Eclética Química
ISSN: 0100-4670
ISSN: 1678-4618
ecletica@journal.iq.unesp.br
Universidade Estadual Paulista Júlio de Mesquita Filho
Brasil

Gonzales-Condori, Elvis Gilmar; Alvarez-Gonzales, Rosa; Ramírez-Revilla, Stamber Alvaro
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Eclética Química, vol. 46, núm. 2, 2021, pp. 57-66
Universidade Estadual Paulista Júlio de Mesquita Filho
Araraquara, Brasil

DOI: https://doi.org/10.26850/1678-4618eqj.v46.2.2021.p57-66

Disponible en: https://www.redalyc.org/articulo.oa?id=42966338006

Eclética Química Journal

Original article

iq.unesp.br/ecletica

| Vol. 46 | n. 2 | 2021 |

Ultrasound-assisted extraction for the determination of α-linolenic and linoleic acid in vegetable oils by high performance liquid chromatography

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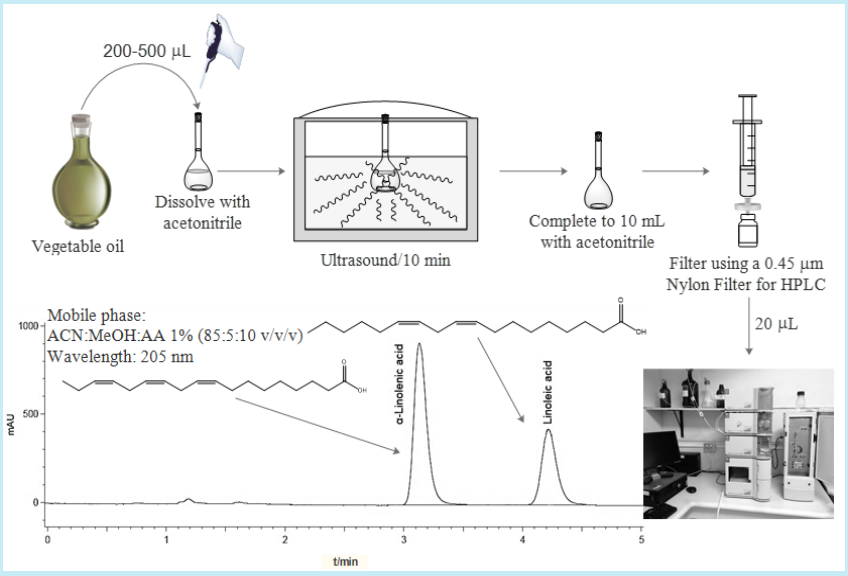
ARTICLE INFO

Article history:
Received: March 26, 2020
Accepted: December 03, 2020
Published: April 01, 2021

Keywords
1. α-linolenic acid
2. linoleic acid
3. vegetal oils
4. HPLC

ABSTRACT: The present research consisted in determining α-linolenic acid (ALA, ω-3) and linoleic acid (LA, ω-6) in vegetable oils with ultrasound-assisted extraction. The analytical method was validated by high performance liquid chromatography (HPLC) for the quantification of both polyunsaturated fatty acids (PUFA). The determination was made at a wavelength of 205 nm, mobile phase composed of acetonitrile: methanol: acetic acid 1% (85:5:10), with a flow of 1 mL min^{-1} and during 5 min. The calibration graph data (50 to 300 mg L^{-1}) adjusted to the linear regression model with a coefficient of determination (R^2) of 0.99914 and 0.99986 for ALA and LA, respectively. The repeatability, reproducibility, accuracy and percentage of recovery complied with international guidelines for analytical methods.



The proposed method is simple, fast, linear, precise and accurate for the quantitative determination of ALA and LA in vegetable oils. The previous ultrasound application during 10 min achieves significantly higher concentrations ($p < 0.05$) when ultrasound is not applied. In conclusion, the present method can serve as a useful alternative for routine determinations of ALA and LA in vegetable oils, as it proved to be an easy, fast and reliable method.

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1. Introduction

Fatty acids (FA) are carboxylic acids with a hydrocarbon chain that can vary from C_6 to C_{24} and can be present in nature as pure free fatty acids (FFA), FA salts or as part of more complex molecules known as lipids. Fatty acids can be unsaturated and sub-classified as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)[1]. In general, MUFA and long-chain PUFA are involved in neuronal processes: their deficiency and other nutrients between the last trimester of pregnancy and the first two years of childhood could damage brain function[2]. On the other hand, they are important for the treatment of eye, skin, central nervous system and viral diseases[3], prevention and management of violence in schizophrenia[4]. In addition, mitochondrial FA (β oxidation) are crucial for the maintenance of thermogenesis[5].

Cis, cis-9, 12-octadecadienoic acid or linoleic acid (LA) and cis, cis, cis-cis, 9, 12, 15-octadecatrienoic acid or α-linolenic acid (ALA) are among the best-known PUFAs present in plants, nuts, seeds and vegetable oils[6,7]. These PUFAs are considered essential FAs: they are not synthesized by humans, hence it is necessary to include them in the diet[2,8,9]. Other FA present in vegetable oils are palmitic acid, stearic acid, palmitoleic acid, oleic acid, arachidic acid, behenic acid, lignoceric acid, etc[10]. On the other hand, a group of PUFA is known as Omega 3, which includes ALA, stearidonic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid present in various sources, such as plants, fish, fish oils, seaweed[11] and as omega 6 to LA[12]. Also, vegetable oils can be composed of phenolic acids, flavonoids, vitamins A and E[13].

Concerning LA (Omega 6, ω-6) benefits, it was found to effectively reversed the inflammatory responses induced by palmitic acid treatment in microglial cells[14]. Likewise, it has been reported that this acid constitutes a safe, easy to apply, well tolerated and effective anti-inflammatory prophylactic treatment of chronic migraine[15]. Furthermore, the intake of LA is associated with a lower risk of suffering from diabetes mellitus type 2 (DM2), as well as a better glycemic control and/or insulin sensitivity[16].

On the other hand, ALA (Omega 3, ω-3), a known precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is considered as a nutraceutical/pharmaceutical candidate due to its safe use as a food ingredient. Regarding the benefits of ALA, cardioprotective, anticancer, neuroprotective, anti-osteoporotic, anti-inflammatory and antioxidant effects have been reported[17], as well as bone protector[18] and coronary heart disease prevention capacities[19]. Alfa-linolenic acid could be involved in the preventive effect on cognitive impairment and dementia[20] and other researches consider it as a nutraceutical to prevent devastating damages caused by cerebral ischemia[21,22].

Hence, fatty acids such as ALA and LA are plant-origin dietary components of great nutritional interest, which justifies the necessity of research concerning their concentration in vegetable oils. Developing faster techniques for ALA and LA quantification is of crucial importance. Currently, emerging (non-thermal) technologies, such as high-pressure processing (HPP), ultrasound treatment (US), ionizing radiation (IR), pulsed electric field (PEF) and cold plasma, are applied for the preservation of food components[23]. Moreover, studies report that ultrasound-assisted oil extraction increases yield and reduces extraction time without affecting the quality of oil, compared to other extraction methods[24,25]. In addition, it was shown that the sample exposure time to ultrasound gives more optimal results when analyzing short-chain fatty acids (SCAF) compared to shaking the sample in a solvent[26], this is because ultrasound in the immiscible liquid-liquid-solid system produces high-speed impacts between solid particles and liquid phases and therefore promotes mass transfer between phases[27,28]. To date there are various methods for determining FFA, such as thin layer chromatography (TLC)[29], volumetric methods, gas chromatography (GC)[30] and particularly high performance liquid chromatography (HPLC)[31], which would provide various advantages over the official GC method, especially in terms of simplicity, separation times, sensitivity, speed[32] and its importance in many different fields[33,34].

Therefore, the present research proposes an easy methodology for the determination of ALA and LA in vegetable oils by HPLC with diode array detector

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(HPLC-DAD) with ultrasound-assisted determination (UAD). The use of ultrasound as a method for the preparation of samples for solid-liquid extraction is widespread among laboratories and can be considered fast and effective. This opens a gateway to new perspectives, mainly in relation to analytes that are strongly bound to the matrix[35]. Therefore, the goal of this research was to compare concentrations of ALA and LA with and without UAD in soybean, Sacha Inchi, linseed oils that are highly commercialized and Loche pumpkin and Macre pumpkin oils that present scarce studies in relation to the composition of ALA and LA.

2. Materials and methods

2.1 Chemical reagents and samples

Analytical standards of ALA and LA were obtained from Sigma-Aldrich. Likewise, acetonitrile (ACN) and methanol (MeOH) HPLC grade and acetic acid (AA) 99.8%, were obtained from Merck. Ultrapure water (18.2 MΩ cm) was produced by a Thermo Scientific Barnstead Easypure II - RF purifier. The samples of vegetable oils (soybean, Sacha Inchi, linseed, Loche pumpkin and Macre pumpkin) were provided by the Quality Control Laboratory of the Universidad Católica de Santa María.

2.2 Chromatographic conditions

A HPLC Hitachi LaChrom Elite with diode array detector (DAD), Merck LiChroCART 250-4 RP-18e column (5 µm), ACN mobile phase:MeOH:AA 1% v/v/v (85:5:10) was used. The system was kept in isocratic conditions exposed to a 205 nm wavelength and with a flow of 1 mL min^{-1} during a period of 5 min.

2.2.1 Evaluation of the linearity of the ALA and LA quantification method by HPLC

To prepare the calibration graph, a standard stock solution was prepared weighing 10.0 mg of the ALA and LA standards in a 10.0 mL flask, achieving a concentration of 1000 mg L^{-1} in ACN. Six calibration solutions were prepared from the stock solution: 50, 100, 150, 200, 250 and 300 mg L^{-1}. Finally, 20 µL were injected into the HPLC column under the established chromatographic conditions. This procedure was performed three times. Linear regression was performed using the method of the least squares in order to find the equation of the straight line.

$$y = a + bx \quad (1)$$

Where, y is the absorbance, x is the concentration, a is the intercept and b is the slope.

Then, the coefficient of determination R^2, which should be greater than 0.99, was calculated[36].

2.2.2 Determination of the detection and quantification limits

The limit of detection (LOD) that corresponds to the minimum amount of analyte that the method can determine, but not quantify, was calculated using Eq. 2[36].

$$LOD = \frac{Y_{bl}+3(Sbl)}{b}\frac{1}{\sqrt{n}} \quad (2)$$

On the other hand, the limit of quantification (LOQ) that corresponds to the minimum amount of analyte that the method can quantify with precision and accuracy was calculated with Eq. 3[36]:

$$LOQ = \frac{Y_{bl}+10(S_{bl})}{b}\frac{1}{\sqrt{n}} \quad (3)$$

Where, Y_{bl} (blank response) corresponds to the intercept that relates the concentration and the average response (area) and S_{bl} (standard deviation of the blank) to the intercept corresponding to the calculated line of the concentration versus the standard deviation[36].

2.2.3 Precision

Six samples of vegetable oil were prepared and quantified (ALA and LA) through HPLC. To evaluate repeatability, the six analysis were performed by the same analyst; for reproducibility, another six analysis were performed by two analysts. Accuracy was

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expressed as the relative standard deviation (RSD) using Eq. 4[36]:

$$RSD = \frac{s}{\bar{x}} \times 100 \quad (4)$$

Where $\bar{x}$ is the average of the six measurements and *s* corresponds to the standard deviation of those measurements.

2.2.4 Evaluation of accuracy

Accuracy was calculated using the percentage recovery method *(%R*). To do this, the concentration of ALA and LA in a sample of vegetable oil was quantified and then, the sample was enriched with 100 mg L^{-1} of ALA and 150 mg L^{-1} of LA. Once analyzed by HPLC, the *%R* was calculated using Eq. 5:

$$\%R = \frac{C_e - C}{C_{st}} \times 100 \quad (5)$$

where, *C* is the concentration of ALA and LA in the vegetable oil, C_e is the concentration after enrichment with ALA and LA standards in vegetable oil and C_{st} is the concentration of ALA and LA standards with which the vegetable oils were enriched.

2.3 Samples preparation

For the analysis of the vegetable oils, 200 µL of each of soy, Sacha Inchi and linseed oils and 500 µL of each pumpkin oil, Loche and Macre, were poured in 10 mL volumetric flasks. Then, 5 mL of ACN were added to the samples, which were covered with aluminum foil and exposed to ultrasound during 10 min in the Branson 2510 ultrasound. Finally, the flasks were made up to with ACN, filtered using 0.45 µm nylon membranes and 20 µL of the solution was injected to HPLC. Also, analyses of the same oils were performed without the application of ultrasound. The volume of oil analysis was higher for pumpkin oils because it gave results below the LOQ.

2.3.1 Concentration

Alfa-linolenic acid and LA concentrations were expressed in g L^{-1} using Eq. 6:

$$Concentration(g/L) = \frac{Concentration(mg/L)}{1000} \times \frac{10}{v} \quad (6)$$

where, concentration *(*mg L^{-1}*)* is obtained by replacing the area in the equation of the line, which is divided by 1000 to transform mg to g, (*10*) corresponds to the volume of the flask where the vegetable oil is dissolved, and (v) corresponds to the volume of vegetable oil in milliliters.

2.4 Statistics

The data obtained were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. On the other hand, the calibration equation was established by applying a linear regression model that relates the analyte concentrations to the signals (area)[37]. The concentrations of ALA and LA were expressed in g L^{-1} ± SD. For the comparison of the results, a two-way analysis of variance (ANOVA) with a significance of 95% was used and then Tukey's test post-hoc method was used. All results were obtained using GraphPad Prism 6.0 and OrginPro 9.0 software.

3. Results

3.1 The ALA and LA determination method by HPLC

The chromatogram (Fig. 1) shows the retention times for ALA and LA, which are 3.2 and 4.3 min, respectively, at the established chromatographic conditions.

To evaluate the linearity of the method, the areas of the ALA and LA calibration solutions were measured from 50 to 300 mg L^{-1}. Concerning ALA, it resulted with a coefficient of determination R^2 of 0.99914, a slope "*b*" of 228877 and an intercept "*a*" of 1850196. Knowing that the y axis corresponds to the area in mAU and the x axis to the concentration of ALA in mg L^{-1}, the Eq. 7 is obtained.

$$Area = 228877[Concentration] + 1850196 \quad (7)$$

Likewise, concerning LA, it resulted with coefficient of determination R^2 of 0.99986, a slope "*b*" of 111061 and an intercept "*a*" of 430963. Knowing

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that the y axis is the area in mAU (milli-Absorbance Units) and the x axis is the concentration of LA in mg L^{-1}, Eq. 8 is obtained:

$$Area = 111061[Concentration] + 430963 \quad (8)$$

Concerning the results of the precision evaluation, the repeatability tests resulted in an RSD of 0.77 and 0.72% for ALA and LA, respectively. For reproducibility, the RSD was 1.27 and 1.34% for ALA and LA, respectively.

Regarding the sensitivity of the method, the LOD for ALA and LA were 6.14 and 3.16 mg L^{-1}, respectively, and the LOQ for ALA and LA were 12.77 and 6.84 mg L^{-1}, respectively.

The accuracy assessed by the percent recovery method (*%R*) resulted in values of 100.50 and 100.19% for ALA and LA, respectively.

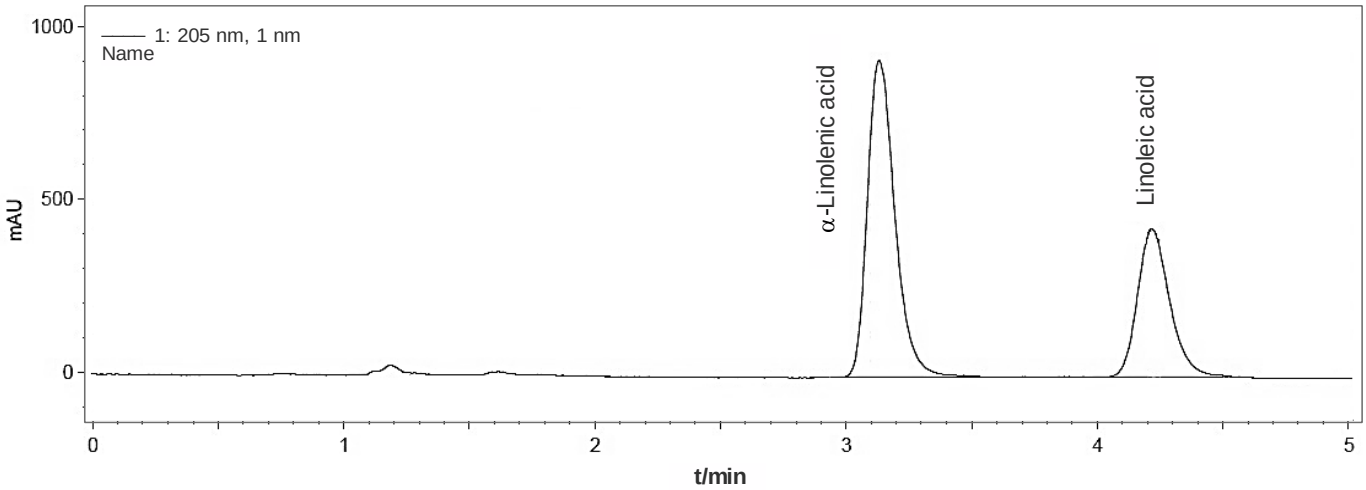

Figure 1. The ALA and LA chromatogram at 205 nm.

3.2 Quantification of ALA and LA in vegetable oils with ultrasound-assisted determination

In the present investigation, ultrasound was used to accelerate the preparation and the determination of fatty acids from samples of vegetable oils. The areas determined in the quantification of ALA and LA in the studied vegetable oils are shown in Tabs. 1 and 2, which also show the concentration in mg L^{-1} calculated with Eqs. 7 and 8, the average concentrations ± SD and the concentrations in g L^{-1} ± SD of both acids under study, which was calculated with Eq. 6. Likewise, the same table shows the results with and without UAD.

Table 1. Concentration (g L^{-1}) of free α-linolenic acid (ALA) in vegetable oils.

Vegetable oil	With UAD		Without UAD	
	Average concentration mg L^{-1} ± SD	Concentration g L^{-1} ± SD	Average concentration mg L^{-1} ± SD	Concentration g L^{-1} ± SD
Soybean oil	16.2 ± 0.2	0.81 ± 0.01[a]	13.2 ± 0.4	0.66 ± 0.02[a]
Sacha Inchi oil	44.5 ± 0.1	2.23 ± 0.01[b]	38.1 ± 0.5	1.91 ± 0.03[b]
Linseed oil	54.8 ± 0.7	2.74 ± 0.03[c]	50.1 ± 0.7	2.51 ± 0.03[c]
Loche pumpkin oil	17.9 ± 0.4	0.36 ± 0.01[d]	15.7 ± 0.6	0.31 ± 0.01[d]
Macre pumpkin oil	15.1 ± 0.5	0.30 ± 0.01[e]	13.5 ± 0.4	0.27 ± 0.01[e]

*__UAD:__ ultrasound-assisted determination, __different letters:__ significant difference.

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Table 2. Concentration (g L^{-1}) of free linoleic acid (LA) in vegetable oils.

Vegetable oil	With UAD		Without UAD	
	Average concentration mg L^{-1} ± SD	Concentration g L^{-1} ± SD	Average concentration mg L^{-1} ± SD	Concentration g L^{-1} ± SD
Soybean oil	47.1 ± 0.5	2.36 ± 0.02[a]	40.0 ± 0.6	2.00 ± 0.03[a]
Sacha Inchi oil	16.0 ± 0.4	0.80 ± 0.02[b]	13.0 ± 0.6	0.65 ± 0.03[b]
Linseed oil	16.8 ± 0.1	0.84 ± 0.01[b]	13.2 ± 0.2	0.66 ± 0.01[b]
Loche pumpkin oil	45.9 ± 1.0	0.92 ± 0.02[c]	41.7 ± 1.0	0.83 ± 0.02[c]
Macre pumpkin oil	55.0 ± 1.0	1.10 ± 0.02[d]	50.7 ± 0.6	1.01 ± 0.01[d]

***UAD:** ultrasound-assisted determination, **different letters:** significant difference.

The ANOVA performed on the data in Tabs. 1 and 2 showed that the concentration of ALA and LA differ in at least one sample of vegetable oil ($p < 0.05$). On the other hand, statistical analysis also indicates that the application of ultrasound reports significantly higher results ($p < 0.05$) than when no ultrasound was applied.

In relation to the results obtained by UAD, the Tukey's test showed that the concentration of ALA in all the studied oils was different from 95% confidence. The concentration of ALA in oils in descending order is: linseed (2.74 ± 0.03 g L^{-1}) > Sacha Inchi (2.23 ± 0.01 g L^{-1}) > soybean (0.81 ± 0.01 g L^{-1}) > Loche pumpkin (0.36 ± 0.01 g L^{-1}) > Macre pumpkin (0.30 ± 0.01 g L^{-1}). On the other hand, Tukey's test showed that there is no significant difference between the concentration of LA in linseed oil and Sacha Inchi oil at 95% confidence. These concentrations differ from the ones of the other oils studied. The order of concentration of LA in the studied oils is the following: soybean (2.36 ± 0.02 g L^{-1}) > Macre pumpkin (1.10 ± 0.02 g L^{-1}) > Loche pumpkin (0.92 ± 0.02 g L^{-1}) > linseed (0.84 ± 0.01 g L^{-1}) = Sacha Inchi (0.80 ± 0.02 g L^{-1}).

4. Discussion

The International Conference on Harmonization (ICH) standards establish that, for the validation of the method, linearity, precision, accuracy, LOQ and LOD must be determined[38,39]. A method is considered linear if the coefficient of determination R^2 is greater than 0.99, precise when the RSD is less than 2% and accurate as long as the recovery percentage (*%R*) is close to 100% (90–110%)[36]. The method turned out to be linear with an R^2 = 0.99914 for ALA and 0.99986 for LA; precise with an RSD of 1.27% for ALA and 1.34% for LA; and in reproducibility and repeatability tests, the RSDs for ALA and LA were 0.77 and 0.72%, respectively. The LODs were 6.14 for ALA and 3.16 mg L^{-1} for LA, and LOQs were 12.77 and 6.84 for ALA and LA, respectively.

Nowadays, ultrasound is used for the isolation of organic compounds present in solid samples, such as soils, animal tissues, plants, etc., since they are comparable to methods that involve more intensive treatments and with various solvents[35,40,41]. In addition, the application of ultrasound showed that in terms of yield and extraction time, it does not affect the quality of the oil[25]. Several investigations were developed with different methods of extraction and determination of fatty acids. The ALA was found in linseed oil[17,42], in linseeds[18] and perilla, canola and soybean oil[20]. It is also reported that LA was not found in soybeans oil[43], however Sacha Inchi did show the presence of ALA and LA[44,45]. In the present investigation, ALA (± SD) and LA (± SD) were found in Sacha Inchi oils (2.74 ± 0.03 g L^{-1}; 0.80 ± 0.02 g L^{-1}), linseed oil (2.74 ± 0.03 g L^{-1}; 0.84 ± 0.01 g L^{-1}), Loche pumpkin oil (0.36 ± 0.01 g L^{-1}; 0.92 ± 0.02 g L^{-1}), Macre pumpkin oil (0.30 ± 0.01 g L^{-1}; 1.10 ± 0.02 g L^{-1}) and soybean oil (0.81 ± 0.01 g L^{-1}; 2.36 ± 0.02 g L^{-1}).

In relation to HPLC methods, some use direct derivatization for the determination of both FFA with and without saponification of the samples, showing recoveries in the range 98.30 – 103.40% and the coefficients of variation were in the range 0.70 – 3.10%[31]. In the present investigation, it also turns out to be a simple, fast and accurate method that resulted in recoveries of 100.19 and 100.50% for LA and ALA, respectively, with a determination coefficient of less

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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66

than 1.50 % as opposed to the derivation method that gave a maximum result of 3.10%.

In another study, a method was developed and validated by HPLC-DAD for the determination of short chain fatty acids such as: acetic, propionic, butyric, valeric and isovaleric acids in newborn meconium. The method presented a high precision (RSD ≤ 2.5%), high coefficients of determination of the calibration curves (R^2 > 0.997). Assay recovery ranged from 90 ± 2 and 106 ± 2%. The validation parameters obtained in said study showed that the HPLC-DAD method is a reliable and useful tool for the determination of short chain fatty acids in meconium samples, where the samples were exposed to ultrasound energy at 40 °C for 80 min and subsequent shaking for 60 min[46]. This study showed that the application of ultrasound for only 10 min optimizes the determination process of FFA from vegetable oils giving results significantly higher than when no ultrasound is applied, in addition, the method by HPLC-DAD developed also presents high accuracy, with coefficients greater than 0.997.

Another method by HPLC with conductivity detector for the separation and simultaneous determination of myristic, palmitic, stearic, oleic and linoleic acids presented calibration curves of the five well correlated FA (R^2 > 0.999)[32]. In addition, 2- (7H-dibenzo [a, g] carbazol-7-il) ethyl 4-methylbenzenesulfonate (DBCETS) was proposed as a new fluorescent labeling reagent to detect FFA by HPLC in serum sample analysis resulting in low detection limits of 0.28–0.57 ng mL^{-1} and guaranteed high accuracy and reliability of results[47]. Unlike the present investigation where the detection limits for ALA and LA were 6.14 and 3.16 mg L^{-1}, respectively. Clearly the method developed by HPLC-DAD offers high detection limits compared to the method by HPLC with fluorescence detector; however, both analyses are oriented to different matrices and the concentration of FA in oils is relatively higher than in serum, demonstrating that the methodology developed provides reliable results in the analysis of ALA and LA in vegetable oils.

5. Conclusions

A method for the determination of ALA and LA by HPLC at 205 nm in vegetable oils has been developed. For this method, the sample preparation uses between 200 to 500 µL of oil, a 10-min ultrasound application during a short run time (5 min), at a flow of 1 mL min^{-1}, obtaining retention times of 3.2 and 4.3 min for ALA and LA, respectively. The method for determining ALA and LA was found to be linear (R^2 > 0.99), precise (RSD < 2.0%) and accurate (90% < *%R* <110%). The validated method allowed the determination of ALA and LA concentrations in samples of vegetable oils (Sacha Inchi, linseed, Loche pumpkin, Macre pumpkin and soybean). Therefore, the present method can serve as a useful tool for the routine determinations of ALA and LA in vegetable oils since it turned out to be an easy, fast and reliable method.

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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66