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Study of the operational conditions for ethyl esters production using residual frying oil and KF/clay catalyst in a continuous system

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SUMMARY: The transesterification of residual frying oil (RFO) with pressurized ethanol was carried out in a continuous reactor containing KF/clay as a heterogeneous catalyst. In the experiments, different oil:ethanol mass ratios were evaluated at 275 and 300 ºC and 20 MPa. In the sequence, the operational stability of the catalyst was evaluated for 8 hours, as well as the conduct of the reaction in two steps (testing new and recycled catalyst). An esters yield of ~90% was achieve at 275 ºC, for15 min and at 1:1.5 oil:ethanol mass ratio. Under these conditions, the catalyst provided a stable yield in the first 3 hours of operation, and a total decrease of 29% after 8 hours. This result can be attributed mainly to the leaching of the K^+ cations for the reactions in which the catalyst was exposed to long operating times. The two-step reaction served to increase the RFO conversion to esters, with low thermal decomposition.

KEYWORDS: *Ethyl esters; Heterogeneous catalyst; Residual frying oil.*

RESUMEN: ***Estudio de las condiciones para la producción de ésteres etílicos utilizando aceite de fritura desechado y catalizador KF/arcilla en un sistema continuo.*** La transesterificación de aceite de fritura desechado (AFD) con etanol presurizado se llevó a cabo en un reactor continuo que contenía KF/arcilla como catalizador heterogéneo. En los experimentos, se evaluaron diferentes relaciones de cantidad de aceite:etanol a 275 y 300 ºC y 20 MPa. En la secuencia, se evaluó la estabilidad operativa del catalizador durante 8 horas, así como la conducta de la reacción en dos pasos (prueba de catalizador nuevo y reciclado). Se logró un rendimiento de ésteres de ~ 90% a 275 ºC, 15 min y una relación de aceite:etanol de 1:1,5. En estas condiciones, el catalizador proporcionó un rendimiento estable en las primeras 3 horas de funcionamiento y una disminución total del 29% después de 8 horas. Este resultado se puede atribuir principalmente a la lixiviación de los cationes K^+ para las reacciones en las que el catalizador estuvo expuesto durante las condiciones de funcionamiento. La reacción de dos pasos permitió aumentar la conversión de AFD a ésteres, con baja descomposición térmica.

PALABRAS CLAVE: *Aceite de fritura desechado; Catalizador heterogéneo; Ésteres etílicos.*

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1. INTRODUCTION

The search for renewable fuels, less polluting and more economically and technologically viable has intensified, especially those that meet the worldwide demand and replace fossil fuels. Biodiesel is an attractive option to meet this demand, and has similar physicochemical properties to mineral diesel (Shehata, 2013). Transesterification, which involves the reaction between triglycerides and a short-chain alcohol to produce esters, as the main product, and glycerol, is an attractive method for converting oils and fats into biodiesel. Residual frying oils (RFO) are an interesting alternative for making biodiesel economically competitive, with its economic viability restrained by the high prices of raw materials, even to a greater extent than technological or processing limitations. It is estimated that about 75% of the cost of production is related to the processing of raw material (Mahlia *et al*., 2020).

In addition, the improper disposal of RFO leads to environmental damage, where harmful discharge pollutes large amounts of water, which is exacerbated by high production, which in Brazil reached around 1.2 million tons per year (of waste oils and fats) (Aprobio, 2018). Another advantage to using RFO is the significant decrease in agricultural land which is necessary for the planting of oilseeds used for the extraction of the oil used in the production of biodiesel.

Although RFO is attractive, the frying process leads to changes in its properties due to exposure to high temperatures, and the transfer of water from the food to the oil, resulting in the hydrolysis of triacylglycerols, and formation of free fatty acids (Ouanji *et al*., 2016). RFO may also contain polar compounds, which associated with the presence of a high concentration of free fatty acids and water, hinders the progress of the reaction (Fonseca *et al*., 2018). In a study by Campanelli *et al*. (2010) the authors found a low reaction rate by applying the conventional transesterification process, requiring high residence times to achieve complete conversion. There are also reports on the formation of oxidized polymers, dimers, triacylglycerol, and diacylglycerides, where some of these components cannot be converted into esters (Gonzalez *et al*. 2013.).

The presence of water and free fatty acids in the RFO triggers hydrolysis and saponification reactions, respectively (Álvarez-Mateos *et al.,* 2019). When the conventional method is used for homogeneous alkaline catalysis for biodiesel synthesis, in addition to consuming catalyst, it causes low ester yield (Kusdiana and Saka, 2004). The method using alcohol under pressurized conditions was reported by some authors as tolerant to the presence of these compounds in the oil (Kusdiana and Saka, 2004; Tan *et al*., 2010), without compromising the yield of the reaction. In this method alcohol is used in conditions close to or above the critical point of temperature and pressure. Under these conditions, modifications occur in the properties of the solvent, such as increased density, solubility and mass transfer and decreased polarity of alcohol, in addition to reducing the viscosity of the oil (Kusdiana and Saka, 2004; Osmieri *et al*., 2017). Thus, high yields are obtained in short reaction times without the use of catalyst (He *et al*., 2007), and it is possible to maximize the ester yield by adjusting the temperature, pressure and amount of alcohol employed in the process, as they influence the solubility between phases (Hegel *et al*., 2008).

However, in order to obtain high conversion values of triacylglycerols in esters, the use of this method requires operation at high temperatures and pressures, together with a large amount of alcohol used in the reaction medium (Silva and Oliveira, 2014). The combination of the residence time and the reaction temperature is fundamental for the effectiveness of the process, since an increase in both results in better yields, but also propitiates parallel reactions of thermal decomposition. The increase in temperature causes isomerization reactions of the type cis to trans in the double bonds of the esters, polymerization (formation of dimers), pyrolysis of fatty acids in smaller compounds and hydrogenation (Liu *et al*., 2016; Quesada-Medina and Olivares-Carrillo, 2011). Regarding the composition of the oil, it is emphasized that the soybean oil commonly used for frying food can contain from 52 to 54% linoleic acid (Gonzalez *et al*., 2013), and according to a study carried out by Quesada-Medina and Olivares-Carrillo (2011), for the transesterification of soybean oil with methanol, the thermal decomposition reactions of the linoleic ester intensified as the temperature increased, with a reduction in ester content reported at 300 °C.

Some studies have investigated improvements in the process, so that it is possible to work under milder conditions of temperature and pressure, thus mitigating the hindrance of thermal decomposition and using lower ethanol volumes in the process, without compromising the high efficiency in reaction yield. To that end, researchers in the area have evaluated the use of heterogeneous catalysts under pressurized conditions in the batch reactor, such as Nano-MgO (Wang and Yang, 2007), CaLaO (Teo *et al*., 2015), carbohydrate-derived (Lokman *et al*., 2016) and CH_3ONa (Zeng *et al*., 2017), perceiving high catalytic efficiency, reusability, low mass loss, possibility for reuse of catalyst without loss of catalytic efficiency, and elevation of ester yield even with small amounts of catalyst.

The transesterification under pressurized conditions conducted in a continuously operated reactor applying heterogeneous catalysts was poorly reported. Among the available studies, McNeff *et al.* (2008) made use of a zirconia-based catalyst, noting an increase in the conversion to esters, from 14.1 to 98% with the use of catalyst. Mazanov *et al*. (2016) reported a yield of 77.51% in a non-catalytic reaction, and 97.46% with the use of SrO/Al_2O_3. Krohn *et al.* (2011) and Maçaira *et al.* (2011) also investigated the use of heterogeneous catalysts in a continuous system under supercritical conditions, using titania porous catalyst and acid resin, respectively. Both concluded that the use of heterogeneous catalysts resulted in shorter operating time. The application of the heterogeneous catalyst has also been successfully reported by Visioli *et al.* (2019) in a reaction using methyl acetate. The authors reported that the use of γ-alumina catalyst increased the production of esters by 33%.

Alves *et al.* (2014) describe the efficiency of using inorganic salts such as potassium fluoride (KF) impregnated with clay as a catalyst in the transesterification to obtain biodiesel. The catalyst was used in a low-pressure batch reactor and the catalyst showed high efficiency in terms of ester yield, low mass loss and morphological stability. Catalysts prepared from clay have a crystalline structure that can be modified in a controlled manner by means of various treatments to optimize their use as catalysts. Such treatments consisted of the modification of the specific area and porosity, thus facilitating the access of the molecules of reagents to the active sites (Alves *et al*., 2014; Centi and Perathoner, 2008). In addition, smectites are abundant in nature and have a high surface area (Nagendrappa, 2011), an important feature in heterogeneous catalysts, which, together with its versatility and low cost, makes clay a promising catalyst or catalytic support in several processes.

The use of KF/clay catalyst in a pressurized medium for the synthesis of ethyl esters was reported in a previous study (Zempulski *et al*., 2020) in which the performance of the catalyst was verified for 3 h of operation in 225 to 300 °C, verifying low variation in the ester yield at 275 and 300 °C. Moreover, after the transesterification, it was verified that the potassium resulting from the impregnation remained in the catalyst, and that the roughness of the particles and agglomerates of the catalyst were not affected by the experimental conditions employed. and the morphological characteristics of the catalyst were maintained.

Thus, the application of a low-cost residual oil for the production of biodiesel is currently of special interest especially from the environmental perspective concerning the re-use of waste, as well as the production of renewable fuel. In addition, through technology that uses pressurized conditions, the reaction takes place in short residence times, and the use of clay-based catalysts that are easy to obtain contributes to higher ester yields and allows for milder operating conditions. Based on the context described, the purpose of this work was to evaluate the continuous production of ethyl esters from residual frying oil (RFO), using ethanol under pressurized conditions in the presence of a heterogeneous catalyst based on clay impregnated with potassium fluoride. The effects of the oil to ethanol mass ratio at 275 and 300 ºC and different residence times were evaluated. Under the conditions of higher ester yield, the stability of the catalyst was evaluated for 8 hours of operation. The reaction was further carried out in two stages, using new catalyst and recycled in the second step. The catalyst was characterized after its use and compared with the catalyst inserted into the reactor.

2. MATERIALS AND METHODS

2.1. Materials

The substrates used were residual frying oil obtained from a local restaurant (Umuarama - PR) and ethanol (Panreac, 99.9% purity). The composition of the RFO was previously reported by Zempulski *et al.* (2020), and its free fatty acid and water contents were 1.16 wt% and 0.16 wt%, respectively. For the preparation of the catalyst, Brazilian sodium smectite clays were used from deposits located in the states of Paraná and Paraíba, and potassium fluoride $KF.2H_2O$ (Synth, 98% purity). For the characterization analyses methyl heptadecanoate (Sigma-Aldrich, > 99% purity), heptane (Anhydrol), potassium hydroxide, methanol (Panreac, 99.9%), sulfuric acid (Anidrol, 98%), boron trifluoride (Sigma-Aldrich, 14% in methanol), monolein (Sigma Aldrich, ≥ 98%), 1,3-diolein (Sigma Aldrich, ≥ 95%), glycerol trioleate (Sigma Aldrich, ≥ 98.5%), derivative N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA, Sigma Aldrich, ≥ 98.5%) were used.

The catalyst used in this work was based on the study of Alves *et al.* (2014), where the clay was crushed (< 60 mesh) and wet-impregnated with potassium fluoride at the concentration of 1.7 $mol \cdot L^{-1}$. The suspension, a 15% clay and potassium fluoride solution (mass concentrate), was maintained in a blanket and reflux (80 °C, 30 min), followed by drying (110 °C, 24 h). The mass obtained from clay and KF was de-agglomerated in a mortar until a fine powder was obtained. The final stage consisted of the formation of granules, where the powder was compacted in a hydraulic press with a 5-ton pressure, de-agglomerated, and classified in sieves. The granules used in the work were in the range of 710 µm to 1 mm obtained with the aid of sieves.

2.2. Experimental procedures

The reactions, carried out in duplicate, served to investigate the influence of the oil-to-ethanol mass ratio of 1:1, 1:1.5 and 1:2 (equivalent to the oil-to-ethanol molar ratios of 1:20, 1:30 and 1:40, respectively). The tests were conducted using a catalyst mass of 2 g in the catalyst bed (these conditions correspond to an oil-to-catalyst ratio of 8:1, 6.4:1 and 5.4:1, respectively) at 275 and 300 °C. The catalyst mass and temperatures were defined based on the results obtained by Zempulski *et al.* (2020), which provided the experiments the highest ester content and in the case of the selected temperature range did not show any loss in catalytic activity (in 3 h of reaction). The pressure was maintained at 20 MPa based on previous studies (Gonzalez *et al.* 2013; Trentini *et al.* 2018), and the residence times evaluated were 15, 30 and 45 min.

The experimental apparatus where the reactions were performed was previously described (Zempulski *et al.*, 2020), and operated in continuous mode. The reaction mixture (RFO + ethanol) was kept under constant stirring (mechanical stirrer, IKA RW20) and pumped into the system using an isocratic pump (Waters 515 HPLC). The heating and maintenance of the operating temperature was done in a furnace (Sanchis, BTT1050-00), where the reactor was placed. The reactor consisted of 3 parts: pre-heating zone, catalytic tubular reactor (internal diameter of 9.5×10^{-3} m), and non-catalytic tubular reactor, whose total volume was 35.15 mL. The catalytic tubular reactor was filled with glass beads of medium diameter of 4 mm at the ends and the catalyst (previously activated in a muffle - 48 h, 200 °C). To avoid mass losses to the system, sintered steel filters (2 µm, Allcron) were arranged at the ends. Before collection sampling, the samples were cooled by indirect heat exchange, using a thermostatic bath (Tecnal TE - 184). The residence time was calculated by dividing the empty volume of the reactor (mL) by the flow of substrates (mL min^{-1}) and counted after the system entered steady state, considering 2.5 times the residence time. After the reaction, unreacted ethanol was recovered using a rotary evaporator (Marconi, MA 120) coupled with a vacuum pump (Quimis®, 0955V).

The stability of the catalyst was performed under the experimental conditions (catalyst mass, oil-toethanol mass ratio, temperature and residence time) that provided not only high yield but also low thermal decomposition, resource savings (energy for temperature rise and ethanol employed). This step was performed from the addition of 2 g of catalyst in the catalytic tubular reactor and as soon as the adopted experimental conditions were reached the reaction mixture (oil + ethanol) was then pumped into the system. The reaction consisted of operating the reactor for a period of 8 h with sample collection every 30 min after reaching steady state.

Under the same experimental conditions as those selected to assess catalyst stability, the reaction was carried out in two steps. The procedure previously described (Abdala *et al.*, 2014; Trentini *et al.*, 2018) was used, which consisted primarily of collecting the sample and removal of glycerol. The removal of glycerol was performed by adding distilled water and *n*-hexane to the reaction product obtained to form immiscible phases. The mixture was centrifuged and the phase containing esters was carried to the oven for evaporation of ethanol and *n*-hexane. Then, ethanol was added to the sample (without glycerol) and the mixture was pumped into the reaction system. In the second step, the system was filled with fresh catalyst and recycled catalyst (used for a period of 3 h). After the reaction, the recycled catalyst was washed with ethanol and *n*-hexane to remove possible products adhered to the catalyst. It was then dried in an oven (100 °C for 24h) for later re-use.

2.3. Analytical methods

The collected samples were monitored for preparation: evaporation of unreacted ethanol, removal of glycerol, addition of internal standard (methyl heptadecanoate) and dilution in heptane. One µL of the diluted sample was injected into a gas chromatograph (Shimadzu, GC-2010 Plus) equipped with an automatic injector (Shimadzu, AOC-20i Autoinjector), Shimadzu Rtx-Wax capillary column (30 m x 0.32 mm x 0.25 µm) and flame ionization detector. Nitrogen was used as the carrier gas and the samples were injected in split mode (1:20). The injector and detector were kept at a temperature of 250 °C and the heating ramp for the oven was as follows: it started at 120 °C, followed by an increase to 180 °C at 15 $°C·min^{-1}$ and then to 240 °C at 6 $°C·min^{-1}$, as described by Trentini *et al.* (2018).

The ester yield (Equation 1) was obtained through the convertibility of the RFO (89.52± 0.88 wt%) as determined using methodology proposed by Gonzalez *et al.* (2013), which refers to the maximum conversion of ethyl esters that can be achieved from the RFO.

$$FAEE\ yield\ (\%) = \frac{esters\ content}{convertibility} \times 100 \quad (1)$$

Thermal decomposition was determined according to Vieitez *et al.* (2009), and consisted of derivatizing of the compounds (mono, di and triglycerides and ethyl esters) present in the samples with methanolic solution of BF_3 and analyzed by gas chromatography. The decomposition of the samples was obtained from Equation 2:

$$Decomposition\ (\%) = \left[1 - \left(\frac{\sum Pi}{P_{16}}\right)_S x \left(\frac{P_{16}}{\sum P_i}\right)_0\right] x\ 100 \quad (2)$$

Where was the sum of all ethyl ester percentages, P_{16} was the percentage of 16 ethyl ester (considered the most stable), and the subscripts 0 and S indicated the original oil and sample, respectively.

For the quantification of mono-, di- and triglycerides, the sample was derivatized with MSTFA (15 minutes at room temperature) and diluted in heptane (Trentini *et al.*, 2019). The prepared samples were injected (2 µL) into a gas chromatograph (Shimadzu, GC-2010 Plus) equipped with capillary column Zebron ZB-5HT inferno™ (10 m × 0.32 mm × 0.10 µm), flame ionization detector and column injector. The following oven temperature gradient was applied: initially the column was held at 50 °C for 1 minute, and then heated to 180 °C at a rate of 15 $°C·min^{-1}$, then at 230 °C at a rate of 7 $°C·min^{-1}$ and at 380 °C at 10 $°C·min^{-1}$ and held at this temperature for 5 min. The detector temperature was 380 °C and the heating program of the injector was: initial temperature of 60 °C for 1 min. then heated to 380 °C at a rate of 10 $°C·min^{-1}$ and held for 10 min at this temperature. For the quantification of the compounds, a calibration curve with the standards of triolein, diolein and monolein were used (Trentini *et al.*, 2019).

2.4. Catalyst characterization

After the reaction, the catalyst recovered from the reactor was washed with ethanol and *n*-hexane and dried in an oven (Zempulski *et al.*, 2020). The characterization of the catalysts was obtained by X-ray fluorescence analysis (XRF, Spectro Philips MagiX); X-ray diffraction (using the XRD apparatus, Siemens Kristalloflex), in the range of $4° < 2\Theta < 40°$, using CuKα radiation (λ = 1.54056 nm, 40 kV, 40 mA as radiation incident) with a nickel filter, and speed of 0.5^{o} min^{-1} and; Fourier transform infrared spectroscopy (spectrometer FTIR Bomem MB-Series) in the range of 4000 and 500 cm^{-1}, using a resolution of 4 cm^{-1}; and scanning electron microscopy (SEM) (Quanta 440 microscope FEI), coupled to an energy dispersive spectroscopy (EDS). In the SEM analysis, the powder samples were dispersed onto a double-sided carbon tape, and subsequently metallized with a thin layer of gold by sputtering.

2.5. Analysis of data

All reactions and analyzes were performed, at least, in duplicate. The means and standard deviation of each assay were submitted to ANOVA using Excel® 2010 software and Tukey's test (95% confidence) was applied to determine whether there was a significant difference between samples from the different operating conditions studied. The principal component analysis (PCA) was carried out using the Statistica 8.0 software (StatSoft™, Inc.). The principal components were derived from a correlation matrix.

3. RESULTS

3.1. Effect of mass ratio oil to ethanol

Figure 1 shows the values obtained in terms of ethyl ester yield for the reactions conducted by varying the oil-to-ethanol mass ratio (1:1, 1:1.5 and 1:2) at different residence times. It can be observed at 275 ºC (Figure 1a) that the addition of ethanol to the reaction had a greater influence on the initial minutes of the reaction (15 min), for the other evaluated times there was an increase in the ester yield only when the oil-to-ethanol mass ratio was increased from 1:1 to 1:1.5. The highest yield obtained at this temperature was ~90% in 15 min of reaction. At a temperature of 300 ºC (Figure 1b), the highest ester yield, obtained in 15 min, was for the oil-to-ethanol mass ratio of 1:2. Similarly, for other ratios and residence times, the increase in ethanol did not influence the yield of esters. From the general analysis of the data obtained in Figure 1, it appears that higher yields were obtained in short residence times and that the addition of a high concentration of ethanol to the reaction (above 1:1.5) was not necessary to obtain a high yield of esters at the evaluated temperatures, which is advantageous, considering the lower mass ratio of oil-to-ethanol and low reaction time to obtain high ester yields.

Increasing the proportion of ethanol in the reaction mixture benefits the conditions of mass transfer in the reaction medium (Biktashev *et al*., 2011). It can also increase the contact area between the alcohol and triglycerides, thus shifting the balance of transesterification to the formation of products (Campanelli *et al*., 2010; Silva and Oliveira, 2014). In addition, the addition of alcohol to the reaction medium decreases the critical temperature of the reaction mixture (Osmieri *et al*., 2017). As an example, for the mass ratios of 1:0.5, 1:1 and 1:2 (oil:methanol), the critical temperatures are 345.93, 305.85 and 282.20 °C, respectively (Bunyakiat *et al*., 2006). Thus, the greater availability of alcohol in the reaction medium, the better the possibility of conducting the reaction at a milder temperature, still resulting in a high reaction rate (Hegel *et al*., 2008).

Silva *et al.* (2010) reported that the mass ratio 1:1 seemed to be the most adequate in terms of ester yield and reagent savings for the reaction between soybean oil and ethanol at 300 °C. He *et al.* (2007) found that the increase in ethanol-to-oil mass ratio resulted in an increase in yield, and this behavior was maintained until the ratio of ~1:1.5. Xu *et al.* (2016) indicated that when working with excess alcohol in supercritical conditions, it must be considered that alcohol at its critical point acts not only as a reagent, but also as a solvent, which dissolves the oil present in the reaction mixture and produces an almost homogeneous reaction. The same researchers found that an excess of alcohol could impair the reaction yield because the reagents were too diluted. High concentrations of ethanol can lead to a reduction in reaction rates, which prevents the chemical balance of the reaction from being achieved. It is also important to mention that a high mass ratio may be unfavorable, as more intense processes are required for the separation and evaporation of alcohol (Bunyakiat *et al*., 2006).

Regarding the temperature applied to the reaction, it was noted that in general, raising the temperature from 275 to 300 °C resulted in a low increase in ester yield for the evaluated residence times. Lin and Tan (2014) also found that even by raising the operating temperature (from 280 to 300 °C), ester yields remained similar (~95%) for the reaction between coconut oil and methanol (mass ratio 1:1), with 40 g of MnO_2 catalyst and 11 MPa. Visioli *et al*. (2019) reported ~60% ester content for the reaction between macauba oil and methyl acetate (mass ratio 1:2.5), with 2 g of γ-alumina catalyst, at 275 and 300 °C.

Figures 1c and 1d show the percentages of thermal decomposition of the fatty acids present in the samples obtained and it was verified from these data that thermal decomposition is influenced by residence time and temperature. For the initial minutes of reaction, there was low decomposition, and by increasing the residence time, a thermal decomposition of up to 15% was noted, similarly, by raising the operating temperature from 275 to 300 °C (45 min); in the mass ratio of 1:2, thermal decomposition went

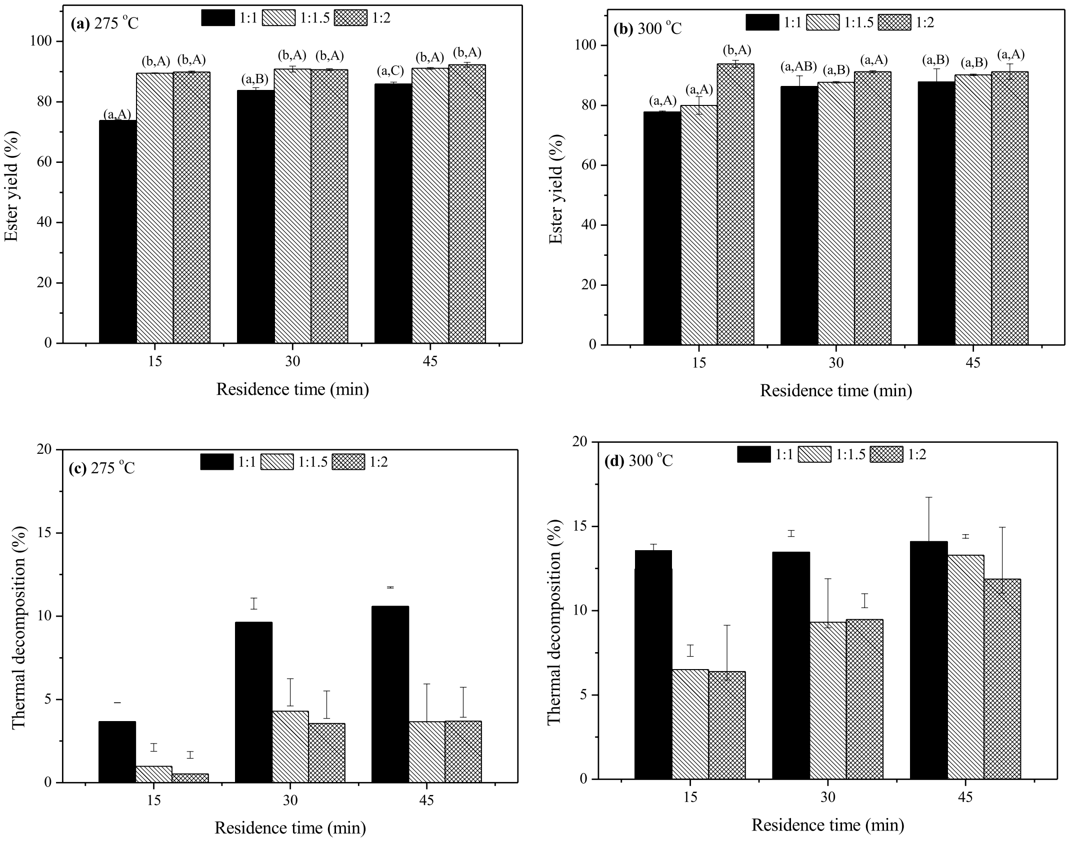

FIGURE 1. Effect of oil-to-ethanol mass ratio on the ester yield for the reactions conducted at 20 MPa using 2 g catalyst and thermal decomposition. Means followed by same letters do not differ statistically ($p > 0.05$) in lower case for the oil-to-ethanol mass ratio (with the same residence time), and upper case for the residence time (with the same oil-to-ethanol mass ratio) using ANOVA (Tukey's test). Values are presented as mean ± standard deviation (n=4).

from ~5 to 15%, respectively. It was also possible to verify that a greater amount of ethanol in the reaction mixture contributed to lower percentages of thermal decomposition at the temperatures evaluated. Thermal decomposition for reactions with waste oil and ethanol has also been reported by Trentini *et al.* (2020), with values of ~20% for 275 and 300 °C, and by Abdala *et al.* (2014), who obtained ~ 6 and 12% decomposition at 275 and 300 °C, respectively.

The observed thermal decomposition intensified at 300 °C when the oil used for the reactions was composed mainly of linoleic fatty acid, which is less stable at high temperatures compared to saturated and monounsaturated esters, as found by Trentini *et al.* (2019). They reported ~23% reduction in the amount of linoleic ester used by raising the operating temperature from 275 to 300 °C, thus verifying that the decomposition of esters is strongly dependent on the operating temperature. Therefore, temperatures above 300 °C should be avoided, as close to this temperature, unsaturated esters are consumed due to thermal decomposition.

Regarding the reactions that occur with thermal decomposition, linear thermal dimerization generally occurs in monounsaturated and polyunsaturated esters, forming acyclic structures of the dimers. A mixture of monocyclic and cyclic dimers of six members can cause other compounds to be formed, such as oligomers, polymers, isomers and thermal cracking products (Quesada-Medina and Olivares-Carrillo, 2011). In addition to reducing the yield in esters, thermal decomposition can influence the quality of the biodiesel obtained due to the increase in viscosity and crystallization temperature related to the accumulation of decomposition products (Liu *et al.*, 2016).

In order to reduce the dimensionality of the data set related to the effect of operational variables on the ester yield (EY) and thermal decomposition (TD) of the reaction products, the principal component analysis (PCA) was performed, using the data from the Table 1. Figure 2 shows the biplot (PC1xPC2) of scores and loadings obtained from a correlation matrix. The two main components (PCs) explained a total cumulative variance of 100% of the data. The vectors of the variables under study form an angle of 90 °, which indicates that there is no correlation between EY and TD.

The PCA formed 2 trend groups. Group A is formed by samples that presented higher EY, since it is close to the vector of this variable. This group is mainly composed of samples obtained at 275 °C, in residence times of 15 to 45 min and oil-to-ethanol mass ratio of 1:1.5 and 1:2, except for sample 6 (obtained at 15 min and 300 °C). These results confirm that the increase in residence time (30 to 45 min) and oil-to-ethanol mass ratio (1:1 and 1:2) did not affect EY, since under these conditions, samples with similar characteristics were obtained. Group B contains samples that showed higher TD and was mainly secreted by reaction products obtained at a temperature of 300 °C and residence time of 45 min, regardless of the oil-to-ethanol mass ratio. This indicates that

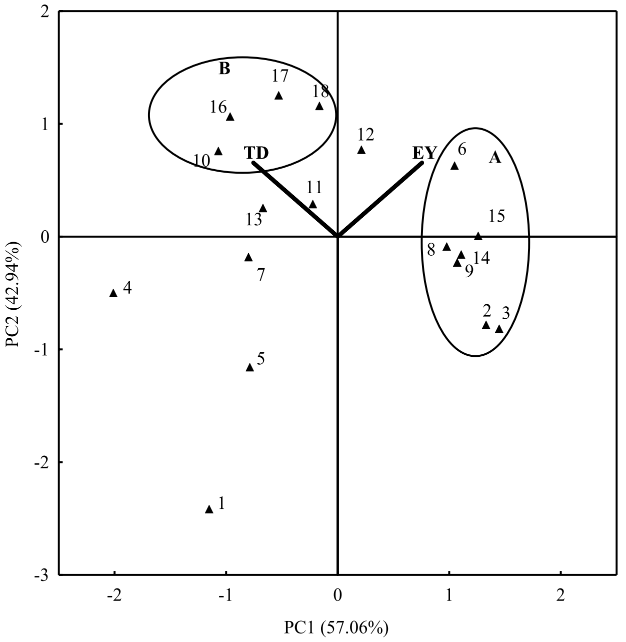

Figure 2. Principal component analysis (PCA) of the ester yield (EY) and thermal decomposition (TD) of reaction products obtained under different conditions of temperature (275 and 300 °C), residence time (15 to 45 min) and oil-to-ethanol mass ratio (1:1 to 1:2). The conditions for 1 to 18 are described in Table 1.

Table 1. Experimental data set of the effect of operational variables on esters yield and thermal decomposition used in the principal component analysis.

Run[1]	Residence time (min)	Temperature (°C)	Oil-to-ethanol mass ratio	EY[2] (%)	TD[3] (%)
1	15	275	1:1	73.77±0.40	4.80±0.01
2			1:1.5	89.50±0.10	2.12±0.23
3			1:2	89.85±0.32	1.67±0.20
4		300	1:1	77.80±0.31	13.58±0.36
5			1:1.5	79.97±2.96	7.62±0.34
6			1:2	93.85±1.21	7.51±1.62
7	30	275	1:1	83.68±1.00	10.76±0.33
8			1:1.5	90.85±0.97	5.43±0.82
9			1:2	90.65±0.32	4.68±0.82
10		300	1:1	86.24±3.61	14.58±0.18
11			1:1.5	87.68±0.25	10.44±1.46
12			1:2	91.21±0.37	10.59±0.42
13	45	275	1:1	85.86±0.67	11.72±0.04
14			1:1.5	91.08±0.30	4.79±1.13
15			1:2	92.27±0.78	4.83±0.90
16		300	1:1	87.83±4.41	15.22±1.51
17			1:1.5	90.19±0.28	14.41±0.11
18			1:2	91.25±2.58	12.99±1.95

[1]all experiments were conducted at 20 MPa, [2]ester yield and [3] thermal decomposition. Each value in the table represents the mean ± standard deviation (n = 4).

the increase in temperature and residence time contributed to TD. Sample 10 (obtained at 300 °C, 30 min and oil-to-ethanol mass ratio 1:1) is also represented by this group due to the lower mass of ethanol used at a high temperature. Samples 2 and 3 showed lower TD, since the opposite variable was found in the opposite quadrant. This result was attributed to the lower temperature and residence time employed in this case.

In short, from the results obtained (Figures 1 and 2), it was possible to verify that similar ester yields were obtained with oil-to-ethanol mass ratio of 1:1.5 and 1:2. In addition, the thermal decomposition became accentuated by increasing the residence time, as well as the operating temperature from 275 to 300 °C. It was also noted that raising the operating temperature resulted in a maximum percentage increase of only 5% in ester yield and intensified thermal decomposition (2.6 times). Thus, the operating conditions of 275 °C, 15 min and oil-to-ethanol mass ratio of 1:1.5 were promising to conduct the reaction and verify the stability of the catalyst.

3.2. Catalyst stability

The stability of the catalyst was evaluated for the reaction conducted at the residence time of 15 min, for a period of 8 h after reaching a steady state, at 275 ºC, using 2 g of catalyst and oil-to-ethanol mass ratio of 1:1.5. The results for yield in ethyl esters are presented in Figure 3.

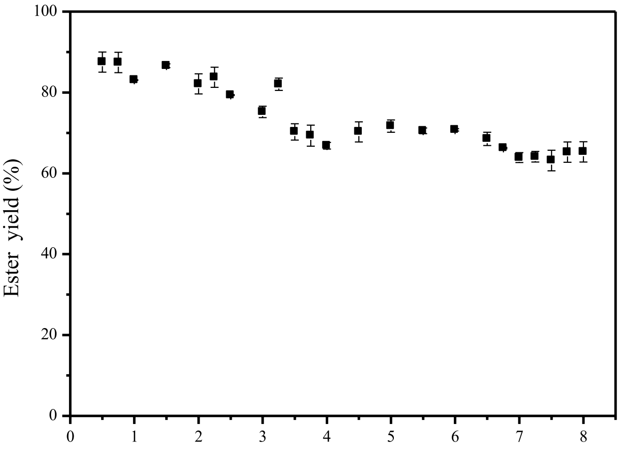

FIGURE 3. Stability of the KF/clay catalyst at 275 ºC in terms of ethyl ester yield, using oil-to-ethanol mass ratio of 1:1.5, 2 g of catalyst and residence time of 15 min. Values are presented as mean ± standard deviation (n=4).

In the first 3 hours of operation, the catalyst remained stable, providing average values for ester yield of 84.26 ± 2.75%. After the mentioned period, there was a slight reduction in the yield, ~15%, and the values remained stable at the time of 3 to 6 h of operation, when again a reduction of ~29% was verified in relation to the initial yield (30 min), and from this period the yield remained close to 65.30 ± 1.60% until the evaluated operation time (8h). According to Simões *et al.* (2020) with a loss of up to 15% in catalyst activity, it can still be reused. Ensuring catalyst stability is of great importance for industrial applications and reducing the cost of biodiesel production (Zhang *et al.*, 2018). Therefore, the results obtained suggest that the KF/clay catalyst has considerable stability and can be used continuously for ~6 hours of operation.

The stability of the catalyst used in the transesterification reactions in a continuous and pressurized reactor was investigated by other researchers. Jesus (2010) used 4 g of hydrotalcites in pressurized methanol at 300 ºC, 15 MPa and residence time of 7 min, and observed that the ester content remained unchanged for 24h. Visioli *et al.* (2019) analyzed the reaction of macauba oil and methyl acetate using 2 g γ-alumina catalyst and found that the catalyst remained stable in the evaluated period (8 h) at 225 and 275 °C, with no loss in activity of the catalyst.

Regarding the catalyst stability in reactions conducted in a batch reactor, Teo *et al.* (2015) evaluated the activity of the catalyst for 5 cycles of 10 min and found a reduction of ~25% in the esters yield in the fourth cycle, for the reaction between *Jatropha curcas* L. oil and methanol, with 1 wt.% of CaLaO catalyst, at 240 °C. In the study by Ribeiro *et al.* (2018) the reuse of the γ-alumina catalyst at 300 °C was reported for 8 cycles of 60 min each, with a reduction of 12.47% in ester content after the seventh cycle.

3.3. Two-step reaction

Based on the results obtained in the first step of the reaction with KF/clay catalyst, a second step was conducted to investigate the influence of the recycled catalyst and the new catalyst in the reaction. The first-step sample (time 0) was obtained in 15 min of reaction, 275 °C, oil-to-ethanol mass ratio 1:1.5 and 2 g of catalyst, and used to conduct the second step, under the same operating conditions with residence times of 15, 30 and 45 min. The esters and inter-

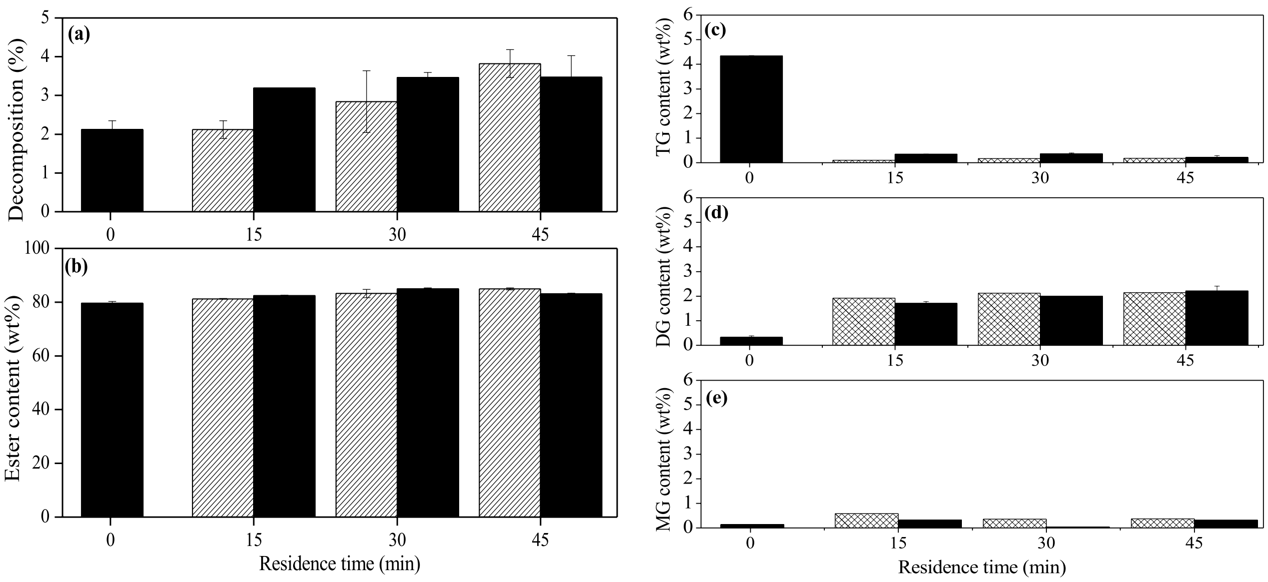

FIGURE 4. Reaction products obtained in two-step reaction at 275 °C, 20 MPa, oil-to-ethanol mass ratio 1:1.5 and applying 2 g of recycled catalyst (▨) and new catalyst (■): (a) ester content, (b) thermal decomposition, and content of (c) triglycerides, (d) diglycerides and (e) monoglycerides. Values are presented as mean ± standard deviation (n=2).

mediate compound contents obtained are presented in Figure 4, as well as the values for percentage of thermal decomposition.

Applying the second reaction step, it was possible to verify that using recycled and new catalysts, they provided an increase of ~6.6% in the ester content, respectively. Trentini *et al.* (2018) carried out the reaction in two steps, using lipids from grease trap waste and ethanol, in which the ester content increased from ~70 to 72%. Regarding thermal decomposition (Figure 4b), this was higher than that found in the first reaction step, reaching values of ~3.8%. However, these decomposition values are still low, since numbers between 6-7% are reported in the literature for reactions with residual oils and the same temperature range as in this study (Abdala *et al.*, 2014; Trentini *et al.*, 2020).

Figure 4c shows a reduction in the triglyceride content (TG), compared to the first reaction step (time 0), with > 99% consumption. In contrast, increases in diglyceride (DG) and monoglyceride (MG) contents were observed, (Figures 4d and 4e, respectively). The contents in intermediate compounds (DG and MG) obtained using a reaction step was 0.46 wt%, which increased to 2.50-2.53 wt%; while the content in triglycerides decreased from 4.34 to 0.19-0.36 wt%. Santos *et al.* (2018) reported the presence of ~12 wt% of intermediate compounds, in ~ 30 min, 279 °C and 20 MPa for the reaction between acidic oil and ethanol, and Trentini *et al.* (2020) reported that 11.23 wt% unreacted compounds remained from the reaction with residual oil/crambe oil mixture (27/75) at 300 °C, 20 MPa and 30 min. Thus, the low levels found for the components in this study indicate the high conversion of residual frying oil into ethyl esters. The esters present in the samples, together with the intermediate components and the decomposed components total the convertibility content of the residual frying oil.

The execution of the second reaction stage for both tested catalysts was effective, and resulted in an increase in the ester content, low decomposition and still low content of unreacted compounds, proving the efficiency of the application of this process. According to the ANP 45/2014 (Brazil) (ANP, 2014) the limit for MG, DG and TG in biodiesel is 0.70, 0.20 and 0.20 wt%, respectively, so the values obtained for MG and TG are in accordance with the legislation.

3.4. Comparison to the literature

Table 2 shows the comparison of the results obtained in this study with reports regarding the use of heterogeneous catalyst under pressurized conditions using a continuous reactor to obtain esters, as well as

TABLE 2. Comparison of the results obtained in this study with those reported in the literature for transesterification in continuous process.

Oil	Alcohol	T (ºC)	P (MPa)	Oil-to-ethanol molar ratio	Catalyst/mass	Ester yield (%)	Reference
Frying oil	ethanol	275	20	1:20	KF/clay (2g)	94.85	This work
Soybean	methanol	300	15	1:30	Hydrotalcites (4g)	91.8	Jesus (2010)
Alga	methanol	340	15	1:31	Porous titania[2]	94.7	Krohn *et al.* (2011)
Soybean	methanol	440	22.7	1:73.7	Base Modified Zirconia[2]	98.2	McNeff *et al.* (2008)
Sunflower	methanol	205	25	1:253	acidic catalyst resin (Nafion SAC-13) 9g	88.2	Maçaira *et al.* (2011)
Rapessed	ethanol	350	30	1:20	Al_2O_3 (2%wt)	97.86	Mazanov *et al.* (2016)
Frying oil	ethanol	300	20	1:204	Catalyst free	87	Abdala *et al.* (2014)
Frying oil	ethanol	325	15	1:305	Catalyst free	82.2	Gonzalez *et al.* (2013)
Waste oil	ethanol	300	15	1:156	Catalyst free	74	Fonseca *et al.* (2018)
Waste oil	ethanol	300	20	1:39	Catalyst free	~50.53	Trentini *et al.* (2020)

[1] *n*-hexane as co-solvent in the process, molar ratio of 1:90 methanol:*n*-hexane; [2] The authors did not inform the mass or concentration of catalyst employed; [3] CO_2 was employed in the process (mass ratio of 25:75 methanol:CO_2); [4] *n*-hexane was used as co-solvent in the process (20% wt in relation to ethanol); [5] water was employed in the process, 5% wt (in relation to ethanol); [6] Hydroesterification - a previous hydrolysis step was carried out with a mass ratio of 1:1 (oil:water) and 50% *n*-hexane (in relation to the oil mass) and subsequent esterification reaction

research using RFO in pressurized conditions, without using a catalyst.

As seen in Table 2, for reactions conducted using catalysts and refined oils, ester yields close to this research were obtained; however, the use of residual frying oil represents an ecologically better, low-cost option (Hajjari *et al*., 2017). In the studies carried out by Jesus (2010) and Krohn *et al.* (2011) methanol, alcohol of better efficiency in transesterification, was used, due to the lower activation energy compared to ethanol, which implies a higher reaction rate. However, they were not able to obtain results higher to those reported in this study, in addition, the production of biodiesel through ethanol is more attractive, as it is a reagent obtained from renewable sources (Soares and Andreozzi, 2011). McNeff *et al.* (2008) and Mazanov *et al.* (2016) reported higher yields than this study, although the reactions were carried out at higher temperatures (440 and 350 °C, respectively).

The researchers Maçaira *et al.* (2011), also using a catalyst, reported a lower yield than that obtained in this research for the reaction at ~200 °C, lower than the critical alcohol temperature, which may have influenced the low ester yield when compared to this study. The esters yield obtained in this study was high when compared to other studies (Table 2) that also used residual frying oil (Abdala *et al*., 2014; Fonseca *et al*., 2018; Gonzalez *et al*., 2013; Trentini *et al*., 2020). Fonseca *et al*. (2018) reported 74% esters through hydro-esterification at 300 ºC. However, a previous hydrolysis step was performed in this study. It is important to mention that not even the addition of co-solvent (*n*-hexane) and water to the reaction, as performed by Abdala *et al.* (2014) and Gonzalez *et al.* (2013) respectively, resulted in a higher yield than reported in this study. Therefore, the presence of the KF/clay catalyst in the reaction was essential to achieving high ester yield.

In this work, oil-to-catalyst mass ratios of 8:1, 6.4:1 and 5.4:1 (oil to ethanol mass ratio 1:1, 1:1.5 and 1:2, respectively) were used, and high performance was achieved with the application of a low cost and residual oil for the production of renewable fuel, and under milder temperature conditions. It should be noted that for most cases, reactions using catalysts seek to conduct the reactions under less severe conditions, as it allows to reduce energy consumption (Andreo-Martínez *et al*., 2020). In this way, the conditions reported in this study, which promoted high yield in esters, applying residual oil, ethanol and lower temperature are advantageous, highlighting the effectiveness of the KF/clay catalyst in the production of biodiesel. In addition, the catalyst used is easy to obtain and comes from clay, a cheap and natural source which can be of great interest for the various benefits linked to its use.

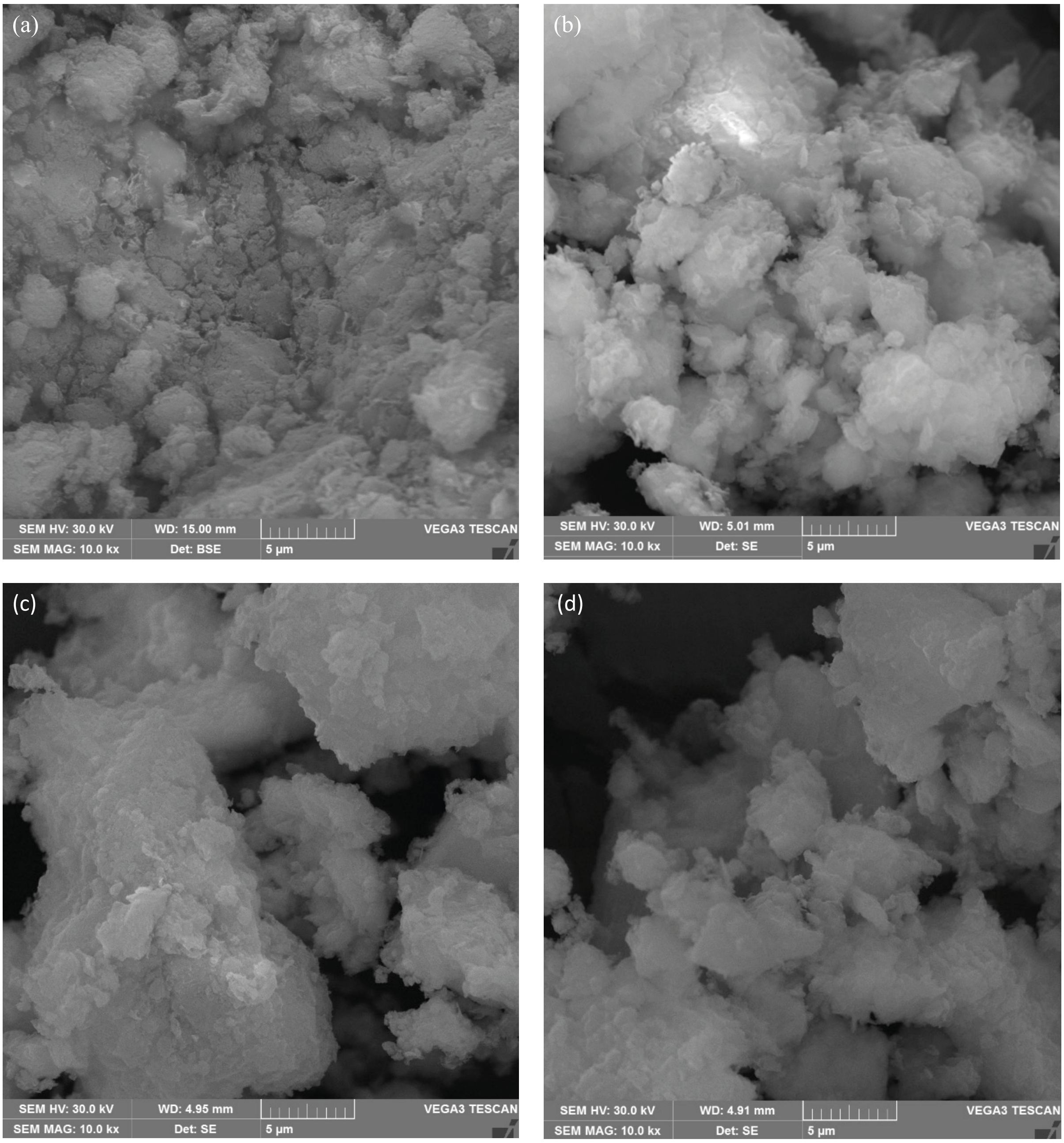

Figure 5. Micrographs: (a) catalyst before use, and catalyst recovered after the reactions of (b) stability (8 h); (c) two steps (recycled) and (d) two-step (new catalyst).

3.5. Catalyst characterization

The micrographs of the catalyst employed in the reactions, before and after the reactions of stability and two steps (recycled and new catalyst) are set forth in Figure 5. Note that the recovered catalyst samples (Figures 5b-d) resemble the catalyst before being used in the processes (Figure 5a), maintaining the rough-appearance characteristic of this process. Even after exposure to the conditions of temperature and pressure employed, the granules of the catalyst were not destroyed, which suggests that the stages of compaction and granulation were successful, and that the material performed well during the reaction time. Thus, the conditions employed in the studied reactions did not significantly modify the morpho-

Table 3. Analysis of energy dispersive spectroscopy (EDS) of catalyst and recovered catalyst.

Elements (%)	Catalyst (KF/Clay)	Recovered catalyst		
		Estability	Two steps (recicle)	Two steps (new catalyst)
C	3.6	15.55	12.4	5.73
O	36.2	46.39	45.58	44.95
Na	0.7	0.71	0.57	0.56
Mg	1.2	1.24	1.01	1.08
Al	3.6	3.03	2.66	2.80
Si	18.5	11.88	13.92	17.87
Ca	ND	0.33	0.39	0.28
Fe	0.6	0.16	0.14	0.19
K	16.5	4.58	6.27	8.46
F	19.4	16.03	17.06	18.08

logical characteristics of the catalyst, indicating that the compacting process was effective for the possibility of its application in pressurized and continuous processes.

The quantification of the elements present in the catalyst before and after the reactions is in Table 3, obtained from the semi-quantitative analysis of energy dispersive spectroscopy (EDS).

The presence of fluoride (19.4%) and potassium (16.5%) in the catalyst sample is due to the impregnation process with potassium fluoride salt. It can be observed that in the recovered samples there was a decrease in these components (mainly potassium), being more accentuated for the catalysts that were exposed for the longest time to the operational conditions (stability reaction and 2 stages with recycle). The activity of the catalysts found is due to the basic sites generated precisely in the impregnation with KF, since the active sites correspond to the fluoride ions themselves and to the negatively charged oxygen atoms located in their vicinity, and the presence of K^+ cations distributed around the active sites generating the basicity of the catalyst (Alves *et al.*, 2014; Boz *et al.*, 2009; Endalew *et al.*, 2011). Thus, the results from the EDS analysis (Table 3) indicate that the leaching of these elements resulted in a drop in yield over the 8 h operation in the stability reaction (Figure 3). When the two-step reactions were compared (Figure 4), the catalyst submitted to reuse showed lower yield than the reaction with a new catalyst, showing the occurrence of leaching of these elements (F and K). The other elements present in the catalyst originated from clay, which is composed of several types of minerals and their composition can vary. The catalyst recovered after the reactions had a higher carbon content, which may be due to remaining oil residues and esters in the sample, even after washing. It is worth noting that the EDS results are semi-quantitative, and that the percentages shown in Table 3 represent the average composition of five selected regions within each analyzed micrograph.

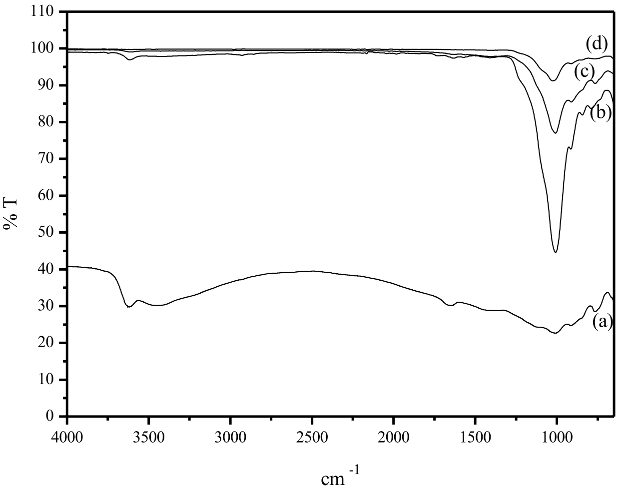

Figure 6. Infrared spectra for (a) catalyst and recovered catalyst after the transesterification reaction: (b) stability; (c) two steps, new catalyst and (d) two steps, recycled catalyst.

Figure 6 s shows the infrared spectra for the catalyst samples before (a) and after the stability reactions (b), two steps with new catalyst (c) and two steps using recycled catalyst (d). The vibration bands that appear between 650 and 1200 cm^{-1} correspond to Si-O and Si-O-Al vibrations, and are typical of quartz and montmorillonite clay minerals, with bands appearing between 700 and 900 cm^{-1} to the octahedral layers of clay. These vibrations remain even on the catalysts subjected to the reactions. In the range of 900 to 1100 cm^{-1} there is the characteristic SiO bond elongation, being more evident in the catalyst after use in the reactions (b-d), probably due to the leaching of the K^+ ion, which is responsible for the catalyst activity. Thus, the structure of the clay becomes more evident and for this reason, the increase in the band attributed to the SiO bond present in the minerals of the clay occurs. The 3500 to 3800

cm^{-1} range corresponds to the O-H elongation. The bands in the region of 1250-1750 cm^{-1} correspond to the treatment of the clay with KF. Bands in the range of 1600-1700 represent H-O-H and correspond to adsorbed water, present only in sample (a).

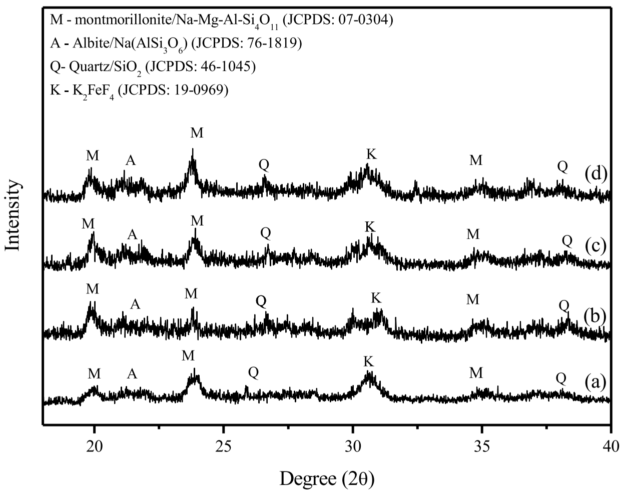

Figure 7. X-ray diffractometry for (a) catalyst and recovered catalyst after reactions from: (b) stability (c) Two steps, recycled catalyst (d) two-step, new catalyst.

In Figure 7 the XRD for the catalyst is presented before and after the use of stability reactions, two steps with new catalyst and recycled catalyst. The crystalline phases identified in the samples were montmorillonite ($Na\text{-}Mg\text{-}Al\text{-}Si_4O_{11}$), quartz ($SiO_2$) and albite ($Na(AlSi_3O_8)$), a type of feldspar. These phases were already predicted, as they are crystalline phases commonly found in bentonite clays. The peaks present in the catalyst (Figure 7a) remain similar in the catalysts recovered after use in the reactions (Figure 7b-d). There is also a crystalline phase in the catalyst, corresponding to the treatment with potassium fluoride, and it can be observed that the leaching shown in the EDS (Table 3) and FTIr (Figure 6) of the K^+ element is also evident in the XRD, mainly in the reaction which stood exposed for the longest operating conditions (stability, Figure 7b).

It is worth mentioning that there is some possibility for the development of a more stable catalyst in order to reduce the leaching of active species such as K^+ and F^-. According to the results obtained in this work, given the reactor operating conditions (temperature, flow and pressure practiced), it is believed that there is an “excess” of K^+ and F^-, which eventually are not interacting properly with the surface of the clay particles (weak interaction), in order to withstand the experimental conditions of temperature and pressure, which in turn, are not mild. Thus, it is necessary to conduct a future study to evaluate the amount of the KF saline precursor used in the impregnation, which can guarantee that the ideal content of K^+ and F^- ions capable of forming stable active sites on the catalyst surface.

4. CONCLUSIONS

In this work, the production of ethyl esters using residual frying oil and ethanol in a pressurized medium was investigated in the presence of the KF/clay catalyst. The results indicate that increasing the mass ratio of oil-to-ethanol resulted in ester yields close to those for the ratios of 1:1.5 and 1:2, in 30 and 45 min, with a mass ratio of 1:1.5 being sufficient to achieve balance. Raising the operating temperature from 275 to 300 °C resulted in greater thermal decomposition of the esters. Conducting the reaction in one step at 275 °C, 20 MPa, mass ratio of oil-to-ethanol of 1:1.5 and 15 min provided maximum ester yield of ~90%. Under the best operating conditions, the stability of the catalyst was evaluated for a period of 8 h, which provided consistent results in the first 3 h, and the yield decline in esters by 29% throughout the reaction, mainly due to potassium leaching. Conducting the reaction in two stages made it possible to obtain a higher yield in esters (~94.85%), using recycled catalyst and new catalyst, in addition to low thermal decomposition and higher consumption of TG. The conditions employed in the reactions did not cause significant changes in the morphology of the catalyst.

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Chemical-functional composition of *Terminalia catappa* oils from different varieties

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SUMMARY: This study aimed to extract and physical-chemically characterize *Terminalia catappa* L. kernel oil from purple (CR) and yellow (CA) varieties. Physical-chemical parameters, composition of fatty acids, nutritional quality indices, bioactive compounds and antioxidant capacity of both oil varieties were evaluated according to the literature. Both oils presented low levels of acidity and peroxides, besides the predominance of unsaturated fatty acids, ~63% of oleic and ~26% of linoleic acids, which influenced its nutritional indices. The CR oil variety exhibited a higher content in anthocyanin (18.3 ± 1.5 mg·100 g^{-1}), ascorbic acid (68.4 ± 2.02 mg·100 g^{-1}) and total polyphenol contents (152.3 ± 2.4 mg GAE·g^{-1}), and a good antioxidant activity (38.6 ± 2.2 µg TE·g^{-1}) determined by TEAC assay, when compared to the CA oil ($p < 0.05$). Therefore, the results confirm the importance of *T. catappa* as a lipid source for human consumption to be used in the development of food products.

KEYWORDS: *Antioxidant activity; Bioactive substances; Linoleic acid; Oleic acid; Tropical almond; Vegetable oil.*

RESUMEN: ***Composición química-funcional de aceites de* Terminalia catappa *de diferentes variedades***. El objetivo de este estudio fue extraer y caracterizar físico-químicamente aceite de semilla de *Terminalia catappa* de las variedades violeta (CR) y amarilla (CA). Se evaluaron parámetros fisicoquímicos, composición de ácidos grasos, índices de calidad nutricional, compuestos bioactivos y capacidad antioxidante de ambas variedades de aceite de acuerdo con a la literatura. Como resultado, ambos aceites presentaron bajos niveles de acidez y peróxidos, y predominio de ácidos grasos insaturados, ~63% de ácido oleico y ~26% de ácido linoleico, lo cual influyó en su perfil nutricional. La variedad de aceite CR presentó un mayor contenido de antocianina (18,3 ± 1,5 mg·100 g^{-1}), ácido ascórbico (68,4 ± 2,02 mg·100 g^{-1}) y contenido total de polifenoles (152,3 ± 2,4 mg GAE·g^{-1}), y una alta actividad antioxidante (38,6 ± 2,2 µg TE·g^{-1}) determinado por ensayo TEAC, en comparación con el aceite CA ($p<0.05$). En conclusión, los resultados presentados refuerzan la importancia de *T. catappa* como fuente de lípidos para la ingesta humana y para su uso en el desarrollo de productos alimenticios.

PALABRAS CLAVE: *Aceite vegetal; Ácido linoleico; Ácido oleico; Actividad antioxidante; Almendra tropical; Sustancias bioactivas.*

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1. INTRODUCTION

Among the diversity of fruit species in Brazil there are underexploited species that can be used for human nutrition, as well as for the extraction and isolation of functional/bioactive compounds, which can play an important role in maintaining human health. In this context, a native species from tropical and subtropical regions, *Terminalia catappa* L., stands out as an innovative source of fruits and their derivatives. It belongs to *Combretaceae* family, and produces glabrous, rounded and flattened drupaceous fruits. These fruits are commonly named Sea-almond, Tropical-almond, Indian-almond or Malabar-almond (Abdulkadir, 2015).

The Tropical almond fruits initially exhibit a green color, which during the maturation process becomes red-purple and, also may turn yellow (Salawu *et al.,* 2018). The fruits measure 5-7 cm long and 3-6 cm in width, have an exocarp (bark) adhered to an edible fibrous pulp (mesocarp), and a single rigid seed. The fruit pulp is a good source of carbohydrates, up to 76%, and low in lipids, less than 3% (Ladele *et al*., 2016). The seed has an oily endocarp (kernel), containing up to 52% lipids, 38% proteins and minerals, which is covered by a thin peel (Agu *et al*., 2019; Abdulkadir, 2015; Souza *et al*., 2016). Both pulp and seeds are edible (Ladele *et al*., 2016). The lipids extracted from the kernel almond have great potential for application as an edible vegetable oil due to their elevated lipid yield, up to 60%, which has higher value when compared to main commercial oilseeds, such as soybeans, palm, and peanuts (Ladele *et al*., 2016; Jokić *et al*., 2015).

Terminalia catappa can be considered a fruit tree with high economic potential because its fruiting starts at around 3 to 5 years of age, with two harvests per year, producing up to 30 kg fruits per year, reaching an estimated world production of more than 700,000 tons in 2004 (Agu *et al*., 2019; Singh and Choudhary, 2012). However, its productivity needs further assessment. In addition, this species is commonly cultivated in several countries as an ornamental tree, a fruit tree and as a vegetable oil source for different applications (Agu *et al*., 2019; Janporn *et al*., 2015; Menkiti *et al*., 2015). The Tropical almond cultivation generally needs low maintenance, since it has a simple propagation from seeds and can grow quickly in different soils and environments (Ladele *et al*., 2016).

Investigations have been carried out to characterize the Tropical almond fruit and to determine and quantify its bioactive compounds as a source of natural antioxidants (Abdulkadir, 2015; Huang *et al*., 2018). These bioactive compounds have been studied for complementary functions and actions of insulin in the treatment of diabetes, to act regulating dietary constituents in human daily intake and as potential anti-inflammatory agents (Ben *et al*., 2019; Huang *et al*., 2018). A recent study has focused on the nutritional and functional properties of the pulp and kernel oils of *Terminalia catappa* L. obtained by supercritical fluids (Santos *et al*., 2021). In another work from Agu *et al.* (2019), the *T. catappa* kernel oil was chemically modified and characterized as a potential replacement for mineral transformer fluid. In a similar application, the oil from *T. catappa* was used by Silva *et al.* (2020b) to synthetize biodiesel (via methyl route).

It is worth mentioning that in the works in the literature, a few of them have identified which variety of the Tropical almond, purple or yellow, was used in their research. Thus, investigations comparing different varieties of Tropical almond fruit can increase knowledge based on its different chemical compositions and bioactive/nutritional constituents, and guide new applications for the food and chemical industries.

The aim of this research was to evaluate the functional chemical composition of *Terminalia catappa* L. kernel oil, and to compare its purple (CR) and yellow (CA) varieties. Fatty acids and triacylglycerol profile, nutritional quality parameters, bioactive compounds, such as anthocyanins, ascorbic acid and polyphenol contents, and antioxidant activity were investigated.

2. MATERIALS AND METHODS

2.1. Raw material and oil extraction

Fruit seeds of *Terminalia catappa L.* from purple (CR) and yellow (CA) varieties were harvested on the campus of Federal University of Pará (UFPA) in 2020. The fruits were collected in the following geographical coordination: latitude 01º 27 ‘21”S, longitude 48º 30’ 16”W and altitude 10 m. To ensure the proper taxonomic identification of this plant, some parts of it, such as leaves and fertile material, were collected and deposited in the herbarium Professor Normélia Vasconcelos/UFPA under the code MG nº 3791.

2.2. Sampling and oil extraction

The fruits of *Terminalia catappa* L. from purple and yellow varieties were washed to remove any physical dirt, gently peeled off, and then the seeds were manually cracked, and their kernels were removed. The kernels were dried at 60 °C for 24 h in an air-circulation oven (model 81-150, New Lab Equipamentos, Piracicaba, SP, Brazil), and milled in a Willey miller (TE-650 model, Tecnal, SP, Brazil). Subsequently, a solid-liquid extraction was carried out in a Soxhlet apparatus, using hexane as solvent according to the methodology of Silva *et al.* (2020b). All analyses were performed in triplicate. The oil yield (%) was calculated according to the Eq. 1.

$$Oil\,yield\ (\%) = \frac{\mathrm{W_{oil}}}{\mathrm{WT}_g} \times 100 \quad (1)$$

where $\mathrm{W_{oil}}$ is the extracted mass of oil (g) and the WT_g is the total mass of seeds (g).

2.2. Physical-chemical analysis of *T. catappa* kernel oils

2.2.1. Quality parameters

The physical-chemical quality parameters of the *Terminalia catappa* L. kernel oils from purple (CR) and yellow (CA) varieties were determined according to the official methods from the American Oil Chemist's Society (AOCS), as follows: acidity, peroxide and saponification values were determined according to AOCS methods Cd 3d-63, Cd 8-53 and Cd 3-25, respectively (AOCS, 2004). The true density (ρ, g m^{-3}) was measured using a DA–130 digital density meter (Kem Kyoto Electronics, Japan) at room temperature (~25 °C) and the refractive index was investigated according to the Cc 7-25 method (AOCS, 2004).

2.2.2. Fatty acids profile

The fatty acid (FAs) profile of *Terminalia catappa L.* oils CR and CA was determined as fatty acid methyl esters (FAMEs) according to the established procedure ISO 5509:2000 reported by the International Organization for Standardization (ISO, 2000). After phase separation, the supernatant was collected for subsequent gas chromatographic analysis with flame ionization detector (GC-FID) (Thermo Scientific Trace GC Ultra) using a wall-coated open-tubular column (WCOT). The analysis was performed in a gas chromatograph (Varian 430 model, Agilent Technologies, CA, USA) equipped with a microcomputer with the software Galaxie Chromatography under the following parameters: fused silica SP®-2560 capillary column (Merck, USP-G5, SUPELCO, USA) of 100 m in length and 0.25 mm internal diameter, containing 0.2 µm of polyethylene glycol. The operation conditions were: 50:1 split injection ratio, column temperature at 140 °C for 5 min programmed with an increasing rate of 4 $°C\cdot min^{-1}$ up to 240 °C, helium as carrier gas in 37 psi isobaric pressure, 20 $cm\cdot sec^{-1}$ linear velocity, make up gas: 29 $mL\cdot min^{-1}$ helium flow, 250 °C injector temperature, autosampler model Varian CP8410, detector temperature 250 °C. The peaks were identified by comparing peak retention time to the known FAMEs standard (37-Component FAME Mix - methyl esters of fatty acids ranging from C_4 to C_{24} CRM47885, Supelco). The quantitative composition was carried out by area normalization, and expressed in mass percentage as established by the official method Ce 1-62 (AOCS, 2004). The samples were analyzed in triplicate.

2.2.3. Nutritional quality indices

The nutritional quality indices in the *Terminalia catappa L* oils from purple and yellow varieties were established based on their respective FAs profiles, which were classified according to the presence and number of double or triple bonds: saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The following indices were used to investigate its nutritional quality: atherogenicity (AI) and thrombogenicity indices (TI) were determined according to Ulbricht, Southgate (1991), and calculated according to Eq. 2 and Eq. 3, respectively. The hypocholesterolemic/hypercholesterolemic ratio (h/H) was determined as defined by Santos-Silva *et al.* (2002) and calculated using the Eq. 4. The calculated oxidative stability value (COX) was defined according to Silva *et al.* (2020a) as expressed in the Eq. 5.

$$AI = \frac{[(\mathrm{C_{12:0}} + (4 \times \mathrm{C_{14:0}}) + \mathrm{C_{16:0}})]}{(\mathrm{PUFA} + \mathrm{MUFA})} \quad (2)$$

$$TI = \frac{(\mathrm{C}_{12:0} + \mathrm{C}_{16:0} + \mathrm{C}_{18:0})}{[(0.5 \times \mathrm{MUFA}) + (0.5 \times \mathrm{n6-PUFA}) + (3 \times \mathrm{n3-PUFA}) + (\frac{\mathrm{n3-PUFA}}{\mathrm{n6-PUFA}})]} \quad (3)$$

$$h/H = \frac{\mathrm{C}_{18:1} + \mathrm{PUFA}}{(\mathrm{C}_{14:0} + \mathrm{C}_{16:0})} \quad (4)$$

$$COX = \frac{(\mathrm{C}_{18:1}) + (10.3 \times \mathrm{C}_{18:2}) + (21.6 \times \mathrm{C}_{18:3})}{100} \quad (5)$$

2.2.4. Triacylglycerol composition

The triacylglycerol composition in the *Terminalia catappa L.* oils from purple and yellow varieties was estimated based on the 1,3-random-2-random distribution hypothesis using the software PrOleos®, which predicts the molar percentage of triacylglycerols present in oil based on its fatty acid composition (Antoniosi Filho *et al*., 1995). This software is available online at the website "https://lames.quimica.ufg.br/p/4035-apoio-didatico".

2.2.5. Analysis of bioactive compounds and antioxidant capacity

The bioactive compounds and antioxidant activity in *Terminalia catappa* L. kernel oils from purple and yellow varieties were investigated. The bioactive compounds were analyzed as anthocyanin, ascorbic acid and total polyphenol contents, and the antioxidant activity was determined based in the Trolox Equivalent Antioxidant Capacity (TEAC) assay. Prior the analysis, the oil samples were solubilized in isopropyl alcohol at 320 mg·mL^{-1} concentration.

Anthocyanin content. The content of anthocyanins was determined as reported by Silva *et al.* (2014). About 1 g of each sample was mixed with 10 mL of a 1.5N HCl in 85% ethanol solution. The samples were homogenized and left to rest overnight under refrigeration and dark covered. Then, the absorbance of the samples was measured at 535 nm wavelength using an UV-Vis spectrophotometer (model UV-1800, Shimadzu, Tokyo, Japan). The analysis was performed in triplicate. The anthocyanins' content was calculated using the Eq. 6 and results were expressed as mg·100g^{-1}.

$$Anthocyanin\ content = \frac{[Abs\ \times dilution\ factors] \times 1000}{[W_{sample} \times \varepsilon^{1\%}_{1cm,535}]} \quad (6)$$

where Abs is the measured absorbance of the sample at 535 nm, W_{sample} is the weight of the sample and $\varepsilon^{1\%}_{1cm,535}$ is the absorption coefficient for anthocyanins, which is equal to 982 g·100 mL^{-1}cm^{-1}.

Ascorbic acid content. The ascorbic acid content was determined by the reduction of the 2,6-dichlorophenol-indophenol compound, according to the adapted methodology of Cunha-Santos *et al.* (2019). About 10 mL of sample were mixed with 2 mL of a 0.03g·mL^{-1} metaphosphoric acid diluted in an acetic acid aqueous solution and titrated with 0.2% 2,6-dichlorophenol-indophenol solution with sodium bicarbonate at 0.21 mg·mL^{-1} concentration, until the appearance of a pink color was persistent for more than 5 s. The 2,6-dichlorophenol-indophenol solution was standardized with an ascorbic acid solution prior to analysis. The sample was analyzed in triplicate and the results were expressed as mg of ascorbic acid per 100 g of sample (mg·100g^{-1}).

Total polyphenol content. The total polyphenol content of these fractions was analyzed following the Folin-Ciocalteu assay as reported by Aliakbarian *et al.* (2011). Initially, 0.2 mL sample, 4.8 mL deionized water, and 0.5 mL Folin-Ciocalteu reagent (Sigma-Aldrich) were transferred to a 10 mL volumetric flask, and vigorously mixed. Then, 1 mL of a 20% sodium carbonate solution was added, followed by deionized water until reaching a final volume of 10 mL. The solutions were mixed and left to rest at room temperature in the dark for 1 h. An aliquot (~2 mL) of sample was used for the determination of total polyphenols using a UV-Vis spectrophotometer (model UV-1800, Shimadzu, Tokyo, Japan) at a wavelength of 725 nm. Distilled water was considered as blank. The sample was analyzed in triplicate, and the results were calculated based on a standard curve of gallic acid (Sigma-Aldrich) and expressed as mg GAE·g^{-1}.

Antioxidant capacity. The determination of the antioxidant capacity from the samples was performed according to the Trolox equivalent antioxidant capacity (TEAC) assay using the ABTS (2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid, from Sigma-Aldrich) reagent as described by Chen *et al.* (2011). The absorbance was measured at 734 nm wavelength using UV-Vis spectrophotometer (model

UV-1800, Shimadzu, Tokyo, Japan). The assay was performed in triplicate against a calibration curve of Trolox (μg Trolox equivalent·L^{-1}) and calculated using the following linear equation:

$$TEAC = (0.6239 - Absorbance_{734nm})/0.3364 \quad (R^2 = 0.997)$$

2.3. Statistical analyses

The results were statistically analyzed using the Statistica software version 7.0 (Statistica, 2000), by analysis of variance (ANOVA) and Tukey's test at the significance level of 5% ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Oil extraction

The solid-liquid extraction of the *Terminalia catappa* L. kernel oils from purple (CR) and yellow (CA) varieties showed a lipid yield of 57 and 54%, respectively. These results are higher than the value of 52% found by Silva *et al.* (2020b) and lower than the value of 61.7% obtained by Ladele *et al.* (2016) for kernel oil yield from *Terminalia catappa* seeds. It is worth mentioning that in both studies, hexane was used as solvent for solid-liquid extraction, and the seeds were harvested in Brazil and Benin, respectively.

3.2. Physical-chemical analysis of the *T. catappa* kernel oils

3.2.1. Quality parameters

The results from the quality parameters of the *Terminalia catappa* L. kernel oils from purple (CR) and yellow (CA) varieties are shown in Table 1. The quality parameters in vegetable oils, acidity, and peroxide values, are ruled by the Codex Alimentarius (2001). This institution recommends the maximum values for acidity and peroxide, in crude vegetable oils as 4 mg KOH·g^{-1} and 15 meq·Kg^{-1}, respectively.

Table 1. Quality parameters of *Terminalia catappa* kernel oils from purple (CR) and yellow (CA) varieties.

Quality parameters	**Oil samples**	
	CR	**CA**
Acidity value (mg KOH·g^{-1})	1.25 ± 1.05^{a}	1.55 ± 0.47^{a}
Peroxide value (meq O_2·Kg^{-1})	2.05 ± 1.17^{a}	3.43 ± 0.72^{a}
Saponification value (mg KOH·g^{-1})	185.5 ± 0.18^{a}	180.7 ± 1.05^{a}
Refractive index	1.50 ± 0.01^{a}	1.45 ± 0.00^{a}
Density (g·m^{-3})	0.91 ± 0.00^{a}	0.90 ± 0.00^{a}

Data represent the mean ± standard deviation of triplicate analyses (n = 3). Different superscript lowercase letters in the same line represent significant differences ($p < 0.05$) at 95% confidence interval according to Tukey's test.

The acidity values for the CR and CA oils are in accordance with the Codex Alimentarius (2001) standard values for crude vegetable oils. When comparing this result to the acidity value for *T. catappa* kernel oil from Benin and Congo, ~2.24 and ~2.42 mg KOH·g^{-1} respectively, from the work of Ladele *et al.* (2016), it was observed that the CR and CA oils presented a lower value. In the work of Janporn *et al.* (2015), the acidity value for the *T. catappa* oil from Thailand was around 2.4 mg KOH·g^{-1}, a higher value when compared to the CR and CA oils. The determination of acidity in vegetable oils is an important indicator of the presence of free fatty acids, which can be associated with lipid hydrolytic degradation and quality loss (Ghafoor *et al.*, 2019).

The peroxide values determined for CR and CA oils were below the maximum value recommended by the Codex Alimentarius (2001), demonstrating its good quality. It was observed that the CA oil presented a higher peroxide value than CR, although with no statistically significant difference ($p > 0.5$). When investigating the peroxide value of *T. catappa* oils from Benin, Nigeria and Congo extracted using organic solvents, Ladele *et al.* (2016), found similar values of 3.7, 2.8 and 0.5 meq O_2·Kg^{-1}, respectively. In another work, the crude oil of *T. catappa* from Thailand presented a lower value for peroxides at 0.65 meq O_2·Kg^{-1} (Janporn *et al.*, 2015). The peroxide value is a crucial factor in the quality evaluation of edible oils as it can be correlated to the presence of secondary lipid oxidation products and may cause rancidity. Besides, it is well established that high temperatures during processing, storing, as well as long-time exposures to light, humidity and atmospheric oxygen are key factors to lipid oxidation, which is reflected in high levels of acidity and peroxides.

The saponification value for *T. catappa* CR and CA oils was higher than the amount of ~175 mg KOH·g^{-1} obtained by Ladele *et al.* (2016), and ~179 mg KOH·g^{-1} as determined in the work of Janporn *et al.* (2015). When comparing both varieties of *T. catappa* oils, purple and yellow, the first one was found to present a higher saponification value, although with no significant difference ($p > 0.05$). In the Codex Alimentarius (2001) there is no indication of maximum value for saponification in crude vegetable oils, but there is a recommended value for virgin olive oil of 184 – 196 mg $KOH \cdot g^{-1}$. When considering these limits, the CR and CA oils presented lower values, which is a good indication of quality. The saponification value is commonly used to estimate the average length of FA chains, which may indicate a high percentage of short-chain ester bonds and a higher saponification value.

The physical properties of vegetable oils, such as density, refractive index, viscosity, and other rheological parameters are factors to be considered when considering the processing design of equipment, as well as its proper function, e.g., pumping, settling and filtration (Freitas *et al.*, 2018). Nevertheless, the density and the refractive index of CR and CA are in accordance with the literature (Ghafoor *et al.*, 2019; Freitas *et al.*, 2018). For both results, the CR and CA oils presented no significant difference ($p > 0.5$) between each other. Furthermore, the quality parameters of CR and CA oils are in accordance with the literature and international standards (Codex Alimentarius, 2001).

3.2.2. Fatty acid profile and nutritional quality indices

The composition on FAs of *Terminalia catappa* kernel oils from purple (CR) and yellow (CA) varieties is shown in Table 2, and for comparison purposes the FA profiles of authentic vegetable oils from maize, soyabean and palm kernel determined by the Codex Alimentarius (2001) were listed. The FA profiles of CA and CR oils exhibited the predominance of unsaturated fatty acids (UFAs), up to 62.9%, mainly represented by oleic acid. The major proportions of FAs in both samples were oleic acid (up to 39%), follow by palmitic acid (~33%), then linoleic acid (~26%). When comparing the CR and CA oils, the percentages of oleic and linoleic acids presented significant differences ($p < 0.05$), a behavior that was not observed for the other FAs. Furthermore, the FA profiles of CR and CA are in accordance with the literature. In the work of Janporn *et al.* (2015), the oil from *T. catappa*, extracted by solvent using the Soxhlet apparatus, presented a remarkably similar FA profile. For these authors, the major proportions of FAs were oleic acid (~31.7%), followed by palmitic acid (~31.4%), then linoleic acid (~23%).

TABLE 2. Comparative profiles of fatty acids in *T. catappa* kernel oils from purple (CR) and yellow (CA) varieties and other oilseeds.

% Total fatty acids	***Terminalia catappa* oils**		**Edible vegetable oils***		
	CR	**CA**	**Maize**	**Soyabean**	**Palm kernel**
Myristic acid ($C_{14:0}$)	0.10 ± 0.00	---	< 0.3	< 0.2	0.5−1.5
Palmitic acid ($C_{16:0}$)	31.08 ± 0.00^a	33.4 ± 0.9^a	8.6−16.5	8−13.5	38−43.5
Palmitoleic acid ($C_{16:1}$)	0.38 ± 0.00^a	0.29 ± 0.00^a	< 0.5	< 0.2	< 0.6
Stearic acid ($C_{18:0}$)	5.72 ± 0.01^a	5.62 ± 0.01^a	<3.3	2−5.4	3.5−5
Oleic acid ($C_{18:1}$ *cis* ω-9)	39.08 ± 0.02^a	33.9 ± 2.3^b	20−42.2	17−30	39.8−46
Linoleic acid ($C_{18:2}$ *cis* ω-6)	22.80 ± 0.03^a	26.0 ± 2.6^b	34−65.6	48−59	10−13.5
α-linolenic acid ($C_{18:3}$ ω-3)	0.06 ± 0.01^a	0.04 ± 0.05^a	<2	4.5−11	<0.6
Arachidic acid ($C_{20:4}$ ω-6)	0.62 ± 0.00^a	0.55 ± 0.01^a	----	----	----
Behenic acid ($C_{22:0}$)	0.19 ± 0.01^a	0.16 ± 0.06^a	<0.5	----	----
Σ SFAs	37.10	39.20	----	----	----
Σ UFAs	62.90	60.80	----	----	----
Σ MUFAs	39.46	34.20	----	----	----
Σ PUFAs	23.50	26.70	----	----	----
Σ ω-6	23.42	26.65	----	----	----
Σ ω-3	0.06	0.04	----	----	----
Total	100.00	100.00	----	----	----

*Values determined from authentic samples by Codex Alimentarius (2001). ---- = Non-defined. Data represent the mean ± standard deviation of triplicate analyses (n = 3). Different superscript lowercase letters in the same line represent significant differences ($p < 0.05$) at 95% confidence interval according to Tukey's test.

The composition of FAs in the *T. catappa* kernel oil from Benin, investigated by Ladele *et al.* (2016), also displayed a similar profile, in which the palmitic (~40%), linoleic (~26.6%) and oleic acids (~26.2%) stood out.

When comparing the FA composition determined by the Codex Alimentarius to the *Terminalia catappa* kernel oils, major differences are found. However, palm kernel oil presented the closest FA profile to the CR and CA oils, mainly due to relatively similar amounts of palmitic and oleic acids, around 40%. Palm kernel oil also presented a significant proportion of linoleic acid, around 13%, which was two times lower than the CR and CA. These results corroborate the edibility of *Terminalia catappa* kernel oils from purple and yellow varieties.

The evaluation of the FA composition of vegetable oils can provide a vital classification of its lipids related to nutritional indices, mainly due to the presence of essential fatty acids, which can be used to correlate it to the prevention of cardiovascular diseases. The nutritional quality indices of *T. catappa* kernel oils from purple (CR) and yellow (CA) varieties are presented in Table 3. For comparison purposes the indices from other tropical fruit oilseeds, *Caryocar villosum*, *Bactris gasipaes*, and *Oenocarpus bacaba*, are displayed in the same table.

The ratio between polyunsaturated and saturated acids (P/S) is a relevant index of the nutritional quality of oils intended for human consumption, as a higher proportion of PUFAs may prevent the increase in body weight in high-fat diets. Nutritional regulations suggest a P/S ratio above 0.4, although this index cannot be taken into account alone for a healthy diet (Domínguez *et al*., 2016). the P/S value determined for the CR and CA oils were inferior to the value determined by Ladele *et al.* (2016), 0.84, and by Janporn *et al.* (2015), 1.4. However, when considering the recommend value of 0.4 by European legislations (Domínguez *et al*., 2016) the CR and CA still exhibited superior values.

Table 3. Nutritional quality indices of *Terminalia catappa* kernel oils from purple (CR) and yellow (CA) varieties and other tropical fruit oilseeds

***T. catappa* oils**		**Other tropical fruit seed oils**		
CR	**CA**	***Caryocar villosum*[1]**	***Bactris gasipaes*[2]**	***Oenocarpus bacaba*[3]**
0.63	0.68	0.61	ND	0.43
0.50	0.54	0.38	1.10	0.30
1.16	1.27	0.75	2.04	0.67
0.75	0.79	2.58	0.84	3.32
2.75	3.05	ND	ND	ND

Data represent the calculated results from mean values (n = 3) of FA fractions, according to the equations previously presented. P/S – Polyunsaturated/saturated fatty acid ratio, AI – Atherogenicity index, TI – Thrombogenicity index. *h/H* – Hypocholesterolemic/hypercholesterolemic ratio, COX - calculated oxidation value. ND – non-determined. [1]Lorenzo *et al.* (2020); [2]Santos *et al.* (2020); [3]Pinto *et al.* (2018).

The atherogenicity (AI) and thrombogenicity (TI) indices in human intake can linked to an increase in cardiovascular and other chronic non-transmissible diseases, when these values are not as low as possible (Santos *et al*., 2021; Ulbricht and Southgate, 1991). The AI and TI of the *T. catappa* kernel oils from purple (CR) and yellow (CA) varieties were higher than the values from *Caryocar villosum* and *Oenocarpus bacaba* oils determined in the works of Lorenzo *et al.* (2020) and Pinto *et al.* (2018), and lower than *Bactris gasipaes* oil (Santos *et al*., 2020). The replacement of animal fats in reformulated meat products by vegetable oils with lower AI and TI have demonstrated a significant improvement from a nutritional perspective (Domínguez *et al*., 2016). The h/H ratio of CR and CA oils were similar, and lower than the other tropical fruit oilseeds. A high value for this index in lipid intake may be advantageous to reducing low-density lipoproteins (LDL) in cholesterol fractions (Santos-Silva *et al*., 2002).

The calculated oxidation capacity value, COX, has a strong correlation with the proportion of PUFAs in lipid sources, and therefore, is expected to be higher in oils with high contents of PUFAs because they are more susceptible to oxidation. The COX value was lower in the CR oil than CA oil, which could be explained by the significant difference in the amount of linoleic acid between them, higher in CA than CR. The COX values for both samples, CR and CA, were lower than the indices of 6.6, 7.3, 6.5 and 7.8, as determined in oils from non-conventional sources, black cumin seeds (*Nigella sativa*), grape seeds (*Vitis vinifera*), tomato seeds (*Lycopersicon esculentum*) and wheat germ (*Triticum vulgare*), respectively (Hassanien *et al.,* 2014). The CA and CR oils displayed lower COX values when compared to conventional oilseeds, such as linseed (12.6 - 13.9), sunflower (1.94 - 9.16), rapeseed (4.2 - 4.4), and camelina oils (8.7 - 9.4) (Symoniuk *et al*., 2018).

Furthermore, these data corroborate the advantageous use of the *T. catappa* kernel oils from purple (CR) and yellow (CA) varieties for agro-industrial applications.

3.2.3. Triacylglycerol composition

The composition of triacylglycerides (TAGs) in CR and CA oils is displayed in Table 4. Both oils exhibited a quite similar proportion of triacylglycerols. The most frequently estimated triacylglycerols in CR were PLO, POO, POP, OLO, PLP, OLL and OOO, which represent 74.73% of the total. On the other hand, the predominant triacylglycerols in CA oil were almost the same, but slightly different with PLO, POO, POP, OLO, PLP, OLL and PLL representing 74.74% of the total. It was observed that the composition of TAGs was mainly composed of unsaturated acylglycerols, SU_2 and U_3, as can be seen in Table 4, which should be expected considering that the oleic and linoleic acids were most frequently in its FAs profile. Triglycerides are an important group of lipid sources for human nutrition. The TAG composition of CR and CA oils presented the predominance in ECN52 followed by ECN54, which can be linked to a large amount of long-chain triglycerides and, therefore, their inclusion in human intake can be helpful for preventing cardiovascular diseases.

Table 4. Estimated percentage of triacylglycerol composition of *Terminalia catappa* kernel oil from purple (CR) and yellow (CA) varieties.

ECN	**Triacylglycerol**	***Terminalia catappa* oil % (normalized)**	
		CR	**CA**
C48:0	PPP	3.13	3.87
C50:0	SPP	1.75	1.98
C50:1	POP	11.80	11.77
C50:2	PLP	6.94	9.06
C52:1	SOP	4.41	4.02
C52:2	SLP	2.59	3.09
C52:2	POO	14.85	11.95
C52:3	PLO	17.47	18.39
C52:4	PLL	5.14	7.08
C54:2	SOO	2.77	2.04
C54:3	SLO	3.26	1.14
C54:3	OOO	6.22	4.04
C54:4	SLL	0.96	1.21
C54:4	OLO	10.98	9.34
C54:5	OLL	6.46	7.19
C54:6	LLL	1.27	1.84
Triacylglycerol classes		**%**	
	S_3	4.88	5.85
	S_2U	25.74	27.94
	SU_2	44.45	41.81
	U_3	24.93	22.41

ECN: equivalent carbon number. P - Palmitic acid, S - Stearic acid, O - Oleic acid, L - Linoleic acid.

S = saturated acylglycerol and U = unsaturated acylglycerol. Data represent the calculated results from mean values (n = 3) of FA fractions, according to the software PrOleos®.

3.2.4. Analyses of bioactive compounds and antioxidant capacity

The bioactive compound analyses for anthocyanin, ascorbic acid and total polyphenol contents, and the antioxidant activity (TEAC assay), in the *Terminalia catappa* L. kernel oils from purple and yellow varieties are presented in Table 5. The analyses were used to investigate the presence of these bioactive compounds, and their potential antioxidant action as preserving agents in CR and CA oils. It was observed that the CR oil displayed higher values for bioactive compounds and antioxidant activity than CA oils ($p < 0.05$), which can be related to the difference in its variety.

In another work, the antioxidant activity of oils from *T. cattapa* was evaluated by the DPPH assay and its EC50 was found to be close to 7 $mg \cdot mL^{-1}$, indicating a potential antioxidant action (Ladele *et al.*, 2016). Castelo-Branco, Torres (2012) investigated the antioxidant activity by TEAC assay of conventional oilseeds, such as soyabean, maize, sunflower, and canola, and found values close to 7.1, ~4.5, ~4.3 and ~5.3 mmol of Trolox $eq \cdot Kg^{-1}$ of oil, respectively.

A precise comparison of data from bioactive compounds and antioxidant activity in *T. catappa* oil was

Table 5. Bioactive substances and antioxidant capacity of *Terminalia catappa* kernel oils from purple (CR) and yellow (CA) varieties.

Assays	**Samples**	
	CR	**CA**
Anthocyanin content ($mg \cdot 100\ g^{-1}$)	18.3 ± 1.5^a	2.55 ± 1.03^b
Ascorbic acid content ($mg \cdot 100\ g^{-1}$)	68.48 ± 2.02^a	38.7 ± 1.5^b
Total polyphenols content ($mg \cdot GAE\ g^{-1}$)	152.3 ± 2.4^a	127.3 ± 3.0^b
Antioxidant activity ($\mu g \cdot TE\ g^{-1}$)	38.6 ± 2.2^a	31.1 ± 1.6^b

Data represent the mean ± standard deviation of triplicate analyses (n = 3). Different superscript lowercase letters in the same line represent significant differences between samples ($p < 0.05$) at 95% confidence interval according to Tukey's test.

difficulted because of the scarce information available. Furthermore, different results found in the literature can be related to differences in protocol, sample preparation, solvents used, variations among species, harvest season, environmental conditions, and others.

4. CONCLUSIONS

The solvent extraction of *Terminalia catappa* L. kernel oils from purple (CR) and yellow (CA) varieties showed a good yield, above 54% lipids. These unconventional oils presented high-quality physical-chemical parameters, mainly observed by low levels of acidity and peroxides. Both oils, purple and yellow varieties, exhibited the predominance of unsaturated fatty acids (UFAs), with almost 63% oleic and 26% linoleic acids, which influenced its nutritional quality index values. These oils presented higher values for polyunsaturated and saturated acids ratio, which is relevant to human diets. The atherogenicity and thrombogenicity indices were higher in the *T. catappa* oils when compared to other tropical oilseeds. The calculated oxidation capacity values for both oils were lower than other non-conventional oil sources, even with a high proportion of PUFAs. The composition of triacylglycerols was mainly composed of unsaturated acylglycerols, which may be helpful for preventing cardiovascular diseases with their inclusion in human intake or in a potential use in formulated products with improvements in their nutritional profiles. Besides the nutritional quality properties, *T. catappa* oils from both varieties exhibited significative contents in anthocyanin, ascorbic acid and total polyphenol contents, and good antioxidant activity as determined by the TEAC assay. Thus, the presented results confirm the importance of *T. catappa* as a lipid source for human intake and to be used in the development of food products.

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Color of extra virgin olive oils enriched with carotenoids from microalgae: influence of ultraviolet exposure and heating

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SUMMARY: A carotenoid-rich extract containing 2.5 mg/mL of lutein and 3.3 mg/mL of β-carotene from the microalga *Scenedesmus almeriensis* was added to ten extra virgin olive oils from four Spanish cultivars with differing degrees of ripeness, obtaining carotenoid enriched oils with lutein and β-carotene concentrations of 0.082 and 0.11 mg/mL, respectively. Extra virgin olive oils enriched with carotenoids from microalgae were studied by analyzing the effect on color of three different treatments: ultraviolet exposure, microwave heating and immersion bath heating. The methodology was designed to simulate, in controlled laboratory conditions, the effects of household treatments. Spectrophotometric color measurements were then performed to monitor color changes in the enriched and non-enriched extra virgin olive oil samples. Enriched oils are much more chromatic, darker and redder than natural oils. After 55 days UV irradiation, 40 min microwave heating, and 72 hours thermostatic heating, the average color differences for natural/enriched extra virgin olive oils were 98/117, 15/9 and 57/28 CIELAB units, respectively. In general, increasing temperature and ultraviolet exposure produced higher CIELAB color differences in the non-enriched samples. The addition of microalga extracts to extra virgin olive oils was found to induce some color stability and may constitute a future way of increasing the daily intake of beneficial bioactive compounds such as carotenoids.

KEYWORDS: *Extra virgin olive oil;* Scenedesmus almeriensis; *CIELAB system; Carotenoids.*

RESUMEN: ***Color de aceites de oliva virgen extra enriquecidos con carotenoides procedentes de microalgas: influencia de la exposición a la radiación ultravioleta y al calentamiento.*** Añadimos un extracto rico en carotenoides, que contiene 2,5 mg/mL de luteína y 3,3 mg/mL de β-caroteno, procedente de la microalga *Scenedesmus almeriensis*, a diez aceites de oliva virgen extra de cuatro variedades con diferentes grados de maduración, obteniéndose aceites enriquecidos en carotenoides con concentraciones de luteína y β-caroteno de 0,082 y 0,11 mg/mL respectivamente. Se han estudiado aceites de oliva virgen extra enriquecidos con carotenoides procedentes de microalgas, estudiando el efecto producido sobre el color de los mismos como consecuencia de irradiación ultravioleta, calentamiento en microondas y en baño termostático, reproduciendo en el laboratorio los efectos de los tratamientos domésticos. Se ha determinado el color para monitorizar los cambios de las muestras control y enriquecidas de los diferentes aceites. Los aceites enriquecidos son mucho más cromáticos, oscuros y rojizos que los naturales. Tras 55 días de irradiación UV, 40 minutos de calentamiento por microondas y 72 horas de calentamiento termostático, las diferencias medias de color para los aceites de oliva virgen extra naturales/enriquecidos fueron de 98/117, 15/9 y 57/28 unidades CIELAB, respectivamente. En término generales, el incremento en la temperatura y la exposición a la radiación ultravioleta produce diferencias de color más grandes en las muestras no enriquecidas. El enriquecimiento de los aceites virgen extra con extractos procedentes de microalgas, induce estabilidad en el color y puede constituir una vía para incrementar la ingesta diaria de compuestos bioactivos beneficiosos como son los carotenoides.

PALABRAS CLAVE: *Aceite de oliva virgen extra; Carotenoides;* Scenedesmus almeriensis*; Sistema CIELAB.*

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1. INTRODUCTION

In contrast to the reduced consumption of fruits and vegetables, the production and consumption of premium olive oil has been steadily increasing worldwide in recent years as indicated by the International Olive Oil Council (IOC, 2013; IOC, 2018). Extra virgin olive oil (EVOO) is an important component of the Mediterranean diet, and is obtained exclusively by mechanical methods which conserve nutritional and organoleptic properties *(Garcia-Oliveira et al.*, 2021; Peñalvo *et al.*, 2016). In addition to its unique sensorial attributes, EVOO has been credited with multiple health benefits, such as preventing coronary and degenerative diseases (Borges *et al.*, 2017; Garcia-Oliveira *et al.*, 2021). Unfortunately, olive oil has only a few months' shelf-life and is easily prone to oxidation and changes in quality. The addition of antioxidants to olive oil, however, may prolong its shelf-life, improve acceptability and enhance its nutritional value (Limón *et al.*, 2015; Murillo-Cruz *et al.*, 2021a; Murillo-Cruz *et al.*, 2021b).

There is currently a growing interest in the relationships between food and health, well-being, and quality of life. Fruit and vegetable consumption is a pressing concern, and it is known that insufficient intake of these foods causes around 14% of gastrointestinal cancer deaths, about 11% of ischemic heart disease deaths and about 9% of stroke deaths worldwide (World Health Organization, 2014). Consequently, the decrease in the consumption of fruits and vegetables implies that several bioactive food components are not being ingested in sufficient amounts, with negative repercussions on human health. Carotenoids are a group of pigments that are widely extended in nature, but, unfortunately, the amounts of carotenoids found in human tissues are almost exclusively of dietary origin, mainly from fruits, vegetables and from other products like algae (Rodríguez-Concepcion *et al.*, 2018). Carotenoids can be classified into two categories on the basis of their functional groups: xanthophylls (containing oxygen); and carotenes (without oxygen). Xanthophylls include lutein and zeaxanthin, while carotenes include α-carotene, β-carotene and lycopene. Regulation aspects about carotenoids have been developed by the European Commission through the European Food Safety Agency (EFSA, 2012). In addition, there is much evidence that carotenoids (and their derived products and metabolites) may be involved in health-promoting biological activity in humans (Meléndez-Martínez, 2019; Meléndez-Martínez *et al.*, 2020; Van Hoang *et al.*, 2018).

Foods fortified with β-carotene have been used to prevent vitamin A deficiency in pre-school children and pregnant women, although this particular carotenoid is liposoluble and weakly dispersed in foods (Turner *et al.*, 2013). In addition, β-carotene has antioxidant properties that suppress oxygen or free radicals, reducing the risk of chronic diseases, inflammation and cancer (Syamila *et al.*, 2019). It can be found in vegetables such as carrots, pumpkins and sweet potatoes and colors them orange, as well as in purple carrots and purple tomatoes, which also contain anthocyanins (Limón *et al.*, 2015). Lutein, on the other hand, is a xanthophyll compound with an antioxidant activity recommended for preventing some types of cancer and cardiovascular diseases. It also has a protective effect with regard to age-related eye diseases and contributes to maintaining cognition (Ozawa *et al.*, 2012). Lutein is commercially produced from marigold (*Tagetes erecta*), although the lutein content of marigold flowers is very low, whereas microalgae such as Chlorella and *Scenedesmus almeriensis* can contain large amounts of this compound (Limón *et al.*, 2015). Specifically, *Scenedesmus almeriensis* contains up to 1.5% d.w. of lutein, together with other carotenoids such as β-carotene, and can be efficiently produced in closed tubular photobioreactors in continuous mode and on a large scale. Currently, the use of microalgae as a source of carotenoids is a research line in continuous expansion (Meléndez-Martínez *et al.*, 2020). As for the bioaccessibility of carotenoids, several studies have examined potential dietary and host-related factors, and have concluded that dietary lipids can significantly enhance carotenoid absorption by fostering micellization (Iddir *et al.*, 2019).

Color is one of the most important properties in foods for its relationship to chemical and nutritional composition as well as its strong influence on consumer emotions and preferences. Various scales have been proposed for specifying the color of EVOOs in connection with the standard colorimetric system of the International Commission on Illumination (CIE) (Gutiérrez and Gutiérrez, 1986; Moyano *et al.*, 1999; Salmerón *et al.*, 2012; Yu *et al.*, 2020). Attention has also been given to the color emotions aroused by natural antioxidant-enriched virgin olive oils in both the Spanish and Japanese populations (Montoya *et al.*, 2018; Limón, 2017).

According to the definition of the European Union Commission, an extra virgin olive oil must be extracted "only from olives with a superior quality, cannot undergo any treatment other than washing the fruits, and decanting, centrifuging and filtering the extracted olive oil". Therefore, from a legal point of view, extra virgin olive oil (EVOO) enriched in carotenoids cannot be called "extra virgin olive oil", and should be named as olive oil that has been processed with vegetables, algae, etc (Issaoui *et al*., 2016). For instance, currently it would also be possible to use the designation "olive oil enriched in carotenoids". Currently, efforts are being made for the approval and registration of *Scenedesmus almeriensis* as a component of functional foods. In the future this may be an additional value to the well-known qualities of natural EVOOs.

In summary, we feel that EVOOs with carotenoid-rich extracts from the microalga *Scenedesmus almeriensis* (henceforth referred to as "enriched EVOOs", as opposed to "natural EVOOs") could be a good convoy for enhancing the daily intake of carotenoids. However, the color of natural and enriched EVOOs may vary, and this may influence consumer preferences. The main objective of this study was to evaluate whether the enrichment of olive oil with carotenoids extracted from microalgae produces changes in its color and protects its color during UV exposure and heating.

2. MATERIALS AND METHODS

2.1. Production of *Scenedesmus almeriensis* microalgae

Scenedesmus almeriensis microalgae (CCAP 276/24) were produced at the Chemical Engineering Department of the University of Almería (Spain). The culture medium used was prepared in freshwater using fertilizers ($NaNO_3$, KH_2PO_4 and micronutrients). The cultures were performed at pH=8.0 by on-demand injection of CO_2 and below 30 ºC, by passing thermostated water through a heat exchanger located inside the reactor. The biomass was harvested daily by centrifugation, then lyophilized and stored at -18 ºC (Acién *et al*., 2012). This lyophilized biomass was used as raw material.

2.2. Extraction of carotenoids

The carotenoid extract was obtained by following a specific methodology for recovering these compounds from the lyophilized *Scenedesmus almeriensis* biomass. The first step was a cell disruption process with alumina in a 1:1 w/w proportion, using a mill with beads of 28 mm in diameter and a rotation speed of 120 rpm for 5 min to remove fatty acid soaps. The second step was saponification, using an aqueous solution with KOH and biomass concentrations of 40 g/L and 100 g/L, respectively, for 5 min. Finally, extraction was performed using a 1:1 ratio of hexane to sample volume. By this 3-step method it was possible to recover more than 90% of the carotenoids contained in the processed biomass (Cerón *et al*., 2008). At each step a volume of ethanol equal to 1% of the total volume was added to avoid emulsion. Hexane was removed from the extract by high vacuum distillation. The carotenoid extract produced was a concentrated oily solution containing lutein and β-carotene (2.5 mg of lutein/mL and 3.3 mg of β-carotene/mL, respectively). Regarding carotenoid quantification in the extract, it was performed using a methodology based on HPLC chromatography. A C18 reverse reversed-phase chromatographic column was used (250 mm x 2.5 mm) (Phenomenex Luna C18 column). The analysis was carried out at 23 ºC with a linear gradient for 30 minutes. The mobile phase was water-methanol (80%) with 0.05% triethylamine and 20% ethyl acetate (with 0.05% triethylamine) with a flow rate of 1 mL/min. From the chromatogram obtained, analyzing the peaks corresponding to the different carotenoids, the amounts of these compounds were obtained (Limón, 2017). Using this methodology, it was found that the composition of the carotenoids extract was β-carotene (56%), lutein (43%) and other undefined carotenoids (1%).

2.3. Addition of the carotenoids extract

Figure 1 shows CIELAB color differences produced in a Picual October EVOO during an enrichment process in which different lutein concentrations were achieved by adding an increasing volume of carotenoid extract (2.5 mg of lutein/mL and 3.3 mg of β-carotene/mL). As expected, parallel to this increase in carotenoid concentration, the color differences also increased. With the first addition of carotenoid extract (0.1 mL) a lutein concentration of 0.02 mg/mL was achieved, producing a significant color change from that of the original oil ($\Delta E^*_{ab.10}$ =14.7). Successive additions of extract produced an

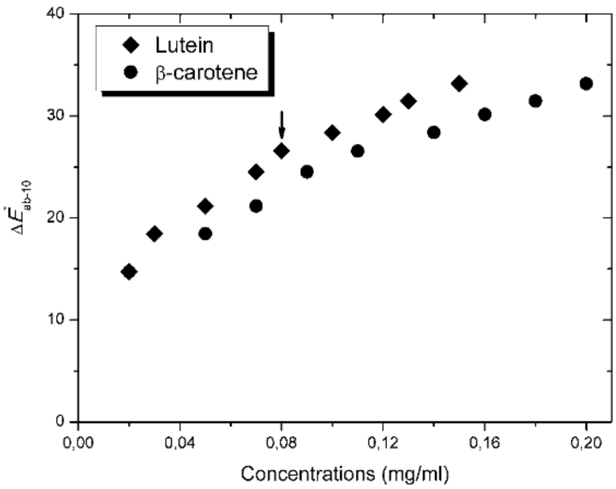

Figure 1. CIELAB color differences for a Picual EVOO enriched with increasing quantities of carotenoid extract (2.5 mg of lutein/mL and 3.3 mg b-carotene/mL). The x-axis shows concentration values reached in oil by increasing the amount of carotenoid extract added. The arrow indicates the concentration of the extract used in experiments performed in the current paper.

almost linear growth of the CIELAB color difference (see Figure 1). It is clear that the amount of carotenoid extract added increased color changes in natural EVOOs.

With reference to lutein, it is important to note that the concentration of this carotenoid chosen for this study was 0.082 mg/mL of oil because average olive oil intake (person/day) was estimated at 36 mL/day (data for Spain from IOC, 2018), and thus our enriched EVOOs should provide each consumer with 3 mg of lutein/day. With regard to β-carotene, 4 mg would be ingested from our enriched EVOOS (0.11 mg β-carotene/mL oil). These values should be 50 and 27% of the recommended intake, respectively, which is 6 and 15 mg/day for lutein and β-carotene, respectively (Meléndez-Martínez, 2019; EFSA 2012). On the other hand, it is necessary to point out that there are also carotenoids which are naturally present in olive oil (5-100 mg/kg) and they can also be incorporated with other foods through the diet (Cichelli and Pertesana, 2004). Obviously, it should be possible to use more concentrated EVOOs to increment carotenoid intake, but it should be remembered that the cost of the carotenoid extract is very high. Therefore, using enriched EVOOs with a concentration of 0.192 mg carotenoids/mL of oil (0.082 mg lutein/mL + 0.11 mg β-carotene/mL) would be acceptable for providing an appropriate dose at a reasonable price. Regarding lutein, this concentration is shown in Figure 1 by an arrow (0.082 mg lutein/mL of oil).

Carotenoids are present in olive oils in a range of 5-100 mg/kg and their lutein content usually ranges from 2-8 mg/kg (Cichelli and Pertesana, 2004). Thus, our enriched EVOOs, which contain 0.082 mg lutein/mL of oil, are 10-40 times more concentrated in this kind of carotenoid than natural EVOOs. Concerning β-carotene, this compound content ranges from 1-4 mg/kg, with our enriched oils (0.11 mg β-carotene/mL of oil) being 28-110 times more concentrated in this carotenoid (Limón *et al*., 2015). Here the values expressed in mg/mL were obtained assuming a density of 0.918 mg/mL for EVOOs. Because of the minor amount of carotenoids contained in the control (non-enriched) oils, the total amount of carotenoids in the enriched oils may be considered as the one from the added extract.

2.4. Extra-virgin olive oils

Four cultivars of representative EVOOs from northeastern Jaén (a province in Andalusia, Spain) were selected for the present study: Picual, Arbequina, Royal, and Frantoio. The EVOO samples were provided by the Castillo de Canena Olive Juice Company, from the same crop season (2018/2019) but harvested in different months (October-January), which implies olives with different ripening degrees. All EVOOs were initially filtrated in the presence of anhydrous sodium sulphate. After filtration, samples were homogenized and stored at 4 °C in the dark, using amber glass bottles without head space until analysis. For each type of oil, two 50-mL samples were constituted: natural EVOO (without the carotenoid extract); and enriched EVOO (with 0.082 mg of lutein per mL of oil and 0.11 mg β-carotene per mL of oil).

Concerning the quantification of the carotenoid content in the oil after the enrichment process, it is important to highlight that the extract added to the oils dissolved perfectly. We can assume that the concentration of carotenoids in the oils corresponds to the total amount of carotenoids from the extract which was contained in the volume of extract used for enrichment. The corresponding extract volume was added at room temperature to the oil sample and manually stirred until it was completely dissolved, waiting overnight to ensure adequate stabilization of the enriched samples.

2.5. Color measurement

The colors of both the natural and enriched EVOOs were measured with a CM-5 Konica Minolta spectrophotometer using appropriate 14-mL cuvettes with 10-mm path lengths. In this way, the CIELAB coordinates (L^*_{10}, a^*_{10} and b^*_{10}) of the samples were measured by the spectrophotometer, after proper calibration, assuming the CIE D65 illuminant and CIE 1964 standard colorimetric observer. L^*_{10} is a measure of lightness, usually ranging from 0 to 100 (corresponding to black and white, respectively). The a^*_{10} coordinate ranges from negative (green) to positive (red) values, while the b^*_{10} coordinate also ranges from negative (blue) to positive (yellow) values. Color features were obtained as the average of three measurements performed on each sample. In the current paper CIELAB cylindrical coordinates (lightness L^*_{10}, chroma $C^*_{ab,10}$, and hue-angle $h_{ab,10}$) computed by conventional equations from the International Commission of Illumination (CIE, 2018) were used, as these are more intuitive than CIELAB Cartesian coordinates (L^*_{10}, a^*_{10}, b^*_{10}). The intensity of color is related to $C^*_{ab,10}$, which has a null value for achromatic colors (i.e. white, black, and gray). The color attribute denominated as hue is related to hue-angle, $h_{ab,10}$, which has values of 0, 90, 180, and 270° for red, yellow, green, and blue samples, respectively.

Color change was quantified using the CIELAB color-difference ($\Delta E^*_{ab,10}$), defined as follows:

$$\Delta E^*_{ab,10} = [(\Delta L^*_{10})^2 + (\Delta a^*_{10})^2 + (\Delta b^*_{10})^2]^{\frac{1}{2}} = \left[(\Delta L^*_{10})^2 + \left(\Delta C^*_{ab,10}\right)^2 + \left(\Delta H^*_{ab,10}\right)^2\right]^{\frac{1}{2}}$$

The total CIELAB color differences $\Delta E^*_{ab,10}$ can be split into lightness, chroma, and hue differences, with the corresponding percentages defined as follows:

$$\%\Delta L^*_{10} = 100\left(\Delta L^*_{10}/\Delta E^*_{ab,10}\right)^2$$

$$\%\Delta C^*_{ab,10} = 100\left(\Delta C^*_{ab,10}/\Delta E^*_{ab,10}\right)^2$$

$$\%\Delta H^*_{ab,10} = 100\left(\Delta H^*_{ab,10}/\Delta E^*_{ab,10}\right)^2$$

$$\%\Delta L^*_{10} + \%\Delta C^*_{ab,10} + \%\Delta H^*_{ab,10} = 100$$

2.6. Ultraviolet exposure

An 18-W source provided by a portable Verivide CAC 60 color cabinet was used. The spectral irradiance of this UV light has a main peak at 367 nm with full width at half maximum of 16.9 nm. Individual EVOOs were placed in special 14-mL color buckets positioned in the center of the cabinet floor, where the UV source provided average irradiance and illuminance of 0.311 W/m^2 and 4.01 lx, respectively.

All experimental color measurements were taken at room temperature, with time 0 weeks corresponding to the unexposed EVOOs. For this photostability study, times of 1, 2, 4, 6, and 8 weeks (a total of 55 days) were selected for color measurements during exposure of the EVOOs to UV. These times were chosen by visual inspection of color change in the samples, noting that after 55 days of UV exposure all the EVOOs became nearly achromatic.

2.7. Heating by microwave and thermostatic immersion bath

For the study of microwave heating, the color of the samples was measured every 5 minutes over a total time of 40 min, simulating conventional times in household microwave conditions. After 40 min, the colors of all the EVOOs were almost achromatic. For each EVOO a sample of 20 mL was heated in appropriate glass test tubes using a domestic microwave oven (LG) at maximum potency (700 W). The temperature of the oil samples inside the tubes was measured with an appropriate probe (Crison, 638 Pt thermometer) showing values ranging from 70 ºC (1 min) to 190 ºC (40 min), with a non-linear dependence on the heating time. The color of unheated (0 min) EVOOs was used as reference.

For the thermostatic immersion bath study, color measurements were performed on the oil samples at a constant temperature of 120 ºC and for a period of 70 hours, as after this time the color of all EVOOs became nearly stable and close to neutral. For each oil tested, a sample of 20 mL was immersed in a thermostatic immersion bath (Julabo, SE-Z) using glass test tubes and a special synthetic thermostatic liquid (Thermal H5S liquid) operating at 120 ºC. Similar to microwave heating, the temperature of the oil samples inside the tubes (120 ºC) was measured with an appropriate probe. This temperature was used as it is typically applied in stability studies involving fatty food matrices (Rancimat methodology).

3. RESULTS AND DISCUSSION

3.1. Initial color evaluations

It is well known that consumers judge foods and beverages according to external appearance, and color plays a key role in their final preference (Cavallo and Piqueras-Fiszman, 2017). Consequently, to characterize the natural and enriched EVOOs we studied, their colors were measured. The results are shown in Table 1. From the Wilcoxon nonparametric test at the 0.05 level, it was determined that the differences in average values for L^*_{10}, $C^*_{ab,10}$, and $h_{ab,10}$ for the natural and enriched EVOOs in Table 1 are statistically significant.

Figure 2 shows color shifts from natural to enriched EVOOs in two CIELAB planes, $C^*_{ab.10}$-L^*_{10} and $h_{ab.10}$-L^*_{10}. As can be seen in this figure, the addition of carotenoid extract always increased chroma (with the exception of sample 9) and decreased both lightness and hue-angle in natural EVOOs. Enriched EVOOs were therefore more chromatic, darker, and redder than natural EVOOs, which agrees with the visual perception of our samples by untrained observers with normal color vision. The highest shift produced in the samples was in chroma (Figure 2, top), as expected from the high color intensity of the added carotenoid extract. Specifically, the greatest variation in chroma was found for oil sample number 3 with initial and final chroma values of 75.9 and 134.7, respectively, and the lowest chroma variation was found for oil sample number 1 with initial and final chroma values of 121.8 and 124.2, respectively.

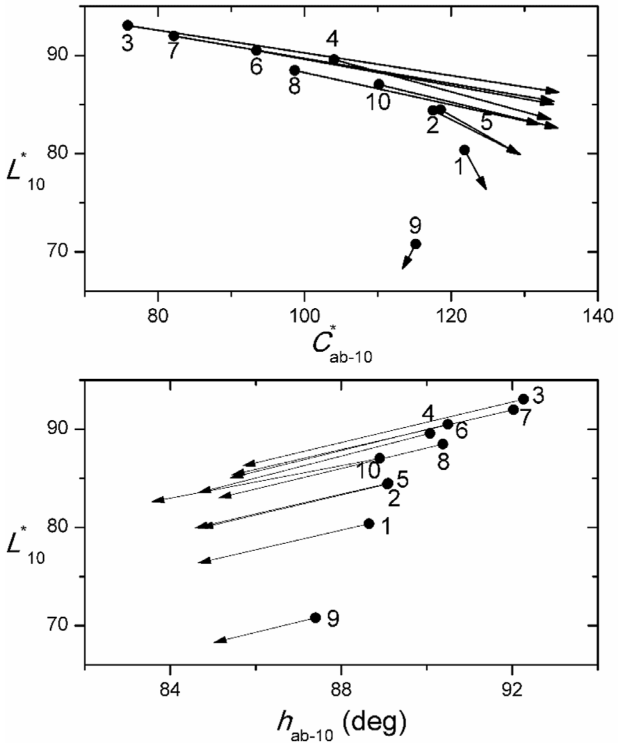

FIGURE 2. Color shifts from natural (circles) to enriched (arrowheads) EVOOs in the CIELAB planes $C^*_{ab.10}$-L^*_{10} (up) and $h_{ab.10}$-L^*_{10} (down). The numbers of the samples correspond to those indicated in TABLE 1

Figure 3 shows the total color differences and their three components (lightness, chroma and hue) in CIELAB space, as a consequence of adding the carotenoid extract to the 10 natural EVOOs. It can

TABLE 1. CIELAB cylindrical coordinates (lightness L^*_{10}, chroma $C^*_{ab,10}$, and hue-angle $h_{ab,10}$) of natural and enriched EVOOs. The enriched EVOOs were produced by adding carotenoid extract from the microalga *Scenedesmus almeriensis* (0.082 mg lutein/mL of oil, 0.11 mg β-carotene/mL of oil). Results are expressed as mean ± standard deviation of three sample replicates.

Sample Number	Variety	Harvest Month	Natural EVOOs			Enriched EVOOs		
			L^*_{10}	$C^*_{ab.10}$	$h_{ab.10}$ (deg)	L^*_{10}	$C^*_{ab.10}$	$h_{ab.10}$ (deg)
1	Picual	October	80.4±1.8	121.8±1.6	88.6±1.5	76.4±1.6	124.2±1.4	84.7±0.7
2	Picual	November	84.4±1.1	117.5±0.3	89.1±0.9	79.9±1.0	129.4±1.2	84.7±0.5
3	Picual	December	93.0±0.3	75.9±1.5	92.3±0.2	86.3±0.5	134.7±0.3	85.7±0.2
4	Picual	January	89.6±0.2	104.0±1.1	90.1±0.1	83.5±1.0	133.5±0.6	84.7±0.8
5	Arbequina	October	84.5±0.8	118.6±0.6	89.1±0.7	79.9±0.8	129.3±0.8	84.6±0.3
6	Arbequina	November	90.5±0.4	93.4±1.7	90.5±0.2	85.1±0.4	133.9±0.3	85.4±0.2
7	Arbequina	December	92.0±0.3	82.2±1.5	92.0±0.4	85.3±1.3	134.0±0.4	85.4±0.7
8	Arbequina	January	88.5±0.2	98.7±1.6	90.4±0.2	83.0±0.5	132.1±0.2	85.1±0.4
9	Royal	October	70.8±1.6	115.2±1.4	87.4±1.5	68.3±1.5	113.4±1.5	85.0±1.3
10	Frantoio	October	87.0±0.5	110.2±1.5	88.9±0.2	82.6±1.9	134.5±0.6	83.6±2.0

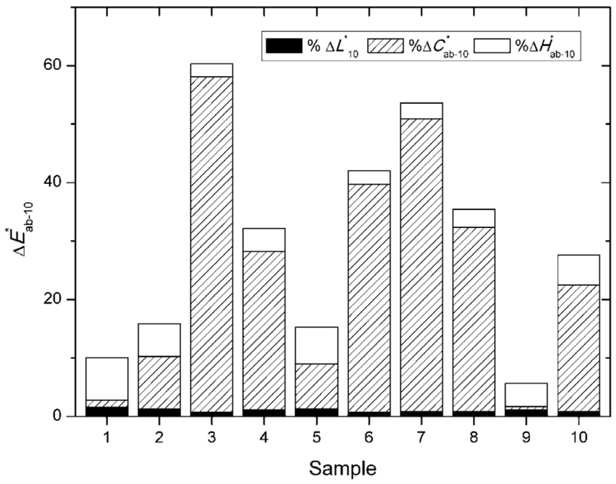

Figure 3. Total CIELAB color differences between natural and enriched EVOOs with their percentages of lightness, chroma, and hue differences. The numbers of the samples correspond to those indicated in Table 1.

be seen that color differences between natural and enriched EVOOs were quite high, ranging from 5.7 CIELAB units (sample number 9) to 60.4 CIELAB units (sample number 3), with an average of 29.8 CIELAB units. Differences in the chemical composition of the natural EVOOs tested may explain these discrepancies, since the carotenoid extract added was always the same. Similar values were found in a previous study with a similar extract but different olive oils (Limón *et al.*, 2015).

Average threshold color-difference values in visual experiments were below 1.6 CIELAB units (Melgosa *et al.*, 1992), and in a recent experiment it was reported that such values were even lower, in the range 0.55-1.10 CIELAB units (Huang *et al.*, 2015). Regarding foods, it has been reported that for red wines a 50% acceptance percentage resulted from a color difference of 2.8 CIELAB units, using a reference anchor-pair of wine samples with 4.0 CIELAB units (Martínez *et al.,* 2001). For orange juice, it has been stated that untrained assessors can easily distinguish color differences of 2.8 CIELAB units (Fernández-Vázquez *et al.*, 2013). As far as we know, magnitudes of just perceptible or acceptable color differences in EVOOs have not been reported in previous literature. However, we can assume that the minimum difference shown in Figure 3 (i.e. 5.7 CIELAB units) is above the human visual threshold, and we can conclude that any observer with normal color vision will easily perceive the color differences among the 10 natural and enriched oils considered in the current paper.

3.2. Color change during UV-light exposure

One of EVOO degradation variables that must be taken into account is exposure to electromagnetic radiation, which promotes changes during olive oil storage until use (Luna *et al.*, 2006). Natural and enriched EVOOs follow similar trends under UV irradiation: increase in lightness (L^*_{10}), increase in hue-angle ($h_{ab.10}$), and decrease in chroma ($C^*_{ab.10}$), the last of these presenting the greatest change (about 110 and 100 units for natural and enriched EVOOs, respectively) (Figure 4). It can also be noted that the chroma of enriched EVOOs was nearly constant during the first 15 days of irradiation. Total color change ($\Delta E^*_{ab.10}$) after 55 days of irradiation was also very high (97.7 and 116.7 CIELAB units for natural and enriched, respectively), and during the first 30 days of irradiation it was lower in enriched than in natural EVOOs. It can be noted as well that UV irradiation produces greater color variability in the 10 enriched EVOOs than in the 10 natural EVOOs (i.e. larger error bars in the plot in Figure 4D for $\Delta E^*_{ab.10}$ change). At the 0.05 level, the Wilcoxon rank test for paired samples indicated that the total CIELAB color differences produced by UV irradiation (Figure 4D) were statistically significant for natural and enriched EVOOs only for the irradiation times below 20 days.

3.3. Color change during microwave heating

Another oil degradation variable to consider in food is temperature, with microwave heating being a common methodology for studying oil stability (Malheiro *et al.*, 2013). As can be seen in Figure 5, microwave heating for 40 min had a very small influence on lightness (L^*_{10}) and hue-angle ($h_{ab.10}$), and slightly reduced chroma ($C^*_{ab.10}$) in a nearly linear way for both the enriched and natural oils. It is also observed that the change in chroma was smaller for enriched than for natural EVOOs, without any overlapping of error bars for the 10 tested samples. Specifically, for enriched oils the average chroma changed from 129.5 to 121.6 (i.e. 7.9 CIELAB units) while for natural oils the average chroma changed from 103.6 to 89.6 (i.e. 14.0 CIELAB units). For enriched and natural EVOOs the total color differences after 40 min were high (Figure 5D) in comparison with human-vision color-threshold values (Huang *et al.*, 2015), and always smaller for enriched than for natural EVOOs. Specifically, after 40 min the total color differences for the enriched and

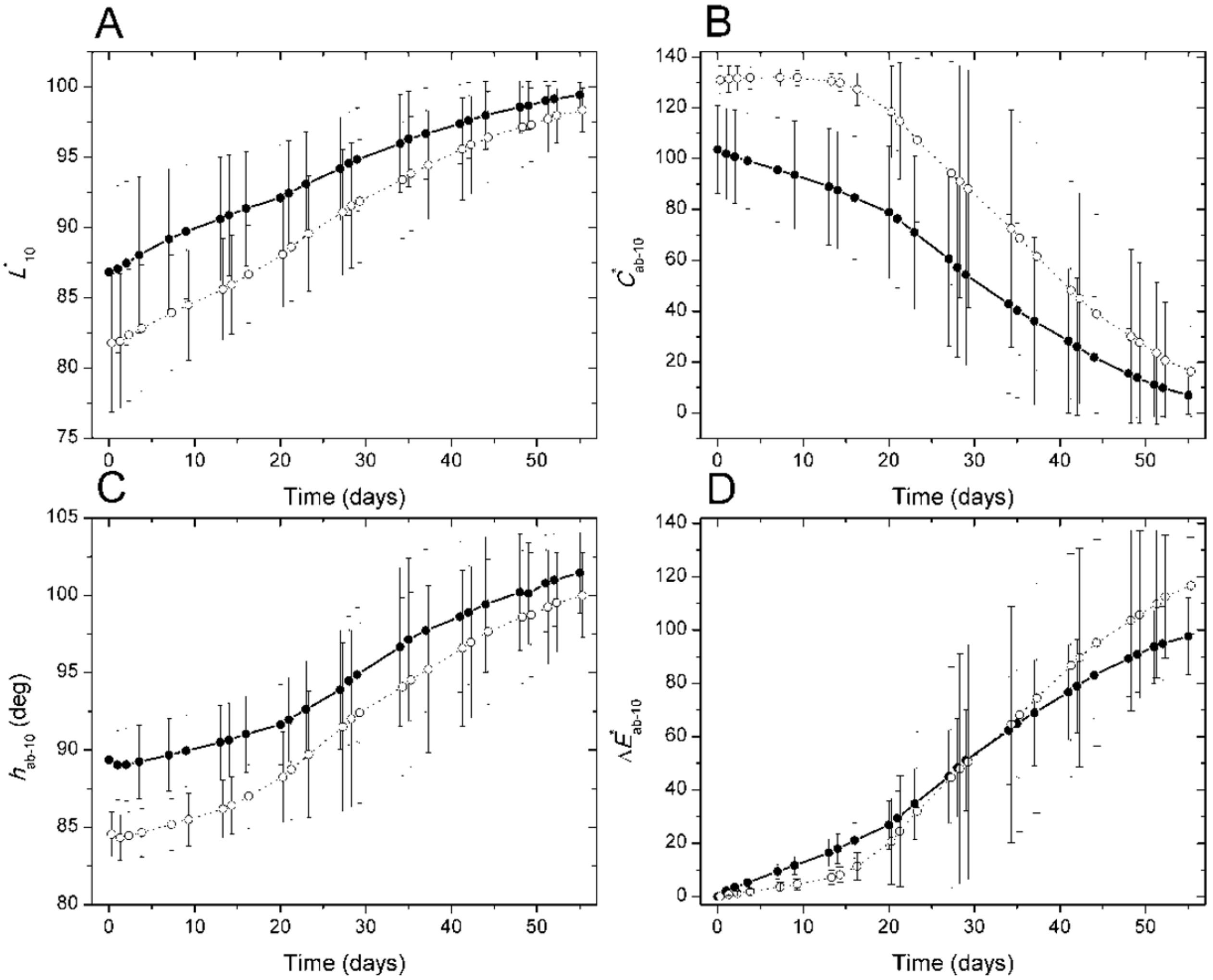

FIGURE 4. Color change as a consequence of UV exposure over 55 days for natural (black) and enriched (white) EVOOs. Error bars indicate standard deviations of 3 independent measurements of all EVOOs. Changes in the CIELAB color attributes of lightness L^*_{10} (A), chroma $C^*_{ab.10}$ (B), and hue-angle $h_{ab.10}$ (C), as well as total CIELAB color differences with respect to the oils without UV exposure $\Delta E^*_{ab.10}$) (D), are shown.

natural EVOOs were 8.7 and 14.5 CIELAB units, respectively. At the 0.05 level, the Wilcoxon rank test for paired samples indicated that the total CIELAB color differences produced by microwave heating (Figure 5D) were statistically significant for natural and enriched EVOOs at all measured times above 1 min. Total color differences after 40 min microwave (Figure 5D) heating were 6.7 and 13.4 times lower than those after 55-day UV exposure (Figure 4D) for natural and enriched EVOOs, respectively.

3.4. Color change during thermostatic immersion bath

The technique of immersion in a thermostatic bath is a practice which is quite common in conventional household food treatments (Nogueira-de-Almeida and de Castro, 2018). As can be seen in Figure 6, this heating method increased lightness and hue-angle in a nearly linear manner, and this is similar for both natural and enriched EVOOs. Chroma, however, decreased much more dramatically for natural than for enriched EVOOs (after 72 hours, chroma change in natural EVOOs was about 2.2 times higher than that of enriched EVOOs). Specifically, for enriched oil the average chroma value changed from 129.5 to 104.3; while for natural oils the average chroma change was from 104.0 to 47.8 CIELAB units. The heating process made EVOOs lighter, less reddish, and considerably less chromatic. In particular, after 72 hours the natural EVOOs came to resemble neutral transparent liquids. Overall, total CIELAB color differences (Fig-

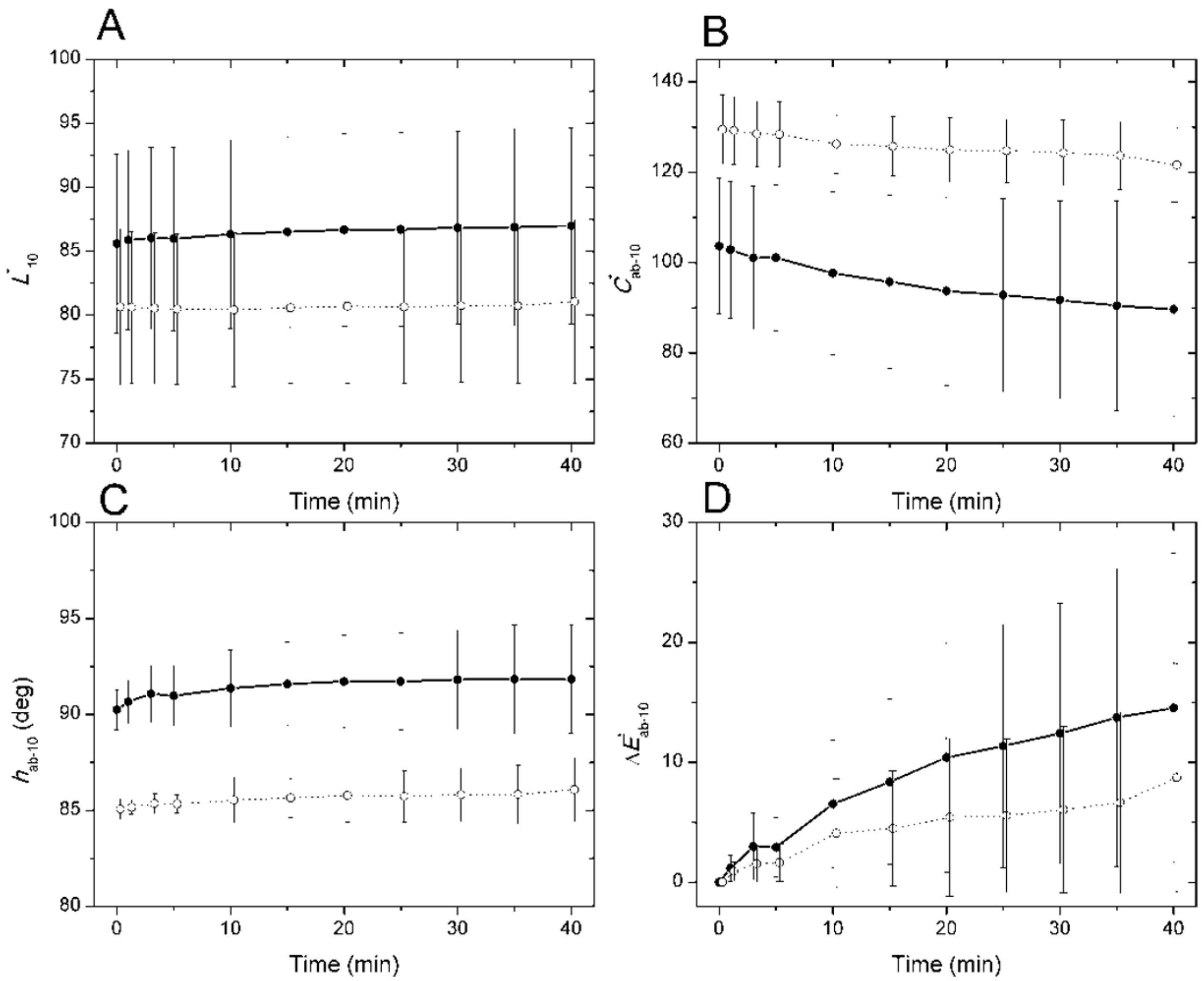

FIGURE 5. Color change as a consequence of microwave heating over 40 min for natural (black) and enriched (white) EVOOs. Error bars indicate standard deviations of 3 independent measurements of all EVOOs. Changes in the CIELAB color attributes of lightness L^*_{10} (A), chroma $C^*_{ab.10}$ (B), and hue-angle $h_{ab.10}$ (C), as well as total CIELAB color differences $\Delta E^*_{ab.10}$ (D), are shown.

ure 6-D) produced by thermostatic bath heating were 2.0 times lower for enriched than for natural EVOOs. At the 0.05 level, the Wilcoxon rank test for paired samples indicated that the total CIELAB color differences produced by thermostatic immersion bath heating (Figure 6D) were statistically significant for natural and enriched EVOOs at all measured times. Specifically, after 70 h the average total color difference for the enriched and natural EVOOs were 27.9 and 57.1 CIELAB units, respectively.

4. CONCLUSIONS

Spectrophotometric color measurements were made for a set of 10 natural and 10 enriched EVOOs, the latter being obtained by the addition of a carotenoid extract (0.082 mg lutein per mL of oil and 0.11 mg of β-carotene per mL of oil) from the microalga *Scenedesmus almeriensis*. Enriched EVOOs were much more chromatic, darker, and redder than natural EVOOs, and the average color difference between the two types of EVOOs was high (29.8 CIELAB units). This may affect consumer preferences for EVOOs, although this point was not tested here. UV irradiation made natural and enriched EVOOs lighter, less chromatic, and less reddish. The average color difference after 55 days was very high (97.7 and 116.7 CIELAB units for natural and enriched EVOOs, respectively). However, during the first 20 days of UV irradiation, the color change was statistically significantly lower in enriched than in natural EVOOs. Micro-

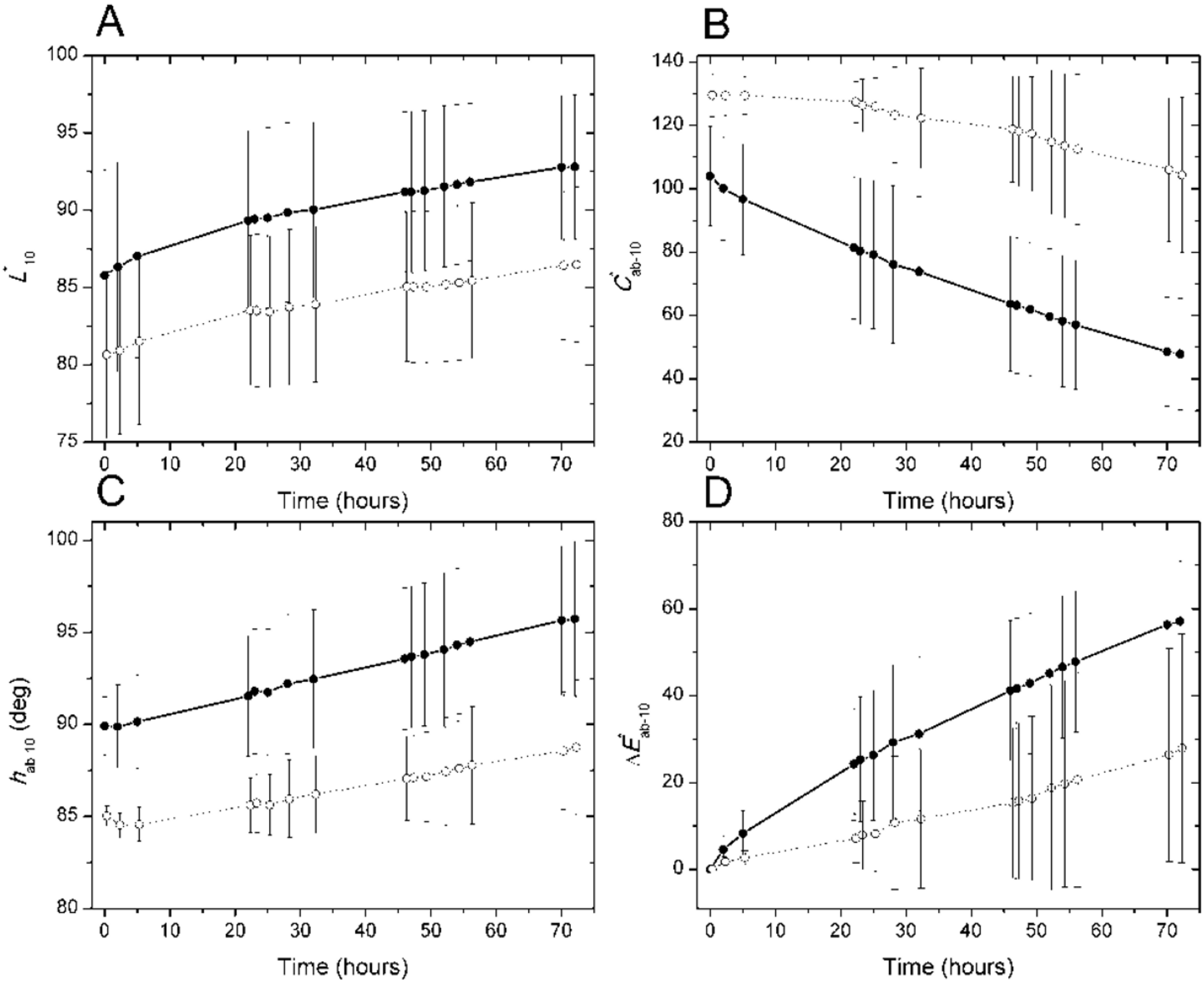

FIGURE 6. Color change as a consequence of immersion bath heating over 72 h for natural (black) and enriched (white) EVOOs. Error bars indicate standard deviations of 3 independent measurements of all EVOOs. Changes in the CIELAB color attributes of lightness L^*_{10} (A), chroma $C^*_{ab.10}$ (B), and hue-angle $h_{ab.10}$ (C), as well as total CIELAB color differences $\Delta E^*_{ab.10}$ (D), are shown.

wave heating for 40 min did not change lightness or hue-angle, but did decrease the chroma of natural and enriched EVOOs. It could be stated that the chroma difference for natural oil was higher than that of enriched oil after 40 min of microwave heating. After 40 min of microwave heating the total color differences for the enriched and natural EVOOs were 8.7 and 14.5 CIELAB units, respectively. Thermostatic bath heating for 72 hours made EVOOs lighter, less reddish, and considerably less chromatic, and the total CIELAB color difference was higher for natural than for enriched EVOOs (57.1 and 27.9 CIELAB units, respectively). It can be concluded that color change produced by increasing temperature was lower in enriched than in natural EVOOs. These colorimetric analyses may be relevant with regard to the potential future use of enriched EVOOs as functional foods, increasing the daily intake of beneficial bioactive compounds such as carotenoids. In addition, these enriched oils could have a longer half-life, since carotenoids are antioxidants.

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The effect of replacing red palm stearin with red palm olein in baked potato cookies

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SUMMARY: Potato cookies were formulated by replacing red palm stearin (RPS) by red palm olein (RPOL) at 0, 17 and 35%, and then baked at 160, 180 and 200 °C for 10, 12 and 15 min. The sensory analysis, using an orthogonal test, showed that a RPS-RPOL ratio of 65:35, baking temperature of 160 ºC, and baking time 12 min were the optimal conditions. Cookies made from 65% RPS + 35% RPOL composition exhibited 0.6 times less squalene, but 1.5 times more β-carotene, tocopherols and tocotrienols than the mixture of RPS and RPOL at 100:0. In addition, cookies with superior oxidative stability were obtained at a lower temperature (160 ºC) and short baking time (10 min). This study demonstrates that the application of RPOL and RPS blending can positively enhance the nutritional properties and oxidative stability of baked food, and that using potato in the baking processing may be beneficial.

KEYWORDS: *Micronutrients; Oxidative stability; Potato; Red palm olein; Red palm stearin*

RESUMEN: ***Efecto de reemplazar estearina por oleína de palma en galletas de papa horneadas.*** Las galletas de papa fueron formuladas reemplazando estearina de palma roja (RPS) por oleina de palma roja (RPOL) al 0, 17 y 35%, y posteriormente horneadas a 160, 180 y 200°C durante 10, 12 y 15 min. El análisis sensorial utilizando una prueba ortogonal mostró que la relación RPS-RPOL 65:35, la temperatura de horneado 160 ºC, y el tiempo de horneado 12 min fueron las condiciones óptimas. Las galletas hechas de 65% RPS + 35% RPOL presentan 0,6 veces menos de escualeno, pero 1,5 veces más β-caroteno, tocoferoles y tocotrienoles que la mezcla de RPS y RPOL en 100:0. Por otra parte, las galletas con mayor estabilidad oxidativa se obtuvieron a menor temperatura (160 ºC) y menor tiempo de horneado (10 min). Este estudio demuestra que la mezcla RPOL y RPS puede mejorar positivamente las propiedades nutricionales y la estabilidad oxidativa de los alimentos horneados, y que el uso de papa en el procesamiento de hornear puede ser beneficioso.

PALABRAS CLAVE: *Estabilidad oxidativa; Estearina de palma roja; Micronutrientes; Oleina de palma roja; Patata*

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1. INTRODUCTION

The potato, an all-around nutritious food which is rich in protein, vitamins, dietary fiber, minerals, and micronutrients (Singh, 2016) is grown in 149 countries and ranks as the fourth-most important food crop worldwide. This high-yielding, adaptable, nutritional crop has been utilized many times during periods of food shortage. As an economic crop, the value of potato has gradually increased in the past two decades. The international trade of potato has risen considerably because of the growing demand by the food processing industry (Birch *et al.*, 2012). More and more research has been focused on the potato staple food processing and industrial development, such as potato steamed bread, rice noodles, bread and so on (Zhang *et al.*, 2017). The potato could also be a good nutritional additive to foods that are highly desirable in a diet because of their beneficial effects on human health, such as cookies. Using the potato as a raw material for making biscuits and cookies can not only enrich the types of potato and increase consumption, but can also ameliorate the disadvantages of single nutritional components of existing products. This could be of great significance for improving people's nutrition and dietary balance. Nevertheless, studies on potato cookies are still limited. The development of potato cookies can increase the variety of cookies, enhance the living standards of people and contribute to theoretical reference for the development of potato leisure food.

Cookies are a type of convenient baked food which is loved by consumers. It could be made from different dough comprising of majorly wheat flour and/or sugar, milk, egg, salt, and flavor added during production (Oluwamukomi *et al.*, 2010). Extensive efforts have been made to improve the nutritional value of cookies. The lipids applied in cookies and other baking products are mainly animal fats and hydrogenated vegetable oils, which are solid at room temperature and have good processing performance. However, animal oils are expensive. Hydrogenated vegetable oils, on the other hand, are low in price, but contain trans fatty acids which may increase the risk of cardiovascular disease, cancer, diabetes and other diseases (Kong *et al.*, 2011). The low-cost, large-scale production palm oil, with approximately 48% saturated fatty acids, is expected to replace hydrogenated oils (Mba *et al.*, 2015).

In addition, palm oil is the vegetable oil with the largest volume of production, consumption and trade in the world at present. It is cheap and stable. Palm oil is also widely known for its high contents in carotenoids, squalene, tocopherols and tocotrienols. It is processed by advanced technologies such as molecular distillation (Mayamol *et al.*, 2007). While removing impurities in the oil, the product retains most of its nutrients. These nutritional substances are especially good for the eye and skin, protect biological systems against oxidation and prevent various types of cancer and other diseases (Mayamol *et al.*, 2007). Red palm olein (RPOL) and red palm stearin (RPS) are deep red palm oils with different melting points processed by fractionation. RPOL is liquid at room temperature, with poor processing properties but abundant micronutrients. On the contrary, RPS is semi-solid at room temperature, with relatively low nutrient content, but suitable for the processing of cookies. (Nor and Miskandar, 2007). The application of RPOL and RPS blending in baking processes not only gives baked foods an ideal natural color and enhances their nutritional value, but it also expands the application of palm oil and increases the number of consumers.

Thus, this research is devoted to the development of potato cookies with different RPS-RPOL ratios. The micronutrients, oxidative stability and sensory attributes of the samples were determined. Baking processing parameters such as baking temperature and baking time affect the physicochemical properties of products. To obtain a better understanding of these changes in red palm oil that occur in the baking process in this study, samples were baked at three temperatures (160, 180, 200 °C) for three different durations (10 min, 12 min, 15 min), which are the usual production conditions for baked foods (the cookies were fully cooked but not burnt). This study may provide reference values for the selection of formulae for baking products, the optimization research of baking parameters in industrial production and the prediction of shelf-life.

2. MATERIALS AND METHODS

2.1. Materials

Potato flour (homemade), wheat flour, sugar, and egg yolk were purchased from Wal-Mart supermarket (Shanghai, China). RPS (50 ºC) and RPOL

(24 ºC) were donated by Palm Oil Research and Technical Service Institute of Malaysian Palm Oil Board (PORTSIM) (Shanghai, China).

2.2. Sample preparation

The pre-optimized recipe for potato cookies was used: potato flour 200 g, wheat flour 300 g, fats (RPS-RPOL) 300 g, sugar 200 g, egg yolk 150 g. Melted RPS (heated at 55 ºC) and RPOL were well mixed, followed by the addition of sugar and egg. The mixture of potato flour and wheat flour was added last, and the dough was kneaded and sheeted to a uniform thickness of 5 mm. Then, the dough was cooled at 4 °C for 20 min and cut into the size of 30 mm × 30 mm. Baking was carried out in a SM-522 baking oven (Xinmai machinery Co., Ltd., Wuxi, China). After that, the cookies were cooled at room temperature, then sealed and stored at -20 ºC.

The fats in the formula were blended with RPS and RPOL at three different ratios (RPS-RPOL: 100:0, 83:17, and 65:35) (the dough becomes difficult to form and oil flows out when a larger proportion of RPOL is added, hence the ratio of 65:35 was chosen as the maximum). The three levels of baking temperature (160, 180, and 200 ºC) and three levels of baking time (10, 12, and 15 min) were used. Under these usual baking conditions, the cookies were fully cooked but not burnt.

2.3. Sensory analysis

In order to simplify the sensory analysis, the L_9 (3^3) orthogonal experimental design was adopted. The factors and levels are shown in Table 1. The 9 products were put into randomly numbered containers. Fifteen semi-trained panel members (all from the Food Science Department, Shanghai University, Shanghai, China) evaluated the color, aroma, taste, texture, and overall acceptability of the products through a 7-point hedonic scale, from 1 (strongly dislike) to 7 (strongly like) (Harianti *et al.*, 2018).

Table 1. Orthogonal experimental design (three factors and three levels) for sensory analysis

Levels	Factors		
	A (RPS-RPOL)	B (Temperature/ºC)	C (Time/min)
1	100:0	160	10
2	83:17	180	12
3	65:35	200	15

2.4. Oil extraction

100 g of cookies were finely ground before extraction and the lipids were extracted by 500 mL petroleum ether in a DK-S12 electric-heated thermostatic water bath (Hualian Medical Equipment Co., Ltd., Shanghai, China) at 65 °C for 20 min. The micronutrients were stored in a deep freezer for further analysis after the rotary evaporation of the solvent at 35 °C.

2.5. β-Carotene

A β-Carotene analysis was performed on UV-1800PC Ultraviolet spectrophotometer (Mapada Instrument Co., Shanghai, China) (Pan *et al.,* 2016). 0.1 g of cookies was accurately weighed, the volume was made constant with n-hexane to 25 mL, and then placed in a colorimetric dish. The absorbance of the sample was measured by UV-1800PC at 446 nm wavelength with a blank as control. The calculation method of carotene was as follows:

$$Co = \frac{383E}{IC} \quad (1)$$

Where *E* represents the sample absorbance, *I* represents the width of the colorimetric dish, *C* represents the content of red palm oil in the sample (g/100mL), *Co* represents the content of β-carotene in red palm oil (mg/kg).

2.6. Squalene

Squalene was analyzed by a LC-20A High Performance Liquid Chromatography (HPLC) coupled with an SPD-M20A UV detector (Shimadzu Co., Japan) (Pan *et al.*, 2016). The unsaponifiables were extracted from 3 g oil samples, then dissolved and diluted to 25 mL with n-hexane. The extracts were filtrated through a 0.22 µm membrane filter and chromatograms were acquired at 204 nm using an injection volume of 25 µL. An Inertsustain C18 column (250 mm × 4 mm, 5 µm; Shimadzu Co., Japan) was equilibrated using acetonitrile/methanol 4:0 (v/v) at a flow rate of 1 mL/min. Squalene was identified and quantified by comparison to the corresponding standards (Aladdin Industrial Co., Shanghai, China) as external standards.

2.7. Tocotrienols and tocopherols

Tocotrienols and tocopherols were analyzed according to a modified procedure of Yui *et al.* (2016) using the external standard (α-, β-, γ-, and δ-isoforms, Solarbio Co., Beijing, China) method on an LC-20A HPLC equipped with a RF-10AXL Fluorescence detector (Shimadzu Co., Japan). 2 g of oil sample were dissolved in methanol and sonicated for 20 min, centrifuged at 500×g for 10 min. Excitation and emission absorbance were set at 290 nm and 330 nm, respectively. Separation was done on an Inertsustain C18 column (250 mm × 4 mm, 5 µm; Shimadzu Co., Japan) thermoset at 30 °C. The mobile phase comprised a mixture of methanol-water (98:2, v/v) at a flow rate of 1 ml/min (injection volume = 5 µL).

2.8. Schaal oven test

The Schaal oven test was used for sample pre-treatment to accelerate oxidation (Michotte *et al.*, 2011). The samples were placed in the electro-thermal blower drying box (63 ± 1°C) for 40 days to measure the β-carotene content at 3-day intervals. The determination of β-carotene was performed according to the method of Pan *et al.*, (2016).

2.9. Statistical analysis

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

3. RESULTS AND DISCUSSION

3.1. Sensory characteristics

The effects of different RPS-RPOL ratios, baking times and baking temperatures on the color, aroma, taste, texture and overall acceptability of cookies were studied by L9 (3^3) orthogonal tests (Table 1), and the results of orthogonal tests are shown in Table 2.

TABLE 2. Orthogonal test results for sensory characteristics of potato cookies

Samples		A (RPS-RPOL)	B (Temperature/ºC)	C (Time/min)	Color	Aroma	Taste	Texture	Overall
1		1	1	1	5.1	5.1	5.0	5.9	5.0
2		1	2	2	5.4	5.2	5.8	6.8	6.2
3		1	3	3	1.8	3.6	3.4	5.1	2.3
4		2	1	2	5.3	4.9	5.5	5.8	6.0
5		2	2	3	3.5	4.4	4.5	5.1	4.5
6		2	3	1	5.2	4.9	4.5	4.9	4.1
7		3	1	3	5.7	5.4	5.5	5.3	5.6
8		3	2	1	5.9	5.1	5.6	5.4	5.8
9		3	3	2	3.3	4.3	4.1	4.5	3.2
Color	k_1	4.1	5.4	5.4					
	k_2	4.7	5.0	4.7					
	k_3	5.0	3.4	3.7					
	R	0.9	1.9	1.7					
Aroma	k_1	4.6	5.1	5.0					
	k_2	4.7	4.9	4.8					
	k_3	4.9	4.3	4.5					
	R	0.3	0.8	0.6					
Taste	k_1	4.7	5.3	5.0					
	k_2	4.8	5.3	5.1					
	k_3	5.1	4.0	4.5					
	R	0.3	1.3	0.7					
Texture	k_1	5.9	5.7	5.4					
	k_2	5.3	5.8	5.7					
	k_3	5.1	4.8	5.2					
	R	0.8	0.9	0.5					
Overall	k_1	4.5	5.6	5.0					
	k_2	4.9	5.5	5.1					
	k_3	4.9	3.2	4.2					
	R	0.4	2.3	1.0					

RPS, red palm stearin; RPOL, red palm olein.

According to the R values, the order of importance of the variables on the color, aroma, taste and overall acceptability was as follows: baking temperature (B) > baking time (C) > RPS-RPOL ratio (A), and the optimal condition was A3B1C1 or A3B1C2; that is, the optimal conditions were RPS-RPOL 65:35, baking temperature 160 ºC, baking time 10 min or 12 min. However, the factors influencing the cookie texture were as follows: baking temperature (B) > RPS-RPOL ratio (A) > baking time (C) and the optimal condition was A1B2C3. Different from other sensory qualities, the RPS-RPOL ratio had great influence on the texture of cookies. The optimal RPS-RPOL ratio was 100:0, which means that the high content in RPS is beneficial to the crisp texture of cookies. In general, considering the influence of all sensory properties, the most significant factor affecting the quality of cookies was baking temperature (B), followed by baking time (C), and then RPS-RPOL ratio (A). The better sensory evaluation scores for the cookies were obtained when the baking condition was A3B1C2 (RPS-RPOL ratio 65:35, baking temperature 160 ºC, and baking time 12 min). These conditions caused evenly-colored cookies, with no incomplete cooking, no charring, and a crisp taste.

3.2. β-Carotene

The contents of β-carotene in RPOL and RPS were 454.735 ± 1.986 mg/kg and 224.401 ± 1.704 mg/kg, respectively. The content in β-carotene in RPOL was 15 times higher than that in carrot and 300 times higher than that in tomato (Radhika *et al.*, 2017). The effects of different RPS-RPOL proportions, baking temperatures and baking times on the content in β-carotene in oil are presented in Table 3. There was a significant effect of different RPS-RPOL ratios and baking conditions on β-carotene content (mg/kg) in the oil of potato cookies. The content in β-carotene in the oil of potato cookies significantly decreased as the baking time and baking temperature increased ($p < 0.05$). Without adding RPOL, β-carotene was retained the most at 160 ºC for 10 min (81.7% of the oil), and the least at 200 ºC for 15 min (73.9% of the oil). Siti *et al.* (2018) conducted a deep-frying experiment at 180 ºC, and after 10 times of repeated frying, the contents in β-carotene in red palm oil decreased from 294 to 143 mg/kg, and from 119 to 82 mg/kg in yellow palm oil. It can be seen that the content in β-carotene in red palm oil after thermal processing is still higher than that in ordinary yellow palm oil.

Added red palm oil to cookies can effectively increase the β-carotene content in cookies. El-Hadad *et al.* (2010) used 40% shortening and 60% RPOL, and the β-carotene content in cookie samples was 188 mg/kg, 14.8 times higher than in 100% shortening cookies. Researchers have used red palm oil as a substitute fat for a variety of functional food processing, in which the β-carotene can be retained better in red palm oil; the retention in cookies was better than that in bread (Sidhu *et al.*, 2004; Marjan *et al.*, 2016). Thus, the consumption of red palm oil can supplement β-carotene. It was reported that the long-term intake of RPOL as a source of carotenoids, can improve regional population problems (Sobhana *et al.*, 2019), improve the biological characteristics of animal plasma glucose, and cholesterol, and prevent diabetes and cardiovascular diseases. Red palm olein improve the levels of catalase and glutathione peroxidase in red blood cells, which is beneficial to antioxidation (Szulczewska-Remi *et al.*, 2019). In addition, the combined effects of carotene, tocopherols, tocotrienols and other natural antioxidants in red palm olein cannot be underestimated (Yanishlieva *et al.*, 1998).

TABLE 3. The effects of different RPS-RPOL ratios, baking times and baking temperatures on β-carotene content (mg/kg) in the oil of potato cookies

RPS-RPOL	160 ºC			180 ºC			200 ºC		
	10 min	12 min	15 min	10 min	12 min	15 min	10 min	12 min	15 min
100:0	183.2 ± 1.9^{aC}	170.9 ± 2.5^{bcC}	169.8 ± 0.5^{cdC}	175.0 ± 2.2^{bC}	165.6 ± 5.7^{d}	166.7 ± 1.9^{cdC}	171.6 ± 0.8^{bcC}	165.7 ± 0.4^{dC}	165.6 ± 0.9^{dC}
83:17	229.0 ± 2.3^{aB}	226.3 ± 2.7^{abB}	213.8 ± 4.0^{cB}	227.5 ± 4.1^{abB}	221.3 ± 1.9^{bB}	203.5 ± 5.1^{deB}	223.4 ± 1.9^{abB}	208.9 ± 2.0^{cdB}	198.2 ± 3.1^{eB}
65:35	281.1 ± 4.5^{aA}	263.5 ± 8.9^{bcA}	255.5 ± 3.4^{cdA}	274.9 ± 5.6^{abA}	255.4 ± 7.0^{cdA}	245.4 ± 8.0^{deA}	268.7 ± 5.0^{bA}	238.4 ± 3.1^{efA}	233.6 ± 0.1^{fA}

*Results are expressed as mean ± standard deviation (n=3).

*Labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test.

3.3. Squalene

The squalene content in RPOL and RPS were 185.7 mg/kg and 233.3 mg/kg, respectively. The squalene content in RPS was higher than in RPOL, which is the opposite of β-carotene. Kumar and Krishna (2014) carried out the dry fractionation of crude red palm olein, and obtained the squalene contents of crude red palm olein and crude red palm stearin as 360 mg/kg and 16.4 mg/kg, which were inconsistent with the results of this paper, and may be related to the special processing technology of the oil. Another research showed that the processing method of palm oil can affect the micronutrient content in the oil (Mayamol *et al*., 2007).

There is a significant ($p < 0.05$) influence of different RPS-RPOL ratios, baking times and baking temperatures on squalene content (mg/kg) in the oil of potato cookies (Table 4). The squalene content (mg/kg) in the oil of potato cookies was found to vary significantly under different baking conditions and showed a decreasing trend ($p < 0.05$). The content in squalene descended with the elevation of baking temperature and extended time and the decreased trend observed in the amount of squalene under different baking conditions was the same as that of β-carotene. Without the addition of RPOL, most squalene was retained at 160 ºC for 10 min, accounting for 95.7% of the oil content. At 200 ºC for 15 min, the retention rate of squalene was the lowest at 58.7%. With the addition of RPOL, the amount of squalene in the oil decreased accordingly. Applying red palm oil as a squalene source may increase the amount of squalene for humans as squalene in food can be well absorbed (the absorption amount reaches 60-85%). Therefore, food with red palm oil as the source of squalene is expected to improve the content in human squalene after intake. Squalene has strong antioxidant effects, which can quench free radical oxygen, prevent lipid peroxidation, promote anti-aging and improve immunity. It can also protect normal cells, inhibit the growth of tumor cells and reduce the risk of breast cancer, colon cancer, pancreatic cancer and other cancers. Squalene can promote metabolism of the body, and protect the heart, liver, kidney and other organs (Reddy and Couvreur, 2009).

3.4. Tocotrienols and tocopherols

The data pertaining to tocotrienol and tocopherol contents (ppm) in the oil of potato cookies is presented in Table 4. The total amounts of tocopherols and tocotrienols in RPOL and RPS were 664.3 mg/kg and 254.7 mg/kg, respectively. In addition, different RPS-RPOL ratios and different baking conditions showed significant differences in tocotrienol and tocopherol contents (ppm) in the oil of potato cookies ($p < 0.05$). The content in tocotrienols was higher than that of tocopherols, and the β/γ- isomer was higher than of other isomers. The most serious losses were presented in β/γ-tocotrienol, in agreement with the report for baking, deep-frying, and other thermal processing (Rossi *et al*., 2007).

The retention of tocotrienols and tocopherols was under the influence of process parameters. It was obvious that the δ-, β/γ-tocotrienol, δ-, β/γ-tocopherol and total tocotrienol, tocopherol tendencies resembled β-carotene. The loss in vitamin E was minimum at a low temperature for a short time (160 ºC, 10 min). The thermal degradation of vitamin E in oils may have been aggravated by the increases in temperature and time, as clarified by Hamid *et al.* (2014).

What's more, the results from sample baking under the same conditions but with different RPS-RPOL proportions showed that the content in vitamin E in cookies could be increased by adding

Table 4. The effects of different RPS-RPOL ratios, baking times and baking temperatures on squalene content (mg/kg) in the oil of potato cookies

RPS-RPOL	**160 ºC**			**180 ºC**			**200 ºC**		
	10 min	**12 min**	**15 min**	**10 min**	**12 min**	**15 min**	**10 min**	**12 min**	**15 min**
100:0	223.9 ± 2.4^{aA}	196.8 ± 5.3^{bA}	194.1 ± 2.2^{cA}	159.7 ± 1.4^{cA}	133.7 ± 3.0^{eB}	133.1 ± 1.2^{eA}	147.9 ± 1.0^{dA}	135.2 ± 4.2^{eA}	131.2 ± 1.4^{eA}
83:17	151.3 ± 3.9^{aB}	140.5 ± 4.1^{bB}	128.7 ± 1.6^{cB}	143.9 ± 5.5^{abB}	138.2 ± 7.1^{bA}	122.7 ± 2.0^{cB}	139.9 ± 2.1^{bB}	130.0 ± 4.4^{cB}	126.8 ± 0.8^{cB}
65:35	135.2 ± 1.7^{aC}	127.8 ± 2.2^{bC}	115.6 ± 4.3^{cC}	122.7 ± 2.4^{bC}	108.6 ± 0.3^{dC}	99.1 ± 4.3^{fC}	114.3 ± 2.5^{cC}	105.7 ± 2.5^{deC}	101.2 ± 1.6^{efC}

*Results are expressed as mean ± standard deviation (n = 3).

*Labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test.

RPOL, which contains higher vitamin E content. RPOL and RPS are not only rich in natural antioxidant vitamin E, but have also been applied in the production of sugar-snap cookies and bread with good sensory characteristics and consumer acceptability (Al-Saqer *et al.*, 2004). The presence of tocotrienols and tocopherols retards oil autoxidation greatly and protects fatty foods from off-flavors. In addition, these compounds possess gene regulatory functions (α-tocopherol), anti-inflammatory, antitumor activities (γ-tocopherol), neuroprotective properties, preventive effect on cholesterol biosynthesis, and anticancer effects (Kamaleldin and Appelqvist, 1996). In short, considerable retention of tocotrienols and tocopherols in food is beneficial to both food storage and human health.

3.5. Schaal oven test

To characterize the oxidative stability of the products, the change in trends of β-carotene content during the oven test are presented in Figure 1. Generally, combined with the ordinates of Figure 1a, 1b, 1c, samples with a higher ratio of RPOL showed superior properties in the oven test since the β-carotene concentration in RPOL was higher than that in RPS. Besides, the β-carotene contents increased, then decreased and became steady at last with the prolongation of oxidation time, indicating that the oxidation of oil proceeded continuously. The concentration of β-carotene increased from day 0 to day 3, decreased slowly from day 3 to day 12, decreased rapidly from day 12 to day 20, and remained unchanged after day 33. Lee (1986) reported a slight increase followed

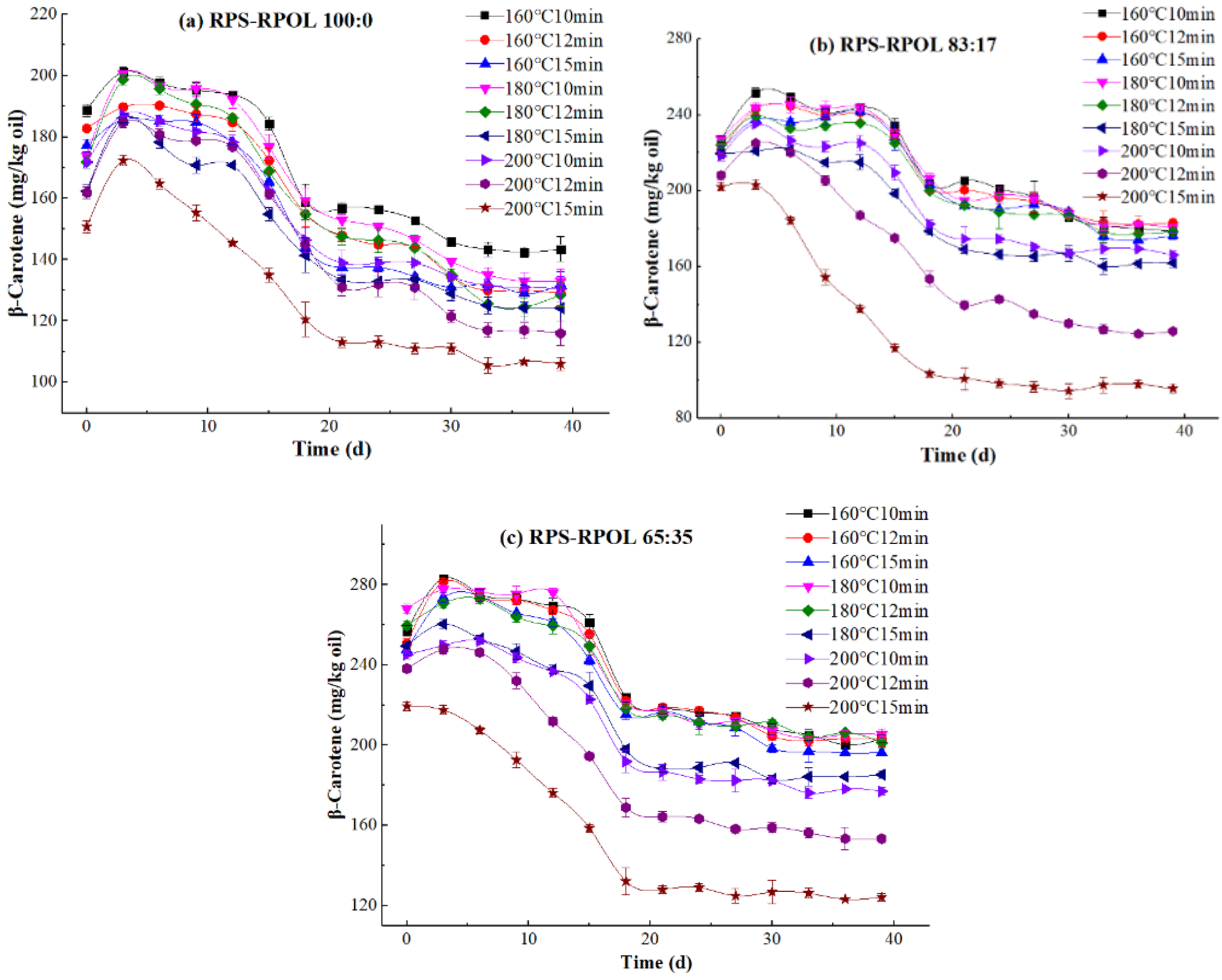

FIGURE 1. The β-carotene contents in the oils extracted from potato cookies during the oven test at 63 ºC for 39 days. The results are expressed as mean ± standard deviation (n = 3). (a. RPS-RPOL, 100:0; b. RPS-RPOL, 83:17; c. RPS-RPOL, 65:35).

Table 5. Effect of different RPS-RPOL ratios, baking times and baking temperatures on tocotrienol and tocopherol contents (ppm) in the oil of potato cookies

RPS-RPOL	Temperature	Time	δ-T_3	β/γ-T_3	α-T_3	δ-T	β/γ-T	α-T	Total
100:0	160 ºC	10 min	33.4 ± 1.1^{cde}	98.0 ± 2.8^{fgi}	15.7 ± 0.9^{ij}	20.8 ± 1.6^{cd}	41.1 ± 2.2^{def}	16.0 ± 0.4^{n}	225.0 ± 9.0^{def}
		12 min	29.9 ± 0.7^{efghi}	93.8 ± 4.1^{fghi}	23.3 ± 2.2^{fgh}	18.5 ± 1.4^{def}	34.2 ± 2.8^{ghij}	$24.2\ \pm1.3^{ghij}$	223.9 ± 12.5^{def}
		15 min	27.9 ± 0.5^{ghij}	89.9 ± 2.6^{ghij}	22.9 ± 1.8^{fgh}	19.6 ± 1.6^{cde}	34.7 ± 1.5^{ghi}	26.7 ± 1.9^{fgh}	221.7 ± 9.9^{defg}
	180 ºC	10 min	$32.0\pm0.^{1def}g$	90.6 ± 5.8^{ghij}	20.9 ± 1.5^{h}	20.9 ± 0.8^{c}	36.1 ± 3.3^{fgh}	18.1 ± 1.9^{lmn}	218.6 ± 13.4^{defgh}
		12 min	25.2 ± 1.2^{jkl}	84.5 ± 4.4^{ij}	23.9 ± 1.0^{fgh}	17.0 ± 0.5^{fghi}	30.1 ± 1.0^{ijkl}	21.8 ± 2.2^{ijkl}	202.5 ± 9.3^{efghi}
		15 min	25.9 ± 1.3^{ijkl}	85.9 ± 8.2^{hij}	22.3 ± 0.5^{gh}	16.9 ± 0.3^{fghi}	29.5 ± 0.7^{ijkl}	21.0 ± 1.2^{jklm}	201.5 ± 12.2^{efghi}
	200 ºC	10 min	23.4 ± 1.2^{klm}	82.0 ± 4.6^{j}	19.8 ± 1.2^{hi}	15.0 ± 1.0^{i}	28.9 ± 0.1^{jkl}	21.1 ± 1.7^{jklm}	190.2 ± 9.8^{hij}
		12 min	22.5 ± 4.8^{lmn}	71.2 ± 6.1^{k}	20.3 ± 3.8^{h}	16.0 ± 2.4^{ghi}	25.4 ± 2.2^{lm}	18.5 ± 1.5^{lmn}	173.9 ± 20.8^{ij}
		15 min	19.5 ± 0.1^{n}	60.4 ± 5.1^{l}	14.0 ± 1.3^{j}	11.8 ± 0.8^{j}	22.8 ± 1.7^{m}	17.0 ± 3.0^{mn}	145.5 ± 12.0^{k}
83:17	160 ºC	10 min	36.7 ± 1.7^{bc}	130.5 ± 8.9^{bc}	36.8 ± 3.0^{c}	23.6 ± 1.8^{b}	54.1 ± 3.5^{c}	33.2 ± 3.0^{bcd}	314.9 ± 21.9^{b}
		12 min	32.1 ± 1.9^{def}	$116.0\pm3.^{7d}e$	30.3 ± 2.8^{de}	19.4 ± 0.8^{cde}	38.2 ± 2.9^{defg}	30.0 ± 2.0^{def}	266.0 ± 14.1^{c}
		15 min	30.3 ± 1.2^{efgh}	112.5 ± 2.4^{e}	27.1 ± 3.2^{def}	17.7 ± 0.3^{efgh}	35.1 ± 3.1^{ghi}	24.9 ± 2.4^{ghij}	247.6 ± 13.6^{cd}
	180 ºC	10 min	36.2 ± 2.2^{bc}	126.8 ± 1.1^{c}	37.6 ± 1.7^{c}	20.4 ± 2.1^{cd}	43.5 ± 0.2^{d}	29.9 ± 1.0^{def}	294.4 ± 14.1^{b}
		12 min	30.9 ± 3.0^{defg}	111.8 ± 7.4^{e}	31.6 ± 0.5^{d}	19.7 ± 0.8^{cde}	41.9 ± 5.4^{de}	31.6 ± 1.2^{cde}	267.5 ± 21.7^{c}
		15 min	26.7 ± 2.8^{hijk}	99.7 ± 2.4^{fg}	29.1 ± 0.7^{de}	17.4 ± 1.1^{efgh}	36.9 ± 2.1^{efg}	28.4 ± 0.3^{efg}	238.2 ± 9.4^{d}
	200 ºC	10 min	$29.2\pm5.^{0fgh}i$	101.2 ± 1.9^{f}	26.2 ± 2.8^{efg}	16.6 ± 1.0^{fghi}	33.0 ± 3.4^{ghijk}	23.3 ± 3.0^{hijk}	229.5 ± 17.1^{de}
		12 min	$24.6\pm2.^{4jk}l$	84.3 ± 4.2^{ij}	23.7 ± 2.0^{fgh}	16.7 ± 2.6^{fghi}	30.6 ± 4.1^{hijkl}	24.7 ± 1.3^{ghij}	204.6 ± 19.7^{efgh}
		15 min	22.3 ± 0.3^{lmn}	81.9 ± 6.9^{j}	21.2 ± 1.4^{h}	15.4 ± 0.4^{hi}	27.7 ± 4.9^{klm}	25.6 ± 2.8^{ghi}	194.1 ± 19.7^{ghij}
65:35	160 ºC	10 min	41.4 ± 0.6^{a}	153.6 ± 8.6^{a}	48.9 ± 3.2^{a}	29.0 ± 0.6^{a}	66.9 ± 5.8^{a}	33.2 ± 0.3^{bcd}	373.0 ± 19.1^{a}
		12 min	37.8 ± 3.0^{b}	136.9 ± 5.0^{b}	44.6 ± 5.9^{b}	27.9 ± 0.5^{a}	61.9 ± 3.2^{b}	45.0 ± 6.5^{a}	354.1 ± 24.1^{a}
		15 min	31.8 ± 3.4^{defg}	123.5 ± 3.5^{cd}	$39.6\pm0.^{7}c$	24.2 ± 1.3^{b}	55.6 ± 1.7^{c}	35.3 ± 2.6^{bc}	310.0 ± 13.1^{b}
	180 ºC	10 min	34.6 ± 2.1^{bcd}	129.8 ± 5.4^{bc}	40.9 ± 3.0^{bc}	25.1 ± 0.6^{b}	55.7 ± 4.9^{c}	35.8 ± 1.2^{bc}	321.9 ± 17.2^{b}
		12 min	32.2 ± 0.7^{def}	122.8 ± 2.9^{cd}	39.1 ± 0.4^{c}	25.2 ± 1.5^{b}	56.0 ± 2.6^{c}	36.7 ± 3.7^{b}	311.9 ± 11.8^{b}
		15 min	22.4 ± 0.9^{lmn}	82.6 ± 7.9^{j}	$24.2\pm0.^{9fg}h$	17.9 ± 0.7^{efg}	36.5 ± 2.5^{efg}	11.9 ± 0.3^{o}	195.6 ± 14.2^{fghij}
	200 ºC	10 min	29.9 ± 2.8^{efghi}	116.5 ± 9.2^{de}	36.9 ± 5.2^{c}	24.4 ± 0.2^{b}	54.2 ± 1.7^{c}	34.8 ± 2.8^{bc}	296.7 ± 21.9^{b}
		12 min	24.6 ± 0.2^{jkl}	94.4 ± 2.3^{fgh}	29.1 ± 3.7^{de}	18.9 ± 1.4^{cdef}	43.0 ± 2.4^{d}	27.6 ± 0.9^{efgh}	237.6 ± 12.9^{d}
		15 min	$19.0\pm1.^{5}n$	65.6 ± 2.7^{kl}	$20.6\pm1.^{4}h$	14.8 ± 0.8^{i}	30.0 ± 0.6^{ijkl}	19.7 ± 1.6^{klmn}	169.8 ± 10.6

*Results are expressed as mean ± standard deviation (n = 3). The isomers of tocotrienols and tocopherols are shown as δ, β/γ, α.
*Labelled with the same letter did not differ significantly ($p \leq 0.05$) according to Duncan's test.

by a decrease in β-carotene content during the storage of fresh carrots. This phenomenon may relate to processing methods, the influence of lipoproteins on the separation of carotenoids, and the distribution of different carotenoids during storage. However, it is difficult to ascribe the reason for the increase in β-carotene to only one of them. Research showed that carotene was almost completely lost under high-temperature accelerated oxidation, but tended to be stable in this study because the egg lecithin in cookie formula had a protective effect on carotene at 60 ºC (Yanishlieva *et al.*, 1998).

When RPS-RPOL was 100:0 (Figure 1a), the β-carotene content in the samples at 200 ºC for 15 min decreased rapidly from day 3, and it was significantly lower than that of the samples under other conditions and after dozens of days. Such a phenomenon revealed that the samples under this condition (200 ºC, 15 min) were the most unstable. In addition, as shown in Figures 1b and 1c (RPS-RPOL were 83:17, 65:35), β-carotene concentration in samples baked at 200 ºC (10 min, 12 min, 15 min) and 180 ºC (15 min) were significantly lower than that of other samples, however, there was no visible difference in the samples at 160 ºC. The results illustrated that temperature was the dominant factor that affected oxidative stability negatively at temperatures above 200 ºC. In addition, long baking time played a more decisive role when temperature was less than or equal to 180 ºC.

4. CONCLUSIONS

In this study, cookies with better sensory properties were obtained when the baking conditions were RPS-RPOL ratio 65:35, baking temperature 160 ºC, and baking time 12 min. Formulated cookies with an RPS-RPOL ratio of 65:35 exhibited 0.6 times less squalene, but 1.5 times more β-carotene, and tocopherols and tocotrienols than the mixture of RPS and RPOL at 100:0 ratio. In addition, the results indicated that products with high micronutrients and oxidative stability can be obtained with low temperature and short baking time (160 ºC, 10 min). Baking time played a more decisive role in oxidative stability when the temperature was below 180 ºC. However, temperature was the dominant factor that affected the oxidative stability at temperatures above 200 ºC. The total properties as measured in this research is important for formulating, investigating baking conditions, and predicting the shelf-life of red palm oil-potato containing functional baked food. Red palm oil has high nutritional value, which can be used as a healthy food oil and health care product, and can also be used as a substitute for pigment and a supplementary food for some nutritional functional components, thus having a high development and utilization prospect. The development of potato cookies with red palm oil can not only enrich the variety of cookies, make up for the defects of single raw materials and insufficient varieties of baked food, but also have important practical significance for improving the utilization rate of potatoes and meeting the market demand for convenient leisure food.

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Optimization of date seed oil extraction using the assistance of hydrothermal and ultrasound technologies

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SUMMARY: The date seed is a by-product from the date industry. Its use as a source of added-value compounds is of great interest. Oil accounts for 5-13% of the seed's weight. Soxhlet extraction with organic solvents is the traditional method for obtaining oil from seeds. In this work, hydrothermal pre-treatments and sonication are proposed to make the extraction a more environmentally friendly process. Factors such as sonication time and temperature and hexane-to-seed ratio (H/S) have been considered. Response surface methodology was applied for optimization. Hydrothermal treatments increased oil recovery. H/S was the most influential factor, and was close to 7 mL/g seeds for both samples. 71% recovery was achieved for native seeds after 15 min sonication at 45 ºC, and 80% for 180 ºC-treated seeds after 45 min at 35 ºC when compared to Soxhlet extraction. These conditions comply with our initial aim. Pre-treatments seem to have a negative effect on oil stability, although this observation needs to be confirmed.

KEYWORDS: *Date seed; Hydrothermal technology; Oil extraction; Oxidation stability; Response surface methodology; Sonication*

RESUMEN: ***Optimización de la extracción de aceite de semilla de dátil mediante la ayuda de tecnologías hidrotermales y de ultrasonido.*** La semilla de dátil es un subproducto de la industria datilera. Su uso como fuente de compuestos de valor añadido sería muy interesante. El aceite representa el 5-13% del peso de la semilla. La extracción mediante Soxhlet con disolventes orgánicos es el método tradicional para obtener aceite de semillas. En este trabajo se proponen pretratamientos hidrotérmicos y sonicación para diseñar un proceso de extracción más respetuoso con el medio ambiente. Se han considerado factores como el tiempo y la temperatura de sonicación y la proporción hexano/semilla (H/S). La optimización del proceso se llevó a cabo por el método de superficie de respuesta. Los tratamientos hidrotérmicos aumentaron el porcentaje de recuperación. El factor más influyente fue H/S, y su valor óptimo estuvo cerca de 7 mL/g para ambas muestras. La recuperación óptima fue del 71% para las semillas sin tratamiento tras 15 min de sonicación a 45 ºC, y del 80% para las semillas tratadas a 180 ºC después de 45 min a 35 ºC. Estos resultados cumplen con nuestro objetivo inicial. Sin embargo, los pretratamientos parecen tener un efecto negativo sobre la estabilidad del aceite, lo que se confirmará en estudios posteriores.

PALABRAS CLAVE: *Estabilidad oxidativa; Extracción de aceite; Método de superficie de respuesta; Semilla de dátil; Sonicación; Tratamientos hidrotérmicos*

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1. INTRODUCTION

The date palm has long been one of the most important fruit crops and it is widely distributed in many regions of the world, including Middle Eastern countries and North Africa (El-Rahman and Al-Mulhem, 2017; Djaoudene *et al.*, 2019). The date palm is a multipurpose tree with nutritional, therapeutic, and environmental attributes (Daoud *et al.*, 2015), and plays an important role at economic and social levels for people from arid and semiarid regions of the world (Besbes *et al.*, 2004). At present, its production and consumption are continuously increasing: in 2018, more than 8.5 million tons of dates were produced, with an annual by-product yield of approximately 2.5 million tons of date seeds from date fruit processing industries (pitted dates, date powders, date syrup, date juice, chocolate-coated dates and date confectionery) (Basuny and Al-Marzooq, 2011). The fruit from the date palm is composed of a fleshy pericarp and a seed, which constitutes 10 to 15% of the date's weight. Although the date seed is a good source of cattle food, Kchaou *et al.* (2013) reported that the disuse of this by-product represents a real economic loss since it offers a promising raw material for the extraction of bioactive compounds. In addition to dietary fiber, proteins and minerals, date seeds present a content in oil between 5-13% on dry weight basis, which is rich in phenolics, tocopherols and phytosterols (Besbes *et al.*, 2004; Mrabet *et al.*, 2015). The composition of date seed oil has been studied by other authors, and its content in antioxidants, vitamins, minerals and fatty acids (50% oleic acid, 19% linoleic acid, 10% lauric acid, and 10% palmitic acid) makes it valuable for food formulation and cosmetic and pharmaceutical applications (Al Ghezi *et al.*, 2020; Mrabet *et al.*, 2020).

Extraction is the most important step in the recovery of this bioactive oil. In comparison to other seeds, the process for date seeds is hindered by the hardness of the raw material. Therefore, the application of a pre-treatment would be useful. A wide range of pre-treatment techniques for date seed oil extraction has already been reported (Kareem *et al.*, 2021), but they are all chemical-consuming processes. One of the most interesting environmental-friendly processes is based on hydrothermal pre-treatments which enable the extraction of soluble compounds into the liquid phase, leaving an oil-enriched fibrous material as a solid fraction (Fernández-Bolaños *et al.*, 2004). This technology has been successfully applied to several agricultural by-products, such as sugarcane bagasse (Boussarsar *et al.*, 2009) or corn cobs (Egües *et al.*, 2012) to obtain sugars, oligosaccharides, or phenols. Our research team has designed a 100-L reactor for the treatment of lignocellulosic material (Lama-Muñoz *et al.*, 2011) which works at mid pressures (up to 9 Kg/cm2) and can be applied to a wide range of agricultural by-products (Mrabet *et al.*, 2015; Rubio-Senent *et al.*, 2015; Fuentes-Alventosa *et al.*, 2013) without the addition of chemicals.

The most widely used procedures for oil extraction from a solid plant matrix are cold extraction and conventional Soxhlet extraction. However, these methods use large amounts of organic solvents which are released into the atmosphere and are very time- and energy-consuming (Wang and Weller, 2006). Nowadays, there is a need to use new technologies that can reduce extraction time and solvent consumption. Ultrasound-assisted extraction is based on the energy that ultrasound waves bring to an extraction medium and improves the mass transfer phenomena by cavitation, as well as promoting biomass diffusion, cell disruption, and solvent penetration (Barba *et al.*, 2016; Kaufmann and Christen, 2002). Several process variables such as extraction solvent, time, temperature, and liquid-to-solid ratio have great impacts on process optimization. Therefore, the different interactions among these factors could have significant effects on extraction yields. The optimization of oil extraction conditions is commonly achieved by statistical and mathematical approaches, usually by the use of response surface methodology (Bassani *et al.*, 2014; Sharif *et al.*, 2014). This statistical implement has been successfully used for optimizing the oil extraction conditions from avocado pulp (Xuan *et al.*, 2017), papaya seeds (Samaram *et al.*, 2015), winter melon seeds (Bimakr *et al.*, 2012) or capper seeds (Ara *et al.*, 2014).

The main objective of this work is to study the oil extraction from date seeds by the application of two environmentally friendly technologies (hydrothermal pre-treatments for conditioning the raw material and sonication for improving the oil yield) in comparison to traditional Soxhlet extraction. We studied several parameters, such as liquid-to-solid ratio, sonication time, and sonication temperature, and maximized the extraction process using response surface methodology. In addition, the oxidative stability of the optimized oils was presented as an initial assessment of oil quality.

2. MATERIALS AND METHODS

2.1. Sample preparation

Date seeds were collected randomly from the Nefzaoua area, located in the South West of Tunisia. The selected seeds were soaked in water, and then washed to remove any adhered date flesh and air dried. They were further oven-dried at 60 ºC for 24 h. The dried seeds were milled and sieved using a 1-mm mesh sieve and stored at -20 ºC until use.

2.2. Hydrothermal treatments

Steam treatments were carried out using a 100-L capacity reactor that can work at temperatures between 50 and 190 ºC by direct heating with saturated steam, and at a maximum pressure of 9 kg/cm^2. Date seed samples (10 Kg) were treated at temperatures between 140 and 190 ºC, as preliminary experiments. After treatment, the samples were collected, vacuum filtered through filter paper, dried, milled and sieved using a 1-mm mesh sieve and stored at -20 ºC.

2.3. Conventional soxhlet extraction

Date seed oil extraction was carried out on a solvent extractor equipped with six Soxhlet posts. 10 g of date seed powder were added to a cellulose thimble and placed into a Soxhlet apparatus that was connected to a 250-mL round-bottom flask containing 200 mL hexane. The extraction process lasted for 8 h and until the solvent in the reflux pipe became colorless. The extract was evaporated in a rotary vacuum evaporator (model R-210, Buchi, Flawil, Switzerland) at 40 °C, after which the collected oil was stored in vials at -20 °C until further analysis.

2.4. Ultrasound-assisted extraction (UAE)

The date seed powder was put into a screw-cap flask (250 mL), made up to required volume with hexane, and sonicated in an ultrasonic bath (Thermo-10D; 40 kHz frequency; 240 W ultrasonic output power; 500×300×150 mm internal dimensions). The seed and solvent mixture was irradiated at different levels of sonication time, temperature, and hexane-to-seed (H/S) ratio. The bath water was placed in an external thermostatic device to avoid auto-heating effects during sonication. Upon extraction completion, the extract was passed through filter paper in a funnel and the solvent was removed using a rotary vacuum evaporator (model R-210, Buchi, Flawil, Switzerland) at 40 °C. The extracted oils were stored at -20 °C until further analysis.

2.5. Experimental design

Response surface methodology with a three-factor, three-level Box–Behnken design was employed to study the effect of UAE parameters on the recovery of date seed oil. Three independent factors, namely sonication time (X_1, min), sonication temperature (X_2, °C), and H/S ratio (X_3, mL/g) were used. Three levels were applied to each factor (Table 1). The ranges of the variables studied were chosen according to the preliminary experiments. The response was oil recovery (%) referred to Soxhlet extraction yield. The experimental design and statistical analysis were created and performed using the Statgraphics Plus Version 2.1 software (Statgraphics Technologies Inc., The Plains, Virginia). The experimental design was composed of 15 experimental runs. The three replicates of the center points evaluated the repeatability of the designed method and were used to examine experimental error. A second-order polynomial model was used to fit the experimental data.

Table 1. Levels and values of independent variables

Variables	**Symbol code**	**Levels**		
		-1	**0**	**1**
Sonication temperature (ºC)	X_1	25	35	45
Sonication time (min)	X_2	15	30	45
Hexane (mL)/seed(g) ratio	X_3	3	5	7

The experiments were randomly conducted in order to avoid the effects of unexpected variability in the observed responses which resulted from extraneous factors. Analysis of variance (ANOVA) was used to analyze the individual linear, quadratic and interaction regression coefficients. The values of R^2 (coefficient of determination) and F-test were used to examine the adequacy of the designed model. Regression analysis and response surface plot were used to examine the optimal conditions and the effects of the interactions among independent variables. The confidence level was set at 95%. The validity of the designed model was confirmed by performing triplicate analyses on the generated optimal parameters and one-sample t-test was used to verify the differences between experimental and predicted values.

2.6. Oil oxidative stability

The oxidative stability of the oil (OOS) was evaluated by applying a modification of the thiobarbituric acid (TBA) reactive species method (Rodríguez *et al.*, 2007). The degradation compounds (aldehydes, ketones, etc.) synthesized during oil oxidation reacted with TBA to give a pink-colored complex whose concentration was measured at 540 nm. Sixty microliters of oil and 5 µL of ABAP were added to an Eppendorf tube (1.5 mL capacity) and made up to 0.1 mL with distilled water (in quadruplicate). Afterwards, 150 µL 20% acetic acid (pH 3.5) and 150 µL 0.8% w/v TBA in 1.1% sodium dodecylsulphate w/v were measured into each tube. This mixture was stirred in a Vortex and heated at 80 ºC for 1 h. After cooling at room temperature, 0.5 mL of 1-butanol were added, stirred and centrifuged at 12,100 g for 3 min. The absorbance of the butanol layer was measured at 540 nm. High absorbance values indicated high concentrations of TBA-reactive species quantified in the reaction medium and therefore low oxidative stability in the assayed oil. As the lower the absorbance the higher the stability, for reasons of clarity the final oil oxidative stability (OOS) was expressed as:

$$\mathrm{OOS} = 1/\mathrm{Abs}_{540}$$

2.7. Statistical analysis

To assess the differences among samples, a multiple sample comparison was performed using the Statgraphics® Plus program Version 2.1. A multifactorial analysis of variance (ANOVA), followed by Duncan's multiple comparison test was performed to differentiate the samples. The level of significance was $p < 0.05$.

3. RESULTS AND DISCUSSION

The ultrasound technique was studied as assistance for a less contaminating and less energy-consuming hexane extraction compared to the traditional Soxhlet extraction. In addition, hydrothermal pre-treatments were applied to the date seeds in order to disorganize their structure and to ease the oil release from the seed tissue. Statistical designs were developed to study the significance of each parameter (sonication temperature and time, H/S ratio, and the implementation of a pre-treatment). Multifactorial ANOVA and response surface models were applied for identifying significant factors, formulating regression equations and finding the optimized conditions for each experiment.

3.1. Oil yield of the native and pre-treated date seeds

The control and hydrothermally-treated samples were subjected to Soxhlet extraction and the obtained yields were considered as reference values for the ultrasound-assisted experiments. After preliminary experiences, the highest oil yield by the Soxhlet method was obtained at 180 ºC. This fact suggests a very intense disorganization of seed tissues which enabled the extraction of higher amounts of oil, together with the enrichment in oil of the solid recovered after pre-treatment (Fernández-Bolaños *et al.*, 2004) due to the partial solubilization of the solid material into the treatment liquid. In a previous work (Mrabet *et al.*, 2015), cellulose, lignin and mannans coming from date seeds were quantified in the solid residue recovered after hydrothermal treatments of complete date fruits, pointing to a partial degradation of seed structure. This disorganization of lignocellulosic material (the solubilization of lignin and hemicelluloses and the enrichment with cellulose and oil of the resultant solid) have been described when working with other agricultural by-products (Rubio-Senent *et al.*, 2015; Fuentes-Alventosa *et al.*, 2013). For the native date seed, a lower oil yield was obtained (7.67 ± 0.06%) compared to pre-treated ones (Besbes *et al.*, 2004) or 11.8-15.6% (Mrabet *et al.*, 2015) for some Tunisian varieties, and the lowest for some Algerian ones (5.4-5.6%) (Boukouada *et al.*, 2014). Surface response methodology was applied to samples, both the native seeds and 180 ºC-treated ones, for oil recovery optimization.

3.2. Oil recovery for the different experiments

The different parameters and levels summarized in Table 1 were applied to both samples (native date seeds and with hydrothermal pretreatments at 180 ºC), and two different Box-Behnken designs were developed (Tables 2 and 3). Each model was composed of fifteen experiments plus three at optimal conditions to validate the designed model. The oil yield was the response variable studied because it is the main parameter considered to study the economic viability of the extraction process. The obtained oil recovery by UAE was between 42-73% and 57-81% of the oil extracted by Soxhlet for native seeds and those treated at 180 ºC, respectively. In spite of these yields, the savings in energy, solvent, and working time could make this system valuable. In addition, the chemical composition of UAE oils should be less modified when compared to the corresponding Soxhlet ones, probably due to a gentle exposure to temperature; but it is also important to keep in mind that ultrasound application could decrease the oxidative stability of oil (Samaram *et al*., 2015; Böger *et al*., 2018). The extra energy applied to plant materials by ultrasound is one of its advantages, because interesting biomolecules are extracted in higher concentrations, e.g. phenols and other antioxidants (Bimakr *et al*., 2012). However, this energy is also the activation energy for the appearance of free radicals, molecules that trigger oxidation chain reactions, which are responsible for the characteristic rancid color and flavor. These defects negatively affect the shelf-life and consumer acceptance of food products and are a major cause of food waste (Böger *et al*., 2018).

The high energy of ultrasonic sound waves provokes the disruption of the cell wall, thus making the oil more accessible and more easily extracted (Vinatoru, 2001). During sonication, cycles of compression and decompression are originated in the liquid. During compression, little bubbles are generated in the solvent that lead to localized pressure increases. But during decompression, the bubbles implode, collapse and originate rarefactions. These cycles are also known as cavitation phenomenon, which is responsible for cell wall disruption, oil release into the solvent and destruction of oil emulsion (Lou *et al*., 2010). The date seed is a very hard by-product from date fruit processing with a chemical composition that suggests a very complex and closed cell wall structure: 20-25% lignin, 17-20% cellulose and 11-20% hemicelluloses, 60% of which are mannans (Mrabet *et al*., 2015). Mannans establish many closed complexes with cellulose microfibers, which tighten the cell wall network (Whitney *et al*., 1998). These facts could hinder oil extraction. Hydrothermal pre-treatments change the structure of the plant material, dissolving most of the lignin and loosening the lignocellulosic materials (Fernández-Bolaños *et al*., 2004; Jaramillo-Carmona *et al*., 2019). The added effects of both processes, ultrasound and hydrothermal treatments, could be the reason for the high-

Table 2. Box-Behnken design with the different combinations of the three independent variables (sonication temperature X_1, sonication time X_2, and hexane/seed ratio X_3) for ultrasound-assisted hexane extraction optimization of natural date seeds.

Run order	**Independent variables**			**Oil recovery (%)**
	X_1	**X_2**	**X_3**	
1	25	30	7	73.27
2	25	15	5	61.93
3	25	30	3	48.89
4	35	30	5	55.41
5	35	30	5	55.41
6	35	15	7	65.19
7	45	30	7	70.01
8	45	45	5	60.23
9	45	15	5	61.93
10	35	45	7	65.19
11	25	45	5	61.93
12	35	30	5	55.41
13	35	15	3	45.63
14	45	30	3	50.46
15	35	45	3	42.37

Table 3. Box-Behnken design with the different combinations of the three independent variables (sonication temperature X_1, sonication time X_2, and hexane/seed ratio X_3) for ultrasound-assisted hexane extraction optimization of date seeds treated at 180 ºC.

Run order	**Independent variables**			**Oil recovery (%)**
	X_1	**X_2**	**X_3**	
1	25	30	7	73.73
2	35	30	5	75.30
3	35	45	3	64.70
4	45	15	5	66.27
5	35	45	7	81.33
6	25	30	3	63.25
7	45	30	3	64.70
8	35	15	7	75.30
9	45	45	5	78.31
10	45	30	7	73.73
11	35	15	3	57.23
12	35	30	5	72.29
13	35	30	5	72.29
14	25	45	5	72.29
15	25	15	5	70.72

er extraction yields and oil recoveries in the sample treated at 180 ºC.

A multifactorial ANOVA was applied to all these assays, and the application of a pre-treatment was included as a factor. The results are presented in Table 4. The sonication temperature was the only factor that did not show significance, although it effects were variable when working with other vegetal products, e.g. winter melon seeds (Bimakr *et al*., 2012) and caper seed (Ara *et al*., 2014). In the cited works, the yield increased with temperature until it reached its maximum. This fact could be related to an increased diffusivity of the solvent into cells and to an enhanced solubility of oil from the cells into solvent. At higher temperatures, the yield decreases were probably due to a decrease in the number of acoustic bubbles generated by sonication. H/S ratio and the pre-treatment had the greatest influence (Table 4). At a high H/S ratio, there was sufficient solvent capacity for oil transfer and enhanced diffusion through a viscosity reduction. Both circumstances led to increases in the concentration gradient and to a more efficient extraction (Samaran *et al*., 2015). All these phenomena were eased by the disruption of the matrix structure caused by hydrothermal pre-treatments. The effect of the different significant factors is graphically displayed in Fig 1 as the recovery average values and LSD intervals.

Table 4. Multifactorial ANOVA results for oil recovery (%) and four factors (sonication temperature, sonication time, hexane/solid ratio, and pre-treatment).

Source	**Sum of squares**	**DF**	**Mean square**	**F-ratio**	**p-value**
Temperature	30.8169	2	15.4085	1.11	0.3489
Time	45.9242	2	22.9621	2.38	0.0467
H/S ratio	1266.38	2	633.19	45.41	0.0000
Pretreatment	1180.39	1	1180.39	84.65	0.0000
Residual	306.758	22	13.9435		
Cor. total	2836.76	29			

3.3. Statistical models for date seed oil recovery

The best-fitting models were generated by including only the significant terms ($p < 0.05$) in the generated quadratic models. Table 5 shows the results of the ANOVA analysis for the reduced quadratic model of the samples analyzed. These models can explain most of the variations in oil recovery, as shown by the high F values in the models (816.22 and 25.40 for native and 180 ºC-treated date seeds, respectively). This value decreased with pretreatment, and the same happened with the coefficient of determinations (R^2 and adjusted R^2). This suggested a lower fit for treated seeds than for the native ones. Nevertheless, the models displayed high adjusted R^2, with values > 0.80.

Table 5. Analysis of variance (ANOVA) for the response surface quadratic model of date seed oil recovery.

Native date seeds					
Source	**Sum of squares**	**DF**	**Mean square**	**F-ratio**	**p-value**
X_1	1.436	1	1.436	7.57	0.0284
X_2	3.075	1	3.075	16.21	0.0050
X_3	931.177	1	931.177	4908.31	0.0000
X_1^2	137.191	1	137.191	723.14	0.0000
X_1X_3	5.832	1	5.832	30.74	0.0009
X_2X_3	2.657	1	2.657	14.00	0.0072
X_3^2	2.574	1	2.574	13.57	0.0078
Pure error	1.328	7	0.190		
Cor. total	1088.66	14			
R^2 99.90; adjusted R^2 97.33					

180 ºC-treated date seeds					
Source	**Sum of squares**	**DF**	**Mean square**	**F-ratio**	**p-value**
X_2	91.858	1	91.858	10.16	0.0078
X_3	367.434	1	367.434	40.65	0.0000
Pure error	108.473	12	9.039		
Cor. total	567.765	14			
R^2 87.89; adjusted R^2 80.89					

For native date seeds, the H/S ratio (X_3) was the most influential factor (Table 5), followed by the second-order term of sonication temperature (X_1^2) and interaction term between temperature and H/S ratio (X_1X_3). In addition, sonication time (X_2), interaction term between time and H/S ratio (X_2X_3), second-order term of H/S ratio (X_3^2), and sonication temperature (X_1) were significant. The significant interactions among variables are presented in Figure 2 as response surfaces. The interaction between temperature and H/S ratio (X_1X_3) (Sub-Figure 2.1) showed that at low and high temperatures the recovery increased, especially at a high H/S ratio. The interaction between time and H/S ratio (X_2X_3) (Sub-Figure 2.2) was less significant, and the recovery decreased at a low H/S ratio and high sonication time, showing the opposite effect at a high H/S ratio. The reduced quadratic model that shows the relationship between the different factors and oil recovery is:

$$Y = 93.927 - 3.995X_1 - 0.177X_2 + 8.774X_3 \\ +0.061X_1^2 - 0.060X_1X_3 + 0.027X_2X_3 - 0.208X_3^2$$

For pre-treated seeds, the results were much simpler. For 180 ºC-treated seeds, only sonication time (X_2) and H/S ratio (X_3) showed significance. The reduced quadratic model that shows the relationship between the different factors and the oil recovery is:

$$Y = 47.045 + 0.226X_2 + 3.388X_3$$

In Figure 3, the main effects are presented graphically. It is clear that for native seeds, sonication temperature and H/S ratio led to variations in oil recovery. By examining the H/S ratio, it seems that recovery did not reach its maximum and that, at higher ratios, recovery would increase. This aspect needs further confirmation. This factor was also the most influential for treated seeds, reaching maximum recovery at close to 7. For 180 ºC-treated seeds, sonication time also produced significant increases in recovery, as addressed in Table 5.

In summary, the H/S ratio was the most influential factor, although in some cases it was the time and/or temperature during sonication treatments as well. This ratio is also discussed in most published works, and is identified as the most significant fac-

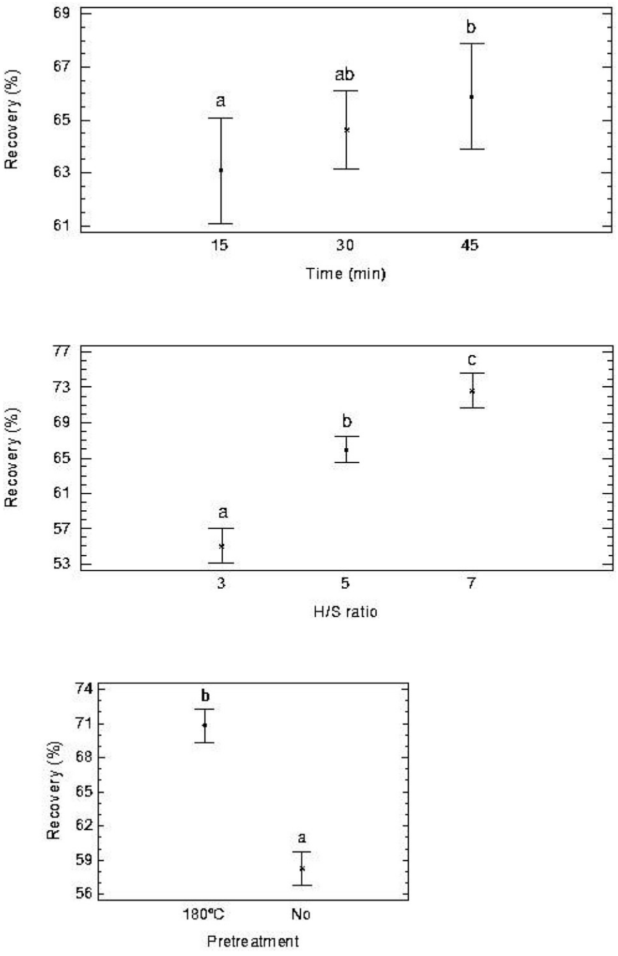

FIGURE 1. ANOVA average values for oil recovery and LSD intervals for the different significant factors: A, sonication time; B, H/S ratio; C, pretreatment.

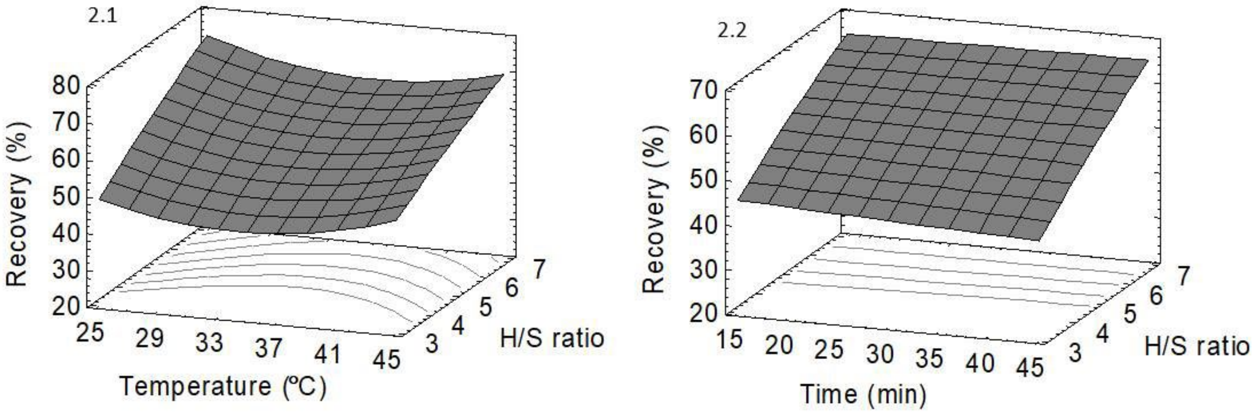

FIGURE 2. Response surface plots of interactions between sonication temperature (ºC) and hexane/solid ratio (2.1), and sonication time (min) and hexane/solid ratio (2.2) on oil extraction yield (%) for native date seed sample.

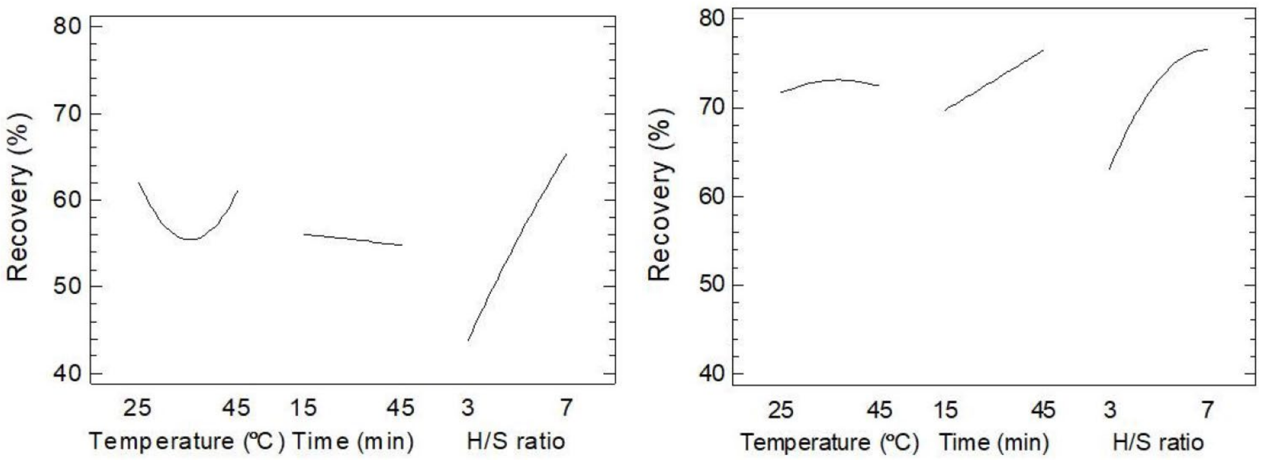

FIGURE 3. Main effect plots showing the effect of sonication temperature, sonication time and hexane/solid ratio on date seed oil recovery.

tor in the ultrasound-assisted extraction of oil from capers (Ara *et al*., 2014), black seeds (Abdullah and Koc, 2013), and rapeseed flakes (Perrier *et al*., 2017). Sonication time and temperature, and their interactions with the H/S ratio had different effects, depending on materials and other experimental conditions, as occurred in our study.

3.4. Optimization and validation of the different models presented

A statistical study of optimization was carried out to find the optimal extraction conditions for each sample. To validate the model, three experiments were developed at optimal conditions and a t-test was used to verify significant differences (confidence level 95%) between the predicted values and the experimental ones.

For native seeds, the highest predicted recovery (74.32%, i.e. 5.42% oil yield) was found at sonication temperature and time of 45 ºC and 15 min, and at the H/S ratio of 7. The experimental average was 71.03±2.33% (5.18±0.17% as oil yield). For treated seeds at 180 ºC, the highest recovery (80.93%, i.e. 6.71 oil yield) was found with 45 min sonication, at 35 ºC and H/S ratio of 7. A mean value of 79.28±0.83% recovery (6.58±0.07% oil yield) was obtained. In any case, there were no significant differences between predicted and experimental values for recovery percent, so the validity of the designed models was confirmed. The recovery increased with the hydrothermal pre-treatment, increasing from 71% in untreated date seeds to 80% in those treated at 180 ºC. This higher oil yield could be related to the disorganization of the seed matrix due to hydrothermal pre-treatment, which facilitated oil extraction.

Similar recoveries (70-80%) were found in the bibliography when working with black seeds, 74.77% (Abdullah and Koc, 2013) or grape seeds, 74.77-82.9% (Böger *et al*., 2018; Malicanin *et al*., 2014), the highest recoveries reported. Oils from winter melon (Bimakr *et al*., 2012) or pomegranate seeds (Goula, 2013) were recovered in lower amounts, 43.2 and 59.8%, respectively. For H/S ratio, most authors found that the bests conditions were at high ratios, e.g. 20 for kiwi and pomegranate seeds (Cravotto *et al*., 2011; Goula, 2013), or even higher (40 for rapeseed flakes (Perrier *et al*., 2017)). It is clear that the volume of hexane used for extraction has a positive influence on oil recovery, but some authors pointed to its negative effect on the oxidative stability of the oil (Samaram *et al*., 2015). Sonication temperature and time at optimum conditions were very similar to ours, varying between 20 and 50 ºC and 20 and 45 min.

3.5. Oxidative stability of extracted oils

As pointed out throughout this work, ultrasound could affect the oil's oxidative stability under certain conditions. To confirm the influence of ultrasounds on the stability of date seed oil, an assay was developed in which the absorbance at 540 nm was

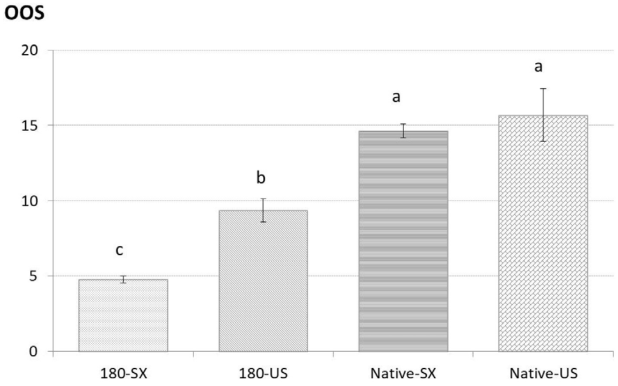

FIGURE 4. Oxidative stability of optimized oils from native date seeds and seeds treated at 180 ºC. The extractions were performed by Soxhlet (SX) or ultrasound-assisted (US). The results are the average values of four replicates. Different letters mean significant differences at $p < 0.05$.

inversely proportional to oxidative stability. The compounds resulting from oil oxidation (aldehydes, ketones, etc.) are TBA-reactive species, therefore unstable oils are easily oxidized at TBA assay conditions and give high 540 nm absorbance values. The results for OOS are shown in Figure 4. For native seeds, no differences were found between Soxhlet and UAE oils. The extractions at relatively low H/S ratio, as presented in this work, could maintain oil stability better than when extracted at higher ratios (>10) (Samaram *et al*., 2015).

Significant decreases in oil stability were quantified in the case of 180 ºC-treated seeds. These results suggested that, in any case, ultrasounds did not lead to a loss in stability when compared to Soxhlet, but hydrothermal pretreatment did. With 180 ºC pretreatment, the oil recovered by both sonication and Soxhlet was less stable. The higher resistance to oxidation in UAE oils from pre-treated seeds (180-US) when compared to its corresponding Soxhlet oil (180-SX) could be due to a more effective extraction of antioxidant molecules which are originated during the hydrothermal pre-treatment of date by-products (Mrabet *et al*., 2015). Pre-treatments could also destroy the naturally present antioxidants in date seed oil, but this loss could be partially counteracted by the appearance of new antioxidant compounds during hydrothermal treatments.

4. CONCLUSIONS

The extraction of oil from date seeds can be a profitable way to give added value to this by-product from date fruit processing industries. By applying environmentally friendly technologies (hydrothermal treatments and sonication), oil yield would be close to that obtained by traditional extraction methods (Soxhlet). With sonication alone, a recovery of nearly 75% was achieved, working at 45 ºC for 15 min, instead of 8 h at 70-80 ºC (Soxhlet conditions). In addition, the volume of solvent is much lower in assisted extractions (optimized H/S ratio near 7) than in the traditional ones (H/S ratio 20) and, under these conditions, the OOC did not decrease, probably due to the low H/S ratio. These results suppose savings in working time, energy and solvent, which would counter the lower sonication extraction yields. Higher recoveries were reached when working with pre-treated seeds, but the oil's stability decreased. From these results, the sonication-assisted extraction of untreated seeds seemed to be the most adequate system for developing an energy- and solvent-saving process, with valuable extraction yield and good oil stability. It would be interesting to study the fatty acid composition and bioactive compounds present in the oil (tocopherols, sterols, and phenols) in order to monitor the effects of the applied treatments to the chemical composition of the oils. Knowledge about how the different parameters affect oil composition and stability is of great interest for the industrial application of the proposed technology.

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Determination of nutritional health indexes of fresh bovine milk using near infrared spectroscopy

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SUMMARY: Bovine milk is one of the most complete foods that exist. During the last decades, milk FA have shown to improve human health due to the reduction in risk of cardiovascular disease and related pathologies. The aim of this study was to evaluate the feasibility of near infrared spectroscopy (NIRS) reflectance analysis to predict the nutritional value, fatty acid (FA) composition, and health index of fresh milk from dairy cows of pastoral systems. The prediction of Atherogenicity and Thrombogenicity indexes, along with other FA ratios in fresh milk samples by NIRS were precise and accurate. In addition, the calibration model obtained by NIRS provides an opportunity for the routine quantification of milk's healthy FA such as omega-3 and conjugated linoleic acid (CLA), with applications in the dairy industry for food labeling, and at the farm level for management of the dairy cow's diet.

KEYWORDS: *Chemometrics; Conjugated linoleic acid; Dairy fat; Health indexes; Human nutrition.*

RESUMEN: ***Determinación de los índices de salud nutricional de la leche fresca de bovino mediante espectroscopía de infrarrojo cercano.*** La leche bovina es uno de los alimentos más completos que existe. Durante la última década, se ha demostrado que los ácidos grasos de la leche pueden mejorar la salud humana, a través de la reducción del riesgo de enfermedades cardiovasculares y patologías asociadas. El objetivo de este estudio fue evaluar la factibilidad del análisis de reflectancia NIRS para predecir valor nutricional, composición de ácidos grasos e índices de salud de leche fresca de vacas de sistemas lecheros pastoriles. La predicción por NIRS del índice aterogénico y trombogénico, de ácidos grasos en muestras de leche fresca, fueron precisos. Por tanto, el modelo de calibración obtenido por NIRS representa una oportunidad para la cuantificación rutinaria de los ácidos grasos saludables de la leche como omega-3 y CLA, con aplicaciones en la industria lechera para el etiquetado nutricional y a nivel de lechería para el manejo de la alimentación de las vacas.

PALABRAS CLAVE: *Ácido linoleico conjugado; Grasa láctea; Índices de salud; Nutrición humana; Quimiometría.*

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1. INTRODUCTION

Bovine milk is one of the most complete foods that exists. It provides energy as lactose lipids, protein, and vitamins and minerals. Particularly, milk fat is made up of a complex mixture of lipids that mainly include triglycerides, phospholipids, and cholesterol; and it is considered an essential milk constituent in terms of its nutritional value, technological aptitude for manufacturing dairy products, and the palatability that it delivers to dairy products (Rodríguez-Alcalá *et al*., 2009).

During the last decades, it has been shown that milk FA improves human health (Shingfield *et al*., 2013) due to a reduction in the risk of atherosclerosis, hypercholesterolemia and other factors related to cardiovascular disease (Salter, 2013). Nonetheless, from a nutritional perspective, the effects of individual conjugated linoleic acids (CLA) are not well elucidated due to the difficulty in isolating individual CLA isomers. Therefore, most studies have used predominantly 18:2*cis*-9, *trans*-11 (9,11 CLA or rumenic acid) or 18:2*trans*-10, *cis*-12 (10,12 CLA) within a mixture of CLA isomers and other FA. Both 9,11 CLA and 10,12 CLA are the most abundant CLA isomers in milk, accounting for approximately 85 and 10% of all CLA isomers naturally present in milk, respectively (Den Hartigh, 2019). Recent research suggests that the beneficial effects of CLA are mainly related to rumenic acid (c9t11, RA) and its precursor (t11 18:1, vaccenic acid), and that RA and t10c12 would exert different physiological effects (Gómez-Cortés *et al*., 2018). Moreover, conjugated linoleic acids have been shown to reduce the risk of cardiovascular disease, type 2 diabetes, rheumatoid arthritis, asthma, degenerative diseases associated with age, and some types of cancer (Preble *et al*., 2019). At the same time, 10,12 CLA has shown an anti-lipogenic effect in lipogenic tissues such as liver, mammary and adipose tissue (Park and Pariza, 2007). Omega-3 FA are essential FA that are found in bovine milk, and possess well-known anti-inflammatory properties (Den Besten *et al*., 2013). The contents in healthy or beneficial FA in bovine milk depend mostly on the composition of the dairy cow's diet, with greater milk CLA and n-3 FA when the diet is based on pasture grazing as compared to mix diets with preserved forage and grains (Morales *et al*., 2015).

In addition, milk is also an important source of saturated FA, especially whole milk and high-fat dairy products (e.g., cream, butter). Saturated FA have been claimed to be harmful due to the association between saturated fat intake and cardiovascular disease. However, this harmfulness has been recently challenged by new research (Siri-Tarino *et al*., 2010). In this sense, there is evidence that dietary exposure to whole dairy products can substantially affect several health conditions, even chronic disease by reducing risk in later life (Markey *et al.,* 2014; Givens, 2020). Yet, it is now unclear whether saturated FA are harmful or not to human health, and therefore the use of low-cost indexes that may better characterize the diet in human population studies is timely.

Ulbricht and Southgate (1991) proposed two indexes that characterize the atherogenic and thrombogenic potential of the diet based on the content in saturated (SFA) and unsaturated FA, in addition to the polyunsaturated (PUFA) to SFA ratio. The atherogenic (AI) and thrombogenic index (TI) consider the effects of FA on human health, as well as the probability of an increase in incidence of injuries such as atheroma and/or thrombus formation (Pilarczyk *et al*., 2015). Another index regarding the profile of FA is the n-6 to n-3 ratio, which is a numerical balance between these FA, as n-6 and n-3 have distinct metabolic pathways, both necessary for physiological functions (Simopoulos, 2002).

For the analysis of this type of compounds, gas chromatography has traditionally been used as a technique of proven specificity and robustness even when it requires considerable time, highly trained personnel, use of many solvents and reagents, and therefore, is an expensive analysis.

In the last decades, new instrumental methods that are as robust and reliable as conventional methods have been developed. One of them is Near-Infrared Spectroscopy (NIRS), a method that captures the reflectance spectrum of a sample in a range of 780-2500 mm, corresponding to NIR. Briefly, the radiant energy of a sample is absorbed, according to the vibration frequency of the molecules present, which generates an overtone in the spectrum (Conzen, 2006). Vibrations in C-H, O-H, N-H chemical bonds produce reflectance signals which serve to identify the relative proportion of each element in the analyzed sample (Cécillon *et al*., 2009). NIRS technology has been reported to be a rapid, consistent, and inexpensive tool for predicting authenticity control, sensory evaluation, rheological and technological properties, and physical attributes in solid, dry, paste, and liquid samples in diverse matrices (Porep *et al.*, 2015). NIRS has been used in the dairy industry for over 30 years, in liquid and oven-dried milk samples for the analysis of major components (fat, protein, lactose, moisture, etc.) without sample pre-treatment, and recently for FA composition in liquid and dry milk (Coppa *et al.*, 2010; Coppa *et al.*, 2014). In addition, the prediction of AI or TI by the use of NIR has only been reported by Nuñez-Sanchez *et al.* (2016), Nuñez-Sanchez *et al.* (2020) and Llano-Suarez *et al.* (2018).

The objective of this study was to evaluate the feasibility of NIRS reflectance analysis to predict the nutritional value, FA composition, and health indexes of fresh milk from cows of pastoral systems.

2. MATERIALS AND METHODS

2.1. Milk sampling collection

A total of 175 fresh milk samples were used in this study, obtained from 2 dairy farm studies conducted in Los Lagos Region, Chile, between 2018 and 2020 as follows.

Set 1: Between October 2018 and March 2019, 133 bulk tank milk samples were collected from a dairy farm in Fresia, Los Lagos Region, Chile (41 ° 28′18 ″ S 72 ° 56′12 ″ W). During this period, the tested dairy farm had 43 lactating Holstein Friesian cows with an average production of 21 L/cow/day. Their diet was composed of grazed pasture [*Lolium Perenne* (54%), *Lolium Trifolium* (34%), *Holcus Lanatus* (7%), Trifolium repens (2%) and 3% of other species] and 2 kg of a pelleted concentrate (2 kg/cow/day).

Set 2: Between August 2019 and February 2020, 42 milk samples were collected from milk collection trucks at a dairy processing facility in La Araucanía region. The sample collection period represented the pasture-grazing period of the year, and all milk sampled from the trucks was produced in pasture-grazing based dairies.

All milk samples collected were kept at 4°C during transport to the NIR spectroscopy laboratory of INIA-Remehue. Each fresh milk sample was registered by NIRS and subsequently stored at -80 ° C until FA analysis by gas chromatography was carried out.

2.2. Fatty acids analysis

The derivatization of milk fat was performed with 3 mL of fresh milk at room temperature using the double fat extraction method of chloroform and methanol 1:1 (Kramer *et al.*, 2008). For FA methylation, a base-catalyzed methylation procedure with sodium methoxide was used (i.e. 0.5N methanolic base #33080, Supelco Inc., Bellefonte. PA) as described by Cruz-Hernandez *et al.* (2006). Prior to methylation, 1 mL of internal standard was added to the sample for FA quantification (1 mg/mL of 23:0 methyl ester, n-23-M, Nu-Chek Prep Inc., Elysian. MN. USA). The contents in FA methyl esters (FAME) were expressed as g per 100 g of FAME quantified and as mg of FAME per 100 mL of fresh milk.

The FAME were analyzed using a GC equipped with a flame ionization detector (GC-2010 Plus; Shimadzu®. Kyoto. Japan), a capillary column (SP-2560; 100 m × 0.25 mm (i.d.) with 0.2-μm film thickness; Supelco Inc., Bellefonte. PA. USA) and an ionic liquid capillary column (SLB-IL111; 100 m × 0.25 mm (i.d.) with 0.2-μm film thickness; Supelco Inc., Bellefonte. PA. USA) to confirm the identification of several biohydrogenation intermediates such as CLA isomers and other *trans* FA (Delmonte *et al.*, 2011). The samples were analyzed with two GC temperature programs that plateaued at 175 °C and 150 °C (Kramer *et al.*, 2008). Hydrogen was used as carrier gas in both columns, with a constant flow rate of 1 mL/min. and the injector and detector temperatures were set at 250 °C.

For peak identification, one reference standard (GLC463), individual FAMEs (21:0. 23:0. 26:0), and a CLA mixture (9c,11t-/8t,10c-/11c,13t-/10t,12c-/8c,10c-/9c,11c-/10c,12c-/11c,13c-/11t,13t-/10t,12t-/9t,11t-/8t,10t-18:2; UC-59M) were used, all of which were obtained from Nu-Chek Prep Inc. (Elysian, MN, USA). Isomerized mixtures of linoleic (18:2*n*−6) and linolenic (18:3*n*−3) acids were purchased from Sigma-Aldrich, and branched-chain FA (BCFA) were identified using a bacterial FAME mixture from Matreya (Pleasant Gap, PA, USA).

2.3. Health index calculations

Atherogenic (AI) and Thrombogenic (TI) indexes were calculated as in Ulbricht and Southgate (1991), as follows:

AI = [12:0 + (4 × 14:0) + 16:0] / [Σ Monounsaturated (MUFA) + PUFA-*n6* + PUFA-*n3*]

TI = (14:0 + 16:0 + 18:0) / [(0.5 × ΣMUFA + 0.5 × PUFA-*n6* + 3 × PUFA-*n3*) + (PUFA-*n3* / PUFA-*n6*)]

2.4. NIRS and chemometric analysis

For spectral analysis, 175 fresh milk samples in reflectance mode were scanned using NIR spectroscopy (MPA-FT NIR, Bruker Optik GmbH, Ettlingen, Germany). Spectral data were transformed to absorbance (A) according to the equation: A = log10 (1/R), where R is the reflectance obtained at each wavenumber from 12.000-4.000 cm^{-1} (NIR region) with 16 cm^{-1} resolution and 64 scans (Figure 1). Partial least-squares regression (PLSR) with leave-one-out (LOO) cross validation was performed to fit predictive models using chemometrical software OPUS version 6.5 (Bruker Optik GmbH, Melvyn Becerra Cia. Ltda). For the cross-validation method, the selection of validation samples was based on spectral information as described by Conzen, (2006).

In addition, external validation was performed with a random subset of 10 samples which were not included in the calibration and cross validation method.

The software OPUS was used to apply different preprocessing to spectra:vector normalization (VN), multiplicative scatter correction (MSC), straight line subtraction (SLS), first derivative (FD), and second derivative (SED). Outliers were identified and removed during the calibration process to improve precision and model performance.

The criteria used in choosing the best prediction model considered: i) low root mean square error of cross validation (RMSECV), ii) high coefficients of determination in cross-validation (R^2cv), iii) root mean square error of estimation (RMSEE); iv) residual predictive deviation (RPD: ratio between the

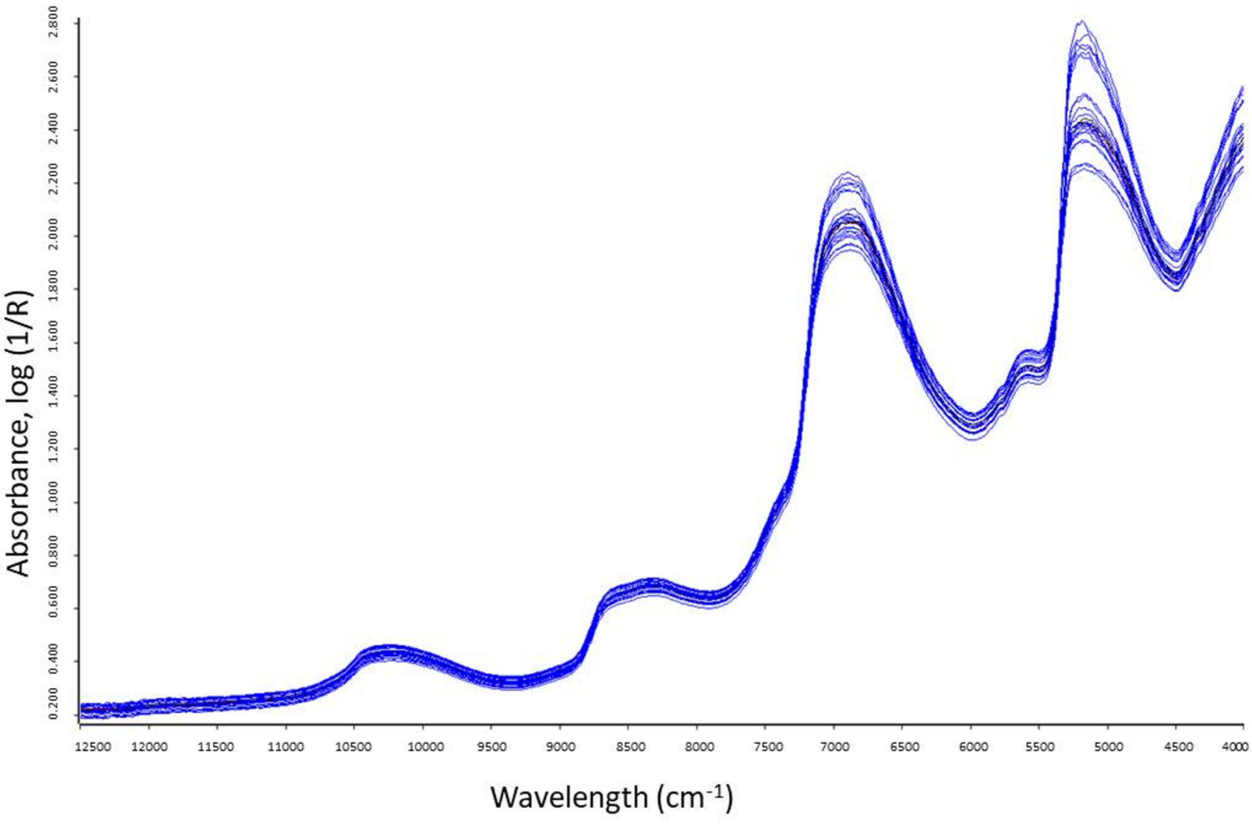

Figure 1. Average absorbance (log (1/R)) of near-infrared spectra for liquid milk NIR calibration results. PLS: Number of PLS factors; Treatment: [(MSC:Multiplicative Scatter Correction); (VN:Vector Normalization); (FD+VN: First Derivate + Vector Normalization); (COE: Constant Offset Elimination); (NSDP: No Spectral Data Preprocessing)]; R_c^2: Coefficient of determination of the calibration set; RMSEE: root mean square error of estimation; RPD: residual prediction deviation; R_{cv}^2: Coefficient of determination of cross validation; RMSECV: root mean square error of cross validation.

standard deviation -SD- of the reference values and the error of prediction), and v) number of factors (Conzen, 2006).

In this study the criteria for selection were the lowest RMSECV, the lowest number of PLS factors and the highest RPD. Small error of cross validation was desired, as this would result in greater RPD values, and a better prediction model.

3. RESULTS

3.1. Milk fatty acid composition

The fatty acid methyl ester contents in the milk of the 2 sets of samples used in this study are expressed as % of total FAME (g/100g) and mg FAME per 100 mL of milk and shown in Table 1. The ratio of n-6 to n-3 was 1.69 ± 0.5 with a range between 1.14 and

TABLE 1. Fatty acid methyl esters (FAME) and health indexes of milk samples quantified by gas chromatography.

FAME[a]	**g/100g FAME**			**mg FAME /100mL milk**		
	Mean ± SD	Min	Max	Mean ± SD	Min	Max
C 8:0 Caprylic acid	0.6 ± 0.3	0.0	1.3	26.7 ± 16.6	0.0	82.6
C 10:0 Capric acid	2.7 ± 0.5	1.0	4.1	107.6 ± 35.2	34.3	282.1
C 12:0 Lauric acid	3.8 ± 0.5	2.8	5.4	151.7 ± 44.0	85.7	396.9
C 14:0 Myristic acid	12.6 ± 0.9	10.1	15.5	503.4 ± 144	319.8	1382.2
C 16:0 Palmitic acid	31.4 ± 0.3	25.4	36.8	1260.4 ± 373.3	734.6	3506
C 18:0 Stearic acid	10.3 ± 1.3	7.3	14.7	411.8 ± 112.6	252	1044
C 9-18:1 Oleic acid	18.8 ± 1.5	14.4	23.7	753.2 ± 204.6	435.3	1968.5
10 *trans*-18:1	0.32 ± 0.16	0.16	1.61	12.6 ± 7.20	5.3	62.3
11 *trans*-18:1	2.15 ± 0.67	1.03	4.83	86.1 ± 29.94	32.6	200
Linoleic acid (LA)	1.33 ± 0.31	0.88	2.49	53.2 ± 20.61	23.2	162
Linolenic acid (LNA)	0.71 ± 0.10	0.41	1.09	28.2 ± 8.24	15.4	87.3
Eicosapentaenoic acid	0.07 ± 0.02	0.00	0.11	3.02 ± 1.08	0.06	10.3
Docosahexaenoic acid	0.10 ± 0.03	0.04	0.31	4.23 ± 1.52	1.36	12.3
Total n-6	1.50 ± 0.35	0.99	2.84	60.3 ± 24.4	26.0	1867
Total n-3	0.91 ± 0.13	0.54	1.35	36.2 ± 11	20	115
∑ CLA	1.12 ± 0.27	0.57	2.33	44.7 ± 15.5	15.5	129
Saturated FA (SFA)	66.7 ± 2.29	59.3	72.6	2692 ± 777	1658	7366
Monounsaturated FA (MUFA)	28.3 ± 1.84	23.1	32.9	1138 ± 311	648	3009
Polyunsaturated FA (PUFA)	2.42 ± 0.38	1.75	3.64	96.5 ± 32.6	46	3012
Unsaturated FA (UFA)	30.7 ± 1.96	25.7	35.9	1234 ± 340	694	3311
Hypercholesterolaemic FA (HFA)	47.7 ± 2.94	38.9	54.8	1932 ± 570	1160	5284
∑ Odd and branched FA	4.10 ± 0.34	3.36	4.93	163 ± 43.9	102.6	448
Health Indexes						
n-6/n-3	1.69 ± 0.5	1.14	5.01	1.69 ± 0.5	1.19	5.01
UFA/SFA	0.46 ± 0.05	0.35	0.60	0.46 ± 0.05	0.35	0.60
HFA/UFA	1.57 ± 0.2	1.09	2.13	1.57 ± 0.2	1.09	2.13
h/H	0.49 ± 0.1	0.34	0.72	0.49 ± 0.1	0.34	0.72
LA/LNA	1.91 ± 0.6	1.23	5.41	1.91 ± 0.6	1.23	5.41
Thrombogenic index (TI)	3.09 ± 0.3	2.16	3.88	3.09 ± 0.3	2.25	3.98
Atherogenic index (AI)	2.86 ± 0.4	1.96	4.07	2.84 ± 0.4	1.96	4.07
PUFA/SFA	0.04 ± 0.01	0.03	0.06	0.04 ± 0.01	0.03	0.06
MUFA/SFA	0.43 ± 0.04	0.32	0.55	0.43 ± 0.04	0.32	0.55

[a]SFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; total PUFA: polyunsaturated fatty acids; UFA: total unsaturated fatty acids (MUFA+PUFA); HFA: hypercholesterolaemic FA (Σ C12:0+C14:0+C16:0); h/H: hypocholesterolaemic/hypercholesterolaemic ratio [C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)/(C14:0+C16:0) (Santos-Silva *et al*., 2002); Σ Odd and branched FA (Σ C11:0; isoC14:0; isoC15:0; anteisoC15:0; C15:1; iso C16:0; C17:0; isoC18:0; C17:1); EPA: Eicosapentaenoic acid (20:5n-3); DPA: Docosapentaenoic acid (22:5n-3); LA: Linoleic acid (18:2n−6) (LA); LNA: Linolenic acid (18:3n−3); n-6/n-3: ratio omega 6 FA/omega 3FA; Atherogenic Index (AI)= [12:0 + (4 × 14:0) + 16:0] / (ΣMUFA + PUFA-*n6* + PUFA-*n3*) Ulbricht and Southgate (1991); Thrombogenic Index (TI)= (14:0 + 16:0 + 18:0) / [(0.5 × ΣMUFA + 0.5 × PUFA-*n6* + 3 × PUFA-*n3*) + (PUFA-*n3* / PUFA-*n6*)] Ulbricht and Southgate (1991). For the calculation of total conjugated linoleic acids (Σ CLA), all the CLA isomers identified in fresh milk samples by GC were summed.

5 g/100g of FAME. The TI and AI were 3.09 ± 0.3 and 2.86 ± 0.4 g/100g of FAME, respectively, with a range of 2.16 to 3.88 and 1.96 to 4.07 for TI and AI, respectively.

3.2. NIR models

NIR spectral features. Average absorbance of NIR spectra for liquid milk presented two bands with maxima at 7500-6400 cm^{-1} and 5400-4900 cm-1 related to O-H first overtone and O-H combination band (Figure 1).

NIR calibration. The calibration statistics for individual FA and groups of FA and indexes used to evaluate the nutritional health properties of food (considering the potential negative or positive effects) in fresh bovine milk are detailed in Table 2. The coefficient of determination in the calibration sets fluctuated between 0.76 and 0.95. The RPD values varied between 2.1 and 4.3. The number of PLS factors values varied between 2 and 10. All pre-pre-processing methods used were different and in accordance to the main functional groups of FA and health related indexes. The relation between NIRS prediction and composition obtained by the reference methods for all main functional groups of FA and health indicators are shown in Figures 2a and 2b.

External validation. Table 3 shows the means of the residuals and the Root Mean Standard Error (RMSE) obtained from the external validation. The levels of significance obtained were between 0.09 for 10 *trans*-18:1 and 0.88 for LA. Therefore, no differences between the spectroscopic and chromatographic method were detected.

4. DISCUSSION

4.1. Milk fatty acid composition

Regarding the FA composition of milk, the FAME contents in this study were in the same range reported previously for Holstein Friesian's milk from Chilean dairies (Morales *et al*., 2015; Vargas-Bello *et al*., 2015), in milk from dairy processing facilities in Southern Chile (Pinto *et al*., 2002), and in pasture-grazing based dairies from other regions (Nantapo *et al*., 2014). For instance, total SFA (66.6 g/100 g of FAME) was in the same range reported for grazing animals by Morales *et al*. (2015); 67.19 g/100 g, and for TMR-fed Holstein Friesian cows of the control group of Vargas-Bello *et al*. (2015); 68.2 g/100 g.

Our reported milk FAME content is not comparable to other studies where diet relies on a total mixed ration based on preserved forages and grains because diet and fresh forage inclusion in the diet have a major impact on the FA profile of milk (Sun and Gibs, 2012). Although our study only included milk samples of Holstein Friesian cows, greater variances and range of milk FA observed in other studies could be explained, in lesser magnitude, by the inclusion of other dairy breeds. In this sense, Coppa *et al*. (2014) reported milk FA composition for a heterogeneous productive system that included five different dairy cow breeds present in northwest Italy, with total milk SFA and total CLA (64.28 and 0.93 g/100 g of FA, respectively) below our reported values (66.7 and 1.12 g/100 g FA, respectively). On the contrary, this last study reported greater values for MUFA, PUFA, and n-6/n-3 (29.63; 5.17; 3.42 g/100 g of FA, respectively) than our study, which could be explained by the use of TMR and a more diverse set of cow genetics that included Jersey cows.

The nutritional and health indexes PUFA/SFA, n-6/n-3, AI, and TI, are commonly used to evaluate the nutritional value and effects of edible products on consumer health. In general, a ratio of dietary PUFA to SFA above 0.45 and a ratio of *n-6/n-3* below 4.0 are expected to reduce the risk of diseases such as coronary heart disease and cancer (Simopoulos, 2002). Furthermore, the low PUFA/SFA ratio (0.04 g/100g FAME) reported in this study was due to the high SFA content in the two sets of milk samples analyzed. The *n-6/n-3* ratio obtained in the current study (1.69 g/100 g of FAME) is lower than the ratio reported by Morales *et al*. (2015), most likely because of differences in pasture botanical composition. Indeed, some authors have indicated that the PUFA/SFA ratio may not be adequate to evaluate the nutritional value of dietary fat, as it ignores the effects of MUFA and also, some SFA have no effect on plasma cholesterol (Orellana *et al*., 2009).

Milk atherogenic and thrombogenic indices in cattle from different breeds and feed and management systems have been previously reported. Kuczyńska *et al*. (2012) and Pilarczyk *et al*. (2015) reported AI of 2.1 and 2.5 in dairy cows from a pasture-based and a total mixed-ration-based dairy system, respectively, values which are slightly lower than those reported in our study. Nantapo *et al*.

TABLE 2. Statistical descriptors for the partial least-squares regression (PLSR) predictions for fatty acid methyl esters (FAME) in milk samples and corresponding health indexes.

FAME[a]	**#PLS**	**Treatment**	**Spectral region (nm)**	**R_C^2**	**RMSEE**	**RPD_C**	**R_{CV}^2**	**RMSECV**	**RPD_{CV}**	**Range**	**N**
10 *trans*-18:1	6	VN	12489.5-7498.3; 4605.4-4242.9	0.84	0.9	2.5	0.76	1.1	2.0	5.3 - 21.1	96
11 *trans*-18:1	4	FD+VN	6102-5446.3	0.78	9.7	2.1	0.74	10.2	2.0	36.81 - 138.6	104
Linoleic acid (LA)	9	COE	12489.5-7498.3;6102-5446.4;4605.4-4242.9	0.94	4.2	3.9	0.84	6.3	2.5	28.52 - 131.4	118
Linolenic acid (LNA)	9	COE	9295.7-6094.3; 5454-4844.6	0.82	2.6	2.4	0.73	3.2	1.9	19.58 - 61.69	99
Eicosapentaenoic acid	5	MSC	9997.7- 7498.3; 6102- 5770.3	0.77	0.4	2.1	0.71	0.4	1.9	1.40 - 6.35	127
Docosahexaenoic acid	2	MSC	12489.5- 7498.3; 6102- 5446.3	0.82	0.4	2.3	0.80	0.5	2.2	1.66 - 8.11	110
***Total n*-6**	7	VN	12489.5-7498.3; 6102-5446.3; 4428-4242.9	0.91	5.4	3.4	0.82	7.5	2.4	36.78 - 155.9	120
***Total n*-3**	4	MSC	12489.5-7498.3; 5454-4242.9	0.79	3.8	2.2	0.75	4.1	2.0	24.52 - 74.47	105
∑ CLA	6	VN	12489.5-7498.3; 6102-5446.3	0.89	4.4	3.0	0.82	5.3	2.4	22.68 - 104.7	116
Saturated FA (SFA)	5	MSC	12489.5-7498.3	0.89	190	3.0	0.80	249	2.3	1679 - 5442	132
Monounsaturated FA (MUFA)	4	MSC	12489.5-7498.3	0.90	86.9	3.1	0.85	102	2.6	727.8 - 2531	129
Polyunsaturated FA (PUFA)	7	VN	12489.5-7498.3; 6102-5446.3; 4428-4242.9	0.90	9.5	3.1	0.81	12.5	2.3	61.3 - 241.9	127
Unsaturated FA (UFA)	7	NSDP	12489.5-7498.3; 6102-5446.3	0.81	127	2.3	0.71	155	1.9	773.2 - 2773	150
Hypercholesterolaemic FA (HFA)	5	MSC	12489.5-798.3	0.87	155	2.8	0.77	201	2.1	1160 - 3859	140
∑ Odd and branched FA	5	MSC	12489.5-5446.3	0.81	13.4	2.3	0.77	14.5	2.1	113.1 - 307.4	102
Health Indexes											
***n*-6/*n*-3**	8	VN	12489.5-7498.3;6102-5770.3;4605.4-4242.9	0.91	0.1	3.3	0.77	0.1	2.1	1.288 - 2.662	110
UFA/SFA	8	COE	12489.5-9990; 6102-5446.3	0.94	0.0	3.9	0.79	0.0	2.2	0.379 - 0.576	109
HFA/UFA	7	COE	12489.5-7498.3; 6102-5770.3	0,86	0.1	2.7	0,76	0.1	2.1	1.16 - 1.96	114
h/H	6	COE	12489.5-7498.3; 6102-5446.3	0,83	0.0	2.4	0,71	0.0	1.9	0.34 - 0.59	107
LA/LNA	5	VN	12489.5-6094.3; 4605.4-4242.9	0,85	0.1	2.6	0,70	0.1	1.8	1.45 - 2.79	102
Thrombogenic index (TI)	9	NSDP	12489.5-7498.3; 6102-5446.3; 4428-4242.9	0,93	0.8	3.6	0,78	0.1	2.2	2.45 - 3.59	100
Atherogenic index (AI)	9	NSDP	12489.5-7498.3; 6102-5446.3 ;4428-242.10	0,95	0.1	4.3	0,81	0.1	2.3	1.96 - 3.69	109
PUFA/SFA	10	NSDP	9997.7- 7498.3; 6102-5446.3; 4428-4242.9	0,92	0.0	3.5	0,84	0.0	2.5	0.03 - 0.05	101
MUFA/SFA	6	NSDP	12489.5-7498.3; 6102-5446.3	0,76	0.0	2.1	0,62	0.0	1.6	0.36 - 0.49	100

[a] SFA: total saturated fatty acid; MUFA: total monounsaturated fatty acids; total PUFA: polyunsaturated fatty acids; UFA: total unsaturated fatty acid (MUFA+PUFA); HFA: hypercholesterolaemic FA (sum of C12:0. C14:0. and C16:0); h/H: hypocholesterolaemic/hypercholesterolaemic ratio [C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)/(C14:0+C16:0) (Santos-Silva *et al*., 2002); Σ Odd and branched FA (Σ C11:0; isoC14:0; isocC15:0; anteisoC15:0; C15:1; iso C16:0; C17:0; isoC18:0; C17:1); EPA: Eicosapentaenoic acid (20:5n-3); DPA: Docosapentaenoic acid (22:5n-3); LA: Linoleic acid (18:2n−6) (LA); LNA: Linolenic acid (18:3n−3); n-6/n-3: ratio omega 6 FA/omega 3FA; Atherogenic Index (AI)= [12:0 + (4 × 14:0) + 16:0] / (ΣMUFA + PUFA-*n6* + PUFA-*n3*) Ulbricht and Southgate (1991); Thrombogenic Index (TI)= (14:0 + 16:0 + 18:0) / [(0.5 × ΣMUFA + 0.5 × PUFA-*n6* + 3 × PUFA-*n3*) + (PUFA-*n3* / PUFA-*n6*)] Ulbricht and Southgate (1991). For the calculation of total conjugated linoleic acids (Σ CLA), all the CLA isomers identified in fresh milk samples by GC were summed. #PLS: Number of PLS factors; Treatment: ((MSC:Multiplicative Scatter Correction); (VN:Vector Normalization); (FD+VN: First Derivate + Vector Normalization); (COE: Constant Offset Elimination); (NSDP: No Spectral Data Preprocessing))R_c^2: Coefficient of determination of the calibration set; RMSEE: root mean square error of estimation; RPD: residual prediction deviation; R_{cv}^2: Coefficient of determination of cross validation; RMSECV: root mean square error of cross validation.

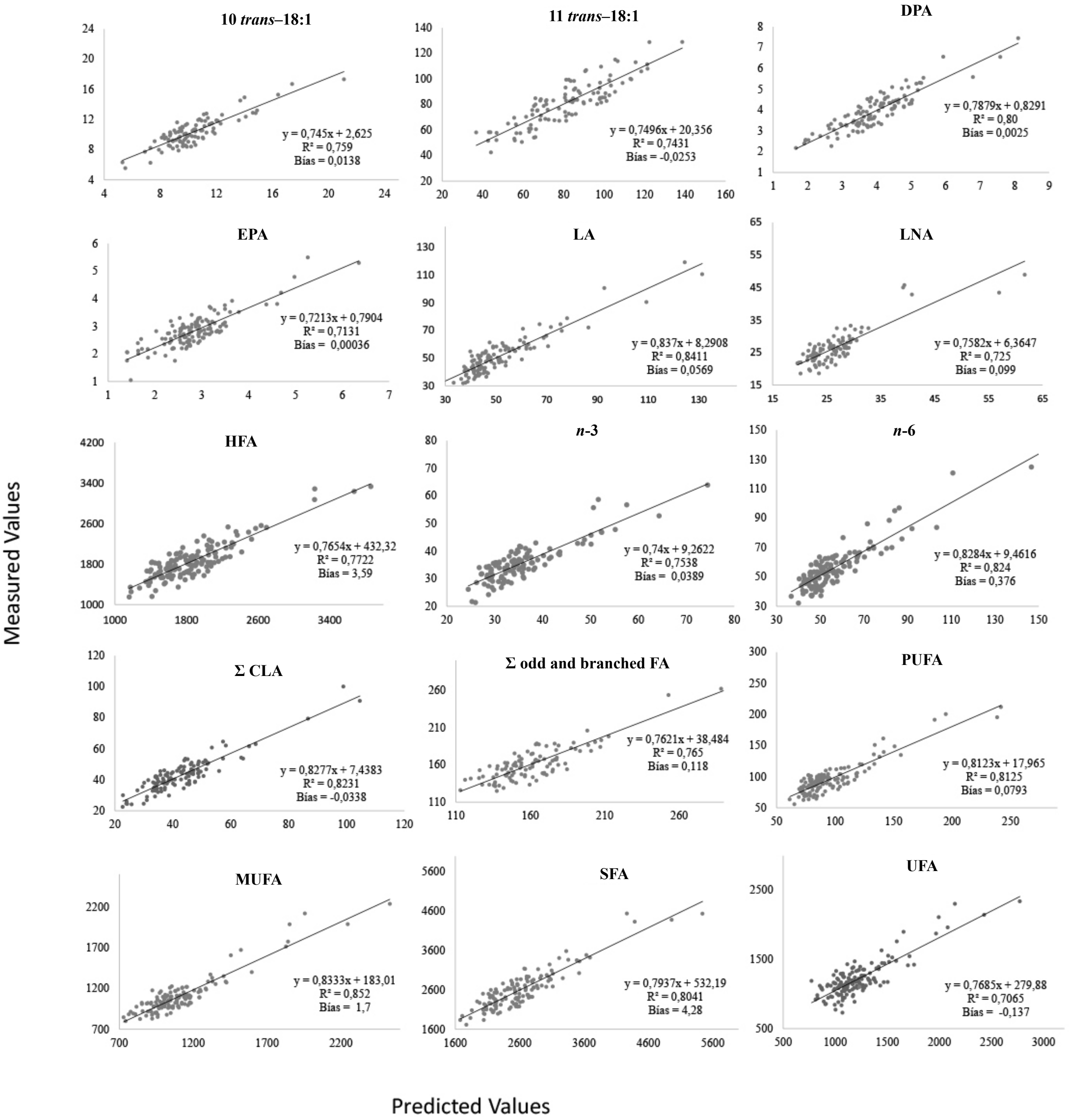

Figure 2A. Validation model performance for the correlation of values obtained in the laboratory with respect to those predicted by NIRS for a) 10 *trans*-18:1; b) 11 *trans*-18:1; c) Docosahexaenoic acid (DPA); d) Eicosapentaenoic acid (EPA); e) Linoleic acid (LA); f) Linolenic acid (LNA); g) Hypercholesterolaemic FA (HFA); h) Total n-3; i) Total n-6; j) ∑CLA; k) ∑ odd and branched FA; l) Polyunsaturated fatty acids (PUFA); m) Monounsaturated FA (MUFA); n) Saturated FA (SFA) and, o) Unsaturated FA (UFA) in fresh bovine milk.

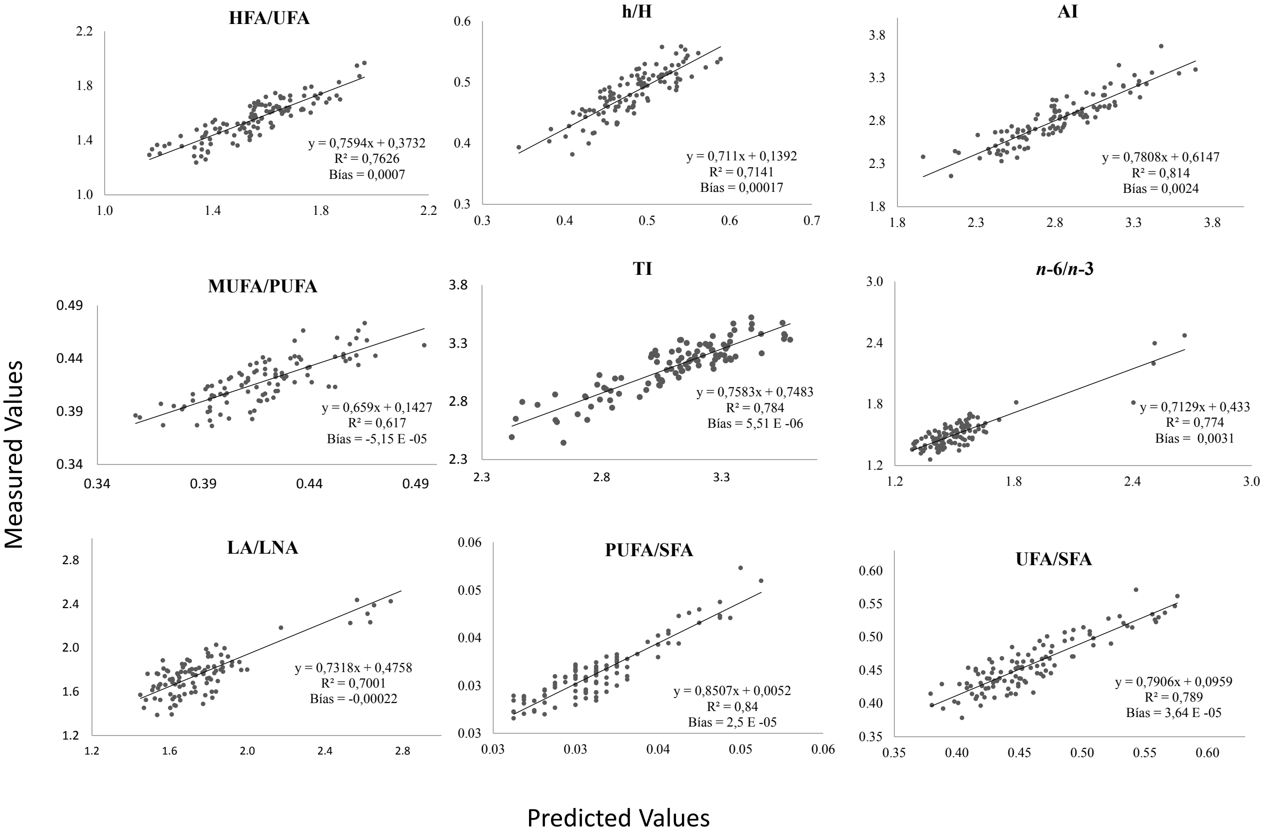

FIGURE 2B. Validation model performance for the correlation of nutritional and health indexes obtained in the laboratory with respect to those predicted by NIRS for a) HFA/UFA; b) h/H; c) AI; d) MUFA/PUFA; e) TI; f) n-6/n-3; g) LA/LNA; h) PUFA/SFA and i) UFA/SFA in fresh bovine milk.

(2014) reported milk AI values of 4.08 and 5.13 for Jersey and Friesian x Jersey cows, respectively. In Tarentaise and Montbeliarde cows, Ferlay *et al.* (2006) reported an AI of 3.14 and 3.43. On the other hand, the TI reported in the present study is within the range of that reported by Vargas-Bello *et al.* (2015), and below that of Thanh and Suksombat (2015), who reported 4.11.

4.2. NIR models

NIR spectral features. Average absorbance of NIR spectra for liquid milk presented small bands corresponding to FA and fat contents and appeared at 8900-7450 cm^{-1} and 6000-5300 cm^{-1}, associated with the first and second overtones from C-H stretching vibration of methyl ($-CH_3$), methylene ($-CH_2-$), and ethenyl (-CH=CH-). In addition, the absorption bands of bovine liquid milk used in this study were similar to those reported by Coppa *et al.* (2010) and Llano-Suaréz *et al.* (2018).

NIR calibration results. For model evaluation, Williams (2014) proposed R^2 y RPD as parameters that serve to classify NIR models into excellent or good, when R^2 fall above 0.91 or between 0.9 and 0.82, respectively. However, when R^2 falls between 0.81 and 0.66, the model will predict approximate values, and only simple discrimination of low, medium and high when R^2 is between 0.65 and 0.5. The RPD value is a measure of comparison between the standard error of the predicted values with the deviation of the references data, and therefore will evaluate the NIR calibration model (Williams, 2014; Nuñez *et al.*, 2016). Therefore, RPD above 4 and 3 are considered excellent and good, respectively, while values between 2.9 and 2 are acceptable for detection, between 2 and 1.5 acceptable for discrimination between low and high concentration, and below 1.5 are not useful.

Regarding R_{CV}^2 statistics, DPA, ∑CLA, SFA, MUFA, PUFA, n-6, AI, PUFA/SFA and LA, showed

Table 3. Level of significance, residual mean and root mean square error (RMSE) of milk fatty acid ethly esters (FAME) and corresponding health indexes. (n=10)

FAME[a]	**p (Level of significance)**	**Residual mean**	**RMSE**	**R^2**	**Bias**	**Slope**
10 *trans*-18:1	0.09	4.62	6.5	0.49	3.02	0.13
11 *trans*-18:1	0.44	22.4	27.8	0.61	2.20	0.43
Linoleic acid (LA)	0.88	9.24	10.8	0.81	0.56	0.95
Linolenic acid (LNA)	0.41	5.99	7.81	0.67	0.99	0.47
Eicosapentaenoic acid	0.17	0.77	0.96	0.72	0.43	0.55
Docosahexaenoic acid	0.22	0.44	0.56	0.94	022	0.89
Total n-6	0.38	9.22	12.8	0.84	3.18	0.98
Total n-3	0.64	5.74	7.69	0.83	1.21	0.67
∑ CLA	0.44	7.09	9.11	0.83	-1.20	0.87
Saturated FA (SFA)	0.36	3389	351.3	0.94	150.00	0.83
Monounsaturated FA (MUFA)	0.33	90	109.4	0.98	70.70	0.89
Polyunsaturated FA (PUFA)	0.45	11.3	14.7	0.92	2.10	1.08
Unsaturated FA (UFA)	0.36	124	143.5	0.94	61.40	0.84
Hypercholesterolaemic FA (HFA)	0.15	255	273.4	0.92	126.00	0.86
∑ Odd and branched FA	0.45	19.8	21.7	0.86	2.43	0.81
Health Indexes						
n-6/*n*-3	0.17	0.32	9.11	0.01	0.14	0.00
UFA/SFA	0.23	0.03	0.04	0.58	-0.01	0.69
HFA/UFA	0.23	0.03	0.04	0.74	0.02	1.00
h/H	0.84	0.01	0.02	0.94	0.00	1.01
LA/LNA	0.72	0.36	0.45	0.37	0.06	0.18
Thrombogenic index (TI)	0.81	0.11	0.13	0.85	0.01	0.97
Atherogenic index (AI)	0.48	0.14	0.15	0.90	0.04	0.85
PUFA/SFA	0.14	0.00	0.00	0.51	0.00	0.47
MUFA/SFA	0.27	0.02	0.03	0.73	0.01	0.52

[a] SFA: total saturated fatty acid; MUFA: total monounsaturated fatty acids; total PUFA: polyunsaturated fatty acids; UFA: total unsaturated fatty acid (MUFA+PUFA); HFA: hypercholesterolaemic FA (sum of C12:0. C14:0. and C16:0); h/H: hypocholesterolaemic/hypercholesterolaemic ratio [C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)/(C14:0+C16:0) (Santos-Silva *et al*., 2002); Σ Odd and branched FA (Σ C11:0; isoC14:0; isocC15:0; anteisoC15:0; C15:1; iso C16:0; C17:0; isoC18:0; C17:1); EPA: Eicosapentaenoic acid (20:5n-3); DPA: Docosapentaenoic acid (22:5n-3); LA: Linoleic acid (18:2n−6) (LA); LNA: Linolenic acid (18:3n−3); n-6/n-3: ratio omega 6 FA/omega 3FA; Atherogenic Index (AI)= [12:0 + (4 × 14:0) + 16:0] / (ΣMUFA + PUFA-*n6* + PUFA-*n3*) Ulbricht and Southgate (1991); Thrombogenic Index (TI)= (14:0 + 16:0 + 18:0) / [(0.5 × ΣMUFA + 0.5 × PUFA-*n6* + 3 × PUFA-*n3*) + (PUFA-*n3* / PUFA-*n6*)] Ulbricht and Southgate (1991). For the calculation of total conjugated linoleic acids (Σ CLA), all the CLA isomers identified in fresh milk samples by GC were summed.

good predictive capacity, with R_{CV}^2 values between 0.80 and 0.85. The R_{CV}^2 for EPA, n-3, n-6/n-3, TI, UFA, UFA/SFA, HFA, HFA/UFA, h/H, LNA, LA/LNA, 10 *trans*, 11 *trans*, and ∑ odd and branched FA were very acceptable with values from 0.7 to 0.8. The ratio MUFA/SFA had a R_{CV}^2 of 0.6. Therefore, this model is considered not suitable to be used; however, discriminations between high, medium and low concentrations could still be made. Regarding our data, RPD values ranged from 1.6 to 2.5, and were in accordance with those detailed above when comparing R_{CV}^2 values.

Possible explanations for our low NIR prediction for individual FA and some ratios may include: i) Low FA variability in the sample sets used in this study (Table 1) which may have limited their prediction from NIR spectra. The generation of a successful statistical model requires wider data sets, or data sets covering a wide range of concentrations. When random samples are used for the purpose of calibration, as was the case in this study, performance may be constrained by narrow data sets. Also, ii) the complexity of the aqueous matrix of liquid milk (minerals in solution, proteins in a colloidal dispersion, and lipids in emulsion), hinder the NIR analysis (Marinori *et al.*, 2013). The highwater content of milk in the fresh state could limit the detection capacity of other constituents, since the absorption bands of water in NIRS are strong. Calibration models could be improved by the use of a bigger set of samples and with a greater range in concentration of analyzed FA.

Our results suggest that water content can often mask NIR signals and generate a limited predictive model (Reeves, 2000). It is necessary to work on reducing the difference between RMSEE and RMSECV values, which will serve to strengthen the calibration models (González-Sáiz *et al*., 2007).

The R^2_{CV} and RPD obtained in this study for SFA, MUFA, PUFA, n-6, n-3, n6/n3, AI, LNA and 10 *trans* were higher than those reported by Núñez-Sanchez *et al.* (2016), in calibration models using dry and fresh samples of goat milk measured in reflectance and transflectance mode, respectively. The R^2_{CV} obtained by Andueza *et al.* (2013) in samples of dry goat milk were higher than our reported R^2_{CV}, except for n6 (0.26), LA (0.42), LNA (0.49) and ∑odd and branched fatty acids (0.38). In addition, our study obtained greater R^2_{CV} and RPD values than those reported by Núñez-Sanchez *et al.* (2020) for EPA, DPA, MUFA, n-6, n-3 and h/H in thawed ewe's milk samples.

As compared to our study, the models generated by Coppa *et al.* (2010) with samples of fresh cow's milk measured in transflectance mode obtained lower R^2 coefficients in set validations for: ∑CLA (0.60); PUFA (0.65); n-6 (0.00); n-3 (0.20) and ∑odd and branched FA (0.57). Similarly, Coppa *et al.* (2014), using samples of fresh cow's milk in reflectance mode, reported validation R^2 values for ∑CLA (0.71); MUFA (0.79); PUFA (0.77), n-6 (0.43); n-3 (0.72) and n-6/n-3 (0.66). In addition, our study obtained greater RCV2 and RPD values than those reported by Llano-Suárez *et al.* (2018) for SFA, MUFA, and PUFA in liquid milk samples.

In addition, only some authors have determined AI and TI by NIRS: i) Nuñez-Sanchez *et al.* (2016) in oven-dried goat milk samples in reflectance mode (AI=0.77; TI=0.74) and liquid goat milk samples in transflectance mode (AI=0.68; TI=0.75); ii) Llanos-Suárez *et al.* (2018) in liquid milk samples using a near infrared handheld spectrometer (AI=0.85; TI=0.89); and iii) Nuñez-Sanchez *et al.* (2020) in thawed ewe's milk samples previously oven-dried on glass fiber filters (AI=0.75; TI=0.87). In comparison to these reports, our calibration models presented greater R^2_{CV} (AI=0.95; TI=0.93) and RPD (AI=4.3; TI=3.6) values.

External validation. Finally, the robustness of our model was checked with an independent subset of 10 milk samples which were not included initially in the calibration set of samples. Briefly, these samples were analyzed both by GC, and by NIR, and resulting NIR prediction values were compared to values obtained by GC with a Student t-test for paired values. There were no differences between the predicted values by NIRS and the values reported by gas cromatography obtained for this set of 10 samples which were not included in the calibration model (P=0.05). Therefore, it can be concluded that the NIRS method provides significantly identical data to the reference data for individual FA, groups of FA and health indexes.

Therefore, the development of a fast, reliable method to routinely monitor milk FA individual or as FA groups (PUFA, MUFA, etc) could be applied on a larger scale in the dairy industry in order to promote farmers, and improve dairy systems regarding factors which affect milk's FA composition. The use of NIRS as a rapid method provides an opportunity for the routine quantification of healthy milk FA such as omega-3 and CLA, with applications in the dairy industry for food labeling, and at the farm level for management of the dairy cow's diet.

5. CONCLUSIONS

The coefficient of determination of the calibration sets fluctuated between 0.76 and 0.95, while RPD values varied between 2.1 and 4.3. The R^2_{CV} and RPD statistics were proven to have an excellent predictive capacity of the models for DPA, ∑CLA, TSFA, TMUFA, TPUFA, n-6, AI, PUFA/SFA and LA. The results obtained for EPA, n-3, n-6/n-3, TI, UFA, UFA/SFA, HFA, HFA/UFA, h/H, LNA, LA/LNA, 10-*trans*, ∑odd and branched FA, and 11-*trans* displayed very acceptable R^2_{CV} and RPD statistics but it was not possible to generate a robust calibration model for MUFA/SFA. Also, based on the external validation, it can be stated that NIRS can predict individual and grouped FA, as well as health indexes based on FA content in fresh milk samples. Therefore, models of NIRS calibration can be used for predicting the nutritional and health values of fresh milk from cows from pastoral systems.

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An advanced aqueous method of recovering pumpkin seed kernel oils and de-oiled meal: Optimization and comparison with other methods

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SUMMARY: The optimal process conditions of the advanced aqueous method for recovering oil and de-oiled meal from pumpkin seed kernels were: baking the kernels at 110 °C for 1 min, grinding them to pass through a sieve of 150 μm pore size, adding 1.60 ml brine to 10.00 g ground kernels, stirring for 30 min at 30 °C, centrifuging at 4000 r/min for 30 min and cold-pressing the residue from centrifugation. This method recovered > 94% oil. Its oil recovery rate was comparable to that of solvent extraction and higher than that of enzyme-assisted aqueous method or hot-pressing. It recovered edible oil with higher quality and level of coenzyme Q10, tocopherols, carotenoids, total phytosterols and squalene as compared to solvent extraction or hot-pressing and requirements of China's national standard. It is superior to enzyme-assisted aqueous method or hot-pressing for recovering de-oiled meal which is suitable for making texturized protein.

KEYWORDS: *Optimizing parameters; Comparing methods; Water; Bioactive compounds; Green technology.*

RESUMEN: ***Método acuoso avanzado para recuperar aceites de pepitas de calabaza y harina desengrasada: optimización y comparación con otros métodos.*** Las condiciones óptimas del proceso del nuevo método acuoso para la recuperación de aceite y harina desengrasada de las pepitas de calabaza fueron: horneado a 110 °C durante 1 min, molienda para que pasen por un tamiz con un tamaño de poro de 150 μm, adición de 1,60 ml de salmuera a 10,00 g de pepita molida, agitando durante 30 min a 30 °C, centrifugación a 4000 r/min durante 30 min y presión en frío del residuo de la centrifugación. Este método recuperó > 94% de aceites. Esta tasa de recuperación de aceite fue comparable a la de la extracción con solvente y más alta que la del método acuoso asistido por enzimas o prensado en caliente. Se recuperó aceite comestible con mayor calidad y nivel de coenzima Q10, tocoferoles, carotenoides, fitoesteroles totales y escualeno en comparación con la extracción con solvente o prensado en caliente cumpliendo los requisitos de la norma nacional de China. La extracción es superior a la obtenida mediante el método acuoso asistido por enzimas o al prensado en caliente por lo que se recupera una harina desengrasada adecuada para hacer proteínas texturizadas.

PALABRAS CLAVE: *Agua; Comparación de métodos; Compuestos bioactivos; Optimización de parámetros; Tecnología verde.*

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1. INTRODUCTION

The annual consumption of edible oil per capita in 2019 have been up to 27 kg per capita (OECD/FAO, 2020). Large amounts of oils or fats may also be applied to other industries, for example, efficient solvents for extracting nonpolar bioactive substances and materials for making many valuable products (lubricants, biodiesel, linoleum, etc.) (Pal *et al.*, 2019; Kumar *et al.*, 2016).

The efficient utilization of proteins in defatted meal is essential for obtaining the maximum profit from the oilseed processing industry for the production of oils. For food security, 31 kg per capita per year by 2029 is projected to be required while actual annual consumption per capita may be up to 41 kg (OECD/FAO, 2020). Furthermore, large quantities of proteins may be consumed by food and other industries, for example textile or medicinal industries (Stenton *et al.*, 2021; DeFrates *et al.*, 2018; Asgar *et al.*, 2010).

Pumpkin seed kernels (PSKs) contain 36-50% oil, 36-50% protein and significant amounts of other bioactive compounds (Veronezi and Jorge, 2012). Therefore, pumpkin seed kernels are a very good source of oil and protein and the simultaneous production of oil and de-oiled meal which are rich in protein is important. This means that an efficient, environmentally-friendly, safe, un-costly and sustainable method for recovering oil from PSKs is in high demand.

Recently, the enzyme-assisted aqueous method (EAAM) has been studied extensively because it has advantages over solvent extraction (SE)/ or cold-pressing-solvent extraction (CP-SE) (e.g. hexane emitting harmful solvent, terpenes (e.g. α-pinene, p-cymene and d-limonene) being expensive and hardly available in large quantities), critical CO_2 extraction (small scale and high pressure needed) and other methods (e.g. hot-pressing, HP; low value of de-oiled meal) which have disadvantages (Environmental Protection Agency, 1999; Yusoff *et al.*, 2014). EAAM uses large quantities of water and enzymes to disperse (or solubilize) hydrophilic compounds such as proteins, phospholipids and free fatty acids and separate oils. The application of EAAM in oilseed processing on an industrial scale is still not successful because of the use of expensive enzymes, quite low oil recovery rate (ORR; 64.2 to 89.1% (Zhang *et al.*, 2018; Konopka *et al.*, 2016; Li *et al.*, 2016; Jiao *et al.*, 2014; Hu and Zou, 2013) caused by serious emulsion, difficulty and high cost of treating large quantities of water, losses in large quantities of valuable compounds with waste water, and high production cost as compared to other methods (Yusoff *et al.*, 2014).

Recently, an advanced aqueous method (AAM) has been developed for efficiently processing tea seeds (Lv and Wu, 2019a), almonds (Fu and Wu, 2019), white sesame (Lv and Wu, 2020), soybeans (Tu and Wu, 2019a), rapeseeds (Lv and Wu, 2019b), sunflower seeds, peanuts, or walnuts (Tu *et al.*, 2017; Tu and Wu, 2019b), respectively. A comparison of optimized process conditions of the advanced aqueous method for different oilseeds is shown in Table 1. The table indicates that different oilseeds had different optimized conditions for obtaining the maximum oil recovery rate (ORR). The variation should be caused by the variation in the chemical composition and physicochemical properties of different oilseeds.

This research aimed to optimize process conditions of AAM for recovering high quality edible oil and de-oiled meal from PSKs and evaluate them as comparing with HP, EAAM and SE. All experiments carried out, their results and discussion with respect to industrial processing are reported here.

2. MATERIALS AND METHODS

2.1. Materials

Raw pumpkin seeds bought from Inner Mongolia, China via Mobile Phone Taobao (https://www.taobao.com/) were manually peeled without any heat treatment. All reagents used in the experiments were analytically pure.

2.2. Optimization of process conditions for recovering oils by AAM

Figure 1 shows the flow chart for recovering oils by the AAM studied. All measurements were carried out in triplicate.

2.2.1. Division 1 Optimization by single factor experiments.

Only one process condition varied with all others fixed and its level producing the maximum ORR was selected for the subsequent experiment. AAM procedures for investigation into the effect

Table 1. Comparison of optimized process conditions for different oilseeds by the new aqueous method

Oilseeds	Process conditions							
	H or P[a]	BT[b]	Bt[c]	PSSP[d]	W or B[e]	AT[f]	At[g]	ORR[h]
Peanut[1]	Hulled and peeled	110	90	150	1.5 ml H_2O+0.1 g NaCl per 10 g kernel slurry	20	Till free oil observed	96
Walnut[1]	Hulled	115	90	150	1.5 ml H_2O+0.1 g NaCl per 10 g kernel slurry	20	Till free oil observed	97
Sunflower[1] seed	Hulled	115	110	48	1.8 ml H_2O+0.1 g NaCl or 0.03 g Na_2CO_3 per 10 g kernel slurry	70	Till free oil observed	95
White sesame[2]	-	115	1	154	1.95 mL 6.0% (w/w) salt solution per 10 g ground seeds	65	25	96.06
Almond[3]	Hulled	110	3	58	1.37 or 1.40 ml H_2O per 10 g kernel slurry	RT[i]	40 or 45	96.38
Tea seed[4]	Hulled	115	90	74	1.2 ml H_2O+0.1 g NaCl or 0.08 g Na_2CO_3 per 10 g kernel slurry	65	40	94.47
Rape seed[5]	Hulled	115	2	61	1.5 ml H_2O per 10 g kernel slurry	50	30	94.64
Soybean[6]	Peeled	120	5	150	1.3 ml H_2O+0.08 g NaCl per 10 g kernel slurry (3 parts oil+5 parts powder)	75	30	81

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

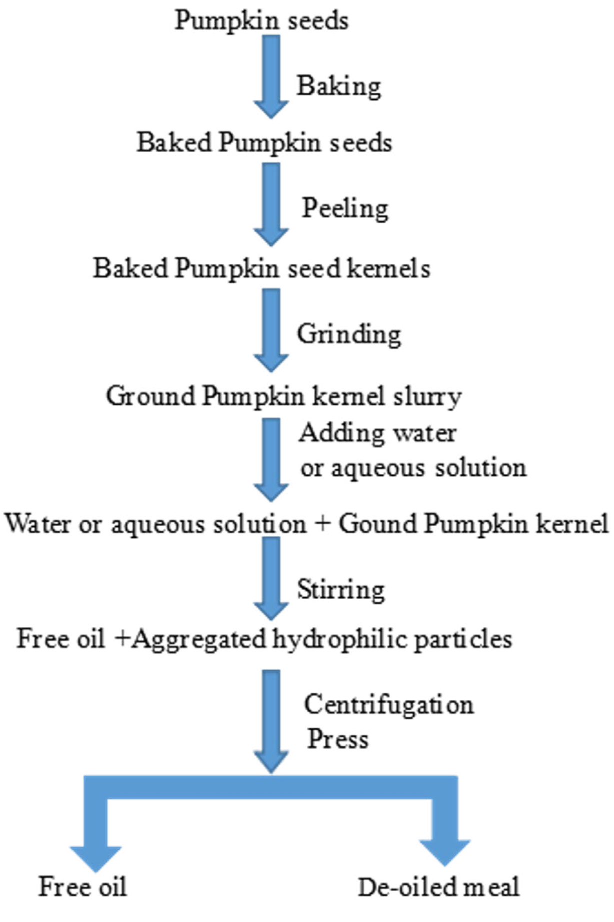

Figure 1. Flow chart of recovering pumpkin seed kernel oil and de-oiled meal by using a small amount of aqueous solution.

of baking time on ORR were as follows: "Raw PSKs were baked for 0, 1, 2, 3, 4, or 5 min, respectively, at 105 ºC, peeled, crushed and ground to pass through a sieve with 105 μm pore size producing ground PSK (GPSK). Water (1.50 ml) was added to a 20 ml centrifugal tube with 10.00 g GPSK. After stirring at room temperature (25 ºC) for 30 min, the oil was collected by centrifuging at 4000 r/min (1435 g) for 30 min thrice with 80-2 centrifuger (manufactured by Honghua Instrument Factory, China) and weighed. All residues from centrifugation were quantitatively collected and cold-pressed once with a presser (made by Ai Bang Agricultural and Horticultural Machinery Plant, China). All the de-oiled meal from the presser was quantitatively collected, dried, weighed and ground to pass through a sieve with 154 μm pore size for determining oil content according to the Soxhlet method. The extraction yield of oil (EYO) was calculated as follows:

$$EYO\ (\%) = (X_1 - X_2) \div X_1 \times 100$$

In the formula, X_1 (g) represents the total oils of GPSK [10 g × crude oil fraction of GPSK], while X_2 (g) represents the total oils of de-oiled meal (the amount of de-oiled meal (g, dry basis) × its oil fraction). For AAM, EYO is the same as ORR.

AAM procedures for the investigation into the effect of baking temperature on ORR. Raw PSKs were baked for 1 min at 100, 105, 110, 115, 120, or 125 ºC, peeled, crushed and ground to pass through a sieve of 105 µm pore size to produce ground PSK (GPSK). Water (1.50 ml) was added to a 20-ml centrifugal tube with 10.00 g GPSK. After stirring at room temperature (25 ºC) for 30 min, the rest of the procedure was the same as that described above in "AAM procedures for the investigation into the effect of baking time on ORR".

AAM procedures for the investigation into the effect of water added on ORR. Raw PSKs were baked for 1 min at 110 ºC, peeled, crushed and ground to pass through a sieve of 105 µm pore size to produce ground PSK (GPSK). Water (0.00, 0.50, 1.00, 1.20, 1.40, 1.50, 1.60, 1.70, 1.80, or 1.90 ml) was added to a 20-ml centrifugal tube with 10.00 g GPSK. After stirring at room temperature (25 ºC) for 30 min, the rest of the procedure was carried out in the same manner as that described above in "AAM procedures for the investigation into the effect of baking time on ORR".

AAM procedures for the investigation into the effect of sieve pore size on ORR. Raw PSKs were baked for 1 min at 110 ºC, peeled, crushed and ground to pass through sieves with 550, 270, 250, 212, 160, 150, 105, 75, or 58 µm pore sizes to produce ground PSK (GPSK). Water (1.60 ml) was added to a 20-ml centrifugal tube with 10.00 g GPSK. After stirring at room temperature (25 ºC) for 30 min, the rest of the procedure was the same as that described above in "AAM procedures for the investigation into the effect of baking time on ORR".

AAM procedures for investigation into effect of stirring time on ORR. Raw PSKs were baked for 1 min at 110 ºC, peeled, crushed and ground to pass through a sieve with 150 µm pore size to produce ground PSK (GPSK). Water (1.60 ml) was added to a 20-ml centrifugal tube with 10.00 g GPSK. After stirring at room temperature (25 ºC) for 0, 15, 20, 25, 30, 35, 40, or 45 min, the rest of the procedure was carried out in the same manner as that described above in "AAM procedures for the investigation into the effect of baking time on ORR".

AAM procedures for the investigation into effect of stirring temperature on ORR. Raw PSKs were baked for 1 min at 110 ºC, peeled, crushed and ground to pass through a sieve with 150 µm pore size to produce ground PSK (GPSK). Water (1.60) was added to a 20-ml centrifugal tube with 10.00 g GPSK. After stirring at 25, 30, 35, 40, 45, or 50 ºC for 30 min, the rest of the procedure was the same as that described above in "AAM procedures for the investigation into the effect of baking time on ORR".

AAM procedures for the investigation into the effect of salt added on ORR . Raw PSKs were baked for 1 min at 110 ºC, peeled, crushed and ground to pass through a sieve with 150 µm pore size to produce ground PSK (GPSK). Water (1.60 ml) and salt (0.00, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, or 0.12) were added to a 20-ml centrifugal tube with 10.00 g GPSK. After stirring at 30 ºC for 30 min, the rest of the procedure was the same as that described above in "AAM procedures for the investigation into the effect of baking time on ORR".

AAM procedures for the investigation into the effect of water added with the presence of 0.08 g salt on ORR. Raw PSKs were baked for 1 min at 110 ºC, peeled, crushed and ground to pass through a sieve with 150 µm pore size to produce ground PSK (GPSK). Water (0.00, 0.50, 1.00, 1.20, 1.40, 1.50, 1.55, 1.60, 1.65, 1.70, 1.75, or 1.80 ml) was added to a 20-ml centrifugal tube with 10.00 g GPSK plus 0.08 g salt. After stirring at 30 ºC for 30 min, the rest of the procedure was the same as that described above in "AAM procedures for the investigation into the effect of baking time on ORR".

2.2.2. Division 2 Further optimization by response surface method

According to the results of the single factor experiments, sieve pore size (A), stirring time (B), water addition (C), and baking temperature (D) were selected for further optimization using the response surface method with all other process conditions fixed. For this purpose, Box-Benhnken's central combined experimental design was employed.

2.3. Recovering oils through SE

Pumpkin seeds were baked at 105 ºC for 1 min, cooled, pulverized, and extracted for 10 h at 85 ºC in a water bath using a Soxhlet extractor with n-hexane as solvent. The extract was evaporated in a rotary evaporator until the n-hexane was completely removed. Free oil was vacuum dried at 50 ºC to a constant weight. The oil content in the de-oiled meal was determined. EYO was calculated following a method similar to that described in "*2.2.1. Division 1*". The crude oil obtained was refined by a method similar to that optimized by Ma *et al.* (2017). ORR was calculated by: [total refined oil obtained (g)/total crude oil extracted (g)] x 100.

2.4. Recovering oils by HP

Pumpkin seeds were baked at 105 ºC for 1 min and hot-pressed twice. Oil content in dark de-oiled meal was determined by the Soxhlet method. EYO was calculated by the method similar to that described in "*2.2.1. Division 1*".

2.5. Analytical methods

Transparency or smell or taste, solvent content, water content, phosphorus content, peroxide value, acid value, crude oil content, crude protein content, total phytosterols and carotenoids were determined by using Chinese National Standard Analytical Methods: GB/T5525-2008, GB5009.262-2016, GB5009.3-2016, GB5009.87-2016, GB5009.227-2016, GB5009.229-2016, GB5009.6-2016, GB5009.5-2016, GB/T25223-2010 and GB5009.83-2016, respectively. Tocopherols and squalene were estimated according to Wong *et al.* (1988) and LS/T6120-2017, respectively.

2.6. Statistical analysis

One-way ANOVA was conducted with IBM SPSS Statistics 23. The P-value determined significant difference between two groups. Significance between two groups of data was obtained by post-test and multiple-comparison. Response surface analysis was conducted with Design-Expert 8.0.6 software.

3. RESULTS AND DISCUSSION

Some important compositions of experimental materials (PSKs) were as follows: contained 44.68% crude fat (dry basis), 31.24% crude protein (dry basis) and 6.18% water. ORR calculation was based on these measurements.

3.1. Optimization of process conditions of recovering oils by AAM

3.1.1. Division 1. Optimization by single factor experiments

Figure 2 shows the effect of water addition, baking time or temperature and sieve pore size on

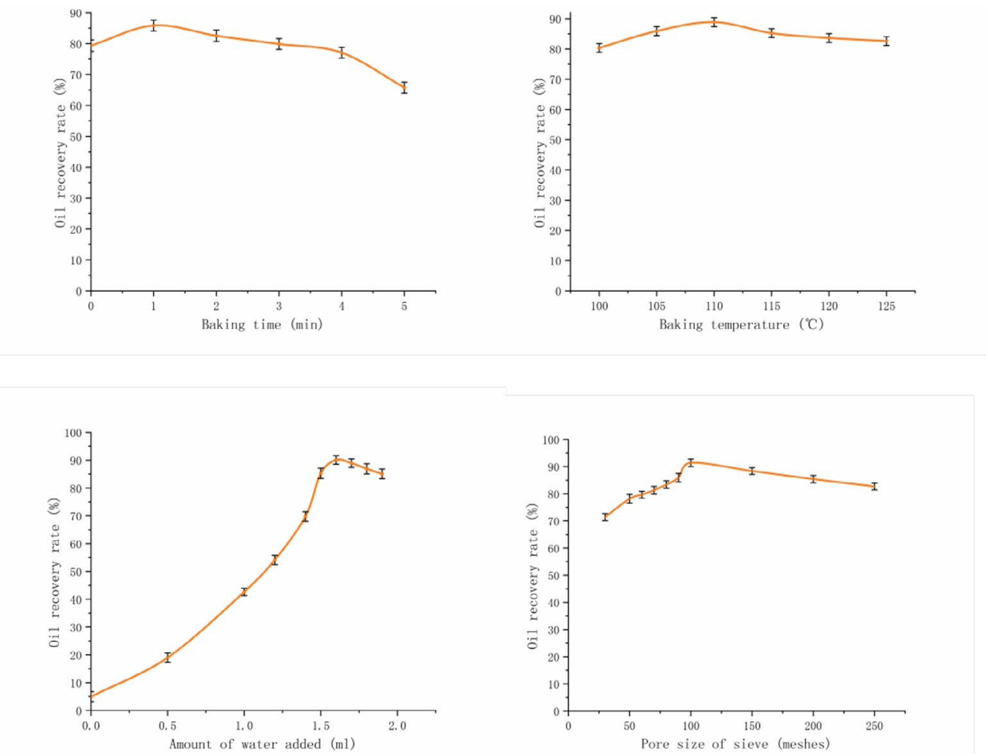

FIGURE 2. Effect of baking time or temperature, amount of water added and sieve pore size (meshes) on oil recovery rate (mean ± SD; n=3).

ORR. The effects of all these three factors were significant with different priorities.

ORR increased from 5.03 to 90.11% as water addition increased from 0 to 1.60 ml, but it continuously decreased when > 1.60 ml was added. The ORR of only 5.03% without water addition indicated that water addition was the major, essential and critical factor for efficiently separating oils. When water addition was at the level of 1.60 ml, solid particles absorbed all added water and aggregated together and the maximum release of free oils occurred. According to quantum chemistry theory, the aggregation of solid particles should be caused by the formation of hydrogen bonds because of water addition. However, the addition of too much water might cause the solubilization of some hydrophilic compounds, increase their movability, reduce the size of solid particles and therefore facilitate the formation of emulsion so that ORR decreased. The maximum ORR obtained was meaningful since it was higher than that obtained by EAAM (Hu and Zou, 2013). The amount (1.60 ml) of water added to 10.00 g raw materials (GPSK) for obtaining the maximum ORR did not cause any free water or result in waste water during the extraction of oils by AAM.

ORR increased from 79.01 to 86.02% as baking time increased from 0 to 1 min, but it continuously decreased for longer than 1 min while it increased from 80.31 to 89.02% as baking temperature increased from 100 to 110 ºC, although it continuously decreased at temperatures higher than 110 ºC. Proper heating treatment should deactivate lipase, which may reduce the losses in neutral oils during oil extraction and therefore elevate ORR. On the other hand, excessive heating (> 1 min and > 110 ºC) may cause the denaturation of proteins and increase their oil absorption capacity, which could therefore reduce ORR. The extent of variation in ORR caused by the variation in the heat treatment was much smaller than that caused by the variation in water addition, which means that heating treatment is a minor or an assistant factor.

ORR increased from 71.4 to 91.36% as sieve pore size reduced from 550 to 150 μm, but it continuously decreased when < 150 μm was used. Grinding PSKs to pass through a sieve with proper pore size should be necessary for fully breaking oil bodies and cell walls to release oils so that proper grinding increased ORR. However, excessive grinding might produce fine particles which are too small in size, which could increase the surface areas of the particles, elevate their ability to stabilize the emulsion (Dickinson, 2006) and therefore decrease ORR. This factor also had a smaller extent of variation in ORR compared to water addition, which should also be an assistant factor.

Figure 3 indicates the effect of stirring temperature or time, NaCl addition and water addition with the presence of 0.08 g NaCl on ORR. These three factors also significantly affected ORR to different extents.

ORR increased from 91.30 to 92.48% as stirring temperature increased from 25 to 30 ºC, but it continuously decreased at temperatures higher than 30 ºC. A reduction in oil viscosity and an increase in the intensity of molecular motion of oils and hydrophilic compounds might occur when stirring temperature is elevated, which might facilitate the aggregation of hydrophilic groups via hydrogen bonds as well as oil coalescence and release from the surface of aggregated solid particles so that ORR increased. On the other hand, too high a temperature (≥ 30 ºC) might lead to an increase in dispersity of surfactants, which might increase the formation of emulsion and thus reduce ORR. This factor also produced a smaller extent of variation in ORR compared to other factors, which should therefore be an assistant factor. ORR increased from 58.52 to 92.56% as stirring time increased from 0 to 30 min, but it continuously decreased after longer than 30 min. Proper stirring time should be necessary for the excess of water to hydrophilic groups of solid particles and the formation of hydrogen bonds. This might facilitate the aggregation of solid particles and the release of free oils from their surface. Excessive stirring might elevate the dispersity of surfactants which might cause emulsion and thus reduce ORR.

ORR increased from 92.40 to 94.07% as NaCl addition increased from 0.00 to 0.08 g, but it continuously decreased when > 0.08 g was added. With the presence of 0.08 g NaCl, ORR increased from 4.83 to 94.14% as water addition was increased from 0.00 to 1.60 ml, but it continuously decreased when > 1.60 ml was added. The addition of NaCl might increase the density or surface charge of aggregated solid particles and therefore facilitate the separation of oils. The addition of NaCl only increased ORR by 1.67% compared to the addition of pure water. Therefore, AAM without the addition of NaCL might also be meaningful in pumpkin seed processing practices.

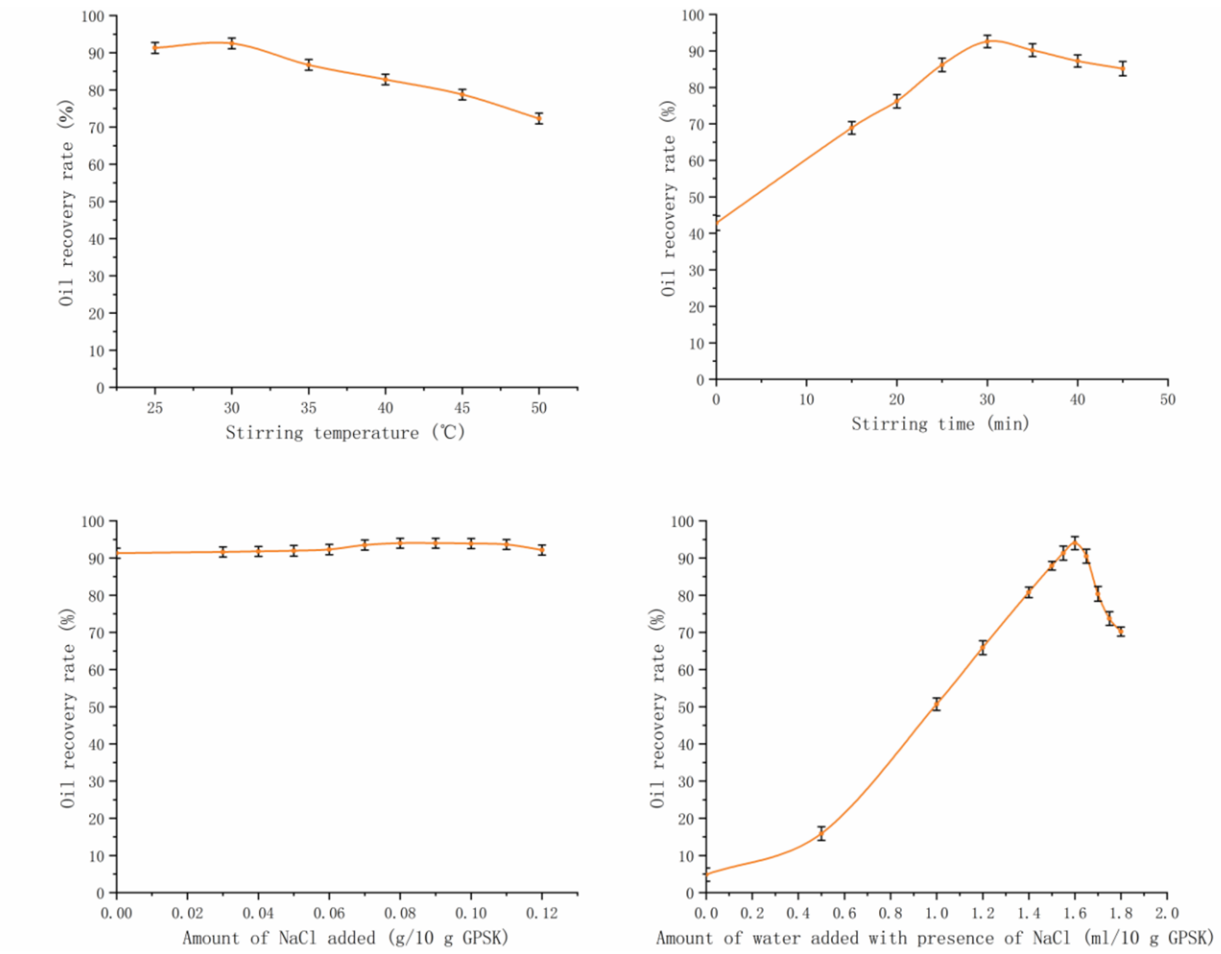

FIGURE 3. Effect of amount of salt added, amount of water added with the presence of NaCl, stirring temperature or time on oil recovery rate (mean ± SD; n=3).

Therefore, the optimal process conditions of AAM obtained by single factor experimentation were as follows: baking Pumpkin seeds at 110 °C for 1 min, crushing PSKs and grinding them to pass through a sieve with 150 µm pore size to obtain GPSK, adding 1.60 ml water and 0.08 g NaCl to 10.00 g GPSK, stirring for 30 min at 30 °C, centrifuging at 4000 r/min (1435 g) for 30 min thrice, and cold-pressing the residue from centrifugation once. The verification test proved that ORR was 94.08% under the optimal process conditions with the general procedures (see “2.2”) followed. Under the optimum conditions, centrifugation and the subsequent cold-pressing recovered 90.15% and 3.93% of total oils from GPSK, respectively. The AAM process conditions optimized in this study for obtaining the maximum ORR from PSKs were significantly different from those for processing other oilseeds previously published (Table 1).

3.1.2. Division 2. Further optimization by response surface method

The response surface method associated with sieve pore size (A), stirring time (B), water addition with the presence of NaCl (C) and baking temperature (D) did not improve ORR as compared to single factor experimentation. The results obtained by the response surface method are indicated in Table 2. However, Figures 4-6 show that AB, AC and AD had significant cross-effects on ORR. The second-order response surface equation ($R^2 = 0.9943$) was obtained to represent the final oil yield.

$$ORR = +93.65 + 5.15*A+0.1667*B + 0.5517*C + 0.1217*D - 0.0525*AB - 0.5525*AC - 0.17*AD + 0.1725*BC - 0.18*BD + 0.365*CD - 6.2*A^2 - 0.9242*B^2 - 1.834*C^2 - 0.7643*D^2$$

Table 2. Design and results of response surface experimentation

Run	A: Sieve pore size (meshes)	B: Stirring time (min)	C: Water added (ml) with 0.08 g NaCl	D: Baking temperature (ºC)	*ORR (%; mean ± SD; n=3)
1	100	25	1.55	110	90.02±1.16
2	100	25	1.60	105	91.01±1.18
3	120	25	1.60	110	92.19±0.65
4	80	25	1.60	110	81.82±1.28
5	100	25	1.60	115	91.89±0.62
6	100	25	1.65	110	90.79±0.92
7	100	30	1.55	105	91.14±1.12
8	80	30	1.55	110	78.82±0.42
9	120	30	1.55	110	90.53±1.13
10	100	30	1.55	115	90.82±1.15
11	80	30	1.60	105	81.48±0.88
12	120	30	1.60	105	91.87±0.66
13	100	30	1.60	110	93.57±0.79
14	100	30	1.60	110	94.08±0.64
15	100	30	1.60	110	93.68±0.61
16	100	30	1.60	110	93.72±0.67
17	100	30	1.60	110	93.18±0.62
18	120	30	1.60	115	91.33±1.38
19	80	30	1.60	115	81.62±1.33
20	100	30	1.65	105	91.31±0.81
21	120	30	1.65	110	90.72±0.76
22	80	30	1.65	110	81.22±0.96
23	100	30	1.65	115	92.45±1.03
24	100	35	1.55	110	90.42±0.90
25	100	35	1.60	105	91.82±0.88
26	120	35	1.60	110	91.89±0.95
27	80	35	1.60	110	81.73±0.84
28	100	35	1.60	115	91.98±0.98
29	100	35	1.65	110	91.88±1.03

*Oil recovery rate.

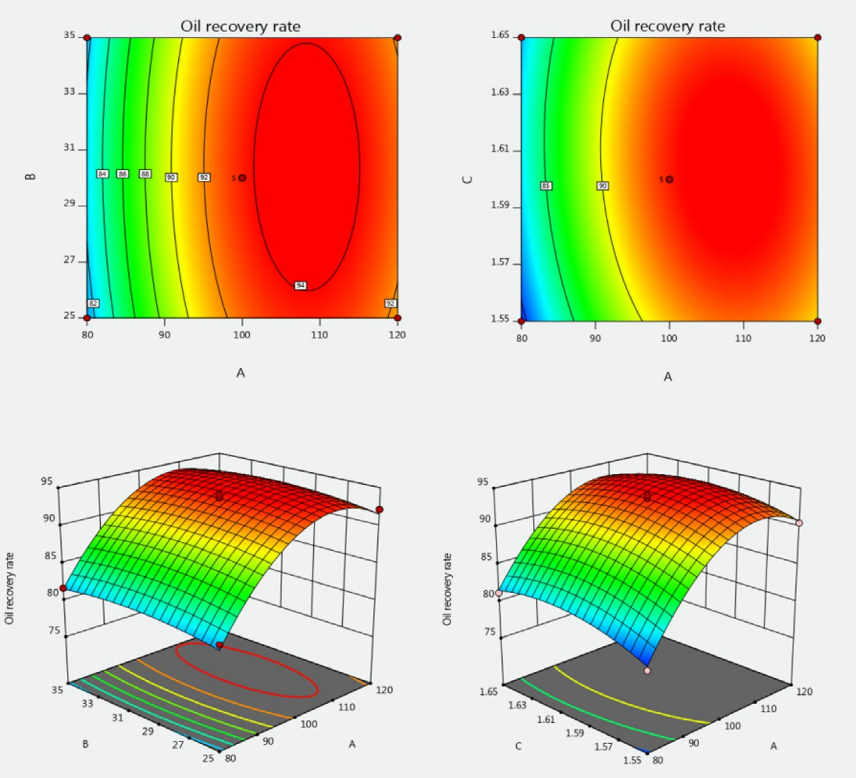

FIGURE 4. Effect of interaction between sieve pore size (meshes; A) and stirring time (B) or water addition with the presence of NaCl (C) on oil recovery rate (%; mean ± SD; n=3).

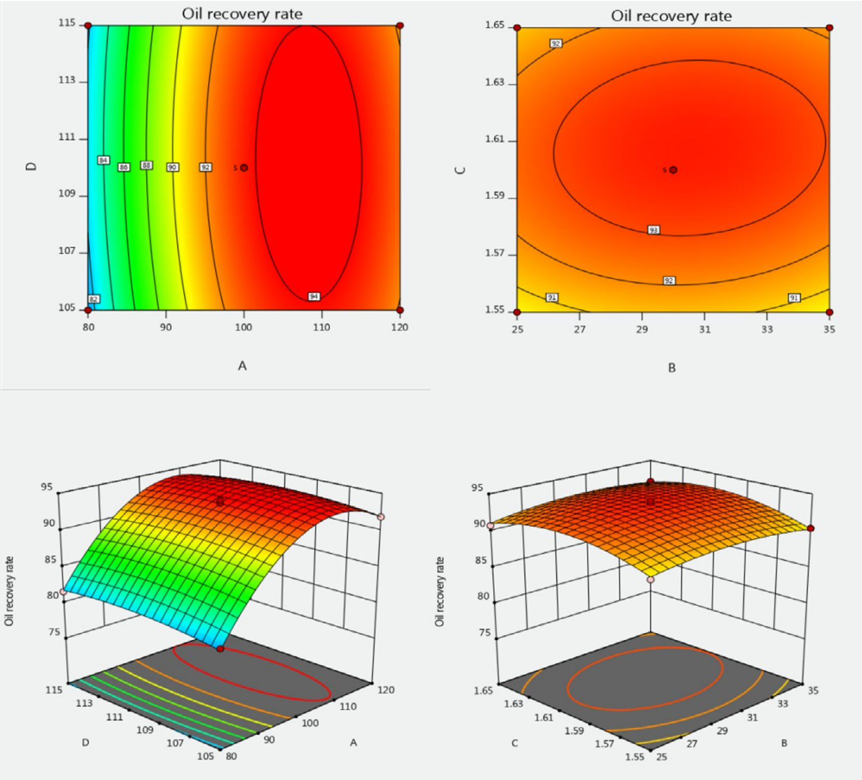

FIGURE 5. Effect of interaction between sieve pore size (meshes; A) and baking time (D) or between stirring time (B) and water addition with the presence of NaCl (C) on oil recovery rate (%; mean ± SD; n=3).

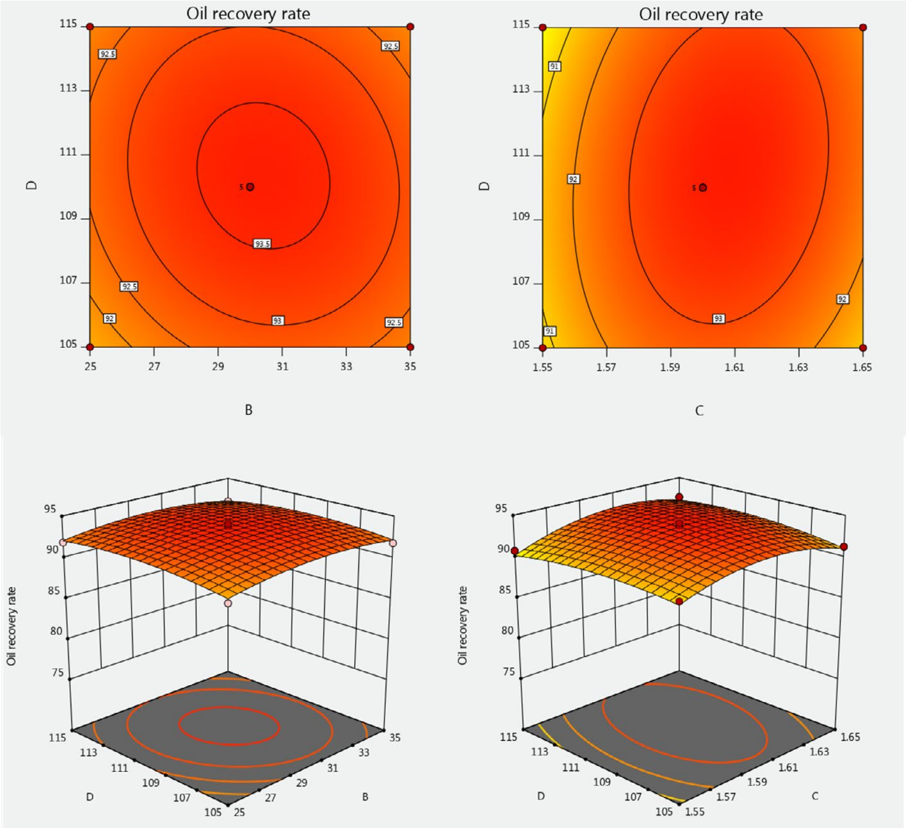

Figure 6. Effect of interaction between stirring time (B) and baking time (D) or between water addition with the presence of NaCl (C) and baking time (D) on oil recovery rate (%; mean ± SD; n=3).

Single factor experimentation adequately optimized process conditions since the surface response method did not find different process conditions to give higher ORR. Adding the proper amount of water was essential and critical while baking seeds, grinding PSKs, stirring GPSK and adding NaCl significantly affected ORR. The optimal conditions established can provide useful scientific basis for process engineering purposes. According to the results of some (including experiments on baking, water or salt addition, sieve pore size and stirring) of this study, bakers (e.g. tunnel furnace) with the accurate control of heating temperature (110 °C) and time (1 min), grinders (e.g. roller-type) which can grind PSKs to pass through a sieve with 150 μm pore size (or destruct all oil bodies and cell walls without production of too fine solid particles) and stirrers (e.g. worm-type) with automatically quantitative addition of water or brine solution and feeding of GPSK are necessary for producing free oils and aggregated solid particles from PSKs on an industrial scale. Centrifugers and cold-pressers are good selections for collecting free oils and de-oiled meal.

3.2. Comparison of efficiency of different methods

Table 3 compares the efficiency of AAM, SE, EAAM and HP for recovering PSK oil. It was found that ORR of AAM was comparable to that of SE and significantly higher than that of EAAM or HP. Although AAM had a lower EYO compared to SE, the crude oil obtained by SE needs to be refined for safe consumption. The oil refinery process can cause losses in neutral oil so that the ORR of SE was much lower than its EYO, which can increase the production cost of edible oil. The main purpose of extensive studies on EAAM is to improve the ORR of the aqueous method, but EAAM has not been successful yet because of a serious un-resolved emulsion problem. Therefore, the AAM for recovering PSK oil developed in this study was advanced. AAM should have the potential to replace SE for efficiently recovering PSK oil with acceptable ORR.

TABLE 3. Comparison of efficiency of recovering PSK oil by AAM and other methods or Chinese National Standard (CNS)

Items	CNS	AAM (mean ± SD; n=3)	EAAM	SE (mean ± SD; n=3)	HP (mean ± SD; n=3)
Extraction yield of oils (%)	_	94.08±0.52	-	98.34±0.43	87.14±0.79
Oil Smell, taste[b]	b	b	_	b	b
Water in oil (%)	-	0.058±0.011	-	0.037±0.012	0.065±0.021
Phosphorus (mg/kg oil)	-	40±1	-	400±9	405±9
Oil transparency	C,T	C,T[c]	_	C,T	C,T
AV (mg KOH/g oil)	2.5 (pressing) 2.0 (solvent)	0.25±0.01	_	0.63±0.01	0.41±0.02
PV (mmol/kg oil)	6.2 (pressing) 4.1 (solvent)	2.58±0.14	_	2.95±0.19	4.08±0.16
Residual solvent (mg/kg oil)	ND[d]	ND[d]	ND[d]	25±1	ND[d]
Oil content in de-oiled meal	_	4.60±0.15	_	1.32±0.09	9.41±0.31
ORR (%)	_	94.08±0.37	89.12[a]	93. 87±0.27	82.69±0.19
Coenzyme Q10 (mg/kg edible oil)	_	146±1	_	105±1	68±1
Tocopherols (mg/kg edible oil)	-	150±2	-	110±2	115±2
Carotenoids (mg/kg edible oil)	-	82±1	-	62±1	71±1
Total phytosterols in edible oil (%)	-	0.5±0.02	-	0.30±0.01	0.40±0.02
Squalene (mg/kg edible oil)	-	4562±111	-	2500±121	3541±112

[a] The highest extraction yield of PSK oil by EAAM reported (Hu and Zou, 2013). [b] Having the inherent smell and taste of PSK oil, no adverse smell. [c] C,T-Clarify, transparent. [d] ND-undetectable.

Table 3 also indicates that AAM-recovered oils with a significantly lower acid value or peroxide value, significantly higher concentrations of tocopherols, carotenoids, coenzyme Q10, total phytosterols or squalene, low water or phosphorus content and other better quality indexes compared to SE or HP, which are currently used as commercial production methods. In particular, the acid value or peroxide value of the oil obtained by AAM was better than the mandatory China National Standard (CNS) requirement, although this was not normally achieved by SE or HP. Although the level of fat-soluble bioactive compounds (including tocopherols, etc. mentioned above) in crude oils recovered by SE was significantly higher, it was significantly lower because oil refinery required for edible purpose caused losses compared to AAM. Furthermore, high temperatures treatment during the refining process can significantly increase the trans-fatty acid content, which is harmful to the cardiovascular system. With regard to these aspects, AAM should be superior to SE, HP and EAAM.

De-oiled meal obtained by AAM contained 4.6% residual oil and 60.7% proteins (dry-weight basis). This level of residual oil content and protein content met the requirements for making texturized protein (Crowea and Johnson, 2001). This means that the whole PSKs can be completely utilized by employing AAM to recover oil. On the other hand, HP produced dark de-oiled meal with completely denatured proteins which had little value for applying to the food industry. EAAM was not able to achieve the complete utilization of whole PSKs since significant amounts of compounds were discarded with large quantities of waste water. With regard to this aspect, AAM should be better than EAAM and HP.

AAM used only a small amount of water and caused no waste water during oil separation and NaCl to be consumable without desalting. Since only water vapor goes into the atmosphere, recovering oil by AAM may be considered a method with zero discharge of wastewater. Therefore, this method should be a green technology. EAAM discharges large quantities of waste water with high COD or BOD or alkali or acid or other chemicals during oil extraction. The refinery process of crude oil obtained by SE also produces significant amounts of waste water. With regard to this aspect, AAM should be superior to SE and EAAM.

4. CONCLUSIONS

Process conditions for AAM were optimized by single factor experimentation followed by the surface response method. A liquid:solid (water:raw material (GPSK)) ratio of 1.6:10 was able to recover more than 94% oils. The addition of water was found to be the major, essential and critical factor for efficiently separating oils while other conditions were minor or assistant factors. The AAM recovered oils with higher quality or level of fat-soluble bioactive compounds from PSKs compared to SE, EAAM and HP, which should be green technology. With regard to producing de-oiled meal suitable for making texturized protein, AAM should be better than EAAM and HP.

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Chemical characterization of baru oil and its by-product from the northwest region of Minas Gerais, Brazil

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SUMMARY: This study investigated baru oil and partially defatted baru flour from the northwest region of Minas Gerais, Brazil. The physicochemical characterization of the oil was made by determining the fatty acid profile using gas chromatography, lutein, and α- and β- carotenes by means of high-performance liquid chromatography, and total carotenoids by spectrophotometry. The flour was analyzed for its chemical composition, fiber, and mineral contents. Baru oil presented excellent quality parameters and high contents in unsaturated fatty acids and carotenoids. The flour showed relevant levels of proteins, lipids, and dietary fiber, in addition to having representative mineral contents for food such as manganese, magnesium, and copper. Thus, baru oil and the by-product of its extraction offer a rich chemical composition, and their application may add nutritional value to foods in addition to reducing negative environmental impacts.

KEYWORDS: *Agricultural waste valorization; Brazilian Cerrado; Carotenoids; Fatty acid composition*

RESUMEN: ***Caracterización química del aceite de barú y su subproducto de la región noroeste de Minas Gerais, Brasil.*** En este estudio se investigó el aceite de barú y la harina de barú parcialmente desengrasada de la región noroeste de Minas Gerais, Brasil. La caracterización físico-química del aceite se realizó mediante la determinación del perfil de ácidos grasos mediante cromatografía de gases, luteína y α- y β- carotenos mediante cromatografía líquida de alta resolución y carotenoides totales mediante espectrofotometría. En la harina se analizó su composición química, fibra y contenido mineral. El aceite de barú tiene excelentes parámetros de calidad, un buen contenido de ácidos grasos insaturados y carotenoides. La harina presentó niveles relevantes de proteínas, lípidos y fibra dietética, además de tener un contenido representativo de minerales para la alimentación, como manganeso, magnesio y cobre. Así, el aceite de baru y el subproducto de su extracción tienen riqueza en su composición química y su aplicación puede agregar valor nutricional a los alimentos, además de reducir los impactos ambientales.

PALABRAS CLAVE: *Carotenoides; Cerrado Brasileño; Composición de ácidos grasos; Valorización de residuos agrícolas*

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1. INTRODUCTION

The Brazilian savanna or Cerrado is the second largest biome in Brazil and is considered one of the richest savannas in the world and is recognized for its biodiversity due to its great plant diversity (Oliveira-Alves *et al*., 2020). However, a large number of endemic fruit species remain unexplored despite their high nutritional and economic potential (Schiassi *et al*., 2018). Because of the area's rich biodiversity, it is important to investigate the nutritional composition and technological and commercial potential of exotic fruits in order to contribute to the preservation of native biomes and the diversification of the human diet.

The baru (*Dipteryx alata* Vog.) is an oleaginous which is native to the Cerrado that deserves to be considered. It produces a light brown drupoid fruit that contains an elliptical dark brown edible seed called the baru almond (Bento *et al*., 2014). Studies have reported that baru almonds are rich in high-quality proteins, lipids, unsaturated fatty acids (especially oleic and linoleic acids), dietary fibers, phenolic compounds (e.g., gallic acid, caffeic acid, rutin, vanillin, and catechin), tocopherols, and minerals (such as iron, zinc, and magnesium) (Campidelli *et al*., 2020; Lima *et al*., 2020; Oliveira-Alves *et al*., 2020). Among the constituents present in the baru almond, the oily fraction has quality characteristics and a good balance of fatty acids, especially those of highly unsaturated fatty acids which are required by the food industry (Pineli *et al*., 2015a).

In addition, the extraction of baru oil by mechanical pressing generates processing residues, known as partially defatted bran. This by-product contains a large amount of unsaturated fatty acids, dietary fibers, proteins, and minerals such as iron (Caetano *et al*., 2017). Thus, baru bran has nutritional relevance as human food, and prevents the improper disposal of large amounts of waste into the environment. In addition, it contributes to the preservation of native species and sustainable regional development. Lima *et al*. (2020) developed and evaluated the technological quality and sensory profile of a nutritive bar produced from baru almond by-products, and reported that the product maintained the nutritional characteristics and bioactive compounds. Formulations with 75 and 100% baru almond obtained favorable results in sensorial tests. Caetano *et al*. (2017) prepared oat cookies by replacing 100% soy oil with baru oil and 30% wheat flour with partially defatted baru flour. They reported that the cookies offered high protein and dietary fiber contents, along with substantial concentrations of oleic acid and iron. Therefore, the results from these studies reinforce the possibility of using this residue in human nutrition.

It is worthwhile to consider the applicability of baru bran and oil in food formulations and to study the nutritional potential of these alternative vegetable sources. Prior to this study, there were no studies on these raw materials from the Brazilian Savanna in the northwest of Minas Gerais, which strengthens the originality of this study. This study aimed to investigate the quality, carotenoid content, and fatty acid profile of baru almond oil from the northwest region of Minas Gerais, and to evaluate the chemical composition and nutritional potential of partially defatted baru flour.

2. MATERIALS AND METHODS

2.1. Plant material

The baru (*Dipteryx alata* Vog.) used to carry out the experiments was donated by an agro-industry located in the north of Minas Gerais, Brazil. The samples were obtained during the harvest season (between August and September 2017) from the Brazilian Savanna, in Arinos Town located in the northwest of the Minas Gerais State (coordinates: 15° 55' 01" S and 46° 06' 21" W, and altitude of 927 m). The procedure described below was carried according to the processes used for extraction in communities where the collection and sustainable management of baru are common practices.

The fruits that fall to the ground were collected and only ripe fruits of uniform size and without injuries were selected. In other words, when the baru fruit is at its proper ripening point, the plants are practically leafless; the fruits detach from the trees and fall naturally to the ground. After harvesting, the fruits were dried naturally in the sun for 1–3 days (28 ± 2 °C). The producers had already established the practice of collecting baru for extraction, by observing whether the fruits were properly dry and suitable for the removal of the almond. Otherwise, the fruits were left a little longer in sun to dry, the time for this may vary.

The almonds were then removed with a manual breaker consisting of a handmade guillotine with a blade fitted to a wooden structure. The baru fruit was placed under this structure and was broken with the aid of the blade to remove the almond. Subsequently, the almonds were packed in low-density polyethylene bags and immediately sent for oil extraction.

2.2. Extraction of oil and obtaining partially defatted baru flour

The oil was extracted and the partially defatted baru flour (PDBF) was obtained at the Cooperativa dos Agricultores Familiares e Agroextrativistas Grande Sertão Ltda, Brazil. The almonds were subjected to a drying process, which was carried out in an industrial machine called grain conditioner (SMR 610, Scott Tech, São Paulo, Brazil), built specifically used to promote the pre-heating of oily matrices to facilitate oil extraction. The samples were kept at 55 °C for 15 min. After that, the baru oil was extracted by cold mechanical pressing using a continuous press type "Expeller" (Scott Tech) with an extraction capacity of 200 mL of oil/min, at the nominal power of 1.5 kW, and electrical voltage of 220 V. Pressing was performed with 5 kg of almonds and the oil extraction yield was calculated based on the total mass of almonds in relation to the mass of oil obtained. The final yield of the process was 22% crude oil. After pressing, the crude oil was submitted to decantation (3 h) and then filtered through filter press (Ecirtec, São Paulo, Brazil). The baru oil was packed in hermetically sealed bottles of 250 mL and stored (7 ± 1 °C) until analysis. The partially defatted baru bran was ground, packed in low-density polyethylene bags and frozen (- 18 ± 1 °C) until use.

2.3. Oil quality

The oil quality parameters were determined according to the Official Methods of the American Oil Chemist's Society (AOCS, 2009): acid value (mg $KOH\cdot g^{-1}$), saponification value (mg $KOH\cdot g^{-1}$), peroxide value (meq $O_2\cdot kg^{-1}$), iodine value (g $I_2\cdot 100\,g^{-1}$) and colors (yellow, red and blue).

2.4. Determination of the fatty acid profile in baru almond oil

The analysis of fatty acid methyl esters (FAME) (tetradecanoic, palmitic, heptadecanoic, stearic, arachidic, behenic, lignoceric, palmitoleic, *cis*-10-heptadecenoic, oleic, linoleic, linolenic and eicosenoic acids) was conducted using a gas chromatograph Agilent 68650 with plus detector (quadrupole, electron impact) and autoinjector. Briefly, chromatographic analyses were performed using a column DB-23 Agilent (cyanopropil-methylsiloxane) of 60 m in length, 0.25 mm internal diameter and 0.25 mm film thickness. Identification of the peaks was performed by comparison with the retention time of standards (Supelco 37 component FAME Mix, Sigma-Aldrich) of known concentrations of each and mass spectra (ratio m/z) and compared to the internal database. The fatty acids were quantified by peak areas correlated to the response factors of the detector. These factors were determined for each FAME in the internal standard mix by comparing the unit area of each peak to the unit area of the fatty acid methyl ester peak investigated.

2.5. Determination of the carotenoids and vitamin A content in baru almond oil

Carotenoids were extracted according to the methodology proposed by Rodriguez-Amaya (2001) and the analysis followed the chromatographic condition developed by Pinheiro-Sant'ana *et al.* (1998). The analysis were performed by High Performance Liquid Chromatografy (HPLC), using a Phenomenex Gemini RP-18 chromatography column (250 mm × 4.6 mm, 5 µm i.d.), fitted with a Phenomenex ODS guard column (C18) (4 mm × 3 mm), at room temperature and the injection volume varied from 5 to 100 µL, according to the sample. The mobile phase consisted of methanol:ethyl acetate:acetonitrile (80:10:10, v/v). The mobile phase flow rate was 2.0 mL/min, isocratic, during 12 min. The quantification was performed by comparing peak areas to those obtained from the analytical curve constructed from the injection, in duplicate, of six different concentrations of standard solutions (lutein, α-carotene and β-carotene).

The total carotenoids in the oil samples were determined according to the procedure described by Rodriguez-Amaya (2001). The spectrophotometer used was Thermo Scientific (Evolution 60S, USA) and the absorbance of extracts was measured at 449 nm using n-hexane as blank.

The vitamin A content in baru oil was determined from each precursor carotenoid, considering the conversion rate of 6 µg β-carotene to 1 µg retinol and 12 µg α-carotene to 1 µg retinol, as advised by the Codex Alimentarius (FAO, 1985). The results were expressed in µg Retinol Equivalent – $RE\cdot 100\,g^{-1}$.

2.6. Centesimal composition and total phenolics of the partially defatted baru flour

The centesimal composition of the PDBF was obtained based on the methods recommended by the Association of Official Analytical Chemists (AOAC, 2016). The moisture analysis was performed by drying in an oven at 105 ºC until constant weight. Protein content was determined using the Kjeldahl method with a correction factor of 6.25. The lipids were quantified by direct extraction in a Soxhlet apparatus for 6 h, using petroleum ether as solvent. The ash analysis was done by muffle incineration at 550 °C. Soluble and insoluble dietary fibers were determined according to the gravimetric-enzymatic method using enzymes (α-amylase, protease and amyloglucosidase). Digestible carbohydrates were obtained by difference. The determination of the energy value was performed using the Atwater conversion factors for carbohydrates (4.0 $kcal\cdot g^{-1}$), proteins (4.0 $kcal\cdot g^{-1}$) and lipids (9.0 $kcal\cdot g^{-1}$) (FAO, 1985). Food components and energy values were expressed in % and $kcal\cdot g^{-1}$, respectively.

Total phenolics were determined by the Folin–Ciocalteau reagent method (Waterhouse, 2002), using gallic acid (0.2–1.4 $mg\cdot ml^{-1}$) as the standard for the calibration curve. The absorbance was measured at 750 nm with a spectrophotometer, and the results were expressed in mg of gallic acid equivalent (GAE)$\cdot$100 g^{-1}.

2.7. Mineral characterization of the partially defatted baru flour

The methodology used to quantify the minerals was based on acidic digestion overnight according to Kumari and Platel (2017). For that, 5 mL of nitric acid were added to 0.5 g of each ground sample for pre-digestion overnight. The following day, the samples were heated to 90-120 ºC on a heating plate for complete digestion. The obtained products were filtered with ultrapure water in a volumetric flask and the separated extract was analyzed in an atomic absorption spectrophotometer (Varian AA 240 FS), as well as the standard solutions of each element. The minerals evaluated were Calcium (Ca), Copper (Cu), Iron (Fe), Magnesium (Mg), Manganese (Mn) and Zinc (Zn), and the results were expressed in mg$\cdot$100 g^{-1} of sample.

2.8. Statistical analysis

The results were expressed as a mean value ± standard deviation (SD) for three measurements (n = 3), and calculated using SISVAR Software, version 5.6 (Lavras, Minas Gerais, Brazil).

3. RESULTS AND DISCUSSION

3.1. Physicochemical characteristics of baru almond oil

The results for the acidity and peroxide index obtained in this study (Table 1) comply with the standards established by Codex Alimentarius (FAO, 2019) for cold-pressed oils, giving values lower than the recommended maximum limits (4.0 mg $KOH\cdot g^{-1}$ for acidity and 15 meq $O_2\cdot kg^{-1}$ for peroxide). According to Siqueira *et al.* (2016), a low acidity index indicates that a high-quality raw material and processing method were used. In addition, the peroxide content expresses the presence of hydroperoxides, the primary oil oxidation products formed during the initial stages of oxidation that are toxic to humans. According to the Codex Alimentarius (FAO, 2019), the saponification values are 168–181, 186–198, and 188–194 mg $KOH\cdot g^{-1}$ for rapeseed, safflower, and sunflower oils, respectively, which have similar degrees of unsaturation to that of baru oil. The iodine index measures the degree of unsaturated fatty acids (Siqueira *et al.*, 2016); the value for baru oil in this study (92.50 g$\cdot$100 g^{-1}) was lower than that of rapeseed (94–120 g$\cdot$100 g^{-1}), safflower (136–148 g$\cdot$100 g^{-1}), and sunflower (118–141 g$\cdot$100 g^{-1}) oils (FAO, 2019).

TABLE 1. Physicochemical properties of baru almond oil

Analysis	Baru almond oil[a]
Acidity (mg $KOH\cdot g^{-1}$)	0.39 ± 0.42
Saponification value (mg $KOH\cdot g^{-1}$)	187.27 ± 0.64
Peroxide value (meq $O2\cdot kg^{-1}$)	4.78 ± 0.08
Iodine value (g$\cdot$100 g^{-1})	92.50 ± 0.05
Color yellow	70.6 ± 0.12
Color red	4.4 ± 0.10
Color blue	0.7 ± 0.06

[a]Mean value ± standard deviation (n = 3).

Although physicochemical characteristics vary according to the plant variety and environmental and post-harvest conditions (Soares *et al.*, 2021), the iodine, acidity, and peroxide index values for baru oil were similar to those reported in other studies. Pineli *et al.* (2015a) and Siqueira *et al.* (2016) extracted baru oil by cold pressing and obtained values ranging from 72.90 to 97.68 g $I_2 \cdot 100\ g^{-1}$, 0.28 to 0.44 mg $KOH \cdot g^{-1}$, and 1.61 to 4.33 meq $O_2 \cdot kg^{-1}$, for iodine, acidity, and peroxide indices, respectively. The results obtained in our study demonstrate that baru fit in with the quality standards; this can be mainly attributed to post-harvest conditions.

Crude vegetable oils generally have different colors compared to commercial and refined oils, mainly because of the presence of unremoved pigments like carotenoids (Sulihatimarsyila *et al.*, 2020). This is likely because of the carotenoid content in baru oil, as these are the main source of the yellow/red color in oils.

3.2. Fatty acids profile of baru almond oil

Baru almond oil mainly contained monounsaturated fatty acids (MUFA), followed by polyunsaturated fatty acids (PUFA) and a significant amount of saturated fatty acids (SFA), with values of 48.77, 29.09, and 21.84%, respectively (Table 2). The high degree of unsaturation was due to the predominance of oleic (45.83%) and linoleic (28.93%) acids. This result was expected, considering that unsaturated fatty acids are most commonly found in vegetable oils. The considerable PUFA content may cause lipid oxidation due to the presence of double bonds which affect oil stability. However, the presence of tocopherol (vitamin E) in the oil acts as an antioxidant because this molecule donates its phenolic hydrogens to free radicals, thus mitigating the oxidation process in the product (Lemos *et al.*, 2016). Palmitic acid was the SFA with the highest concentration (6.37%) in baru oil, followed by stearic acid (5.28%). The values found in this study are in line with the range applied by Codex standards for peanut oil: palmitic acid (5–14%), oleic acid (35–80%), and linoleic acid (4–43.0%) (FAO, 2019).

The levels of most of the fatty acids in this study corroborate with the range reported for baru oil from other regions of the Brazilian savanna: palmitic (5.51–6.40%), stearic (3.59–6.66%), oleic (37.48–49.20%), linoleic (25.59–39.40%), and linolenic (0.15–4.2%) (Pineli *et al.*, 2015a; Siqueira *et al.*, 2016; Caetano *et al.*, 2017). The growing region, soil type, cultural practices, and climatic conditions may affect fatty acid biosynthesis (Kaseke *et al.*, 2020), thus explaining these differences.

Among the fatty acids found in baru oil, the percentage of oleic acid (ω-9) was the highest. According to Pereira *et al.* (2018), oils that have monounsaturated fatty acids in the carbon chain have received increasing interest because they help preserve the characteristics of the oil and provide high stability, making them less prone to oxidative reactions. A high consumption of oleic acid decreases the con-

TABLE 2. Fatty acids profile of baru almond oil

Common and systematic names	Carbon numbers	Chemical formula	Baru almond oil[a] (%)
Tetradecanoic acid	C14:0	$C_{14}H_{28}O_2$	0.04 ± 0.01
Palmitic acid	C16:0	$C_{16}H_{32}O_2$	6.37 ± 0.06
Heptadecanoic acid	C17:0	$C_{17}H_{34}O_2$	0.08 ± 0.01
Stearic acid	C18:0	$C_{18}H_{36}O_2$	5.28 ± 0.04
Arachidic acid	C20:0	$C_{20}H_{40}O_2$	1.38 ± 0.02
Behenic acid	C22:0	$C_{22}H_{44}O_2$	3.90 ± 0.17
Lignoceric acid	C24:0	$C_{24}H_{48}O_2$	4.79 ± 0.19
Palmitoleic acid	C16:1	$C_{16}H_{30}O_2$	0.04 ± 0.01
Cis-10-heptadecenoic acid	C17:1	$C_{17}H_{32}O_2$	0.21 ± 0.14
Oleic acid	C18:1	$C_{18}H_{34}O_2$	45.83 ± 0.36
Linoleic acid	C18:2	$C_{18}H_{32}O_2$	28.93 ± 0.12
Linolenic acid	C18:3	$C_{18}H_{30}O_2$	0.16 ± 0.01
Eicosenoic acid	C20:1	$C_{20}H_{38}O_2$	2.69 ± 0.02
Unidentified acids			0.37 ± 0.03
Total saturated fatty acids			21.84
Total monounsaturated fatty acids			48.77
Total polyunsaturated fatty acids			29.09

[a] Mean value ± standard deviation (n = 3).

centrations of plasma triglycerides and low-density lipoprotein cholesterol (LDL-c) and is a protective factor against the development of cardiovascular diseases (Marcelino *et al.*, 2019).

Linoleic acid is considered to be essential since it cannot be synthesized by the human body. In this study, the percentage of linoleic acid was significantly higher than that recommended by the Food and Agriculture Organization (FAO). This, in association with presence of fibers and bioactive compounds, may contribute to reducing the risk of cardiovascular disease (Bento *et al.*, 2014). The essential fatty acids, linoleic (ω-6) and linolenic (ω-3) acids, affect various physiological processes and act in the prevention and treatment of cardiovascular disease, reducing atherosclerotic plaque and thrombosis, and consequently, the risk of stroke (CVA) (Lemos *et al.*, 2016). Thus, baru oil can be added to other commercial oils in food preparation to provide health benefits.

3.3. Carotenoids and vitamin A content in baru almond oil

As shown in Table 3, the sum of carotenoid values did not correspond to the total carotenoids. This can be explained by the use of different analytical techniques, e.g., the greater HPLC method sensitivity, and by the possible presence of other carotenoids than lutein, and α- and β-carotenes in baru oil. The total carotenoid content was lower in baru almond oil (15.68 mg·100 g^{-1}) than in virgin palm oil (55.34 mg·100 g^{-1}) but higher than in refined canola oil (0.0084 mg·100 g^{-1}) (Mba *et al.*, 2017). Differences in cultivar type, climatic and growing conditions, maturation degree, oil processing, extraction and quantification methods, storage conditions, and other factors might explain these variations in total carotenoid values.

The lutein value (0.91 μg·100 g^{-1}) in this work was higher only in comparison to commercial canola and sunflower oils that exhibit nondetectable levels of lutein (Flakelar *et al.*, 2017). However, the lutein content of baru oil was much lower than that of crude canola oil (3160 μg·100 g^{-1}) and commercial olive oil (976 μg·100 g^{-1}) (Flakelar *et al.*, 2017). The β-carotene content in baru oil was higher (0.24 mg 100 g^{-1}) than in commercial sunflower (0.14 mg·100 g^{-1}) and canola oils (not detected) (Flakelar *et al.*, 2017). However, the β-carotene content was slightly lower compared to crude canola oil (0.41 mg·100 g^{-1}) and commercial olive oil (0.31 mg·100 g^{-1}) (Flakelar *et al.*, 2017). The α-carotene content of 1.05 mg·100 g^{-1} was observed in the present study. This fraction was not detected in the research conducted by Soares *et al.* (2021) in the oil extracted from buriti, which is another fruit commonly found in the Cerrado.

Table 3. Carotenoid and vitamin A content in baru almond oil

Analytical Determinations	Baru almond oil[a]
Total carotenoids (mg·100g^{-1})	15.68 ± 6.78
Lutein (μg·100g^{-1})	0.91 ± 0.81
α-carotene (mg·100g^{-1})	1.05 ± 0.35
β-carotene (mg·100g^{-1})	0.24 ± 0.01
Vitamin A (μg RE·100g^{-1})	127.5 ± 0.41

[a]Mean value ± standard deviation (n = 3). Data are represented in fresh weight.

The quantification of carotenoids is necessary because these compounds have antioxidant properties, and provide photoprotection and provitamin A activity (Resende and Franca, 2019). Furthermore, studies suggest an essential role of dietary intake and nutritional supplementation of carotenoids in the protection against eye diseases including macular degeneration (Arunkumar *et al.*, 2020). Campidelli *et al.* (2020) reported high levels of antioxidants (measured by the β-carotene/linoleic acid system) in baru almonds; this is an important result for the attenuation of oxidative reactions. The vitamin A content found in the baru oil of the present study was 127.5 μg RE·100 g^{-1}. According to the Codex Alimentarius (FAO, 1985) this value supplies approximately 16% of the Reference Daily Intake (RDI) for individuals older than 36 months. It is important to highlight that this content may be higher, considering that baru oil may contain other provitamin-A carotenoids that were not quantified in this study. Added to food, baru oil can increase vitamin A intake in the diet.

3.4. Centesimal composition and total phenolics of the partially defatted baru flour

As seen in Table 4, the moisture content in PDBF (5.10%) is close to that found (6.53%) by Caetano *et al.* (2017) in baru bran from Goiás State, an area in the Cerrado. This variation is probably associated with the differences in the processes used to obtain flour. The dehydration process applied to baru almonds to remove excess free water can increase flour preservation by disfavoring microbial growth. The ash content (4.12%) was higher than that of

Table 4. Centesimal composition and total phenolics of partially defatted baru flour (PDBF)

Component	PDBF[a]
Moisture (%)	5.10 ± 0.10
Ash (%)	4.12 ± 0.04
Lipids (%)	25.12 ± 0.50
Proteins (%)	34.42 ± 0.68
Total dietary fiber (%)	18.31 ± 5.09
Soluble dietary fiber (%)	4.07 ± 0.05
Insoluble dietary fiber (%)	14.24 ± 0.08
Digestible carbohydrates (%)	12.93
Energy value (kcal·100g^{-1})	415.49
Total phenolics (mg GAE·100 g^{-1})	28.09 ± 5.61

[a]Mean value ± standard deviation (n = 3).

Except for moisture, data are expressed on a dry basis.

nuts such as Brazil nuts (3.4%), cashew nuts (2.6%), roasted, salted almonds (1.5%), and walnuts (2.1%) (NEPA, 2011). The ash percentage reflects the total amount of minerals present in the PDBF. The details of the most important minerals in this fraction are discussed below.

The percentage of lipids determined in this study (25.12%) was higher than the value (12.59%) reported by Siqueira *et al.* (2015) but lower than the value (56.12%) reported by Caetano *et al.* (2017) for baru bran obtained from Goiás. Although baru almonds were subjected to oil extraction, the lipid content in PDBF was not low because mechanical pressing was unable to remove all the oily content from this product. The mechanical pressing, considered a "clean technology," is an interesting alternative because it does not use toxic chemicals and does not alter the structure of the extracted oil. The lipid content found in this study can be interesting from a nutritional point of view because according to the Food and Drug Administration (FDA, 2020), 100 g of PDBF can supply 32.2% of RDI for adults and children aged 4 years and older, and for pregnant and lactating women. However, high-fat levels can favor hydrolytic and oxidative rancidity that can cause sensory changes in flour. Owing to its large amount of lipids, PDBF can become more susceptible to rancidification if improperly stored. Therefore, efficient conservation techniques must be implemented.

The protein content in PDBF (34.42%) detected in this study was higher than the value (22.96%) verified by Campidelli *et al.* (2020) in baru almond obtained from Mato Grosso, another region of the Cerrado. Moreover, the results of the present study indicate much higher protein levels than those found for other fruit by-products of the Cerrado such as pequi peel (5.30%) (Bemfeito *et al.*, 2020). In addition, the flour obtained in this study showed a higher protein content than other commercial flour such as wheat (9.8%), corn (7.2%), and rye (12.5%) (NEPA, 2011). It should also be noted that the protein content in 100 g of PDBF supplies 68.84% of RDI (FDA, 2020). Thus, PDBF has great potential in food preparation as a partial replacement for cereal flour because of its high protein content.

The total dietary fiber value (18.31%) present in PDBF in this study was close to that (16.12%) reported by Siqueira *et al.* (2015) in baru bran but significantly lower than those found for other fruit residues such as banana (40.94%), mango (39.25%), and watermelon (46.20%) peels (Garcia-Amezquita *et al.*, 2018). However, PDBF showed a higher fiber content than cashew nuts (3.7%) and Brazil nuts (7.9%) (NEPA, 2011). The dietary fiber content in 100 g of PDBF supplies 65.39% RDI (FDA, 2020), more than half of the recommended daily value. The percentage of soluble fiber (4.07%) was higher in PDBF than in fruit by-products such as orange (3.74%), tamarind (3.86%), and watermelon (3.17%) (Garcia-Amezquita *et al.*, 2018). The value for insoluble fibers (14.24%) obtained in this study was higher than that observed in pumpkin pulp flour (11.25%) (Bemfeito *et al.*, 2020). Thus, the dietary fiber content indicates that PDBF is a good source of fiber. It is important to mention that these components are associated with many benefits to human health including reduced risk of coronary heart diseases, type 2 diabetes, and cancer (Resende and Franca, 2019), reduction in glycemic response, and improvements in intestinal functions, among others.

As shown in Table 4, PDBF had a digestible carbohydrate content (12.93%) higher than the value (11.59%) reported by Caetano *et al.* (2017) for baru bran. This variation in the carbohydrate profile is probably due to the different origins of the raw material and its maturation degree (Caetano *et al.*, 2017). Being a flour with a high lipid, protein, and carbohydrate contents, it also has a high energy value (415.49 kcal·100 g^{-1}). This value is higher than those found by Santiago *et al.* (2018) for baru peel (240 kcal·100 g^{-1}) and pulp (276 kcal·100 g^{-1}). Notably, 100 g of PDBF supplies approximately 21% of RDI (FDA, 2020). Thus, when marketed at a low cost, PDBF can be part of fiber-rich products, making a positive impact on the human diet.

For phenolic compounds, PDBF showed low levels in this study (Table 4) compared to those reported by Siqueira *et al.* (2015), with a value of 588.11 mg GAE·100 g^{-1}. However, the value found in the present work is close to the range (11.52–44.53 mg GAE·100 g^{-1}) reported by Cangussu *et al.* (2021) for pequi peel flours. The low phenolic compound content can be attributed to the fact that many phenolics are found in the exocarp of the fruit as a defense mechanism of plants against aggressors such as bacteria and insects (Cangussu *et al.*, 2021). Although PDBF contains a small number of phenolics, the content is interesting to increase these bioactive compounds when associated with other flours. According to Oliveira-Alves *et al.* (2020), baru is an important source of these compounds, especially gallic acid and its derivatives (such as gallic acid esters and gallotannins), which are responsible for the high antioxidant activity. These authors also reported that the baru nut has the potential to inhibit colorectal cancer cell proliferation. Thus, PDBF, when used as an ingredient, can provide health benefits.

3.5. Mineral profile in partially defatted baru flour

Table 5 shows the mineral content of PDBF together with the recommended daily intake (RDI) for each mineral. Except for calcium, all the minerals evaluated in this study are present in amounts which meet the nutritional needs of an adult person. In addition, PDBF can be considered to contain high levels of Cu, Fe, Mg, and Mn, because its values supply at least 30% of the nutrient reference values (NRVs) (FAO, 1997). Pineli *et al.* (2015b) found much higher values in baru bran for calcium (200.91 mg·100 g^{-1}), copper (2.04 mg·100 g^{-1}), iron (13.29 mg·100 g^{-1}), and zinc (7.62 mg·100 g^{-1}) than reported in the present study. In contrast, PDBF showed higher levels of minerals than those reported by Schiassi *et al.* (2018) when investigating fruits from the Brazilian Savanna such as araça (42.29 mg Ca·100 g^{-1}, 0.18 mg Fe·100 g^{-1}, and 15.28 mg Mg 100 g^{-1}), cagaita (22.50 mg Ca·100 g^{-1}, 0.33 mg Fe·100 g^{-1}, and 5.79 mg Mg·100 g^{-1}), and mangaba (31.01 mg Ca·100 g^{-1}, 0.50 mg Fe·100 g^{-1}, and 12.80 mg Mg·100 g^{-1}). Moreover, as shown in the Brazilian Table of Food Composition (NEPA, 2011), wheat flour has amounts of Mn, Mg, Fe, and Ca at approximately thirty-, six-, five-, and three-fold less than the PDBF, respectively. The comparison of these results suggests that the by-product from the extraction of baru almond oil has relevant mineral content and has great potential to fortify food formulations and prevent micronutrient deficiency.

Table 5. Mineral composition of partially defatted baru flour (PDBF) and the Reference Daily Intake (RDI) contribution per 100 g of PDBF

Mineral	PDBF[a] (mg·100g^{-1})	RDI[b] % in 100 g (PDBF)
Manganese (Mn)	14.00 ± 0.44	608.70
Copper (Cu)	1.54 ± 0.05	171.11
Magnesium (Mg)	194.20 ± 8.66	46.24
Iron (Fe)	5.50 ± 1.16	30.60
Calcium (Ca)	47.20 ± 9.22	3.63
Zinc (Zn)	2.75 ± 0.07	25

[a]Mean value ± standard deviation (n = 3).
Data are represented in fresh weight. [b]FDA (2020).

The intake of minerals is crucial to a healthy diet because they are a part of well-functioning biological mechanisms (Weyh *et al.*, 2022). For example, iron is essential for transporting oxygen to the tissues from the lungs by red blood cell hemoglobin (Gupta and Gupta, 2014). Magnesium is involved in energy metabolism, protein synthesis, RNA, and DNA synthesis (Weyh *et al.*, 2022). Calcium is associated with bone maintenance, helping to prevent osteoporosis in women, and zinc acts as a cofactor of several enzymes (Gupta and Gupta, 2014; Weyh *et al.*, 2022).

4. CONCLUSIONS

Baru oil from the northwest region of Minas Gerais offers quality that meets the required standards because it has a high content in unsaturated fatty acids (mainly oleic acid), and total carotenoids, confirming the possibility of using it as a partial replacement for other oils. Partially defatted baru flour has high levels of proteins, lipids, and dietary fiber, in addition to the mineral contents, especially manganese, magnesium, and copper. Therefore, the addition of this flour to food formulations may produce nutritionally valuable products. Thus, baru may be used as an alternative source of oil together with offering an advantage of an agro-industrial by-product such as baru bran that can be transformed into flour. These products may be beneficial to human health, the environment, and the food industry.

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Application of MOFs and natural clays for removal of MCPD and GEs from edible oils

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SUMMARY: The aim of this study was to investigate the removal of 3-monochloropropane-1,2-diol (3-MCPD) and glycidyl esters (GEs) from edible oils by using Metal Organic Frameworks (MOF) and natural clays. First, the model oil was treated with adsorbents and titanium (IV) butoxide-terephthalate MOF (Ti-MOF) and kaolin were selected as the best performing MOF along with natural clay, respectively, for the removal of 3-MCPD and GEs. The effects of treatment conditions were also investigated, 6.0% adsorbent level, 120 min treatment time and 95 ºC temperature were determined to be the best treatment parameters. Finally, palm oil samples were treated with Ti-MOF and kaolin under the selected conditions and removal of 3-MCPD and GEs was obtained at up to 27% and 58%, respectively. In conclusion, MOFs and natural clays showed good potential for the removal of 3-MCPD and GEs, and the efficiency of the treatment can be improved by modifying the adsorbents.

KEYWORDS: *3-MCPD; Glycidyl esters; MOFs; Natural clays; Palm oil*

RESUMEN: ***Aplicación de EOMs y arcillas naturales para la eliminación de MCPD y EG de aceites comestibles.*** El objetivo de este estudio fue investigar la eliminación de 3-monocloropropano-1,2-diol (3-MCPD) y ésteres de glicidilo (EG) de aceites comestibles mediante el uso de estructuras orgánicas metálicas (EOMs) y arcillas naturales. El aceite modelo se trató en primer lugar con adsorbentes, se seleccionaron titanium (IV) tereftalato de butóxido (Ti-EOM) y caolín como EOM y arcilla natural, respectivamente, para el mejor rendimiento en la eliminación de 3-MCPD y EG. También se investigaron los efectos de las condiciones de tratamiento y se seleccionaron como los mejores parámetros un nivel de adsorbente de 6,0%, un tiempo de tratamiento de 120 min y temperatura de tratamiento de 95ºC. Finalmente, las muestras de aceite de palma se trataron con Ti-EOM y caolín en las condiciones seleccionadas y se obtuvo una eliminación de 3-MCPD y EG de hasta 27% y 58%, respectivamente. En conclusión, los EOMs y las arcillas naturales mostraron un buen potencial para la eliminación de 3-MCPD y EG, y la eficiencia del tratamiento se puede mejorar modificando los adsorbentes.

PALABRAS CLAVE: *3-MCPD; Aceite de palma; Arcillas naturales; EOM; Ésteres de glicidilo*

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1. INTRODUCTION

The fatty acid esters of glycidol (GEs) and 3-monochloropropane-1,2-diol (3-MCPD) are food-processing contaminants. 3-MCPD, which is a type of chloropropanol, is formed as a result of the interaction of acylglycerol with chloride ions at high temperatures (Arisseto *et al.*, 2018). In the review of Rahn and Yaylayan (2011), four proposed mechanisms for 3-MCPD ester formation are mentioned: direct nucleophilic attack on glycerol carbon carrying an ester group, direct nucleophilic attack on glycerol carbon carrying a hydroxyl group, the formation of acyloxonium ion and the formation of an epoxide ring. GEs are generally formed from monoacylglycerols and diacylglycerols at high temperatures without chloride (Cheng *et al.*, 2017a). In the review of Cheng *et al.* (2017a), four mechanisms for GE formation are mentioned: common reactive intermediate formation by the deacidification of 1,2-diacylglycerols, common reactive intermediate formation by the dehydration of monoacylglycerols, direct intramolecular rearrangement followed by the elimination of fatty acids for diacylglycerols, direct intramolecular rearrangement followed by the elimination of water for 1-monoacylglycerols. Generally, these contaminants are formed during the refining of oils, during the deodorization step (Larsen, 2009). They are present in all refined vegetable oils according to the surveys carried out by MacMahon *et al.* (2013) and Custodio-Mendoza *et al.* (2019). These contaminants have also been detected in oil-based foods such as margarine, potato chips, bread, crackers, infant formulas, breakfast cereals, coffee creamer, roasted malt and cooked meats. The reported levels of these contaminants in foodstuffs are generally much lower than the levels in refined vegetable oils. Therefore, studies mostly focus on refined vegetable oils, especially palm oil (Larsen, 2009).

Studies have shown that free 3-MCPD and glycidol, which are the free forms of 3-MCPD esters and GEs, can be harmful to human health, and even if they are present in ester form, they can be hydrolyzed to their free forms in the gastrointestinal tract. According to the International Agency for Research on Cancer (IARC), free 3-MCPD is classified as a possible human carcinogen (group 2B), while glycidol is classified as probably carcinogenic to humans (group 2A) (IARC, 2000; IARC, 2013; Arisseto *et al.*, 2018). JECFA has established a provisional maximum tolerable daily intake of 4 µg/kg body weight for 3-MCPD and 3-MCPD esters singly or in combination (JECFA, 2016). According to Commission Regulations (EU) (2020), the maximum level of GEs should be 1000 µg/kg in vegetable oils and fats for general uses and 500 µg/kg in vegetable oils and fats for infant formulas. The maximum level of 3-MCPD should be 1250 µg/kg in vegetable oils and fats for general uses, 2500 µg/kg in pomace olive oils for general uses and 750 µg/kg in vegetable oils and fats for infant formulas.

When the health effects of 3-MCPD and glycidyl esters and their levels in oils are considered, the removal of these contaminants or the prevention of their formation becomes an important issue. The main factors affecting 3-MCPD and GEs formation in oils can be listed as follows: the presence of glycerol, mono-, di- and tri-acylglycerols, the presence of chloride ions (originating from water, steam, bleaching earth or other materials used in refining, natural organochloride and pesticides), bleaching earths activated by acids (especially HCl), and the time and temperature of thermal treatments. Considering these factors, three main approaches are recommended to reduce the levels of 3-MCPD and GEs: the removal of the precursors, modifications to oil processing parameters, and the removal of contaminants (Larsen, 2009; Matthaus and Pudel, 2013; Cheng *et al.*, 2017a; Arisseto *et al.*, 2018). Some methods studied in the literature to mitigate 3-MCPD and GEs levels in foodstuffs are as follows: the use of natural and synthetic antioxidants (Wong *et al.*, 2019), the removal of precursors and reorganization/optimization of the refining process (Matthäus *et al.*, 2011; Ramli *et al.*, 2011; Zulkurnain *et al.*, 2012; Zulkurnain *et al.*, 2013; Li *et al.*, 2016), the enzymatic removal of contaminants (Bornscheuer and Hesseler, 2010), the removal of contaminants by adsorbent materials (Strijowski *et al.*, 2011; Shimizu *et al.*, 2012) and the removal of contaminants by oil modifications (Kyselka *et al.*, 2018).

Metal–organic frameworks (MOF) are synthesized polymers constructed through chemical reactions between metal-containing units and organic linker molecules to create open crystalline structures with permanent porosity, dynamic flexibility and diverse morphologies. They have been used for gas adsorption and storage, separation, sensors, drug delivery, and selective adsorption applications (Stock and Biswas, 2012; Furukawa, *et al*., 2013; Ma *et al*., 2014). To the best of our knowledge, there is only one study about MOF application for the removal of 3-MCPD and related substances from oils (Ahn *et al*., 2020). In this study, a series of Fe-MIL-88s with different ligand types, which were modified by carboxylation and deprotonation processes, were synthesized and used for the removal of 3-MCPD and glycidol. They found that, among different types of this MOF, Fe-MIL-88 BDC showed the most efficient adsorption performance. The present study differs from the study of Ahn *et al*. (2020) in that seven different MOFs and four different natural clays were used for the same purpose. Hence, this study expands the knowledge concerning this subject.

The main objective of this study was to evaluate the usage of MOFs as new and potential adsorbent materials in the removal of 3-MCPD and GEs from edible oils. For this purpose, seven different MOFs and four different natural clays were used and their structural properties were determined. After the treatments, the remaining 3-MCPD and GE contents in the model oil samples were measured and the efficiencies of the adsorbents were compared. Some process parameters like level of adsorbent addition, temperature and time were also studied with the two best-performing adsorbents in order to determine the optimum conditions for the adsorption treatments. Finally, the best-performing adsorbents were used under the best treatment conditions for the removal of 3-MCPD and GEs from commercial palm oil, which is a natural source of these contaminants.

2. MATERIALS AND METHODS

2.1. Materials

The seven different MOFs used in this study were synthesized and characterized in our laboratory. These MOFs were as follows: titanium (IV) butoxide-terephthalate MOF (Ti-MOF), synthesized according to Vlasova *et al*., (2016); gamma-cyclodextrine-potassium hydroxide MOF (γ-CD-MOF), synthesized by the method described in Moussa *et al*., (2016); chrome nitrate-terephthalate MOF (Cr-MOF), synthesized by the method of Li *et al*. (2015); aluminum chlorate-terephthalate MOF (Al-MOF), synthesized by the method of Ma *et al*. (2014); zinc nitrate-2,5-furandicarboxylicacid MOF (Zn-MOF), synthesized according to Bu *et al*. (2012); magnesium-MOF (Mg-MOF), synthesized by the method described in Spanopoulos *et al*., (2015); and zinc-2-methylimidazole zeolitic-type MOF (ZIF-8-MOF), synthesized according to Park *et al*. (2006). After synthesis, these MOFs were placed into amber-colored glasses and stored at room temperature throughout the study. The abbreviated MOF names given in parentheses are used throughout the paper.

The commercial pre-activated bleaching earth (CBE) was provided by the Trakya Birlik Oil Processing Factory (Tekirdağ, Turkey); natural zeolite and natural kaolin clay were provided by the Türkzeolit Mining Ind. and Trade. Inc. (Istanbul, Turkey); and natural sepiolite was provided Anadolu Industrial Mine Inc. (Istanbul, Turkey). Extra virgin olive oil and commercial palm oils were bought from Komili Oil (Balıkesir, Turkey) and Besler Oil Factory (Istanbul, Turkey), respectively. Glycidyl palmitate (99.1%, High Purity Compounds, Cunnersdorf, Germany), glycidyl stearate (96.5%, High Purity Compounds, Cunnersdorf, Germany) and 3-MCPD (98%, Sigma- Aldrich, St. Louis, USA) were purchased. All chemicals, solvents, and standards used in the analyses were of analytical or chromatographic grade and purchased from Sigma (St. Louis, MO, USA), Merck Co. (Darmstadt, Germany) and local stores.

2.2. Characterization of the adsorbents

The surface areas and pore properties of the adsorbents were determined using a Quantachrome Nova 4000E instrument (Quantachrome Instruments, Boynton Beach, FL, USA) with nitrogen gas and the Brunauer-Emmett-Teller (BET) and Langmuir models (Moussa *et al*., 2016).

The morphologies of the adsorbents were determined with a JSM-7100F (JEOL, Japan) scanning electron microscope (SEM). First of all, samples were placed onto a specimen holder carbon band with double-sided scotch tape. Then, they were coated with Au-Pd (80–20%) under vacuum (0.8 mbar) and 10 mA voltage using a Quorum coating device. Finally, samples were examined under accelerated voltage of 15 kV and 150–15,000-fold magnifications (Peerajit *et al*., 2012).

The X-ray diffraction (XRD) patterns of the samples were determined using a PANalytical Empyrean model (Netherland) X-ray diffractometer under 40 kV and 40 mA CuKa (λ = 0.1546 nm), within the range of 4-40° at a scanning rate of 0.02-0.6 (sec^{-1}).

2.3. Preparation of the model oil

First, active silica gel was added to extra virgin olive oil (EVOO) at a ratio of 0.5:1 (w/w), stirred at 50 °C for 3 hours, and filtered with filter paper (Whatman no. 1 filter paper, 11 µ pore, 125 mm diameter) for the removal of naturally-present impurities from the EVOO. Then, 3-MCPD, glycidyl palmitate and glycidyl stearate were added to this oil at a ratio of 125 mg/kg, 72 mg/kg and 72 mg/kg, respectively. Finally, this model oil was placed into amber-colored glass bottles and stored in a cool, dark place for the duration of the study.

2.4. Adsorbent treatments of the model oil

15 g of model oil was weighed for each treatment, and each of the adsorbents was added into the oils at a ratio of 3% (w/w). They were then shaken in an incubator (Certomat IS, Sartorius Stedium Biotech, Germany) at 95 °C for 1 h. Finally, the treated oils were filtered through a Whatman no. 1 filter paper (11 µ pore, 125 mm diameter), placed into amber-colored glass bottles, and stored in a refrigerator in the dark for the duration of the study.

2.5. 3-MPCD and GEs analyses of the treated oils

The amounts of 3-MPCD and GEs in the samples were measured according to the AOCS Cd 29c-13 Method (AOCS, 2017). The analysis was carried out with a GC-MS (GC/MS-QP2010, Shimadzu Co., Nishinokyo, Japan) equipped with a Rxi-5MS column (30m x 0.25mm ID x 0.25µm film thickness, Restek Co.). The working conditions of the GC were as follows: splitless, 2 µl injection volume, 0.83 ml/min flow rate, 250 °C inlet temperature and helium as carrier gas. The oven temperature program was as follows: held at 100 °C for 1 min, increased to 160 °C (20 °C/min) and held at that temperature for 1 min, increased to 180 °C (4 °C/min), then to 330 °C (30 °C/min) and held at that temperature for 4.70 min. The working conditions of the MS detector were as follows: 200 °C ion source temperature, 280 °C interface temperature, 5 min solvent-cutoff-time. 3-MCPD-d5 was used as internal standard.

2.6. Effects of the process parameters on the adsorption treatment

As a result of the analyses, Ti-MOF and kaolin were selected as the best MOF and natural clay for removing 3-MPCD and GEs, respectively. With these two adsorbents, the effects of adsorbent addition level (0.5%, 1.5%, 3.0%, 6.0%), treatment temperature (25 °C, 50 °C, 95 °C, 150 °C) and time (15, 45, 60, 120 min) were evaluated under the same treatment conditions described in the adsorbent treatments of the model oil section. After treatment, the 3-MPCD and GE contents in the treated and control samples were measured and the best process parameters were determined.

2.7. Adsorbent treatments of commercial palm oils

Finally, Ti-MOF and kaolin were used under the best treatment conditions (6.0%, 95 °C, 120 min), for the removal of 3-MCPD and GEs from commercial palm oil samples (RBD: Refined, Bleached and Deodorized; DEO: Deodorized), which are natural sources of these contaminants. After the treatments, the 3-MPCD and GE contents in the treated and control samples were measured.

2.8. Statistical analysis

The whole study was repeated twice. Each analysis for each replicate was done in triplicate. The collected data were analyzed using one-way ANOVA and treatment groups were compared with Tukey's tests. Minitab Ver. 16.1.1 (Minitab, 2010) and SPSS (SPSS, 1994) package software programs were used for statistical analyses. There was a minimum 95% level of confidence in this study.

3. RESULTS AND DISCUSSION

3.1. Structural properties of the adsorbents

The surface areas and pore properties of MOFs and natural clays are shown in Table 1. Surface area and pore volume are important parameters which affect the treatment efficiency of adsorbents. Clearly, there is a great variation among the adsorbents in terms of surface area values. The highest surface area was measured for Al-MOF (1415.53 m^2/g), while the lowest value was for γ-CD-MOF (4.55 m^2/g). There is also a great variation among the adsorbents in terms of pore vol-

Table 1. The surface areas and pore properties of MOFs and natural clays

	Surface Area (m^2/g)	Pore Volume (cm^3/g)	Pore Radius (Å)
Ti-MOF	226.65 ± 3.21^{e}	0.27 ± 0.01^{h}	44.45 ± 0.89^{f}
γ-CD-MOF	4.55 ± 0.07^{k}	1.24 ± 0.00^{a}	707.63 ± 1.55^{a}
Cr-MOF	717.45 ± 15.90^{c}	0.77 ± 0.00^{d}	33.95 ± 0.87^{g}
Al-MOF	1415.53 ± 57.07^{a}	0.46 ± 0.01^{f}	19.37 ± 0.40^{i}
Zn-MOF	19.70 ± 2.33^{i}	1.05 ± 0.01^{b}	203.20 ± 0.94^{b}
Mg-MOF	66.50 ± 1.89^{h}	0.65 ± 0.00^{e}	96.85 ± 0.08^{d}
ZIF-8-MOF	1020.25 ± 48.84^{b}	0.55 ± 0.01^{f}	18.42 ± 0.01^{i}
Sepiolite	314.57 ± 13.90^{d}	0.91 ± 0.01^{c}	114.67 ± 0.05^{c}
Zeolite	181.25 ± 9.82^{f}	0.24 ± 0.00^{h}	62.05 ± 2.23^{e}
Kaolin	9.625 ± 1.44^{j}	0.03 ± 0.00^{i}	20.29 ± 0.01^{h}
CBE	143.20 ± 7.32^{g}	0.36 ± 0.01^{g}	99.89 ± 1.47^{d}

*Results are expressed as mean ± SEM. Different superscript letters in the same columns indicate statistically significant differences. The whole study was replicated two times and each analysis for each replicate was done in triplicate (n = 6). The data were analyzed by using one-way ANOVA and groups were compared with Tukey's tests ($P \leq 0.05$). Ti-MOF, titanium (IV) butoxide-terephthalate MOF; γ-CD-MOF, gamma-cyclodextrine-potassium hydroxide MOF; Cr-MOF, chrome nitrate-terephthalate MOF; Al-MOF, aluminum chlorate-terephthalate MOF; Zn-MOF, zinc nitrate-2,5-furandicarboxylicacid MOF; Mg-MOF, magnesium-MOF; ZIF-8-MOF, zinc-2-methylimidazole zeolytic type MOF; CBE commercial bleaching earth.

umes. The highest pore volume was measured for γ-CD-MOF (1.24 cm^3/g), while the lowest value was for kaolin (0.03 cm^3/g). As a result, it can be said that γ-CD-MOF and kaolin seem insufficient for utilization as adsorbents because of their low surface area values. In one study, the surface areas of Zn-MOF, Al-MOF and Ti-MOF were found to be 380, 1196 and 1310 m^2/g and the pore volumes were measured at 0.29, 0.76 and 0.97 cm^3/g, respectively (Vlasova *et al.*, 2016). In the study of Ahn *et al.* (2020), the surface areas of different versions of Fe-MIL-88 MOF varied between 2.58 and 13.74 m^2/g. In a review, the structural properties of different MOF types were listed and it was observed that the surface areas varied between 46 and 2222 m^2/g (Du *et al.*, 2018). Obviously, there are significant differences among MOF types in terms of surface areas and pore properties. These differences are even observed in the same MOF types synthesized in different studies. Overall, it was clear that the surface areas and pore properties of adsorbents depend on ligand type, synthesis conditions and the modifications applied.

The SEM images of MOFs and natural clays are presented in Figure 1. Obviously, the morphological properties of the adsorbents are different from each other. Ti-MOF has clustered spheres, Al-MOF has needle-like crystals, Zn-MOF has cubic crystals and ZIF-8-MOF has hexagonal crystals. Besides these certain geometric shapes, there are also amorphous bulk structures. In one study, the morphology of Ti-

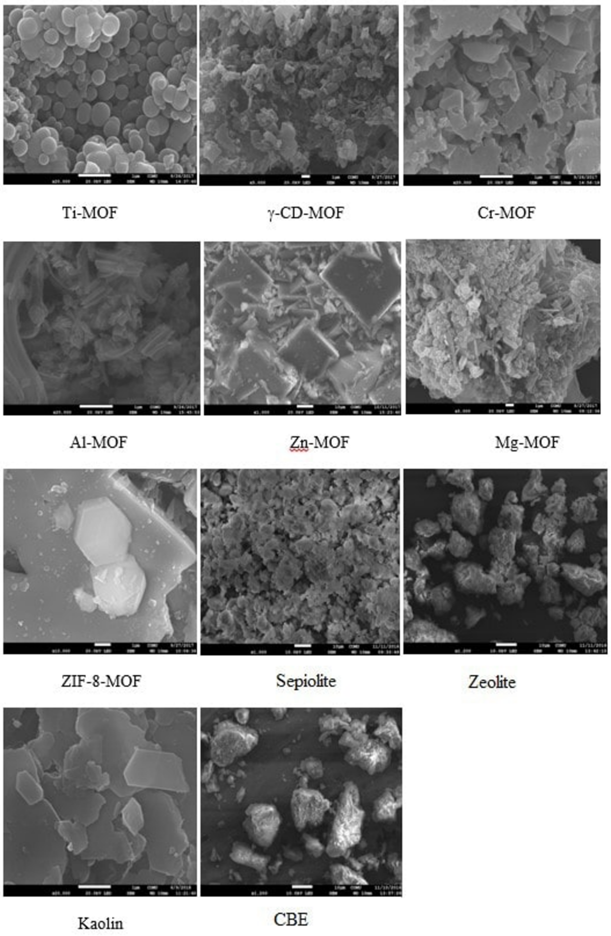
Ti-MOF γ-CD-MOF Cr-MOF
Al-MOF Zn-MOF Mg-MOF
ZIF-8-MOF Sepiolite Zeolite
Kaolin CBE

Figure 1. The scanning electron microscopy (SEM) images of MOFs and natural clays. Ti-MOF, titanium (IV) butoxide-terephthalate MOF; γ-CD-MOF, gamma-cyclodextrine-potassium hydroxide MOF; Cr-MOF, chrome nitrate-terephthalate MOF; Al-MOF, aluminum chlorate-terephthalate MOF; Zn-MOF, zinc nitrate-2,5-furandicarboxylicacid MOF; Mg-MOF, magnesium-MOF; ZIF-8-MOF, zinc-2-methylimidazole zeolytic type MOF; CBE commercial bleaching earth.

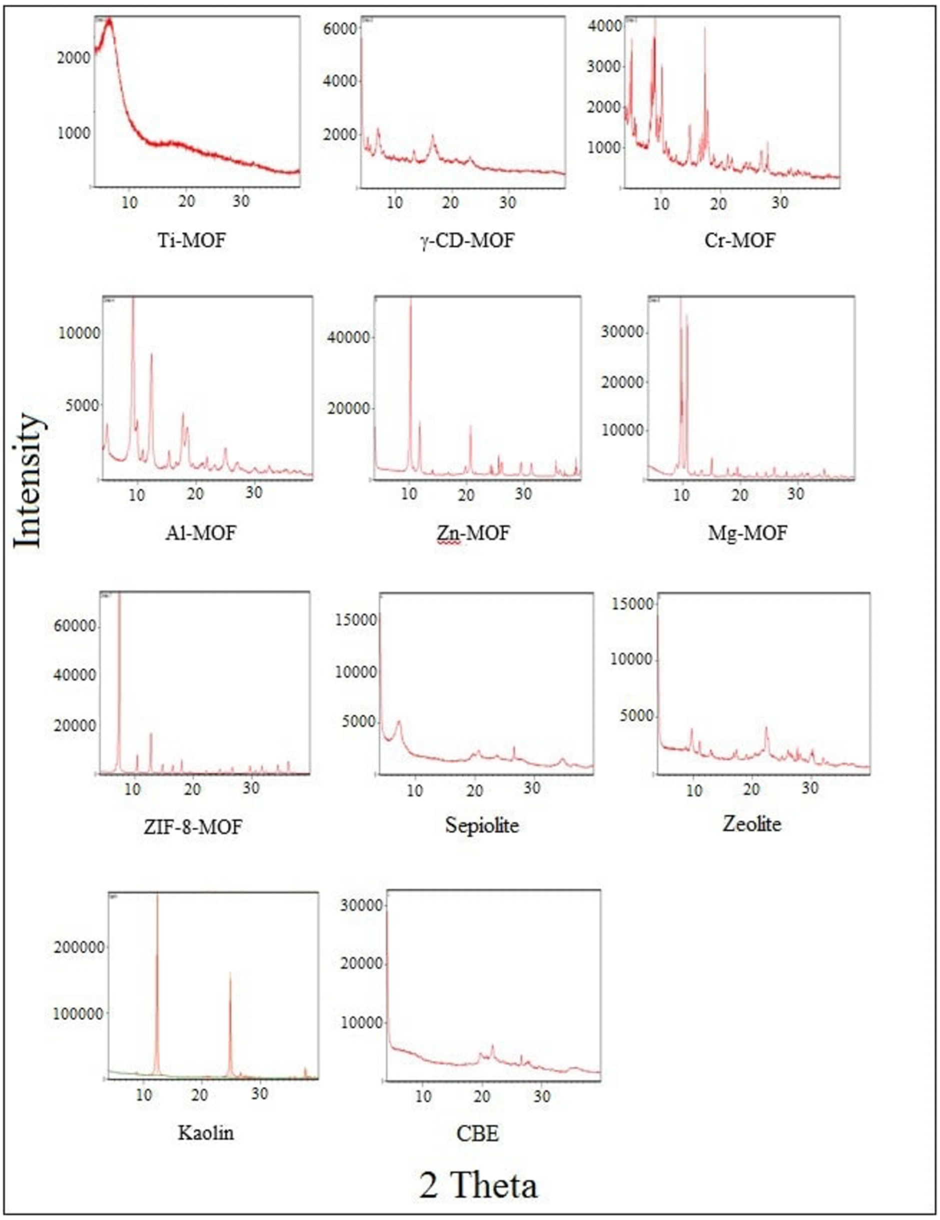

FIGURE 2. The X-ray diffraction (XRD) patterns of MOFs and natural clays. Ti-MOF, titanium (IV) butoxide-terephthalate MOF; γ-CD-MOF, gamma-cyclodextrine-potassium hydroxide MOF; Cr-MOF, chrome nitrate-terephthalate MOF; Al-MOF, aluminum chlorate-terephthalate MOF; Zn-MOF, zinc nitrate-2,5-furandicarboxylicacid MOF; Mg-MOF, magnesium-MOF; ZIF-8-MOF, zinc-2-methylimidazole zeolytic type MOF; CBE commercial bleaching earth.

MOF was determined as ovoidal or flat-cubed with round corners, like in our study (Xiao *et al*., 2019). In the study of Fallah and Sohrabnezhad (2019), the SEM image of Cr-MOF was similar to the one in this study. In the study of Lee *et al*., (2015) MOFs with different morphologies from amorphous spheres to crystalline hexagonal rods were presented. Naturally, there is a great variation among the adsorbents in terms of morphological properties.

X-ray diffraction (XRD) patterns of the adsorbents are presented in Figure 2. This analysis provides information about the crystal structures of solids. In the study of Vlasova *et al*. (2016), the XRD patterns of Al, Zn, and Ti-MOF were obtained. A major peak was observed at about 4-6° for Ti-MOF and the same peak was also detected in this study. In addition to this peak, there were two peaks at around 11 and 18° in the study of Vlasova *et al*.

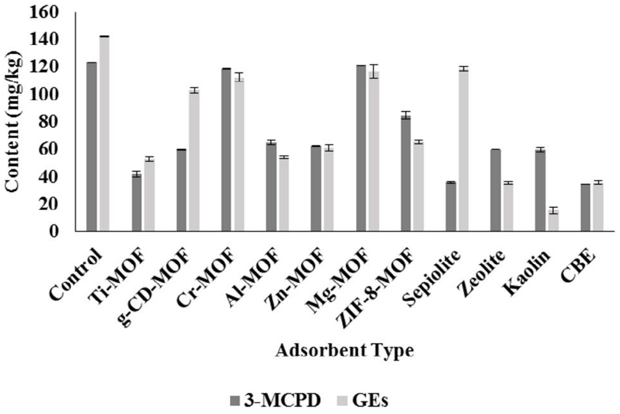

Figure 3. 3-MCPD and GE contents in the control and treated model oil samples. Results are expressed as mean ± SEM. Error bars represent standard error of mean. The whole study was repeated twice and each analysis for each replicate was done in triplicate (n = 6). The data were analyzed using one-way ANOVA and the groups were compared with Tukey's tests (P ≤ 0.05). 3-MCPD, 3-monochloropropane-1,2-diol; GEs, glycidyl esters; Ti-MOF, titanium (IV) butoxide-terephthalate MOF; γ-CD-MOF, gamma-cyclodextrine-potassium hydroxide MOF; Cr-MOF, chrome nitrate-terephthalate MOF; Al-MOF, aluminum chlorate-terephthalate MOF; Zn-MOF, zinc nitrate-2,5-furandicarboxylicacid MOF; Mg-MOF, magnesium-MOF; ZIF-8-MOF, zinc-2-methylimidazole zeolytic type MOF; CBE commercial bleaching earth.

(2016), but not detected in this study. The XRD patterns of Zn-MOF and Al-MOF in this study were also similar to that study. In another study, the XRD patterns of γ-CD-MOF were presented (Moussa *et al*., 2016). Although there were some differences, similar peaks were observed for γ-CD-MOF in our study. According to Li *et al*., (2015), Cr-MOF showed peaks at around 3°, 5°, 6°, 9°, 10° and 17° and similar peaks were also observed in our study. The XRD patterns for ZIF-8-MOF were presented in the study of Park *et al*. (2006) and it was very similar to this study. Although there are some slight differences, the results generally concur with the literature. The determined peaks in the samples indicate that they all included certain types of crystal structures. In addition, the XRD patterns of natural clays indicate the presence of some amorphous structure besides crystallinity.

3.2. 3-MCPD and GE contents in the treated model oil samples

The 3-MCPD and GEs contents of the samples after the adsorbent treatments, and the removal, based on the calculation over the control sample, are presented in Figure 3. In the control (not treated with any adsorbent) oil sample, 3-MCPD and GE contents were determined as 123.51 mg/kg and 142.44 mg/kg, respectively. These contents were lower than the added amounts, probably because of losses during operation or the level of purity of the added compounds. These differences do not matter because the removal was calculated over these control values. Among MOFs, the highest removal was observed for Ti-MOF (65.97% and 62.95%) for 3-MCPD and GEs. Among natural clays, CBE yielded the lowest remaining 3-MCPD (71.86% reduction), and kaolin yielded the lowest remaining GEs (89.09% reduction). Obviously, there is a variation among the adsorbents in terms of removal. Although the mechanism of the interaction between adsorbent and 3-MCPD/GEs is not investigated in this study, it is thought that selective adsorption of 3-MCPD and GEs from oils mostly depends on the presence of high affinity groups in adsorbents and structural properties (pore size, specific volume, surface area etc.).

In another study, activated bleaching earth was used to remove glycidyl esters from diacylglycerol oils and it was observed that glycidyl esters in varying amounts were reduced to the limit of quantification value (0.1 mg/kg) (Shimizu *et al*., 2012). In addition, a glycerol diolate model oil system containing glycidyl palmitate was used to investigate the elimination mechanism and it was determined that the elimination of glycidyl esters by activated bleaching earth occurs by transformation rather than adsorption. In another study, acid-washed oil-palm-wood-based activated carbon was investigated for the removal of glycidyl palmitate from a hexadecane solution, as a model oil system, and it was observed that removal can reach up to 95% depending on process conditions (Cheng *et al*., 2017b). In a study about MOF treatment, carboxylated and deprotonated Fe-MIL-88s with different ligand types were used for the removal of 3-MCPD and glycidol from model media (isopropyl alcohol/toluene, 1:1, w/w) and removal reached up to 95%, depending on process conditions and MOF type (Ahn *et al*., 2020). Generally, higher removal was achieved in these studies compared to the present study. In the study of Ahn *et al*. (2020), the adsorption mechanism of 3-MCPD and glycidol was investigated and they found that carboxylated Fe-MIL-88s had higher adsorption capacity for both 3-MCPD and glycidol compared to non-functionalized MOFs, probably due to the esterification of 3-MCPD and glycidol with deprotonated carboxyl groups. It is obvious that in addition to the structural properties of the adsorbents, the functional groups on the surface were also effective. This also explains why the kaolin and Ti-MOF used in this study were more effective despite having a lower surface area than some other absorbents.

The MOFs and natural clays used in our study showed good potential for the removal of 3-MCPD and GEs, and the efficiency of the treatment can be improved by adding some functional groups onto the surface of the adsorbents and optimizing the process conditions.

3.3. Effects of the treatment parameters

Ti-MOF and kaolin were selected as the best performing adsorbents among MOFs and natural clays, respectively, to investigate the effects of adsorbent addition levels, treatment temperatures and treatment times.

Figure 4 presents the effect of the level of adsorbent addition (0.5%, 1.5%, 3.0%, 6.0%) on the removal of 3-MCPD and GEs with the two selected adsorbents at 95 °C treatment temperature and 60 min treatment time. As the addition level increased, the removal of 3-MCPD and GEs increased for both adsorbents. Therefore, higher adsorbent addition levels could be used for more efficient 3-MCPD and GEs removal, but the cost of the adsorbent must also be considered.

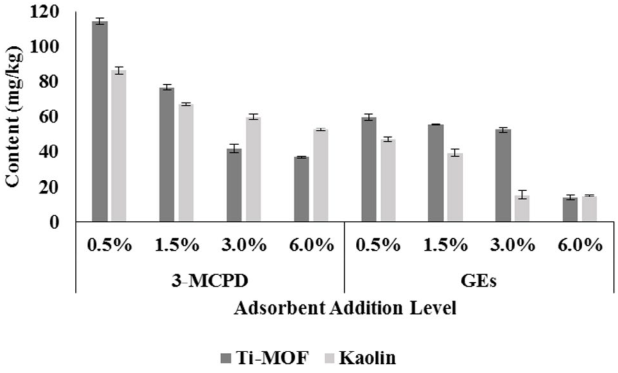

Figure 4. The effect of the level of adsorbent addition on the removal of 3-MCPD and GEs. Results are expressed as mean ± SEM. Error bars represent standard error of mean. The whole study was repeated twice and each analysis for each replicate was done in triplicate (n = 6). The data were analyzed using one-way ANOVA and groups were compared with Tukey's tests (P ≤ 0.05). 3-MCPD, 3-monochloropropane-1,2-diol; GEs, glycidyl esters; Ti-MOF, titanium (IV) butoxide-terephthalate MOF.

The effect of treatment time (15, 45, 60 and 120 min) on the removal of 3-MCPD and GEs at 95 °C treatment temperature and 3.0% adsorbent addition level is shown in Figure 5. Clearly, the removal of 3-MCPD and GEs increased for both adsorbents with the increase in treatment time. Hence, the effectiveness of the treatment can be improved by increasing treatment time but the processing price should also be considered.

Figure 6 shows the effect of treatment temperature (25 °C, 50 °C, 95 °C, 150 °C) on the removal

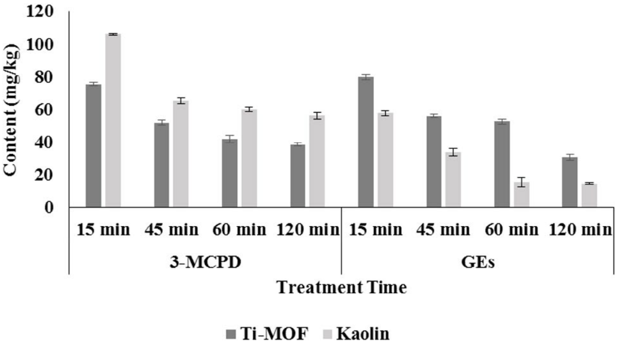

Figure 5. The effect of treatment time on the removal of 3-MCPD and GEs. Results are expressed as mean ± SEM. Error bars represent standard error of mean. The whole study was repeated twice and each analysis for each replicate was done in triplicate (n = 6). The data were analyzed using one-way ANOVA and groups were compared with Tukey's tests (P ≤ 0.05). 3-MCPD, 3-monochloropropane-1,2-diol; GEs, glycidyl esters; Ti-MOF, titanium (IV) butoxide-terephthalate MOF.

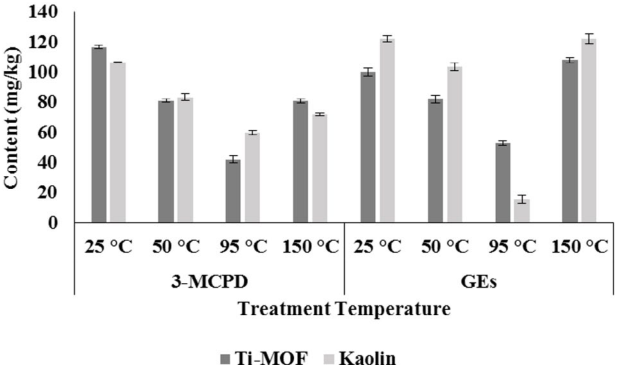

Figure 6. The effect of treatment temperature on the removal of 3-MCPD and GEs. Results are expressed as mean ± SEM. Error bars represent standard error of mean. The whole study was repeated twice and each analysis for each replicate was done in triplicate (n = 6). The data were analyzed using one-way ANOVA and groups were compared with Tukey's tests (P ≤ 0.05). 3-MCPD, 3-monochloropropane-1,2-diol; GEs, glycidyl esters; Ti-MOF, titanium (IV) butoxide-terephthalate MOF.

of 3-MCPD and GEs with 3.0% adsorbent addition level and 60 min treatment time. The highest removal was obtained at 95 °C and it was observed that the removal decreased at lower and higher treatment temperatures. It is thought that at lower temperatures, the solubilities of 3-MCPD and GEs were not enough to penetrate into the adsorbents or kinetic energy was not enough for adsorption; while at higher temperatures, adsorbent affinity decreased or adsorbed contaminants dissolved back into the oil with increasing solubility and kinetic energy. As a result, 6.0% adsorbent level, 95 °C treatment temperature and 120 min treatment time were selected as the best treatment conditions for Ti-MOF and kaolin.

In one study, inorganic adsorbent materials were used to remove 3-MCPD esters and related substances from palm oil and the effects of process parameters on the efficiency of the treatment were investigated with the two most promising adsorbent materials (Strijowski *et al*., 2011). It was determined that as adsorbent level increased, the removal of the contaminants increased for both adsorbents. It was also observed that increasing treatment temperature and time improved the efficiency of synthetic magnesium silicate, while calcinated zeolite was not affected by these parameters. In another study, carboxylated and deprotonated Fe-MIL-88s with different ligand types were used for the removal of 3-MCPD and glycidol from model media (isopropyl alcohol/toluene, 1:1, w/w) (Ahn *et al*., 2020). It was observed that the removal of 3-MCPD and glycidol increased as adsorbent level, treatment temperature and time increased. Although there are some slight differences, the results generally concur with the literature.

3.4. Removal of 3-MCPD and GEs from palm oil

RBD and DEO palm oils were treated with Ti-MOF and kaolin for the removal of 3-MCPD and GEs, and the results are presented in Figure 7. Clearly, there are no significant differences in terms of removal between Ti-MOF and kaolin for both RBD and DEO palm oils. In RBD palm oil, the removal of 3-MCPD and GEs was measured at 26 and 56% for Ti-MOF and 28 and 58% for kaolin, respectively. In DEO palm oil, the levels of 3-MCPD and GEs were reduced by 26 and 56% with Ti-MOF and 25 and 56% with kaolin, respectively. It is obvious that these values are lower than the removal in the model oil system but this is a quite expected result because commercial palm oil is a more complex system than model oil.

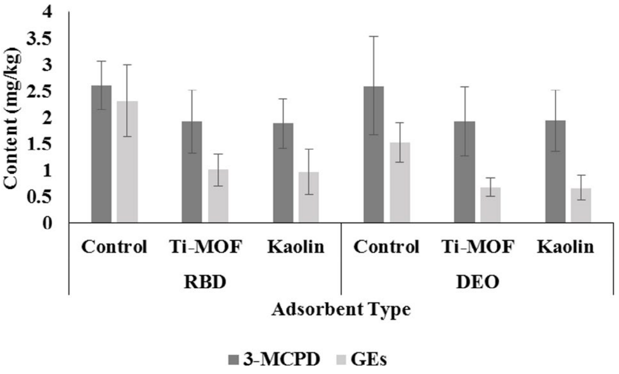

FIGURE 7. 3-MCPD and GEs contents in the control and treated palm oil samples. Results are expressed as mean ± SEM. Error bars represent standard error of mean. The whole study was repeated twice and each analysis for each replicate was done in triplicate (n = 6). The data were analyzed by using one-way ANOVA and groups were compared with Tukey's tests ($P \leq 0.05$). 3-MCPD, 3-monochloropropane-1,2-diol; GEs, glycidyl esters; Ti-MOF, titanium (IV) butoxide-terephthalate MOF; RBD, refined, bleached and deodorized; DEO, deodorized.

In one study, nine different commercially-available inorganic adsorbent materials were investigated for the removal of 3-MCPD esters and related substances from palm oil (Strijowski *et al*., 2011). Among all adsorbents, calcinated zeolite and the silicon calcium silicate were selected as the most promising adsorbents whose removal reached up to 40%. In another study, the carboxylated and deprotonated form of Fe-MIL-88-BDC, which is a type of MOF, was used for the removal of 3-MCPD and glycidyl ester from refined palm oil and both of these contaminants were reduced by 90% (Ahn *et al*., 2020). It is thought that the efficiency of the treatment in our study should be improved by modifying the adsorbents.

4. CONCLUSIONS

In this study, selected MOFs and natural clays were evaluated for the removal of 3-MCPD and GEs from edible oils. First, the structural properties and morphologies of the adsorbents were determined. Then, the 3-MCPD and GE removal capacities of the adsorbents were investigated using a model oil, and Ti-MOF and kaolin were selected as the best performing MOF and natural clay, respectively. The effects of treatment conditions on treatment efficiency were also analyzed and 6.0% adsorbent level, 95 °C treatment temperature and 120 min treatment time were selected as the best treatment conditions. Furthermore, palm oils were treated with Ti-MOF and kaolin at selected treatment conditions and removal of about 25 and 55% were achieved for 3-MCPD and GEs, respectively. Overall, this study shows that MOFs and natural clays have good potential for the removal of 3-MCPD and GEs from edible oils, and for further studies, adsorbent modification is recommended in order to improve the efficiency of the treatment. Especially synthesis chemists may pay attention to adding specific ligands to the adsorbents, which have high affinity for chloride compounds to selectively remove those process contaminants from edible oils.

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Acidic and enzymatic pre-treatment effects on cold-pressed pumpkin, terebinth and flaxseed oils

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SUMMARY: Oil yield and the properties of oil can be improved with various enzymatic pre-treatments before obtaining oil from oilseeds by cold-press extraction. A commercial mixture of pectolytic enzymes was used in this study. In addition, apple seed meal as a source of β-glucosidase enzyme and citric acid were applied to oilseeds (pumpkin, terebinth and flaxseed) as pre-treatments. The results were evaluated by comparing the effects of the pre-treatments on oil yield and properties. Enzyme preparate could increase the oil yield of pumpkin seeds (~300%) and flaxseed (151%). Significant increases in the phenolic contents of terebinth (from 91.67 to 319.33 mg GAE/kg) and flaxseed oils (from 12.03 to 40.47 mg GAE/kg) were achieved by citric acid and enzymatic pre-treatments. These two pre-treatments were also effective in terms of peroxide formation and oxidative stability in terebinth oil. With the help of the pre-treatments applied to oilseeds it was possible to increase the transition of phenolics from seeds to oil for terebinth oil with increase ratios of 245% for citric acid, 248% for the enzymatic process compared to the control.

KEYWORDS: *β-glucosidase; Cold-pressed oil; Enzymatic pre-treatment; Flaxseed; Pumpkin seed; Terebinth*

RESUMEN: ***Efectos de pretratamientos ácido y enzimático sobre los aceites de calabaza, terebinto y linaza prensados en frío***. El rendimiento y las propiedades del aceite se pueden mejorar con varios pretratamientos enzimáticos antes de obtener el aceite de las semillas oleaginosas mediante extracción por prensado en frío. En este estudio se utilizó una mezcla comercial de enzimas pectolíticas. Además, se aplicó, como pretratamientos, harina de semilla de manzana, como fuente de enzima β-glucosidasa y ácido cítrico, a semillas oleaginosas (calabaza, terebinto y linaza). Los resultados se evaluaron comparando los efectos de los pretratamientos sobre el rendimiento y las propiedades del aceite. El preparado enzimático puede aumentar el rendimiento de los aceite de las semillas de calabaza (~ 300%) y la linaza (151%). Se lograron aumentos significativos en los contenidos fenólicos de terebinto (de 91,67 a 319,33 mg GAE/kg) y aceites de lino (de 12,03 a 40,47 mg GAE/kg) mediante pretratamientos con ácido cítrico y enzimas. Estos dos pretratamientos también fueron efectivos en términos de formación de peróxido y estabilidad oxidativa en aceite de terebinto. Con la ayuda de los pretratamientos aplicados a las semillas oleaginosas, fue posible aumentar el traspaso de fenoles de las semillas al aceite, para el caso del aceite de terebinto, con porcentajes de aumento del 245% con el tratamiento con ácido cítrico y 248% para el proceso enzimático, en comparación con el control.

PALABRAS CLAVE: *Aceite prensado en frío; β-glucosidasa; Pretratamiento enzimático; Semilla de calabaza; Semilla de lino; Terebintos*

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1. INTRODUCTION

Oilseeds are a rich source of bioactive components. Their direct consumption is widespread and the oils of these seeds have recently attracted attention due to their protective, restorative effects and flavors. The processes used to obtain oil from oilseeds affect oil quality (Siger and Józefiak, 2016). Various methods are used for oil extraction. These usually include distillation, solvent-supercritical fluid extraction and the pressing method. In the pressing method, the yield is lower than other methods, but the quality of the oil obtained is high (Çalıkoğlu *et al.*, 2006). Advantages to this method are the small amount of energy consumed in oil production from oilseeds, easy application, no chemical or heat treatment required and high oil quality. This method is quite valuable in terms of obtaining oil quality; however, the yield is much lower than the solvent extraction method (Maier *et al.*, 2009). High polyphenol content, which is one of the important quality parameters of oil, can pass from seed to oil by a cold-press method without deterioration. The press method has different applications such as mechanical pressing, hydraulic pressing or pressing with gear grinders for oil extraction (Sevindik and Selli, 2016).

To increase the yield, pre-treatments of oil samples before extraction have been evaluated in many studies (Anwar *et al.*, 2013; Karataş, 2015; Koubaa *et al.*, 2016; Mazaheri *et al.*, 2019). The most common among them is the heat treatment. However, in some oils, heat treatment decreases the quality of the oil and even causes the oil unsuitable to be defined in the cold-pressed oil range. Other pre-treatments are roasting, ultrasound or microwave application, maceration and irradiation (Şeran, 2011). Another pre-treatment that is thought to be effective in increasing oil quality is enzyme addition. Enzymes are used in the food industry to increase yields in the production of fruit juice, oil and sugar by affecting polymers on the cell wall. However, using enzymes was a major disadvantage that leads to a high priced process. But now, thanks to advances in biotechnology, low price and high-quality enzyme formulations can be obtained.

In this paper, pumpkin seed, flaxseed and terebinth were used. Pumpkin seed is a widely produced oilseed with high commercial value. In particular, the raw form of pumpkin seeds without a shell has the highest bioactive component (tocopherols, polyphenols, squalene, piperazine) among the pumpkin species (Nakić *et al.*, 2006). The range of oil varies from 40 to 60% depending on the type of pumpkin. Pumpkin seeds are rich in unsaturated fatty acids, especially oleic (30.35%–42.07%) and linoleic acid (43.68%–52.15%) (Akin *et al.*, 2018). This oil is preferred in salads (Jafari *et al.*, 2012). The use of terebinth seed spreads over a wide range of applications, from cosmetics to food. The reasons for selecting the terebinth seed in this study are the high oil content of the terebinth and its richness in oleic acid, high number of phenolic compounds and its potential as a commercial product. The highest fatty acid content in the terebinth seeds was found as monounsaturated oleic acid with a range of 51.2–67.5 g/100 g (Ertaş *et al.*, 2013). Oleic acid (45.8%), linoleic acid (23.93%) and palmitic acid (24.27%) have been identified as the main fatty acids of terebinth oil (Kaya and Özer, 2015). Flaxseed, which is rich in quality protein, is a natural source of phytochemicals such as flavonoids, lignans and phenolic acids. Cold-pressed flaxseed oil contains about 50% α-linolenic acid (ALA), which is one of the omega-3 fatty acids. A low temperature operation should be preferred for long shelf-life and high quality due to its high ALA content (Wiesenborn *et al.*, 2005).

Phenolic compounds found in oilseeds are available in free, soluble conjugates and insoluble-bound forms. The distribution of phenolic compounds varies in different parts of the seeds (Rahman *et al.*, 2018). These plant-derived phenolic compounds are in conjugated form with sugar functional groups. During the process or metabolism, these glycoside forms are converted to aglycones by enzymatic or chemical means. This transformation can increase the concentration and bioactivity of phenolic compounds in free form (Küçükhüseyin, 2012). Few studies have reported the application of enzymes to increase phenolic production in processes using organic solvents or water in the extraction of phenolic compounds from vegetable samples (Laroze *et al.*, 2010). The use of enzymes in oil extraction processes provides a high number of antioxidant compounds and an increase in the yield by disrupting the plant cell wall (García *et al.*, 2001). β-glucosidases are enzymes capable of hydrolyzing β-glucoside bonds in oligosaccharides or phenolics with glycosidic compounds (Ergöçen, 2013). The β-glucosidase enzyme is commonly found in many fruit seeds (fig, apricot, grape, papaya) and bread yeast (Sirilun *et al*., 2016). The study by Yu *et al.* (2007) revealed that the β-glucosidase enzyme obtained from apple and peach kernels was more suitable than that of commercially supplied β-glucosidase. The dry matter of apple and peach kernels was determined as the most suitable enzyme source due to their high activity, broad substrate specificity and strong stability. In addition, in the same study, the total activity and specific activity of β-glucosidase in the crude extract of apple seed were given as 54.8 U and 0.38 U per mg, respectively. Besides, the half-life of apple seed β-glucosidase at 50 °C was 42.9 h. It was stated that this result showed that the glucosidase obtained from apple seeds had higher thermal stability than commercial almond glucosidase. These two enzymes with similar pH preference (apple seed β-glucosidase and commercial almond β-glucosidase) were most active at pH 6.0 and remained stable up to 24 hours in the pH range of 5.0-9.0. It was concluded that apple seed glucosidase is an easily accessible natural catalyst and can be reused for about for one month without immobilization. The crude meal from apple seed was used for the first time in the study by Tong *et al*., (2004) as an easily available and inexpensive biocatalyst in the large-scale synthesis of alkyl-β-glucopyranosides. The world yield of apples was approximately 76 million metric tons in the 2019/20 season, and the majority of these are used for juice production, which generates a large amount of waste including seeds and pomace. If these fruit wastes can be used as enzyme sources, the waste will be valued and the price of the enzyme can also be reduced greatly. Hence, in this study, apple seed meal was used as a simple and cost-effective source of β-glucosidase enzyme. Alternatively, the acid hydrolysis of glycosides and phenolics has also been a common practice. Usually, HCl has been used for this procedure (Watson *et al*., 2014). Here, in our study, citric acid was preferred for acid pre-treatment since it is not harmful, easy to access, economical and widely used in the food industry.

The current study investigates the effect of a commercial enzyme mixture, apple seed meal and citric acid pre-treatments applied to oilseeds on oil yield and oil properties (peroxide value, FFA, free radical scavenging activity). With the commercial enzyme mixture, which is a pectinase complex, it is aimed to degrade the cell wall and hydrolyze glycosidic aglycons with apple seed meal; while the objectives of the citric acid pre-treatment are cell wall disruption and the hydrolysis of aglycones.

The effect of enzyme pre-treatment on oil yield and properties in oil extraction has been investigated in many studies (Latif *et al.,* 2007; Latif and Anwar, 2009; Latif *et al.,* 2011; Liu *et al.,* 2016). However, the number of studies using enzyme treatment before cold press oil extraction is limited (Soto *et al.,* 2008). No studies which utilize apple seed β-glucosidase and citric acid as pre-treatments are available. Citric acid and apple seed β-glucosidase provide an easily accessible and inexpensive source for industrial applications. In addition, the different effects of pre-treatments originating from the seeds were revealed by using three different oil seeds (pumpkin, terebinth and flaxseed) in the study.

2. MATERIALS AND METHODS

2.1. Materials

Terebinth and flaxseed were obtained from local markets in Konya, Turkey. Hulled pumpkin seeds harvested in Ürgüp (Turkey) were purchased from a local market. All chemicals and standards were of analytical grade and purchased either from Merck (Darmstadt, Germany) or Sigma- Aldrich (Germany).

2.2. Preparation of oilseeds for cold pressing

The oilseeds were divided into equal batches (500 g) and stored in a cool, dry place till pressing. The seeds were milled to a particle size of approximately 2–4 mm to increase surface area before cold pressing. The grinding process was conducted with an Arsel industrial kitchen grinder (220 V, 0.8 kg capacity, 0.3 kW, 9000 rpm/9 min motor power). The milled seeds were evenly distributed on a tray (40x60x5 cm, 0.2 cm thick). 0.1 M (pH=6) Na_2HPO_4 buffer solution was used in all pre-treatments.

Citric acid pre-treatment*:* a citric acid pre-treatment was performed to oilseeds by spraying with a ratio of 1% (by seed weight). For this pre-treatment, 5 g of citric acid were dissolved in 25 mL purified water. The resulting citric acid solution was sprayed into the ground seeds homogeneously in the presence of 100 mL buffer solution.

Commercial enzyme*:* Olivex (Advanced Enzyme Technologies Ltd. India), a pectinase complex produced by the fungus *Aspergillus aculeatus* was added to the batches at a ratio of 1%. For this purpose, 5 g enzyme were weighed for 500 g sample and dissolved in 100 mL buffer solution. The solution was sprayed onto the seeds.

Apple seed meal: To produce a meal rich in the β-glucosidase enzyme, 'Golden Delicious' apples (*Malus domestica* Borkh.) which were harvested in 2016–2017. The fresh apple seeds were powdered via a coffee grinder, washed three times with ethyl acetate and two times with acetone and filtered under vacuum (Yu *et al*., 2007). The aim of this procedure was to remove some phenolic compounds and lipids from the apple seeds with the help of acetone. For a convenient and low-cost material in industrial application, only the isolation stage was used to produce β-glucosidase-rich meal from apple seeds. After the process, the solid extract remaining on the filter paper was left at room temperature for 24 h to remove the residual acetone (Ergöçen, 2013). The dry residue was stored in the refrigerator at +4 °C until use. 50 g of apple seed meal was used for 500 g oilseeds (10%) in the presence of 100 mL buffer solution.

The samples were individually incubated with each of the three pre-treatment methods for 3 h, at 60 °C (Emir *et al*., 2015). The samples were mixed at regular intervals during incubation. A control oil sample was also prepared by pressing the seed material under the specified conditions without any pre-treatment.

2.3. Cold pressing of oil seeds

A manual screw-press extractor (Karaerler NF 500, Turkey, 50 kg seed/h capacity, single head, 1.5 kW power) was used for the cold-pressed oil extraction. The screw rotation speed in a cold-press extraction was determined as 20 Hz for flaxseed, and 10–15 Hz for pumpkin and terebinth seeds. During the cold-pressing process, the temperature did not exceed 50 °C. The oils and press cake samples were taken and weighed, the oil phases were filtered and placed in dark colored glass bottles and kept at +4 °C. Defatted meal samples were tightly packed in plastic bags and stored in the dark at +4 °C.

2.4. Determination of oil content and yield

Oil seeds which were cleaned and brought to dry weight were ground using a mill. The total oil content of the ground samples was determined in the oil analyzer (Velp Scientifica Ser 148/6 Series) with n-hexane. According to the Randall method (Medvedevskikh *et al.,* 2021), 2.5 g ground seeds were weighed into the device cartridges and extracted with n-hexane for 1 h. At the end of the period, the solvent was removed from the extraction vessels and weighed and the oil content in the seeds was determined as a percentage. The analyses were conducted twice in parallel.

Oil yield was calculated as the ratio of the weight of the oil obtained at the end of the extraction to the initial weight of the seed. The extraction yield is the amount of oil obtained from the cold pressing of 100 kg oilseeds. The yield was calculated using the following equation:

$$\text{Extraction yield} = (A_{oil} / A_{seed}) \cdot 100$$

A_{oil} is the mass of the extracted oil (kg) and A_{seed} is the mass of the oilseed (kg).

2.5. Quality indices

The determination of oil quality indices was performed according to Regulation EEC/2568/91 (EU, 1991) by determing free fatty acid (FFA) (mg KOH/g) and peroxide value (PV) (meqO_2/kg).

2.6. Determination of total phenolic compounds (TPC) and DPPH radical scavenging capacity

The concentration of TPC was determined colorimetrically using FCR (Folin-Ciocalteu reagent) (Singleton *et al*., 1999). 6 g of oil sample were weighed, dissolved in 3 mL n-hexane and vortexed for 2 min with the addition of 6 mL methanol/water (80:20 v/v) for extracting phenolic substances. The n-hexane and methanol/water phases were separated via centrifuge (at 3000 rpm for 5 min). This process was repeated three times, and the methanolic phases were evaporated using a nitrogen evaporator. The methanolic extracts were stored in the refrigerator until analysis. To obtain the methanolic extract from press cake samples, 10-g samples were weighed into a 100 mL Erlenmeyer flask and adjusted to volume with methanol/water (80:20 v/v). The flasks were shaken at 250 rpm for 2 h. After shaking, the supernatants were taken and centrifuged at 10000 rpm for 10 min. This process was repeated twice and the supernatants were filtered and concentrated up to 15 mL in a rotary evaporator at 40 °C. Methanol extracts obtained from oil and press cake were also used to calculate the DPPH free radical scavenging capacity.

For the determination of the total number of phenolics, the extracts were taken to the test tube at appropriate concentrations and their volumes were made up to 5 mL with ultra-pure water. 0.5 mL of FCR/distilled water (1: 3 v/v) was added and stirred for 30 min in the dark. At the end of this period, 1 mL of a sodium carbonate solution (35%) and 3.5 mL of purified water were added and the mixture was kept in the dark again for 2 h. Absorbance values were measured at 725 nm in a spectrophotometer (Biochrom Libra S22, UK) against a blank sample.

For the determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of oil and press cake extracts prepared at proper concentrations were placed in test tubes and the volume was completed to 1 mL with buffer solution (Tris-HCl). 2 mL of a methanolic solution of DPPH were added and left to react in the dark at room temperature. The decrease in absorbance at 517 nm was recorded in a spectrophotometer (Biochrom Libra S22, UK) after 30 min. Results were calculated based on the percent inhibition of the DPPH radical (Blois, 1958).

2.7. Oxidation stability of cold-pressed oils

892 Professional Rancimat apparatus and StabNet 1.0 software were used for induction time measurements. Oil samples (3 g) were weighed into reaction vessels and heated at 120 °C under a dry airflow of 20 L/h. The volatile compounds released during oxidation were collected into a cell containing distilled water, and the increasing water conductivity was continually measured. The time taken to reach the conductivity inflection point was recorded as the induction period (IP), and expressed in hours (h). All determinations were conducted in triplicate.

2.8. Statistical analysis

The data obtained as a result of the research were subjected to analysis of variance “General linear model multivariate analysis”; significant differences between the mean values of the main sources were compared with Duncan’s multiple comparison tests (Zolman, 1993). SPSS 10.0 for Windows (v.10) was used for statistical analysis and the significance level was given as $P \leq 0.05$. This research was performed in three duplicates with a replicate.

3. RESULTS AND DISCUSSION

3.1. Oil yield and quality

The total oil contents of the oilseeds were 46.97, 43.86, and 37.80% on a dry weight basis for pumpkin seed, terebinth and flaxseed, respectively, which were determined by the rapid oil extraction method. The lowest oil yield was obtained by cold pressing untreated pumpkin seeds (around 16% of the initial oil content). The low oil yield of hulled pumpkin seed was known to us according to previously published data. For instance, in the study conducted by Neđeral and coworkers (2012), it was reported that the oil yield of untreated hulled pumpkin seed from the cold-press process was 48% lower than the initial oil content for husked pumpkin seeds. On the other hand, the highest increase in oil yield due to the pre-treatments was obtained for pumpkin seeds. For all three pre-treatment (apple seed meal, citric acid and enzyme), the increase was around 300%. Although not at the levels seen in pumpkin seeds, pre-treatments also caused increases in oil yield for flaxseed. The highest oil yield increase in this oily seed was achieved by enzymatic pre-treatment (an increase from 16 to 24.23% , which corresponds to a 151% increase). Anwar *et al.* (2013) evaluated the effect of three enzymes on the flaxseed cold-press extraction and reported a remarkable effect on oil recovery with 8,12, and16% increase rates. Ranalli *et al.* (2005) and Latif *et al.* (2011) explained the enhancement in oil yield with the help of enzymatic treatment by using rupturing cell walls and expelling some more free oil. Moreover, Ezeh and coworkers (2016) reported that they achieved the highest oil recovery of 90% by applying enzymatic pre-treatment on tiger nut tubers. The average moisture content in hulled pumpkin seeds and terebinth were 7.2 (Neđeral *et al.,* 2012) and 6.17% (Dalgıç *et al.,* 2011), respectively. Oil yields were different as a result of cold-press extraction of these two oilseeds, whose total oil contents were close to each other. The physical and chemical properties of the seeds, such as moisture, crude oil, crude fiber and diameter-to-length ratio may be effective in the formation of this difference. The pre-treatments for terebinth seed negatively affected the oil yield and even caused decreases of 22-27% compared to the control. The emulsion formation caused by the mechanical effect of pre-treatments and pressing was responsible for the decrease in oil yield from terebinth seeds. The negative effect of pre-treatments in this case could be attributed to the specific physical structure of the mass. A slurry texture was formed after mechanical pressing due to its rich content in resinous substances and this phenomenon triggered the emulsion formation in terebinth paste. Particularly, when seeds with high oil content are ground into very small particle sizes, the particles can easily stick together (Liu *et al.*, 2016).

Although enzymatic pre-treatment led to higher values and apple seed meal and citric acid resulted in lower values, these changes in FFA were not significant in the pumpkin seed oil (Table 1). Because of the apple seed meal pre-treatment (1.77 mg KOH/g oil), FFA in flaxseed oil significantly decreased compared to that of the control (2.14 mg KOH/g oil). The application of apple seed meal was thought to have inactivated the lipase enzyme. This inhibition effect was not statistically significant for pumpkin seed oil, but a significant effect was determined for the flaxseed oil. On the other hand, the pre-treatments showed the most spectacular changes in the FFA of terebinth seed oils. The highest increase was determined in the samples treated with enzyme preparate, as the average FFA values were 11.98 mg KOH/g oil. Note that the FFA of untreated terebinth oil (control sample) was also higher compared to untreated flaxseed and pumpkin seed oils. The relatively higher moisture content and specific cell structure of terebinth seeds were probably responsible for these high FFA levels. The incubation step in the pre-treatment triggers lipolytic activity due to moisture and impurities results in the hydrolysis and formation of free fatty acids. The cellulolytic effect of the enzyme mixture should also be taken into account. Similar findings were reported by Emir *et al.* (2015) that enzymatic pre-treatment increased the FFA of cold-pressed poppy seed oil. Codex standards indicate that the acid value of cold-pressed oils and edible fats should be less than 4.0 mg KOH/g.

The PV of the oil samples ranged from 1.51–5.79 meqO_2/kg (Table 1). The PVs of pumpkin and flaxseed oils were higher in pre-treated samples. Among

TABLE 1. Oil yields and quality indices of pre-treated oil samples obtained by cold press extraction.

Pre-treatments	**Oil seeds**		
	Pumpkin seed	**Flaxseed**	**Terebinth**
Oil yield (%)			
Control	7.52 ± 0.34[†]c C	16 ± 0.49 c** B	26.3 ± 0.69 a A*
Citric acid	22.58 ± 0.80 a A	16.4 ± 0.33 c C	20.46 ± 0.44 b B
Commercial enzyme	20.07 ± 1.07 b B	24.23 ± 0.20 a A	20.73 ± 0.92 b B
Apple seed meal	22.92 ± 0.80 a A	20.44 ± 0.36 b AB	19.39 ± 1.80 b B
Free fatty acid (mg KOH/g)			
Control	1.95 ± 0.46 B	2.14 ± 0.13 ab[ii] B	3.91 ± 1.00 b A
Citric acid	1.58 ± 0.27 B	2.13 ± 0.52 ab B	7.05 ± 0.47 b A
Commercial enzyme	2.04 ± 0.58 B	2.88 ± 0.26 a B	11.98 ± 2.77 a A
Apple seed meal	1.58 ± 0.35 B	1.77 ± 0.35 b B	5.47 ± 0.36 b A
Peroxide value (meq O_2/kg)			
Control	2.35 ± 0.26 c B	1.51 ± 0.79 b B	4.79 ± 0.23 a A
Citric acid	5.79 ± 0.67 a A	1.99 ± 0.59 ab C	3.68 ± 0.39 b B
Commercial enzyme	3.77 ± 0.92 b	2.53 ± 0.23 ab	3.49 ± 0.24 b
Apple seed meal	4.8 ± 0.20 ab A	3.27 ± 0.63 a B	4.61 ± 0.41 a A
Induction Period (h)			
Control	6.05 ± 1.22 B	0.44 ± 0.31 C	8.27 ± 0.57 b A
Citric acid	5.34 ± 0.53 B	0.46 ± 0.26 C	9.12 ± 0.39 b A
Commercial enzyme	4.85 ± 0.30 B	0.50 ± 0.31 C	11.12 ± 0.79 a A
Apple seed meal	5.00 ± 0.37 B	0.75 ± 0.38 C	6.18 ± 0.32 c A

Note: [†]Data represent mean ± SD of 3 replicates in duplicate. [*]Lowercase letters show the significant differences between pre-treatment applied and non-applied samples ($P \leq 0.05$) and [**]Uppercase letters show the significant differences among oilseeds ($P \leq 0.05$) using Duncan's multiple comparison test.

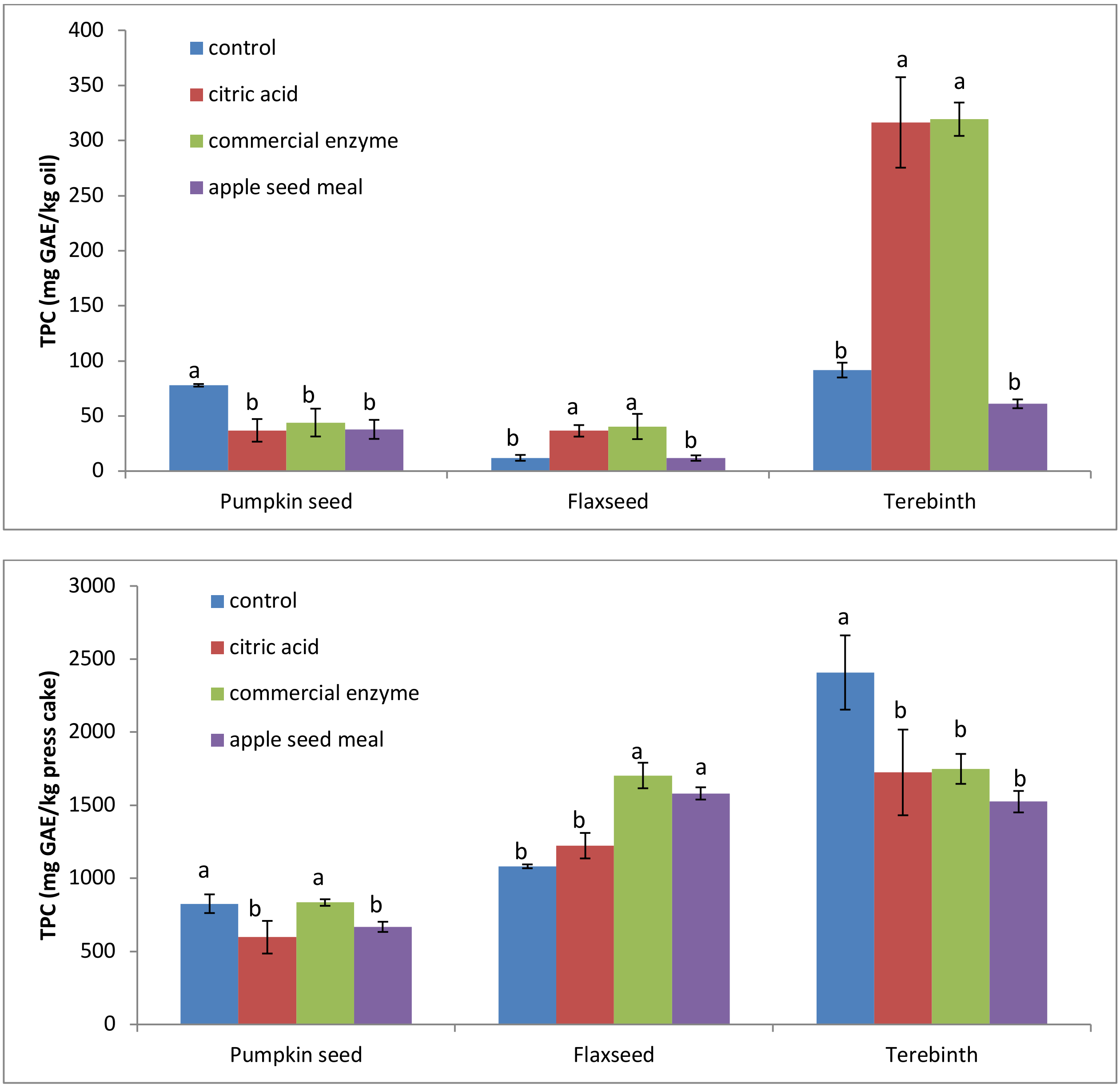

FIGURE 1. Total phenolic contents in oil and press cake samples obtained by cold-press extraction. Data represent mean ± SD of 3 replicates in duplicate. Lowercase letters show the significant differences among pre-treatments ($P \leq 0.05$) using Duncan's multiple comparison test.

the treatments, the increase in PV was more evident for apple seed meal than treated flaxseed oil. However, it was citric acid which considerably increased the PV of pumpkin seed oil. It was obvious that the incubation process performed within the scope of pre-treatments triggered the oxidation of these oils. In contrast, citric acid and commercial enzyme pre-treatments provided a significant reduction in the PV of terebinth oil compared to that of the control sample ($P \leq 0.05$). It might be possible for the polar phenolic components in the natural structure of the terebinth seed to better exert their antioxidant activity thanks to the pre-treatments. In addition, the transition of apolar phenolics or other antioxidants to oil occurred at a higher rate thanks to the citric acid and enzymatic pre-treatments (Figure 1). The fact that this was not the case for flaxseed may be attributed to its rich α-linolenic acid (ω-3) content. Pumpkin seed oil, on the other hand, did not show resistance to oxidation as much as terebinth oil due to its fatty acid composition and its relatively low phenolics and antioxidant content. Considering the oxidative stability of the oil, the presence of some natural compounds with antioxidative capacity is a crucial factor (Vujasinovic *et al*., 2010). Tuberoso *et al*. (2007) found that the correlation between antioxidant activity and oilseed composition was variable and that this variability was caused by squalene, chlorophyl, carotenoid, phenolics and their mutual interactions. In addition, the oxidative stability of oil relies on the fatty-acid profile. The correlation between fatty acids and oxidative stability correlates positively with oleic acid with a negative correlation with linolenic and linoleic acid content (Tańska *et al*., 2016).

3.2. TPC and DPPH radical scavenging capacity

The use of acid or alkaline for hydrolysis is a general approach to the cleavage of glycosides from phenolic acid esters, phenolic acids and flavonoids. Enzymatic hydrolysis is another method applied as an alternative to acid hydrolysis. Glucosidases, pectinases, cellulases and amylases are some of the types of enzymes used for enzymatic hydrolysis (Adlard *et al*., 2011). This pre-treatment is a suitable alternative for increasing the recovery of polyphenols (Laroze *et al*., 2010). Moroever, the enzymatic hydrolysis of glycosides increases the hydrophobicity and bioactivity of the extracts since aglycones have a strong hydrophobicity (Do *et al.,* 2009).

Citric acid and commercial enzyme pretreatments led to significant increases in the number of TPC in terebinth and flaxseed oils ($p \leq 0.05$). This enhancing effect has been noted for terebinth seed oil with a much more significant difference between untreated and treated samples. Apple seed meal pre-treatment did not show any positive effect on the transition of phenolics into the oil (Figure 1).

However, regarding pumpkin seed oil, all the pre-treatments showed negative effects on the recovery of phenolic compounds, causing a ~76% reduction. The physical structure, moisture content and the character of the phenolic compounds in the ground seed may be effective in the reduction in the amount of phenolic components transferred to pumpkin seed oil as a result of the pre-treatments applied. Due to its unique tissue and cell structure, each oilseed is shredded to different sizes even if the same conditions are applied. Therefore, the penetration of the applied acids and enzymes may have remained at different levels. Since citric acid, enzyme preparation and apple seed enzyme extract were applied to the ground seed in a buffer solution, the moisture content of the seed mass was increased. During the subsequent incubation, the humidity remained at relatively high levels compared to the control, although there was moisture loss. Besides, the phenolic components in these oilseeds have different properties. Due to their features, such as containing more or less glycosidic bonds, having a large polyphenolic molecular structure or a simple phenolic acid structure, the amount of phenolics that pass into the oil and that remain in the paste may vary. In summary, the physical structure of the ground seed, its moisture content and the molecular structures of the phenolic components contained in the seed were effective on hydrolysis. Andjelkovic *et al.* (2010) reported the total phenol content in raw pumpkin seed oil to be between 24.71–50.93 mg GAE/kg, which was lower than the values determined in our study. They also stated that this difference in phenolic concentrations might be caused by variations in the harvesting and production conditions of the seeds. The higher values determined in our study may be due to cold pressing and lack of roasting step. Flaxseed is a rich source of flavonoids, lignans (especially secoisolariciresinol diglucoside (SDG)) and phenolic acids. The lignans are conjugated with carbohydrates and mostly located in the fiber part of flaxseed. Alkaline treatment breaks the link and releases free SDG, which is also used to obtain the aglycone form of lignans (Sainvitu *et al*., 2012). The increase in the total phenolic content of flaxseed oil samples (from 12.03 mg GAE/kg to 40.47 mg GAE/kg with commercial enzyme pre-treatment) was due to the release of phenolics from conjugated to free form via acidic and enzymatic hydrolysis.

In the study of Yıldız (2013), the total flavonoid content of terebinth fruit was reported as 76.01 mgCE/g extract. As a result of the HPLC analysis, to evaluate the flavonoid composition, luteolin, luteolin-7-glucoside and apigenin-7-glucoside compounds were determined to be the major compounds (Kavak *et al*., 2010). These compounds exist in the form of glycosides in plants. These flavonoids in the glycoside form can be released by separating the conjugated aglycones with acid and enzyme application. However, some glycosides show strong resistance to acid hydrolysis. For example, 7-*O*-glucuronides and 7-*O*-glucosides are glycosides that show

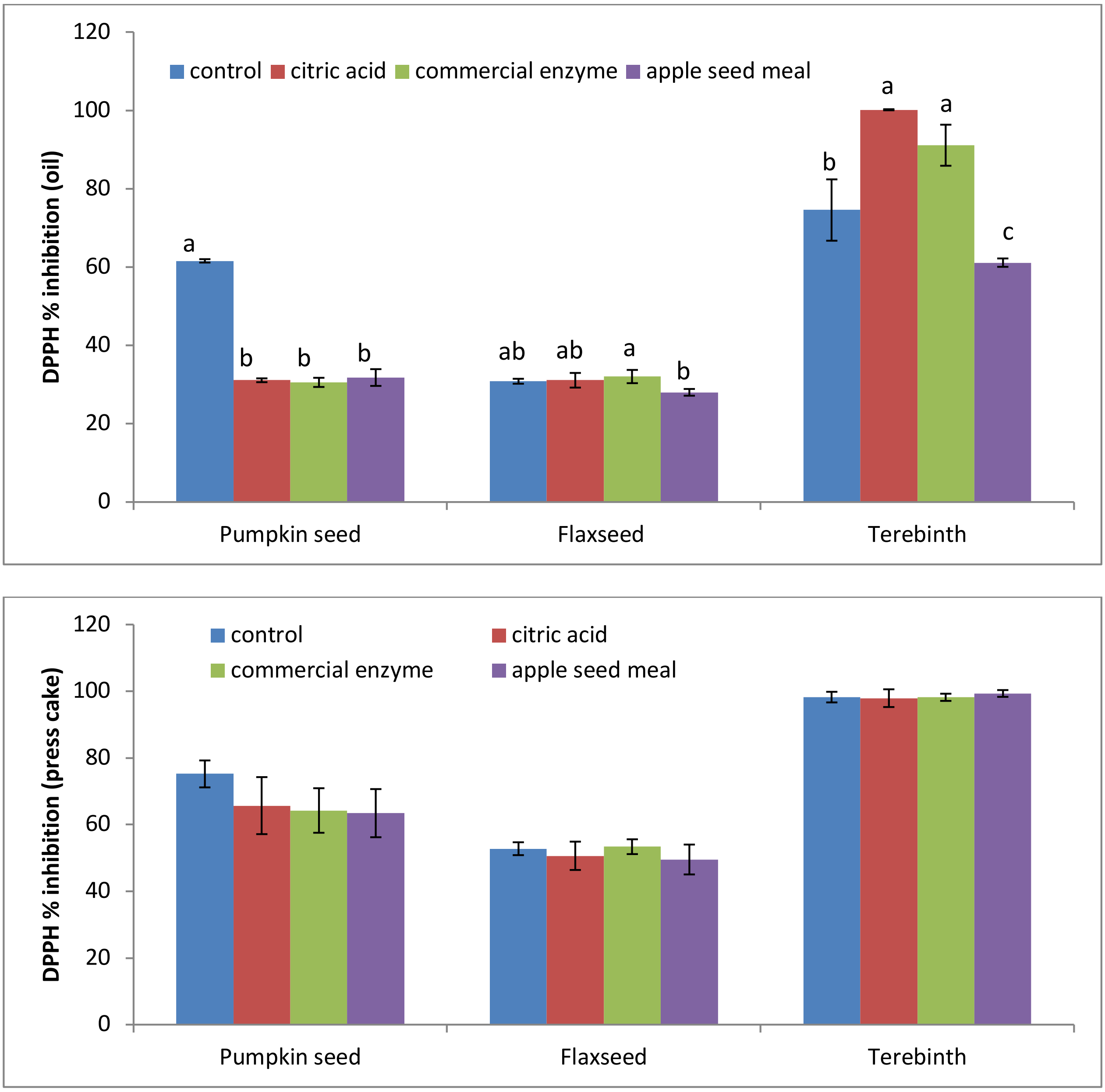

FIGURE 2. DPPH radical scavenging activity values in oil and press-cake samples obtained as a result of cold-press extraction. Data represent mean ± SD of 3 replicates in duplicate. Lowercase letters show the significant differences among pre-treatments ($P \leq 0.05$) using Duncan's multiple comparison test.

the most resistance to acid hydrolysis. Alternatively, these glucosides are rapidly hydrolyzed with suitable enzymes. Some *O*-glycosides have been reported to require prolonged heating (4–6 h) as they are difficult to dissolve with the effect of aqueous acid. For instance, luteolin 7-glucoside requires 6 h of heating with 30% H_2SO_4 for complete hydrolysis (Harborne, 1965). Considering this information, the increase in the number of TPC in terebinth oil due to enzyme and acid pre-treatments can be explained (from 91.67 mg GAE/kg to 319.33 and 316.4 mg GAE/kg, respectively). In order for apple seed meal to be effective in increasing the level of TPC as well, pre-treatment conditions could be rearranged (e.g., long incubation time or high incubation temperature).

Preceding works on oils such as flaxseed, olive, hemp seed and cottonseed pointed out that an enzyme pre-treatment notably enhanced the recovery of polyphenolic compounds and improved antioxidant activities in oils (Ranalli *et al*., 2005; Latif *et al*., 2007; Latif and Anwar, 2009; Anwar *et al*., 2013). Moreover, Soto *et al*. (2008) demonstrated that enzyme-aided cold-pressed borage seed oil had three times more antioxidant content than the values obtained from a non-enzyme-aided process. In our study, apple seed meal pre-treatment did not show any positive effect on the transition of phenolics to the oil. Apparently, beta glucosidase enzyme obtained from apple seed pulp was at a level to increase the oil yield of the processing steps, but not at a level to allow phenolic transition.

It could be said that the relationship between the TPC remaining in the press cake and the amount passing into the oil was meaningful for terebinth seeds. That is, press cakes from citric acid and enzyme-treated oil samples containing much higher amounts of TPC than the control also contained significantly lower amounts of TPC compared to the control. Acid and enzyme pre-treatments provided the formation of aglycones in the terebinth seed, thus increasing the amount of phenolic components transferred to the oil. Regarding the flaxseed samples, the opposite relationship, although expected, was not seen. As could be seen from the much lower number of flaxseed phenolics that transferred to oil compared to terebinth oil, this might be because most of the phenolics contained in flaxseeds were polar. As mentioned above, the release of phenolics from conjugated to free form and the formation of aglycones via acidic and enzymatic hydrolysis was more evident for terebinth oil due to higher transition ratios (245% for citric acid, 248% for enzyme pre-treatment compared to the control).

The DPPH inhibition ratios of the oil samples showed a direct correlation with their TPC contents. Commercial enzyme and citric acid pre-treatments for flaxseed and terebinth oil improved DPPH radical scavenging activity. The antioxidant effect of citric acid addition against hydrolytic and oxidative rancidity (Arawande *et al.,* 2011) or its ability to chelate metal ions (Hraš *et al.,* 2000) may explain the increase in antioxidant activity due to citric acid pre-treatment. In addition, an increase in pH due to the addition of citric acid may have created a pro-oxidant effect by enabling peroxides to be decomposed more easily.

DPPH analysis results performed on press cakes did not reflect the effect of the pre-treatments applied (Figure 2). After the oil was extracted, the number of phenolic components in the press cake increased proportionally. The phenolic content of press cake could not reveal the differences between pre-treatments, probably due to the small number of phenolics transferred to the oil. That is, phenolics transformed into oil could be considered significant for oil, but these were not significant amounts for press cake. Fruehwirth *et al*. (2020) reported that the phenolic content in flaxseed oil as 0.27 mg GAE/100 g seeds and only 0.1% of polyphenols were transferred from seeds into the cold pressed oil, which was explained by the hydrophilic properties of most polyphenols (Tsao, 2010).

3.3. Oxidative stability of cold-pressed oils

Pumpkin seed oil is a member of the oleic-linoleic-type oils. The oils with a high degree of unsaturation are more susceptible to oxidative degradation. Squalene is considered important because of the antioxidant activity in pumpkin seed oil. The antioxidant activity of squalene is against polyunsaturated fatty acids (Dessi *et al*., 2002). The oxidative stability of cold-pressed pumpkin seed oils determined by the rancimat test was 4–4.5 h (IP) (Gorjanović *et al*., 2011), which was lower than the values of the present study (6.05 h). Vujasinovic *et al*., (2010) reported the oxidative stability index of cold-pressed pumpkin seed oil at 100 °C to be 18.4 ± 0.3 h (at an airflow of 18 L/h). The study by Murkovic and Pfannhauser (2000) revealed that the induction time of pumpkin seed oils, measured at 120 °C, was 6.83 h, similar to the result in our study.

The differences in the induction times of the pre-treated pumpkin seed and flaxseed samples compared to untreated samples were not statistically significant. On the other hand, in accordance with PV values, enzyme and citric acid pre-treatments gave significantly higher induction times for terebinth oil (11.2 h). As mentioned above, being rich in monounsaturated oleic acid and its endogenous antioxidant compounds gave terebinth oil more stability against oxidation. The remarkably low oxidative stability of flaxseed (0.44 h for the untreated sample) is not surprising due to its high content in α-linolenic acid, which is more prone to oxidation.

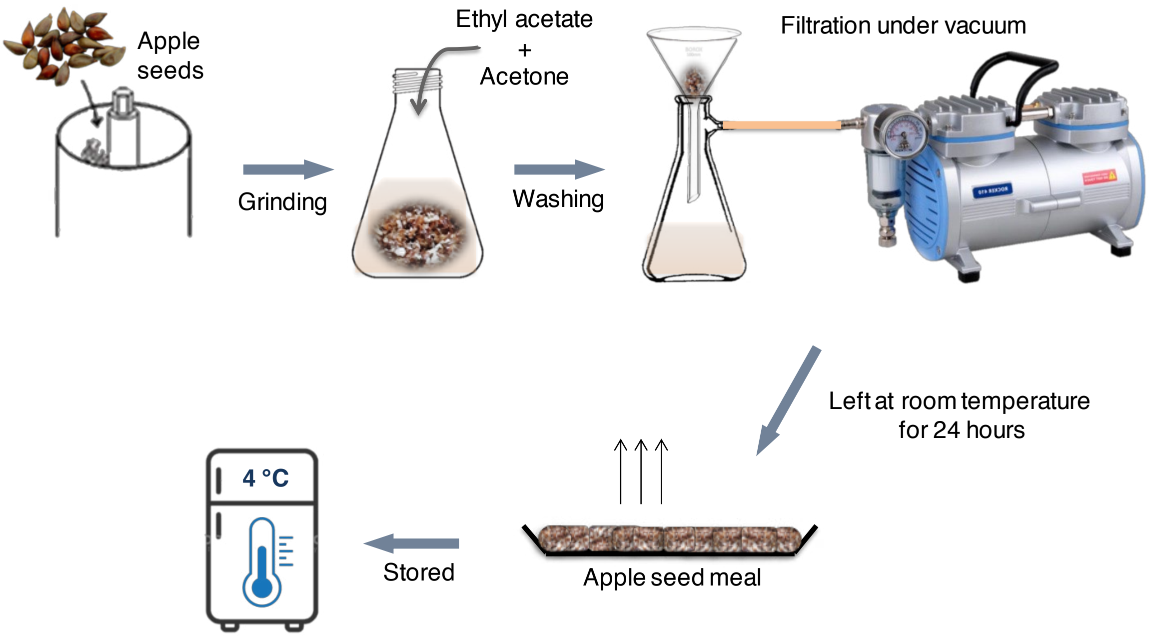

Figure 3. Flow diagram of the preparation of apple seed meal.

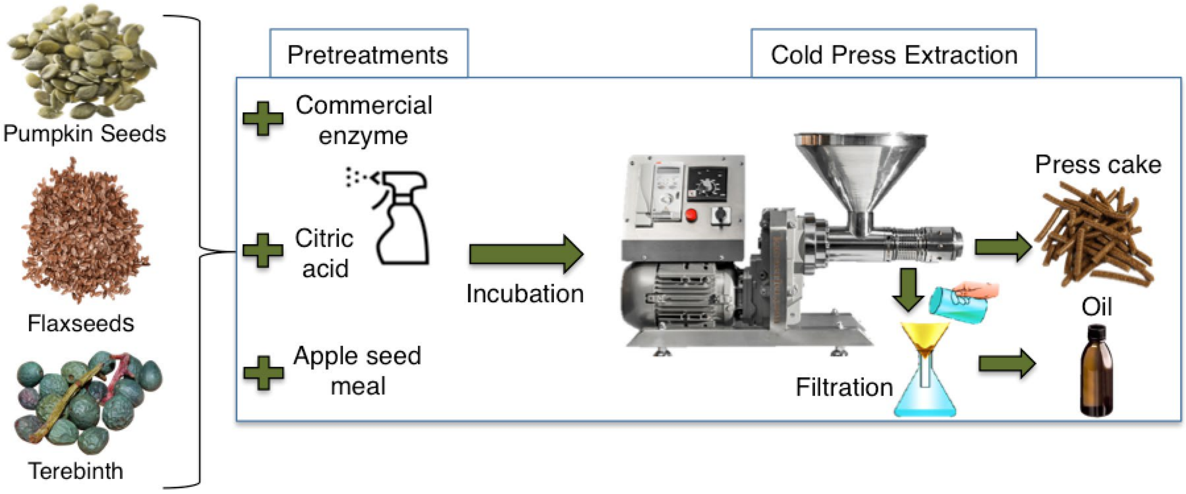

Figure 4. Scheme of seed oil extraction line.

4. CONCLUSIONS

Pre-treatments have provided an oil yield increase of up to 300% in pumpkin seeds, oil yield increased by 151% in flaxseeds with the aid of enzyme pre-treatment. With the mechanical effect of pre-treatment and pressing, the yield diminished due to the emulsion formation in terebinth seed paste.

Along with no significant effect on induction time, pre-treatments increased the development of peroxide values in pumpkin and flaxseed oils, probably due to the effect of the incubation process. In the case of terebinth oil, pre-treatments had a positive effect on peroxide values. The antioxidant compound contents and fatty acid compositions of the oils were the influencing factors on peroxide value.

The expected opposite relationship between the press cake and the phenolic content in the oil was not observed for flaxseed oil, since most of the phenolics it contained were polar. The effect of pre-treatments on the release of phenolics from the conjugated form was through acidic and enzymatic hydrolysis and the formation of aglycone oil. The observation of this relationship in terebinth oil can be attributed to the increase in the transition of apolar aglycones to this oil.

Citric acid and enzyme pre-treatments are recommended for higher transfer of phenolics to oil. These two applications are also effective in terms of peroxide and oxidative stability in terebinth oil. The application of apple seed meal helped reduce free acidity in oil. In addition, it was as successful as citric acid in increasing the oil yield from pumpkin seeds.

This study demonstrated that enzyme and acid pre-treatments can be explored as a feasible alternative to conventional cold pressing for improving not only the oil yield but also the nutritive and functional quality of oils. Future studies can be conducted to get more effective results by modifying the process conditions, particularly incubation, and merging them with different treatments. Citric acid and enzyme pre-treatment methods can also be modified to have positive effects in terms of yield, so that it would be possible to produce improved terebinth oil in terms of yield, oxidative stability and functionality.

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Recent advances in plant-based fat formulation as substitute for lard

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SUMMARY: Lard is one of the main animal fats used as shortening and frying medium. Religious prohibitions and negative health perceptions regarding animal fats have caused concerns about the consumption of lard among communities living around the world. Various research efforts have been made in the past to formulate plant-based fats and shortenings as substitutes for the exclusion of lard from food. This would eventually help countries to regularize food formulations according to their religious compliance. As the existence of a single plant fat as substitute for lard has not been discovered from nature, researchers attempted to study the possibility of mixing native fats and oils such as enkabang fat, canola oil, guava oil, palm oil, palm stearin, soybean oil and cocoa butter as raw materials. The compatibility of the formulated plant-based fat substitute for lard was assessed in terms of chemical composition and thermo-physical properties. The formulated plant-based shortenings and lard shortening were simply plastic fats based on their consistency value and existence of β' and β-form polymorphs of which the β' -form was dominant. The functional properties of formulated plant-based shortenings and lard were also compared in the formulation of cookies. Although a substantial amount of work has been done over the past decade, there was hardly any discussion on the pros and cons of the approaches used for raw material selection and the criteria adopted in the assessment of the formulated products. Hence, this review intended to bring an update of the progress of studies with regard to these two aspects.

KEYWORDS: *Avocado fat; Cocoa butter; DSC; Lard substitute; Palm stearin; Thermal analysis*

RESUMEN: ***Avances recientes en la formulación de grasas a base de plantas como sustituto de la manteca de cerdo.*** La manteca de cerdo es una de las grasas animales que se utiliza principalmente como manteca y como medio para freír. Las prohibiciones religiosas y las percepciones de salud negativas con respecto a las grasas animales han causado preocupación sobre el consumo de manteca de cerdo entre las comunidades que viven en todo el mundo. Se han realizado varios esfuerzos de investigación, en el pasado, para formular grasas y mantecas vegetales como sustitutos de la exclusión de la manteca de cerdo de los alimentos. Esto eventualmente ayudaría a los países a regularizar las formulaciones de alimentos de acuerdo con el cumplimiento religioso. Como todavía no se ha descubierto en la naturaleza la existencia de una sola grasa vegetal como sustituto de la manteca de cerdo, los investigadores intentaron estudiar esta posibilidad mezclando grasas y aceites nativos como grasa enkabang, aceite de canola, aceite de guayaba, aceite de palma, estearina de palma, aceite de soja y manteca de cacao como materia prima. Se evaluó la compatibilidad del sucedáneo de grasas vegetales formuladas con la manteca de cerdo en términos de composición química y propiedades termofísicas. Las mantecas vegetales formuladas y la manteca vegetal eran simplemente grasas plásticas basadas en su valor de consistencia y la existencia de polimorfos de las formas β 'y β de las cuales la forma β' era dominante. También se compararon las propiedades funcionales de las mantecas vegetales formuladas y la manteca de cerdo en la formulación de galletas. Aunque se ha realizado una cantidad considerable de trabajo durante la última década, apenas hubo discusión sobre los pros y los contras de los enfoques utilizados para la selección de materias primas y los criterios adoptados en la evaluación de los productos formulados. Por tanto, esta revisión pretendía aportar una actualización del avance de los estudios en relación con estos dos aspectos

PALABRAS CLAVE: *Análisis térmico; DSC; Estearina de palma; Grasa de aguacate; Manteca de cacao; Sustituto de manteca de cerdo*

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1. INTRODUCTION

In recent times, animal fats have received much attention from the scientific community throughout the world. Lard and tallow, in particular, have been used in international trade over several decades due to their widespread availability and use in food-processing. Various research reports have highlighted the use of lard in Europe (Paleari *et al.*, 2004), North America (deMan *et al.*, 1991) and Asia (Hsu and Yu, 2002). Apart from being used as a medium for deep-frying, animal fats are also used as shortening in bakery products due to their lower cost. According to a food analysis report, some commercial biscuit formulations were found to contain lard as an ingredient (Yanty *et al.*, 2014b). According to Kamel (1992), the inclusion of lard in bread making formulations was mainly due to the unique liquid-to-solid content ratio. Its performance characteristics during food processing as well as flavors imparted to fried products are said to be other reasons (Marikkar and Yanty, 2014). In a separate study, Seriburi and Akoh (1998) formulated a variety of plastic shortenings by blending lard with sunflower oil in different ratios due to these reasons. Although the use of lard has always been popular among certain ethnic groups, its consumption has become a controversy for communities whose religious restrictions command the exclusion of swine-based products (Regenstein *et al.*, 2003). Other than this, the negative health implication of animal fats in general has caused some concern about the consumption of lard among various communities. One proactive approach for the exclusion of lard in food is to provide a potential alternative substance which would mimic its properties. During the last couple of years, a number of research reports have emerged to study the partial replacement of lard in various meat products (Delgado-Pado *et al.*, 2011; Rodriguez–Carpena *et al.*, 2011; Choi *et al.*, 2010; Muguerza *et al.*, 2003). A brief survey of the recent literature would give evidence for initiatives taken to fully replace lard by screening various raw materials and evaluating formulated substitutes (Table 1). However, there was hardly any attempt to revisit the pros and cons of the approaches used in raw material selection and the criteria adopted for the assessment of these efforts. Hence, it is pertinent to critically analyze these as-

Table 1. Lard substitute formulations using a variety of native fats and oils[1]

Raw materials	Blending types	Formulated blending ratios	Assessment methods	Observations	References
MF and PS	Binary	MF:PS (99.5:0.5); MF:PS (99:1); MF:PS (98:2)	SMP and IV; Chemical compositional data; DSC thermal properties; SFC and polymorphic profiles	MF:PS (99:1) and lard had closest compatibility	Yanty *et al.,* 2014a
EF and CO	Binary	EF:CO (25:75); EF:CO (30:70); EF:CO (35:65); EF:CO (40:60)	-do-	EF:CO (35:65) and lard had closest compatibility	Nur Illiyin *et al.,* 2014; Nur Illiyin *et al.,* 2013
PS and GO	Binary	PS:GO (40:60); PS:GO (45:55); PS:GO (50:50); PS:GO (55:45)	-do-	PS:GO (55:45) and lard had closest compatibility	Raihana *et al.,* 2017a
Avo, PS and CB	Ternary	Avo:PS:CB (88:7:5); Avo:PS:CB (86:7:7); Avo:PS:CB (84:7:9)	-do-	Avo:PS:CB (84:7:9) and lard had closest compatibility	Yanty *et al.,* 2017a
PO, PS, SBO and CB	Quaternary	PO:PS:SBO:CB (38:5:52:5); PO:PS:SBO:CB (36:5:54:5); PO:PS:SBO:CB (34:5:56:5)	-do-	PO:PS:SBO:CB (38:5:52:5) and lard had closest compatibility	Marikkar *et al.,* 2018

[1]Abbreviations: MF, mee fat; PS, palm stearin; GO, guava seed oil; CO, canola oil; Avo, avocado oil; CB, cocoa butter; EF, engkabang fat; PO, palm oil; PS, palm stearin; SBO, soybean oil; LD, lard.

pects by bringing an update of the progress of studies in this arena.

More than 100 different varieties of plant species in nature with oil-bearing seeds and fruits (Raihana *et al.,* 2015) have been detected. Among these, the availability of a single plant fat as substitute for lard is yet to be discovered mainly due to the natural differences in compositions. Nevertheless, the judicious mixing of different plant fats and oils in appropriate ratios could yield a formula that would mimic lard. Producing novel fat products by blending oils and fats is the easiest and most economical way without involving any chemical treatment. In addition, this approach could preserve their natural flavors, organoleptic characteristics and nutritional attributes. Fractional crystallization and interesterification could become complementary to these endeavors if they could help modify physical properties by changing either fatty acid or TAG compositions (Marikkar and Ghazali, 2011). The outcome of any effort in this direction needs to be assessed according to some meaningful criteria. It should be devoid of any component of animal origin and mimic lard in composition as well as physical characteristics. The analytical approaches used should be comparable to those adopted for the assessment of *trans*-free fats (Miskandar, and Nor Aini, 2010), structured lipids (Norizzah *et al.*, 2004), and cocoa butter equivalents (Jahurul *et al.*, 2014; Wassell and Young, 2007). Generally, in the assessment of any novel fat formulation, researchers pay attention to aspects such as fatty acid and triacylglycerol compositions, melting and crystallization behavior, solidification and polymorphic characteristics (Jun Jin *et al.*, 2018). The nutritional status of fats along with their other functional characteristics can also be decided using fatty acid and triayclgylcerol compositions. In the fractionation of fats and interesterification of structured lipids or formulation of cocoa butter equivalents, these two aspects have been investigated (Ramli *et al.*, 2008; Jahurul *et al.*, 2014; Marikkar and Yanty, 2014). According to some previous reports, thermal analysis by DSC has been shown to be a useful approach to determine a variety of thermal properties involving fat modification (Yanty *et al.*, 2013a; Yanty *et al.*, 2013b; Nur Illiyin *et al.*, 2014). Other parameters such as solid fat content (SFC) measurements by pulse NMR spectroscopy have helped to monitor the changes in hardness as a function of the percentage of solids at different temperatures (Nor Lida *et al.*, 2002). Crystal morphology, using polarized light microscopy and polymorphic behavior using the powder X-ray diffraction method are examined to identify their suitability as shortenings. In this article, we intend to review the usefulness of different analytical approaches, namely GC/GLC, HPLC, DSC, and pulsed nuclear magnetic resonance spectroscopy in the assessment of the formulated plant-based fat substitutes for lard.

2. CHEMICAL COMPOSITION AND PHYSICAL CHARACTERISTICS OF LARD

A great deal of effort has been devoted to compiling the chemical composition and physical properties of lard and its modified forms such as its fractions (Nina Naqiyah *et al.,* 2017; Marikkar and Yanty, 2014). Similar to most oils and fats, lard and its fractionated component are mainly composed of TAG molecules. Unlike other oils and fats, TAG species occurring in higher amounts in lard are esterified with palmitic acid, predominantly in the sn-2 position of the glycerol backbone (Marikkar *et al.,* 2021; Rashood *et al.,* 1996). According to previous studies, lard generally consists of more unsaturated fatty acids (51.3 to 65.9%) than saturated (34.1 to 48.7%) fatty acids (Marikkar and Yanty, 2014). Among its saturated fatty acids, palmitic and stearic acids are more prominent and among the unsaturated fatty acids, oleic acid (40.17%) has emerged as the most dominant fatty acid, followed by linoleic acid (17.25%) (Table 2). The observed variations in fatty acid composition are said to be due to sample-to-sample differences caused by the diet of the animals, breed type, etc. Native lard usually exists in semi-solid form at room temperature and its slip meting point is found to vary from 28 to 32 °C (Marikkar *et al.*, 2017; Marikkar *et al.*, 2021). Its shorter plastic range, sharp melting point and larger crystal size are said to be due to the unique composition of lard.

In the study on the physical properties of lard, DSC thermal analysis is one of the approaches adopted by several research groups. As of date, this has contributed immensely to the understanding of the thermal behavior of several lipids, including the melting and crystallization behavior of both plant and animal fats (Marikkar, 2015). Over the years, the DSC thermal curves of various animal fats have been compiled by several research groups, and are

Table 2. Basic physico-chemical characteristics and fatty acid compositions (%) of formulated substitutes for lard from different studies[1]

Parameter	EF:CO (30:70)	PS:GO (55:45)	MF:PS (99:1)	Avo:PS:CB (84:7:9)	PO:PS:SBO:CB (38:5:52:5)	LD
SMP	29.3^{a}	49.70^{d}	38.50^{b}	40.50^{c}	41.25^{c}	29.5^{a}
IV	101.85^{f}	42.78^{a}	56.04^{b}	65.47^{c}	92.26^{e}	73.68^{d}
Fatty acids						
Lauric	n.d.	0.12^{a}	n.d.	n.d.	0.11^{a}	n.d.
Myristic	n.d.	0.74^{a}	0.02^{a}	0.03^{a}	0.54^{a}	n.d.
Palmitic	8.10^{a}	37.82^{e}	23.53^{b}	32.97^{d}	27.88^{c}	23.78^{b}
Palmitoleic	0.17^{a}	0.08^{a}	n.d.	2.66^{a}	0.11^{a}	2.00^{a}
Stearic	16.32^{d}	4.86^{a}	21.29^{e}	4.57^{a}	7.83^{b}	12.96^{c}
Oleic	51.92^{e}	18.05^{a}	47.05^{d}	44.04^{c}	29.39^{b}	40.17^{c}
Linoleic	15.06^{b}	37.84^{e}	8.11^{a}	14.28^{b}	30.78^{d}	17.25^{c}
Linolenic	4.94^{b}	0.11^{a}	n.d.	1.02^{a}	2.96^{a}	n.d.
Arachidic	1.06^{a}	0.39^{a}	n.d.	0.44^{a}	0.43^{a}	0.74^{a}
Gadoleic	0.86	n.d.	n.d.	n.d.	n.d.	-
Others	-	-	-	-	-	2.46
Reference	Nur Illyin *et al.*, 2013	Raihana *et al.*, 2017a	Yanty *et al.*, 2014a	Yanty *et al.*, 2017a	Marikkar *et al.*, 2018	Nur Illyin *et al.*, 2013

[1]Each value in the table represents the mean of three replicates. Means within each row bearing different superscripts are significantly ($p < 0.05$) different.

Abbreviations: MF, mee fat; PS, palm stearin; GO, guava seed oil; CO, canola oil; Avo, avocado oil; CB, cocoa butter; EF, engkabang fat; PO, palm oil; PS, palm stearin; SBO, soybean oil; LD, lard; SMP, slip melting point; IV, iodine value; n.d, not detectable.

now available for authentication purposes (Marikkar *et al.*, 2021; Dyszel and Baish, 1992). Chemical compositional differences arising from adulteration practices are known to cause clear deviations in the original DSC profiles of pure lipids (Marikkar and Sohel Rana, 2014). According to several reports, the DSC cooling and heating thermograms of lard were unique when compared to those of other common animal fats (Marikkar *et al.*, 2021). According to Yanty *et al.* (2011a), lard was found to have two major exothermic thermal transitions, which are widely separated into two distinct temperature regions (Table 3). Unlike other animal fats, lard is found to display two sharp thermal transitions as the peak at the region below 10 °C in the cooling thermogram is designated as high-melting group (HMG); while the peak at the region above 10 °C can be termed as low-melting TAG group, and vice versa in the

Table 3. DSC thermal transitions of formulated substitutes for lard reported from different studies[1]

DSC Curve type	Sample	Thermal transition (°C)				Reference
		1	2	3	4	
Cooling	EF:CO (30:70)	4.4^{a}	-	-	-46.5^{c}	Nur Illyin *et al.*, 2013
	PS:GO (55:45)	28.2^{e}	-	-4.5^{c}	-	Raihana *et al.*, 2017a
	MF:PS (99:1)	23.0^{d}	-	0.0^{a}	-30.6^{b}	Yanty *et al.*, 2014a
	Avo:PS:CB (84:7:9)	20.1^{c}	-	-2.0^{b}	-	Yanty *et al.*, 2017a
	PO:PS:SBO:CB (38:5:52:5)	-	-	-	-	Marikkar *et al.*, 2018
	LD	16.8^{b}	10.3	-	-18.7^{a}	Yanty *et al.*, 2011a
Heating	EF:CO (30:70)	28.6^{a}	-	-6.9^{c}	-19.9^{b}	Nur Illyin *et al.,* 2013
	PS:GO (55:45)	50.0^{c}	-	-		Raihana *et al.*, 2017a
	MF:PS (99:1)	30.1^{a}	5.0	-5.0^{b}	-	Yanty *et al.*, 2014a
	Avo:PS:CB (84:7:9)	37.1^{b}	-	-	-18.5^{a}	Yanty *et al.*, 2017a
	PO:PS:SBO:CB (38:5:52:5)	29.1^{a}	-	-	-20.0	Marikkar *et al.*, 2018
	LD	29.0^{a}	-	-3.6^{a}	-21.4^{c}	Yanty *et al.*, 2011a

[1]Abbreviations: DSC, differential scanning calorimetry; MF, mee fat; PS: palm stearin; GO, guava seed oil; CO, canola oil; Avo, avocado oil; CB, cocoa butter; EF, engkabang fat; PO, palm oil; PS, palm stearin; SBO, soybean oil; LD, lard.

melting thermogram (Table 2) (Marikkar and Yanty, 2014; Yanty *et al.*, 2011a).

The solidification behavior of any semisolid fat in a temperature region is characterized by its solid fat content (SFC), which is the solid-to-liquid ratio of a fat at a particular temperature (Mahjoob *et al.,* 2018). As such, SFC curves of fats are found to serve as useful guides to determine the suitability of a fats or oil in a particular application (Graef *et al*., 2012). Until today, several reports have appeared on the SFC profile of lard to understand its thermal behavior under varied circumstances. According to Ospina-E *et al*. (2010), the SFC curve of lard displayed a gradual drop in SFC values in the initial phase starting from 0 to 25 °C; SFC values at 0, 20 and 40 °C were 30.8, 18.0 and 0%, respectively. Somewhat similar results were later found by Yanty *et al*. (2011a) while studying the fractional crystallization behavior of lard. This later study showed that the SFC value of lard at 0 °C was 30.8% and tended to display a gradual drop in the initial phase starting from 0 to 25 °C and become 0% at 40 ºC. According to Nur Illyin *et al.* (2013), the hardness value of lard was 7.7 g, which was found to correlate well with the increasing SFC values. With regard to polymorphism, lard generally possesses larger crystals of β and β' polymorphs which are important in lubricity since they impart tenderness and richness while improving eating qualities with a feeling of satiety after eating (O'Brien, 2005).

3. CHEMICAL COMPOSITION AND PHYSICAL CHARACTERISTICS OF SUBSTITUTES

In pursuit of finding a substitute for lard, a variety of native plant fats and oils were chosen to blend with a set of liquid oils. According to Table 1, fats and oils from various plants such as oil palm (PO and PS), cocoa (CB), engkabang juntung (EF), avocado (Avo), *Madhuca longifolia* (MF), canola (CO) and soybean (SBO) were used for this purpose. Among these, PS, CB and EF are hard fats since they usually exist in solid form at tropical temperature conditions. PO, Avo, and MF are grouped together as semisolid fat category while canola oil, soybean oil and guava oil represent the liquid oil category. The blending of fats and oils has been done in the form of binary, ternary and quaternary mixtures in different ratios to formulate novel fat substances. The evaluation of the physical properties of the formulated plant-based fat blends was based on parameters such as melting point (SMP) and iodine value (IV), solid fat content (SFC) profile and thermal properties. However, the analysis of fatty acid and triacylglycerol (TAG) compositions was also inevitable as they were keys to understand the physical behaviors of formulated plant-based fat substitutes. According to several other reports, this approach has already proven to be sound for assessing the suitability of plant fat blends in numerous other industrial applications (Miskandar and Nor, 2010; Ribeiro *et al*., 2009).

3.1. Binary mixtures of EF:CO

EF is a tropical hard fat extracted from the seeds of *Shorea macrophyilla.* This has been previously known for its characteristic properties being similar to cocoa butter. Canola oil, a major vegetable oil, has been previously identified as a suitable candidate for novel fat formulations (Campbell *et al*., 2002; Marangoni and Rousseau, 1998). Several efforts have been made in the past to expand the use of EF as it is considered an under-utilized plant lipid (Yanty *et al.,* 2013b; Nesaretnam and Mohd Ali, 1992). In a previous attempt, Gani *et al*. (2011) demonstrated the usefulness of EF as a raw material for preparing moisturizers used in cosmetic products as well as hair conditioners. In a separate study, EF was fractionated into a hard stearin and a liquid olein to explore its suitability in other applications (Yanty *et al.*, 2013b).

In the search for lard substitutes, engkabang fat was mixed with canola oil in proportions ranging from 25 to 40% (w/w) (Table 1). The formulated blends appeared pale yellow in color, but displayed varying degrees of unsaturation (IV from 104.7–92.5). In fact, the IV of the formulated blends of EF:CO resulted higher than that of lard (IV, 73.6), affecting the oxidative stability. Among the physical characteristics, SMP is a parameter of considerable importance as it is indicative of the point of transition from solid to liquid. According to Nur Illyin *et al.* (2013), the SMP of the formulated blends of EF:CO fell within the range of 24.8–31.9; while the SMP of lard was 29.5. As shown in Table 2, the SMP of the EF:CO=30:70 (w/w) blend was found to tally with that of lard, despite some deviation in the degree of unsaturation.

Blending canola oil with engkabang fat brought significant deviations in chemical compositions, as shown in Tables 2 and 4. According to several previous reports, trioleoyl glycerol (OOO), dioleoyl-3-linoleoyl glycerol (OOL), and dioleoyl-3-linolinoyl glycerol (OOLn), were the major TAG molecular species of canola oil (Marikkar and Sohel Rana*,* 2014; Nur Illyin *et al.,* 2013; Marikkar *et al.,* 2005). The addition of engkabang fat into canola oil caused gradual but significant increases in the proportions of palmitoyl-oleoyl-stearoyl glycerol (POS) and disearoyl-2-oleoyl glycerol (SOS) with concurrent decreases in the proportions of OOO, OOL and OOLn (Table 4). For instance, the proportions of triunsaturated TAG molecular species decreased remarkably as the proportion of engkabang fat in canola oil was increased from 25 to 40% (w/w) (Nur Illyin *et al.,* 2013). When compared to canola oil, the binary blends of EF:CO were found to possess a high amount of saturated fatty acid with reduced amounts of unsaturated fatty acids. From the nutritional point of view, this would have a negative effect on the availability of essential fatty acids. This was mainly due to the increases in the proportions of palmitic and stearic acids with concurrent reductions in the amounts of oleic and linoleic acids (Nur Illyin *et al.*, 2013). Similar situations were reported previously for blending canola oil with butter oil (Marangoni and Rousseau, 1998) and canola oil with lard stearin (Marikkar and Sohel Rana*,* 2014). In addition to this, blending canola oil with other fats was found to steadily change the unsaturated to saturated fatty acid ratio.

The impact on DSC thermal profiles and solidification characteristics of canola oil caused by changing chemical compositions is another important aspect. As reported previously, the DSC curve of canola oil is characterized by the occurrence of two overlapping endotherms: a large-higher temperature transition at -17.86 ^{o}C and a small -lower temperature transition at -28.50 ^{o}C (Marikkar and Rana, 2014). Since the end-set of the melting of canola oil was at -6.79 ^{o}C, thermal transition beyond this point was hardly seen. All formulated fat blends, however, displayed a high-melting thermal transition in the temperature region above 10 °C and the enthalpy and peak temperature of this high-melting peak were found to increase proportionately with the increasing proportion of EF in the fat blends. This would be most probably due to the increases noticed earlier in the proportion of saturated TAG molecules in the

Table 4. TAG composition of formulated fat substitutes for lard from different studies[1]

TAG	EF:CO (30:70)	PS:GO (55:45)	MF:PS (99:1)	Avo:PS:CB (84:7:9)	PO:PS:SBO:CB (38:5:52:5)	LD
LLLn	2.49^{a}	n.d.	n.d.	1.56^{a}	3.93^{a}	0.93^{a}
LLL	5.51^{b}	22.47^{d}	n.d	0.58^{a}	12.23^{c}	0.68^{a}
OLL	n.d.	6.69^{c}	0.51^{a}	2.72^{a}	9.28^{d}	4.68^{b}
OLLn	6.06^{b}	n.d.	n.d.	n.d.	1.97^{a}	n.d.
OOLn	7.70^{a}	n.d	n.d	n.d	n.d.	n.d
PLL	n.d.	8.63^{c}	0.43^{a}	3.58^{b}	8.23^{c}	7.05^{c}
OOL	16.04^{d}	0.16^{a}	2.91^{b}	6.98^{c}	5.58^{c}	6.93^{c}
POL	3.6^{a}	8.26^{c}	5.11^{b}	16.18^{e}	11.31^{d}	20.00^{f}
PPL	n.d	4.24^{b}	1.66^{a}	2.62^{a}	5.02^{b}	2.62^{a}
OOO	20.18^{d}	1.59^{a}	9.11^{c}	9.62^{c}	3.14^{b}	4.33^{b}
POO	4.54^{a}	7.59^{b}	23.57^{e}	19.36^{d}	10.87^{c}	20.67^{d}
PPO	2.51^{a}	17.26^{e}	15.29^{d}	13.28^{c}	13.48^{c}	10.63^{b}
PPP	n.d	14.68	2.51^{b}	7.07^{d}	4.9^{c}	0.38^{a}
SOO	1.53^{b}	0.48^{a}	9.84^{d}	0.71^{a}	1.85^{b}	3.62^{c}
SPO	11.1^{d}	2.23^{a}	18.41^{e}	10.56^{c}	4.58^{b}	12.52^{d}
PPS	0.18^{a}	2.78^{a}	0.79^{a}	0.79^{a}	0.84^{a}	0.81^{a}
SOS	15.44^{d}	0.18^{a}	5.61^{c}	3.54^{b}	1.59^{a}	0.83^{a}
SSS	0.27^{a}	0.34^{a}	0.59^{a}	0.21^{a}	0.02^{a}	1.31^{a}
Others	n.d.	n.d.	3.71^{a}	0.69^{a}	n.d.	1.41^{a}
Reference	Nur Illyin *et al.*, 2013	Raihana *et al.*, 2017a	Yanty *et al.*, 2014a	Yanty *et al.*, 2017a	Marikkar *et al.*, 2018	Nur Illyin *et al.*, 2013

[1]Abbreviations: TAG, triacglycerol; O, oleic; P, palmitic; L, linoleic; Ln, linolenic; St, stearic; U, unsaturated; S, saturated; MF, mee fat; PS: palm stearin; GO, guava seed oil; CO, canola oil; Avo, avocado oil; CB, cocoa butter; EF, engkabang fat; PO, palm oil; PS, palm stearin; SBO, soybean oil; LD, lard; n.d, not detectable.

admixtures. Out of the four blends, the peak maxima of EF:CO (35:65) showed the closest value to that of lard, which had its maximum peak at 29.3 °C (Table 3). The SFC profiles usually describe the solidification behavior of fatty substances within a specified temperature range. For fat blends of the EF:CO series, the SFC values tended to increase throughout the temperature range with the increasing proportion of EF. Further, the increase in SFC values were found to correlate well with the increasing proportion of disaturated TAG molecules in the fat blends (Table 4). According to Rao *et al.* (2001), the SFC of a fat at 25 °C should be within the range of 15-35% to achieve the desired spreadability and texture. Further, SFC at 20 °C needs to be adjusted to more than 10% in order to avoid any oil separation in the blend. Although the SFC values of EF:CO (30:70) and lard were not similar at 0 °C, they were found to be quite similar within the range of 30-40 °C and become tallied at 25 °C. Apart from this, the results from X-ray diffractograms also showed both EF:CO (30:70) and lard possessed both β and β' polymorphs, which were ideal for a plant-based shortening (Nur Illyin *et al.*, 2013). This was because both lard and EF:CO (30:70) had roughly similar amounts of SPO (Table 4). According to Timms (1984), the presence of SPO could significantly contribute to the β' crystal polymorph's development in lard.

3.2 Binary mixtures of PS:GO

Guava seeds (*Psidium guajava* L.), discarded after juice production can be a useful material for oil extraction. According to a previous study reported from India, the Guava seed is rich in essential fatty acids such as linoleic acid (76.4%) (Prasad and Azeemoddin, 1994). Although guava oil is known for this important nutritional attribute, its commercial exploitation has not been advanced to product formulations. In search for a substitute for lard, mixing guava oil with palm stearin has been performed in varying proportions ranging from 45 to 60% (w/w) (Table 1). The SMP of all fat blends were found to be higher (SMP = 43.6 to 50.3) than that of lard (29.25), but lower than that of palm stearin (52.7) (Table 2). With regard to the degree of unsaturation, all fat blends displayed significantly lower IV values ($p < 0.05$) (IV= 42.3 to 50.6) than that of lard (73.76) (Table 2). Hence, none of the binary mixtures of PS:GO was found to have either SMP or IV similar to those of lard. As the added portion of palm stearin into guava oil increased from 45 to 60%, a clear change in the unsaturated to saturated fatty acid ratio was noticed. For instance, there was a gradual decrease in linoleic (from 46.16 to 33.91%) acid content with a concurrent increase in palmitic acid content (from 31.35 to 40.72%) (Table 2). This pattern of change was very similar to those previously observed for the EF:CO blend formulation (Nur Illyin *et al.,* 2013). Simultaneously, decreases in the proportions of TAG molecules such as LLL, OLL, PLL and POL occurred with concurrent increases in the amounts of PPO, tripalmtoyl glycerol (PPP), POS and dipalmitoyl-3-stearoyl glycerol (PPS) (Table 4). The changes were happened in such a way that the proportions of triunsaturated TAG molecular species declined from 36.79 to 27.79 % (Table 4). In fact, increases in the proportions of disaturated and trisaturated TAG molecular species led to the increase in palmitic acid content as noticed before in the overall fatty acid distribution (Table 2). As noted previously in EF:CO blend formulations (Nur Illyin *et al.*, 2013), the proportions of POS and SOS were increased while those of OOO, OOL and OOLn were decreased. In this aspect, both of these formulation efforts yielded somewhat similar patterns of changes (Table 4).

Serval previous studies indicated that the SFC values of fats are mainly influenced by their respective fatty acid and TAG compositions. According to Graef *et al.* (2012), the proportional distribution of disaturated and trisaturated TAG molecular species would have greater influence on the SFC values of fats as more saturated TAG molecules would create a stronger crystal network. Raihana *et al.* (2017a) observed that the SFC value at 5 °C of the formulated blends of PGO-1 (PS:GO=60:40), PGO-2 (PS:GO = 55:45), PGO-3 (PS:GO = 50:50) and PGO-4 (PS:GO = 45:55) were 47.19, 47.19, 38.16 and 34.38%, respectively. The increasing values of SFC presented herein were found to correlate well with the increasing proportion of disaturated TAG molecules in the fat blends (Table 4). This observation is in conformity with the previous findings reported on EF:CO fat blends (Nur Illyin *et al.,* 2013; Nur Illyin *et al.,* 2014). Of these blends, PGO-2 was found to display a SFC value similar to lard at temperatures 15, 20, and 25 °C. Particularly, at 30 °C, the SFC value for lard (12.43%) was between the SFC values for PGO-

3 (13.70) and PGO-4 (11.81%). Based on some calculations performed with regard to least difference in SFC values, out of the four blends, PGO-1 and PGO-2 were found to display the smallest differences compared to lard in terms of SFC values.

Several previous studies pointed out that even a little change in fatty acid and TAG compositions would affect the DSC thermal profile of pure oils (Marikkar, 2015). Aziz *et al.* (2011) indicated that the mixing of palm stearin with palm oil would affect the DSC cooling profile of the formulated shortening because the high melting TAGs are introduced by palm stearin. Separately, Nur Illyin *et al.* (2013) and Nur Illyin *et al.* (2014) noticed that a little mixing of enkabang fat into canola oil caused fatty acid and TAG compositional changes which affected the thermal profile of canola oil. Likewise, the mixing of palm stearin into guava oil affected the DSC profile in such a way that all fat blends displayed high melting thermal transitions above 10 °C. The maximum peak of the high-melting thermal transitions corresponding to PGO-1, PGO-2, PGO-3 and PGO-4 shifted from 46.26 to 50.32 °C. According to this observation, out of the four formulated-blends, the peak maxima of PGO-4 (PS:GO=45:55) was at 44.20 °C and that of PGO-2 (PS:GO=55:45) was at 50.0 °C (Table 3). The results of the SMP and SFC, as well as fatty acid composition indicated that PGO-2 would become a better fat blend to substitute lard. Apart from this, the result of X-ray diffractograms also showed both PGO-2 and lard possessed with β and β' polymorphs, which were ideal for a plant-based shortening. This was quite similar to what was previously observed in the case of EF:CO (30:70), which exhibited both β and β' polymorphs (Nur Illyin *et al.*, 2013). The occurrence of a large amount of disaturated TAG molecules such as PPO in PGO-2 (Table 2) contributed to the existence of β and β' polymorphs in PGO-2.

3.3. Binary mixtures of MF:PS

Mee fat extracted from the seeds of *Madhuca longifolia* is said to be an analogue of Indian Mahua butter (Ramadhan *et al.*, 2016; Marikkar *et al.*, 2010). Although mee fat is preferred as a fat ingredient in the formulations of balms and ointments used in folk medicine (Ramadhan *et al.*, 2016), its edible uses are yet to be realized (Marikkar *et al.*, 2010). In an effort to find a substitute for lard, Marikkar and Yanty (2012) compared the solid and liquid fractions of mee fat with those of lard. Although both of these exhibited compatibility in terms of SFC values at some temperatures, they displayed some disparity at certain other temperatures. Despite this, these findings provided the impetus to formulate three fat blends by mixing mee fat with palm stearin in proportions ranging from 0.5 to 2 % (w/w) (Yanty *et al.*, 2014a). Although the IV of formulated-blends of MF:PS were significantly lower (54.27 to 57.81) ($p < 0.05$) than that of lard (73.76), their SMP were significantly higher (37.25 to 40.25) ($p < 0.05$) than that of lard (29.5 °C) (Table 2). In conformity to this, Raihana *et al.* (2017a) also noticed that the fat blends of PS:GO displayed significantly lower IV values ($p < 0.05$) (IV= 42.3 to 50.6) than that of lard (73.76). However, Nur Illyin *et al.* (2013) and Nur Illyin *et al.* (2014) reported that all formulated blends of EF:CO were found to display (104.7–92.5) higher IV values than lard (73.76). This could be due to the high contribution of monounsaturated fatty acids such as oleic in the formulated blends. When considering the thermal properties, the SMP of all fat blends of MF:PS were found to be higher (37.25 to 40.25) than that of lard (29.25) in conformity with Raihana *et al.* (2017a), who also subsequently noticed that all fat blends of PS:GO had significantly higher SMP values ($p < 0.05$) (SMP= 43.60 to 50.3) than that of lard (29.5). In contrast to these two studies, some formulated blends of EF:CO were found to display roughly similar SMP (24.8 – 31.1.5) to that of lard (29.5) (Table 1). As the proportion of palm stearin mixed into mee fat increased from 0.5 to 2%, the proportions of POL, dioleoyl-3-palmitoyl glycerol (POO), dioleoyl-3-steroyl glycerol (SOO) and SOS were decreased in such a way that the proportions of di-unsaturated TAG molecular species declined from 43.13 to 34.65 % (Table 4). In the meantime, the proportions of disaturated TAG molecular species increased, leading to the increase in palmitic acid in the overall fatty acid distribution.

As fractional crystallization would yield novel solid and liquid fractions of mee fat and lard, they were compared in terms of their physicochemical parameters and thermal properties (Marikkar and Yanty, 2012). According to this study, the mee fat and lard used had displayed compatibility in terms of SFC profiles, especially, SFC values at 5, 20 and 25 °C. However, their SFC values differed within the temperature ranges from 10 to 15 °C as well as 30 to 35 °C, which could be due to their differences in TAG distributional pattern as discussed previously (Yanty *et al.*, 2012). Owing to this fact, Yanty *et al.* (2014a) hypothesized that mixing a small amount of palm stearin into mee fat would adjust the SFC values of mee fat at those temperatures. With increasing proportion of palm stearin from 0.5 to 2%, the SFC values of the three formulated blends increased gradually. The observed increases in SFC values were well-correlated with the increasing proportions of di- and tri-saturated TAG molecules (PPO, PPSt, and StPO) in the binary blends (Table 4). Out of the three formulated-blends, MF:PS (99:1) showed better compatibility with lard at most temperatures within the specified range. The pattern of changes in the SFC profiles of these fat blends was more or less comparable to those of the binary blends of EF:CO (Nur Illyin *et al.*, 2013; Nur Illyin *et al.*, 2014) and PS:GO (Raihana *et al.*, 2017a) as reported previously. However, changes in the fatty acid composition during blend formulation were only marginal (Table 2). For instance, with increasing proportions of palm stearin in the blends, only slight changes occurred in the contents of total SFA (from 44.25 to 45.77%) and total USFA (from 55.75 to 54.23%). This led to slight increases in the amount of palmitic acid with concurrent decreases in the amounts of stearic, oleic and linoleic acids.

The DSC thermal profiles of pure lipids are generally influenced by chemical compositional changes. Hence, the changes in both fatty acid and TAG compositions would affect the DSC thermal profiles of the formulated binary blends in such a way that the maximum peak of all melting transitions were shifted to higher temperature regions, resulting in higher end-sets of melting (T_{endset}) (Yanty *et al.,* 2014a). The T_{endset} value is often regarded as an alternative for slip melting point. Nur Illyin *et al.* (2013) previously noticed a high-melting thermal transition in the temperature region above 10 °C in all binary blends of EF:CO. Since there was hardly any thermal transition in the original DSC curve for canola oil above 10 °C, it was considered as a remarkable feature in the formulated-blends. Owing to the increasing amounts of more saturated TAG molecules, the maximum peak of the high-melting thermal transitions present in the blends were increased gradually (Table 3). This is in conformity with the previous findings reported by Raihana *et al.* (2017a), who observed that the maximum peak of the high-melting thermal transition of PS:PGO blends also shifted from 46.26 to 50.32 °C. In a separate study, Norizzah *et al.* (2004) made a similar observation in the case of palm olein blended with palm stearin, giving rise to the appearance of a broad peak in the high-melting region of the thermal curve of palm stearin. Yanty *et al.* (2017b) conducted a X-crystallographic study and reported that both LD and MF:PS (99:1) blends displayed both β' and β-form polymorphs, of which the β' form was dominant. These researchers believed that the occurrence of the disaturated TAG (USS) molecular species such as PPL, PPO, StOP, and StOSt in both of these could be the probable reason for this similarity.

3.4. Ternary mixtures of Avo:PS:CB

In recent years, the oil of avocado pulp has received much attention due to its uses in food, cosmetics and health care products (Qin and Zhong, 2016). Although avocado oils from some varieties exist in liquid form, avocado lipids of some Malaysian cultivars were found to exist in a semi-solid state at room temperature. This difference in their physical state could be due to the compositional differences based on TAG and fatty acid profiles (Yanty *et al.,* 2011b). Qin and Zhong (2016) made some further comments regarding this matter in a survey presented on the efficiency of different extraction methods of avocado fat. A plant-based substitute for lard was proposed by Yanty *et al.* (2017a) by making use of avocado fat as the major component and palm stearin / cocoa butter as minor components. According to a previous study by Yanty *et al.* (2013b), Malaysian cocoa butter was found to possess POP, POS, and SOS as its major TAG molecular species. Likewise, another previously mentioned that when compared to lard, Avo of different Malaysian cultivars exhibited lower SFC values throughout the temperature range (Yanty *et al.,* 2014a). As such, the addition of hard fats such as cocoa butter and palm stearin is inevitable to complement or adjust the SFC profiles.

When studying the properties of novel fat blend formulations, the degree of unsaturation is an index of considerable importance as it is an indicator of the oxidative stability of food lipids. With regard to degree of unsaturation, Avo:PS:CB blends displayed slightly lower IV (65.47 to 70.27) ($p < 0.05$)

than the lard (73.76) (Table 2). This characteristic feature was somewhat similar to those reported earlier by Yanty *et al.* (2014a) and Raihana *et al.* (2017a) in binary blends of MF:PS and PS:GO, respectively. The SMP of the ternary blends studied by researchers were also found to be higher (38.25 to 40.5 °C) than that of lard (29.25) (Table 2). Hence, the SMP and IV of these ternary blends and lard were not found to be similar. Additions of palm stearin and cocoa butter into avocado fat caused slight increases in the amounts of palmitic (from 30.37 to 33.08%), stearic (from 1.30 to 4.57%) and oleic (from 43.64 to 44.04%) acids with concurrent decreases in the amounts of linoleic acids (from 17.45 to 14.28%). The increases in palmitic and stearic acids of these ternary blends was probably due to the presence of higher amounts of palmitic and stearic acids in palm stearin and cocoa butter, respectively.

DSC thermograms are generally helpful in monitoring the thermal properties of natural fats and their blends (Marikkar, 2015). The addition of palm stearin and cocoa butter into avocado fat would affect the melting peaks of avocado fat throughout the temperature region. Compositional changes such as increases in saturated fatty acids (Table 2) and disaturated/trisaturated TAG contents in the formulated blends are attributed to this (Table 4). On a comparative basis, the melting transitions of lard and the formulated ternary blends were found to display more differences than similarities. For instance, the end-sets of melting (T_{endset}) transition of all three Avo:PS:CB blends were higher (at around 47 °C) than that of lard (37.5 °C). However, there was a similarity noticed between Avo:PS:CB (84:7:9) blends and lard with regard to the maximum peak of thermal transitions at around -3.59 °C. Although it was a positive attribute, this alone cannot be sufficient to make a decision regarding which blend would be best in compatibility. A more relevant parameter to measure the compatibility of the ternary blends with lard would be the SFC profile as monitored by NMR. The sample containing the NMR tube was melted at 70 °C for 15 min, followed by chilling at 0 °C for 60 min, and then held at each measuring temperature for 30 min prior to measurement.

Past studies found that the SFC values of avocado fat of Malaysian cultivars were always lower than those of lard throughout the specified temperature range (Yanty *et al.*, 2012). The incorporation of a hard fat like palm stearin or cocoa butter could help enhance the SFC values of avocado fat to a level compatible with those of lard. By the addition of palm stearin (7%) and cocoa butter (5 to 9%) in small quantities into avocado fat, some of the TAG molecular groups were increased (e.g. PPO, PPP, SOO, SPO and PPS) while others tended to decrease (e.g. LLn, LLL, OLL, PLL, OOL, POL, PPL, OOO and POO) (Table 4). As PPP, PPO and SPO were originally present in high amounts in PS, their amounts would certainly go up. The excessive presence of these TAG molecules in PS had been previously confirmed by other researchers (Podchong *et al.*, 2018; Miskandar and Nor, 2010). In addition, Segall *et al.* (2005) stated that the excessive amounts of SPO, PPO and SOS TAG molecules present in cocoa butter would create a strong fat crystal network. With the gradual increases in the proportion of cocoa butter and palm stearin, the SFC values of the ternary fat mixtures tended to increase throughout the temperature region (Yanty *et al.*, 2017a). In fact, there was a good correlation ($r=+0.97$; $p < 0.05$) between the increasing SFC values of the fat blends and the increasing proportion of disaturated/trisaturated TAG molecules (Yanty *et al.*, 2017a). Of the three fat blends formulated, Avo:PS:CB (84:7:9) showed better compatibility to lard at most of the temperatures in the range. In addition to this, both lard and Avo:PS:CB (84:7:9) were found to possess almost similar proportions of PPO and SPO (Table 4). The occurrence of these two TAG molecular species in significant amounts caused both LD and Avo:PS:CB (84:7:9 blends to display both β' and β-form polymorphs, of which the β' form was dominant.

3.5. Quaternary mixtures of PO:PS:SBO:CB

Palm oil is a semi-solid that has been used worldwide for its multifaceted food and nonfood applications. The ever-increasing popularity of palm oil across the globe has resulted in its inclusion in various products. As palm oil can undergo fractionation, it would be an ideal substance for novel product formulation for specific needs (Nor Aini and Miskandar, 2007). For instance, palm oil has been used for different types of shortenings, margarines, vanaspati, deep-frying fat and other specialty fats. Nevertheless, it cannot become a direct substitute for lard due to the occurrence of PPO and POO in high amounts, which can cause an unusually high solidification profile (Siew, 2002). The SFC values for palm oil were found to be higher than that of lard within the temperature ranges of 0 to 20 °C (Yanty *et al.,* 2012). In fact, a tremendous gap existed between these two at the beginning of solidification and melting end-point. For instance, at 0 ºC the SFC of lard and palm oil were 30.8 and 68.63%, respectively, but their SFC values tended to become 0% at 40 and 55 ºC, respectively. It is generally assumed that by blending an appropriate amount of liquid oil such as soybean oil with palm oil, the SFC value can be adjusted to similar to that of lard at all temperatures (Siew, 2002).

Soybean oil can be an oil of choice to be used for blending with palm oil because the SFC value of soybean oil at 0 ºC was 0.31%, and would become 0% at 5 ºC. Based on this hypothesis, an attempt was made to develop a quaternary fat blend comprising of palm oil and soybean oil as its major components (Marikkar *et al.*, 2018). The question related to how much soybean oil would be just enough to make a proper blending with palm oil indeed needs to be worked out experimentally. For this purpose, the SFC profiling of fat blends at various temperatures would be inevitable. SFC is generally expressed as the percentage that corresponds to the total meltdown of a fat into liquid (Madison and Hill, 1978). The SFC profiles of three binary blends of PO:SBO made at the beginning are shown in Figure 1, which might give some indication for the selection of the best blending proportions. Out of these three binary blends prepared, the addition of 50% of soybean oil into palm oil resulted in SFC values similar to lard from the range 30 to 45 °C, but lower than that of lard within the temperature range 0 to 30 °C (Figure 1B). This deviation, however, could be overcome by adding a hard fatty substance like palm stearin or cocoa butter in smaller amounts due to their role in the adjustment of various physical and functional properties. Although the additions of 5% of PS into PO:SBO blends tended to increase the SFC values at 0 to 27 °C, the SFC values were found to increase higher than those of lard in the range of 27 to 35 °C, as shown in Fig. 1C. However, the addition of 5% CB into PO:SBO blends was found to help adjust the SFC values of the formulated fat blends and lard to become similar within the range of 27 to 35 °C, as shown in Figure 1A.

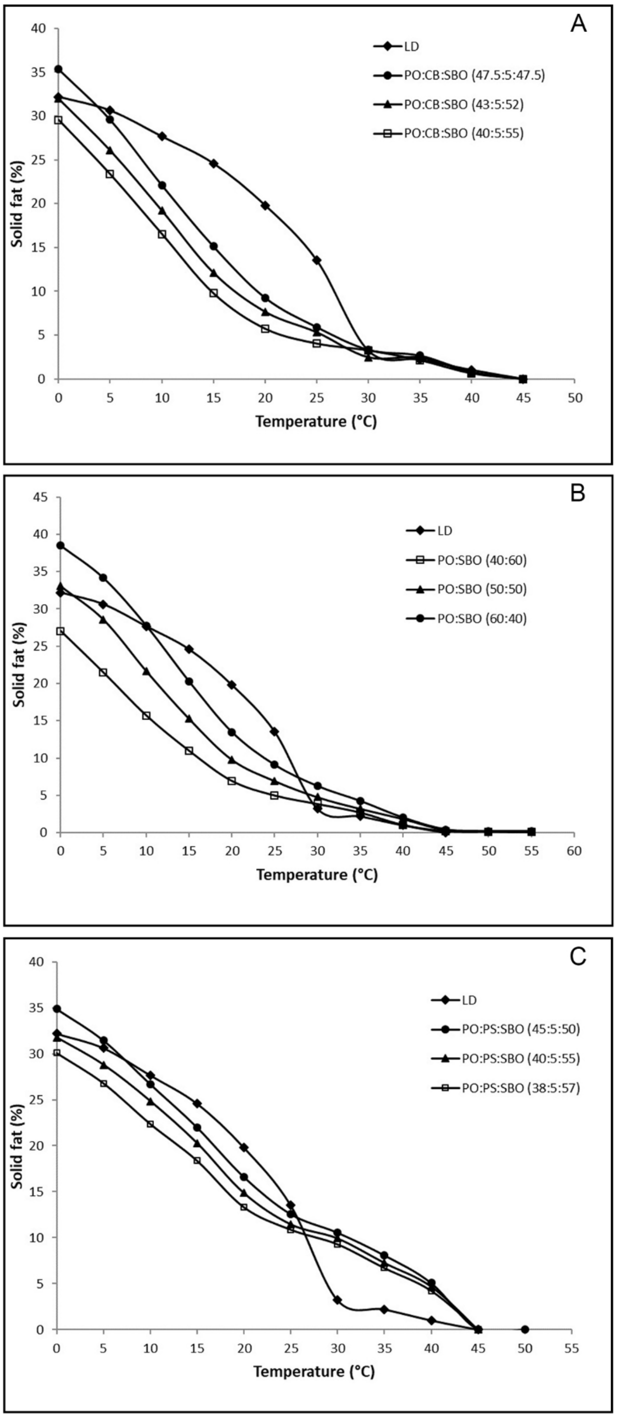

FIGURE 1: Comparison of solid content profiles of different binary and ternary blends and lard

[1]Abbreviations: MF, mee fat; PS: palm stearin; CB, cocoa butter; PO, palm oil; PS, palm stearin; SBO, soybean oil; LD, lard.

With regard to degree of unsaturation, IV of all quaternary fat blends were found to be significantly ($p < 0.05$) higher (92.26 to 96.95) than that of lard (73.76) (Table 2). This characteristic feature was somewhat similar to the observation made previously in the case of EF:CO binary blends (Nur Illyin *et al.*, 2013). This was primarily due to the presence of a high amount of soybean oil in the blends. On the other hand, SMP values for all quaternary blends (18.0 to 21.25 °C) were lower than that of lard (29.25) (Table 2). Hence, the SMP and IV of none of the quaternary blends were found to become similar to that of lard (Marikkar *et al.*, 2018). The additions of soybean oil into palm oil would cause significant increases in the amounts of linoleic acid (from 10.25 to 34.44%) with concurrent decreases in the proportions of palmitic (from 43.99 to 25.72%) and oleic acids (from 39.24 to 27.56%). The palmitic and linoleic acid contents in the quaternary blends were significantly higher ($p < 0.05$) than in lard. These changes in the degree of unsaturation and fatty acid composition led to changes in the IV of these mixtures as noted in Table 2.

The preparation of quaternary fat blends by adding palm stearin, soybean oil and cocoa butter into palm oil caused some increases in certain TAG molecules (Marikkar *et al.*, 2018). Increases in the proportions of PLL, OOL and POL could be due to the influence of soybean oil in the blends as soybean oil possesses TAG molecules namely, LLL (23.56%), OLL (17.77%), PLL (15.82%) and POL (13.69%) in high amounts (Table 4). Significant increases in the proportions of PPP and SOS were also noticed in the fat blends due to the fact that these two were major TAG species of palm stearin and cocoa butter (Nor Aini and Miskandar, 2007; Segall *et al.*, 2005). Among the TAG molecular species, PPO (ranging from 13.48 to 12.09%) and POO (from 10.87 to 9.70%) showed reductions in proportions (Table 4). These changes in composition would certainly have an impact on the melting peaks of the DSC thermograms of the fat blends.

The melting profiles of the formulated fats blends were considerably different from that of the original palm oil sample. For instance, they have one additional minor peak at around -20 °C with a shoulder peak (Table 3). This emerging feature in the melting curve of quaternary blends could be due to compositional changes caused by soybean oil. According to previous studies, soybean oil was reported to have all of its thermal peaks in the low-melting region of the DSC curve. Moreover, the T_{endset} was found to shift to the higher temperature region after the addition of palm stearin and cocoa butter into palm oil. When compared to the melting profile of lard (T_{endset} =37.5 °C), all three quaternary blends had higher end-set of melting (T_{endset}) (at around 44 °C) as well as lower onset of melting T_{onset} (at around -45 °C). These DSC features could be attributed to the influence of the low melting TAG molecules of soybean oil, which tended to crystallize at lower temperatures. Although there were great differences in melting transitions between lard and the three quaternary blends, a closer similarity was seen between them at the maximum peak of some peaks at around -3.59 °C (Marikkar *et al.*, 2018).

4. FOOD APPLICATIONS

Generally, in compatibility assessments, chemical composition and physical properties such as melting point, DSC thermal profiles, solid fat content profile, and polymorphic forms were considered in order to select the best fat blend. Nevertheless, it is essential to apply the formulated plant-based fat blends in real foods such as formulated meat products, bakery and confectionary products. This type of assessment would evaluate the actual performance of the plant-based fats in comparison to lard. Ultimately, it is the desired sensory attributes and consumer acceptance which will guarantee the commercial success of any formulated fat substitutes. Researchers in the past considered only partial replacers for lard in meat product formulations as total replacement would affect the meat products' sensorial attributes. For instance, investigations on the partial replacement of lard with plant fats were reported on products like fermented sausages (Ospina *et al.*, 2010), Pâtés (Delgado-Pado *et al.*, 2011; Rodriguez-Carpena *et al.*, 2011), low-fat Kung-wans (Hsu and Yu, 2002) and frankfurters (Choi *et al.*, 2010). As the primary motive behind the partial replacement of lard in these products was to provide more nutritious food for consumers, this approach would not help to comply with the requirements set by the food laws namely *kosher* and *halal.* Since inclusion of lard in biscuit formulations is strictly prohibited in *halal* and *kosher* food regulations, researchers attempted to test the formulated lard substitute on cookies. After ascertaining that PGO-2 [45:55 (GO:PS)] was the closest substitute for lard, Raihana *et al.* (2017b) converted the formulated plant-fat blend into a shortening to evaluate it as a fat ingredient in cookies. According to this study, the general acceptability of cookies was highly influenced by parameters such as hardness, fracture-ability and appearance. Among the different samples of shortenings, those prepared with PGO-2 [45:55 (GO:PS)] displayed the best quality attributes such as hardness, size and thickness, cracking size and color. In a separate study, Yanty *et al.* (2019) evaluated the formulated lard substitutes in cookies by following the baking procedure described in the AACC Method 10-50D (AACC, 2000). The results showed that there were no significant ($p > 0.05$) differences among the cookies prepared with all three types of shortenings and lard with regard to width and thickness. This was because the cookies made with formulated plant shortenings and lard shortening expanded uniformly. This could be due to the fact that both of these shortenings exhibited similarities in SFC at 25 °C (working temperature).

5. FUTURE PROSPECTS AND CHALLEGES

The food industry is one the single largest industries which is expanding rapidly worldwide. The recent trend shows that ethnic and religious foods have increasingly become an inevitable part of the total food industry. This is mainly due to the ever-increasing migration from Asia, Africa and the Middle East to America and other Western European countries. This trend coupled with globalization has opened up many doors for a new market segment for religious and vegetarian foods which are devoid of components of animal origin. Religious and ethnic foods, hence, occupy a small but rapidly expanding niche in every level of the food supply chain in these countries. As a result, there has been a growing recognition for ethnic and religious foods in Canada, North America, Australia, New Zealand and other Western European countries. This is evidently seen from the incorporation of ethnic and religious foods as a study component of the food science curricular of some universities. Although the world's major religions do not hold exactly the same ideology, they do embrace some similarities with regard to food consumption and dietary habits. For instance, they do take a common stand on prohibiting the consumption of pork and lard by their adherents. Consequently, any innovation leading to technology development on pork-free products or alternatives for lard will be welcome by all communities. In this context, some resistance from the existing piggery industry cannot be ruled out owing to the risk posed to their supply chain on pork products and lard.

The plant-based fat substitutes for lard are novel innovations, which show high potential in replacing lard extracted from porcine sources. As the technology involved is simple, it will easily fulfill the call for a healthy fat substitute for animal fats including lard and can be utilized widely in the 'halal' food production, bakery, meat, cosmetic products and so on. As all raw materials for the lard substitute are of plant origin, they will have the additional advantages of being rich in fat soluble vitamins and other phyto-nutrients. However, cost and ready availability of fat ingredients such as avocado fat, meet fat and cocoa butter are a real concern. This is mainly due to the imbalance between supply and demand as the plantations producing these fat ingredients are mainly limited to Asia and Africa. Apart from this, the ready-availability of lard alternative fats to some ethnic minorities living in certain countries might not be possible right now due to low demand or smaller market segments.

6. CONCLUDING REMARKS

This review highlighted the effectiveness of various blending strategies and the selection of fats and oils in formulating substitutes for lard. Instead of finding a single lipid source as substitute for lard, trying binary, ternary and quaternary blend formulations was found to be a rewarding experience. The choice of multiple lipids such as enkabang fat, canola oil, guava oil, palm oil, palm stearin, soybean oil and cocoa butter from various sources was indicative of the great benefit of diversity as well as inclusiveness. Evaluating formulated plant-fat blends and lard after processing into shortening to assess their properties in terms of hardness, consistency, microstructure and polymorphism has offered excellent results. Among different binary, ternary and quaternary blends developed so far, MP:PS (99:1), Avo:PS:CB (84:7:9) and PO:PS:SBO:CB (38:5:52:5) were found to be best in terms of melting and solid content profiles. The consistency of formulated plant-based shortenings and lard shortening indicated that they could be categorized as plastic fats, which are spreadable. Although the formulated plant-based shortenings and lard shortening were dissimilar in crystal morphology, they displayed a mix of β' and β-form polymorphs of which the β' form was dominant. When their functional properties were tested as fat ingredients in the production of cookies, the consistency of the dough made using binary blend shortening was found to be closer in value to that of lard shortening. The elasticity value of the dough made out of the binary fat shortening was also found to be similar to that of dough made from lard shortening.

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A novel class of bio-lubricants are synthesized by epoxidation of 10-undecylenic acid-based esters

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SUMMARY: Mineral-based lubricants are being supplanted by bio-based lubricants because of environmental concerns and the depletion of fossil resources. The derivatives of edible and non-edible oils are considered potential alternatives to existing natural mineral oil base stocks in certain lubricant applications, where immediate intraction with the environment is predicted. A new class of epoxides were synthesized from the undecylenic esters of 2-ethyl hexanol, neopentyl glycol (NPG), and trimethylolpropane (TMP). These unsaturated esters were epoxidized by using meta chloro perbenzoic acid. The synthesized epoxides were characterized by spectral studies (^{1}HNMR, ^{13}CNMR, IR) physio-chemical (density, specific gravity) and lubricant properties (kinematic viscosity, viscosity index, flash point, fire point, cloud point, pour point, copper strip corrosion). TMP epoxide has a high viscosity index, high flash point, and low pour point compared to 2-ethyl hexyl epoxide and NPG epoxide.

KEYWORDS: *2-ethyl hexyl epoxide; Biolubricants; Epoxidation; Esterification; NPG epoxide; TMP epoxide.*

RESUMEN: ***Nueva clase de biolubricantes sintetizados mediante epoxidación de ésteres de ácido 10-undecilénico***. Los lubricantes de base biológica están reemplazando a los lubricantes de base mineral debido a preocupaciones ambientales y al agotamiento de los recursos fósiles. Los derivados de aceite comestible y no comestible se consideran alternativas potenciales a las existencias base de aceite mineral natural existentes en ciertas aplicaciones de lubricantes, donde se predice una interacción inmediata con el medio ambiente. Se preparó una nueva clase de epóxidos a partir de ésteres a base de ácido undecilénico de 2-etilhexanol, neopentilglicol (NPG) y trimetilolpropano (TMP) mediante epoxidación con ácido metacloroperbenzoico como catalizador. Los epóxidos sintetizados se caracterizaron mediante estudios espectrales (^{1}HNMR, ^{13}CNMR, IR) las propiedades fisicoquímicas (densidad, densidad específica) y lubricantes (viscosidad cinemática, índice de viscosidad, punto de inflamación, punto de combustión, punto de enturbiamiento, punto de fluidez, tira de cobre). corrosión). El epóxido a base de éster de TMP tiene un índice de viscosidad alto, un punto de inflamación alto y un punto de fluidez bajo en comparación con el epóxido a base de éster 2-etilhexílico y el epóxido a base de éster NPG.

PALABRAS CLAVE: *Biolubricantes; Epoxidación, Epóxido; Epóxido a base de éster TMP; Éster de NPG; Propiedades fisicoquímicas.*

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1. INTRODUCTION

Lubricants have a tremendous application in day-to-day life, machine-driven and automobile industries. Most of the existing lubricant oils are non-degradable, causing a tremendous effect on the environment. There has been considerable interest in biodegradable lubricants from renewable resources as substitutes for conventional non-renewable mineral-based lubricants. Environmental concerns and the decrease of fossil fuel reserves have triggered great interest in developing bio-based lubricants from renewable resources.

The performance of an engine can be improved by using various edible and non-edible oil-based lubricants. Vegetable oils are renewable resources and are eco-friendly. Synthesized or modified vegetable oil lubricants have been proven to possess excellent lubricant properties for various applications (Anjana and Preeti, 2013). Polyol esters from palm and palm kernel oils have excellent oxidative stability (Yunus *et al.,* 2004). Fatty esters from rubber seed oil and thumba seed oil synthesized from 2-ethyl hexanol, neopentylglycol (NPG), trimethylolpropane (TMP) and pentaerythritol (PE) are good bio-lubricant base-stocks (Kamalakar *et al.,* 2013; Kamalakar *et al.,* 2015). High-performance polyol ester lubricants were synthesized from10-Undecenoic acid (Padmaja *et al.,* 2012). The novel class of eco-friendly lubricants was synthesized from undecylenic acid (Cavalcante *et al.,* 2019). The polyol esters of Calophyllum inophyllum oils showed good tribological properties. (Srinivas *et al.,* 2020). Trimethylol tri-esters from waste cooking oils showed good lubrication properties (Lakkoju and Vemulapalli, 2020). Polyol ester-based bio-lubricants and their blends showed tribological properties (Kotturu *et al.,* 2020).

The double bonds present in synthesized esters undergo auto-oxidation which is susceptible to degradation on prolonged use, thus decreasing the lubricant efficiency. To improve the lubricant efficiency, double bonds present in esters can be modified chemically by epoxidation reaction.

Oils with unsaturated fatty acids were epoxidized using different catalysts. H_2SO_4 was an effective catalyst for forming an oxirane ring in the presence of H_2O_2 across the double bonds in vegetable oils (Abdullah and Salimon, 2010). Cottonseed oil was epoxidized using sulphuric acid (Dinda *et al.,* 2008), peracetic acid (Sinadinović-Fišer *et al.,* 2012), and soybean fatty methyl esters by performic acid (Campanella *et al.,* 2008), jatropha and castor oils by performic acid and peroxyacetic acid in acid ion exchange resin (Goud *et al.,* 2007). The epoxidation reaction of mahua oil with hydrogen peroxide (H_2O_2) and glacial acetic acid in the presence of an acidic medium was reported (Goud *et al.,* 2006a). Okieimen reported the epoxidation of rubber seed oil using peroxyacetic acid (Okieimen *et al.,* 2002). Vegetable oils such as oil from the Karanja seed (Goud *et al.,* 2006b), soybean oil (Petrovic *et al.,* 2002; Geethanjali *et al.,* 2013), canola oil, canola oil derivatives (Omonov *et al.,* 2016), jatropha seed oil (Goud *et al.,* 2007) and Parkia biglobosa seed methyl esters (Ikhuoria *et al.,* 2007) were also converted to respective epoxidized oils. Epoxidized vegetable oils have various applications as valuable intermediate or end products due to their biodegradability and non-toxicity (Armylisas *et al.,* 2017).

Epoxidized soybean oil exhibited improved low-temperature stability, thermo-oxidative stability friction and wear properties compared to existing lubricants (Erhan *et al.,* 2008, Adhvaryu *et al.,* 2005). Epoxidized jatropha oil showed better antioxidant properties than butylated hydroxyl toluene and zinc dimethyl dithiocarbamate (Sammaiah *et al.,* 2014). Epoxidised Rapeseed oil had superior oxidative stability, better friction reduction and high-pressure abilities (Wu *et al.,* 2000). Epoxidized fatty methyl esters from castor oil have more thermo-oxidative stability than their esters (Venubabu and Goud, 2014). The present study focused on synthesizing a novel class of epoxides from undecylenic esters of 2-ethyl hexanol, NPG and TMP. The synthesized products were assessed for lubricant properties and correlated with their corresponding esters.

2. MATERIALS AND METHODS

2.1. Materials

2-Ethylhexanol, 2,2-Dimethylpropane-1, 3-diol (NPG) and 2-ethyl2-(hydroxyl methyl)-1, and 3-propanediol (TMP) were supplied by ACRO Organics, India. Basic aluminum oxide, dichloromethane (DCM), ethyl acetate, hexane and toluene were supplied by Finar, India. mCPBA (m-chloro per benzoic acid) was supplied by LOBA Chemie, India. Undecylenic acid (UDA) was supplied by SDFCL, Mumbai, India. Silica gel (60-120 mesh) was supplied by Fisher Scientifics, Ahmadabad, India. Sodium thiosulphate (Hypo) and anhydrous sodium sulphate were supplied by Fisher Scientifics, Mumbai, India. Sodium bicarbonate ($NaHCO_3$) was supplied by Merck Specialties Pvt. Ltd., Mumbai, India. p-toluene sulphonic acid (pTSA) was supplied by AVRA labs, Hyderabad, India. All the above chemicals, required for synthesis, were used directly and without further purification.

2.2. Methods

2.2.1. Spectral analysis

A 1600 FT-IR Perkin-Elmer spectrometer (Norwalk, CT) was used for recording Infrared (IR) spectra with a liquid film between the NaCl cells. The streching frequencies of the IR spectra were measured in cm^{-1}. A Brucker AVANCE 400 MHz spectrometer was used to record proton and Carbon - 13 Nuclear Magnetic Resonance Spectra (^1H and ^{13}C NMR) with respect to tetra methyl silane (TMS) an internal standard. The $CDCl_3$ was used as solvent in ^1H, ^{13}C NMR. The **δ** values were recorded in parts per million (ppm).

2.2.2. Characterization properties

Physico-chemical, and lubricant properties of synthesized esters and epoxy compounds were determined by standard ASTM (American standard testing method) methods. The density and specific gravity were tested by pycnometer at room temperature. The Kinematic viscosity at various temperatures (40 °C, 100 °C) was tested in a Kinematic viscosity bath. Flash point, and fire point were tested in a Clevland flash and fire point apparatus. Cloud, and pour point were tested in the automatic cloud and pour point apparatus. Copper strip corrosion was tested in the Copper strip corrosion bath. All the above apparatus were procured from culture instruments India LLP, Bengaluru, India.

2.2.3. Determination of lubricant properties

Kinematic viscosity and viscosity index. The kinematic viscosity was measured at two different temperatures of 40 and 100 °C using an Ostwald direct flow type viscometer tube (type-C, D, E tubes, having definite constant values) in a 'CI' kinematic viscosity bath Digital and calculated according to the ASTM D 445 method. The viscosity index was calculated according to the ASTM D 2270 method. Measurements were done thrice and average values were taken.

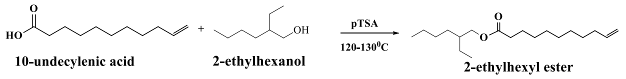
$$\text{Kinematic viscosity} = \text{constant value of viscometer} \times \text{time in seconds}$$

Cloud and pour point. The cloud and pour points were determined according to standard ASTM D 2500, ASTM D 97 methods using a 'CI' ultra crystat cloud and pour point apparatus. The cloud point of the samples was detected automatically in a cloud and pour point apparatus. The pour point of the samples was checked manually for every 1 °C decrease in temperature. Cloud and pour point determinations were made thrice and the average was reported.

Flash and fire points. The flash and fire points were determined according to ASTM D 92 method using a 'CI' Cleveland flash and fire point apparatus. The open-cup method for the detection of flash point was used by means of a vessel, which is exposed to the outside air. The sample was placed in the vessel and the temperature was raised gradually until it flashed and ignited at a certain point. After obtaining the flash point, the sample was heated until the fire came. The flash and fire ponts of all samples were determined thrice and the average was reported.

Copper strip corrosion. The copper strip corrosion of the samples was determined by using a 'CI' copper strip corrosion test apparatus as per the ASTM 130 method. A polished copper strip was immersed in the sample solution and heated at 100 °C for 3 h. After that, the tarnished copper strip was removed and assessed against an ASTM standard.

Synthesis of 2-ethylhexyl undec-10-enoate (2-ethyl hexyl ester) (a). 0.3839 moles of 2-ethyl hexanol (50 g), 0.5759 moles of UDA (106 g), 1% pTSA (total weight of 2-ethyl hexanol and UDA, 1.56 g) and toluene (100 mL) in an RB flask were refluxed at 120-130 °C. The reaction proceeded at 120-130 °C until 7 mL of wáter were collected in the dean-stark apparatus. The reaction progress was checked by observing thin layer chromatography (TLC) with 9:1 v/v hexane and ethyl acetate as elutent at regular intervals. After reaching the required product temperature of the reaction mixture, it was decreased to 23-25 °C. The toluene present in the reaction mixture was removed by a vacuum. The excess or unreacted acid in the reaction mixture was neutralised using a saturated $NaHCO_3$ solution, and ethyl acetate was added to the aqeous solution. The organic (ethyl acetate) layer, which contained crude 2-ethyl hexyl ester, was separated using a separating funnel and thoroughly washed with water twice. Anhydrous sodium sulphate was used to remove moisture from the crude (2-ethyl hexyl ester) present in the organic layer. The crude 2-ethyl hexyl ester was purified by using the column chromatography technique. Basic aluminum oxide was used as a stationary phase, 10% ethyl acetate in hexane was used as a mobile phase in column chromatography. 96.8 g of pure 2-ethyl hexyl ester were obtained. The 2-ethyl hexyl ester was assessed by ^{1}HNMR, ^{13}C NMR and IR spectral studies.

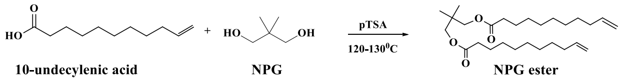


^{1}HNMR spectra: 0.8-9.0 (t, 6H, -C$\underline{H}_3$), 1.2–1.6 (m, 18H, -C$\underline{H}_2$-), 2.0-2.1 (m, 3H, C=C-C$\underline{H}_2$) and -C$\underline{H}$(CH_2)$_3$), 2.3-2.4 (t, 2H, -CO-C$\underline{H}_2$-), 3.9-4.0 (m, 2H, -O-C$\underline{H}_2$-), 4.9–5.0 (m, 2H, CH=C$\underline{H}_2$), 5.7–5.8 (m, 1H, -C$\underline{H}$=CH_2).

^{13}C NMR spectra: 66.5 (O-$\underline{C}H_2$), 115.10 (CH=$\underline{C}H_2$), 139.27 ($\underline{C}$H=CH_2), 173.44($\underline{C}$=O).

IR spectra: 3020 (str, C=C-H), 2929-2857 (str, -C-H), 1727 (str, C=O), 1217 (str, C-O-C), 771 (C-C).

Synthesis of 2,2-dimethylpropane-1,3-diyl diundec-10-enoate (NPG ester)(b). 0.678 moles of NPG (70.6 g), 1.356 moles of UDA (250 g), 1% pTSA (total weight of NPG and UDA, 3.2 g) and toluene (100 mL) were refluxed in a RB flask at 120-130 °C. The reaction took place at 120-130 °C until 24 mL of water were collected in the dean-stark apparatus. The reaction progress was monitored by observing TLC with 9:1 v/v hexane and ethyl acetate as elutent at regular intervals. After the formation of ester, the temperature of the reaction mixture was decreased to 23-25 °C. The toluene present in the reaction mixture was removed by vacuum. Ethyl acetate was added to the neutralized excess or unreacted acid present in the reaction mixture with a saturated $NaHCO_3$ solution in the aqeous solution. The organic (ethyl acetate) layer, which contains crude NPG ester, was separated using a separating funnel and thoroughly washed with water twice. Anhydrous sodium sulphate was used to remove moisture from the crude (NPG ester) present in the organic layer. The crude NPG ester was purified using the column chromatography technique. Basic aluminum oxide was used as stationary phase, 10% ethyl acetate in hexane was used as a mobile phase in column chromatography. 245 g pure NPG ester were obtained. The NPG ester was assessed by ^{1}HNMR, ^{13}C NMR and IR spectral studies.

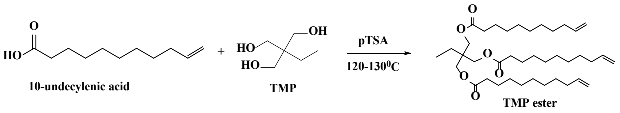


^{1}HNMR spectra: 0.9 (s, 6H, –C$\underline{H}_3$), 1.2–1.4(m, 20H, –C$\underline{H}_2$–), 1.5-1.6(m, 4H, –CO–CH_2–CH_2-), 2.0-2.1(q, 4H, –C$\underline{H}_2$–CH=CH_2), 2.3-2.4(t, 4H, –CO–C$\underline{H}_2$–), 3.9(s, 4H, –O–C$\underline{H}_2$–), 4.9–5.0(m, 4H, –CH=C$\underline{H}_2$), 5.7–5.9 (m, 2H, –C$\underline{H}$=CH_2).

^{13}C NMR spectra: 68.76 (O-$\underline{C}H_2$), 114.78 (CH=$\underline{C}H_2$), 139.09 ($\underline{C}$H=CH_2), 172.76 ($\underline{C}$=O).

IR spectra: 3021(str, C=CH), 2927 (str, C–H), 1736 (str, C=O), 1214(str, COC), 755 (C-C).

Synthesis of 2-ethyl-2-((undec-10-enoyloxy) methyl)propane-1-3-diyl diundec-10-enolate (TMP ester) (c). 0.378 moles of TMP (52 g), 1.356 moles of UDA (250 g), 1% pTSA (total weight of NPG and UDA, 3.02 g) and toluene (100 mL) in a RB flask were refluxed at 120-130 °C. The reaction proceeded at 120-130 °C until 21 mL of water were collected in the dean-stark apparatus. The reaction progress was monitored by observing TLC with 9:1 v/v hexane and ethyl acetate as elutent at regular intervals. The temperature of the reaction mixture decreced to 23-25 °C. The toluene present in the reaction mixture was removed by vacuum. Ethyl acetate was added to the neutralized excess or unreacted acid in the reaction mixture using a saturated $NaHCO_3$ solution, in the aqeous solution. The organic (ethyl acetate) layer, which contains crude TMP ester, was separated using a separating funnel and thoroughly washed with water twice. Anhydrous sodium sulphate was used to remove moisture from the crude (TMP ester) present in the organic layer. The crude TMP ester was purified by using the column chromatography technique. Basic aluminum oxide was used as a stationary phase, 10% ethyl acetate in hexane was used as a mobile phase in column chromatography. 208 g of pure TMP ester were obtained. The TMP ester was assessed by ^{1}HNMR, ^{13}C NMR and IR spectral studies.

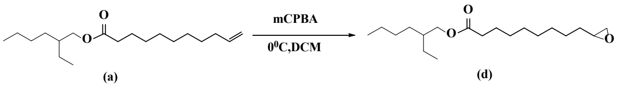


^{1}HNMR spectra: 0.8-1.0 (t, 3H, $–C\underline{H}_3$), 1.2–1.3(m, 30H, $–C\underline{H}_2–$), 1.3-1.4(m, 2H, $CH_3–C\underline{H}_2–$), 1.5–1.7 (m, 6H, $–CO–CH_2–C\underline{H}_2–$), 2.0-2.1 (q, 6H, $–C\underline{H}_2–CH=CH_2$), 2.3-2.4 (t, 6H, $–CO–C\underline{H}_2–$), 3.9 (s, 6H, $–O–C\underline{H}_2–$), 4.9–5.0 (m, 6H, $–CH=C\underline{H}_2$), 5.7–5.8 (m, 3H, $–C\underline{H}=CH_2$).

^{13}C NMR spectra: 63.32 ($O-\underline{C}H_2$), 114.20 ($CH=\underline{C}H_2$), 138.69 ($\underline{C}H=CH_2$), 171.97 ($\underline{C}=O$).

IR spectra: 3021(str, C=CH), 2927 (str, C–H), 1741 (str, C=O), 1162 (str, COC), 758 (C-C).

Synthesis of 2-ethylhexyl 9-(oxiran-2-yl) nonanoate (2-ethyl hexyl epoxide)(d). 2-ethyl hexyl ester (a) (35 g, 0.1146 moles) 175 mL of DCM (10 vol with respect to ester) were taken in a 1L RB flask and cooled to 0 °C. mCPBA (65% purity, 49 g, 0.1720 moles) was added to the reaction mixture at regular intervals for 3 h to maintain the temperature at 0 °C. The reaction was kept at ambient temperature and stirred overnight. The reaction progress was monitored by observing TLC. The reaction mixture was cooled to 0 °C. After completion of the reaction, the precipitated m-chloro benzoic acid was filtered. Ethyl acetate was added to the filtratewith 10% sodium thiosulphate solution, and then stirred thoroughly. The organic (ethyl acetate) layer was separated and washed twice with distilled water. The organic layer was dried over anhydrous sodium sulphate to remove moisture and concentrated under reduced vacuum to obtain crude 2-ethyl hexyl epoxide. Silica gel column chromatography was used to purify crude 2-ethyl hexyl epoxide with 1:1 ethyl acetate and hexane as an elutent to obtain 26 g of pure 2-ethyl hexyl epoxide. The pure 2-ethyl hexyl epoxide was assessed by ^{1}HNMR, ^{13}C NMR, IR spectral studies, and analyzed for physicochemical and basic lubricant properties.

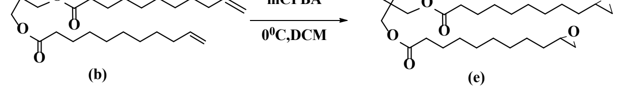


^{1}HNMR spectra: 0.9 -1.0 (t, 6H, $-C\underline{H}_3$), 1.2-1.4 (m, 19H, $-C\underline{H}_2-$ and $-C\underline{H}(CH_2-)_3$), 1.5-1.7 (m, 2H, $\underline{H}_2C—HC\overset{O}{—}CH_2$), 2.0-2.1 (t, 2H, $-C\underline{H}_2C=O$), 2.3-2.5 (m, 2H, $\underline{H}C\overset{O}{—}CH_2$), 2.7 (d, 1H, $HC\overset{O}{—}C\underline{H}_2$), 4.0-4.15 (m, 2H, $-C\underline{H}-O-C=O$).

^{13}C NMR spectra: 68.17 ($O-\underline{C}H_2$), 46.43 ($CHO\underline{C}H_2$), 51.85 ($\underline{C}HOCH_2$), 173.20 ($\underline{C}=O$).

IR spectra: 1724 (str, C=O), 1216 (str, $C\overset{O}{—}C$), 757 (C-C).

Synthesis of 2,2-dimethyl propane-1,3-diyl bis (9-(oxiran-2-yl) nonanoate (NPG epoxide) (e). NPG ester (b) (50 g, 0.1146 moles) 250 mL DCM (10 vol with respect to ester) were taken in a 1L RB flask and cooled to 0 °C. mCPBA (65% purity, 130 g, 0.4535 moles) was added to the reaction mixture at regular intervals for 3 h to maintain the temperature at 0 °C. It was allowed to cool to ambient temperature and stirred overnight. The reaction progress was monitored by observing TLC. The reaction mixture was cooled to 0 °C. After completion of the reaction, the precipitated m-chloro benzoic acid was filtered. A 10% sodium thiosulphate solution was added to the filtrate, along with ethyl acetate and stirred thoroughly. The organic (ethyl acetate) layer was separated and washed twice with distilled water. The organic layer was dried over anhydrous sodium sulphate to remove moisture and concentrated under reduced vacuum to obtain crude NPG epoxide. Silica gel column chromatography was used to purify crude NPG epoxide with 1:1 ethyl acetate and hexane as an elutent to obtain 36 g of pure NPG epoxide. The pure NPG epoxide was assessed by ^{1}HNMR, ^{13}C NMR, IR spectral studies, analyzed for physicochemical and basic lubricant properties.

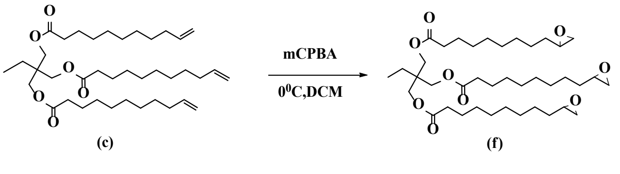


^{1}HNMR spectra: 0.98 (s, 6H, $-C\underline{H}_3$), 1.3-1.4 (m, 24H, $-C\underline{H}_2-$), 1.4-1.5 (m, 4H, $\underline{H}_2C{-}HC\overset{O}{\triangle}CH_2$, 1.6-1.7 (m, 4H, $-C\underline{H}_2-CH_2C{=}O$), 2.3-2.4 (4H, t, $-C\underline{H}_2C{=}O$), 2.7-2.8 (m, 2H, $\underline{H}C\overset{O}{\triangle}CH_2$, 2.9-3.0 (t, 4H, $HC\overset{O}{\triangle}C\underline{H}_2$), 3.9 (s, 4H, $-C\underline{H}_2-O-C{=}O$).

^{13}C NMR spectra: 68.56 ($O-\underline{C}H_2$), 46.44 ($\underline{C}HOCH_2$), 51.86 ($CHO\underline{C}H_2$), 172.94 ($\underline{C}{=}O$).

IR spectra: 1727 (str, C=O), 1258 (str, $C\overset{O}{\triangle}C$), 755 (C-C).

Synthesis of 2-ethyl-2-((9-(oxiran-2-yl) nonanoxyl) methyl) propane-1, 3-diyl (9-(oxiran-2-yl) nonanoate) (TMP epoxide)(f). TMP ester (c) (50 g, 0.0738 moles) 250 mL DCM (10 vol with respect to ester) were taken in a 1L RB flask and cooled to 0 °C. mCPBA (65% purity, 135 g, 0.4737 moles) was added to the reaction mixture at regular intervals for 3 h to maintain the temperature at 0 °C. The reaction was allowed to cool to ambient temperature and stirred overnight. The reaction progress was monitored by observing TLC. The reaction mixture was cooled to 0°C after completion of the reaction, and the precipitated m-chloro benzoic acid was filtered. A 10% sodium thiosulphate solution and ethyl acetate were added to the filtrate and stirred thoroughly. The organic (ethyl acetate) layer was separated and was washed twice with distilled water. The organic layer was dried over anhydrous sodium sulphate to remove moisture and concentrated under reduced vacuum to obtain crude TMP epoxide. Silica gel column chromatography was used to purify the crude TMP epoxide with 1:1 ethyl acetate and hexane as an elutent to obtain 35 g of pure TMP epoxide. The pure TMP epoxide was assessed by ^{1}HNMR, ^{13}C NMR, IR spectral studies, and then analyzed for physicochemical and basic lubricant properties.



^{1}HNMR spectra: 0.9 (t, 3H, $-C\underline{H}_3$), 1.3-1.4 (m, 32H, $-C\underline{H}_2-$), 1.4-1.5 (m, 6H, $\underline{H}_2C{-}HC\overset{O}{\triangle}CH_2$), 1.6-1.7 (m, 6H, $-C\underline{H}_2-CH_2C{=}O$), 2.3-2.4 (t, 6H, $-C\underline{H}_2C{=}O$), 2.7-2.8 (m, 3H, $\underline{H}C\overset{O}{\triangle}CH_2$), 2.9-3.0 (t, 6H, $HC\overset{O}{\triangle}C\underline{H}_2$), 4.0 (s, 3H, $-C\underline{H}-O-C{=}O$).

^{13}C NMR spectra: 68.19 ($O-\underline{C}H_2$), 46.49 ($CHO\underline{C}H_2$), 51.92 ($\underline{C}HOCH_2$), 172.89 ($\underline{C}{=}O$).

IR spectra: 1738 (str, C=O), 1256 (str, $C\overset{O}{\triangle}C$), 751 (C-C).

3. RESULTS AND DISCUSSION

Vegetable oils and synthesized polyol esters possess good lubricity, high viscosity index, and flash point along with lower volatility. In vegetable oils, the presence of a high degree of unsaturation lowers thermal and oxidative stability, which can be improved by the chemical modification of unsaturation such as epoxidation. Epoxidized castor oil possesses enhanced lubricity properties such as high density, viscosity and low pour point (Venubabu and Goud, 2014). Keeping this in mind, undecylenic acid-based epoxides of 2-ethyl hexanol, NPG and TMP based esters were developed. Undecylenic acid, a second-generation product of castor oil, was a suitable feedstock to prepare many novel products for various applications. Upon pyrolysis, castor oil gives undecylenic acid. Various esters are prepared from undecylenic acid using different alcohols such as 2-ethyl hexanol, NPG and TMP in the presence of pTSA. The yields of synthezied esters were in the range of 75-80%. A peak corresponding to ester $O-CH_2$ protons in ^{1}HNMR was observed at 3.9-4.0 ppm in 2-ethyl hexyl ester, 3.9 ppm in NPG ester, and 3.9 ppm in TMP ester. Multiple peaks corresponding to unsaturation were observed at 4.9-5.0 ppm, 5.7-5.8 ppm in 2-ethyl hexyl ester and TMP ester, 4.9-5.0 ppm and 5.7-5.9 ppm in NPG ester was observed (Figure 1). In the ^{13}C NMR spectrum (Figure 2), the carbon signal for unsaturation was observed at the 115.10 ppm $\underline{C}H_2$ peak, 139.27 ppm $\underline{C}H$ peak in 2-ethyl hexyl ester, 114.78 ppm $\underline{C}H_2$ peak and 139.09 ppm for the $\underline{C}H$ peak in the NPG ester, 114.20 ppm $\underline{C}H_2$ peak and 139.69 ppm or $\underline{C}H$ peak for TMP ester. A peak was observed at 173.44 ppm for 2-ethyl hexyl ester, 172.76 ppm for NPG ester, 171.97 ppm for NPG ester-assised r $\underline{C}{=}O$ indicated ester linkage. C=O stretching

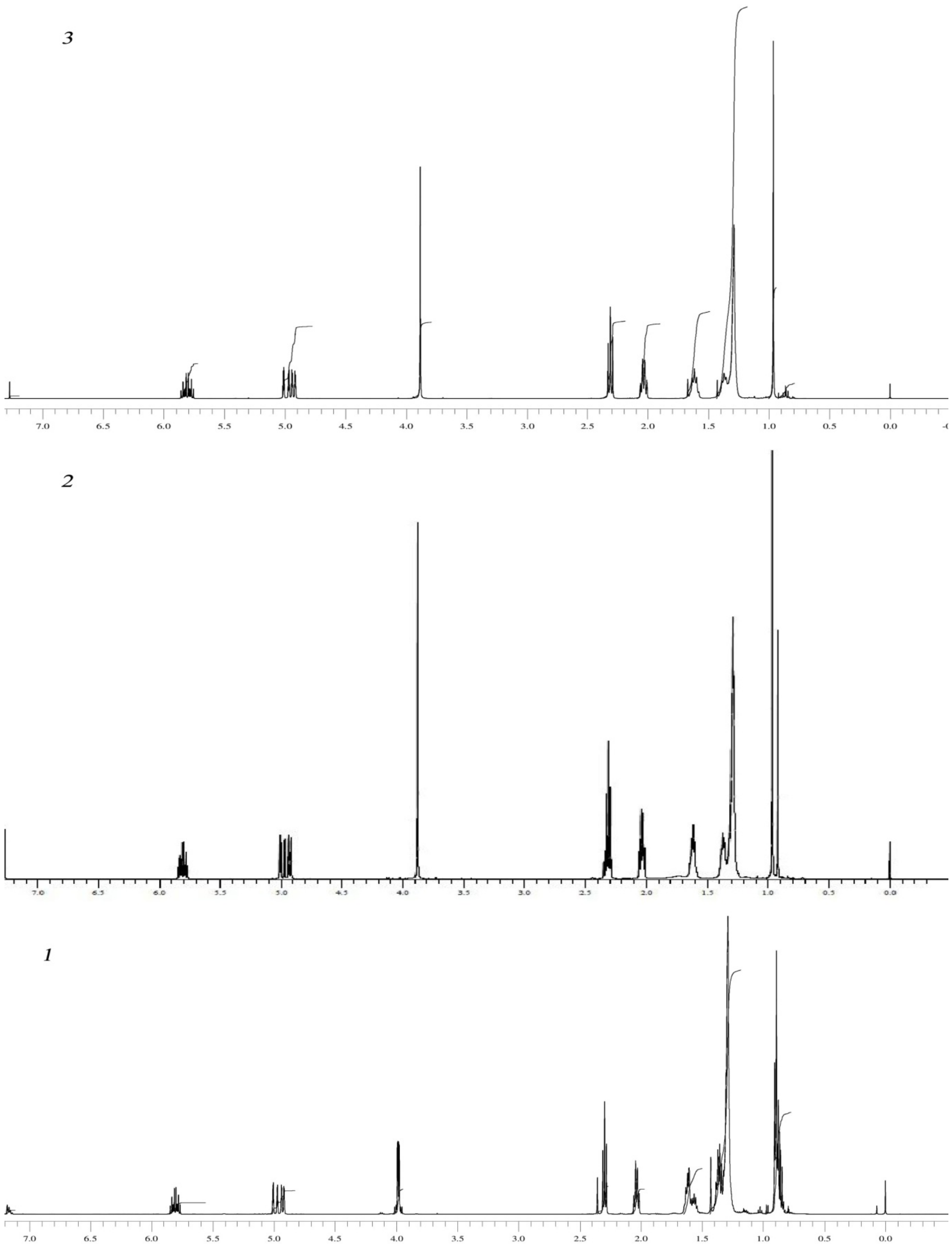


FIGURE 1. ^{1}HNMR spectra of various esters. 1) 2-ethyl hexyl ester, 2) NPG ester and 3) TMP ester

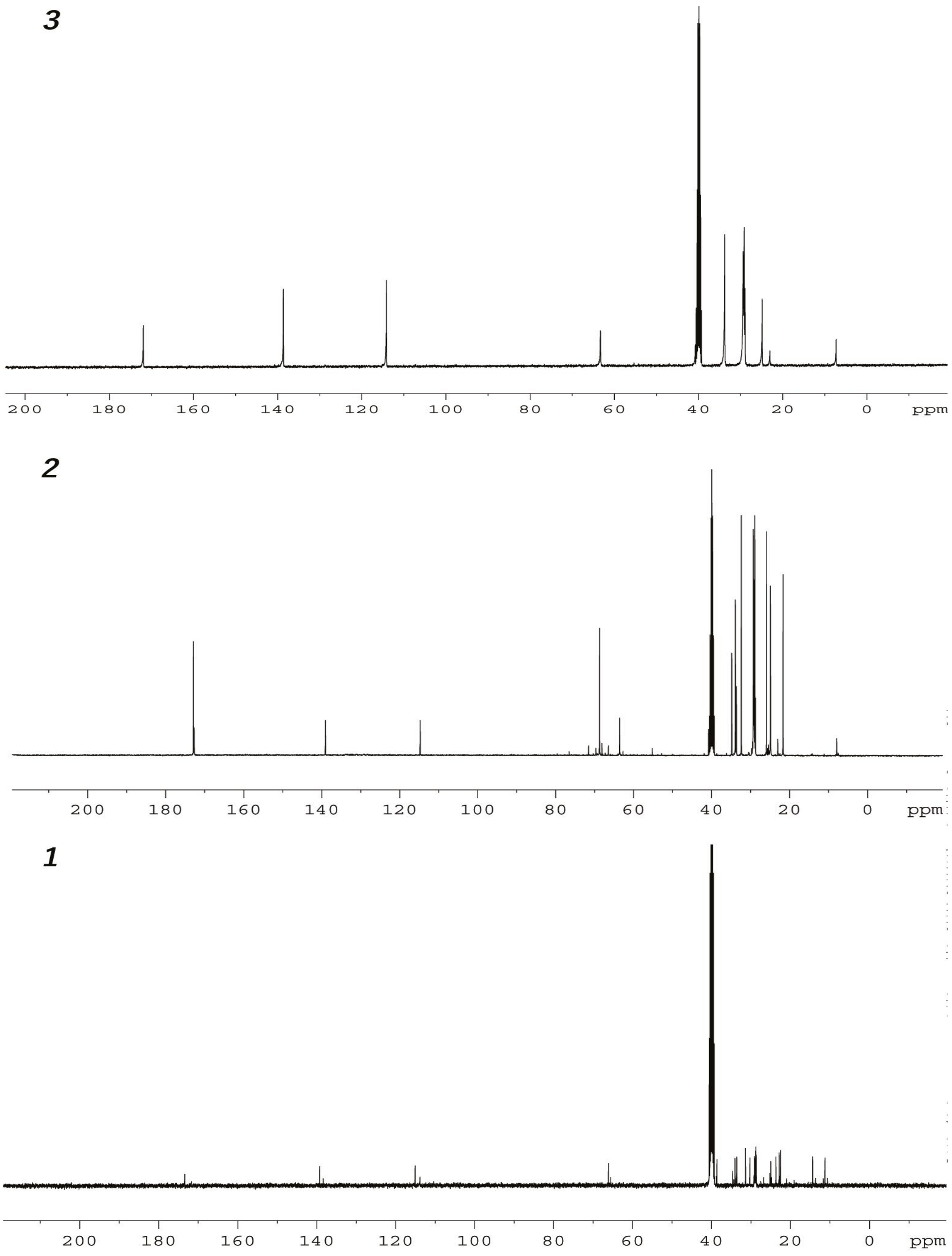


FIGURE 2. ^{13}C NMR spectra of various esters. 1) 2-ethyl hexyl ester, 2) NPG ester and 3) TMP ester

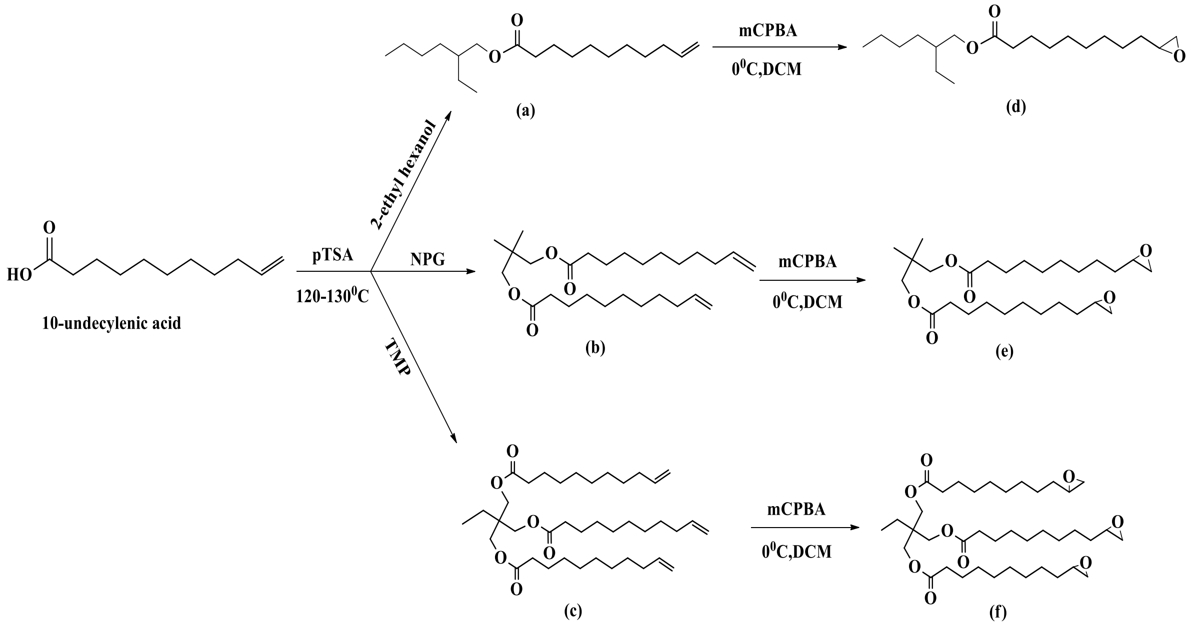
was observed in the t IR spectrum at 1727 cm^{-1} for 2-ethyl hexyl ester, 1736 cm^{-1} for NPG ester and 1741 cm^{-1} for TMP ester. C=C-H stretching at 3020 cm^{-1} for 2-ethyl hexyl ester, 3021 cm^{-1} NPG ester and 3021 cm^{-1} TMP ester were observed in the IR spectra, which confirmed the unsaturation group (Figure 3).

These esters were epoxidized using mCPBA in the presence of DCM. TLC is used to monitor the progress of the reaction. After completion, the reaction mixture was quenched with hypo and extracted with ethyl acetate. The organic layer (Ethyl acetate) was concentrated using a high vacuum to afford crude epoxide, and was purified by column chromatography.

The yields of synthesized epoxides were in the range of 70-75%. The epoxidized 2-ethyl hexanol, NPG and TMP based esters (Scheme-1) were characterized using ^{1}HNMR (Figure 4) ^{13}C NMR (Figure 5) and IR (Figure 6) spectral studies. The presence of epoxide ring protons in ^{1}HNMR was observed at 2.3-2.4 ppm as multiplet, 2.7-2.8 ppm doublet in 2-ethyl hexyl epoxide, 2.7-2.8 ppm as multiplet, 2.9-3.0 ppm as triplet in NPG epoxide, 2.7-2.8 ppm as multiplet, 2.9-3.0 ppm as triplet in TMP epoxide. The multiplet at 5.0-5.5 ppm due to alkene proton in esters (a-c) disappeared in all epoxides, indicating the conversion of the alkene group to oxirane ring (d-f). In the ^{13}C NMR spectrum, two new peaks were observed at 46.43 ppm, 51.85 ppm in 2-ethyl hexyl epoxide, 46.44 ppm, 51.86 ppm in NPG epoxide, 46.46 ppm, 51.92 ppm in TMP epoxide, indicating carbon atoms for oxirane. The carbón signals at 114 ppm and 139 ppm for unsaturation in esters was absent, indicating the conversion of a double bond to oxirane.

The existence of $\overset{\text{O}}{\text{C}\triangle\text{C}}$ stretching at 1216 cm^{-1} in the 2-ethyl hexyl epoxide, 1258 cm^{-1} in the NPG epoxide, and 1256 cm^{-1} in TMP epoxide observed in the IR spectra confirm the presence of the epoxide group. All synthesized epoxides were analyzed for physicochemical and lubricant properties. All properties were triplicated and data correspond to the mean of three determinations plus or minus standard deviation (Table 1).

Density. Density is a crucial property for all lubricant oils. Density was determined by means of a pycnometer at room temperature. The densities of 2-ethyl hexyl, NPG and TMP ester-based epoxides were 1.0351 ± 0.0004, 1.0446 ± 0.0006 and 1.1606



Scheme-1: Preparation of various epoxides from 10-undecylenic acid

(a) 2-Ethylhexyl undec-10-enoate, (b) 2,2-Dimethyl propane-1,3-diyl diundec-10-enoate, (c) 2-Ethyl-2-((undec-10-enoyloxy) methyl) propane-1-3-diyldiundec-10-enolate, (d) 2-ethylhexyl 9-(oxiran-2-yl) nonanoate, (e) 2,2-dimethyl propane-1,3-diylbis(9-(oxiran-2-yl)nonanoate, and (f) 2-ethyl-2-((9-(oxiran-2-yl)nonanoxyl)methyl) propane-1,3-diyl(9-(oxiran-2-yl) nonanoate)

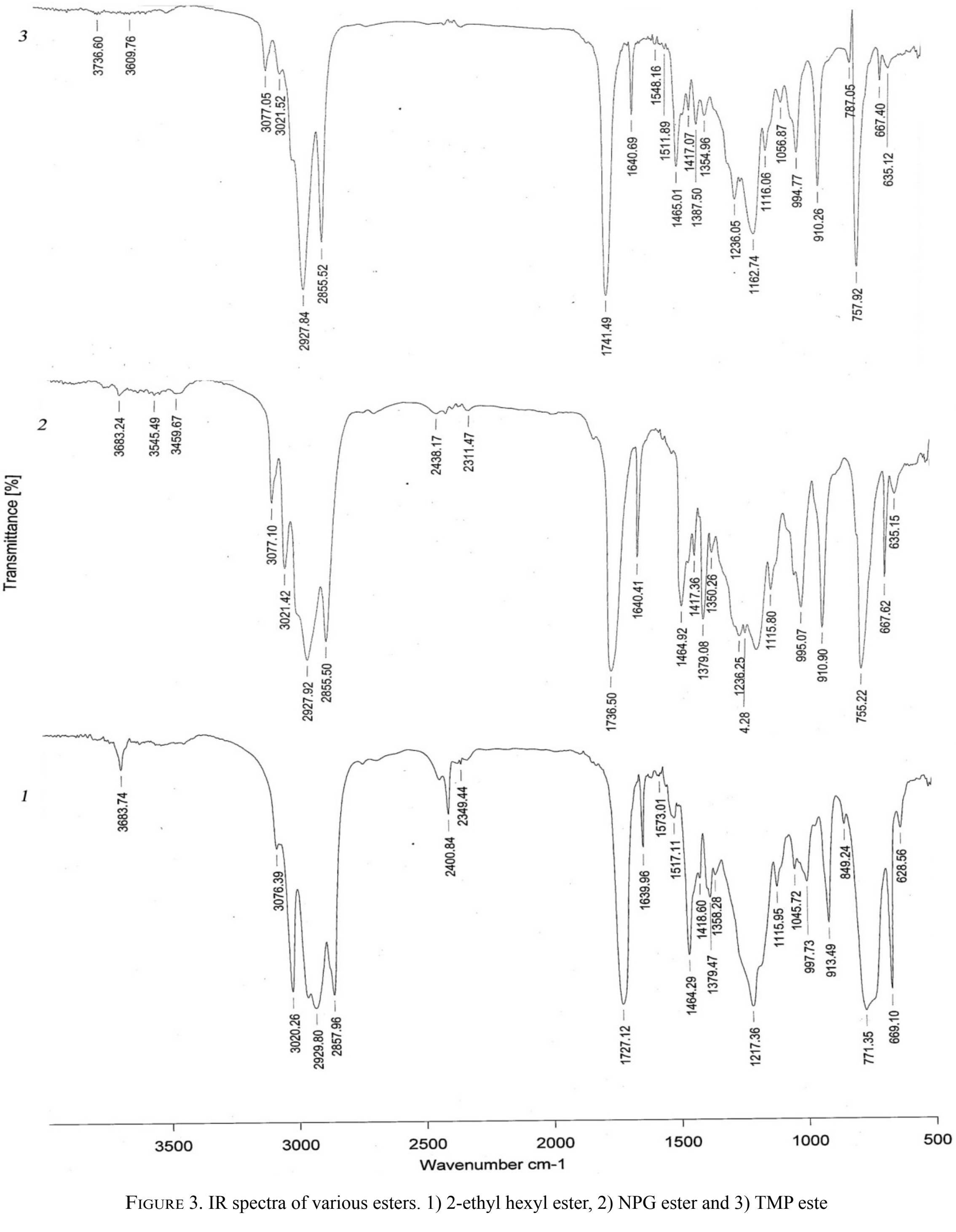


FIGURE 3. IR spectra of various esters. 1) 2-ethyl hexyl ester, 2) NPG ester and 3) TMP este

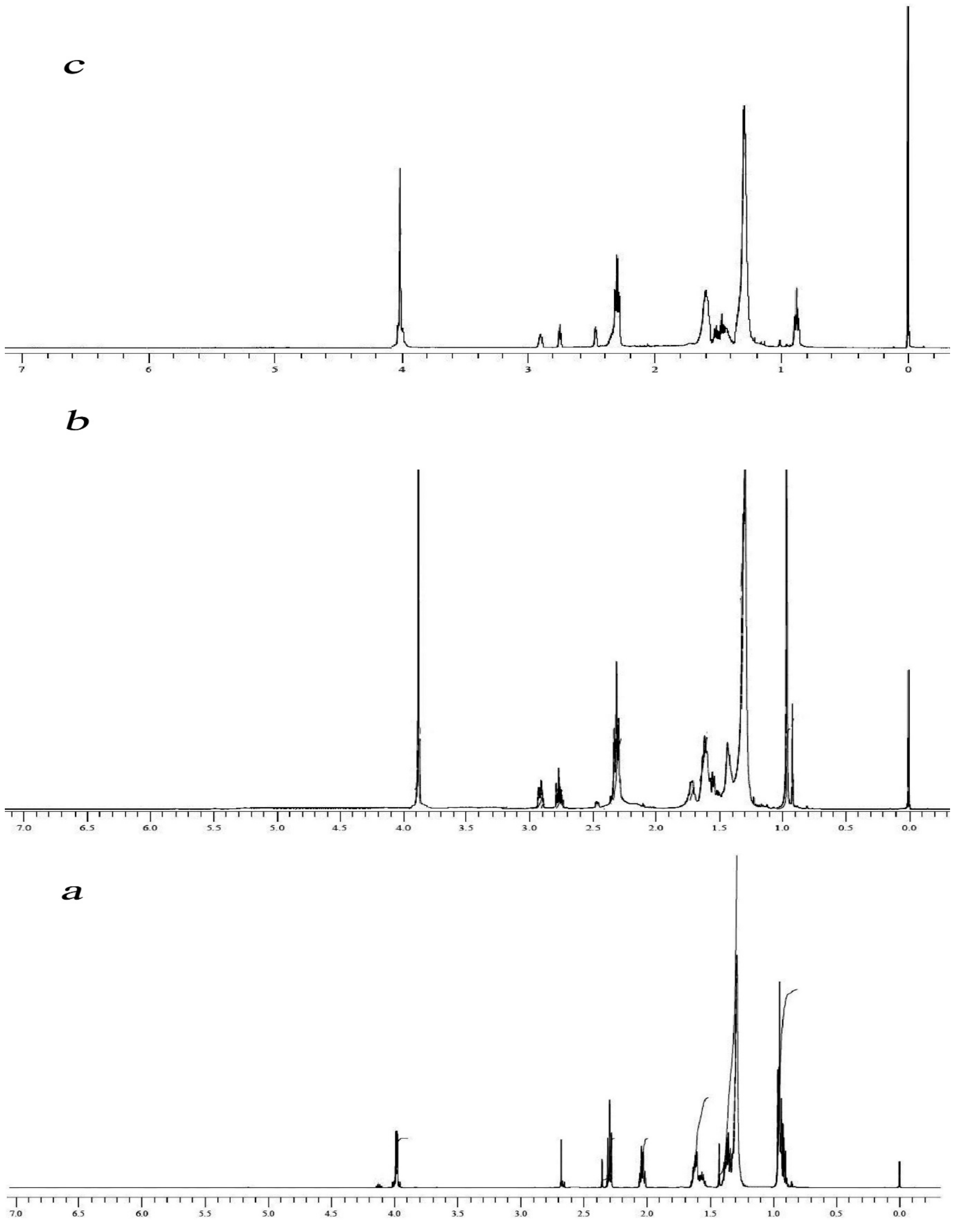


FIGURE 4. ^{1}HNMR spectra of various epoxides. a) 2-ethyl hexyl epoxide, b) NPG epoxide and c) TMP epoxide

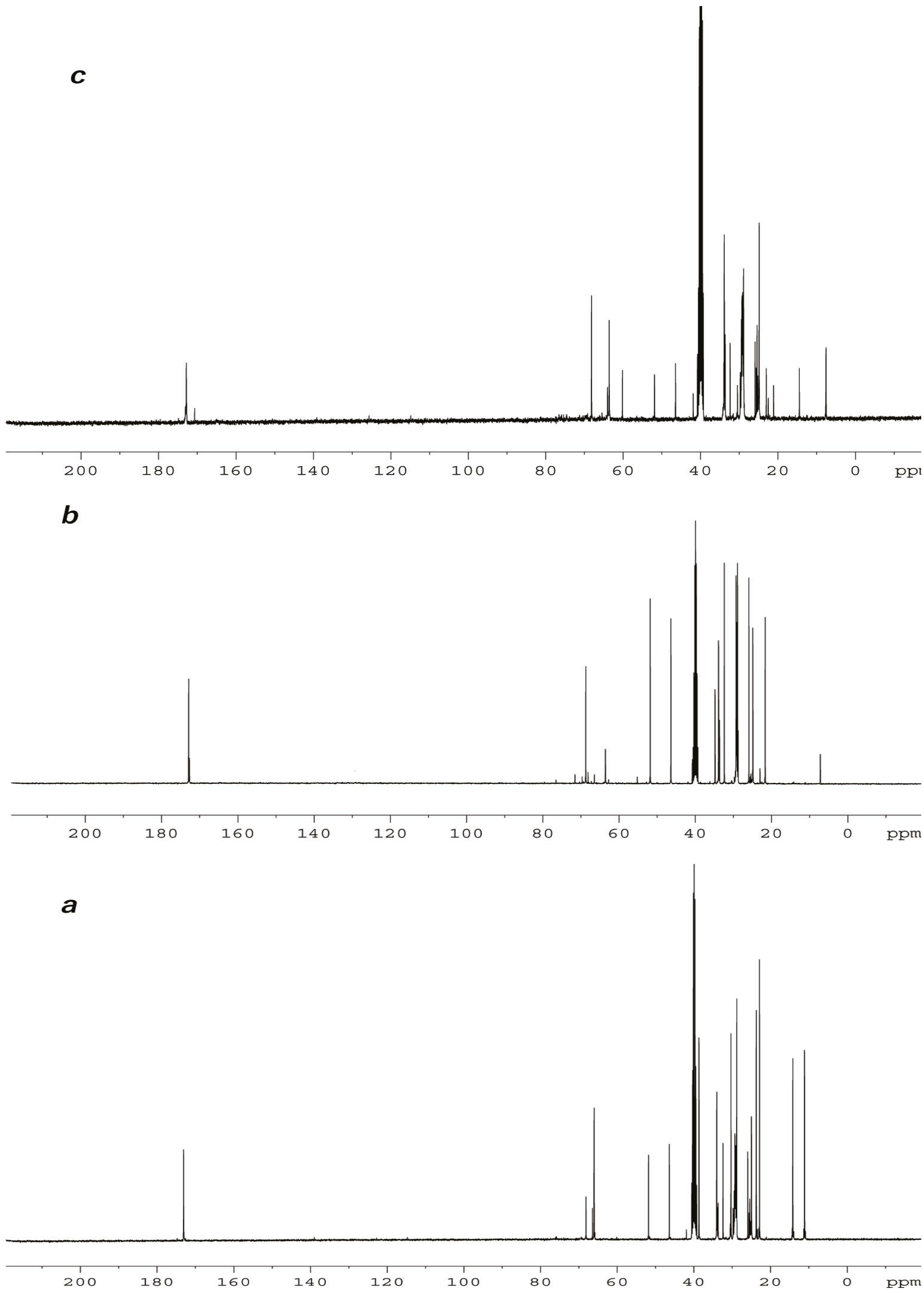


FIGURE 5. ^{13}CNMR spectra of various epoxides. a) 2-ethyl hexyl epoxide, b) NPG epoxide and c) TMP epoxide

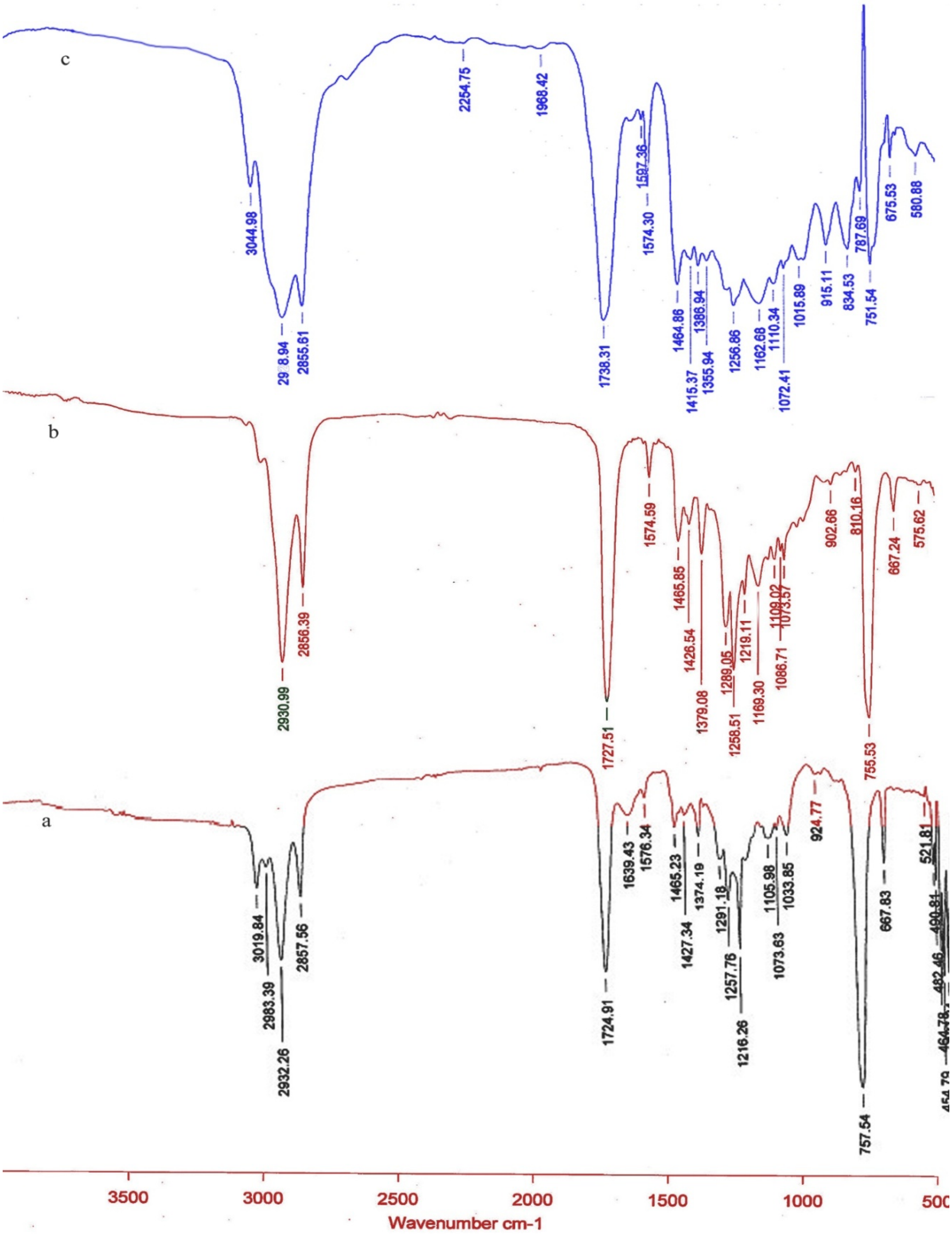


FIGURE 6. IR spectra of various epoxides. a) 2-ethyl hexyl epoxide, b) NPG epoxide and c) TMP epoxide

Table 1. Physico-chemical properties of various epoxides of undecylenic esters

Properties	Methods	2-ethyl hexyl epoxide*	NPG epoxide*	TMP epoxide*
Density (g/cc)	-	1.0351 ± 0.0004	1.0446 ± 0.0006	1.1606 ± 0.0006
Specific gravity	-	1.0351 ± 0.0004	1.0446 ± 0.0006	1.1606 ± 0.0006
Moisture	-	0.0025	0.0021	0.002
Viscosity (40°C) cSt	ASTM D445	10.08 ± 0.025	37.34 ± 0.32	260 ± 0.325
Viscosity (100°C) cSt	ASTM D445	2.98 ± 0.269	7.6 ± 0.16	39.3 ± 0.485
Viscosity index (VI)	ASTM D2270	169	177	204
Flash point (°C)	ASTM D92	164 ± 2	270 ± 6	308 ± 2.1
Fire point (°C)	ASTM D92	173 ± 2.2	285 ± 0.066	314 ± 0.0723
Cloud point (°C)	ASTM D2500	-3.9 ± 0.1	-1.8 ± 0.2	3 ± 0.152
Pour point (°C)	ASTM D97	-35 ± 4	-25 ± 1.96	-12.5 ± 1.75
Copper strip Corrosion	ASTM-D-130	1a	1a	1a

*Data correspond to the mean of three determinations plus or minus standard deviation. The values are expressed as mean ± standard deviation (n=3)

± 0.0006 g/cc, respectively. These synthesized lubricants were referred to as high-density lubricant oils because of their higher density compared to water. So water floats on those lubricants. High-density lubricants are better to control contamination, because of aiding in the suspension, transport and particulate contamination removal. These contamination partials are held in suspension longer to be removed by filtration (Fitch, 1992).

Kinematic viscosity. The kinematic viscosity of 2-ethylhexyl, NPG and TMP-based esters ranges from 1.89 ± 0.01 to 17.52 ± 0.02 cSt at 40 °C, which corresponds to low viscosity grade lubricants, which are useful for industrial applications. 2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide viscosities range from 10 ± 0.025 to 260 ± 0.325 cSt at 40 °C. The viscosities increased with increasing molecular weight, acyl and epoxy groups. The kinematic viscosity at 100 °C of 2-ethyl hexanol, NPG and TMP ester-based epoxides ranges from 2.98 ± 0.269 to 39.3 ± 0.485 cSt (Figure 7). Among all epoxides, the TMP epoxide shows better viscosity, which is suitable for

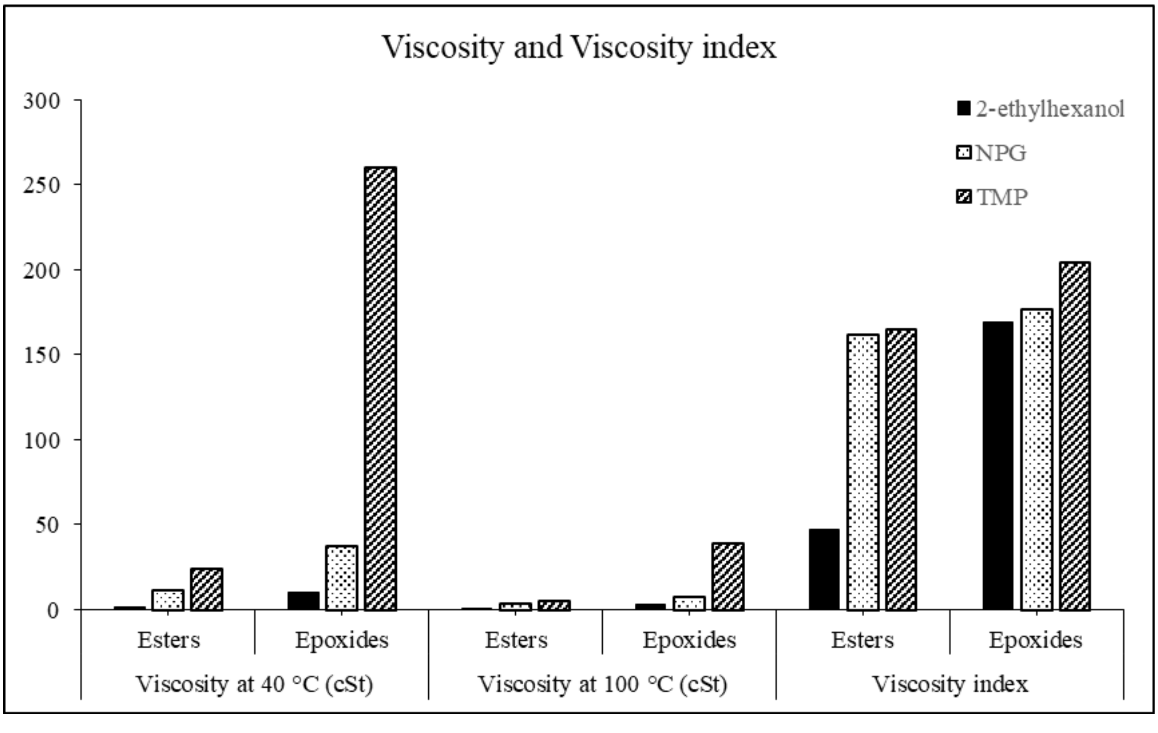


Figure 7. Viscosities of various esters and their epoxides.

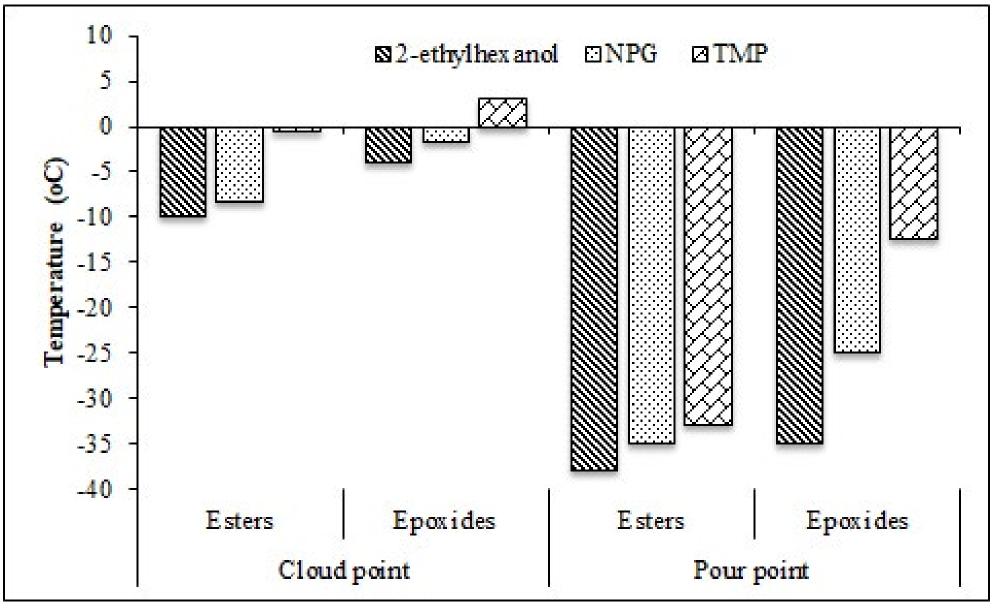


Figure 8. Cloud and pour points of various esters and epoxides.

various industrial applications such as an ISO VG 220 gear oil, metal working fluids and high-temperature applications.

Viscosity index. The viscosity indexes of 2-ethyl hexanol ester, NPG ester and TMP ester range from 47-210, indicating that they are suitable for multi-range applications. 2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide exhibit a viscosity index in the range of 126-204. However, all synthesized ester-based epoxides show viscosity indexes of more than 126, which is a better viscosity index than mineral-based oil.

Flash and fire point. The flash and fire points of lubricants are usually known for higher temperature properties, and are used to determine the volatility and fire-resistance required during storage and transportation. The flash points of 2-ethyl hexanol ester, NPG ester and TMP ester were 138 ± 0.82, 254 ± 0.82, 286 ± 0.82 °C. The fire points were 147 ± 0.96, 263 ± 0.96 and 294 ± 0.82 °C. The flashpoints of 2-ethyl hexanol ester-based epoxide, NPG ester-based epoxide and TMP ester-based epoxide were in the range of 164 ± 2 to 308 ± 2.1 °C. Fire points were 173 ± 2.2 to 314 ± 0.072 °C. All synthesized epoxides showed ≥164 °C flash and fire points, which indicates that they are within the range of standard lubricants. The flash and fire points were increased with increasing molecular weight, carbón chain length and acyl and epoxy groups. So, the synthesized epoxides were stable even at high temperatures and depicted a low tendency to evaporate (Figure 8).

Cloud and pour points. The cloud and pour point are imperative temperature properties at low temperatures for all lubricants. The cloud points of 2-ethyl hexanol ester, NPG ester and TMP ester were – 10 ± 0.19, -8.3 ± 0.10 and -0.6 ± 0.13 °C. The pour points were -38 ± 0.29, -35 ± 0.38, -33 ± 0.28 °C, respectively. The mono-branched alkyl ester exhibited a much lower pour point, which is suitable for many industrial applications. 2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide showed cloud points ranging from -3.9 ± 0.1 to 3.0 ± 0.152 °C and pour points of -35 ± 4 to -12.5 ± 1.75 °C. 2-ethyl hexyl ester-epoxide exhibited a lower pour point, due to its low molecular weight and to being less branched. On the other hand, pour points were increased with increasing molecular weights in NPG and TMP ester-based epoxides. The data reveal that all epoxides showed low pour points, suggesting acceptable lubricants (Figure 9).

Copper strip corrosion. Corrosiveness of 2-ethyl hexanol ester, NPG ester and TMP ester and corresponding epoxides were found to be **1a** and are less corrosive, indicating that esters and their epoxides are resistant to moisture, as these esters and epoxides were prepared from UDA, a derivative of castor oil, which has less moisture content and absence of non-toxic chemicals like sulfur.

Microbial activity. The microbial activity was tested by the agar well diffusion technique accord-

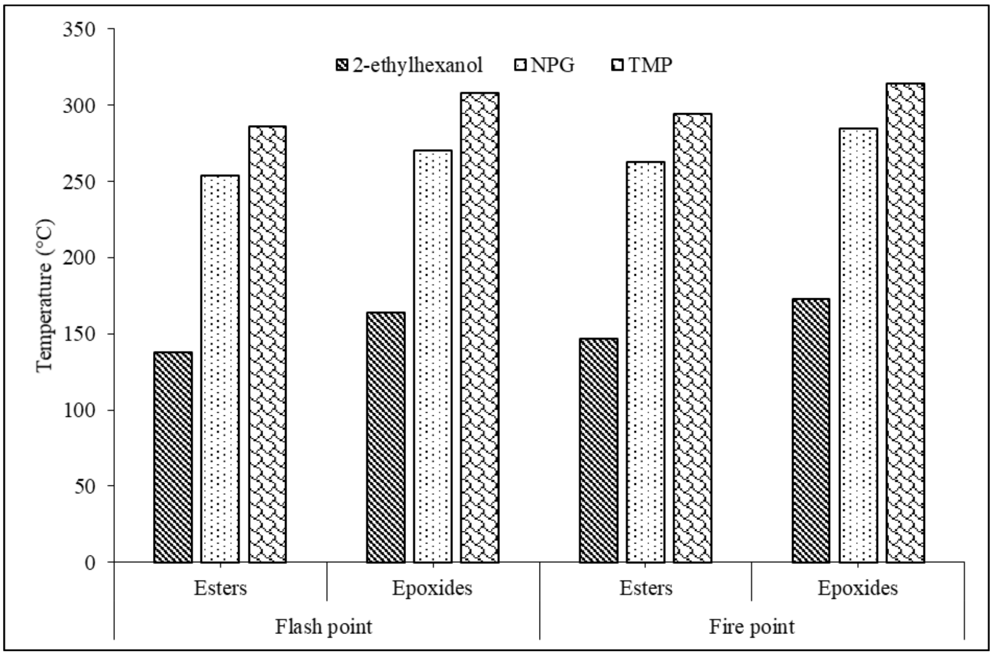
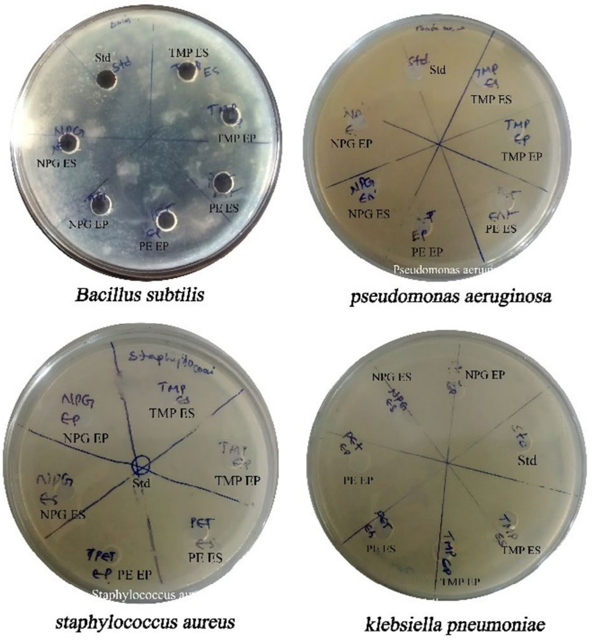


Figure 9. Flash and Fire points of various esters and epoxides. The values are expressed as mean ± standard deviation (n=3).

ing to the standard procedure. The 2-ethyl hexanol ester, NPG ester and TMP ester and corresponding epoxides were treated against two gram-positive bacterial strains: *bacillus subtilis, staphylococcus aureus* and two-gram negitive bacterial stains: *pseudomonas aeruginosa, klebsiella pneumoniae*. In this method, agar plates were inoculated with different strains of bacteria under restraining conditions and borewells (6 mm diameter) and filled with 150 µg/mL of 2-ethyl hexanol ester, NPG ester and TMP ester and corresponding epoxides in a dimethyl sulphoxide (DMSO) solution and incubated at 37 °C for 24 h. After the incubation period, the diameter of the growth inhibition zones was measured. The inhibition zones were reported in millimeters (mm). Vegetable oil was used as standard. All tests were performed in triplicate.



Bacillus subtilis ***pseudomonas aeruginosa***

staphylococcus aureus ***klebsiella pneumoniae***

Figure 10. Zone of inhibition of synthesized esters and their epoxides against bacterial stains such as *staphylococcus aureus, bacillus subtilis, Klebsiella pneumoniae, pseudomonas aeruginosa.*

The results of inhibition zone values for 2-ethyl hexanol ester, NPG ester and TMP ester and corresponding epoxides against bacterial strains such as *bacillus subtilis, staphylococcus aureus*, *pseudomonas aeruginosa, Klebsiella pneumoniae* are shown in Figure 10. According to Figure 10, all 2-ethyl hexanol ester, NPG ester and TMP ester and their corresponding epoxides did not show a zone of inhibition in gram-positive and gram-negative bacterial strains. This strongly suggests that the synthesized 2-ethyl hexanol ester, NPG ester and TMP ester and their corresponding epoxides are non-toxic.

The synthesized epoxides showed an excessive viscosity index, flash point and lower volatility than the respective esters (Table 2). Among all the three synthesized epoxides, TMP epoxide showed

Table 2. Physico-chemical properties of various UDA-based esters

Properties	2-ethyl hexyl ester*	NPG Esters*	TMP ester*
Density (g/cc)	0.9051 ± 0.0002	0.9283 ± 0.0001	1.0046 ± 0.0001
Viscosity (40°C) cSt	1.89 ± 0.01	10.51 ± 0.02	17.52 ± 0.02
Viscosity (100°C) cSt	0.89 ± 0.01	3.31 ± 0.02	4.71 ± 0.02
Viscosity index	47.08	212.89	207.619
Flash point (°C)	138 ± 0.82	254 ± 0.82	286 ± 0.82
Fire point (°C)	147.75 ± 0.96	263.75 ± 0.96	294 ± 0.82
Cloud point (°C)	-10.78 ± 0.19	-8.43 ± 0.10	-0.65 ± 0.13
Pour point (°C)	-38.50 ± 0.29	-35.53 ± 0.38	-33.48 ± 0.28
Copper strip Corrosion	1a	1a	1a

*Data correspond to the mean of three determinations plus or minus standard deviation. The values are expressed as mean ± standard deviation (n=3)

a higher viscosity index, flash point, and lower pour point compared to 2-ethyl hexyl and NPG epoxides. TMP ester-based epoxide has a higher VI compared to various epoxidized vegetable oils such as castor methyl ester (Venubabu and Goud, 2014), rapeseed oil (Wu *et al.,* 2000), Karanja oil (Geethanjali *et al.,* 2013) and HP Lube parthan SL220 (Table 3). Epoxides, thus synthesized, can be a potential base-stock for lubricants. The standard lubricants widely used in the market for machinery are various ISO VG grades. The 2-ethyl hexanol epoxide, NPG epoxide and synthesized TMP epoxide are comparable to standard ISO VG grades.

Kinematic viscosity at 40 °C for NPG epoxide is 37.34 cSt. It is in the range of ISO VG 32 and 46 grades. Standard ISO VG 32 and 46 grades are widely used machinery lubricating oil and turbine oil. Kinematic viscosity at 40 °C for TMP epoxide is 260 cSt, which is in the range of standard ISO VG 220 and 320 grades. The Standard ISO VG 220 and 320 grades are widely used in machinery lubricating oil, bearing oils for steel plants and industrial gear oils.

The synthesized 2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide can replace the existing mineral-based lubricating oils of ISO VG 32-46, ISO VG 220-320 grades in the market for machinery, turbine and industrial gear oil applications. The synthesized 2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide is from a renewable source and is biodegradable without polluting the environment. These are recommended for all industrial machinery and other machine parts lubricated by a thin film of oil for anti-friction bearings, plain bearings, drive gears and the pinions of steel mills, where operating conditions are moderate.

4. CONCLUSIONS

The study involved a new class of epoxides synthesized from undecylenic esters as a bio lubricant base-stock. Undecylenic esters of 2-ethyl hexanol, NPG and TMP were epoxidized using mCPBA. The yields of 2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide were in the range of 70-75%.

Table 3. Physico-chemical properties of various vegetable epoxides and HP Lube parthan SL220

Properties	**Castor methyl ester**[a]	**Rapeseed oil**[b]	**Karanja oil**[c]	**HP Lube parthan SL220**[@]
Viscosity at 40°C (cSt)	35.81	86.74	256	241.9
Viscosity at 100°C (cSt)	-	12.72	28	31.34
Viscosity index	-	145	-	173
Pour point(°C)	8	-12	3	-45
Flash point(°C)	138	239	-	250
Copper strip Corrosion	1[a]	1a	1a	1a

[a]Venu babu *et al.,* 2014, [b]Wu *et al,* 2000, [c]Geethanjali *et al,* 2013, [@]ISO Grade 220.

TMP epoxide showed higher viscosity, higher viscosity indices, higher cloud point, lower pour point, higher flash point, and higher fire point than 2-ethyl hexyl and NPG epoxide. 2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide have superior lubricant properties compared to their corresponding esters.

2-ethyl hexanol epoxide, NPG epoxide and TMP epoxide with high viscosity indices, higher flash point, and lower pour points can be exploited for automotive and hydraulic fluid formulations. Undecylenic esters and their epoxides can be used for numerous applications due to their non-toxicity and biodegradability.

5. ACKNOWLEDGMENTS

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