

Analytical Method Development for Fatty Acid Direct Methylation in Fruits

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Due to the amount of pigments, sugars and fibers found in fruits, the fatty acid quantification process leads to errors, such as its amount underestimation in the matrix. Therefore, the proposed method allows performing experiments in shorter time with reduced amount of sample and reagent. Design Expert software was used to find the validated time for alkaline and acidic reactions, which were six and eight minutes, respectively, and both concentration was 0.5 mol L⁻¹. The validation was made for avocado pulp and different fruits were used for application of the method, such as: apple, mango, coconut and another avocado variety. Accuracy values ranged from 89.05 to 100.21%, being in accordance with acceptable values. Also the relative standard deviation (RSD)_{intra-day} (0.30 to 1.30%) and the RSD_{inter-day} values (0.32 to 1.44%) confirmed that the proposed method for fatty acid derivatization has good precision.

Keywords: lipids, direct derivatization, ultrasound

Introduction

Chemical composition of fruits varies according to species, cultivar, production place, edaphoclimatic factors, cultural treatments, degree of maturation, and harvest season. Due to the health benefits, there is an increase in the consumption of fresh fruits by the population.¹

Among the most consumed fruits, avocado (*Persea americana* Mill) is highlighted since studies have been showing that the extracted oil is significant to prevent cardiovascular diseases, due to the high amount of monounsaturated fat, such as oleic acid.²

Lipid extraction is an essential determination in the characterization of raw material; nevertheless, obtaining this fraction requires attention, such as the usage of cold extraction method to avoid lipid degradation.³

The Bligh and Dyer⁴ method is widely used and it is carried out in room temperature in order to not degrade,

consequently preventing oxidation reactions. This methodology was developed specifically for fish with 1% lipid and 80% moisture; however, it is used for food in general, which may not lead to an entirely extraction of lipid material in sample.⁵

Triacylglycerols (TAG) are the main constituent of the lipid fraction of food, these compounds have an elevated boiling point (about 380 °C), which in order to be analyzed by gas chromatography (GC), it is necessary to be converted into more volatile substances, such as methyl esters of fatty acids.⁶

A correct method that deserves attention is the direct esterification without the previous extraction of lipids, because smaller amount of solvent and sample are applied, as well as the reduction of steps to avoid or reduce experimental errors.⁵ However, esterification time, sample mass, catalyst, and esterifying reagent concentration affect the technique final result, being necessary the optimization of these factors.

Therefore, the aim of this work is to develop a direct

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fatty acids (FA) methylation method in fruit samples using response surface methodology.

Experimental

Reagents, materials and instruments

Chloroform, methanol, *n*-heptane, sulfuric acid and hydrochloric acid were used without further purification and purchased from Millipore Sigma (Darmstadt, Germany). A mixture of fatty acids methyl esters (FAMES, 189-19) and methyl tricosanoate (PI, 23:0 me) were also purchased from Millipore Sigma (St. Louis, MO, USA).

The butter variety avocado (*Persea americana* Mill) was obtained from local market (Maringá City, Paraná State, Brazil). Two kilograms of the avocado were washed in chlorinated water, cut in half to remove the seed, the pulp was removed with a spoon, homogenized in a knife mill, packed in vacuum polyethylene bags and kept in the freezer at $-18\text{ }^{\circ}\text{C}$ until analysis.

Gas chromatography with flame ionization detector analysis (GC-FID)

Chromatographic analysis was performed on a Thermo Scientific GC equipped with FID, split/splitless injector and CP-7420 fused silica capillary column (Select FAME, 100.0 m long, 0.25 mm internal diameter and 0.25 μm thin film of cyanopropyl as stationary phase). Operating parameters are as follow: column temperature of $165\text{ }^{\circ}\text{C}$ for 18 min, then heated to $235\text{ }^{\circ}\text{C}$ ($4\text{ }^{\circ}\text{C min}^{-1}$) for 20 min. Injector and detector temperatures were maintained at 230 and $250\text{ }^{\circ}\text{C}$, respectively. Gas flows were 1.2 mL min^{-1} for carrier gas (H_2), 30.0 mL min^{-1} for make-up gas (N_2), and in the FID 30.0 and 300.0 mL min^{-1} of gas (H_2) and synthetic air, respectively. The samples were injected in split mode, with 1:40 ratio. Injection volume was $1.0\text{ }\mu\text{L}$. FAMES were identified by comparison among the retention time of constituents samples and Sigma FAMES. Theoretical correction factor was used and calculated to obtain FA concentrations according to Visentainer,⁹ and FA amount was calculated in mg g^{-1} of sample. FID correction is necessary because of the magnitude signal generated by detector in proportion to the number of C^+ that are bounded to hydrogen atoms.¹⁰

Analysis of electrospray ionization mass spectrometry (ESI-MS)

Samples were infused into an ESI Xevo Acquity® (Waters, Milford, MA, USA) mass spectrometer with flow

rate of $10.0\text{ }\mu\text{L min}^{-1}$ to evaluate the efficiency of the proposed method in comparison to the traditional methodology for lipid extraction followed by methylation. The spectra were acquired in ratio m/z 500 to 1200 in positive mode. Mass parameters are as follows: capillary voltage, 3.0 kV; cone gas flow, 40.0 L h^{-1} ; desolvation flow, 600.0 L h^{-1} ; desolvation temperature, $600\text{ }^{\circ}\text{C}$; and source temperature, $130\text{ }^{\circ}\text{C}$. Data were processed using MassLynx™ software.

Triacylglycerols identification was carried out as follows: 100.0 mg of oil was weighed; 6.0 mL of solution containing 4.0 mL of chloroform and 2.0 mL of methanol was prepared, 995 μL of this solution was added into the tube containing sample, 20.0 μL of 0.1% ammonium formate was also added, the tube was agitated until the total oil solubilization and then infused.

Validation parameters

Validation parameters of the method were determined in accordance to guidelines of the International Conference on Harmonization.¹¹ Figures of merit used were: precision, accuracy and linearity range, which were obtained with six replicates.

The relative standard deviation (RSD) infer about precision, and the replication was performed in different days. Accuracy was obtained by comparing the results of traditional methodology (TM) with the proposed methodology (PM). Linear range was determined by interpolation of the FA sum obtained by TM in relation to the PM.

Traditional methodology for lipid extraction and esterification / transesterification (TM)

Lipids extractions were conducted according to Bligh and Dyer,⁴ approximately 100.0 g of shredded sample was thawed at room temperature and weighed; 100.0 mL of chloroform and 200.0 mL of methanol were added; the mixture was homogenized for 2 min; 100.0 mL of chloroform was added; the mixture was stirred for 30 s; 100.0 mL of water was added to the mixture aiming the phase separation; mixture was stirred for 30 s; then it was filtered on Whatman No. 1 paper under vacuum and the filtrate was transferred to a funnel of separation. After separation, the organic phase was collected and the solvent was evaporated on a rotary evaporator.

The esterification and transesterification reactions of FA were performed according to Hartman and Lago⁷ method modified by Maia and Amaya.⁸ Approximately 100.0 mg of the extracted lipid was weighed in a test tube and added with 4.0 mL of NaOH/MeOH 0.50 mol L^{-1} ; the tube was

heated in a boiling water bath for 5.0 min with subsequent cooling in running water; 5.0 mL of esterifying reagent ($\text{NH}_4\text{Cl}/\text{H}_2\text{SO}_4/\text{MeOH}$) was added to the tube; it was again heated in water bath for 5.0 min and cooled in running water; 4.0 mL of saturated NaCl solution and 5.0 mL of hexane were added; then the tubes were shaken vigorously for 30 s. Lastly, the internal standard (PI, 23:0 me) was added and after phase separation, the upper phase was collected for injection into GC.

Experimental design

A rotational central composite design was generated by Design Expert[®] 7 software to evaluate the influence of acid and alkaline in reactions time, as well as its concentration (NaOH in methanol and H_2SO_4 in methanol). The alkali time levels -1 and $+1$ were 6.0 to 11.0 min,

respectively, and the acid time levels -1 and $+1$ were 8.0 to 15.0 min, respectively, while the levels of acid and alkaline concentration -1 and $+1$ were 0.2 to 0.5 mol L^{-1} . The axial points ($\pm\alpha$) for rotational system ($k < 5$) were ± 1.4142 , which were used to calculate the quadratic terms, as in Table 1. Five replicates were performed at the central point, totaling 29 experiments.

Proposed method for lipid methylation (PM)

Avocado sample was thawed at room temperature; 100.0 ± 1.0 mg was weighed into a 10 cm test tube; 2.0 mL of NaOH in methanol was added, its concentration being in accordance to the experimental design. Test tubes were placed in ultrasonic bath model Eco-Sonics Q 5.9/25 (Unique, São Paulo, Brazil) with 165 W of power and 25 kHz using different reaction times as determined by

Table 1. Factors, levels and sum of fatty acids (FA) for each procedure of the experimental design

Experiment	Reaction time of NaOH in methanol		Reaction time of H_2SO_4 in methanol		Concentration of H_2SO_4 in methanol		Concentration of NaOH in methanol		Sum of FA / (mg g^{-1} of sample)	
	Real / min	Coded	Real / min	Coded	Real / min	Coded	Real / min	Coded		
Full factorial design	1	6.0	-1	8.0	-1	0.2	-1	0.2	-1	14.6
	2	11.0	+1	8.0	-1	0.2	+1	0.2	-1	14.4
	3	6.0	-1	15.0	+1	0.2	-1	0.2	+1	19.2
	4	11.0	+1	15.0	+1	0.2	+1	0.2	+1	17.1
	5	6.0	-1	8.0	-1	0.5	-1	0.2	-1	18.1
	6	11.0	+1	8.0	-1	0.5	+1	0.2	-1	10.2
	7	6.0	-1	15.0	+1	0.5	-1	0.2	+1	17.2
	8	11.0	+1	15.0	+1	0.5	+1	0.2	+1	11.6
	9	6.0	-1	8.0	-1	0.2	-1	0.5	-1	14.8
	10	11.0	+1	8.0	-1	0.2	+1	0.5	-1	16.7
	11	6.0	-1	15.0	+1	0.2	-1	0.5	+1	14.7
	12	11.0	+1	15.0	+1	0.2	+1	0.5	+1	17.5
	13	6.0	-1	8.0	-1	0.5	-1	0.5	-1	22.1
	14	11.0	+1	8.0	-1	0.5	+1	0.5	-1	11.8
	15	6.0	-1	15.0	+1	0.5	-1	0.5	+1	16.3
	16	11.0	+1	15.0	+1	0.5	+1	0.5	+1	13.5
Axial point	17	3.50	$-\alpha$	11.5	0	0.35	$-\alpha$	0.35	+1	12.5
	18	13.5	$+\alpha$	11.5	0	0.35	$+\alpha$	0.35	+1	12.0
	19	8.5	0	4.5	$-\alpha$	0.35	0	0.35	$-\alpha$	10.3
	20	8.5	0	18.5	$+\alpha$	0.35	0	0.35	$+\alpha$	18.3
Central point	21	8.5	0	11.5	0	0.05	0	0.35	0	17.1
	22	8.5	0	11.5	0	0.65	0	0.35	0	17.1
	23	8.5	0	11.5	0	0.35	0	0.05	0	15.3
	24	8.5	0	11.5	0	0.35	0	0.65	0	18.9
	25	8.5	0	11.5	0	0.35	0	0.35	0	18.9
	26	8.5	0	11.5	0	0.35	0	0.35	0	18.5
	27	8.5	0	11.5	0	0.35	0	0.35	0	20.1
	28	8.5	0	11.5	0	0.35	0	0.35	0	16.9
	29	8.5	0	11.5	0	0.35	0	0.35	0	19.3

± 1 : factor points; $\pm\alpha$: axial points; 0: central points.

experimental design, see Table 1. After alkaline reaction time, 2.0 mL of H₂SO₄ in methanol was added, its concentration is also in accordance to the experimental design; test tubes were placed in ultrasonic bath for a time previously determined by experimental design; 1.0 mL of *n*-heptane was added; tubes were shaken for 30 s; then centrifuged at 2000 rpm for 1.0 min. Thereafter, 500 µL of internal standard (PI, 23:0 me) was added and the upper phase was collected for injection into GC.

Results and Discussion

Experimental design

Table 1 displays the results of FA sums of the avocado pulp for each experimental design procedure.

The highest results correspond to the experiment 13 with FA sum of 22.1 mg g⁻¹ of sample. Results were obtained using the same concentration of H₂SO₄ and NaOH in methanol, conversely, with different times of alkaline and acid reactions. In order to clarify the obtained model and the interactions between factors, the results were evaluated by the analysis of variance (ANOVA) and the response surfaces generated by Design Expert. Among the models indicated by the software (linear, and interaction between two factors (2FI)), 2FI was chosen as the most appropriate due to its high order of significance, low lack of fit, and reasonable agreement between the correlation coefficient obtained and the predicted correlation coefficient for the model. ANOVA parameters are listed in Table 2.

F-Value of the model was 6.96 indicating that it is significant. There is only 0.03% of chance that *F*-value of the model could occur due to noise. Values less than 0.500

indicate that the model terms are significant, values greater than 0.100 indicate that the model terms are not significant.

The lack of adjustment of *F*-value (3.35) implies that it is not relatively significant for pure error. There is 12.49% of chance that *F*-value for lack of adjustment is greater than noise.

Correlation coefficient (R², 0.8165) and coefficient of variation (CV, 12.65%) values indicate that the model obtained is satisfactory.

The model was adjusted based on real values as factor functions studied and indicated in equation 1:

$$\text{FA sum} = 18.0 - 1.0(\text{A}) + 0.8(\text{B}) - 1.8(\text{AC}) - 1.4(\text{A}^2) - 0.9(\text{B}^2) \quad (1)$$

Figure 1a demonstrates the level of acid time while time decreases and acid concentration increases, implicating the highest FA sum. As the acid concentration decreases, the FA sum is reduced since the acid concentration was not efficient to make the methylation reaction occur. For reagent concentration greater than 0.50 mol L⁻¹, saponification reaction favors the reduction of TAG amount available for transesterification. Higher amounts of soap produced difficult the separation of glycerol from methyl esters, reducing FA sum.

Figure 1b illustrates the quadratic effect of acid time in relation to FA sum, increasing with the decrease of time until 8.0 min, providing maximum yield of 22.06 mg g⁻¹ of sample; the reasons were already mentioned before.

Figure 1c shows the quadratic effect of alkaline concentration in relation to FA sum, providing a maximum yield of 22.1 mg g⁻¹ of sample, for reasons previously mentioned.

Table 2. ANOVA parameter models for proposed methodology (PM) in avocado sample

	Square sum	LD ^a	Square average	<i>F</i> -value	Prob > <i>F</i> ^b	
Model	171.83	6	28.64	6.96	0.0003	S ^b
A ^c	26.46	1	26.46	6.43	0.0188	–
B ^d	17.34	1	17.34	4.21	0.0522	–
AC ^{e,f}	52.56	1	52.56	12.77	0.0017	–
A ²	57.55	1	57.55	13.98	0.0011	–
B ²	23.94	1	23.94	5.82	0.0246	–
Residue	90.53	22	4.12	–	–	–
Lack of adjustment	84.90	18	4.72	3.35	0.1249	NS ⁱ
Pure error	5.63	4	1.41	–	–	–
Total color ^g	262.67	28	–	–	–	–

^aLD: degree of freedom; ^bProb > *F*: probability value associated with *F* value; ^cA: reaction time of NaOH; ^dB: reaction time of H₂SO₄; ^eC: concentration of NaOH; ^fAC: interaction between reaction time of NaOH and concentration of NaOH; ^gTotal color: total squares sum corrected to mean; ^hS: significant; ⁱNS: non-significant.

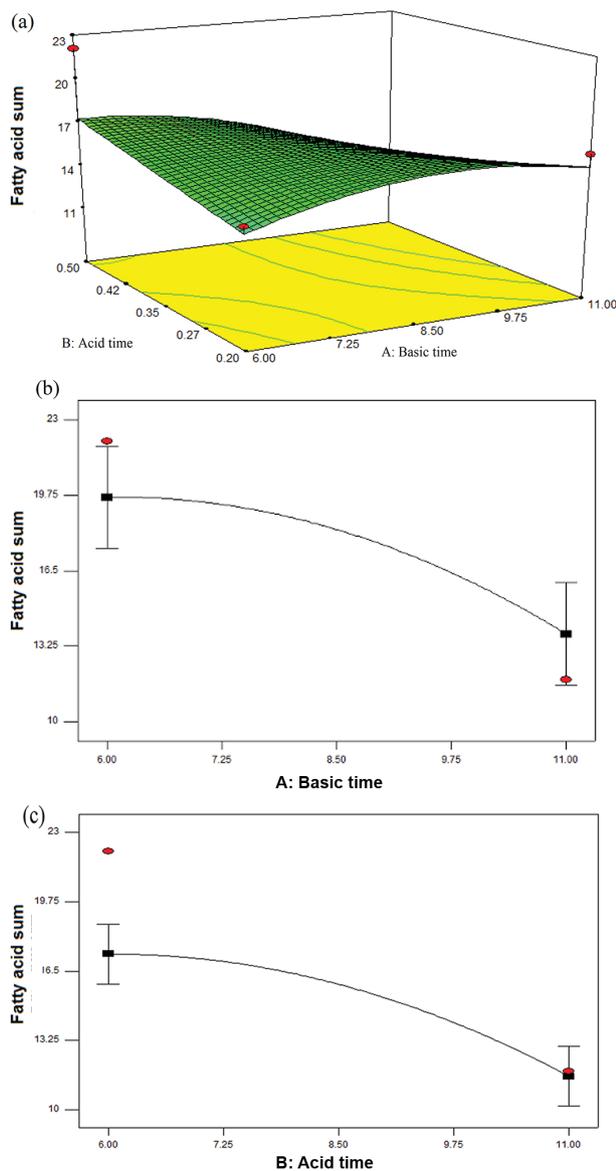


Figure 1. 3D graph of the response surface between the interactions A and B for the alkaline time and the sum of the FA concentration (a), alkaline time influence in FA sum (b) and reaction time influence in relation to yield (c).

Model optimization

In order to optimize PM conditions, parameters of concentration and reaction time, both acid and alkaline, were restricted to values that were sufficient for methylation, since reaction efficiency is essential. With these established criteria, Design Expert predicted the optimum point parameters of 6.00 min for alkaline reaction time and 8.00 min for acid reaction time, and for concentrations the optimum point for acid and alkaline was 0.5 mol L^{-1} , consequently the maximum value obtained with these data was FA sum of $22.16 \pm 2.80 \text{ mg g}^{-1}$, being higher than TM, which was $21.27 \pm 3.01 \text{ mg g}^{-1}$.

Another significant aspect is that the PM reduces the amount of sample and reagents consumed, and the sample preparation for GC analysis became faster, cheaper and with less environmental aggression when compared to TM.

Evaluation of the efficiency of the methylation reaction by ESI-MS

ESI-MS analysis (fingerprinting) by direct infusion was carried out in order to identify secondary metabolites present in extracted oils from avocado pulp, as well as to evaluate TM efficiency in comparison to PM of lipid extraction, esterification and transesterification. Figure 2a displays the mass spectra of the oil extracted from avocado, while Figures 2b and 2c display the mass spectra of non-esterified compounds by PM and TM, respectively.

The main TAGs identified in extracted oil were: triacylglycerol 15:1-15:1-15:1 (m/z 776.74), triacylglycerol 12:0-17:1-17:2 (m/z 790.79), triacylglycerol 16:0-16:0-18:0 (m/z 834.76), triacylglycerol 17:0-17:0-17:1 (m/z 864.81), triacylglycerol 17:0-17:1-18:0 (m/z 878.60), triacylglycerol 18:0-18:0-18:0 (m/z 908.78), triacylglycerol 18:0-18:0-18:1 (m/z 922.83).

Figure 2b represents the amount of non-esterified compounds by PM, while Figure 2c represents the amount of non-esterified compounds by TM, and, as can be observed, PM presented a lower amount of it in comparison to TM. The major diacylglycerols (DAG) detected as non-esterified products were lower in PM, as can be seen in Figure 2b. In $[\text{DAG}]^+$ form, it was detected compounds with m/z 600.73 (16:0 + 17:0), 605.71 (17:0 + 17:0), 647.73 (18:0 + 18:0), 656.86 (17:0 + 20:0), 680.67 (17:2 + 22:0), 682.81 (18:0 + 21:0) and 740.71 (21:0 + 22:0). TAG amount detected for PM was inferior than TM. For PM, the $[\text{TAG}]^+$ encountered were 827.64 (15:0 + 18:1 + 15:0) and 879.04 (16:1 + 18:0 + 18:0), while for TM the $[\text{TAG}]^+$ were 768.93 (12:0 + 12:0 + 20:0), 796.96 (12:0 + 16:0 + 18:0), 822.98 (16:0 + 16:0 + 16:1) and 877.03 (17:0 + 17:2 + 18:0).

The non-esterified products in PM were inferior; it can be observed through the results obtained by GC-FID, since PM sum was $22.16 \pm 5.80 \text{ mg g}^{-1}$ of sample, while TM sum was $21.27 \pm 5.84 \text{ mg g}^{-1}$ of sample. Therefore, results are consistent once in TM is necessary to make a previous lipid extraction, and during this extraction, other non-lipid compounds, which have affinity with the solvents employed, can be also extracted. In PM it does not occur because the esterification and the transesterification are performed directly in the matrix, minimizing the errors.

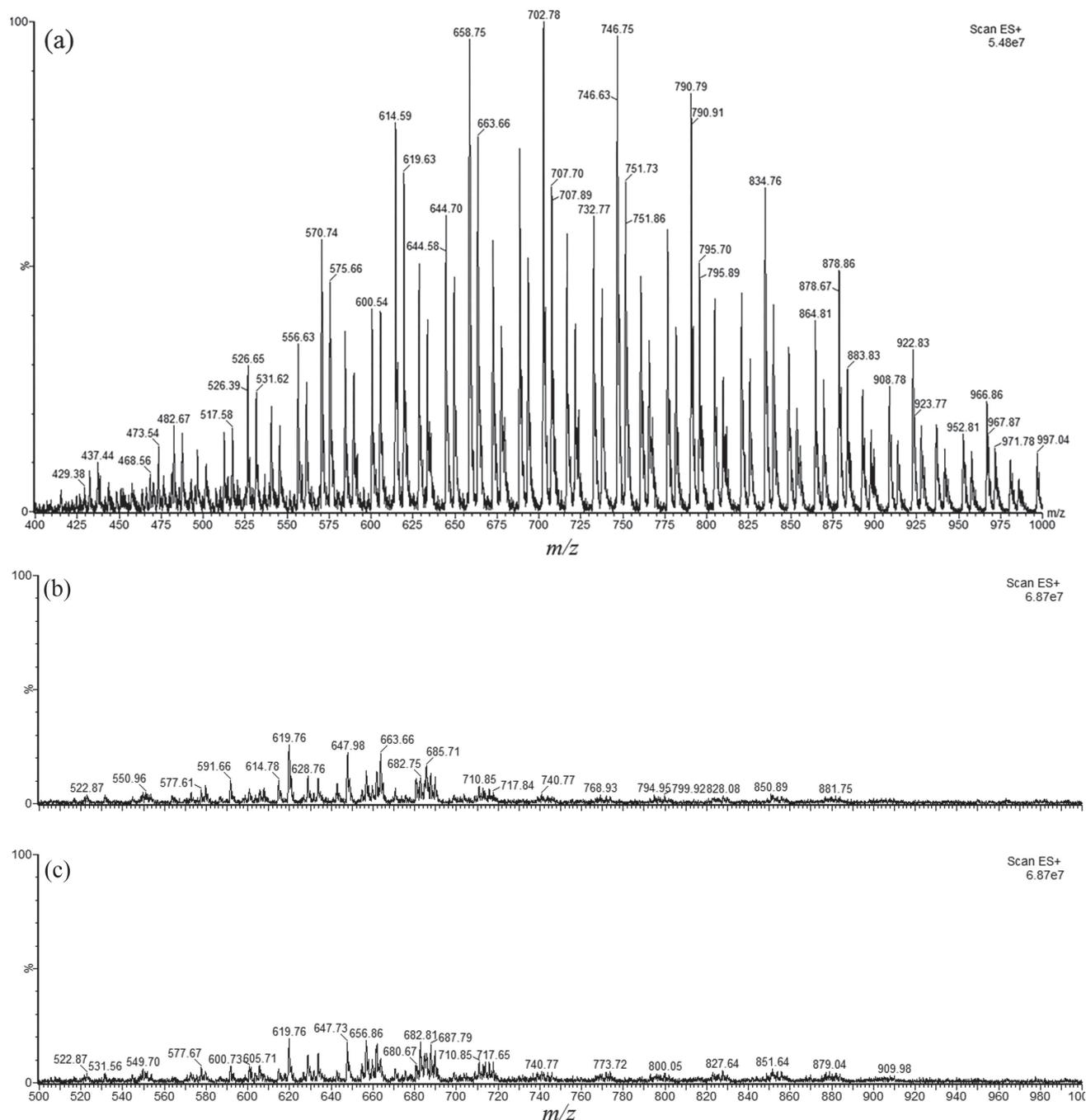


Figure 2. (a) Spectra obtained by ESI-MS(+) from secondary metabolites present in avocado oil; mass spectrum of non-esterified compounds by (b) PM and (c) TM.

Fatty acid analysis

Table 3 expresses the results of FA quantification for avocado pulp carried out by traditional extraction, esterification and transesterification methodology in comparison to PM.

Avocado presents high humidity, fibers, sugars, as well as soluble pigments that difficult the lipids extraction by TM, obtaining considerable deviations in the results. In this

way, results obtained by PM in avocado samples caused a reduction of these procedural errors, due to the withdrawal of some steps. Consequently, PM was very effective when applied to avocado pulp.

Avocado oil is constituted mainly of monounsaturated fatty acids (MUFA, 60%), saturated fatty acids (SFA, 29%) and a significant amount of polyunsaturated fatty acids (PUFA, 11%). The application of the proposed method in avocado oil influenced, mainly, the concentration of

18:2n-6 FA and, consequently, PUFA and n-6, which presented significant difference.

Table 3. Fatty acids quantification by traditional extraction, esterification and transesterification methodology in comparison to proposed method

CN/BD	Avocado / (mg g ⁻¹ of sample)	
	TM	PM
16:0	7.65 ^a ± 1.75	6.68 ^a ± 1.62
16:1n-7	0.85 ^a ± 2.30	1.13 ^a ± 1.55
18:0	0.17 ^a ± 1.30	0.21 ^a ± 1.51
18:1n-9	8.3 ^a ± 1.1	7.50 ^a ± 0.11
18:1n-7	0.75 ^a ± 2.1	1.03 ^a ± 2.50
18:2n-6	3.23 ^b ± 4.4	5.30 ^c ± 3.86
18:3n-3	0.32 ^a ± 1.2	0.31 ^a ± 1.50
SFA	7.82 ^a ± 3.05	6.89 ^a ± 3.13
MUFA	9.9 ^a ± 5.5	9.65 ^a ± 4.16
PUFA	3.55 ^b ± 5.6	5.61 ^c ± 5.36
n-6	3.23 ^b ± 4.4	5.30 ^c ± 3.86
n-3	0.32 ^a ± 1.2	0.31 ^a ± 1.50
n-6/n-3	10.10 ^a ± 3.67	16.99 ^a ± 2.57
PUFA/SFA	0.45 ^a ± 1.84	0.81 ^a ± 3.0
ΣFA	21.27 ^a ± 5.84	22.16 ^a ± 5.80

Results expressed as mean ± variation coefficient of the three replicates. ΣFA: FA sum; SFA: saturated FA sum; MUFA: monounsaturated FA sum; PUFA: polyunsaturated FA sum; n-6: ω-6 FA sum; n-3: ω-3 FA sum; CN/DB: carbon number/double bound; TM: traditional methodology; PM: proposed methodology. Different letters on the same line indicate a significant difference ($p < 0.05$) for *t*-test.

Table 4. Fatty acids (FA) quantification by traditional extraction, esterification and transesterification method in comparison to the proposed method

CN/DB	FA / (mg g ⁻¹ of sample)									
	Apple		Banana		Mango		Coconut		Backyard avocado	
	TM	PM	TM	PM	TM	PM	TM	PM	TM	PM
8:0	–	–	–	–	–	–	2.23 ^e ± 3.2	4.0 ^f ± 3.1	–	–
10:0	–	–	0.042 ^a ± 1.3	0.085 ^a ± 1.7	0.30 ^c ± 1.5	0.097 ^b ± 2.0	8.42 ^e ± 3.5	4.3 ^f ± 3.1	–	–
12:0	–	–	–	–	–	–	32.31 ^e ± 4.3	48.9 ^f ± 2.1	–	–
14:0	–	–	0.020 ^a ± 1.4	–	–	–	13.82 ^e ± 2.1	24.0 ^f ± 2.9	–	–
16:0	0.08 ^b ± 3.8	0.04 ^b ± 4.0	0.61 ^a ± 2.5	0.075 ^a ± 3.0	0.34 ^c ± 1.2	0.2 ^b ± 2.7	8.58 ^e ± 3.8	14.3 ^f ± 4.1	9.8 ^m ± 1.3	12.9 ^m ± 1.6
16:1n-7	–	–	0.046 ^a ± 1.2	–	0.07 ^d ± 0.9	–	–	–	0.90 ^m ± 0.4	2.7 ^m ± 0.5
18:0	0.014 ^a ± 1.4	–	0.024 ^a ± 1.8	0.40 ^a ± 2.0	0.03 ^d ± 1.8	–	2.21 ^e ± 3.3	4.2 ^f ± 3.0	0.25 ^m ± 1.6	0.32 ^m ± 0.9
18:1n-9	0.099 ^c ± 2.3	–	0.26 ^a ± 1.1	0.25 ^a ± 1.5	0.35 ^c ± 0.7	0.22 ^b ± 0.8	8.84 ^e ± 4.2	13.2 ^f ± 4.5	14.4 ^m ± 0.7	13.8 ^m ± 0.5
18:1n-7	–	–	0.32 ^a ± 1.0	0.002 ^a ± 2.2	0.25 ^c ± 1.9	0.12 ^b ± 0.9	1.59 ^e ± 2.6	3.4 ^f ± 2.5	0.46 ^m ± 1.3	0.53 ^m ± 1.5
18:3n-3	–	–	–	–	–	–	–	–	0.06 ^m ± 0.7	0.35 ^m ± 0.4
18:2n-6	0.020 ^b ± 1.1	0.083 ^b ± 0.9	–	–	–	–	–	–	5.49 ^m ± 1.3	6.80 ^m ± 0.9
ΣFA	0.213 ^b ± 8.6	0.12 ^b ± 4.9	1.32 ^a ± 10.3	0.812 ^a ± 9.8	1.34 ^c ± 8.0	0.637 ^b ± 6.5	78.0 ^e ± 27.0	116.3 ^f ± 25.3	31.4 ^m ± 7.5	37.4 ^m ± 7.5

Results are expressed as standard deviation (SD) of the three replicates. Different letters on the same line indicate a significant difference ($p < 0.05$) for *t*-test. CN/DB: carbon number/double bound; TM: traditional methodology; PM: proposed methodology; ΣFA = FA sum.

Application of the proposed method using different samples

Table 4 expresses the results of FA quantification for coconut, banana, apple, mango and another avocado variety, carried out by traditional extraction, esterification and transesterification methodology in comparison to PM.

Fruits containing 1% of lipid content, such as mango, banana and apple, the PM is not applicable, that could be explained by the small amount of sample or the non-sufficient reagent concentration, implying in the not occurrence of methylation, which means that the sample is diluted, consequently, the fatty acids amount identified by GC-FID for PM is lower than TM.

The reduction of procedural errors in the analysis because of the shortened process resulted in better results for avocado and coconut samples in the PM, since in PM it is not necessary the previous lipids extraction causing reduction in the amount of solvent and sample in comparison to TM.

Therefore, PM can be applied to fruits with a lipid percentage greater than 5%.

Method validation

Accuracy values ranged from 89.05 to 100.21% being in accordance with acceptable values. $RSD_{intra-day}$ (0.30 to 1.30%) and $RSD_{inter-day}$ values (0.32 to 1.44%) revealed that PM for FA derivatization has good precision; usually in analysis methods the RSD estimation values should be below 20%.¹²

The linear interval was performed with avocado and coconut samples, indicating the range of 5.0 to 12.0% of lipids.

Conclusion

Through the surface response methodology, it was possible to optimize and validate a new, fast and efficient methylation technique, which was performed in only 14.00 min (6.00 min of sonication after addition of NaOH solution and 8.00 min of sonication after addition of H₂SO₄ solution), and it can be applied efficiently in fruit samples with lipid percentage of 6.0 to 12.0%.

ESI-MS analysis allowed evaluating the PM efficiency in comparison to the traditional lipid extraction, esterification and transesterification methodology. PM occasioned in fewer DAG and TAG residues after reaction, minimizing the usage of sample and reagent amounts. PM also allowed the samples preparation for FA analysis by gas chromatography to be performed faster, cheaper and with less environmental impact when compared to TM.

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