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Research Article

Ultrasound-assisted Extraction and Characterization of Mexican *Moringa oleifera* Seed Oil: Identification of Tocopherols and Phytosterols Using UPLC-APCI+MS/MS

Ivet Gallegos-Marín[1], María L Juárez-Rafael[2], Blanca D Vázquez-Cabral[3], Erasmo Herman-Lara[2], Betsabé Hernández-Santos[2], Jesús Rodríguez-Miranda[2], Alberto Álvarez-Castillo[4], Susana Santoyo[5] and Cecilia E Martínez-Sánchez[2]*

[1]Conacyt-Tecnológico Nacional De México/Instituto Tecnológico De Tuxtepec, Calz, Dr. Víctor Bravo Ahuja Col. Predio el Paraíso, Tuxtepec, Oax, México

[2]Tecnológico Nacional De México/Instituto Tecnológico De Tuxtepec, Av. Dr. Víctor Bravo Ahuja s/n, De Mayo, Tuxtepec, Oax, México

[3]Tecnológico Nacional De México/Instituto Tecnológico De Durango, Department De Ing, Química y Bioquímica, Blvd, Felipe Pescador, Durango, Dgo, México

[4]Tecnológico Nacional De México/Instituto Tecnológico De Zacatepec, Tecnológico, Plan De Ayala, Zacatepec De Hidalgo, México

[5]Instituto De Investigación En Ciencias De la Alimentación CIAL (CSIC-UAM), CEI UAM + CSIC, C/Nicolás Cabrera 9, Universidad Autónoma De Madrid, Madrid, Spain

***Corresponding Author:** Cecilia Eugenia Martínez Sánchez, Tecnológico Nacional De México/Instituto Tecnológico De Tuxtepec, Av. Dr. Víctor Bravo Ahuja s/n, De Mayo, Tuxtepec, Oax, México.

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Abstract

Ultrasound-assisted extraction (UAE) from Mexican *Moringa oleifera* seed oil, characterization, and identification of tocopherols and phytosterols using UPLC-APCI+MS/MS were investigated. The extraction process variables such as sample/solvent ratio (6 - 20 g/mL), temperature (40 - 60°C) and ultrasound time (10 - 20 min) were evaluated on yield, extraction efficiency, peroxide index, p-anisidine, Totox value and free fatty acids. Optimization was carried out by Response Surface Methodology (RSM). The three optimal treatments (A, B and C) were determined by tocopherols and phytosterols content using UPLC-APCI+MS/MS. Antioxidant capacity was also evaluated. Alpha-tocopherol was found in a greater proportion in all three treatments and β-sitosterol was the predominant phytosterol in A and B. Treatment A presented a greater antioxidant capacity. Optimal conditions for UAE were sample/solvent ratio of 1:17.16 (w/v), 50.99 °C and extraction time of 25.95 min.

Keywords: *Moringa Oleifera;* Seed Oils; Tocopherols; Phytosterols; Ultrasound Assisted Extraction

Introduction

Evaluation and applications of plant-derived bioactive compounds are increasing over the past several years mainly as nutraceutical and functional foods for promoting human health. One of the characteristics of edible vegetable oils is their high content of essential fatty acids, however they also contain micronutrient such as tocopherols and phytosterols [1]. Tocopherols, in their different forms (α, β, δ, γ) are precursors of vitamin E (tocopheryl acetate), fundamentally the α-tocopherol form. Its function as lipid oxidation inhibitors in biological systems and in foods is widely known.

Tocopherols exert the antioxidant effect by numerous biophysical and biochemical mechanisms, including the clearance of active oxygen species and free radicals and destruction of the lipid self-oxidation chain. On the other hand, tocopherols have function as inhibitors of cholesterol synthesis [2]. Phytosterols are structurally cholesterol-like molecules and it has been shown that their intake gives a series of benefits to the body like reducing intestinal cholesterol absorption decreasing the concentrations of total cholesterol and LDL cholesterol and potential contributions to prevention of cardiovascular diseases. They also have properties like immunomodulators, anti-inflammatory, antitumor, bactericidal and fungicidal. A daily intake of 2g is recommended.

Moringa oleifera, known as Moringa or Indian tree grows in Asia, South America, Africa and the Caribbean. Moringa belongs to the *Moringaceae* family. Almost all parts of moringa, such as the flower, fruits, leaves, and roots are edible and have been consumed as a vegetable [1]. *M. oleifera* seeds contain 19-47% oil, which is commercially known as Ben oil, and seeds and oil are rich in palmitic, stearic, behenic and oleic acids [2,3]. Ben oil is reported to have a very high level of oleic acid (70%). The oil consists of various sterols, such as campesterol, stigmasterol, clerosterol, β-sitosterol, δ^5-avenasterol, and a lower quantity of 24-methylenecholesterol, campestanol, stigmastanol and 28-isoavenasterol [4]. For this reason, moringa seeds and oil are used in the treatment of arthritis, rheumatism, and hypertension [5].

Oil extraction from *Moringa oleifera* seeds is performed using various techniques, such as solvent extraction, enzyme-assisted aqueous extraction, and supercritical carbon dioxide extraction (SC-CO_2) among others [6,7]. The Soxhlet technique, a conventional solvent extraction method, is widely used to extract oil from *Moringa oleifera* seeds for characterizing the physicochemical properties [8]. However, the application of the Soxhlet technique for the large-scale production of moringa oil is limited due to the long extraction time required (>7 h), possible degradation of labile compounds and limited solvent choice [6,9]. Currently, there are several extraction techniques reported by different scientists in which the advantages and disadvantages of each are discussed [10]. In the ultrasound-assisted extraction (UAE), the ultrasound waves accelerate mass transfer during the process, resulting in the release of proteins and secondary metabolites from the parenchyma cell wall via cavitation, and this effect favors the release of oil from the cells that contain it [11]. Therefore, the objective of this investigation was the UAE of oil from Mexican *Moringa oleifera* seed and characterization, and identification of tocopherols and phytosterols using UPLC-APCI+MS/MS, and optimization of the oil extraction process seeking to obtain the highest yield and oxidative stability as well as the preservation of bioactive compounds found in moringa oil.

Materials and Methods

Materials

Moringa oleifera seeds were acquired in local market of San Juan Bautista Tuxtepec, Oaxaca State, Mexico. All standards of HPLC-grade were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Other reagents used were from J. T. Baker (México City, México).

Extraction of moringa seed oil by ultrasound-assisted solvent extraction

Seed conditioning and oil extraction were performed as described below. The seeds were selected manually, peeled and dried in an oven at 60 °C until the moisture level was constant (1.10% w/w) and reduced to particle size of 0.59 mm. An ultrasonic bath (Elmasonic P 30/80 Khz. Singen, Germany) with a frequency of 80 kHz and power of 100% was used, with 20 g of moringa seed flour used for each treatment and hexane as solvent extraction. The extraction conditions were sample/solvent ratio (6 - 20 g/mL), temperature (40-60 °C) and time (10 - 20 min). After the extraction period, the samples obtained were filtered under vacuum and the oil was recovered in a rotary evaporator (Büchi Rotavapor™ R-100, Thermo Fisher Scientific, USA). Oils recovered were stored in amber bottles under a nitrogen atmosphere at 4 °C until analysis.

Yield and efficiency of the extraction of oil

The yield and efficiency of the extraction were calculated with equations 1 and 2

$$Yield(g/kg) = \frac{extracted\ oil\ (g)}{sample-extracted\ oil} x1000 \text{------}(1)$$

$$Efficiency\ (g/kg) = \frac{yield\ (g)}{total\ lipid\ content} x1000 \text{ ----------}(2)$$

Oxidative stability

Oxidative stability parameters were calculated according to [12]. Peroxide index (PV) (Method Cd 8-53), free fatty acids (FFA) (Method Aa 4-38), p-anisidine (p-A) (Method Cd 18-90) and the TOTOX value (VT) (CG 3-91) was calculated according to the following equation:

TV=*p-A*+ 2PI ----------(3)

Characterization of the optimal products

Identification of tocopherols and phytosterols Oil (100 µL) was added to 900 µL of ethyl acetate, stirred for 1 min, and then filtered on a 0.45-µm membrane and collected in an amber vial to be analyzed later by LC-APC$^+$MS. The analysis of tocopherols and phytosterols was performed according to the method described by [13] with some modifications. The LC equipment was coupled to a mass spectrometer equipped with an APCI interface (in positive ion mode) using the following parameters: a dry gas (nitrogen), flow rate of 0.12 mL/min, a nebulizer gas pressure (bar) at 6 psi, and high-pressure limit of 15,000 psi. A corona discharge of the order 1.0-6.1 kV was applied, and the temperature of the APCI interface was 300 °C. The analytes were separated with an elution phase of water/formic acid (A) and acetonitrile (B) at a flow rate of 0.11 to 0.12 mL/min. The source temperature and solvation temperatures were 150 and 250°C, respectively, with a cone gas flow of 150 L/h. The equipment was programmed with the following conditions: the Acquity column UPLCr BEH ShieldRP C18 (2.1 x 150 x 1.7 µm) temperature was set at 40°C, the voltage was between 30.0-99.19 V and the injection volume was 0.2 µL. The identification of the analytes was based on the comparison of their retention times and their mass spectra with respect to the standards.

Determination of antioxidant activity oxygen radical absorption capacity

Antioxidant capacity determination in the oil was carried out by means of the oxygen radical absorption capacity (ORAC) technique and for this, the extraction of the compounds was first performed by means of two extraction techniques, one in liquid phase (LPE) and another in solid phase (SPE). The first extraction technique (LPE) was performed according to the methodology described by [14]. The second extraction method (SPE) was performed according to the technique described by [15], with some modifications. Cartridges for solid phase extraction with columns packed with Diol (Diol SPE tube-500 mg, 50 µm, pore diameter 70 Å, Supelco, Bellefonte, PA) were pre-conditioned by the addition of 5 mL of methanol and subsequently 5 mL of n-hexane. Then the sample was added, (12 mL of oil dissolved in 12 mL of hexane). Subsequently, the cartridges were washed with 7.5 mL of n-hexane and the sample was eluted with methanol (20 mL). The eluents were evaporated under vacuum in the Centrivap equipment (Labconco console Lab) at a temperature of 30 ° C. Then, the dry residue was dissolved in 2 mL of methanol.

For the determination of radical oxygen absorbance capacity ORAC), a 20 µL of sample was added in a 96-well microplate for fluorescence and then 200 µL of 0.108 µM fluorescein was added; the microplate was incubated at 37 °C for 15 min and subsequently three fluorescence readings were made on a Sinergy HT spectrophotometer at an excitation and emission wavelength of 485/20 and 528/20 µm, respectively; then, 75 µL of the 2,2'-azobis (2-amidinopropane) 79.65 mM radical was added and kinetics were performed for 2 h at 37°C. A standard TROLOX® curve with concentrations of 5, 10, 25, 35 and 50 µM was used; the results obtained were expressed as µM equivalent of TROLOX® (µMET).

Experimental design and data analysis

A central design composed was performed to determine the optimal conditions of the ultrasound-assisted extraction of moringa seed oil. The experimental design consisted of 20 treatments. The sample/solvent ratio (X_1), extraction temperature (X_2) and extraction time (X_3) were chosen as the independent variables (Table 1). Oil yield extracted and some oxidative stability parameters (IP, p-A value, FFA and VT) were selected as the response variables. The actual and coded levels of the independent variables are given in table 1. The experimental runs were randomized, and the experimental data were analyzed with the Response Surface Methodology (RSM) using the Design Expert statistical package (Design Expert 8.0.2, Stat-Ease Inc., Minneapolis, USA).

The analysis of variance (ANOVA), the mean values were considered significantly different when $p < 0.05$, regression analysis and plotting of response surface plots, allowed establishing the optimal conditions for oil extraction. Statistical analysis was performed by using Design Expert program (10.0. version).

Results and Discussions

Effect of independent variables on oil yield and oxidative stability

Table 1 shows the extraction yield in the different treatments, which was found in a range of 389.46 to 438.69 g/kg, with treatments 3, 8 and 10 having the highest yield of extraction. The conditions where a higher extraction yield was obtained were having a sample/solvent ratio of 20 and 17.6g of sample per mL of the solvent (hexane), temperature of 50 and 55.95 °C and time of 14.05, 20 and 25.95 min.

The correlation coefficient of the experimental data was 0.97 for yield extraction, which indicates that the model had a signif-

icant effect ($p < 0.05$) and that it adequately represents the real relationship between the parameters studied. According to the statistical analysis, the oil yield correlates positively with the sample/solvent ratio and temperature in linear terms (Figure 1a); for quadratic terms, the proportion was significant ($p < 0.05$).

Treat Ments	Proportion (g/mL) (X_1)	Temperature (°C) (X_2)	Time (min) (X_3)	Yield (g/kg)	Eficiency (g/kg)	PI (meq O_2/kg)	p-A	FFA (% oleic acid)	Totox value
1	8.84 (-1)	44.05 (-1)	14.05 (-1)	404.84 ± 0.42	922.81 ± 0.95	1.13 ± 0.00	0.88 ± 0.01	0.96 ± 0.00	2.16 ± 0.01
2	17.16 (1)	44.05 (-1)	14.05 (-1)	429.00 ± 0.34	977.90 ± 0.77	1.13 ± 0.00	3.43 ± 0.00	0.97 ± 0.00	4.71 ± 0.00
3	8.84 (-1)	55.95 (1)	14.05 (-1)	414.95 ± 0.06	945.86 ± 0.13	2.26 ± 0.00	5.14 ± 0.00	1.04 ± 0.00	10.23 ±0.00
4	17.16 (1)	55.95 (1)	14.05 (-1)	438.66 ± 0.03	999.91 ± 0.08	2.25 ± 0.00	3.42 ± 0.01	1.21 ± 0.00	8.50 ± 0.01
5	8.84 (-1)	44.05 (-1)	25.95 (1)	410.23 ± 0.42	935.10 ± 0.96	1.13 ± 0.00	3.25 ± 0.00	0.89 ± 0.00	4.52 ± 0.00
6	17.16 (1)	44.05 (-1)	25.95 (1)	430.24 ± 0.34	980.72 ± 0.78	1.13 ± 0.00	3.70 ± 0.03	1.05 ± 0.00	4.97 ± 0.03
7	8.84 (-1)	55.95 (1)	25.95 (1)	417.56 ± 0.31	951.81 ± 0.71	2.26 ± 0.00	0.43 ± 0.00	1.13 ± 0.00	5.52 ± 0.01
8	17.16 (1)	55.95 (1)	25.95 (1)	438.69 ± 0.01	999.97 ± 0.02	2.26 ± 0.00	1.61 ± 0.00	0.96 ± 0.00	6.70 ± 0.00
9	6 (-1.68)	50 (0)	20 (0)	389.46 ± 0.31	887.76 ± 0.70	1.13 ± 0.00	1.55 ± 0.02	0.97 ± 0.00	2.82 ± 0.02
10	20 (1.68)	50 (0)	20 (0)	438.69 ± 0.00	999.97 ± 0.01	1.13 ± 0.00	1.41 ± 0.00	0.96 ± 0.00	2.68 ± 0.00
11	13 (0)	40 (-1.68)	20 (0)	417.61 ± 0.54	951.93 ± 1.22	1.13 ± 0.00	3.54 ± 0.01	0.96 ± 0.00	4.82 ± 0.01
12	13 (0)	60 (1.68)	20 (0)	427.65 ± 0.24	974.81 ± 0.54	2.25 ± 0.00	3.99 ± 0.01	1.13 ± 0.00	9.07 ± 0.01
13	13 (0)	50 (0)	10 (-1.68)	419.91 ± 0.12	957.16 ± 0.28	1.13 ± 0.00	4.00 ± 0.01	0.97 ± 0.00	5.28 ± 0.00
14	13 (0)	50 (0)	30 (1.68)	428.06 ± 0.25	975.74 ± 0.56	1.13 ± 0.00	3.61 ± 0.01	0.88 ± 0.00	4.88 ± 0.01
15	13 (0)	50 (0)	20 (0)	426.55 ± 0.09	972.31 ± 0.20	1.13 ± 0.00	3.33 ± 0.01	1.13 ± 0.00	4.60 ± 0.01
16	13 (0)	50 (0)	20 (0)	426.34 ± 0.02	971.83 ± 0.04	1.13 ± 0.00	2.71 ± 0.01	1.13 ± 0.00	3.98 ± 0.01
17	13 (0)	50 (0)	20 (0)	426.56 ± 0.07	972.32 ± 0.16	1.13 ± 0.00	3.32 ± 0.00	1.13 ± 0.00	4.59 ± 0.00
18	13 (0)	50 (0)	20 (0)	426.55 ± 0.18	972.29 ± 0.40	1.13 ± 0.00	2.78 ± 0.01	1.13 ± 0.00	4.05 ± 0.01
19	13 (0)	50 (0)	20 (0)	426.48 ± 0.09	972.15 ± 0.20	1.13 ± 0.00	3.00 ± 0.00	1.13 ± 0.00	4.27 ± 0.00
20	13 (0)	50 (0)	20 (0)	426.21 ± 0.14	971.53 ± 0.32	1.13 ± 0.00	3.33 ± 0.01	1.13 ± 0.00	3.87 ± 0.01

Table 1: Experimental design of the independent variables and response of the dependent variables according to the central design composed in the extraction of moringa seed oil.

Values in parentheses are the coded values of the independent variables. PI= Peroxide Index; p-AI= p-anisidine Index; FFA= Free Fatty Acid; TV= Totox Value. The results are the average of 3 determinations ± standard deviation.

Figure 1: Effect of independent variables on a) Extraction Yield, b) Efficiency, c) Peroxide value, d) p-anisidine value, e) FFA, f) Totox value.

In figure 1a, increasing the sample/solvent ratio increases the oil yield. This might be due to that raising the sample/solvent ratio, which leads to a heightened mass transfer, thereby increasing the oil yield. Nevertheless, the phenomena stop as the solvent saturates with the extracted oil making the concentration gradient zero. The current study results of the effect of sample on oil yield agreed with previous findings [16].

At elevated temperature, the oil yield increases, which may be related to a greater solubility of the oil in the solvent. As the temperature increases from 50 to 55 °C, the oil yield gradually increases and reaches its maximum value of 438.69 g/kg. An additional increase in temperature beyond 56 °C causes a decrease in response. These two phenomena can be considered as an effect of diffusion of oil in the solvent [17] In addition, at elevated temperatures, the extraction process can affect bioactive compounds such as the content of tocopherols that are heat sensitive compounds. Therefore, the increase in proportions and temperature increase the oil yield. The solvent improved the diffusion in the walls of the vegetable and reduced the viscosity of the oil, allowing the greater amount of oil present in the sieved flour to flow; further, increasing the temperature and time facilitated the transfer of oil because the temperature softens the vegetable tissues and improves diffusion, thus promoting the oil extraction (Figure 2a and 2b) [18].

Figure 2: Moringa seed meal micrographs before (a) and after UAE (b).

Table 1 shows the results of the oil extraction efficiency of moringa seeds, which varied from 887.76 to 999.97 g/kg with an average of 964.69 g/kg, in the 20 treatments. Treatments 8 and 10 presented a greater efficiency. Table 2 shows the regression model coefficients, the extraction temperature coefficients and the ultrasound time showed a first order behavior,and the sample/solvent ratio variable was of second order. Both were significant ($p < 0.05$), while the interaction between the variables showed no statistical significance. This means that increasing sample/solvent ratio and ultrasound temperature contributed to the extraction efficiency (Figure 1b). The relationship of the concentration of the solvent with the sample had a preponderant effect on the extraction efficiency, since the solvent can absorb and transmit ultrasound energy in cell tissues and accelerate the extraction, thus reducing the time [11].

Figure 1b indicates that predicted values (showed in the plot) were close to the real values (represented as darker squares), the determination coefficient ($R^2 = 0.97$) indicates that the model had a significant effect ($p < 0.05$) and that adequately represents the real relationship between the parameters studied.

Peroxide index values varied from 1.13 to 2.26 meq O_2/kg of oil, with treatments T3, T4, T7, T8 and T12 showing high values; this variation was dependent on the extraction conditions. However, these results indicated that the oil is stable to the different ultrasound extraction conditions; these samples have relatively low values compared to those allowed by [12] which authorizes a maximum value of 20 meq O_2/kg in vegetable oils. The temperature had a significant effect ($p < 0.05$) on the linear and quadratic terms on the PI response, while the proportion and the ultrasound time were only significant ($p < 0.05$) for the quadratic term. The interactions of the variables had no significant effect ($p < 0.05$).

Correlation coefficient of the model adjusted to the experimental data was $R^2 = 0.87$, as shown in table 2. The temperature had a significant effect ($p < 0.05$) in the linear and quadratic terms on the PI response, while the proportion and time were only significant ($p < 0.05$) for the quadratic term. Interaction's variables had no significant effect ($p < 0.05$).

However, the extraction temperature is the main variable that had a positive correlation with negative effects, as shown in figure 1c, as the extraction temperature increases the formation of peroxides and hydroperoxides is also increased, that is, the peroxide index. This behavior is probably since at high temperatures, mono and polyunsaturated fatty acids degrade, since they are susceptible to oxidation due to the presence of double bonds, and as is known, peroxides and hydroperoxides are very unstable compounds easily decomposing into secondary oxidation products, which results in a lower peroxide IP. This decomposition is carried out in the middle and final stages of lipid oxidation [19]. The p-anisidine content of the moringa seed oil varied from 0.43 to 5.14 (Table 1). The correlation coefficient was $R^2 = 0.83$. The results revealed that the temperature and time showed a significant effect ($p < 0.05$) in the linear term, and the interaction of the temperature and ultrasound time variables showed a negative correlation (Fig 1d) with a significant effect ($p < 0.05$); the same behavior was observed in the proportion in quadratic terms. However, the extraction temperature and ultrasound time had a positive correlation, which negatively affect ($p < 0.05$) p-anisidine values, thus indicating that as the time is prolonged the value of *p*-anisidine increases. This event probably occurs because as the time increases, the temperature of the medium increases; this increase in temperature can cause the deterioration of the polyunsaturated fatty acids, whose double bonds are more susceptible to oxidation by thermal effects [20]. This effect has been reported in other studies in which an increase in ultrasound time promotes the oxidation of conjugated double bonds [21].

Regarding the content of free fatty acids table 2 shows that the coefficient of determination was $R^2 = 0.76$, indicating that the model had a significant effect ($P < 0.05$). In table 2 and figure 1e, it was observed that the temperature and ultrasonic time had a significantly negative effect ($P < 0.05$) in linear terms, as the temperature and time increased, the content of free fatty acids increased. Respecting to the proportion and time of extraction manifesting a negative correlation with a significant positive effect ($P < 0.05$) in

the quadratic term. The temperature and time promote the activity of the lipases or other enzymes present in the moringa seed flour that could hydrolyze the triacylglycerides to free fatty acids, diglycerides and monoglycerides, and this hydrolysis could be favored by the light and the ultrasound temperature [21].

Free fatty acid oil content varied from 0.88 to 1.13% (Table 1) under the different conditions of extraction by ultrasound. Treatments 1, 2, 3, 8-11, 13 and 14 are within the parameters established by [12] establishing a maximum of 1%. The rest of the treatments presented values higher than 1%. The VT in the oil of the moringa seeds depended on the different extraction conditions used and ranged from 2.16 to 10.23; T10 (Table 1).

Table 2 shows the correlation coefficient that was R = 0.90, demonstrating that the model had a significant effect ($p < 0.05$), the significant effects ($p < 0.05$) of the independent variables in the VT were also observed being the temperature and time that negatively affected in linear and quadratic terms, with respect to the proportion and significant negative effect in the quadratic term. Also, the interactions of temperature and time in the quadratic term had an effect showing a negative correlation. The behavior of these results can be seen in figure 1e, which show a significant effect ($p < 0.05$) of the interaction of the variables with respect to the TV. Because the TOTOX value is associated with both the p-anisidine and peroxide index, this value is also affected by time and temperature as mentioned above at higher time and extraction temperature, the oxidation of fatty acids and therefore the formation of oxidation compounds.

Optimization and validation of the extraction process

The numerical optimization was carried out based on the experimental results and the statistical analysis, with the objective of maximizing the extraction yield and efficiency as well as minimum values of Index of peroxides, *p*-anisidine, Totox value and free fatty acids (oxidative stability), in ultrasound extraction. In this study, 15 optimal conditions were obtained, of which 3 (A, B and C) were chosen based on the most desirable, as shown in table 3. Experimental values higher in yield and efficiency of extraction than in the values predicted by the model can be observed (Table 3). Of the three treatments selected, the best treatment was A. The extraction conditions were as follows: sample/hexane ratio of 1:17.16 (p/v), 50.99°C and extraction time of 25.95 min with a desirability of 0.8.

Parameters	Yield	Efficiency	PI	p-A	FFA	Totox value
Intercept	241.69	550.93	18.32	-26.32	-2.93	33.19
X_1	9.10	20.74	-0.12	1.73	0.13	1.34
X_2	2.811	6.41	0.70	0.24	0.08	2.14
X_3	1.66	3.79	-0.09	1.24	0.10	0.94
$X_1 X_2$	0.00	0.01	0.00	-0.02	-0.00	-0.09
$X_1 X_3$	-0.03	-0.08	0.00	0.00	-0.00	0.00
$X_2 X_3$	-0.01	-0.03	0.00	-0.03	-0.00	-0.03
X_1^2	-0.21	-0.49	0.00	-0.03	-0.00	-0.10
X_2^2	-0.10	-0.04	0.01	0.01	0.00	0.03
X_2^3	-0.01	-0.01	0.00	0.01	-0.00	0.01
R^2	0.97	0.97	0.87	0.83	0.76	0.90
F	33.90	33.9	7.75	5.35	3.61	10.37
P > F	0.00	0.00	0.00	0.01	0.03	0.00

Table 2: Regression coefficients of the independent variables of the central composite model.

* The highlighted values are statistically significant ($P < 0.05$); X_1= Proportion; X_2= Temperature; X_3= Time. PI= Peroxide Index; p-AI= p-anisidine Index; FFA= Free Fatty Acid; TV= Totox Value.

Identification and quantification of tocopherols

Tocopherol content in moringa seed oil is of the 3 optimal treatments (A, B and C) presented in table 4. No significant differences were found in the three treatments ($p > 0.05$), which indicate that the different extraction conditions had no effect on the tocopherol content. The tocopherol that was found in greater proportion in all the treatments was α-tocopherol (72%), followed by γ-tocopherol (26%) and δ-tocopherol (2%), which was consistent with other investigations carried out in the seed oil of moringa in which the predominant tocopherol was α-tocopherol [7,8]. The average of tocopherols content was higher than data reported in other investigations for moringa seed oil, as the reported values of tocopherols oscillated between 238-248 depending on the extraction method used for obtaining the oil [8].

α-tocopherol content in moringa seed oil was higher than the values reported by other researchers [12], who found values of α-tocopherol of 5.06 and 15.38 mg/kg in *Moringa oleifera* seed oil extracted under cold pressure and hexane. Similarly, the presence of α-tocopherol in quantities of 95.85-103.80 mg/kg and 230.3 mg/kg has been found using the Soxhlet oil extraction method

and supercritical fluids, respectively [8,9]. These variations of the α-tocopherol content at the values found in this work are probably due to the composition of the plant material, which in turn depends on the climatic conditions and geographical conditions as well as the efficiency of the ultrasound-assisted extraction method, as this extraction method allows the cells of the plant tissue to be broken. Because this extraction method decreases the viscosity and increases the diffusivity of the solvent, it leads to the release of the analytes from the matrix and, therefore, allows a greater extraction of the secondary metabolites.

Identification and quantification of phytosterols

The results obtained indicate that the highest concentration of phytosterols was observed in A (4749.54 mg/kg), followed by B (4350.97 mg/kg) and C (3908.17 mg/kg), which indicates that the temperature and extraction time of oil interfered in the entrainment of these analytes (Table 4). This behavior can be due to the increase in the extraction time, since increasing the temperature of the medium in turn induces the vegetable tissues to soften and improves diffusion, thus promoting the extraction of phytosterols in the oil.

Phytosterol content distribution was similar in treatments A and B. β-Sitosterol was found in the greatest proportion, followed by ergosterol, campesterol and stigmasterol; in B the compound found in the greatest proportion was ergosterol, followed by campesterol, β-sitosterol and stigmasterol, which was possibly due to the extraction conditions. The content of ergosterol, campesterol and stigmasterol in the three treatments did not present a significant difference ($p > 0.05$). However, β-sitosterol content showed a significant difference between the three treatments; the highest content of β-sitosterol. B treatment presented the lowest β-sitosterol value of the three. These differences in concentration indicate that oil extraction conditions of treatment A are the best for obtaining a greater amount of β-sitosterol as well as the other phytosterols.

Treatments				D	Yield		Efficiency		Peroxide Index (meq O_2/g)		-anisidine index		Free fatty acids (%)		Totox value	
	Proportion (g/mL)	Temperature (°C)	Time (min)		P	E	P	E	P	E	P	E	P	E	P	E
1	1:17.16	50.99	25.95	0.80	436.41	504.02	994.77	995.30	1.36	1.79	2.30	3.30	0.99	1.60	4.34	6.42
2	1:17.16	44.75	14.09	0.77	429.75	499.49	979.60	986.36	1.07	1.12	2.57	2.80	1.01	2.00	3.65	5.09
3	1:16.58	53.73	25.95	0.76	437.06	497.87	996.25	983.16	1.65	2.24	1.80	3.10	1.01	1.98	4.85	7.59

Table 3: Optimization and validation of extraction conditions according to the central composite design.

D= Desirability; P= Predicted Data; E= Experimental Data.

[7] detected the presence of β-sitosterol (2,310.9 mg/kg), campesterol (1,179.2 mg/kg), Δ5-avenasterol (782.5 mg/kg) and stigmasterol (591.5 mg/kg) in fractions of sterols of *Moringa oleifera* seed oil; the first two values of phytosterols are higher compared to this work, and it is important to emphasize that they did not find the presence of ergosterol but that of Δ5-avenasterol. However, the presence of stigmasterol and β-sitosterol has been established in *Moringa oleifera* oil obtained by subcritical fluid extraction (SFE) with CO_2 [23]. These differences in the concentrations of phytosterols in the oil and the type of isomer present are probably due to the method and conditions of extraction of the oil because these parameters significantly impact the yield of the lipid material and, consequently, the phytosterol content. However, the climatic and agronomic condition of the fruit and tree may contribute to these differences, and they are also involved in the response of plants to stresses due to low and high temperature, drought, high salt concentration and pathogen attacks [24]. This fact would be related mainly to the role of β-sitosterol as a precursor in the biosynthesis of brassinosteroids (plant hormones) and its ability to resist oxidation within the cell membrane [25].

In general, terms, β-sitosterol is the most abundant component in moringa seed oil, being one of the phytosterols of greater importance since it stimulates the secretion of insulin; it also has a regu-

latory effect on the production of antibodies, and it can intervene in the inflammatory processes associated with the initial state of type I diabetes, protecting against the destruction of pancreatic β-cells responsible for the release of insulin. Additionally, β-sitosterol improves some clinical symptoms in the treatment of benign prostatic hyperplasia such as the volume and frequency of urine [26]. β-Sitosterol is also known to be the principal sterol found in many seeds and oil seeds. Studies have shown that people with a diet containing 60-130 mg/day of β-sitosterol have a lower incidence of prostate cancer. Phytosterols also appear to play a role in modulating immune function and inflammation by affecting the production of inflammatory cytokines [27,28].

Antioxidant activity of moringa seed oil extracts

Bioactive compounds were obtained by liquid and solid phase extraction with the purpose of evaluating the efficiency of the extraction method with respect to the ORAC (oxygen radical absorption capacity) method.

A significant difference was found ($p < 0.05$) between the two extraction methods; the liquid phase extraction method had the highest antioxidant activity after the concentration of the secondary compounds (tocopherols and phytosterols) present in the extracts of the oil (Table 4). This difference may be because this method attracted a larger quantity of tocopherols and phytosterols, producing a higher antioxidant activity. The difference between the two methods is that the liquid phase extraction may be using a methanol/water mixture (80:20); the water may have possibly extracted polar compounds, and the less polar solvent (methanol) may have extracted less polar compounds, causing a greater concentration of bioactive components (squalene, β-carotene) in the extracts.

Tocopherol composition (mg/kg)			
	T1	T2	T3
α-tocopherol	321.69 ± 39.60^{a}	279.72 ± 16.65^{a}	362.48 ± 74.40^{a}
γ-tocopherol	116.48 ± 1.77^{a}	105.68 ± 4.62^{a}	129.52 ± 10.72^{a}
δ-tocopherol	7.36 ± 0.20^{a}	6.52 ± 0.67^{a}	9.09 ± 0.84^{a}
Total	445.55	391.93	501.10
Phytosterol composition (mg/kg)			
Ergosterol	1213.93 ± 51.89^{a}	1106.40 ± 25.39^{a}	1176.86 ± 51.03^{a}
Campesterol	1113.00 ± 13.55^{a}	1062.65 ± 109.29^{a}	1104.76 ± 154.30^{a}
Stigmasterol	1001.92 ± 150.51^{a}	689.17 ± 251.58^{a}	892.28 ± 56.68^{a}
β-sitosterol	1420.68 ± 26.11^{a}	1049.94 ± 22.04^{c}	1177.05 ± 23.025^{b}
Total	4749.54	3908.17	4350.97
LPE (μMeq Trolox/L)			
	2454.79 ± 88.92^{aA}	2416.86 ± 107.50^{aA}	2416.86 ± 107.50^{aA}
SPE (μMeq Trolox/L)			
	2017.77 ± 9.00^{aB}	1571.18 ± 30.88^{cB}	1840.42 ± 60.53^{bB}

Table 4: Tocopherol, phytosterol composition and antioxidant capacity of moringa seed oil according to the ORAC assay in moringa seed oil.

Results are expressed as the mean of determinations in triplicate ± the standard deviation. Different lowercase letters indicate significant difference ($p < 0.05$) in oil extraction conditions. Uppercase letters represent significant difference ($p < 0.05$) in the extraction method of the phenolic compounds in the oil.

T= Treatment. LPE= Liquid Phase Extraction. SPE= Solid Phase Extraction.

Regarding the difference in oil extraction treatments, there was no significant difference ($p < 0.05$) between the treatments when the liquid phase extraction was used. However, in the solid phase extraction, a significant difference was found ($p < 0.05$) among the

three treatments, with A showing the highest activity and B presenting the lowest antioxidant activity (Table 4). This difference may be due to the tocopherol content, since these can enhance its antioxidant power exerting a synergistic effect with other compounds, such as carotenes and chlorophylls, which were not determined in this work.

This behavior is also because the solvent and the extraction temperature of the oil interfered with the quantity of extracted secondary compounds; the increase in extraction temperature generates an increase in the yield of pigments, tocopherols and phytosterols, and the proportion of the solvent originated the diffusion of the liquid solvent in the solid matrix that favored the kinetics of desorption of the compounds from the matrix [29].

Conclusion

In this study the optimal conditions for the ultrasound-assisted extraction of moringa seed oil were sample/solvent ratio of 1:17.16 (p/v), temperature at 50.99°C and extraction time of 25.95 min. Characterization of the optimal products showed that moringa seed oil obtained by ultrasound presented high tocopherol and phytosterol contents compared with other conventional extraction methods, which indicates that this technique is effective for oil extraction; C treatment presented the highest phytosterol content and increased antioxidant activity. α-Tocopherol and β-sitosterol were the bioactive compounds that were found in greatest proportions. The high concentration of these bioactive compounds in moringa seed oil suggests that the oil can be used as a natural source of antioxidants.

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Bibliography

1. El Baky., *et al.* "Characterization of Egyptian Moringa peregrina seed oil and its bioactivities". *International Journal of Management and Business Research* 2 (2013): 98-108.

2. Ojiako EN and Okeke CC. "Determination of antioxidant of *Moringa oleifera* seed oil and its use in the production of a body cream". *Asian Journal of Plant Science and Research* 3 (2013): 1-4.

3. Yusoff MM., *et al.* "Aqueous enzymatic extraction of *Moringa oleifera* oil". *Food Chemistry* 211 (2016): 400-408.

4. Brilhante RSN., *et al.* "Research advances on the multiple uses of *Moringa oleifera:* A sustainable alternative for socially neglected population". *Asian Pacific Journal of Tropical Medicine* 10 (2017): 621-630.

5. Bhutada PR., *et al.* "Solvent assisted extraction of oil from *Moringa oleifera* Lam. Seeds". *Industrial Crops Production* 82 (2016): 74-80.

6. Latif S and Farooq A. "Aqueous enzymatic sesame oil and protein extraction". *Food Chemistry* 125 (2011): 679-684.

7. Ruttarattanamongkol K., *et al.* "Pilot-scale supercritical carbon dioxide extraction, physico-chemical properties and profile characterization of *Moringa oleifera* seed oil in comparison with conventional extraction methods". *Industrial Crops Production* 58 (2014): 68-77.

8. Farooq A and Bhanger MI. "Analytical characterization of *Moringa oleifera* seed oil grown in temperate regions of Pakistan". *Journal of Agricultural and Food Chemistry* 51 (2003): 6558-6563.

9. Mani S., *et al.* "Optimization of solvent extraction of Moringa (*Moringa oleifera*) seed kernel oil using response surface methodology". *Food and Bioproducts Processing* 85 (2007): 328-335.

10. Rocchetti G., *et al.* "Impact of conventional/non-conventional extraction methods on the untargeted phenolic profile of *Moringa oleifera* leaves". *Food Research International* 115 (2019): 319-327.

11. Zhong J., *et al.* "The application of ultrasound and microwave to increase oil extraction from *Moringa oleifera* seeds". *Industrial Crops and Products* 120 (2018): 1-10.

12. AOCS. 1999. Official Methods and Recommended Practices of the American Oil Chemists Society. Am Oil Chem Soc, 6th Edn. AOCS Press, Urbana, Illinois USA Hu B., *et al.* "Method for extracting oil from cherry seed by ultrasonic-microwave assisted aqueous enzymatic process and evaluation of its quality". *Journal of Chromatography A* 1587 (2019): 50-60.

13. Zarrouk W., *et al.* "multi-component analysis (sterols, tocopherols and triterpenic dialcohols) of the unsaponifiable fraction of vegetable oils by liquid chromatography-atmospheric pressure chemical ionization-ion trap mass spectrometry". *Talanta* 80 (2010): 924-934.

14. Ramadan MF. "Healthy blends of high linoleic sunflower oil with selected cold pressed oils: Functionality, stability and antioxidative characteristics". *Industrial Crops Production* 43 (2013): 65-72.

15. García-Villalba R., *et al*. "Characterization and quantification of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolutive LC-ESI-TOF MS method". *Journal of Pharmaceutical Biomedical* 51 (2010): 416-429.

16. Senrayan J and Venkatachalam S. "Optimization of ultrasound-assisted solvent extraction (UASE) based on oil yield, antioxidant activity and evaluation of fatty acid composition and thermal stability of *Coriandrum sativum* L. seed oil". *Food Science Biotechnology* 28 (2019): 377-386.

17. Sicaire AG., *et al.* "Ultrasound induced green solvent extraction of oil from oleaginous seeds". *Ultrasonics Sonochemistry* 31 (2016): 319-329.

18. Zhang W., *et al*. "Optimized ultrasonic-assisted extraction of papaya seed oil from Hainan/Eksotika variety". *Food Science Nutrition* 7 (2019): 2692-2701.

19. Samaram S., *et al*. "Optimisation of ultrasound-assisted extraction of oil from papaya seed by response surface methodology: Oil recovery, radical scavenging antioxidant activity, and oxidation stability". *Food Chemistry* 172 (2015): 7-17.

20. Ansorena D., *et al.* "Effect of fish and oil nature on frying process and nutritional product quality". *Journal of Food Science* 75 (2010): 62-67.

21. Yolmeh M., *et al.* "Optimization of ultrasound-assisted extraction of natural pigment from annatto seeds by response surface methodology (RSM)". *Food chemistry* 155 (2014): 319-324.

22. Martins PF., *et al.* "Techno-economic optimization of the subcritical fluid extraction of oil from *Moringa oleifera* seeds and subsequent production of a purified sterols fraction". *The Journal of Supercritical Fluids* 107 (2016): 682-689.

23. Ogbunugafor HA., *et al.* "Physico-chemical and antioxidant properties of *Moringa oleifera* seed oil". *Pakistan Journal of Nutrition* 10 (2011): 409-414.

24. Beleggia R., *et al.* "Effect of genotype, environment and genotype-by-environment interaction on metabolite profiling in durum wheat (Triticum durum Desf.) grain". *The Journal of Cereal Science* 57 (2013): 183-192.

25. Piironen V., *et al.* "Plant sterols: biosynthesis, biological function and their importance to human nutrition". *Journal of the Science of Food and Agriculture* 80 (2000): 939-966.

26. Leone A., *et al.* "*Moringa oleifera* seeds and oil: Characteristics and uses for human health". *International Journal of Molecular Sciences* 17 (2016): 2141.

27. Hendriks HFJ., *et al.* "Safety long-term consumption of plant sterol esters-enriched spread". *European Journal of Clinical Nutrition* 57 (2003): 681-692.

28. Uddin S., *et al.* "Phytosterols and their extraction from various plant matrices using supercritical carbon dioxide: a review". *Journal of the Science of Food and Agriculture* 95 (2014): 1385-1394.

29. Gopalakrishnan L., *et al.* "*Moringa oleifera*: A review on nutritive importance and its medicinal application". *Food Science and Human Wellness* 5 (2016): 49-56.