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Simultaneous Quantitation of FFA, MAG, DAG, and TAG in Enzymatically Modified Vegetable Oils and Fats

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Abstract A novel and easy-to-implement high temperature gas chromatographic procedure for the simultaneous quantitation of free fatty acids (FFA), monoacylglycerols (MAG), diacylglycerols (DAG), and triacylglycerols (TAG) for products arising from fats and oils modification processes has been developed. The method involves silylation in the presence of pyridine at room temperature, standard injection system, and FID detection. Species were separated by carbon number and degree of unsaturation; thus, a total of 53 individual compounds could be quantified. An identification plot for unavailable triacylglycerol standard species relating relative retention time with the number of double bonds was built. Calibration curves for the quantitation of by-products (FFA, MAG, and DAG) and a calibration surface for TAG were constructed using standard solutions. Multiple internal standards were used, achieving good repeatability. The method was tested over a wide range of fatty acid unsaturation level (0 to 3 double bonds) and chain length (C14 to C18). The results achieved in real samples were in agreement with those obtained by fatty acid methyl esters (FAME) analysis and HPLC-RI. The method developed is a rapid and reliable technique to quantify FFA, MAG, DAG, and TAG in substrates and products of modification, ideal to monitor the degree of conversion in fats and oils enzymatic esterification, among others.

Keywords Oils · Triacylglycerols · Diacylglycerols · Monoacylglycerols · Free fatty acids · HTGLC

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Introduction

The physical nature of fats and oils is determined mainly by their chemical compositions, namely, chain length and degree of unsaturation of fatty acids as well as their distribution among the glycerol backbone positions of triacylglycerols (TAG) (da Silva et al. 2010). If hydrolysis has occurred to any extent, by-products such as free fatty acids (FFA), monoacylglycerols (MAG), and diacylglycerols (DAG) could alter their physical properties (Pacheco et al. 2012). During oils and fats technological modification, such as interesterification or acidolysis reactions, the degree of conversion must be carefully controlled in order to obtain products with desired chemical or physical characteristics. Changes in chemical properties (TAG composition; relationship among specific TAG species; by-products composition, FFA, MAG, and DAG; and positional isomers of byproducts ratios, among others) can be used to monitor not only the primary reaction progress but also that of secondary reactions, e.g., acyl migration phenomenon.

TAG are the major components of oils and fats, inclusive after most technological modifications, if hydrolysis is excluded. Nowadays, TAG composition can be determined either by high performance liquid chromatography (HPLC) or by high temperature gas liquid chromatography (HTGLC), making use of different injector or detector types (Rezanka and Rezanková 1999; Aparicio and Aparicio-Ruiz 2000; Laakso 2002; Hammond 2003; Ribeiro et al. 2009a). HPLC—in its two most popular versions, Ag + −HPLC in normal phase mode and non-aqueous reversed-phase HPLC (NARPHPLC)—is the most employed (Lerma-García et al. 2011). Its use is recommended by AOCS Official Methods (AOCS 2006) to analyze TAG composition of liquid vegetable oils (Ce 5b-89), and vegetable and animal oils and fats, paying special attention to the presence of hydrogenated fats whose TAG could not be well resolved

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(Ce 5c-93). High molecular mass TAG are not easy to elute from HPLC columns due to their insolubility in a number of popular mobile phases (Fernández-Moya et al. 2000; Buchgraber et al. 2004a). A further disadvantage is that commonly available HPLC detectors are only compatible with isocratic elution (refractive index (RI) detector) or the detector response is influenced by the unsaturation of the separated substances (UV detector), which renders quantitation unreliable (Buchgraber et al. 2004a). Additionally, HTGLC shows some clear additional advantages over HPLC, like higher resolution, avoidance of toxic chemicals, and lower run times and cost (Buchgraber et al. 2004a; Dijkstra et al. 2007). It has, nonetheless, been accused of having some drawbacks related to deterioration of columns stability with temperature and alteration of compounds during analysis (Aparicio and Aparicio-Ruiz 2000). Much has been done in order to increase temperature stability of columns stationary phases. Polarizable columns show high selectivity, separating acylglycerols primarily by carbon number and secondarily by their degree of unsaturation (Buchgraber et al. 2004a), being stable up to 370 °C (Geeraert and Sandra 1987). Moreover, on a polarizable column, the separation of TAG such as POO and PLSt, which are TAG with equal carbon number and degree of unsaturation but with different polarity, can be achieved (Antoniosi Filho et al. 1995). Regarding the degradation of highly unsaturated compounds, the use of individual response factors (RF) for the quantitation of species helps to overcome this drawback since, if any loss or alteration of compounds occurs during the GLC procedure, its effect would be compensated by the application of the corresponding RF. Although several authors have stressed and demonstrated the need for the application of RF (Rezanka and Mares 1991; Carelli and Cert 1993; Buchgraber et al. 2000; Buchgraber et al. 2004a), various recent works have avoided their use when analyzing TAG containing unsaturated fatty acids (Viera-Alcaide et al. 2007; Silva et al. 2009; Ribeiro et al. 2009b; Ribeiro et al. 2009c; Ruiz-Samblás et al. 2012; Mozzon et al. 2013; Guedes et al. 2014).

Nowadays, industry technicians and researchers require techniques to adequately characterize fats modification products making use of laboratory equipment with inexpensive components or methods including limited intermediate purification steps. Flame ionization detection (FID) is inexpensive and robust; thus, its coupling to a HTGLC would provide an adequate tool to monitor these processes if satisfactory response factors are determined.

Just as with all complex lipid mixtures, it is difficult to separate, identify, and quantify most of these individual components using a single chromatographic method (Mangos et al. 1999).

The objective of the present work is to present a rapid, reliable, and easily applicable method for the simultaneous quantitation of TAG, DAG, MAG, and FFA contained in fats and oils modification products, according to acyl chain length and degree of unsaturation. The fact that no pre-separation step is needed and the use of equipment widely available in common laboratories make this method ideal for routine use. The method was developed taking the one proposed by Plank and Lorbeer (1995) as a starting point. This last method was developed for the determination of glycerol, MAG, DAG, and TAG in vegetable oil methyl esters produced by alkali catalyzed transesterification of rapeseed oil. DAG and TAG were separated only by carbon number, obtaining only three and four peaks for each group of compounds, respectively. No FFA were present in the samples, and three peaks of MAG were distinguished. A huge quantity of modifications was introduced in the present work to obtain a much more detailed technique capable of detecting, identifying, and quantifying up to 53 different species when products from enzymatically interesterified soybean oil and fully hydrogenated soybean oil were analyzed. The method is applicable to refined natural and processed vegetable fats and oils with 0 to 3 double bonds and a fatty acid carbon number of 16 to 18, although some evidence that samples with 14 carbons fatty acids could be also analyzed is presented.

Materials and Methods

Materials

Refined vegetable oils (soybean and sunflower oils) were provided by Molinos Río de la Plata SA (Buenos Aires, Argentina), fully hydrogenated soybean oil was kindly provided by Calsa S.A. (Buenos Aires, Argentina), and palmitic-stearic acids mixture was obtained from Fluka (Buchs, Switzerland). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Fluka (Buchs, Switzerland). All standards used for the development of the HTGLC technique were of purity greater than 96 % and were obtained from Sigma Chemical Co. (St. Louis, USA), while fatty acid methyl esters (FAME) standards were purchased from Supelco (Bellefonte, USA). Pyridine was from J.T. Baker (Philipsburg, USA). All other reagents, gases, and solvents were of analytical or chromatographic grade.

Methods

Sample Derivatization

Free hydroxyl groups of FFA, MAG, and DAG must be silylated in order to increase the volatility and thermal stability of compounds. The application of this procedure ensures good peak formation and low detection limits, thus obtaining a robust chromatographic method (Plank and Lorbeer 1995). In this referenced work, the authors developed four simple and

rapid methods to reach complete silylation reaction of partial glycerides samples choosing the one applied in the present work. It consists of adding MSTFA (silylation reagent) and pyridine (catalyst) to the sample. The use of pyridine allows the reaction to proceed at ambient temperature within only 15 min, a further advantage of the technique when unsaturated samples are being analyzed.

Chromatographic Conditions

Glycerides and FFA quantitation was performed by HTGLC by means of a 4890D series gas chromatograph (Agilent, Hewlett-Packard) equipped with a FID. The injector was used in split mode. A metallic capillary column (MXT-65TG, 30 m×0.25 mm×0.1 μm film thickness; Restek, Bellefonte, USA) with a crosslinked 65 % diphenyl/35 % dimethyl polysiloxane stationary phase was used.

Chromatographic conditions to simultaneously determine the four groups of major components of substrates and interesterified fats and oils (FFA, MAG, DAG, and TAG) were established adapting and merging two techniques: one intended to determine the concentration of interesterification secondary products (FFA, MAG, and DAG) according to Gutiérrez-Ayesta et al. (2007), and the other one implemented to establish TAG composition of samples. The latter was optimized to obtain good peaks separation by testing different operative conditions adapted from published works and official methods (Rezanka and Mares 1991; Buchgraber et al. 2000; Buchgraber et al. 2004b; Restek 2005–2006; AOCS 2006).

Final conditions of chromatographic analyses were as follows: split injector at 360 °C (60:1 split ratio); oven temperature programming, 40 °C (4 min), increment until 350 °C at 25 °C/min, and then to 354 °C at the rate of 0.2 °C/min, total run time 36.4 min; and FID temperature, constant and equal to 380 °C. Hydrogen was used as the carrier gas at a linear velocity of 41 cm/s.

Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, Rev. A.01.01).

Quantitation Method

The detector response was not the same for all analytes; hence, the area percentage method could not be used. The use of an internal standard (IS) enables the degree of determination accuracy to be improved since it accounts for any variations in gas chromatograph performance or analyst precision (Poole 2003). Therefore, the multiple point internal standard method was selected as the quantitation method, and relative response factors (RRF) were obtained relating area and concentration of each analyte to the corresponding area and concentration of the IS.

Calibration Curves FFA, MAG, and DAG. Tetradecane (TD) was selected as the IS and oleic acid (O) was used as the reference compound to construct the FFA calibration curve. It was considered that all FFA in the analyzed interesterified samples had the same RRF. It is to be stated that most of the fatty acids had chain lengths of 16 to 18. If applied to samples with a wider range of chain length, the invariability of RRF within fatty acids could not be guaranteed. Tricaprin (CCC) was used as the IS for both groups of partial glycerides (MAG and DAG), while monopalmitin (MP) and dipalmitin (DP) were used to construct the calibration curves of MAG and DAG, respectively (Gutiérrez-Ayesta et al. 2007).

Stock solutions of standards in pyridine were used to prepare injection solutions. Mass concentration ratios of standard compounds to IS in each injection solution varied between 0.10 and 1.66. The concentration of IS was constant for all points of the calibration curves and equaled 0.842 mg/mL. The required amount of MSTFA depends on the composition and type of compounds in the sample. As it was stated above, FFA, MAG, and DAG are prone to be silylated; therefore, the minimum quantity of MSTFA required augments with the concentration of standard compounds in each injection solution. In previous assays, it was checked that the only effect of an excess of MSTFA was a dilution one when all the compounds were completely silylated (data not shown). As a reference value, 60 μL were added to the highest concentration solution, containing 0.12 mg of each standard (O, MP, and DP). Monopalmitin, being the compound with the highest number of free hydroxyl groups, serves as an indicator of incomplete derivatization. In case of insufficient silylation, this peak appears splitted and reduced in height (Plank and Lorbeer 1995).

TAG. Tripalmitolein (PoPoPo) was selected as the IS to determine TAG composition since palmitolein is not an important constituent fatty acid of the samples. Moreover, the presence of this TAG in the products was evaluated and discarded. If the content of palmitolein in the samples to be analyzed is important, then another TAG should be selected as IS.

Previous assays showed that RRF for TAG with different degrees of unsaturation were significantly different. Therefore, a unique calibration curve for all of them could not be applied. Ten commercial standards were available (PPP, POP, PStSt, OPO, POL, StStSt, StStO, StOO, OOO, and LLL; where P, O, St, and L are palmitic, oleic, stearic, and linoleic acids, respectively), and a maximum of 25 chromatographic peaks were obtained in the TAG range of the interesterification products. Therefore, the concept of relative retention time (rrt) (AOCS 2006; Carelli and Cert 1993) (Method Ce 5b-89), which relates retention time (rt) of the considered analyte to solvent and IS rt, was used according to Eq. 1.

$$\mathrm{rrt} = \frac{\mathrm{rt}_{\mathrm{analyte}} - \mathrm{rt}_{\mathrm{solvent}}}{\mathrm{rt}_{\mathrm{IS}} - \mathrm{rt}_{\mathrm{solvent}}} \quad (1)$$

This parameter, together with the FID response to each of the 10 TAG standards, permitted the construction of a surface according to Eq. 2. Hence, the RRF of any TAG in the analyzed range and, consequently, its concentration, could be obtained knowing its rrt and the parameters A, B, and C of this equation.

$$\frac{1}{\mathrm{RRF}} = \left(\mathrm{A}\ \mathrm{rrt}^2 + \mathrm{B}\ \mathrm{rrt} + \mathrm{C}\right) \quad (2)$$

It can be observed that a calibration surface is obtained, rather than a curve. Standard injection solutions were prepared by previous estimation of the range of expected concentrations for each TAG species in the samples to be analyzed. Purity of standards was calculated by mass balance determining the concentration of probably present FFA, MAG, and DAG in the total weighted mass (according to the method described above). This parameter was taken into account to calculate the true concentration of each TAG in the injection solutions.

Different injection solutions were prepared in order to obtain standard/IS mass ratios varying from 0.158 to 7.481. The IS concentration for all solutions was 0.456 mg/mL. Pyridine was added in order to obtain the desired final concentrations, while the added volume of MSTFA was 5 μL. This volume was established based on previous assays, which showed that it is sufficient to derivatize the small amount of impurities which are present in the commercial TAG standards (data not shown).

Solutions were prepared in duplicate and injected by triplicate (injection volume, 1 μL).

Sample Preparation A stock solution was firstly prepared by weighing at least 3 mg of sample in Eppendorf tubes and adding the corresponding pyridine volume to reach a concentration of 38.2 mg/mL. Afterwards, the injection solution was prepared mixing 15 μL of this stock solution, 4.5 μL of TD solution (10.6 mg/mL in pyridine), 4.5 μL of CCC solution (10.6 mg/mL in pyridine), 5.2 μL PoPoPo solution (5.0 mg/mL in pyridine), and 28 μL MSTFA. The injection volume was 1 μL. Before injection, all solutions were mixed in an ultrasonic bath.

Validation

The developed FFA, MAG, DAG, and TAG quantitation method was validated by calculating linearity, repeatability, by performing a recovery experiment, and by comparing different samples' compositions obtained by HPLC-RI and fatty acid methyl esters (FAME) analysis (GLC). In order to evaluate the instrumental precision, a sample from interesterified soybean oil and fully hydrogenated soybean oil was prepared and consecutively analyzed seven times. On the other hand, the repeatability of the method was established by preparing and analyzing seven samples of the same specimen. For the evaluation of the recovery, a spiked interesterified sample containing known amounts of standard substances was analyzed.

TAG Analysis by HPLC-RI Oil samples were purified according to AOCS Official Method Cd 20–91 (2006). Non-polar fractions were dissolved in acetone:chloroform (50:50 *v*/*v*) and analyzed by HPLC-RI according to AOCS Official Method Ce 5c-93 (2006). A Waters e2695 chromatograph (Waters Corp.) equipped with a RI detector (Waters 2414) and a Lichrosphere 100 RP-18 (5 μm) column (25 cm×4 mm I.D., Merck) was used. The mobile phase, acetone:acetonitrile 60:40 (*v*/*v*), was set at a linear velocity of 1 mL/min, and the injector was used in automatic mode with 10 μL of injection volume. Data acquisition and peak integration were performed using Empower 2 software (Waters Corporation, 2005–2008). Results were expressed as area percentage of the total TAG area.

FAME Analysis by GLC Fatty acids of samples were converted to FAME by cold transesterification with methanolic KOH according to AOCS Official Method Ce 2–66 (2006) and subsequently analyzed by GLC. A 4890D series gas chromatograph (Agilent, Hewlett-Packard) equipped with a fused silica capillary column (SP2380, 30 m×0.25 mm×0.2 mm film thickness; Supelco Inc.) was used. Hydrogen, as the carrier gas, was set at a linear velocity of 18 cm/s, and the injector was used in split mode with a ratio of 1:50. FID was maintained at 220 °C, as well as the injector. The oven temperature programming was as follows: 170 °C for 15 min, ramp temperature to 210 °C at a rate of 4 °C/min, and held at final temperature for 10 min. FAME were identified by comparing their retention times with authentic standards. Data acquisition and peak integration were performed using HP 3398A GC Chemstation Software (Hewlett-Packard, Rev. A.01.01).

Results and Discussion

Considering that the main objective of the present work was the analysis of the composition of semisolid products arising from enzymatic modification of vegetable oils and fats, achieving a comprehensive separation of the different groups of resulting compounds was essential. TAG are the major constituents of substrates and products; hence, the chromatographic conditions (oven temperature programming, carrier gas flow, and injector and detector temperature) were optimized in order to obtain the best possible resolution of the different TAG species present in this type of samples. However, a very good separation and identification of FFA and partial glycerides were obtained. In effect, the method has

Table 1 Parameters corresponding to Eq. 2

Parameter	Value[a]	R^2
A	0.558±0.004	0.9814
B	0.795±0.006	
C	−0.181±0.002	

[a] Average±standard deviation. n=103

been already applied to the quantitation of MAG and DAG obtained by partial hydrolysis of crude sunflower oil lecithin catalyzed by phospholipase A1 (Goñi et al. 2011).

Calibration Curves

The standards for FFA, MAG, and DAG (O, MP, and DP) generated linear calibration curves in the analyzed concentration range. In the case of FFA, its relative response factor (RRF_{FFA}) was found to be near unity (RRF_{FFA}=0.9835, R^2=0.9993) which was in accordance with FAME analysis by GLC (Method Cd 11b-91). MAG showed a RRF higher than unity (RRF_{MAG}=1.5676, R^2=0.9996), while DAG RRF was also close to unity (RRF_{DAG}=1.0979, R^2=0.9995). According to Method Cd 11b-91 (2006), the absence of product decomposition and losses in the chromatographic system could be ensured when all RRF are higher than 0.5. Linearity of TAG standards in the analyzed range was also confirmed, presenting R^2 values higher than 0.9730.

Regarding TAG calibration surface, area ratios (TAG standard/IS), and relative retention times obtained by chromatographic analysis were related to their corresponding mass ratios according to Eq. 2 through no linear regression. As previously mentioned, mass ratios were calculated considering the purity of the commercial standards, which ranged from 96.8 to 98.7 %. The obtained parameters of Eq. 2 are shown in Table 1.

Different samples were analyzed by the procedure described herein for a long time (more than a year) with interruptions in the equipment use (maintaining the column into the equipment). Calibration curves were obtained from time to time to control the system performance. Over more than a year of use, the same calibration curve adjusted new data significantly (95 % confidence).

Peak Identification in Samples

A total of 53 peaks—considering FFA, MAG, DAG, and TAG—was distinguished in interesterification samples. Those peaks whose identification was not possible due to unavailability of their original standards were elucidated analyzing controlled mixtures of glycerides and FFA obtained through interesterification reactions between commercial monoacid standards. In the case of TAG, which are the major components of both substrates and products of the interesterification reactions, an additional resource (adapted from AOCS Official Method Ce 5b-89 (2006)) was also used to ensure a correct identification of all peaks. It consists in the representation of the logarithm of the relative retention time of the different compounds versus their number of double bonds, as it is presented in Fig. 1. Firstly, the monoacid original standards were located in the graph, and straight lines between them were traced. Secondly, corresponding data from both original and generated mixed standard TAG were also located as a form of validation. TAG constituted by just two types of fatty acids were located over the line joining the two corresponding monoacid TAG. Regarding the TAG having three

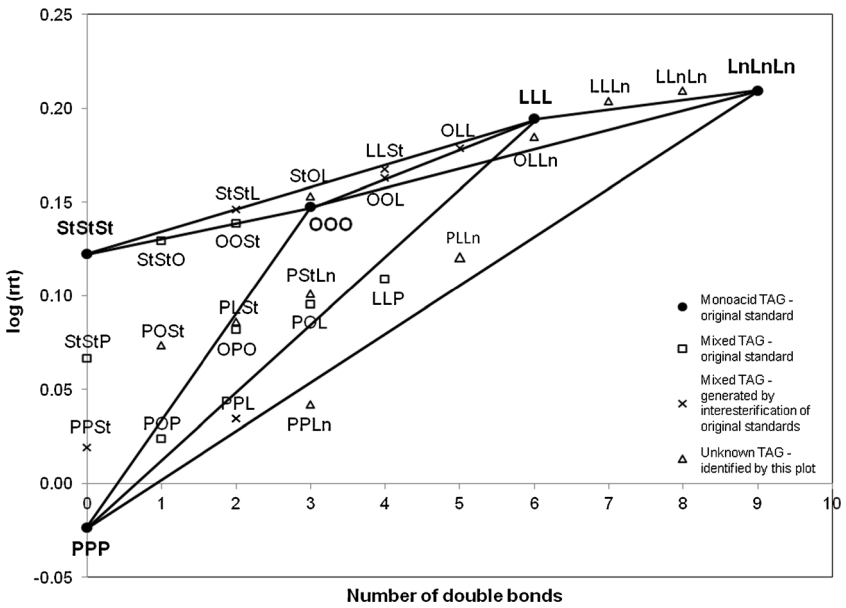
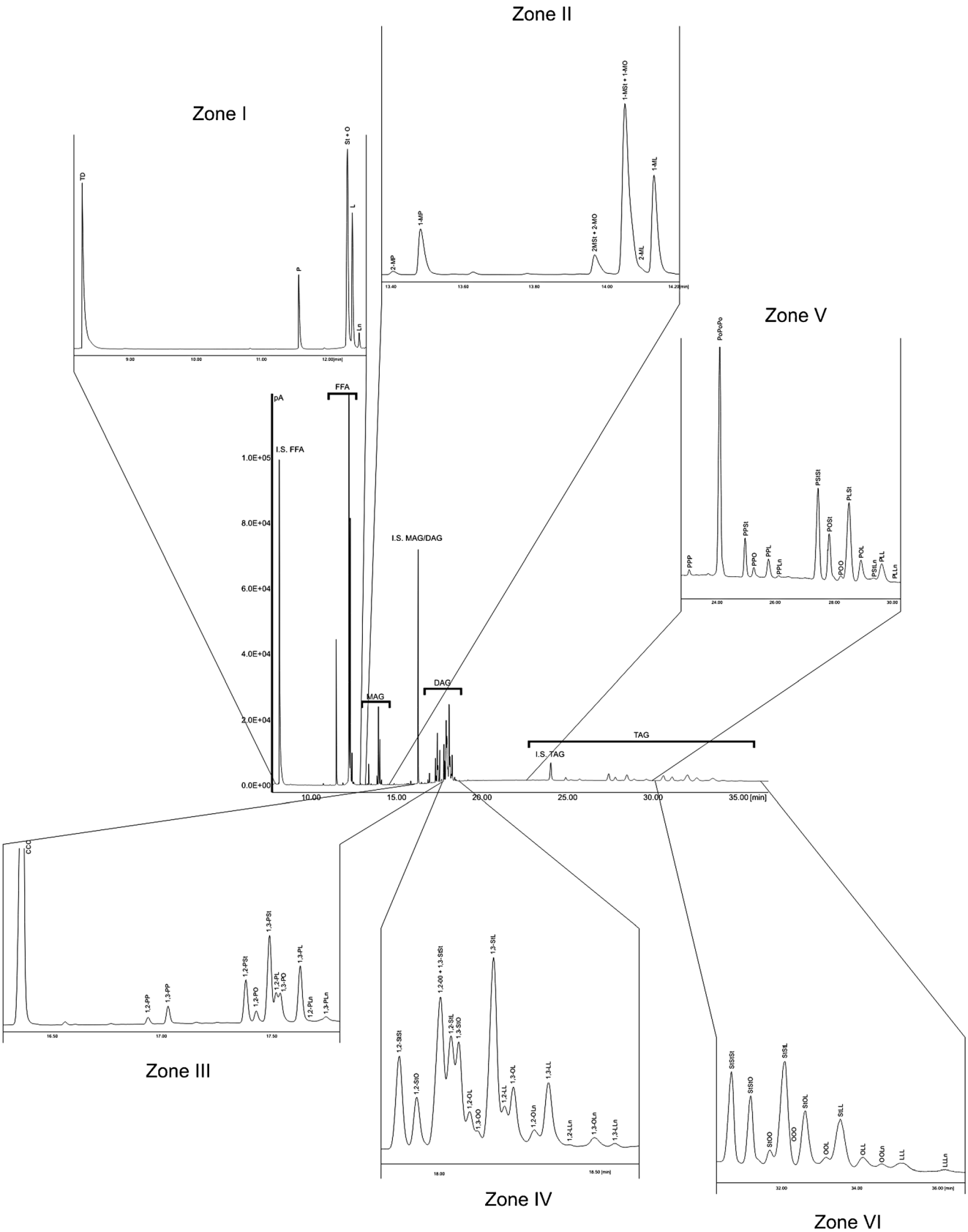


Fig. 1 Plot for the identification of unknown TAG peaks


Zone I
Zone II
Zone III
Zone IV
Zone V
Zone VI
TD
P
St + O
L
Ln
2-MP
1-MP
2MSt + 2-MO
1-MSt + 1-MO
2-ML
1-ML
pA
I.S. FFA
FFA
I.S. MAG/DAG
MAG
DAG
TAG
I.S. TAG
1.0E+05
8.0E+04
6.0E+04
4.0E+04
2.0E+04
0.0E+00
10.00
15.00
20.00
25.00
30.00
35.00 [min]
PoPoPo
PPP
PPSt
PPO
PPL
PPLn
PSISt
POSt
PLSt
POL
PSILn
PLL
PLLn
CCC
1,2-PP
1,3-PP
1,2-PSt
1,2-PO
1,2-PL
1,3-PO
1,3-PSt
1,3-PL
1,2-PLn
1,3-PLn
1,2-StSt
1,2-StO
1,2-00 + 1,3-StSt
1,2-StL
1,3-StO
1,2-OL
1,3-OO
1,2-LL
1,3-StL
1,3-OL
1,3-LL
1,2-OLn
1,2-LLn
1,3-OLn
1,3-LLn
StStSt
StStO
StOO
StStL
OOO
StOL
OOL
StLL
OLL
OOLn
LLL
LLLn

◀ **Fig. 2** Chromatogram of a soybean oil and a fully hydrogenated soybean oil interesterified sample. *P* palmitic acid, *St* stearic acid, *O* oleic acid, *L* linoleic acid, *Ln* linolenic acid, *Zone I* FFA zone, *TD* tetradecane (IS for FFA), *Zone II* MAG zone, *MP* monopalmitin, *MSt* monostearin, *MO* monoolein, *ML* monolinolein. No distinction is done between the isomers 1-MAG and 3-MAG. *Zones III* and *IV* DAG zone, *CCC* tricaprin (IS for MAG and DAG). No distinction is done between 1,2- and 2,3-DAG isomers. *Zones V* and *VI* TAG zone, *PoPoPo* tripalmitolein (IS for TAG). No distinction is done between positional isomers

different fatty acids, they were located in the space limited by the three lines joining the three corresponding monoacid TAG. Finally, by following the same procedure, the unknown peaks were elucidated.

Figure 2 shows the different elution zones of a typical chromatogram of a sample of interesterified soybean oil and fully hydrogenated soybean oil. All peaks were correctly identified. At the established chromatographic conditions, the following pairs of compounds eluted together: stearic and oleic acids (Fig. 2—zone I), 2-monostearin and 2-monoolein, 1-monostearin and 1-monoolein (Fig. 2—zone II), and 1,2-diolein and 1,3-distearin (Fig. 2—zone IV). Regarding TAG zone (Fig. 2—zones V and VI), the only critical pair resulted to be StStL/OOO. Ribeiro et al. (2009b) detected four (OPO/PLSt, OOO/StLO, OLO/StLL, StLSt/StLO) when analyzing products of chemical interesterification of soybean oil and fully hydrogenated soybean oil by HTGLC. It is worth mentioning that, when the chemical catalyzed process is applied, the identification procedure results much simpler because of the randomization of the acyl moieties within the glycerol backbones of the initial TAG mixture (Ribeiro et al. 2009b).

In order to evaluate the lower limit of fatty acid chain length that could be analyzed by this procedure, trimyristin (MMM) and trilaurin (LaLaLa) standard solutions were injected. The former eluted after the DAG zone, perfectly separated from them (trr=0.79), whereas the latter eluted together with DAG (trr=0.71). Moreover, when MMM relative response factor was added to previous data to recalculate Eq. 2 parameters, no variation from those shown in Table 1 was obtained. Although no sample with a considerable amount of myristic acid was analyzed, these results would indicate that the lower limit of the chain length range within which the method is valid could be extended to C14.

Validation

Linearity of FID response of compounds analyzed by the present method was described at the beginning of "Calibration Curves". Injection replications were used to measure the instrumental precision. All values of coefficients of variation (CV) were less than 11.8 %, with only three higher than 10.0 % (Table 2). On the other hand, results from replicates of sample preparation (repeatability of the quantitative method) showed similar values, in general with CV lower than 10.0 % (only three composition values had CV between 10.0 and 15.7 % and they corresponded to concentration values lower than 1 %).

Table 2 Mean composition of an interesterified product[a] obtained from instrumental precision and repeatability analyses

Component	Percentage (wt/wt)[c]			
	Instrumental precision		Repeatability analysis	
	Mean[d]	CV (%)	Mean[e]	CV (%)
FFA	3.60	8.2	3.65	8.9
MAG	0.40	9.7	0.40	9.8
DAG	6.75	3.1	6.68	3.4
TAG[b]				
PPP	1.28	1.7	1.26	3.3
PPSt	2.04	0.8	2.07	2.3
PPO	0.50	4.0	0.50	3.6
PPL	1.56	3.3	1.53	3.2
PPLn	0.16	5.2	0.16	4.0
PStSt	12.11	1.9	12.34	3.4
POSt	3.02	2.8	3.02	2.5
POO	0.83	3.3	0.83	3.2
PLSt	7.52	1.6	7.44	2.9
POL	4.01	2.3	3.95	2.7
PStLn	ND		ND	
PLL	5.00	2.3	4.90	3.3
PLLn	ND		ND	
StStSt	16.19	4.2	16.57	7.1
StStO	4.58	4.2	4.59	2.2
StOO	0.98	9.0	0.95	9.4
StStL	12.76	2.2	12.68	2.7
StOL	5.95	1.7	5.89	2.9
OOL	2.25	5.2	2.23	4.2
LLSt	8.63	1.9	8.50	3.1
OLL	4.62	2.2	4.55	3.6
OOLn	0.25	9.4	0.26	12.0
LLL	4.54	2.8	4.47	2.8
LLLn	1.02	11.8	1.07	13.2
LLnLn	0.21	11.4	0.23	15.6

P palmitic acid, *St* stearic acid, *O* oleic acid, *L* linoleic acid, *Ln* linolenic acid, *CV* coefficient of variation, *ND* not detected

[a] Substrates: soybean oil: fully hydrogenated soybean oil (50:50, wt/wt %); reaction conditions: commercial lipase Lipozyme RM IM, no solvent, 70 °C. For details, see Pacheco et al. (2012)

[b] The positional distribution of FA within TAG is not distinguished

[c] FFA, MAG, DAG are expressed as percentage of the whole sample weight; TAG are expressed as percentage of the whole TAG weight

[d] Mean composition of seven injections from the same solution

[e] Mean composition of seven solutions from the same sample

An interesterified sample was spiked with known amounts of standard substances (1.62 μg O, 2.9 μg MP, 2.9 μg DP, and 9.0 μg StStSt in 50 μL solution 10 % (*w*/*v*) of interesterified sample). The recovery values were 109.9, 100.7, 97.4, and 101.6 % to O, MP, DP, and StStSt, respectively.

Finally, the results obtained from the comparison of fatty acid composition determined by the method developed herein and by analysis of FAME are presented in Table 3. This last method has a high degree of precision (Christie 2005), and it has been widely implemented and used for a long time, being an appropriate additional tool for assessing the performance of the method presented. Samples with rather different origins were evaluated, including natural and processed fats and oils, having different degrees of unsaturation. As can be observed, the results obtained by both methods show excellent agreement for all samples. Moreover, the compositions of sunflower and soybean oil samples were in accordance with the data reported by Padley et al. (1994), while the fatty acid profile of the fully hydrogenated soybean oil sample was comparable to the one presented by López-Hernández et al. (2004).

Comparison of TAG composition by HTGLC and HPLC-RI was performed for the same purpose (Table 4). Soybean and sunflower oils were selected for their compositions to be compared. Samples with a high degree of unsaturation were chosen considering the unquestioned performance of HPLC to quantify this type of materials. Compositions determined by both methods are similar, reaching a linear correlation of HPLC vs HTGLC determinations (slope=0.936, R^2=0.981), being the minor TAG (those with composition lower than 5 %) the ones presenting the higher variations. The agreement of results obtained by the two chromatographic techniques was similar to the one reported by Carelli and Cert (1993) when they analyzed olive and sunflower oils (linear correlation slope=0.947, R^2=0.996). Compositions of major TAG were in excellent agreement (PLL, OOL, OLL, LLL, LLLn); while for peaks which coeluted, compositions should be summed up to be compared. For instance, for the pair POL + LLSt, which is the one with the highest concentration, the composition determined by HTGLC for soybean oil resulted to be 13.35 %, while the one determined by HPLC is 11.93 %. For sunflower oil, the corresponding values resulted in the following: 9.63 and 10.18 %. In the case of OOO, which is one of the major TAG in sunflower oil, its percentage determined by HPLC resulted to be 6.98 %. By HTGLC, it was impossible to quantify it independently from StStL, but if one considered the same proportion between them as the one established by HPLC (OOO/(StStL + OOO)=0.85), the percentage of OOO by HTGLC would be 6.89 %. These results are an additional proof to the accuracy of the method presented herein to quantify TAG, including those with a high level of unsaturation (up to seven double bonds). Moreover, it can be seen that in HPLC-RI, runs two pairs of peaks eluted together (PPO + PLSt and POL + LLSt), while in the HTGLC analyses, just one pair (StStL/OOO) could not be well separated. The composition of both vegetable oils was compared with bibliographical data available in the open literature (Carelli and Cert 1993; Neff and Byrdwell 1995). Considering the different germplasms origins and varieties, it was found that the compositions were in very good agreement.

As mentioned before, the method was originally developed for the analysis of substrates and products of the lipase-catalyzed interesterification reaction between soybean oil and fully hydrogenated soybean oil. The fatty acid profile of their products shows a wide range of unsaturation degree (soybean oil shows a relatively high level of C18:3 (6 %, *w*/*w*), while C18:0 fully hydrogenated soybean oil content amounts to 84 %

Table 3 Mean fatty acid composition (wt/wt %) of different liquid, solid, and semisolid fats and oils determined by the method presented herein (HTGLC) and by their FAME analysis

FA	Sunflower oil		Acidolysis product[a]		Soybean oil		Fully hydrogenated soybean oil		Interesterified product[b]	
	HTGLC	FAME analysis	HTGLC	FAME analysis	HTGLC	FAME analysis	HTGLC	FAME analysis	HTGLC	FAME analysis
P	6.74±0.07	6.50±0.07	20.76±0.17	20.85±0.16	10.72±0.12	11.21±0.10	13.18±0.21	12.52±0.12	12.43±0.05	12.12±0.20
St	2.90±0.05	3.33±0.05	17.70±0.08	18.21±0.06	5.57±0.08	4.81±0.02	85.57±0.33	85.42±0.11	45.65±0.14	45.35±0.13
O	33.69±0.32	31.91±0.05	18.84±0.10	18.56±0.11	22.87±0.22	23.72±0.11	1.01±0.11	1.71±0.01	12.63±0.21	11.89±0.02
L	56.67±2.80	58.26±1.01	42.70±0.12	42.38±0.06	54.78±0.16	53.11±0.06	0.24±0.01	0.31±0.01	27.59±0.04	27.20±0.22
Ln	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.00±0.18	7.07±0.15	0.00±0.00	0.04±0.01	1.71±0.16	3.43±0.04

P palmitic acid, *St* stearic acid, *O* oleic acid, *L* linoleic acid, *Ln* linolenic acid

[a] Substrates: sunflower oil: (P + St) mixture (1:3 molar ratio); reaction conditions: immobilized RM lipase; solvent: hexane, 60 °C (for details, see Palla et al. (2012))

[b] Substrates: soybean oil: fully hydrogenated soybean oil (50:50, wt/wt %), reaction conditions: commercial lipase Lipozyme RM IM, no solvent, 70 °C. For details see Pacheco et al. (2012)

Table 4 TAG composition[a] (wt/wt %) of soybean and sunflower oil determined by HTGLC and HPLC-RI analyses

TAG[b]	Soybean oil		Sunflower oil	
	HTGLC	HPLC-RI	HTGLC	HPLC-RI
PPP	ND	ND	ND	ND
PPSt	ND	0.41±0.08	ND	0.67±0.10
PPO[c]	0.80±0.03	–	0.30±0.00	–
PPL	2.70±0.03	2.12±0.02	0.98±0.02	0.74±0.03
PPLn	0.11±0.03	ND	ND	ND
PStSt	0.07±0.00	0.57±0.02	0.07±0.01	0.28±0.07
POSt	0.78±0.05	ND	0.29±0.01	ND
POO	3.02±0.02	3.43±0.18	2.81±0.03	2.89±0.02
PLSt[c]	2.1±0.07	–	0.85±0.00	–
PLSt + PPO[c]	–	1.53±0.03	–	0.63±0.08
POL[c]	10.16±0.11	–	6.56±0.07	–
PStLn	ND	ND	ND	ND
PLL	14.86±0.06	13.14±0.18	8.32±0.13	7.43±0.02
POLn	ND	0.41±0.08	ND	ND
PLLn	0.55±0.04	3.66±0.04	ND	0.08±0.01
StStSt	0.83±0.01	ND	0.04±0.01	ND
StStO	0.29±0.04	0.23±0.02	0.13±0.03	ND
StOO	0.97±0.05	0.28±0.04	1.26±0.01	0.24±0.03
StStL[c]	–	1.78±0.08	–	1.27±0.18
StStL + OOO[c]	3.53±0.05	–	8.14±0.00	–
StOL	ND	1.87±0.12	2.60±0.43	2.27±0.08
OOO[c]	–	ND	–	6.98±0.04
OOL	8.58±0.24	7.11±0.09	15.27±0.30	14.74±0.17
LLSt[c]	3.19±0.34	–	3.07±0.14	–
POL + LLSt[c]	–	11.93±0.12	–	10.18±0.09
OLL	17.37±0.09	16.14±0.12	27.43±0.06	27.44±0.12
OOLn	1.09±0.02	1.62±0.09	ND	0.42±0.11
PLnLn	ND	0.42±0.03	ND	0.18±0.03
LOLn	ND	3.44±0.11	ND	ND
LLL	20.10±0.03	18.21±0.31	21.87±0.51	20.94±0.21
LLLn	6.48±0.10	7.80±0.09	ND	0.17±0.01
LLnLn	ND	1.28±0.02	ND	ND
NI	–	1.70±0.10	–	2.45±0.17

ND not detected, *NI* not identified

[a] Mean value±SD, $n=4$

[b] For nomenclature, see Table 2

[c] Species that appeared overlapped whether in HTGLC or HPLC-RI analyses

(*w*/*w*)), being ideal substrates to test the capability of the technique to quantify species with such a different chemical identity. Moreover, the interesterification renders a fatty acid rearrangement among different TAG, providing a great quantity of new glyceride molecules with differential unsaturation levels. Additionally, the validated analysis of a sample with a high level of palmitic acid (C16:0) broadens the lower limit of fatty acid chain length from C18 to C16.

Conclusions

The present HTGLC method for the simultaneous determination of FFA, MAG, DAG, and TAG resulted to be effective in analyzing substrates and products of vegetable oils and fats' enzymatic modification processes. The optimized chromatographic conditions permitted a complete group separation and a comprehensive discrimination of the TAG species according to both carbon number and degree of unsaturation of their fatty acids. Only one pair of peaks eluted with equal retention times. Regarding FFA, MAG, and DAG analysis, an advanced degree of separation could be achieved, being 4 out of a total of 35, the number of peaks representing a pair of compounds rather than a unique species.

The method was tested for a wide range of fatty acid unsaturation level (0 to 3 double bonds) and chain length (C16 to C18), rendering a rapid and reliable technique to quantify FFA, MAG, DAG, and TAG in substrates and products of modification processes which involve most vegetable oils and fats.

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