'Picual' (B) and 'Verdial' (C) cultivars. Each point indicates the mean value (bar) ± SD (error bar) of 3 replicates. In each cultivar and each ST the bars with different lowercase letters are statistically different according to the Tuckey test (P < 0.05).

Table 3. Firmness of 'Arbequina', 'Picual' and 'Verdial' cultivars, collected with two different collection methods (R1: Manual Inverted Umbrella; R2: nets) and two storage methods (S1: 5 °C; S2: room temperature) during storage time (ST): 0, 4, 8, 14 days. Each value indicates mean value (N = 100) ± SD.

DAY	R	S	Firmness (N)		
			ARBEQUINA	PICUAL	VERDIAL
0	1	1	46 ± 3	44 ± 5	46 ± 2
		2	46 ± 3	44 ± 5	45 ± 3
	2	1	46 ± 3	44 ± 5	45 ± 3
		2	46 ± 3	44 ± 5	44 ± 4
4	1	1	44 ± 3	44 ± 4 a*	42 ± 4 ab
		2	43 ± 3	41 ± 4 b	41 ± 2 b
	2	1	45 ± 2	44 ± 5 a	43 ± 4 a
		2	43 ± 3	41 ± 4 b	38 ± 3 c
8	1	1	43 ± 3	43 ± 5 a	43 ± 5 a
		2	40 ± 3	35 ± 4 b	41 ± 3 b
	2	1	42 ± 2	43 ± 5 a	44 ± 4 a
		2	41 ± 2	39 ± 5 b	40 ± 4 b
14	1	1	42 ± 3 a	42 ± 6 a	44 ± 3 a
		2	36 ± 3 b	30 ± 8 b	40 ± 6 b
	2	1	42 ± 3 a	43 ± 6 a	42 ± 4 ab
		2	36 ± 3 b	35 ± 6 b	39 ± 6 b
effect of ST		0.000	0.000	0.000	
effect of T		0.957	0.000	0.000	
effect of ST x T		0.000	0.000	0.000	
effect of R		0.034	0.000	0.488	
effect of S		0.000	0.000	0.000	
effect of ST x R		0.786	0.000	0.238	
effect of ST x S		0.006	0.001	0.000	
effect of R x S	0.786	0.000	0.238		
effect of ST x R X S	0.006	0.001	0.000		

* For each storage day, the values for different combinations of treatments followed by different lowercase letters are significantly different according to the Tukey test (P < 0.05). Absence of letter means no significant effect due to treatment according to one-way ANOVA (P < 0.05).

used for harvesting. The firmness of 'Verdial' fruit was not affected by the R. Both 'Arbequina' and 'Picual' fruit firmness harvested by R1 showed significantly higher mean values for this parameter than the R2 harvested fruit. Cold storage allowed for maintaining significantly higher mean values for firmness in the fruit of the three cultivars. As storage time progressed, the effect of factors R and S increased their effects on fruit firmness. The 'Arbequina' and 'Verdial' olives did not show a significant effect of R or storage time. However, both cultivars, as well as 'Picual' were significantly affected by the combined effect of the three factors (ST, R, and S).

3.5. Respiration rate and ethylene production

The production of CO_2 followed the same profile as the other studied parameters, showing a highly significant effect due to storage time, the combination of treatments applied, and the interaction of both of them (Table 4). Storage method resulted in the most important cause of variability for this parameter for the three varieties considered. This effect increased with the progression of the storage time. The fruit stored at 5 ºC showed a significantly higher respiration rate than the one stored at ambient temperature when this parameter was tested at 20 ºC. This effect increased

Table 4. Respiration (mLCO2/kg h) and Ethylene production (uL/kg h) of 'Arbequina', 'Picual' and 'Verdial' cultivars collected with two collection methods (R1: Manual Inverted Umbrella; R2: nets) and two storage methods (S1: 5 °C; S2: room temperature) during storage time (0, 4, 8, 14 days). Each value indicates the mean value of 3 replicates ± SD.

DAY	R	S	ARBEQUINA		PICUAL		VERDIAL	
			CO_2	Ethylene	CO_2	Ethylene	CO_2	Ethylene
0	1	1	263 ± 62	10 ± 1	308 ± 17	19 ± 2 b	653 ± 126	19 ± 2
		2	263 ± 62	10 ± 1	308 ± 17	19 ± 2 b	653 ± 126	19 ± 2
	2	1	343 ± 12	22 ± 7	283 ± 94	39 ± 5 a	488 ± 153	34 ± 14
		2	343 ± 12	22 ± 7	283 ± 94	39 ± 5 a	488 ± 153	34 ± 14
4	1	1	150 ± 45	11 ± 1	163 ± 53 b	18 ± 1 b	405 ± 63 a	10 ± 1
		2	128 ± 14	11 ± 2	226 ± 8 b	21 ± 1 b	150 ± 50 b	11 ± 0
	2	1	233 ± 100	16 ± 2	442 ± 42 a	24 ± 8 ab	383 ± 105 a	15 ± 5
		2	214 ± 76	21 ± 8	188 ± 47 b	45 ± 16 a	138 ± 21 b	15 ± 0
8	1	1	78 ± 11	19 ± 1	324 ± 111 a	16 ± 2 c	354 ± 23 a	7 ± 1 c
		2	88 ± 30	20 ± 3	117 ± 40 b	20 ± 2 c	61 ± 11 c	24 ± 4 a
	2	1	156 ± 50	22 ± 6	371 ± 34 a	48 ± 0 b	300 ± 42 a	15 ± 4 b
		2	118 ± 23	25 ± 7	45 ± 1 b	86 ± 4 a	190 ± 5 b	30 ± 1 a
14	1	1	291 ± 20 a*	14 ± 1	186 ± 27	11 ± 2 b	381 ± 50 a	11 ± 1 c
		2	137 ± 31 b	21 ± 3	152 ± 63	34 ± 8 a	181 ± 56 b	46 ± 9 b
	2	1	378 ± 55 a	22 ± 2	160 ± 56	21 ± 8 ab	426 ± 19 a	16 ± 3 c
		2	84 ± 5 b	26 ± 8	136 ± 31	39 ± 10 a	272 ± 10 b	100 ± 18 a
effect of ST	0.000	0.001	0.000	0.000	0.000	0.000		
effect of T	0.000	0.000	0.000	0.000	0.000	0.000		
effect of ST x T	0.000	0.469	0.000	0.000	0.003	0.000		
effect of R	0.000	0.000	0.343	0.000	0.420	0.000		
effect of S	0.000	0.063	0.000	0.000	0.000	0.000		
effect of ST x R	0.279	0.170	0.007	0.000	0.007	0.001		
effect of ST x S	0.000	0.585	0.000	0.001	0.004	0.000		
effect of R x S	0.094	0.742	0.002	0.002	0.211	0.014		
effect of ST x R x S	0.218	0.669	0.003	0.001	0.497	0.000		

* Each storage day, for each variable the values for different treatments followed by different lowercase letters are significantly different according to the Tukey test ($P < 0.05$). Absence of letter means no significant effect due to treatment according to one-way ANOVA ($P < 0.05$).

with the progression of storage time. Only 'Arbequina' olives showed a significant effect due to R, which did not significantly interact with the progression of storage time. From the beginning of the storage period, the fruit harvested with R2 exhibited a higher respiration rate than the others. 'Picual' olives turned out to be especially sensitive to the combinations of the factors tested, but was not affected by the individual action of the different R.

All the varieties were affected at least very significantly ($P < 0.01$) in the amount of synthesized ethylene due to storage time and the four different treatments applied. Except for 'Arbequina' olives, which did not show significant differences in the amounts of synthesized ethylene induced by the combination of the effect due to storage time and treatment, the rest of the varieties were also affected by the interaction of these two factors. The recollection was mainly responsible for these effects. For all the varieties tested, the fruit harvested with nets showed higher amounts of synthesized ethylene than when picked with MIU. The storage determined an extremely significant effect on the amount of synthesized ethylene ($P < 0.001$) in 'Picual' and 'Verdial' olives, while in 'Arbequina' olives this effect was very near to being significant ($P < 0.063$). 'Picual' and 'Verdial' fruit was also, at least, significantly ($P < 0.05$) affected

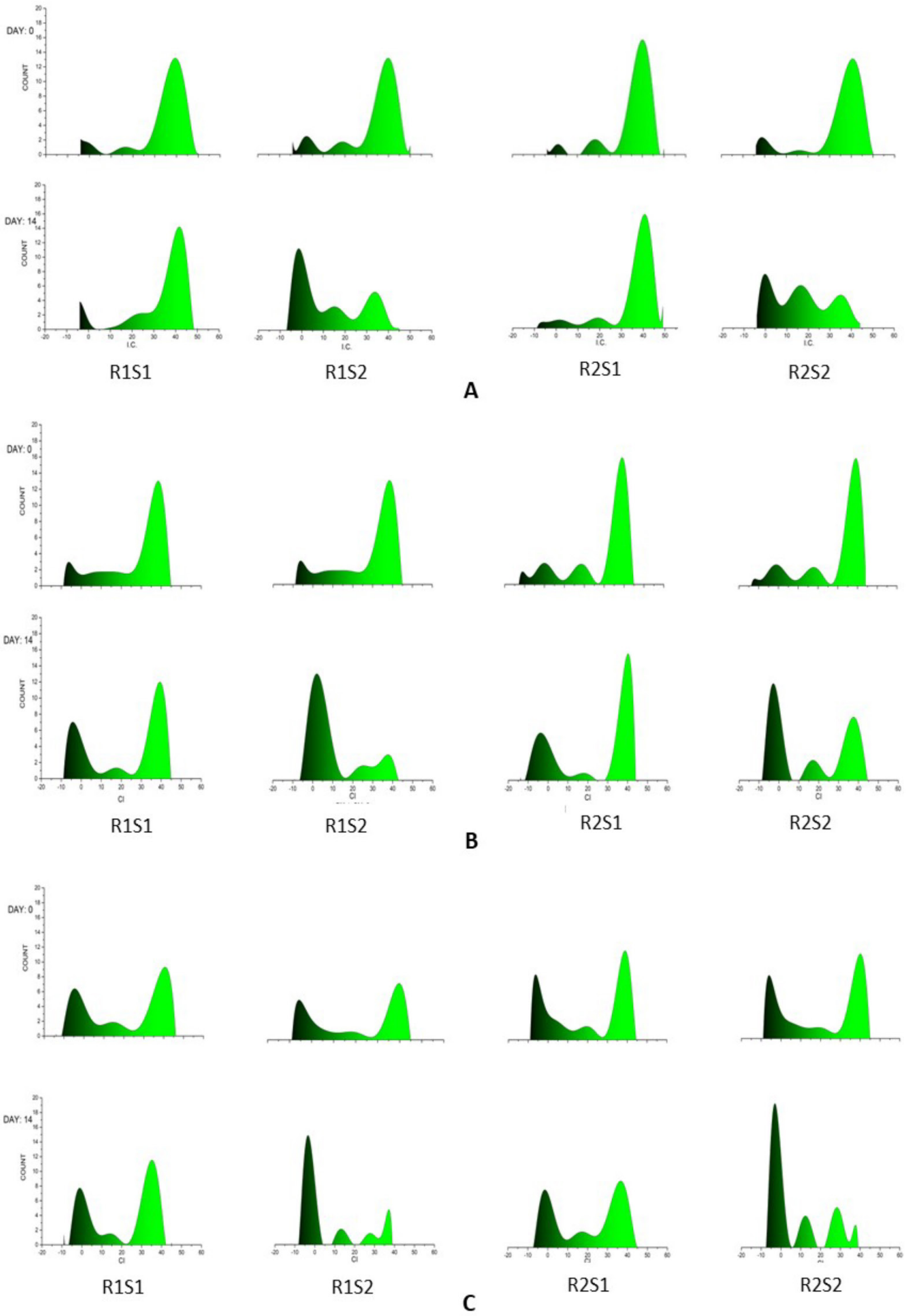

Figure 3. Color index (CI) histogram values (non-linear curve fit) at day 0 and 14 for all possible combinations of two collection methods (R1: Manual Inverted Umbrella; R2: nets) and two storage methods (S1: 5 °C; S2: room temperature) for ‘Arbequina’ (A); ‘Picual’ (B) and ‘Verdial’ cultivar (C).

by the interaction of ST and R, ST and S, R and S, and for the triple interaction of these factors. Both varieties showed a different profile in the changes of these parameters during storage time. From the initial storage time (day 0) all the varieties tested showed higher values for this parameter when they were harvested with nets (R2). However, from the next sampling date (day 4) they showed different profiles. Whereas in 'Arbequina' olives these differences decreased during the following two sampling dates and increased in the last one, in 'Picual' olives the differences had increased on the second and the third sampling dates and decreased on the last one. Finally, in 'Verdial' olives these differences had decreased by the second sampling date of storage, but, subsequently, only increased in the fruit stored at room temperature.

4. DISCUSSION

4.1. Incidence of decay

The fact that the effect of recollection was more evident than storage is not surprising, although the fruit harvested using both systems did not show differences in the incidence of physiological deterioration at the moment of harvesting (data not shown). Yousfi *et al.* (2012). compared the effect of 'Arbequina' olives picked using two different harvesting systems (hand and mechanical with an adapted wine grape harvester) during cold storage. Although they did not appreciate any differences in the appearance of the fruit immediately after harvesting, subsequently they found an exponential increase in decay incidence in mechanically-harvested fruit stored at 3 ºC. In contrast, hand-harvested fruit was not significantly decayed after two weeks of cold storage. In the present study, the differences between R1 and R2 can be explained because the use of R2 inevitably implies the involuntary treading on of the fallen fruit by the operators and the dragging over the ground of the fruit on the nets during its displacement to the next tree until reaching the filling of the boxes. This infers a serious risk of infection with soil pathogens, given the micro-wounds that this activity inevitably provokes. The effect of cold storage (5 ºC), maintaining initial physiological integrity has been observed in different works, using different olive cultivars (Castellano *et al.*, 1993; García *et al.*, 1996; Rinaldi *et al.*, 2010). In this study, the effect of S1 proved to be more evident with the progression of the storage period, whereas the effect of R1 was already noticeable from the second sampling date (4 days). Cold storage requires the combined effect of a careful harvest to maintain a minimum amount of decay in the stored fruit.

4.2. Weight loss

As expected, the storage temperature determined the level of fruit transpiration and, consequently, the olives maintained under refrigeration showed lower weight loss than the ones kept in ambient conditions. In contrast, the harvesting method did not show any effect on this parameter. Yousfi *et al.* (2012) found the same effect of low temperature delaying the progress of weight loss during the storage of 'Arbequina' olives, and they also observed a significant effect due to the recollection method because the use of an adapted grape harvester turned out to be more damaging to fruit than when picked by hand. The results presented in this study should be placed between these two extremes. They are higher than the ones obtained by hand-harvest and lower than when compared with the adapted grape harvester.

4.3. Skin color

As for weight loss, the observed shift in skin color was only produced by storage temperature. Room temperature accelerated changes in the skin color, while refrigeration delayed it. This fact was previously observed in 'Picual' olives by Castellano *et al.* (1993) and by Yousfi *et al.,* (2012) using 'Arbequina' fruit. In this paper, the appearance of the three model profiles when stored at room temperature typified this gradual color shift in the three studied varieties.

4.4. Firmness

The observed changes in firmness showed a similar process as discussed by Yousfi *et al.* (2012). As the storage time progressed, this parameter decreased in all the combinations of treatments, but fruit refrigeration delayed this softening. Furthermore, the damaging action of the harvest method used is related to this process. While in the study by Yousfi *et al.* (2012) the internal partial breaking of the cellulose wall of the olive mesocarp cells was a consequence of the mechanical harvesting method, in this study the responsible agent was related to the use of nets.

4.5. Respiration rate and ethylene production

Low temperature produces a decrease in the respiration rate. Maintaining the fruit at 5 ºC produced a reduction in the metabolism of the fruit cells; while fruit kept at room temperature continued its normal activity, including respiration (Rinaldi *et al.*, 2010). The concentration of substrates for respiration (sugar, fatty acid, etc.) is limited for detached fruit. The olives stored at room temperature consumed these soluble substrates continuously; while refrigerated fruit still disposed of a higher concentration of these metabolites when brought at 20 ºC for respiration measurements. These processes explain the higher CO_2 production levels in refrigerated fruit. Meanwhile, the higher respiration rate showed by 'Arbequina' olives harvested with nets could be attributed to its damaging action, which can accelerate this parameter. Segovia-Bravo *et al.* (2011) found a higher CO_2 production in naturally bruised Manzanilla olives than in the healthy ones. Similarly, Morales-Sillero *et al.* (2015) observed that the fruit of two varieties (Manzanilla de Sevilla and Manzanilla Cacereña), harvested with a grape straddle harvester, showed a higher respiration rate than the hand-picked ones. They related this fact to the damaging action of this harvest method. Jimenez *et al.* (2017) demonstrated that the internal damage at morphological and histological levels differed between these varieties. In this study, 'Arbequina' olives seemed to be more sensitive to the action of the nets during harvest than 'Picual' and 'Verdial' olives.

The significantly higher amounts of ethylene synthesized by R2 harvested fruit indicated that this method was more damaging to the olive physiology than R1. This result coincides with that obtained by Morales–Sillero *et al.* (2017), who studied the ethylene production of Manzanilla and Cacereña olives stored at 2 ºC, which were harvested by hand or mechanically, using a grape straddle harvester. They found a significantly higher ethylene biosynthesis in the mechanically harvested fruit and identified this with the fact that this harvesting method was more detrimental to fruit integrity. Other causes for the increase in ethylene biosynthesis could be the natural progression of fruit ripening (Ardilla *et al.*, 2012; Hyodo, 2018). When the R2S1 'Arbequina' olives were placed at 20 ºC to measure their ethylene production it could be considered normal that they experimented a higher synthesis than the R1 ones because they were more damaged, but they showed significantly lower values for this parameter than R2S2 fruit, which was also damaged but with a higher level of ripening because it was maintained at a higher temperature during storage. R1 'Arbequina' olives showed the same behavior during the first three sampling dates because they were not damaged but the value increased because ripening had progressed. Finally, on the last sampling date, the olives stored at a higher temperature showed a significantly higher ethylene production, evidencing a higher level of ripening. The profile of 'Picual' olives only differed from the one shown by 'Arbequina' olives in the maxima values exhibited by R2 olives in the third sampling date, followed by a clear reduction in this parameter during the last moment of storage. This was probably due to a particular characteristic of this variety, in which ethylene biosynthesis would only coincide with ripening at the beginning of this process. R1S2 'Picual' olives did not show increasing values for this parameter at the end of the storage period when the ripening level of these olives was probably more evident. In contrast, the progression of ripening was better controlled by the cold storage in R1S1 olives, which showed the lowest ethylene production without changes throughout the storage period. The low values for ethylene production shown by S1 'Verdial' olives could be related to better control of the ripening process due to cold storage. In contrast, fruit maintained at ambient temperature from the second sampling date showed increasing values for this parameter, which could be related to an accelerated ripening. Each variety showed a particular profile of ethylene production, due to its characteristics, such as sensitivity to the harvest system, response to cold storage, or the progression of ripening.

5. CONCLUSIONS

The better maintenance of the fruit's physiological conditions due to the harvesting method was demonstrated by the delay that it experienced during storage in its development of decay incidence and the significantly lower release of ethylene, regardless of the variety tested. Furthermore, cold storage induced a delay in weight loss, in changes in skin color, and the softening of the stored fruit. The treatment which used the combination of R1 and S1 systematically showed the best values for the fruit quality parameters tested. This combination of treatments offers a better guarantee than the traditional system (R2 and S2) to obtain a higher level of fruit physiological quality, either for the extraction of its oil, as for its table dressing.

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A novel bleaching approach: Microwave assisted sunflower oil bleaching and optimization

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SUMMARY: The factors affecting the microwave bleaching of sunflower oil and the interaction between them were investigated and optimized by response surface methodology using a three-factor five-level central composite rotatable design. Microwave power, time and the amount of bleaching clay were selected as independent variables studied in the range of 70-120 W, 2-15 min, and 0.01-0.5%. The dependent variables that measure the bleaching efficiency and oil quality were evaluated as hue angle, chroma and totox value. Optimization was carried out by minimizing totox and chroma and maximizing hue angle. Hue angle, chroma and totox were found as 96.91, 37.66 and 23.31 under optimal conditions. Optimal microwave bleaching was successfully performed by using less bleaching clay (0.4%) and a shorter time (8 min) compared to the current industrial application without any adverse effect on oil quality. Hence, microwave bleaching is thought to be an alternative method for the bleaching of edible oils.

KEYWORDS: *Bleaching; Bleaching efficiency; Microwave; Optimization; Sunflower seed oil*

RESUMEN: ***Un enfoque novedoso de blanqueamiento: optimización del proceso asistido por microondas de aceite de girasol.*** Los factores que afectan al blanqueamiento asistido por microondas del aceite de girasol y la interacción entre ellos se investigaron y optimizaron mediante la metodología de superficie de respuesta utilizando un diseño central giratorio compuesto de tres factores y cinco niveles. La potencia del microondas, el tiempo y la cantidad de arcilla blanqueadora se seleccionaron como variables independientes estudiadas en el rango de 70-120 W, 2-15 min y 0,01-0,5%. Las variables dependientes que miden la eficiencia del blanqueamiento y la calidad del aceite se han evaluado como ángulo de tono, croma y valor totox. La optimización se llevó a cabo minimizando el totox y el croma y maximizando el ángulo de tono. El ángulo de tono, el croma y el totox se encontraron en 96,91, 37,66 y 23,31 en condiciones óptimas. El blanqueamiento por microondas óptimo se ha realizado con éxito utilizando arcilla blanqueadora más baja (0,4%) y un tiempo más corto (8 min). En comparación con la aplicación industrial actual sin ningún efecto adverso sobre la calidad del aceite. Por tanto, se cree que el blanqueamiento asistido por microondas es un método alternativo para blanquear los aceites comestibles.

PALABRAS CLAVE: *Aceite de semilla de girasol; Blanqueamiento; Eficacia de blanqueo; Mejoras; Microondas*

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1. INTRODUCTION

Bleaching is the most important step in the edible oil refining process and can be described as a process which is based on removing undesirable color pigments and other undesirable impurities from the neutralized oil by using a certain amount of bleaching earth or active carbon under the determined conditions. Generally, the bleaching process is carried out at temperatures between 90-120 °C, using clay 0.5-2.0%, in times from 20-30 minutes (Zschau, 2001). The quality of the bleached oil depends on the type of earth, dosage, bleaching time, mixing, bleaching temperature and pressure. The surface area of the earth is a critical feature in the bleaching process. Clays are activated by mineral acids in order to obtain better bleaching quality with increasing specific surface area. Clay with 40-60 m^2/g dry clay surface area originally, can be increased up to 200 m^2/g dry clay (Hymore, 1996). The bleaching process in today's edible oil industry is applied batch-wise with long process times and high temperatures by using large amounts of clay. The clay absorbs approximately 0.36 kg of oil/l kg of clay (Gupta, 2017). In the existing conventional method, the desired oil color quality is achieved by increasing the amount of clay, which leads to increasing oil losses and environmental problems. Therefore, the development of new bleaching methods to overcome the problems involved in the process is necessary for the vegetable oil industry.

The use of microwave has become very popular in various industrial applications over recent years. Microwave is used especially in the food industry to reduce process time and increase food quality. In the literature, microwave heating is described as a continued promising method among the other electric heating technologies such as radio frequency, ohmic, and infrared (Torrealba-Meléndez *et al.,* 2015; Zhang *et al.,* 2006). Specific heat is a very important factor in microwave heating (Meda *et al.,* 2017). In particular, materials with low specific heat can be heated rapidly when subjected to microwave. Vegetable oils have a lower specific heat than water so they may be heated by microwave faster than water of the same weight (Schiffmann, 2014). Microwave is preferred for the heating of edible oils because of the reduction in heating time, providing inner penetration and faster heating (Gjorgjevich *et al.,* 2012). Microwave also plays an important role in the sorption capacity of clays. Li *et al.,* 2007 showed that the sorption of cetylpyridinium chloride into bentonite was greatly influenced by microwave. The velocity constant of the sorption reaction was increased 107.6 times, and the free energy of the sorption reaction system was decreased.

There are vast numbers of studies on improving the bleaching process of oils. In all these studies, the type and amount of bleaching agent, mixing time, temperature and pressure are the parameters examined (Skevin *et al.,* 2012; Salawudeen *et al.,* 2014; Chew *et al.,* 2017; Mustafa and Abusabah, 2019; Boroujeni *et al.,* 2020.) by keeping the conventional heating method as the same. In only a few studies, new technologies such as ultrasound and high voltage electric field were used as alternative techniques to the conventional heating process (Su *et al.,* 2013; Abedi *et al.,* 2016; İçyer and Durak, 2018). Although there are a few studies (Boukerroui and Quali, 2002; Foletto *et al.,* 2013) in which the Brazilian bentonite activated by microwave treatment has been used in the bleaching of soybean oil in the literature, this current study is the first one in which the bleaching process has been applied directly with microwave treatment.

Therefore, the main purpose of this study was to evaluate the effect of microwave technology on the bleaching efficiency of sunflower seed oil and to optimize different parameters in the bleaching process, such as amount of bleaching earth, microwave power and process time, based on total oxidation value and color reduction in bleached sunflower oil.

2. MATERIAL AND METHODS

2.1. Materials and chemicals

The non-bleached sunflower oils used in this study were obtained from a local refinery. Sunflower oil samples were placed in dark bottles and stored at –18 °C until bleaching. Acid-activated (Suprefast M1FF) bleaching clay was also procured from same refinery (Gaziantep, Turkey). All chemicals and reagents used in this study were analytical grade and purchased from Sigma-Aldrich.

2.2. Experimental design

A three-level, five factorial central composite rotatable design (CCRD) was employed to analyze the effects of microwave assisted factors on the bleaching and to optimize the bleaching conditions to obtain maximum bleaching quality. The independent variables were selected as microwave power (A), amount of clay (B) and bleaching time (C). The levels of the independent variables were defined as between A, 70-120 W; B, 0.01-0.5% (w/w); C, 2-10 min. Preliminary experiments were carried out to obtain information about the independent variables and their levels prior to the experimental design. The employed CCRD was composed of 20 experiments consisting of 6 center points. At the end of optimization, verification of experiments was carried out under the optimal conditions and the average value for the experiments was compared to the predicted value.

2.3. Microwave-assisted bleaching

Microwave-assisted bleaching (MWB) treatments were performed using a microwave reactor (CEM, Discover SP). Bleaching was carried out in a 35-ml glass vessel. The vessel was not fully filled to avoid oil splash. 20 g sunflower oil were weighed into the vessels in all experiments. The bleaching earth was weighed for each vessel according to the amounts determined in the experimental design. After the addition of clay, the tube was sealed with a rubber lid (CEM) and placed in the microwave reactor. In all experiments, stirring speed mode was set on high. The mixing of clay and sunflower oil was carried out only in the reactor and during the bleaching period. The levels of microwave power and time were adjusted according to the experimental design. This microwave system consisted of an infrared sensor for temperature measurement. Therefore, temperature values varied depending on the microwave power reported during the experiment. At the end of the microwave treatment, the mixture of sunflower oil and clay was centrifuged (EBA 20, Hettich) at 6.000 rpm at 25 °C for 10 min. Sunflower oil was separated easily from the clay because of the clay sticking to the wall of the tube. The sunflower oils bleached by this way were stored in dark amber sample bottles at –18 °C until further analysis.

2.4. Determination of oxidation parameters

Peroxide value (PV) was determined according to AOCS standard method Cd 8–53 (AOCS, 1998). The p-anisidine value (AV) in sunflower oil was determined by the spectrophotometric (Optima, SP-3000nano, Japan) method as described in the AOCS Official Method Cd 18–90 (AOCS, 1998). PV and AV were converted to total oxidation value (Totox) according to Equation 1.

$$\text{Totox value} = 2\ \text{PV} + \text{AV} \qquad (1)$$

2.5. Color measurement

The color of the samples was determined by a HunterLab Colorflex colorimeter with standard illumination D_{65}, and colorimetric normal observer angle of 10°. Color values were evaluated as L*, a* and b*. Measured values L*, a* and b* were also converted to chroma and hue angle values given below in Equations 2 and 3, respectively.

$$\text{Chroma} = \sqrt{(a*)2 + (b*)2} \qquad (2)$$

$$\text{Hue angle} = \tan^{-1}(b^*/a^*) \qquad (3)$$

2.6. Statistical analysis

The experimental design and statistical analysis were conducted using Design Expert version 7.0 software (State-Ease Inc., Minneapolis, Minn., U.S.A.). Statistical parameters such as lack of fit, the coefficient of determination (R^2)

Table 1. Experimental and estimated values obtained for responses

Runs	FACTORS			RESPONSES					
	A (Watt)	B (%)	C (s)	Totox		Hue Angle		Chroma	
				Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	95	0.26	360	22.02±0.61	22.36	96.24±0.05	96.73	41.21±0.03	39.80
2	110	0.11	241	20.50±0.06	19.49	93.55±0.02	93.41	48.02±0.01	49.86
3	120	0.26	360	22.73±0.08	23.82	96.99±0.01	97.30	39.70±0.01	37.37
4	95	0.26	120	19.27±0.01	19.72	92.78±0.02	92.90	50.19±0.01	49.07
5	80	0.11	241	19.28±0.01	19.76	93.56±0.01	93.52	50.05±0.01	50.41
6	110	0.40	503	29.91±0.90	28.69	99.43±0.01	99.47	30.57±0.02	30.41
7	95	0.50	360	21.73±0.24	22.43	97.48±0.01	97.40	37.47±0.01	38.67
8	95	0.26	360	23.14±0.73	22.36	96.84±0.02	96.73	38.97±0.01	39.80
9	95	0.26	360	22.85±0.73	22.36	96.73±0.01	96.73	39.40±0.01	39.80
10	110	0.40	241	21.26±0.01	21.12	96.36±0.01	96.08	40.34±0.05	41.72
11	95	0.26	360	21.69±1.14	22.36	96.63±0.02	96.73	40.44±0.04	39.80
12	80	0.40	503	23.31±0.14	23.59	97.31±0.01	97.44	37.18±0.04	35.60
13	110	0.11	503	26.17±0.01	26.12	96.06±0.01	95.86	42.75±0.01	44.12
14	95	0.01	360	20.55±0.02	20.90	93.67±0.01	93.76	55.48±0.01	53.92
15	95	0.26	360	22.98±0.03	22.36	97.10±0.01	96.73	38.67±0.01	39.80
16	95	0.26	600	28.58±0.02	29.16	97.76±0.01	97.65	34.80±0.01	35.55
17	70	0.26	360	19.84±0.01	19.80	96.00±0.01	95.70	40.15±0.01	42.12
18	95	0.26	360	21.65±0.74	22.36	96.86±0.01	96.73	40.05±0.04	39.80
19	80	0.40	241	19.70±0.07	19.01	94.05±0.02	94.24	47.06±0.02	45.94
20	80	0.11	503	24.01±1.21	23.41	95.56±0.01	95.78	46.34±0.01	45.60

A: Microwave power (W), B: Earth amount (%), C: Time (Sec). ±: Standard Deviation. Data are means of triplicates and the F-test value obtained from the analysis of variance (ANOVA) were used to evaluate the best fitting polynomial model. Regression analysis and three-dimensional surface plots were generated to understand the effect of microwave-assisted conditions on response variables. All experiments were performed in three replicates and the results were given as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Fitting the model

The experimental design and the experimental results for the microwave bleaching of sunflower oil are presented in Table 1. The bleaching efficiency and the oil quality have been analyzed in terms hue angle, chroma and totox value for MWB sunflower oil. The totox value the MWB sunflower oil obtained in this study ranged from 19.27 to 29.91; while the hue angle and chroma ranged from 92.78 to 99.43 and 30.57 to 55.48, respectively.

The multiple regressions and backward elimination were employed to get the best fitting statistical model for each response. Table 2 summarizes the results from the ANOVA and regression analyses for the hue angle, chroma and totox values. Non-significant lack of fit and high R^2 value indicate that the statistical model fits well with data (Içyer and Durak, 2018; Islam *et al.,* 2018). The quadratic model was deter-

Table 2. ANOVA results for responses

Source	Sum of squares	Df	Mean Square	F value	p- value
Model (Totox)	146.07	6	24.35	31.60	< 0.0001
A-MW power	19.73	1	19.73	25.61	0.0002
B-Clay content	2.82	1	2.82	3.65	0.0782
C-Time	107.44	1	107.44	139.45	< 0.0001
AB	2.84	1	2.84	3.69	0.0770
AC	4.45	1	4.45	5.77	0.0319
C^2	8.79	1	8.79	11.41	0.0049
Residual	10.02	13	0.77		
Lack of fit	7.72	8	0.96	2.10	0.2154
Pure error	2.30	5	0.46	31.60	< 0.0001
Core total	156.09	19	-	25.61	0.0002
R^2=0.94, Adj. R^2= 0.91, Pred. R^2=0.83, Adeq Precision =18.37					
Model (Hue Angle)	54.30	7	7.76	95.13	< 0.0001
A-MW power	3.11	1	3.11	38.17	< 0.0001
B-Clay amount	15.99	1	15.99	196.12	< 0.0001
C-Time	27.24	1	27.24	334.08	< 0.0001
AB	1.90	1	1.90	23.32	0.0004
BC	0.44	1	0.44	5.45	0.0378
B^2	2.33	1	2.33	28.57	0.0002
C^2	3.77	1	3.77	46.19	< 0.0001
Residual	0.98	12	0.08		
Lack of fit	0.56	7	0.08	0.97	0.5329
Pure error	0.42	5	0.08	95.13	< 0.0001
Core total	55.27	19	-	38.17	< 0.0001
R^2=0.98, Adj. R^2=0.97, Pred. R^2=0.95. Adeq Precision=36.56					
Model (Chroma)	627.77	6	104.63	36.32	< 0.0001
A-MW power	27.47	1	27.47	9.54	0.0086
B-Clay amount	280.68	1	280.68	97.44	< 0.0001
C-Time	220.64	1	220.64	76.59	< 0.0001
BC	15.25	1	15.25	5.29	0.0386
B^2	76.89	1	76.89	26.69	0.0002
C^2	11.54	1	11.54	4.01	0.0667
Residual	37.45	13	2.88		
Lack of fit	32.85	8	4.11	4.47	0.0580
Pure error	4.59	5	0.92	36.32	< 0.0001
Core total	665.22	19		9.54	0.0086
R^2=0.94, Adj.R^2=0.92, Pred. R^2=0.83, Adeq Precision =22.02					

mined as the best fitting model according to non-significant lack of fit and R^2 values for all responses (Table 2). Adequate precisions for totox, hue angle and chroma were found as 18.37, 36.56 and 22.02. These values were higher than 4. Therefore, it can be said that there is a good correlation between the estimated values and experimental data (Gasemloo *et al.,* 2019). Similarly, Chew *et al.* (2017) and Garcia-Moreno *et al.* (2013) reported that the quadratic model was found suitable for the bleaching studies based on totox and color parameters. The reduced model created with the terms that are only statistically significant are expressed in Equations (4), (5), (6) for totox (Y1), hue angle (Y2) and chroma values (Y3), respectively:

$$Y1 = 22.03 + 1.20xA + 0.45xB + 2.80xC + 0.59xAxB + 0.74xAxC + 0.77xC2 \quad (4)$$

$$Y2 = 99.67 + 0.47xA + 1.08xB + 1.41xC + 0.48xAxB + 0.24xBxC - 0.4xB2 - 0.51xC2 \quad (5)$$

$$Y3 = 39.78 - 1.41xA - 4.43xB - 4.02xC - 1.38xBxC + 2.30xB2 + 0.89xC2 \quad (6)$$

The magnitude of coefficients for bleaching time and amount of clay are higher than the coefficients for current microwave power and other interactions. On the other hand, the magnitude of coefficients for microwave power and amount of clay are higher than the coefficients for current hue angle, chroma and other interactions, indicating that these parameters have a more significant effect on hue angle, chroma and totox values. The other parameters seem to have a quadratic effect on the hue angle, chroma and totox values.

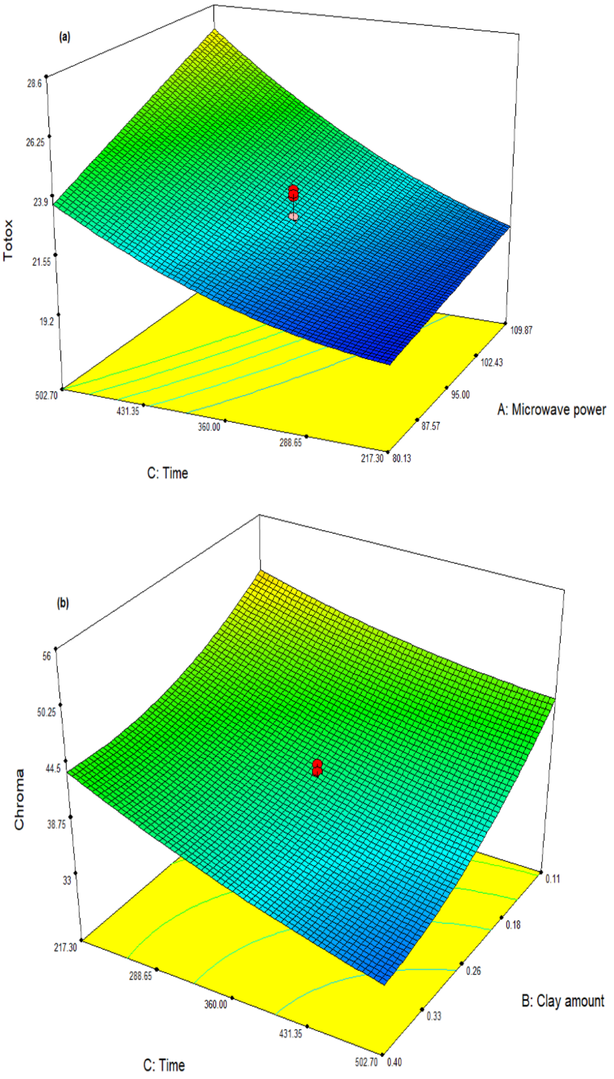

Figure 1. 3D response plots of significant interactions between totox value and chroma.

3.2 Effect of microwave-assisted bleaching parameters on the oxidation of oil

The oxidation of fats and oils during bleaching is one of the important deteriorative reactions. Totox value is one of the important ways to express the total oxidation degree of fats and oils. Hence the totox value was taken as oxidation indicator in this study. Among the independent variables, microwave power, time and their interaction (AC) were found to significantly affect ($p < 0.05$) the Totox value. The second-order term of time (C2) also had significant ($p < 0.05$) effects on the totox value. On the other hand, neither the first-order term of amount of bleaching clay nor its interactions with other responses (AB, BC) had a significant ($p > 0.05$) effect on the totox value of bleached sunflower oil. The 3D response surface plots for the totox value of bleached sunflower oil samples as a function of independent variables are shown in Figure 1a. The totox value for sunflower oil increased with the rise in microwave power and longer irradiation time. The exposure of material to high microwave power increased the temperature of the material. Therefore, the increase in temperature due to high microwave power negatively affected the oxidation stability of the oil. Marrakchi *et al.* (2015) stated that the bleaching temperature significantly affects oxidation stability. In another study in which the optimization of industrial bleaching conditions was studied, it was emphasized that temperature and bleaching earth had significant effects on totox values (Chew *et al.,* 2017). Contrary to the findings of Chew *et al.* (2017) the amount of clay in MWB was not found to be more effective on the totox value than the commercial bleaching process. It may be due to using a small amount of clay and less bleaching time in MWB conditions. İçyer and Durak (2018) emphasized that especially p-anisidine and totox value increased as bleaching time increased in both conventional and ultrasonic bleaching methods. The maximum totox value (29.91) obtained from MWB was found to be comparable to the totox value (31.07) reported by İçyer and Durak (2018) for 30 min conventional bleaching. In general, increasing the amount of clay can facilitate the removal of unwanted compounds in the oil during bleaching. On the other hand, the catalytic properties of acid-activated bleaching earth convert hydroperoxides into secondary oxidation products. Consequently, this situation affects the totox value (Zschau, 2000; Bonveh *et al.,* 2001). It is quite clear from our findings that the low microwave power (80 W) and short processing time (8 min) has no effect on oil quality and only a small effect on peroxide and p-anisidine values of the oil processed by microwave. Even if the totox value increased slightly with increasing peroxide and p-anisidine values, they are similar to the findings obtained by the conventional bleaching process (Chew *et al.,* 2017; Zschau, 2000; Bonveh *et al.,* 2001). In addition, it should be kept in mind that the following deodorization process removes the formed oxidation products which are commonly produced in conventional technology, which has been emphasized in the literature in many sources (Shahidi *et al.,* 1997; Zschau, 2001; Skevin *et al.,* 2012).

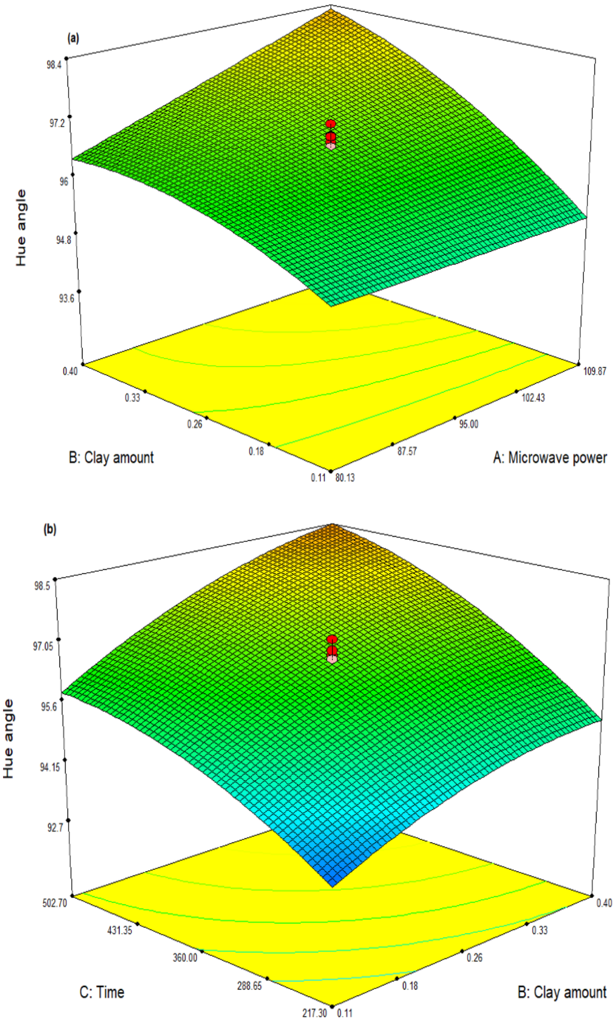

Figure 2. 3D response plots of significant interactions for hue angle.

3.3. Effect of microwave-assisted bleaching parameters on color properties

Although the Lovibond tintometer is widely accepted as a standard method for measuring the color of oils, Hunterlab has been used in many studies to measure the color of oils (Abedi *et al.,* 2015; Abedi *et al.,* 2016; Chew *et al.,* 2017). In addition, Abedi *et al.* (2015) reported a correlation between b* and a* values and carotenoid and chlorophyll concentrations, respectively. Hue angle and chroma were monitored for the prediction of color change during the MWB of sunflower oil. While hue angle represents color appearance (0°: red, 90°: yellow and 180°: blue), chroma is defined as a measurement of whiteness and vividness of color (Mínguez-Mosquera *et al.,* 1991). All independent variables (A, B and C) were found to be significantly effective on the hue angle and chroma value. In addition, some interactions such as microwave power/amount of clay (AB), amount of clay/time (BC) and second-order term of time (C^2) were found to be significantly effective ($p < 0.05$) on the hue angle. García-Moreno *et al.* (2013) found a similar correlation between hue angle and chroma. Response surface plots for chroma and hue angle values for bleached sunflower oil samples as a function of independent variables are shown in Figures 1b, 2a and 2b, respectively. Based on Figure 2a, microwave power and amount of clay had significantly positive effects on hue angle. Moreover, interactions between microwave power and amount of clay had a synergistic effect on hue angle. The interaction of microwave power and amount of clay caused an increase in hue angle. This means that the redness was removed from the sunflower oil. Bleaching time was found as another important parameter for hue angle. In particular, interaction of time and amount of clay caused a positive effect on the hue angle (Figure 2b). Furthermore, the second order effects of these two variables on the hue angle had a negative coefficient which means that these variables had reached an optimum value in the studied range. In other words, the increase in time and amount of clay did not cause further rising in hue angle after a certain point. Interactions of BC and second-order term of amount of clay (B^2) were found significant on the chroma. Chroma value decreased with increasing amount of clay and time and vice versa (Figure 1b). A negative correlation was determined between the hue angle and chroma value. Although the hue angle increased, the chroma values decreased. Marrakchi *et al.* (2015) reported a similar trend in their study.

3.4. Optimization and model verification

The optimization of MWB was carried out by minimizing totox and chroma, maximizing hue angle in sunflow-

TABLE 3. Comparison of experimental and estimated values for each response under optimum conditions

Response	**Experimental**	**Predicted**	**p value**
Totox value	23.31 ± 0.28	22.48	0.13
Hue angle	96.91 ± 0.02	97.28	0.19
Chroma	37.66 ± 0.02	35.67	0.08

± Standard Deviation. Data are means of triplicates.

er oil samples. Under optimal conditions, the temperature started at ambient temperature and reached 100 °C at the end of bleaching. In other words, the temperature increased during the treatment period but remained below 100 °C without applying vacuum. Optimal conditions for MWB were found as 80 W, 8.0 min and 0.4% for microwave power, time and amount of clay, respectively. Compared to studies in the literature on reducing clay and bleaching time, it was shown that optimized MWB conditions provided more effective decreases. For example, Abedi *et al.* (2015) reported that two optimized bleached oils were produced by using 1.22 and 1.35% clay amount in 28 minutes. In a different study on the bleaching of sunflower and soybean oil by using a high voltage electric field, the same researchers (2016) found optimal conditions at 1% clay amount and 20 minutes; 0.5% clay amount and 20 minutes for soybean and sunflower oil, respectively. In another study Asgari *et al.* (2017) studied the bleaching of olive oil and they obtained 13 minutes and 1.21% for bleaching time and amount of clay. The bleaching time could not be decreased below 10 minutes in any of these studies. It ranged from 15 min to 30 min. In these studies, the amount of clay used was at its minimum at 0.8% as determined by İçyer and Durak (2018). Temperature is one of the important parameters for effective bleaching. The temperature values used in the ultrasonic bleaching process can be a limiting factor because with the rapid increase in the liquid temperature, a dense bubble formation begins in the liquid and continues increasingly. But the tendency of the bubbles to collapse becomes harder due to the high pressure inside of them acting as a bed (Thompson and Doraiswamy, 1999). Therefore, in the ultrasound-assisted bleaching process, the bleaching effect at high temperatures needed for obtaining the desired oil quality cannot be due to sonication (Abedi *et al.,* 2015). In addition, in cases where high temperatures are inevitable, unwanted taste and odors have been observed in the ultrasonic method (Jahouach-Rabai *et al.,* 2008). But due to having lower specific heat, vegetable oils can be heated to high bleaching temperatures easily in a short time by microwave, which is an important advantage compared to the ultrasound method. Abedi *et al.* (2016) used 1.5% bleaching earth and 30 minutes bleaching time to imitate the industrial process as a control system. The microwave bleaching results showed that MWB was more advantageous in terms of clay usage (0.4%) and bleaching time (8 min) than those used in industrial processes.

For verification of the response values estimated as a result of the optimization, three independent MWB bleaching studies were carried out under optimal conditions and experimental response values were determined. Experimental and estimated values for each response under optimal conditions are given in Table 3. The adequate precision values obtained from Design Expert for totox, hue angle and chroma were found to be 23.31, 96.91 and 37.66, respectively. One-way t-test showed that there was no statistically significant difference between the experimental data and the predicted values for all three responses ($p > 0.05$).

4. CONCLUSIONS

Microwave was used for bleaching for the first time in this study. The optimization of MWB conditions (microwave power, time and amount of clay) was successfully achieved by using RSM. Optimum values for MWB were found as 80 W, 8 min and 0.4% for microwave power, bleaching time and amount of bleaching, respectively. Under optimum conditions the adequate precision values obtained for hue angle, chroma and totox value were found to be 96.91, 37.66 and 23.31. The microwave power, time and amount of clay used were found as significantly effective parameters on the MWB for color parameters. The amount of clay was not significantly effective on the totox value. However, totox value was also significantly affected by microwave power and time. Bleaching time and the amount of clay used for the MWB of sunflower seed oil were found to be the lowest values compared to new bleaching technologies such as ultrasound and high voltage electric field bleaching methods. Therefore, it can be concluded that MWB seems to be a promising technology for the bleaching of oils.

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Phenolic variability in fruit from the 'Arbequina' olive cultivar under Mediterranean and Subtropical climatic conditions

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SUMMARY: In the present work, we compared the phenol content and composition of fruit from the 'Arbequina' cultivar in four Mediterranean (in Andalucía, Southern Iberian Peninsula) and two Sub-Tropical (Canary Islands) locations throughout the harvest period. Two Mediterranean and two Sub-Tropical locations were maintained with drip irrigation, while the remaining two Mediterranean locations were in dry farming. Water availability and harvest date seemed to play more important roles than air temperature on the phenolic content and most of the studied components. The variability associated with location was a result of the high values observed in the two Mediterranean locations in dry farming, with respect to the other four maintained with drip irrigation. Few differences were found among the four drip-irrigated locations, despite the fact that two were Mediterranean and the other two Sub-Tropical. In addition, a sharp decrease was observed during the harvest period for phenolic content and most of the phenolic compounds.

KEYWORDS: *Environment; Harvest date; Location; Maturity; Olive fruit*

RESUMEN: ***Variabilidad fenólica del fruto en el cultivo de olivo 'Arbequina' en condiciones climáticas mediterráneas y subtropicales***. En el presente trabajo se compara la variabilidad del contenido y composición en fenoles de la variedad de olivo 'Arbequina' en cuatro localidades con clima Mediterráneo y dos con clima Sub-Tropical. Dos de las localidades mediterráneas y las dos Sub-Tropicales contaban con riego por goteo, mientras que las dos mediterráneas restantes estaban en secano. La disponibilidad de agua y momento de recolección parece ser un factor más importante que la temperatura del aire en el contenido y composición de fenoles del fruto. La mayor parte de la variabilidad asociada a la localidad estuvo causada por los altos valores encontrados en las dos localidades mediterráneas en secano, respecto a las otras cuatro localidades en regadío. Solo pequeñas diferencias se encontraron entre las cuatro localidades en regadío, a pesar de que dos eran mediterráneas y las otras dos sub-tropicales. Además, un descenso acusado del contenido de la mayoría de los fenoles analizados se ha observado conforme avanzaba la fecha de recolección.

PALABRAS CLAVE: *Aceituna; Ambiente; Fecha de recolección; Localidad; Madurez*

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1. INTRODUCTION

Olive oil is one of the main components of the Mediterranean diet due to its nutraceutical properties (Piroddi *et al.,* 2017). Among olive oil constituents, phenols play a very important role in its healthy properties (Serreli and Deiana, 2019). For example, olive phenols reduce chronic inflammation and help in the prevention of some diseases, such as Alzheimer's (Omar *et al.,* 2017) or different types of cancer (Piroddi *et al.,* 2017).

The phenolic composition of olive oil depends, mainly on the phenolic composition of the fruit upon arriving to the mill factory, which is then modified by enzymatic processes occurring during oil extraction (Lukić *et al.,* 2017). This initial fruit phenolic composition could be affected by many factors including genotype, harvest date, tree environmental conditions and management practices (Servili and Montedoro, 2002).

The wide olive germplasm has shown a high genetic variability for both phenolic content and composition in the evaluation of cultivar collections (Miho *et al.,* 2018), breeding progenies (Pérez *et al.,* 2014) and comparative trials (Pérez *et al.,* 2018; El Riachy *et al.,* 2013).

Several studies have also attempted to evaluate the phenolic variability of single cultivars in different environments of their country of origin, such as 'Gemlik' (Ben Ghorbal *et al.,* 2018) in Turkey, 'Picholine Marrocaine' in Morocco (Bajoub *et al.,* 2015), 'Chemlali' (Bouaziz *et al.,* 2004) in Tunis and 'Baladi' in Lebanon (El Riachy *et al.,* 2018). Among the environmental variables, phenol composition is mainly influenced by both abiotic and biotic stresses. Among the first ones, water stress has been reported as one of the main factors influencing phenol content and composition (Dabbou *et al.,* 2015; Gómez-Rico *et al.,* 2006; Gucci *et al.,* 2019). In general, an increase in water stress implies an increase in phenolic content in olive oil (García *et al*., 2020). However, some individual phenols, such as lignans, might show the opposite pattern (Ovar *et al.,* 2002). Biotic stresses such as olive fruit fly (Medjkouh *et al.,* 2018) and Verticillium Wilt (Landa *et al.,* 2019) can also increase oil phenolic content and composition.

The total phenolic content and composition normally decreases during the harvest season, as shown for different olive cultivars (Alowaiesh *et al.,* 2018; Bengana *et al.,* 2013; Bodoira *et al.,* 2015; Dag *et al.,* 2011). This includes 'Arbequina', the most widely planted cultivar in the world (Fernández-Escobar *et al.,* 2013), whose phenolic content has been evaluated in olive oil (Abenoza *et al.,* 2015; Morelló *et al.,* 2004) and fruit (Talhaoui *et al.,* 2015) through maturation in single locations in Spain. On the contrary, some phenols such as lignans and flavones have been reported to increase in oil through maturation (Bengana *et al.,* 2013) as well as verbascoside (Bodoira *et al.,* 2015) and hydroxytyrosol (Bouaziz *et al.,* 2004) in fruits. In other cases, irregular patterns of variation in fruit phenols through maturation have been reported (Ben Ghorbal *et al.,* 2018).

All the mentioned works on olive fruit phenols have been performed in Mediterranean climatic conditions. Few reports exist on olive phenols in other climates as is the case of Argentina (Bodoira *et al.,* 2015). However, olive growing is currently expanding worldwide, in many cases outside the Mediterranean climate. Besides, this expansion is mainly based on few cultivars specially adapted to the new trends of olive growing (Fernández-Escobar *et al.,* 2013), with 'Arbequina' being the clearest example. Therefore, it would be of great interest to compare the influence of very different climate conditions (Mediterranean vs. non-Mediterranean) on variation in an important component of olive oil such as phenolic compounds.

In this sense, Tenerife, in the Canary Islands, is one of the non-Mediterranean locations where olives now have some importance (Medina *et al*., 2018). Its Sub-Tropical climate might be of interest as a natural scenario with climatic conditions similar to those of the typical Mediterranean olive growing area in the likely event of a climate warming (Medina *et al*., 2020).

In the present work, we compared the variation in phenolic content and components of 'Arbequina' fruits through maturation in typical Mediterranean locations from Andalusia in Sothern Iberian Peninsula, with others from the Sub-Tropical climatic conditions in Tenerife, Canary Islands. That comparison was used to compare the test of location, harvest date and their interaction on phenolic content and composition.

2. MATERIALS AND METHODS

2.1. Study sites and plant material

The trees of 'Arbequina' olive cultivar were sampled in four locations of Andalusia, Southern Iberian Peninsula from typical olive growing areas and Mediterranean climate: Antequera (37.17N, -4.64W), Baena (37.60N, -4.23W), La Rambla (37.62N, -4.82W) and Úbeda (37.89N, 3.24W); and two locations in Tenerife, Canary Islands, with Sub-Tropical climate: Los Tomillos (28.13N-16.49W) and El Viso (28.30N, -16,37W, Figure 1). The Mediterranean climate is characterized by colder winters and hotter summers than the Sub-Tropical one, which also has a low intraday temperature range. Antequera and Baena are managed in dry farming while the other four with drip irrigation (year amount of irrigation: La Rambla 250 mm, Úbeda 150 mm, Los Tomillos 372 mm and El Viso 312 mm). In the four Andalusia locations, trees were planted in 2008 at 7 x 6 m distance in a clay-loam soils. In the two Tenerife locations, trees were planted in 2010 at 5 x 5 m distance in a sandy-loam soil.

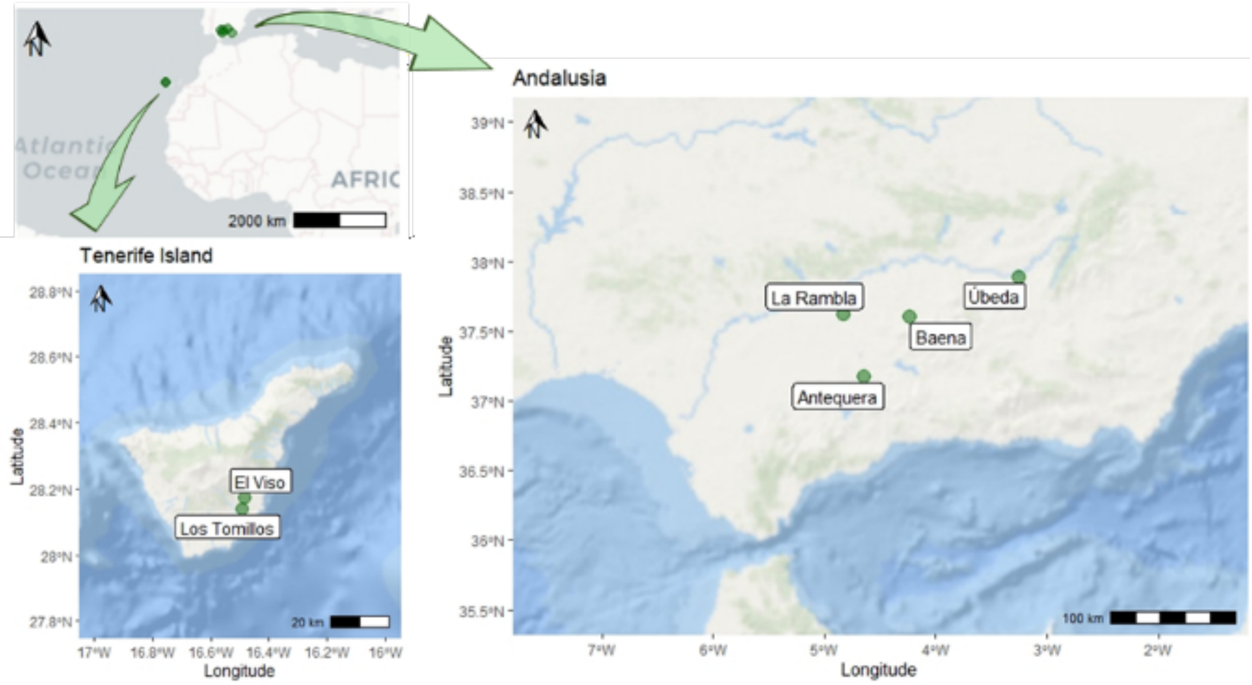

Figure 1.- Geografical location of the different tested orchards

Fertilization was applied on the basis of foliar analysis in order to avoid any limitations on growth and yield.

Four sets of three trees per location with similar fruit load (2-3 in a 0-3 visual scale) were sampled on three dates of the olive maturation period in 2017. In the case of Andalusian locations, sampling was performed in mid-September (harvest 1), mid-October (harvest 2) and mid-November (harvest 3). In the case of Tenerife, as the oil accumulation occurs earlier (Figure 2), harvest dates were mid-August (harvest 1), mid-September (harvest 2) and mid-October (harvest 3).

In each harvest date, a sample of 1 kg fruit was randomly hand-picked for each set of trees and location (4 sets x 3 harvest dates x 6 locations) to evaluate phenolic content and composition as well as fruit traits.

2.2. Phenols analysis

Reagents for extraction and other measurements were supplied by Sigma-Aldrich (St. Louis, MO). Phenolic standards were purchased from Extrasynthese (Genay, France).

Fruit phenolic compounds were extracted from each sample according to a previously developed protocol (García-Rodríguez *et al.,* 2011). Longitudinal pieces of mesocarp were cut at least from twenty olives.

For each sample, fruits were finely chopped and used to prepare phenolic extracts. Representative fruits samples (1 g) were kept at 4 °C for 72 h in dimethyl sulphoxide (DMSO, 6 mL) containing siringic acid as internal standard. The extracts were filtered through a 0.45 μm mesh nylon and kept at -20 ºC until HPLC analysis.

The phenolic extracts were analyzed by HPLC on a Beckman Coulter liquid chromatography system equipped with a System Gold 168 detector, solvent module 126, autosampler module 508 and a Waters column heater module following a previously described methodology (Pérez *et al.,* 2014). A Superspher RP 18 column (4.6 mm i.d. x 250 mm, particle size 4 μm: Dr Maisch GmbH, Germany) at a flow rate of 1 mL/ min and a temperature of 35 ºC was used for all the analyses. A total of 9 phenolic compounds were quantified in the phenolic extracts: hydroxytyrosol-4-glucoside, tyrosol-1-glucoside, demethyloleuropein, verbascoside, luteolin-7-glucoside, rutin, oleuropein, comselogoside, and ligustroside.

Phenolic compounds were monitored at two different wavelengths of 280 nm and 335 nm and quantified taking into account the internal standard and specific response factors for each of them (García-Rodríguez *et al.,* 2011).

The tentative identification of compounds by their UV-vis spectra was confirmed by HPLC/ESI-qTOF-HRMS. The liquid chromatography system was Dionex Ultimate 3000 RS U-HPLC liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a similar Superspher RP 18 column but with 3 μm particle size. Formic acid 1% was used instead of phosphoric acid 0.5% for the mobile phase. A split post-column of 0.4 mL/ min was introduced directly onto the mass spectrometer electrospray ion source. The HPLC/ESI-qTOF operated for mass analysis using a micrOTOF-QII High Resolu-

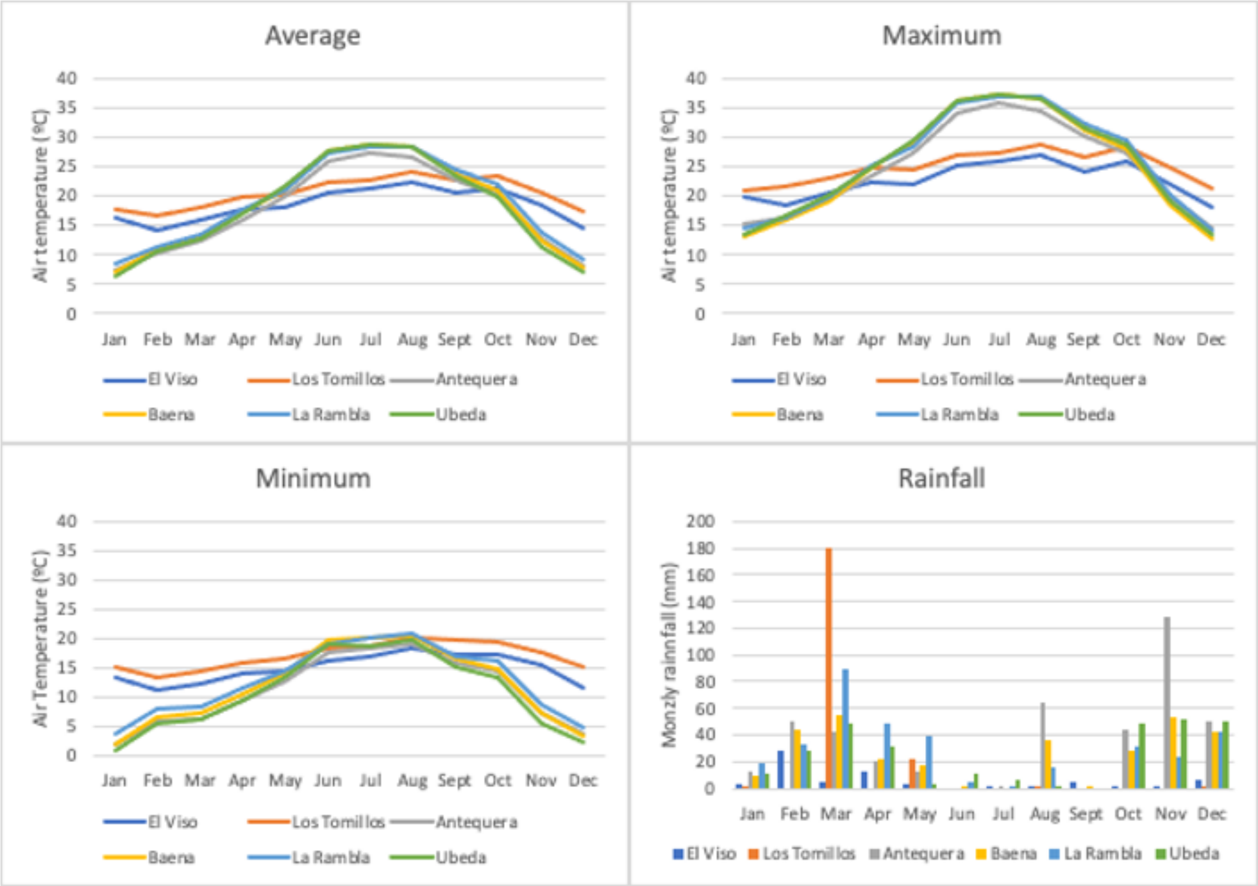

Figure 2. Average, maximum and minimum daily temperature (monthly average in ºC) and monthly rainfall (mm/m^2) of the six locations considered. Temperature data were recorded hourly to make daily statistics

tion Time-of-Flight mass spectrometer (UHRTOF) with qQ-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. Mass spectra were acquired in MS full scan mode and data were processed using Target Analysis 1.2 software (Bruker Daltonics, Bremen, Germany).

2.3. Fruit traits analysis

Ripening index was evaluated according to the procedure described by (Frias *et al.,* 1991). Afterwards, oil content was measured in three random sub-samples of 25 g of each sample. Each sub-sample was oven dried at 105 ºC for 42 h (Río and Romero, 1999) to ensure dehydration, and weighed to measure the percentage of oil content in a NMR fat analyzer.

2.4. Statistics

Analysis of variance was performed to evaluate the relative influence of location, harvest date and their interaction on the variability of phenolic content and composition. Comparison of means (Tukey) was used to test differences among locations, harvest dates and harvest dates-locations, when significant. Previous works have shown that the water availability is the main factor influencing phenol content (Gucci *et al*., 2019). Therefore, in order to test the differences between the Mediterranean and Subtropical conditions of Andalusia and Tenerife regions, a separate analysis was performed with only the four irrigated locations (two in Andalusia and two in Tenerife). Finally, a person correlation was performed to test the correlations among phenol constituents and with fruit traits.

3. RESULTS

The six locations involved in this work showed different climatic conditions during the experimental period, mainly associated with the region level (Figure 2). The two locations in Tenerife, Canary Islands, showed milder temperatures both in summer and winter, with around 7 ºC range of average maximum-minimum temperatures. Temperatures in Los Tomillos (located 200 m.a.s.l.) were 2-3 ºC higher than in El Viso (400 m.a.s.l.). Among the locations in Andalusia, Baena, La Rambla and Úbeda showed quite similar temperature patterns; while only slightly lower temperatures were observed in Antequera. Some differences in

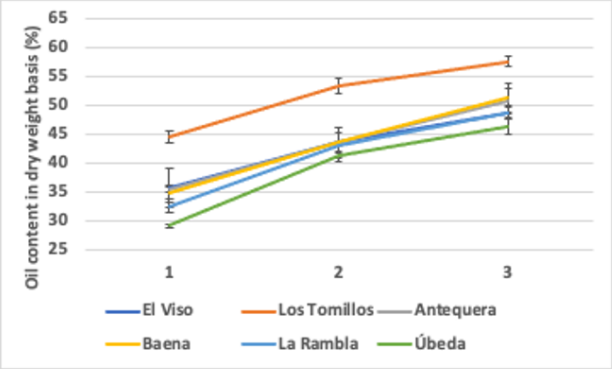

Figure 3. Oil content on dry weight basis of ‘Arbequina’ fruits on three harvest dates in the four locations of Andalusia, Iberian Peninsula (Antequera, Baena, La Rambla and Úbeda) and in two locations of Tenerife, Canary Islands (El Viso and Los Tomillos). In Andalusia, harvests 1, 2 and 3 represent mid- September, mid-October and mid-November at the Iberian Peninsula locations. In Tenerife, oil accumulation occurs one month in advance. For that reason, harvests 1, 2 and 3 represent mid-August, mid- September and mid-October. Three replicates per location and harvest date were averaged.

rainfall distribution among the Andalusian locations were also observed, particularly due to high rainfall in Antequera in the first week of November. Compared to the Peninsula, locations in Tenerife were characterized by limited rainfall throughout the year, with only exceptional rainfall observed in Los Tomillos in March.

The oil accumulation of ‘Arbequina’ in the six locations sampled showed a similar pattern considering that harvest 1 was done in August for El Viso and Los Tomillos (in Tenerife) and in September for the Andalusian locations (Figure 3).

Among all the identified phenols in ‘Arbequina’ fruits, oleuropein represented around half of the total phenolic content (Table 1), and together with demethyloleuropein, comseogoside, ligstroside and verbascoside constituted 95% of the total phenolic content. All the phenols showed high variability (high coefficient of variance), being especially high for verbascoside and oleuropein.

This high variability in phenolic content and composition was mainly due to location effect for phenols and most of the components (Table 2) except for oleuropein and ligstroside, for which location and harvest date showed comparable variance and for demethyloleuropein, which showed a double variance for the harvest date effect (41.9) compared to location (21.2) and their interaction (18.0). The interaction between location and harvest date represented the main contributor to total variance only for hydroxytyrosol-4-glucoside. Error variance represented more than half of the total variance for luteolin-7-O-glucoside. In any case, location, harvest date and their interaction showed a significant effect on phenolic content and all its components except for rutin and luteolin-7-O-glucoside, for which location was the only significant factor.

The phenolic components described were found in all locations and harvest dates (Table 3). The high variability due to location for total phenols was mainly due to the high values observed for Antequera and Baena (Table 3, Figure 4). While the decrease in total phenols with harvest date was, on average, more similar between harvest 1 and 2 than between harvest 2 and 3. However, for Antequera and Baena, this decrease was more evident between harvest 2 and harvest 3 -- just the opposite for the rest of the locations. Oleouropein, the major phenol identified, showed a similar pattern of variation to that of total phenols. The same could be said for ligstroside.

Different patterns of variation were observed for the rest of phenolic components (Table 3, Figure 4). Demethyloleuropein showed an increase between harvest 1 and harvest 2 in all locations except for Los Tomillos. In Antequera, this increase was also maintained between harvest 2 and harvest 3, maybe associated to a heavy rainfall at that time. Few variations among harvest dates were observed for comselogoside and verbascoside. Only in

Table 1. Descriptive statistics (from a total of 54 samples) of the phenols found in ‘Arbequina’ olive pulp. Data are presented in µg/g of fresh olive pulp.

Compound	Average	SDb	CVb	Min	Max
Total phenols	23028	11647	51	8180	51767
Oleuropein	11176	10740	96	585	39985
Demethyloleuropein	7103	3722	52	555	14639
Comselogoside	1470	681	46	462	3279
Ligstroside	1103	972	88	171	4251
Verbascoside	916	927	101	30	4009
Hydroxytyrosol-4-glucoside	431	226	52	75	1061
Rutin	408	252	62	49	1046
Luteolin-7-O-glucoside	367	232	63	15	1335
Tyrosol-1-glucoside	53	18	33	35	97

[a]SD=Standard deviation, [b]CV= coefficient of variance

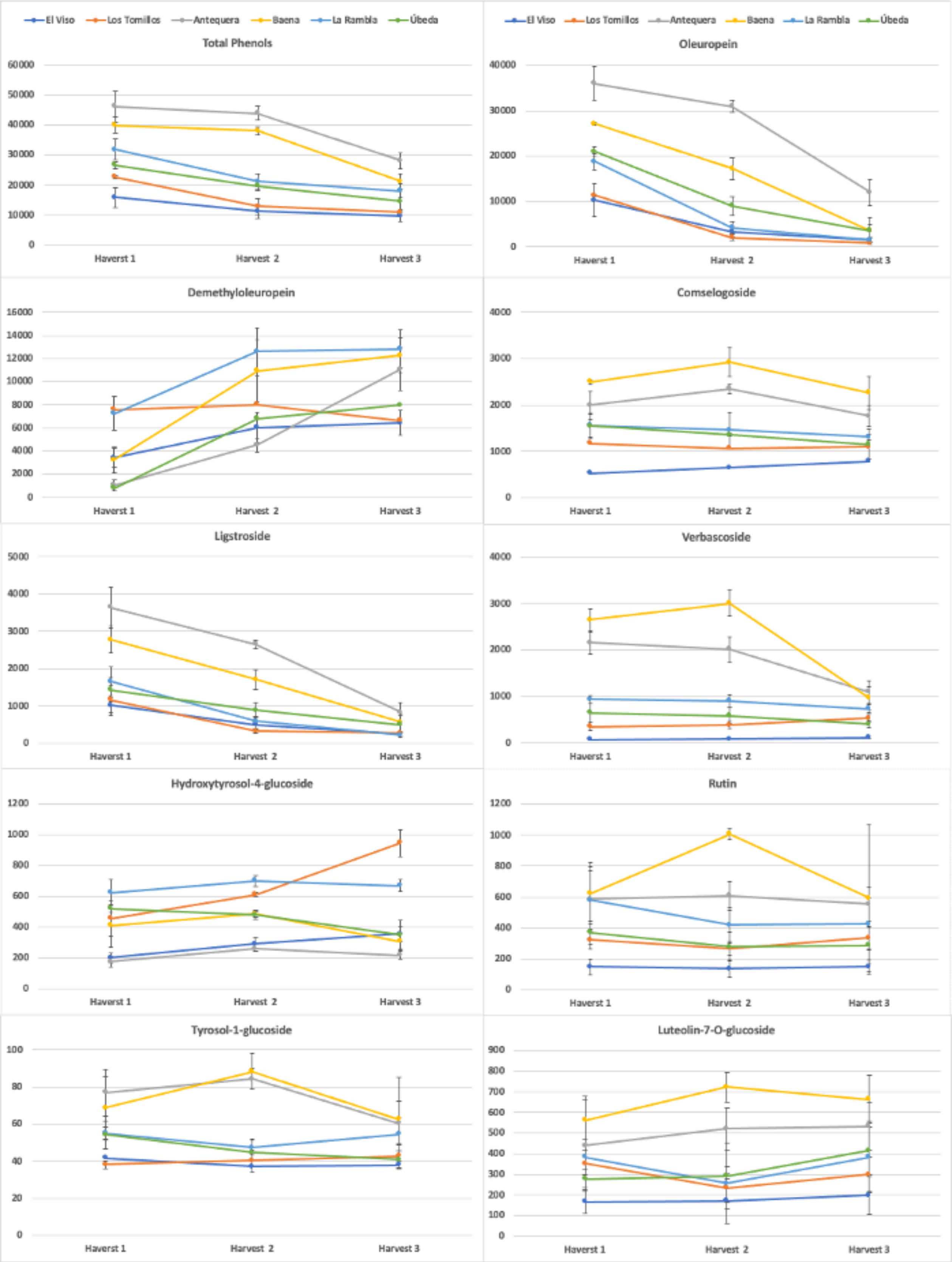

FIGURE 4. Variation in the main phenols found in 'Arbequina' fruits on the three harvest dates of the six locations considered. Data are presented in μg/g of fresh olive pulp. Three replicates per location and harvest date were averaged.

Table 2. Percentage of variance for location, harvest date and their interaction for the phenols found in ‘Arbequina’ olive pulp by the ANOVA analysis. Values in bold indicate significant differences for this source of variation at p<0.01. The analysis included 6 locations, 3 harvest dates and 3 replicates per location and harvest date.

	Total phenols	**Oleuropein**	**Demethyloleuropein**	**Comselogoside**	**Liustroside**	**Verbascoside**	**Hydroxytyro-sol-4-glucoside**	**Rutin**	**Luteolin-7-O-glu-coside**	**Tyrosol-1-glucoside**
Location	66.1	43.2	21.2	87.6	38.7	64.1	40.4	64.0	41.5	65.9
Harvest date	25.0	45.6	41.9	1.0	43.4	4.6	0.0	0.0	0.2	1.2
Location * Harvest date	5.0	5.2	18.0	4.1	8.2	10.7	47.4	4.9	0.0	7.1
Error	3.9	6.1	18.9	7.3	9.7	20.6	12.2	31.0	58.3	25.8

Table 3. Total and main phenol means by harvest date, location and harvest* location in ‘Arbequina’. Different letters indicate significant differences (p< 0.005) among means within each of the three groups of data (Tukey test). Data are presented in µg/g of fresh olive pulp. Three replicates per location and harvest were averaged.

	El Viso		**Los Tomillos**		**Antequera**		**Baena**		**La Rambla**		**Úbeda**		**Average**	
Total Phenols														
Haverst 1	15834.4	ijk	22650.8	fg	46127.2	a	39922.6	bc	31775.7	d	26668.1	ef	30496.5	a
Harvest 2	11258.0	lm	12827.2	klm	43915.2	ab	38081.9	c	21231.0	gh	19747.0	ghi	24510.1	b
Harverst 3	9783.4	m	10911.0	lm	28063.9	de	21206.2	gh	18091.6	hij	14593.6	jkl	17108.3	c
Average	12292.0	d	15463.0	d	39368.8	a	33070.2	b	23699.4	c	20336.2	c		
Oleuropein														
Haverst 1	10242.6	f	11328.1	f	36031.0	a	27113.0	c	18728.1	de	21014.0	d	20742.8	a
Harvest 2	3360.8	gh	2045.8	gh	30914.5	b	17190.7	e	4235.9	g	9018.4	f	11127.7	b
Harverst 3	1454.5	gh	775.7	h	11946.2	f	3501.4	gh	1453.7	gh	3483.5	gh	3769.1	c
Average	5019.3	e	4716.6	e	26297.2	a	15935.0	b	8139.2	d	11172.0	c		
Demetiloleuropein														
Haverst 1	3429.5	d	7593.4	b	1030.6	ef	3226.2	de	7253.3	b	798.0	f	3888.5	c
Harvest 2	6054.5	bc	7995.4	b	4516.2	cd	10959.2	a	12628.2	a	6808.6	b	8160.3	b
Harverst 3	6439.9	bc	6629.7	bc	11057.3	a	12280.1	a	12852.0	a	7963.1	b	9537.0	a
Average	5308.0	d	7406.2	bc	5534.7	cd	8821.9	b	10911.2	a	5189.9	d		
Cosmelogoside														
Haverst 1	531.3	j	1176.1	gh	2002.0	cd	2501.5	b	1562.6	ef	1550.5	ef	1554.0	ab
Harvest 2	653.5	j	1065.3	hi	2357.2	bc	2922.8	a	1460.0	efg	1362.1	fgh	1636.8	a
Harverst 3	790.3	ij	1096.7	hi	1764.8	de	2269.7	bc	1311.2	fgh	1156.3	gh	1398.2	b
Average	658.4	e	1112.7	d	2041.3	b	2564.7	a	1444.6	c	1356.3	cd		
Ligustroside														
Haverst 1	1013.6	e	1155.5	de	3634.6	a	2774.4	b	1653.1	c	1433.6	cd	1944.1	a
Harvest 2	497.2	fg	322.3	g	2647.3	b	1704.9	c	583.5	fg	882.4	ef	1106.3	b
Harverst 3	245.9	g	262.9	g	833.4	ef	561.9	fg	215.6	g	495.0	fg	435.8	c
Average	585.6	d	580.2	d	2371.8	a	1680.4	b	817.4	cd	937.0	c		
Verbascoside														
Haverst 1	71.9	ef	352.2	def	2154.9	b	2646.2	ab	937.4	d	650.6	cde	1135.5	a
Harvest 2	83.4	f	383.5	def	2011.9	b	3005.0	a	901.4	cd	583.5	cdef	1161.5	a
Harverst 3	114.4	f	525.3	cdef	1099.9	c	969.5	cd	731.1	cd	405.2	def	640.9	b
Average	89.9	d	420.3	cd	1755.6	b	2206.9	a	856.6	c	546.4	cd		
Hydroxytirosol-1-Glucoside-1-														
Haverst 1	187.8	h	331.8	fghi	173.7	hi	407.7	efg	622.3	bcd	519.0	cde	373.7	c
Harvest 2	264.9	ghi	473.7	def	256.4	ghi	481.7	def	700.4	b	476.8	def	442.3	ab
Harverst 3	354.5	efgh	942.6	a	215.8	hi	306.6	fghi	665.5	bc	347.4	efghi	472.1	a
Average	269.1	cd	582.7	a	215.3	d	398.7	bc	662.7	a	447.7	b		

	El Viso		Los Tomillos		Antequera		Baena		La Rambla		Úbeda		Average	
Rutin														
Haverst 1	148.5	fg	323.2	defg	585.0	bc	622.3	b	581.1	bc	370.8	cdef	438.5	ns
Harvest 2	135.5	g	268.0	efg	607.9	bc	1007.0	a	419.3	bcde	277.8	efg	452.6	ns
Harverst 3	148.1	fg	335.0	defg	552.9	bcd	591.0	bc	424.8	bcde	287.7	efg	389.9	ns
Average	144.0	d	308.8	cd	581.9	ab	740.1	a	475.1	bc	312.1	cd		
Luteolin-7-O-glucoside														
Haverst 1	167.6	e	352.4	bcde	438.5	abcde	562.7	abc	383.0	bcde	277.3	cde	363.6	ns
Harvest 2	170.9	e	232.8	cde	519.6	abcd	722.2	a	255.0	cde	292.7	cde	365.5	ns
Harverst 3	197.7	e	300.2	cde	533.1	abcd	663.4	ab	383.2	bcde	414.4	abcde	415.3	ns
Average	178.7	c	295.1	bc	497.0	ab	649.4	a	340.4	bc	328.1	bc		
Glucoside-1-tyrosol														
Haverst 1	41.7	ef	38.2	f	76.9	ab	68.7	bc	55.0	cde	54.2	cde	55.8	ns
Harvest 2	37.1	f	40.3	f	84.3	a	88.3	a	47.4	efg	44.7	ef	57.0	ns
Harverst 3	38.0	f	42.8	ef	60.5	cd	62.7	bc	54.5	cde	40.7	ef	49.9	ns
Average	39.0	c	40.4	bc	73.9	a	73.2	a	52.3	b	46.5	bc		

Table 4. Fruit trait means by harvest date, location and harvest* location in ‘Arbequina’. Different letters indicate significant differences (p< 0.005) among means within each of thr three groups of data (Tukey test). Three replicates per location and harvest were averaged.

	El Viso		Los Tomillos		Antequera		Baena		Fuencubierta		Úbeda		Average	
Fruit fresh weight (g)														
Haverst 1	0,86	g	1,29	ef	0,93	g	1,44	cde	1,40	cde	1,08	fg	1,17	c
Harvest 2	1,24	ef	1,66	bc	0,86	g	1,37	def	1,75	ab	1,37	def	1,37	b
Harverst 3	1,32	ef	1,85	ab	1,30	ef	1,76	ab	2,02	a	1,60	bcd	1,64	a
Average	1,14	cd	1,60	a	1,03	d	1,52	ab	1,72	a	1,35	bc		
Oil content in dry basis (%)														
Haverst 1	35,6	g	44,6	e	35,5	gh	34,9	gh	32,4	hi	29,1	i	35,4	c
Harvest 2	43,5	ef	53,4	b	43,6	ef	43,6	ef	43,2	ef	41,2	f	44,8	b
Harverst 3	48,8	cd	57,6	a	50,6	bc	51,4	bc	48,7	cd	46,4	de	50,6	a
Average	42,6	b	51,9	a	43,2	b	43,3	b	41,4	bc	38,9	c		
Fruit Moisture (%)														
Haverst 1	59,7	ab	50,9	fg	45,5	ij	50,0	fgh	59,1	abc	56,7	cde	53,7	a
Harvest 2	57,8	abcd	52,1	f	35,6	k	43,9	j	58,1	abcd	55,2	e	50,4	c
Harverst 3	56,3	de	48,7	gh	44,6	j	47,8	hi	60,5	a	56,2	de	52,4	b
Average	57,9	ab	50,6	c	41,9	e	47,2	d	59,2	a	56,1	b		
Maturity index														
Haverst 1	0,8	gh	1,4	ef	m.d.		1,0	fgh	m.d.		1,5	ef	1,5	c
Harvest 2	1,7	de	2,4	c	0,1	i	1,2	fg	m.d.		3,1	b	1,7	b
Harverst 3	2,0	cd	3,2	b	0,8	h	1,2	fg	m.d.		3,7	a	2,0	a
Average	1,5	b	2,3	a	0,4	c	1,2	b	m.d.		2,7	a		

Antequera and Baena, the two locations with the highest values for both components, a significant decrease was observed in harvest 3.

For the four components with lower contents in ‘Arbequina’ fruits (hydroxytyrosol-4-glucoside, rutin, luteolin-7-O-glucoside and tyrosol-1-glucoside), the highest values were observed in Antequera and Baena samples, probably due to the water stress. The unique exception was hydroxytyrosol-4-glucoside with very high levels in the fruits from La Rambla, and a significantly different accumulation pattern observed in Los Tomillos. This component is probably less influenced by water stress. Few variations with harvest date were observed for rutin, except for the very high values in Baena in harvest 2.

Fruit traits were also evaluated in the six locations and on three harvest dates. Fruit size, moisture and maturity index showed most of the variance due to the location effect. The oil content was mainly influenced by harvest date, as expected (data not shown). Antequera was the location with the smallest fruit size; while Los Tomillos showed higher oil content than the rest of the locations (Table 4). Fruit moisture was much lower in the two dry farming locations (Antequera and Baena), as expected. Maturity index was very delayed in Antequera, and very

Table 5. Percentage of variance of region, harvest date and their interaction for the phenols found in 'Arbequina' olive pulp by the ANOVA analysis. Only data for the four irrigated locations in Andalusia (La Rambla and Úbeda) and Tenerife (Los Tomillos and El Viso) were included. For each location, data of the 3 harvest dates and 3 replicates per location and harvest date were considered. Values in bold indicate significant differences for this source of variation at p<0.01.

	Total phenols	**Oleuropein**	**Demethyloleuropein**	**Comselogoside**	**Liustroside**	**Verbascoside**	**Hydroxytyro-sol-4-glucoside**	**Rutin**	**Luteolin-7-O-glu-coside**	**Tyrosol-1-glucoside**
Region	47,2	19,1	5,9	58,7	13,8	63,1	7,2	45,7	22,3	52,7
Date	38,3	71,0	22,5	3,8	68,1	4,2	8,9	1,8	6,5	4,2
Region * Date	2,4	0,6	11,0	4,1	3,3	4,8	27,3	1,9	7,0	0,3
Error	12,1	9,2	60,5	33,4	14,8	27,9	56,6	50,6	64,2	42,7

Table 6. Pearson correlation coefficients among phenols and fruit characteristics for the data of elementary plots by location and harvest date (18 data points) in 'Arbequina'. Values > 0.7 are highlighted.

	Total phenols	Oleuropein	Demethyloleuropein	Comsegoloside	Ligstroside	Verbascoside	Hydroxytyro-sol-4-glucoside	Rutin	Luteolin-7-O-gluco-side	Tyrosol-1-glucoside	Fruit fresh weight	Fruit dry weight	Oil content in dry weight	Fruit moisture
Oleuropein	0.93													
Demethyloleuropein	-0.20	-0.55												
Comseogoside	0.82	0.61	0.16											
Ligstroside	0.91	0.96	-0.51	0.61										
Verbascoside	0.81	0.65	0.00	0.83	0.69									
Hydroxytyrosol-4-glucoside	-0.28	-0.38	0.31	-0.07	-0.36	-0.10								
Rutin	0.70	0.48	0.20	0.79	0.50	0.74	0.00							
Luteolin-7-O-glucoside	0.49	0.31	0.18	0.62	0.34	0.51	-0.11	0.87						
Tyrosol-1-glucoside	0.83	0.70	-0.02	0.84	0.70	0.80	-0.17	0.63	0.43					
Fruit fresh weight	-0.39	-0.59	0.61	0.00	-0.54	-0.13	0.63	0.09	0.15	-0.27				
Fruit dry weight	-0.19	-0.42	0.56	0.25	-0.36	0.10	0.54	0.31	0.37	-0.02	0.86			
Oil content in dry weight	-0.50	-0.65	0.53	-0.15	-0.61	-0.25	0.33	-0.10	0.05	-0.26	0.63	0.75		
Fruit moisture	-0.59	-0.50	0.04	-0.65	-0.49	-0.59	0.18	-0.53	-0.47	-0.65	0.22	-0.23	-0.23	
Maturity index	-0.62	-0.63	0.07	-0.41	-0.57	-0.39	0.56	-0.36	-0.25	-0.54	0.59	0.35	0.38	0.39

advanced in Úbeda, although intermediate in the rest of the locations.

A separate analysis of variance was performed for the four irrigated locations to better test differences among the Mediterranean and Subtropical conditions of Andalusia and Tenerife regions (Table 5). Region was the main contributor to the total variance for total phenols, comselogoside, verbascoside, rutin and tyrosol-1-glucoside. This effect was mainly due to the higher values for those components in the two Andalusian locations (La Rambla and Úbeda), especially for the first two harvest dates (Figure 4). While oleuropein and lisgtroside showed higher variance for harvest date mainly due to the sharp decrease in their contents, especially between harvests 2 and 3.

The correlation between phenolic content components and with fruit traits were also studied (Table 6). Total phenolic content showed a high positive correlation with most individual phenolic components except for demethyloleuropein, which increased throughout the harvest season, and hydroxytyrosol-4-glucoside and luteolin-7-O-glucoside which did not show a clear decrease during ripening. Among the phenolic components, the stronger correlation was found between oleuropein and ligstroside. Although comselogoside, verbascoside and rutin seemed to also be highly correlated. Besides, tyrosol-1-glucoside showed very high correlation coefficients with verbascoside and comselogoside. Both have a similar chemical structure in which the glucose is directly linked to the phenolic alcohol moiety, hydroxytyrosol and tyrosol, respectively. No high correlations were found between the total phenolic content or specific phenolic components and fruit traits, with most of them being negative, except for demethyloleuropein and tyrosol-1-glucoside. Ripening index showed low correlations with phenol content and composition and with the fruit traits evaluated. In particular, fruits having a high ripening index (more than 2) showed low phenol contents (less than 20,000 µg/g of fresh olive pulp); while fruits having a ripening index lower than 2 showed a very large range in variation in phenol contents (from 8,180 to 51,767 µg/g of fresh olive pulp).

4. DISCUSSION

The nine major phenolic components identified, in all locations and harvest dates, showed high variability (coefficient of variance higher than 50%, except for comselogoside and tyrosol-1-glucoside). Oleuropein was the main phenol as previously reported for olive fruits of other cultivars (Ben Ghorbal *et al.,* 2018; Valente *et al*., 2020).

The high variability found was mainly due to a location effect for total phenols and for most individual phenolic components. Significant the effect of location was previously reported for phenols in several cultivars. This is the case of 'Gemlik' in Turkey (Ben Ghorbal *et al.,* 2018), 'Arbequina', 'Manzanilla' and 'Arauco' in Argentina (Bodoira *et al.,* 2015) and Italian cultivars from a central region of Italy (Mousavi *et al.,* 2019).

Most variability associated with location was caused by the high values observed for Antequera and Baena compared to the other four locations. The most outstanding difference between Antequera and Baena and the rest of the locations is that olive trees are managed in dry farming, while, in Los Tomillos, El Viso, La Rambla and Úbeda, drip irrigation is used. The great influence of water availability on phenolic content and composition of olive fruits and virgin olive oils was previously reported when olive dry farming and irrigation were compared (Gómez-Rico *et al.,* 2006; Cirilli *et al.,* 2017). It seems that water stress periods caused a higher concentration of phenolic compounds in in the dry farming locations of Antequera and Baena, which remained during most of the maturation period. Water availability in the dry farming locations was especially low at the beginning of the oil accumulation period with respect to the four irrigated locations. Previous reports have suggested that water stress in this period has a strong influence on the phenol content (Gucci *et al*., 2019). This is maybe the reason why rainfall at the beginning of November in Antequera had little influence on phenol content. However, in some other works, a decrease in phenol content with higher stress has been reported (Valente *et al*., 2020). Probably, in the latter case, the combination of water and heat stress gave a different response of the olives.

When considering only the four irrigated locations, two in Andalusia and two in Tenerife, higher content for total phenols and some components was observed in Andalusia. These differences were especially important for the first harvest date. The higher summer temperatures in Andalusia with respect to Tenerife could produce higher stress which could be the cause of those differences.

Previously, the Sub-Tropical temperatures of Tenerife have shown a great influence on the flowering phenology of the olive tree (Medina-Alonso *et al*., 2020). This is important, since the Tenerife climatic conditions could help to predict the influence of climate change in the Mediterranean climate. In our case, it seems that the main factor associated with climate change that would impact phenol content is water availability more than changes in air temperature. However, the higher heat stress in summer, predicted in a climate change scenario, could also increase phenol content although have negative influence on other parameters such as oil content (Navas *et al*., 2019). More experimentation is needed to accurately determine the influence of climate change on phenol content and composition.

Harvest date was also showed to influence the variability in total phenols and phenolic components. As observed in our trials, most of the previous works reported a decrease in total phenolic content with maturation (Abaza *et al.,* 2017; Ferro *et al*., 2020), including 'Arbequina' in North-East Spain (Benito *et al.,* 2013). However, different patterns of variation were observed for individual phenolic components. In fact, the decrease in oleuropein content throughout the ripening process was concomitant with a parallel increase in demethyloleuropein, as previously reported (Gómez-Rico *et al.,* 2008). This was the only component with a significant increase with harvest date. Previous reports have also shown different variation patterns for the different phenolic components in fruit (Ben Ghorbal *et al.,* 2018; Talhaoui *et al.,* 2015; Ferro *et al*., 2020).

ANOVA analysis revealed the comparison of the relative influence of location and harvest date. For total phenols and some components such as comselogoside, tyrosol-1-glucoside, verbascoside and rutin, location had a much greater influence than harvest date. On the contrary, in the case of demethyloleuropein, harvest date was more important. And for other components such as oleuropein and ligstroside, both factors were equally important. Therefore, the relative influence of harvest date and location very much depend on the phenolic component. No previous study on the comparative variance analysis has been performed for olive phenols, except for the case of ‘Baladi’ in Lebanon (El Riachy *et al.,* 2018), where a greater influence of location was observed with respect to harvest date for total phenol content determined spectrophotometrically using the Folin-Ciocalteu method. Different influences of location and genotype for the different phenolic components were previously reported in a set of breeding trials (Pérez *et al.,* 2018).

As expected, all phenolic components showing similar behavior across locations and harvest dates were highly correlated. The extremely high correlation found for oleuropein and ligstroside suggest a common biosynthetic pathway for both secoiridoid glucosides. While the negative correlation between oleuropein and demethyloleuropein support the hypothesis on the interconversion of oleuropein into demethyloleuropein (Obied *et al.,* 2008). On the contrary, demethyloleuropein, hydroxytyrosol-4-glucoside and luteolin-7-O-glucoside showed no correlation with the rest as having a different pattern of variation. Different correlations than the ones reported here were found in a previous set of breeding trials (Pérez *et al.,* 2018). This is probably due to the fact that the variability here is only due to environmental factors; while in the previous work, the phenol variability is attributable to different environments but also to different genotypes. The correlation of phenols with oil and moisture contents in the fruit was low and negative, which seems to indicate different metabolic pathways.

Ripening index seems to be also negatively correlated with phenolic content and most of the phenolic components. In fact, ripening index has been proposed as an indicator of fruit composition (Sánchez de Medina *et al.,* 2014). However, the correlation coefficients obtained in this work were not very high and varied greatly across locations. Overall, low phenolic content was found in fruits with high ripening index (2.0 to 4.0). However, at lower ripening indexes (0.8 to 2.0), both fruits with high and low phenolic contents were found. These results could explain some contradictory results reported in previous studies. Indeed, Gomez-Rico *et al.* (2008) and Morelló *et al.* (2004) showed both high correlation and lack of correlation of ripening index with phenolic content and composition in the ‘Arbequina’ cultivar. Therefore, a general relationship between fruit color and phenolic content should be taken cautiously. A strong association of phenol content with harvest time but not with ripening index could be explained by the fact that there is not a consistent pattern of variation in the ripening index with harvest time in the different locations considered.

5. CONCLUSIONS

In summary, water availability and harvest date seem to play a more important role than air temperature on the phenolic content and composition of ‘Arbequina’ fruits, even when very different climatic conditions such as Sub-Tropical and Mediterranean are compared. Therefore, in order to obtain olive fruits with high phenolic content, low water availability together with an early harvest should be considered. Taking into account that those two factors would also reduce the oil content in fruit. Among the phenol components studied, comsegoloside seems to be the one with the greatest variance among locations. Therefore, future studies might consider this phenol as a good marker for plant stresses, especially water availability. Further research is needed to determine the relative influence of water availability and harvest date on other cultivars with different phenolic profiles than the ‘Arbequina’ one here considered.

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Comparative study of the physicochemical properties of a vegan dressing-type mayonnaise and traditional commercial mayonnaise

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SUMMARY: The food industry has developed a vegan dressing-type mayonnaise due to new consumer demands. The aim of this study was to compare three commercial mayonnaise types with a vegan dressing, measuring their physicochemical properties. Four dressing samples were analyzed: vegan, homemade recipe, creamy, and light. The following properties were measured: water activity, color, droplet size, rheological properties, structural analysis, and oxidative stability. A high color difference was observed between vegan and the other samples due to the presence of chickpea protein. The size and distribution of droplets of the vegan sample were greater than the others. The rheological properties indicated that all samples are non-Newtonian pseudoplastic fluids. The FT-IR results indicated that the highest peak for vegan corresponded to its content in mono-unsaturated fat. Therefore, it showed the lowest oxidative stability. In conclusion, the mayonaise formulations were affected by physicochemical properties such as the content and composition of the oil, thickener and protein contents, along with processing technology.

KEYWORDS: *Emulsion; Mayonnaise; Physicochemical properties; Vegan*

RESUMEN: ***Estudio comparativo de las propiedades fisicoquímicas entre una salsa vegana tipo mayonesa con respecto a las mayonesas comerciales tradicionales.*** La industria alimentaria ha desarrollado salsas tipo mayonesa veganas debido a los nuevos requerimientos de los consumidores. El objetivo de este trabajo fue comparar tres tipos de mayonesas comerciales (receta casera, cremosa y ligera) con una salsa vegana tipo también comercial, midiendo sus propiedades fisicoquímicas. Se midió actividad de agua, color, tamaño de gota, propiedades reológicas, análisis estructural y estabilidad oxidativa. Una gran diferencia de color fue observada entre la muestra vegana en comparación con las otras muestras debido a la presencia de proteínas de garbanzo. El tamaño y distribución de gotas y la estabilidad oxidativa de esta salsa fueron mayores en comparación con las otras muestras. Las propiedades reológicas indicaron que todas las muestras son fluidos pseudoplásticos no newtonianos. Los resultados de FT-IR indicaron que el pico más alto de la salsa vegana corresponde a grasas monoinsaturadas por esto mostró la menor estabilidad oxidativa. En conclusión, la formulación de cada tipo de salsa afectó sus propiedades fisicoquímicas.

PALABRAS CLAVE: *Emulsión; Mayonesa; Propiedades físico químicas; Vegano*

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1. INTRODUCTION

An emulsion is a thermodynamically unstable dispersion of two immiscible liquids, usually apolar and polar, which forms small droplets (0.1 to 100 microns); one is called the dispersed or internal phase and the other, continuous or external phase (Muñoz *et al*., 2007). Emulsions are classified according to their composition as simple emulsions such as oil-in-water (O / W), water-in-oil (W / O), or multiple emulsions such as water-in-oil-in-water emulsions (W / O / W) and oil-in-water-in-oil (O / W / O) (Noon *et al*., 2020).

Mayonnaise is a type of oil-in-water (O / W) emulsion. It is defined as a condiment in the form of a dressing obtained by emulsifying edible vegetable oil(s) in an aqueous phase consisting of vinegar, and the addition of egg yolk produces the oil-in-water emulsion; besides, salt and seasonings can be added (Codex-Stan, 1989). Traditional mayonnaise contains 70-80g / 100g of fat and is one of the most commonly consumed dressing worldwide (Chang *et al*., 2017). Its structure, creaminess, appearance, and rheological behavior are of great importance for sensory properties and perceived texture, as well as for physical stability, parameters that represent critical factors in determining consumer choice and satisfaction (Di Mattia *et al*., 2015).

Nowadays, food preferences have been changing, either by controlling and avoiding degenerative diseases associated with food or having healthier and even environmentally-friendly lifestyles (Park *et al*., 2020). In this sense, the consumption of low-fat foods has become a trend, mostly to avoid the development of cardiovascular diseases, obesity, and cancer (Jiménez-Colmenero *et al*., 2013). However, oil is an essential component in the elaboration of mayonnaise, and it plays an important role in its physicochemical and sensory characteristics (Zia-ud-Din *et al*., 2017). That is why the food industry has developed new formulations using additives which allow it to maintain the characteristics of a traditional mayonnaise but with a low oil content (Depree and Savage, 2001; Shen *et al*., 2011). Within traditional mayonnaise, there is also a difference in the type of oil used, the most common being soybean, sunflower, corn and rapeseed oils (Di Mattia *et al.,* 2015).

On the other hand, recent consumer-conscious demands for healthy foods have increased. Moreover, vegan customers cannot eat egg-based foods. These new dietary trends impact mayonnaise formulations (Ali and EL Said, 2020). So it is possible to find mayonnaise on the market which is free of animal components, known as vegan mayonnaise or also called vegan dressings (Cornelia *et al*., 2015). Although this type of dressing is called vegan mayonnaise, international regulations do not define it since the term mayonnaise corresponds to the definition described above. For the formulation of vegan dressing, the use of eggs is replaced by legumes such as beans, soybeans, chickpea, white lupine, wheat protein, and a germ protein isolate (Ali and EL Said, 2020).

Mirzanajafi-Zanjani *et al.* (2019) reported that each dressing component formulation has a specific role, where increasing or decreasing each particle size could influence the mayonnaise's consistency, stability, and the sensory properties of the product's antioxidant stability. Raikos *et al.* (2020) studied a commercially canned chickpea aquafaba as an egg substitute for the development of vegan mayonnaise, showing that the phenolic compounds and saponins of aquafaba have antioxidant potential and rheological properties such as foaming, emulsifying and gelling properties. It demonstrated that changes in formulations influence the physicochemical parameters of mayonnaise type-dressing; however, parameters such as water activity, particle size, color, and lipid oxidation were not determined.

Therefore, the aim of this study was to compare three types of commercial mayonnaise of different formulations (high in fat, traditional homemade recipe, and light) with

Table 1. Nutritional information of different samples.

Nutritional Fact	VEG	HOM	CRE	LIG
Energy (kcal)	615.0	392.0	706.0	140.0
Proteins (g)	0.9	0.7	0.6	0.5
Total fat (g)	63.8	40.5	76.0	11.2
Saturated fat (g)	3.7	4.8	12.0	1.4
Monounsaturated fat (g)	41.4	13.6	17.0	3.8
Polyunsaturated fat (g)	18.7	2.9	43.0	5.9
Trans fatty acids (g)	0.0	0.4	0.9	0.1
Cholesterol (mg)	0.0	21.0	26.0	15.6
Carbohydrates (g)	9.3	6.3	2.0	9.5
Total sugars (g)	1.0	3.5	2.0	4.5
Sodium (mg)	245.0	635.0	549.0	747.0
Dietary fiber (g)	0.0	0.0	0.0	0.0

VEG: vegan dressing-type mayonnaise; HOM: homemade recipe mayonnaise;
CRE: creamy mayonnaise; LIG: light mayonnaise

Table 2. Ingredient lists of different samples.

VEG	HOM	CRE	LIG
Canola oil	Water	Soy oil	Water
Water	Marigold and soy oils	Water	Marigold and soy oils
Chickpea	Pasteurized liquid whole egg	Pasteurized egg	Modified corn starch
Mustard seeds	Modified corn starch	Vinegar	Sugar
Grape vinegar	Sugar	Less than 2% sugar	Pasteurized whole egg
Lemon juice	Alcohol vinegar	Salt	Alcohol vinegar
Salt	Salt	Pasteurized egg yolk	Salt
Brown sugar	Potassium chloride	Natural flavoring	Potassium chloride
White pepper	Sorbic acid	Lemon juice concentrate	Xanthan Gum
Dehydrated garlic	Xanthan Gum	Dehydrated garlic and onion	Sorbic and phosphoric acid
Peppers	Phosphoric acid	Peppers	Lemon juice concentrat
EDTA	Natural flavoring	EDTA	Antioxidants (BHA, propyl gallate, citric acid)
	EDTA	Calcium disodium	EDTA
			Natural identical smell
			Beta carotene (synthetic)

VEG: vegan dressing-type mayonnaise; HOM: homemade recipe mayonnaise; CRE: creamy mayonnaise; LIG: light mayonnaise

a commercial vegan dressing-type mayonnaise, through its physical-chemical properties to determine the differences among the different types of dressings.

2. MATERIALS AND METHODS

2.1. Materials

The different samples were purchased from a local supermarket (Tottus), located in Santiago, Chile. The dressings were as follows: VEG (Notmayo, vegan dressing-type mayonnaise) as a control, HOM (Hellmans, traditional homemade recipe mayonnaise), CRE (Kraft, high-fat-mayonnaise), and LIG (JB, light-mayonnaise). Table 1 and Table 2 show the nutritional information and ingredient list for each of them, reported in labeled brands.

2.2. Methods

Physical properties such as water activity, average droplet size, rheological properties, and color measurement were measured along with the chemical properties FT-IR infrared spectroscopy and oxidative stability.

2.2.1. Water activity (aw)

The water activity meter (AquaLab Pre-water Activity Meter, United States) was used. The samples at 25 ± 0.2 °C were deposited to cover the entire plastic vessel.

2.2.2. Droplet size and distribution

30 mL of sample were placed on a slide and viewed under a binocular vertical light microscope (Zeiss, Primo Star, England) using a 100 x magnification at room temperature. The images were captured with a remotely directed digital camera (Canon EOS Rebel T3, Canon Inc., Tokyo, Japan). The droplet size was determined from the images using the calibrated Motic Images Plus 2.0 software (Causeway Bay, Hong Kong). Drop size values were reported using the Sauter diameter (d32) of at least 200 drops counted from 3 to 8 photos as required for each sample in triplicate (n = 600) (Alarcón-Moyano *et al.*, 2017):

$$d_{32} = \frac{\Sigma_{i=0}^{n} d_i n_i^3}{\Sigma_{i=0}^{n} d_i n_i^2}$$

Where $\Sigma_{i=0}^{n} d_i n_i^3$ is the sum of the d32 corresponding to the drop volume. $\Sigma_{i=0}^{n} d_i n_i^2$ is the sum of the d32 corresponding to the droplet area.

2.2.3. Color measurement

The images of samples were captured on a black background through a calibrated computer vision system (LabVisionQ, Chile), according to the method described by Matiacevich *et al.* (2015). All images were acquired under the same conditions, with a remote camera controlled by the EOS Utility software (Canon Inc., Japan). They were analyzed using Adobe Photoshop v7.0 software to obtain RGB space parameters, converted to CIE L* a* b* standard color space. The L*, a*, and b* parameters represent lightness, the red-green axis, and the blue-yellow axis, respectively. The color difference index (ΔE00) was calculated using the CIEDE2000 equation. The perception of the color difference perceived by the human eye was determined according to the perception table described by (Yang *et al.*, 2012). The yellowness index is measured according to the ASTM E313-73 method of opaque materials close to white.

2.2.4. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) analysis was performed to identify the functional groups of oil and water of the different samples. For the analysis, FT-IR equipment with the attenuated total reflection instrument (ATR) was used, consisting of a diamond with an incidence angle unit of 45 ° (Perkin-Elmer, USA). Enough sample was deposited to cover the diamond. Twenty-four scanners were performed per sample, at a wavelength of 1500 to 1900 cm^{-1} and resolution of 4 cm^{-1}.

2.2.5. Rheological properties

The rheological properties were determined on a rotational rheometer (Rheolab QC, Anton-Paar, Austria), using a suitable concentric cylinder measurement geometry (C27, Anton Paar) to measure this type of more viscous samples. The samples were stabilized for 10 min before starting the measurement to ensure that the molecular structure returned to its initial state caused by placing the samples into the geometry. The temperature was controlled at 25 °C, incorporating a temperature control device (C-PTD 180 / AIR / QC) with a Pt100 temperature sensor into the equipment. Two flow traps with up and down cycles corresponding to cut speeds in the range of 5 to 600 s^{-1} were obtained (Juszczak *et al.*, 2003). The Herschel-Bulkley model described the curves obtained (Liu *et al.*, 2007), whose adjustment and parameter determination was obtained by employing an iterative method with the Solver analysis tool of the Excel 2016 program.

2.2.6. Oxidative stability RapidOxy® setup (pressurized headspace oxygen treatment method)

The oxidative stability study was performed using a RapidOxy test device (RapidOxy, Anton Paar, Graz, Austria). It allowed the study of oxidative degradation reactions in a short period using a pressurized oxygen headspace over a solid sample in a closed oven set at a specific temperature. Experimentally, 5 g of sample were placed in a dish. The method parameters used were oxygen pressure of 700 kPa at 120 °C. The induction period (min) corresponds to the time required to cause the pressure to drop to 10%.

2.3. Statistical analysis

All measurements were performed in triplicate. The results were reported using their average value and standard deviation of at least three measurements. The statistical analysis of these results was evaluated using the one-way ANOVA analysis of variance. For significant differences among samples, multiple comparisons were made using the Tukey test, with a significance level of 95%, using the GraphPad Prism 5.01 software (GraphPad Prism Ink, USA).

3. RESULTS AND DISCUSSION

3.1. Physical properties

Water activity (*aw)* is the water available for microbial development and/or development of biochemical reactions within a food. This property is related to how much food is perishable or not (Fenoglio *et al.*, 2020). Regarding this property, Table 3 shows the results for the different samples, wherein in all cases, their values are high (> 0.9) due to the nature of the mayonnaise which corresponds to an oil emulsion with high water content. Where the CRE sample shows a slight significant ($p < 0.05$) decrease in its water activity (0.938 ± 0.003), which may be because it is the sample with the highest fat content (Table 1). Besides, in the case of VEG, CAS, and LIG, the main ingredient is water ($aw = 0.973 \pm 0.003$); while for CRE, it is oil, so decreasing the *aw* value. This is corroborated in table 2, where the list of ingredients, which are in decreasing order of initial weight can be seen. The results obtained are comparable to the study carried out by Amin *et al.* (2014), where a low-fat mayonnaise was developed with different hydrocolloid gum types and levels. The results showed that water activity depended on oil concentration, where at 75% oil has a lower water activity (0.89 ± 0.01), while the sample with 45% oil increased its water activity (0.94 ± 0.02). Considering that all samples showed high *aw*, it was necessary to add ingredients such as acetic acid, salt, and EDTA (Ethylenediaminetetraacetic acid) to control microbial and biochemical reaction developments. These contents can be observed in Table 2.

Color is one of the most important properties which affects the appearance and acceptability of mayonnaise and the traditional mayonnaise recipe is characterized by a bright yellow appearance (Drozłowska *et al.*, 2020). Table 4 shows the L* a* b* parameters, the color differences between traditional mayonnaise compared to VEG (ΔE (00)), and the yellowness index (Y) of the different samples. For all samples, the color was significantly different ($p < 0.05$) among samples. The highest L* mean value corresponded to HOM, being the sample with the highest lightness, and VEG, the one with the least lightness. According to Mun *et al.* (2009), who mentions that the decrease in L* parameter can be attributed to the presence of solid protein particles, the studied VEG sample showed the highest protein content (Table 1) and therefore, the lowest lightness (Table 4). At the same time, the lower L* parameter of the LIG sample is explained by the higher content of thickeners used in its formulation compared to the other samples (Amin *et al.*, 2014). Low-fat mayonnaise obtained similar results to flaxseed meal extract (Drozłowska *et al.*, 2020). The

TABLE 3. Water activity, Droplet size, and oxidation induction time of different samples.

Sample	Water activity (*aw*) n=3	Droplet size (um) n=100	Oxidation induction time (min) n=3
VEG (control)	0.974 ± 0.004 ^{b}	27.3 ± 3.7 ^{c}	183.8 ± 1.5 ^{b}
HOM	0.973 ± 0.003 ^{b}	15.7 ± 3.2 ^{b}	201.3 ± 4.1 ^{c}
CRE	0.938 ± 0.004 ^{a}	13.8 ± 2.1 ^{b}	144.0 ± 2.7 ^{a}
LIG	0.976 ± 0.003 ^{b}	8.4 ± 1.7 ^{a}	215.6 ± 7.3 ^{d}

* Different letters above the columns indicate a significant difference (p < 0.05) by Tukey test between the samples means (n=3 or 100). VEG: vegan dressing-type mayonnaise; HOM: homemade recipe mayonnaise; CRE: creamy mayonnaise; LIG: light mayonnaise

TABLE 4. Color parameters of different samples.

Sample	Color Parameters				
	L*	a*	b*	Y	$\Delta E_{(00)}$
VEG (control)	80.24 ± 0.02 ^{a}	0.82 ± 0.02 ^{a}	8.47 ± 0.04 ^{a}	22.33 ± 0.06 ^{a}	-
HOM	99.87 ± 0.92 ^{d}	22.70 ± 2.58 ^{d}	14.14 ± 1.38 ^{b}	30.58 ± 1.43 ^{b}	22.34 ± 1.36
CRE	98.19 ± 0.24 ^{c}	8.51 ± 0.18 ^{c}	17.17 ± 0.59 ^{c}	27.31 ± 0.70 ^{b}	14.73 ± 0.24
LIG	92.06 ± 0.25 ^{b}	4.93 ± 0.24 ^{b}	9.14 ± 0.65 ^{a}	21.49 ± 2.11 ^{a}	9.34 ± 0.30

* Different letters above the columns indicate a significant difference (p<0.05) by Tukey test between the samples means (n=3). L: lightness, Y: yellowness index, ΔE(00): color difference index. VEG: vegan dressing-type mayonnaise; HOM: homemade recipe mayonnaise; CRE: creamy mayonnaise; LIG: light mayonnaise

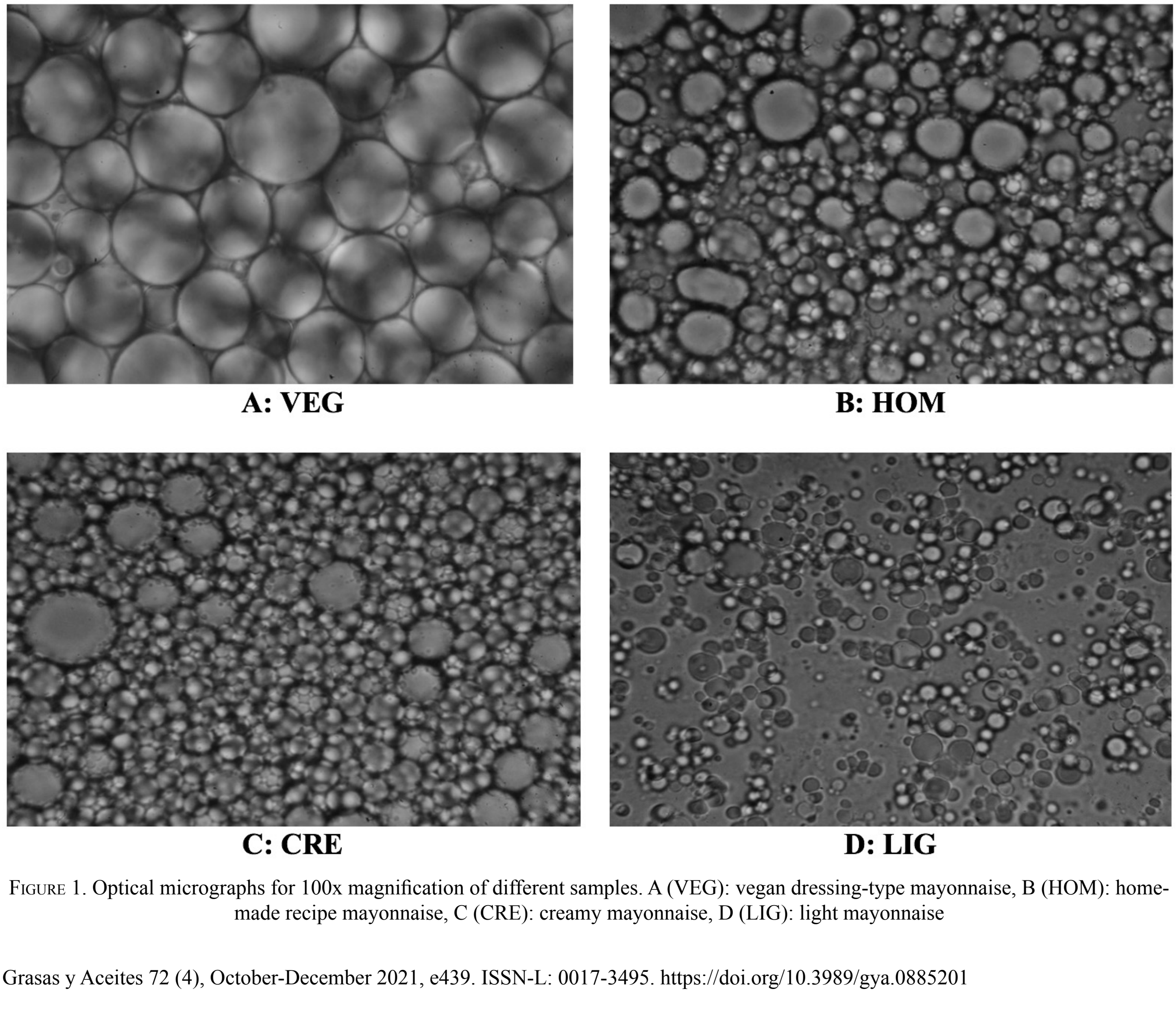
a* and b* parameters indicate the tendency towards red or green and yellow or blue, respectively, if it is a positive or negative value. In all samples, a* and b* showed significant differences ($p < 0.05$) among samples, where positive values were obtained for both parameters, so a tendency towards red and yellow, respectively. In the VEG sample, b* value and yellowness index (Y) were lower than the other samples because the chickpea's protein content controls these color parameters. Similar results were reported by Raikos *et al.* (2020) for a vegan mayonnaise using canned chickpea aquafaba, where the sample that contained a greater quantity of aquafaba indicated a lower value of b* and therefore of Y. On the other hand, the HOM sample showed the highest a* value = 22.70 ± 2.58, but CRE samples showed the highest b* value = 17.17 ± 0.59. Therefore, HOM and CRE samples showed the highest lightness and yellowness index (Table 4). These characteristics belong to a type of traditional homemade mayonnaise with high oil contents (Chang *et al.*, 2017). The color difference (ΔE00) is also observed in Table 4, according to the perceived scale of the color difference among samples proposed by (Yang *et al.*, 2012). A significant color difference in VEG (control) with other samples is between VEG and HOM with an ΔE00 value of 22.34 ± 1.36, which can be due to the highest Y value in sample HOM (31±1). In contrast, a lower value for ΔE00 (9.34 ± 0.30) was determined between VEG and LIG, although notable differences were observed. Finally, the color change and color perception of each formulation can be attributed to the presence of thickeners, fat, and protein contents.

The microstructure of mayonnaise was determined by different factors, including the type and concentration of emulsifiers used to form the emulsion, the viscosity of the aqueous phase, the oil content, and the droplet size (Laca *et al.*, 2010). Droplet size is an important parameter to predict the physical stability of emulsions (Arancibia *et al.*, 2017), with high stability in small droplet size and significant impact on the rheological properties of the final product (McClements, 2012). Table 3 shows the droplet size results, and Figure 1 presents the optical micrographs of the different samples, where significant differences ($p < 0.05$) between samples were observed. For VEG, the largest droplet sizes were obtained with a homogenous monomodal droplet population of 27.3 ± 3.7 µm. Between HOM and CRE the mean sizes were 15.7 ± 3.2 and 13.8 ± 2.1 µm, respectively, with spherical shapes of the oil droplets and different populations; while the smallest size was for the LIG sample of 8.4 ± 1.7 um. In the case of the LIG sample, the droplet size was similar to a study conducted by Drozłowska *et al.* (2020). for a light mayonnaise with substitution of oil for flaxseed protein (5, 10, and 15%), where the droplet sizes of the analyzed samples were 9.4, 9.6 and 8.2 µm, respectively. Similar values were also reported by Worrasinchai *et al.* (2006) (1-9 µm) and Laca *et al.* (2010) (3 to 12 µm). The smaller droplet size can be attributed to two factors, a low oil concentration and the presence of a thickener. In the LIG sample, the main ingredients were water, oil, and modified corn starch (thickener). In the study by Drozłowska *et al*. (2020), it was shown that the droplet size decreased with decreasing oil concentration and when replacing this ingredient with thickeners (proteins or polysaccharides) because the thickeners seemed to affect the development of the system due to their high emulsifying potential. Therefore, the thickener granules kert the oil droplets separated and prevented coalescence (Di Mattia *et al*., 2015). The stabilization of the mayonnaise's oil-water interface was mainly due to microparticles formed from the phosphoprotein and fused low-density lipoprotein constituents of egg yolk (Drozłowska *et al*., 2020).

In addition, Cornelia *et al*. (2015) related the size of the drops in mayonnaise made with egg protein to larger sizes than for vegan dressing-type mayonnaise due to the structure of animal origin proteins; therefore, similar results were expected in this study. However, the unexpected behavior of the VEG sample (the largest droplet size) could be attributed to the technology (type and processing conditions) used for the preparation of this dressing. Drozłowska *et al.* (2020) pointed out that it must be taken into account that the size of the particles and the differences in the colloidal state can be attributed to the different ingredients and preparation processes that are used in the formulation of mayonnaise. Schultz *et al.* (2004) also indicated that the amount of energy required to produce such fine emulsions can generally only be achieved using high-pressure systems. Therefore, the droplet size parameter also depends on technological and process factors during manufacturing. For example, the homogenization technology used for the VEG emulsion might not be sufficient to decrease the droplet size in this mayonnaise. Therefore, greater physical instability, such as droplet agglomeration, is expected in this sample. However, regardless of the droplet size, the observed aggregation droplets (CRE> VEG> HOM> LIG) (Figure 1) increased as the fat content increased (76% > 64% > 41% > 11% (w / w), respectively) in the samples (Table 1).

3.2. Rheological behavior

Figure 2 shows the upward flow curves (upper curve) and downward flow curves (lower curve) of the different samples. The rheological parameters, such as yield stress, consistency index, flow behavior index, coefficient of determi-


A: VEG

B: HOM

C: CRE

D: LIG

Figure 1. Optical micrographs for 100x magnification of different samples. A (VEG): vegan dressing-type mayonnaise, B (HOM): homemade recipe mayonnaise, C (CRE): creamy mayonnaise, D (LIG): light mayonnaise

TABLE 5. Rheological parameters of different samples

Sample	Yield Stress σ_0 (Pa)	Consistency coefficient k (Pa·s^n)	Flow behaviour index n (-)	R^2	Area of hysteresis thixotropy loop (Pa s·cm^3)
VEG	154.4 ± 5.1 ^{c}	10.86 ± 3.42 ^{c}	0.46 ± 0.04 ^{a}	0.99965	105
HOM	143.9 ± 0.7 ^{b}	1.73 ± 0.04 ^{a}	0.70 ± 0.01 ^{c}	0.99687	116
CRE	136.5 ± 4.1^b	5.11 ± 0.82 ^{b}	0.56 ± 0.02 ^{b}	0.99892	110
LIG	85.5 ± 1.1^a	14.72 ± 0.78 ^{d}	0.44 ± 0.01 ^{a}	0.99995	108

* Different letters above the columns indicate a significant difference ($p<0.05$) by Tukey test between the samples means (n=3). R^2 means a determination coefficient. VEG: vegan dressing-type mayonnaise; HOM: homemade recipe mayonnaise; CRE: creamy mayonnaise; LIG: light mayonnaise

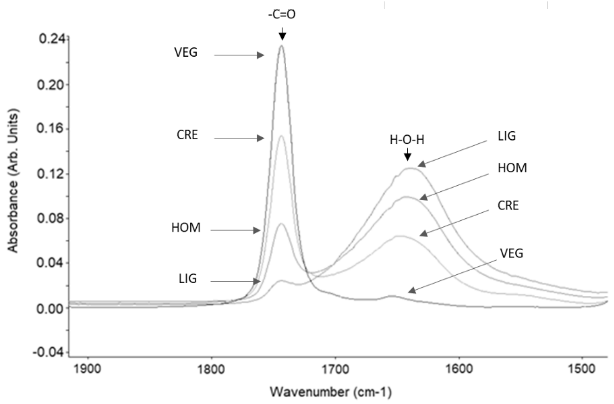


FIGURE 2. Fourier transform Infrared spectra of different samples. VEG: vegan dressing-type mayonnaise; HOM: homemade recipe mayonnaise; CRE: creamy mayonnaise; LIG: light mayonnaise

nation, and relative hysteresis area are detailed in Table 5. All the curves were fitted to the Herschel – Bulkley model and showed an excellent fit to the experimental data ($R^2 > 0.995$). Flow index values were lower than 1, indicating that all the samples had a pseudoplastic flow behavior, typical of mayonnaise, which suggests that the samples were in semi-solid condition with breakable networks, which is in agreement with findings reported by (Liu *et al*., 2007; Li *et al*., 2014; Drozłowska *et al*., 2020). The pseudoplastic properties of mayonnaise may result from the flocculation and deflocculation of oil droplets, leading to the formation of two different size aggregates, which also affects the flow of mayonnaise (Sun *et al*., 2018). As the shear rate grows during the flow, the deflocculation phenomenon intensifies, thus decreasing the system's viscosity (Juszczak *et al*., 2003). Regarding the consistency coefficient, the highest value can be observed for the LIG sample. This behavior is explained by the presence of thickeners in its formulation, where the fat is replaced by a substitute based on carbohydrates such as modified corn starch (see Table 2). Hydrocolloid forms a gel-like structure, which traps oil droplets, slows down their movements, and increases viscosity, which is why this product can resemble the texture of traditional mayonnaise (Amin *et al*., 2014). This same phenomenon is related to the consistency index of the VEG sample corresponding to 10.86 ± 3.42, where this sample does not contain thickeners in its formulation (see table 2). However, this high consistency index is attributed to chickpea protein, a hydrocolloid that causes the same phenomenon as the thickeners.

The yield stress increased when the fat content increased (Peressini *et al*., 1998; Ma and Barbosa-Cánovas, 1995). In this study, LIG had less fat and a lower yield stress value (85.5 ± 1.1 Pa). Ma and Barbosa-Canvas (1995) showed that yield stress for mayonnaise ranged from 23 to 235 Pa, with similar data on yield stress ranging from this study (85.5-154.4 Pa). The comparison between the ascending and descending flow curves determines the thixotropic hysteresis area of mayonnaise (Štern *et al*., 2001). Therefore, the viscosity changes were dependent on time, and related to the structural re-destruction degree of the product due to the shear (Primacella *et al*., 2019). The sample with the largest area was HOM (116). At the same time, VEG (105) had the smallest area, which may be advantageous in technological processes such as pumping, transport, or storage of the sample due to greater capacity to reconstruct damaged structure after removing shear forces. Studies indicated a relationship between fat content with a greater thixotropic area (Juszczak *et al*., 2003). However, this relationship was not found in this study because, in the formulation of the mayonnaises analyzed, the consistency was given mainly by the thickeners used to replace high-fat content. The stress threshold was associated with a three-dimensional network rupture of product structure. The obtained values were similar to the studies by Juszczak *et al.* (2003) for a Polish mayonnaise. The highest threshold stresses correspond to samples VEG 154.4 ± 5.1, HOM 143.9 ± 0.7, and CRE 136.5 ± 4.1, and the lowest stress for LIG 85.5 ± 1.1, which is correlated with the lower fat content and smaller size of oil drops.

3.3. Chemical properties

The FT-IR spectra are presented in Figure 3. The characteristic peak of the H-O-H bands of the water at 1650 cm^{-1} is observed (Daoud *et al*., 2019), where it can be seen that in decreasing order, the peaks correspond to LIG > HOM > CRE > VEG, which is related to the water content

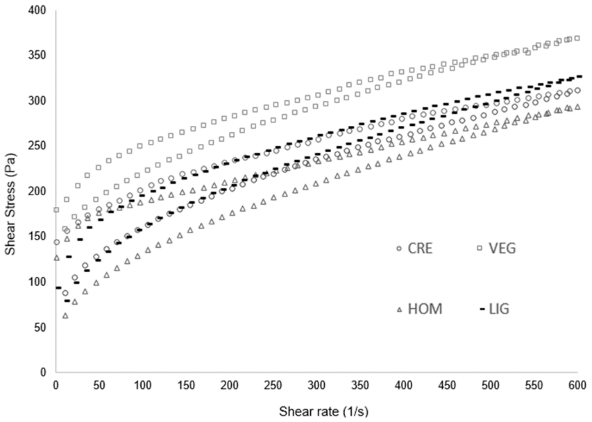


Figure 3. Flow curves of different samples. VEG: vegan dressing-type mayonnaise; HOM: homemade recipe mayonnaise; CRE: creamy mayonnaise; LIG: light mayonnaise

of the samples (Table 1). In contrast, CRE and VEG have water as the second-largest component and oil as the first, which is why it showed lower peak height.

The mono-unsaturated oil characteristic peaks of the bands –C=O at 1750 cm^{-1} (Rohman *et al*., 2011), attributed to the triacylglycerides ester bonds, can be observed in figure 3. The decreasing peak area corresponds to VEG> CRE> HOM> LIG. This relationship was supported by the nutritional information (Table 1) of the different samples of monounsaturated fats, where they contain 41% > 17% > 14% > 4% (w / w), respectively. Similar studies of quantitative analysis of fat and water content in mayonnaise using the FT-IR technique were performed by Chippie *et al.* (2002), and demonstrated a linear relationship between its composition and the height of the peaks.

Oxidative stability is the resistance of samples to be oxidized by the content of fats or other lipids when they come into contact with atmospheric oxygen (Miguel *et al*., 2019). Mayonnaise is a high-fat food (70% – 80% vegetable oil) and therefore is susceptible to oxidative deterioration through the auto-oxidation of the unsaturated and polyunsaturated fats in the oil, which depending on the extent, is likely to have a negative impact on flavor, aroma, color and nutritional value of food (Depree and Savage, 2001). Several strategies can be effective against the lipid oxidation of mayonnaise, such as the addition of antioxidants or the use of a lipid source that is naturally rich in compounds with potent antioxidant activity (Li *et al*., 2014; Di Mattia *et al*., 2015). Table 3 shows the results of the lipid oxidation induction time. These values may be influenced by the antioxidant content used in the different samples. Although all the samples contain EDTA as an antioxidant and preservative component, the labels do not specify the amount incorporated into them. However, it was possible to relate the results obtained with the content of polyunsaturated fats that are available to be oxidized due to instability and greater resonance of the double bonds exposed to oxygen (Roman *et al*., 2019). The LIG sample had the highest induction time of 216 ± 7 min and the lowest content of these fats with only 6% w / w, followed by HOM with a time of 201 ± 4 min with 22% w / w. Similar results were found for the VEG sample with time and content of 184 ± 2 min and 19% w / w. Finally, CRE, with 144 ± 3 min and the highest polyunsaturated fats content, with 43% w / w. Therefore, as the increasing content of polyunsaturated fats in the samples, they will oxidize faster, one factor for this type of product's shelf-life.

4. CONCLUSIONS

A vegan dressing-type mayonnaise (VEG sample), free of animal origin components, showed differences in the physicochemical properties compared to traditional alternatives. It was attributed to its oil composition, the addition of thickeners and proteins, and process conditions. As expected, all the samples showed high water activity (aw > 0.9) and pseudoplastic flow behavior. The addition of vegetable proteins (chickpea) in VEG samples modified the color (being less light) and consistency parameter (increasing consistency index). Regarding the visual appearance, the VEG sample's color parameter presented the lowest L* value, and a notable color difference was observed compared to the LIG and HOM samples. The HOM sample was precisely the yellowest and brightest sample. The largest droplet size was also observed for VEG samples, attributed to its higher oil content and emulsification technology. The high polyunsaturated fat contents led to the VEG and LIG samples showing lower oxidative stability with a shorter induction time. Thus, when a new formulation of vegan dressing-type mayonnaise is designed, it is useful to make the set of measurements described to obtain the same properties, mainly oxidative stability.

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