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Chromatographic Analyses of Fatty Acid Methyl Esters by HPLC-UV and GC-FID

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Um método analítico por cromatografia líquida de alto rendimento com detecção ultravioleta (HPLC-UV) (método A) foi utilizado na determinação simultânea dos valores totais de triacilglicerídeos, diacilglicerídeos, monoacilglicerídeos e ésteres metílicos de ácidos graxos na alcólise de diferentes óleos (algodão, canola, girassol, milho e soja). As análises foram realizadas a 40 °C por 20 min usando gradiente de metanol (MeOH) e 2-propanol-hexano 5:4 (v/v) (PrHex): 100% de MeOH em 0 min, 50% de MeOH e 50% de PrHex em 10 min mantido com eluição isocrática por 10 min. Outro método por HPLC-UV (método B) com eluição isocrática de acetonitrila por 34 min foi utilizado na determinação da composição de ácidos graxos de óleos analisando seus derivados de éster metílico. Os conteúdos foram analisados com satisfatória repetibilidade (desvio padrão relativo, RSD < 3%), linearidade ($r^2 > 0,99$) e sensibilidade (limite de quantificação). O método B foi comparado com o método oficial cromatográfico gasoso com detecção através de ionização por chama (GC-FID) da *American Oil Chemists' Society* (AOCS) na determinação de ésteres metílicos de ácidos graxos (FAME) em amostras reais de biodiesel.

An analytical method using high performance liquid chromatography with UV detection (HPLC-UV) (method A) was used for simultaneous determination of total amounts of triacylglycerides, diacylglycerides, monoacylglycerides and fatty acid methyl esters in alcoholysis of different oil (cotton, canola, sunflower, corn and soybean) samples. Analyses were carried out at 40 °C for 20 min using a gradient of methanol (MeOH) and 2-propanol-hexane 5:4 (v/v) (PrHex): 100% of MeOH in 0 min, 50% of MeOH and 50% of PrHex in 10 min maintained with isocratic elution for 10 min. Another HPLC-UV method (method B) with acetonitrile isocratic elution for 34 min was used to determine the fatty acid composition of oils analyzing their methyl ester derivatives. Contents were determined with satisfactory repeatability (relative standard deviation, RSD < 3%), linearity ($r^2 > 0.99$) and sensitivity (limit of quantification). Method B was compared with an official gas chromatographic method with flame ionization detection (GC-FID) from American Oil Chemists' Society (AOCS) in the determination of fatty acid methyl esters (FAME) in biodiesel real samples.

Keywords: biodiesel, triacylglycerides, gas chromatography, liquid chromatography, methyl ester

Introduction

The direct use of fats and oils (mainly composed by triacylglycerides) in diesel engines is problematic and can lead to many problems, such as carbon deposit due to its poor atomization, high viscosity and thickening of lubricating oil as a consequence of its low volatility.[1,2] There are many possible solutions to address the viscosity problem, which are dilution with diesel fuel, microemulsions with short chain alcohols and pyrolysis or transesterification of triacylglycerides. Transesterification is a widespread process used for obtaining biodiesel and involves the catalyzed reaction of triacylglycerides (TAGs) and short-chain mono-alcohols (such as methanol and ethanol) to produce fatty acid methyl or ethyl esters (biodiesel).[3,4] This process was the chosen technology by several biofuel programs, such as the National Program of Production and Use of Biodiesel (PNPB) in Brazil.[2] Biodiesel is considered the best fuel substitute for diesel due to its physical properties, which

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are close to those of fossil diesel.[1] Advantages of biodiesel include biodegradability, higher flash point and reduction of most regulated exhaust emissions (such as sulfur and aromatic content).[2,5]

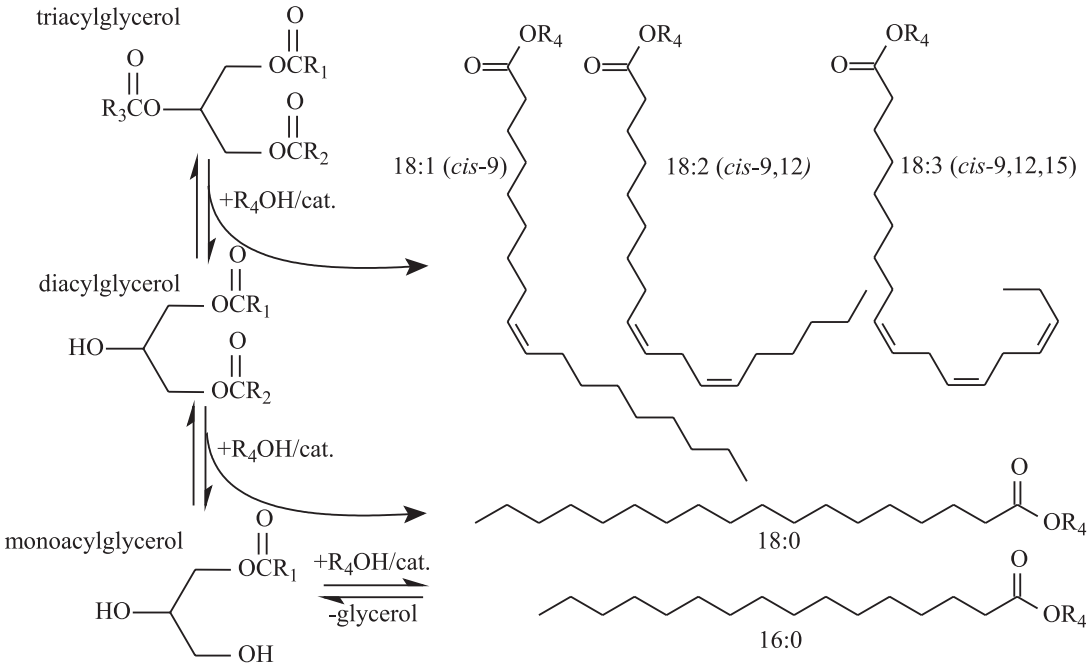
Scheme 1 shows the transesterification reaction of these TAGs with alcohol to obtain the most common fatty esters contained in biodiesel: palmitic (16:0), stearic (18:0), oleic (18:1, *cis*-9), linoleic (18:2, *cis*-9,12) and linolenic (18:3, *cis*-9,12,15).[5] Moreover, the diversity of carbon chains, degree of unsaturation, stereochemistry (*cis*/*trans*) and position of double bonds in the carbon chain make biodiesel as a complex mixture that contains a broad spectrum of fatty acid types, complicating their characterization.

The fatty acid profiles are of considerable importance in the biodiesel analysis. Some chromatographic methods have been created and improved to analyze the biodiesel[6]. Among these, thin layer chromatography (TLC),[7] gas chromatography (GC),[8] high-performance liquid chromatography (HPLC),[9-11] gel permeation chromatography (GPC),[12] nuclear magnetic resonance (NMR)[13] and near infrared spectroscopy (NIR)[14] are included.

The American Oil Chemists' Society (AOCS) recommends in its standard Cd 14c-94 and Ce 1c-89 methods the use of gas chromatography with flame ionization detection (GC-FID).[15,16] Although GC suffers from many drawbacks, it has been the most used technique for analyzing the complex mixture of compounds involved in the transesterification, including triacylglycerides (TAG), diacylglyceride (DAG), monoacylglyceride (MAG), monoalkyl esters, alcohol and free glycerol.[15,17] It is worth to mention that these determinations cannot be carried out in an unique analysis because different methods are required.

HPLC offers an useful alternative to GC and many liquid chromatographic methods have been developed.[9,18] Many workers, however, may find that HPLC offers some advantage and indicate some critical points of GC: (*i*) the presence of heat-labile compounds affects the quantification of FAME (fatty acid methyl esters), (*ii*) the carbon chain polyunsaturated of the fatty acids may undergo structural changes, isomerization and decomposition under high temperatures, (*iii*) it is not possible to collect fractions of the separated fatty acid esters for further analysis, (*iv*) baseline drift, and (*v*) GC analysis frequently requires derivatization step by saponification furthered to methylation, consuming reagents as hexane, BF_3 and NaCl, being time-consuming and labor-intensive.[11,17]

In this work, HPLC (called method B) and GC techniques are compared with respect to the determination of FAMEs in biodiesel. For this study, the main refined vegetable oils used in European Union, United Stated and Brazil were chosen: cotton, canola, sunflower, corn and soybean oils. Although the present study is limited to the investigation of these oils, it is possible to extend our method to other fats and oils if they have their composition based on similar fatty acids. For instance, despite olive and palm-tree oil have not been investigated, their composition usually contains up to 97% of the fatty acids here studied.



Scheme 1. Transesterification of triacylglycerol (any feedstock). R_1, R_2 and R_3 are the carbon chains of aliphatic esters and R_4 are of alcohols. It is illustred the main fatty acid esters in oils used in this work: palmitic (16:0), estearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3).

Moreover, the present investigation deals with the application of an analytical method (called method A) in HPLC in order to determine the total amounts of the chemical substances involved in the reaction between vegetable oils and methanol.

Experimental

Materials

Cotton, canola, sunflower, corn and soybean oils were purchased in local market. HPLC grade acetonitrile, methanol, 2-propanol and hexane were obtained from Vetec Química Fina Ltda (Brazil). Nitrogen (industrial grade) was obtained from White Martins (Brasília-DF, Brazil) and used without further purification. The standards of methyl linolenate (18:3), methyl linoleate (18:2), methyl oleate (18:1, *cis*), methyl elaidate (18:1, *trans*), methyl stearate (18:0) and methyl palmitate (16:0) were purchased from Aldrich (St. Louis, MO, USA). The reference standard FAME mixture (C_4-C_{24}) for GC was obtained from Supelco (Bellefonte, PA, USA).

Methyl ester preparation

Methyl esters were synthesized using potassium hydroxide (KOH) as catalyst and methanol at room temperature. After 1 h, the stirring was stopped and the reaction product was separated into two phases by simple decantation. FAME (upper phase) was further purified by washing ten times with water. Then, FAME was dissolved in dichloromethane, kept standing over magnesium sulfate, filtered and the volatiles removed under reduced pressure. The transesterification reaction utilized a total molar ratio of methanol:oil:KOH of 7:1:0.01.[11]

GC chromatographic instruments and procedures

The analyses were conducted with a GC 3400cx gas chromatograph (Varian Star) equipped with a split/splitness injector and flame ionization detector (FID). A capillary fused silica column SP™ 2380 (30 m × 0.25 mm × 0.2 μm film thickness) with matrix active group stabilized poly(90% biscyanopropyl/10% cyanopropylphenyl siloxane) phase was operated under programmed temperature conditions: 140-240 ºC at 5 ºC min^{-1} in 30 min (detector and injector temperatures of 260 °C), injection volume and mode of 0.4 μL and split (100:1), and nitrogen gas as carrier gas (20 cm min^{-1}). The desired amount (*ca.* 20 mg) of samples was dissolved in 2 mL in hexane before injection.

GC quantitative analysis

FAME was identified by direct comparison with the standard mixture and the percentage of individual FAME was made in relation to total area of the chromatogram. As far as the different studied fatty acid methyl esters have similar carbon chain length, it was assumed that they have the same response factor and volatility, allowing to make a direct comparison of the peak areas to determine the sample composition.

HPLC chromatographic instruments and procedures

The analyses were carried out in CTO-20A (Shimadzu, Tokyo, Japan) high-performance liquid chromatography (HPLC) equipment with an ultraviolet detector (UV) setted at 205 nm. All chromatograms were generated by LabSolutions software (Shimadzu, Japan).

Method A:[9] it was used a single Shim-Pack VP-ODS C_{18} reversed-phase column (250 mm × 4.6 mm, 5 μm) kept at 40 °C. The injection volume of 10 μL and the flow-rate of 1 mL min^{-1} with gradient elution of methanol (MeOH) and 2-propanol-hexane 5:4 (v/v) (PrHex) were used: 100% of MeOH in 0 min, 50% of MeOH and 50% of PrHex in 10 min maintained with isocratic elution for 10 min. The yield of the transesterification reaction was calculated by comparing the sum of the peak areas of the chromatogram components. Desired sample amount (*ca.* 25 mg) was dissolved in 2 mL of 2-propanol-hexane 5:4 (v/v).

Method B:[18] it was used two Shim-Pack VP-ODS C_{18} reversed-phase columns (250 mm × 4.6 mm, 5 μm) in series kept at 40 °C. The injection volume of 10 μL and the flow-rate of 1 mL min^{-1} with isocratic elution of acetonitrile for 34 min were used. Desired sample amount (*ca.* 25 mg) was dissolved in acetonitrile and injected without previous treatment.

HPLC quantitative analysis

The external calibration method was adopted for standardization, as previously determined.[18] The FAME peak identification was determined by the comparison of retention time of the reference standards in the same condition. The HPLC method was validated by the determination of the linearity, limits of detection and quantification and repeatability.

The calibration curves were constructed in a FAME desired concentration and the integrated peak areas *versus* the amounts of methyl ester standard were plotted for each FAME. It was used the software Origin 7.5 (OriginLab Corporation) to make the calibration curves, applying the

method of least-squares to calculate the parameters a, b, r^2 and s_y (a is the angular coefficient or slope, b is the linear coefficient or intercept, r^2 is the correlation coefficient and s_y is the standard deviation of axes y). Standard solutions of each FAME were prepared by the dilution of the desired weight of standard FAME in acetonitrile and analyzed in the concentration range. The solutions for 18:3 were 3.4, 13.4, 67.8, 202.9, 503.8 and 952.0 mg L^{-1}. The solutions for 18:2 were 5.4, 1437.0, 3482.6, 20255.9 and 68411.1 mg L^{-1}. The solutions for 18:1, *cis*, were 161.2, 1556.1, 2789.3, 3985.4, 104736.1 and 131316.0 mg L^{-1}. The solutions for 18:1, *trans*, were 159.9, 676.7, 1005.7, 10829.4 and 24950.2 mg L^{-1}. The solutions for 16:0 were 156.3, 452.8, 4320.8, 20064.3, 35761.0 and 43808.5 mg L^{-1}. The solutions for 18:0 were 133.6, 333.8, 666.9, 810.9, 9089.7 and 18138.3 mg L^{-1}.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined by the successive decrease of the concentration of the prepared standards until the smallest detectable peak. This concentration was multiplied by 3 and 10 to obtain the limits of detection and quantification, respectively.

The repeatability (n = 6) of the HPLC method B for the retention time and integration area was tested for the determination of a standard mixture solution of all FAMEs used in this work: 202 mg mL^{-1} for 18:3, 3482 mg mL^{-1} for 18:2, 2790 mg mL^{-1} for 18:1, *cis*, 20000 mg mL^{-1} for 16:0, 676 mg mL^{-1} for 18:1, *trans*, and 810 mg mL^{-1} for 18:0. This mixture was six times injected in the same day.

NMR spectroscopy

^1H and ^{13}C NMR data were recorded in a Varian spectrometer (Mercury plus model, 7.04 T) operating at 300 and 75 MHz, respectively, using $CDCl_3$ as solvent and the chemical shifts are expressed in parts *per* million (ppm). The absence of unsaturations in carbon chain was determined by quantitative ^1H NMR and ^{13}C NMR analyses at 25 °C and compared to the literature data.[19]

Results and Discussion

Initial considerations: method A

The transesterification of triacylglycerides with methanol involves three consecutive reversible reactions: triacylglycerides to diacylglycerides and to monoacylglycerides and, finally, to glycerol and FAME, as depicted in Scheme 1. Note that because of its reversibility, it is not possible to complete the transesterification reaction resulting in a mixture of TAG, DAG, MAG, FAME (for methanol) and glycerol is obtained. It is also important to note that if there is water in the reaction media, the hydrolysis of the different ester may take place affording free fatty acid (FFA) and the respective alcohol.[1] Thus, the analysis of the reaction yield in these different products is a major challenge.

The HPLC analysis of the methanolysis of soybean, cotton, canola, corn and sunflower oils in the final mixture results in several peaks corresponding to FFA, MAG, DAG, TAG and FAME, as shown in Figure 1 for methanolysis soybean oil in low reaction yield. As can be depicted in Figure 1, the only difficulty of this method was the separation of FFA and MAG, and a good resolution for the other components was achieved. Table 1 shows the results of the HPLC method A from methanolysis of different vegetable oils with high reaction yields in FAME. These results exhibit an important advantage of HPLC when compared with GC: the possibility of analyzing all fatty acids and their derivatives in only one analysis.

HPLC separation and quantitave analysis: method B

Soybean biodiesel analyzed by method B is illustrated in Figure 2. The relative standard deviations (RSD) obtained

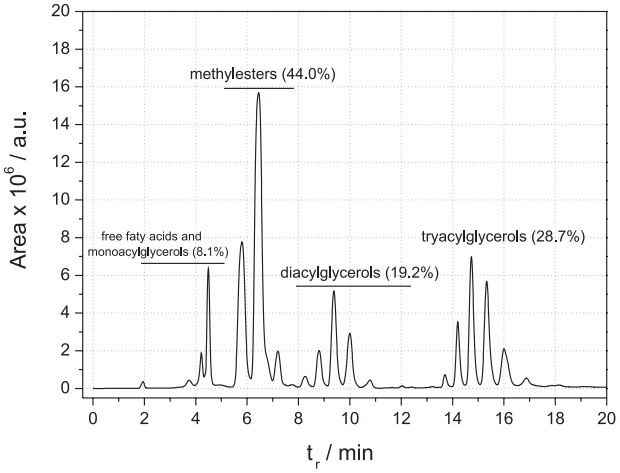


Figure 1. HPLC analysis of low yield of methanolysis of soybean oil in the final mixture.

Table 1. Composition of the final mixture obtained after methanolysis of different vegetable oils

Feedstock	Composition / %			
	FFA + MAG[a]	FAME	DAG	TAG
Cotton	2	94	2	2
Canola	1	97	1	1
Sunflower	2	94	3	1
Corn	1	97	2	0
Soybean	2	95	1	2

[a]In this method, it was not possible to separate free fatty acid and monoacylglycerol; FFA: free fatty acid; MAG: monoacylglyceride; FAME: fatty acid methyl esters; DAG: diacylglyceride; TAG: triacylglycerides.

for testing the repeatability (n = 6) for the retention time were less than 1% and for the area less than 3%. As can be depicted in Figure 2, a good separation can be achieved within 34 min with the conditions used in this method. Note that the separation obtained in this method was only possible using two C_{18} columns in series. The retention time increases with the increase of the chain length and the decrease of the double bond number: 18:3 < 18:2 < 18:1, *cis ca.* 16:0 < 18:1, *trans* < 18:0, being in accordance with previous works reported in the literature.[20] Unfortunately, it was not possible to separate all the methyl ester, and it was observed the pair 18:1, *cis* and 16:0 coelutes, which is probably a result of this opposing tendencies cited above.[17]

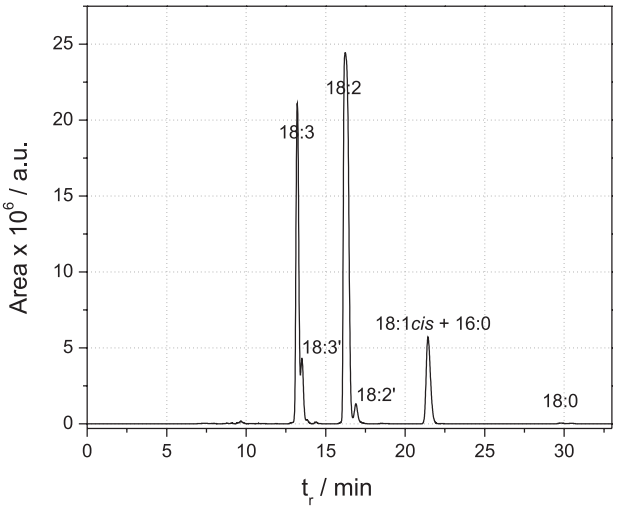


Figure 2. HPLC chromatogram of soybean biodiesel using method B (as described in the Experimental section: UV detection at 205 nm, flow-rate of 1 mL min^{-1}, column temperature at 40 °C and injection volume of 10 μL).

Different approaches have been described in the literature[1,19,22] aiming to separate and quantify *cis*/*trans* fatty acids, but few ones have been completely satisfactory. For instance, the standard American Oil Chemists' Society method uses infrared.[21] However, long packed columns with GC seem to be more targeted in the literature.[3] Cation-exchange column using silver ions has been often used for separation of lipids.[22] In this work, it was possible to separate and quantify the *trans* isomer forms of 18:3, 18:2 and 18:1 designate 18:3', 18:2' and 18:1, *trans*, respectively. These *trans* isomers are eluted soon after the correponding *cis* isomers due to a preferential π-π interaction between acetonitrile and *trans*-olefins. This π-π interaction is smaller for *trans* configuration than *cis* configuration due to a major steric hindrance. These *trans* isomers of 18:3 and 18:2 may be formed in their refinement during the oil separation process involving heating or isomerization in the course of the hydrogenation reactions.[23]

The calibration curve for each fatty acid ester is described in Table 2 and Figure 3. Table 2 reports the parameters a, b, r^2 and s_y of the calibration curves, range of concentration and retention time. Table 2 also shows the LOQ values of the individual compound, which were estimated by the successive decrease of the concentration of the prepared standards until the smallest detectable peak (relation signal:noise = 3). This concentration was multiplied by 10 to obtain the limits of quantification.[24] Figure 3 shows the calibration curves of FAME as a function of integrated peak area *versus* concentration

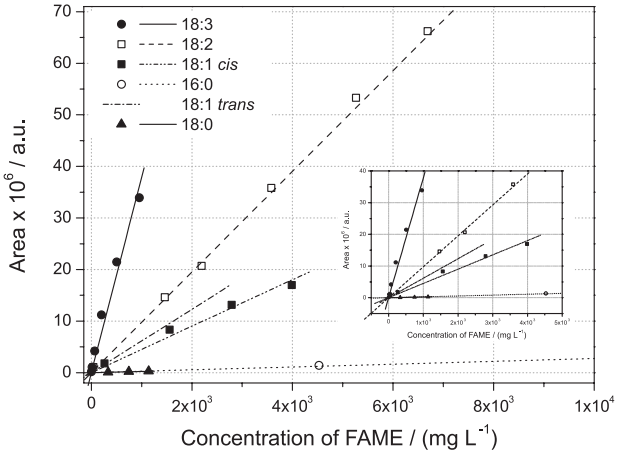


Figure 3. Calibration curves of pure fatty acid methyl ester (FAME) standards: (●) 18:3, (□) 18:2, (■) 18:1, *cis*, (○) 16:0, (◇) 18:1, *trans* and (▲) 18:0 (HPLC conditions of method B as described in the Experimental section: UV detection at 205 nm, flow-rate of 1 mL min^{-1}, column temperature at 40 °C and injection volume of 10 μL).

Table 2. Parameters slope, intercept, correlation coefficient (r^2) and standard deviation (s_y) of calibration plot, time retention (t_r) and limit of quantification (LOQ) for each studied fatty acid methyl esters (FAME)

FAME	Slope / (area mg^{-1} L)	Intercept / a.u.	r^2	s_y	Range of concentration / (mg L^{-1})	t_r / min	LOQ / (mg L^{-1})
18:3	37816.0	0	0.991	2.3	3.4 - 952.0	13.26	2.9
18:2	9761.4	0	0.990	5.2	5.4 - 6.8 × 10^4	16.33	12.7
18:1 *cis*	4507.4	0	0.995	1.1	161.2 - 13.1 × 10^4	21.06	92.1
18:1 *trans*	6146.9	0	0.999	0.9	159.9 - 2.4 × 10^4	21.00	90.6
16:0	236.0	0	0.996	0.9	156.3 - 4.3 × 10^4	22.42	216.5
18:0	273.2	0	0.996	1.2	133.6 - 1.8 × 10^4	30.46	198.1

Table 3. Chemical composition of biodiesel from selected oils in % obtained by gas (GC) and high-performance liquid chromatographies (HPLC)

FAME	Cotton		Canola		Sunflower		Corn		Soybean		PHS[a]	
	GC	HPLC	GC	HPLC	GC	HPLC	GC	HPLC	GC	HPLC	GC	HPLC
18:3	0.2	0.2	5.7	6.0	0.5	0.5	1.3	1.5	6.3	6.2	0	0
18:3'[a]	–	0	–	1.0	–	0.1	–	0.2	–	1.3	–	0
18:2	55.6	55.9	22.4	22.0	61.9	59.2	48.6	51.4	53.6	50.6	5.8	4.4
18:2'[b]	–	0.1	–	0.9	–	0.7	–	1.9	–	1.9	–	6.7
18:1 *cis*	15.4	16.3	60.9	62.8	24.6	21.1	33.4	30.7	23.5	21.2	65.6	38.3
16:0	22.2	19.6	4.9	4.2	6.2	5.3	12.6	12.7	10.7	11.4	11.1	11.6
18:1 *trans*	–	0	–	0	–	0	–	0	–	0	–	32.4
18:0	2.6	2.3	2.0	2.2	4.1	4.2	2.3	2.1	3.3	3.2	6.4	5.7

[a]PHS: partially hydrogenated soybean oil

of standard (mg L^{-1}). Note that these results show good linearity ($r^2 > 0.99$) for all the compounds in the range of concentration tested.

As shown in Table 2 and Figure 2, the detection of the various FAMEs are more sensitive when double bonds are present in FAME. Thus, the response factor (or angular coefficient) and limit of quantification of standard FAME follow the order: 18:3 > 18:2 > 18:1, *cis* > 18:1, *trans* > 18:0 > 16:0.

As can be depicted in Figure 2, unlike provided by other authors,[9] saturated fatty acids have significant absorbance at 205 nm and can be measured by the present method. Indeed, 18:0 and 16:0 FAMEs exhibit a similar behavior in the UV detector at 205 nm, as a consequence of their structural similarity, practically resulting in coincident calibration curves. It is also worth to mention, that *trans* 18:1 isomer has a higher slope than the *cis* form due to its higher absorption of energy for π-π^* transitions.[9]

Despite a hard work performed in order to find a chromatographic resolution for 18:1 *cis* and 16:0, it was impossible to avoid the co-elution of these compounds. It was only possible to quantify 18:1 *cis* and 16:0 after hydrogenation of biodiesel using Pd/C as catalysts (conditions: temperature of 80 °C and hydrogen pressure of 100 atm).[25] This procedure leads to a total hydrogenation of biodiesel, resulting in only 18:0 and 16:0 chains, confirmed by ^{1}H NMR and ^{13}C NMR. The total elimination of unsaturations was confirmed by the absence of the olefinic (–CH=CH–) signal at 5.35 ppm for ^{1}H NMR and at 134.12 ppm for ^{13}C NMR. Thus, analyzing once again the mixture, it was observed an increase of the peak of 18:0 and a drastic decrease of the peak area at 21 min, which is reduced to only 16:0 chains.

Determination of fatty acid compositions of real samples

In order to validate the method here presented, HPLC method B was tested in real samples of methyl fatty acid ester of cotton, canola, sunflower, corn and soybean oils and compared to GC analysis and the results are presented in Table 3. As expected, five fatty acid chains were found in esters, totalizing more that 95% of total composition.

The comparison of the HPLC and GC methods for the methyl ester determination using the same samples of biodiesel shows that both methods give similar results. RSD values ranged from 0 to 10%. Therefore, the proposed method in HPLC is comparable to the GC standard method and can be used in the analysis of fatty acid methyl esters.

On the other hand, it becomes clear from Table 3 that GC was not efficient to separate *trans* and *cis* isomers in the partial hydrogenated soybean oil. In contrast, HPLC using method B shows a good resolution for *trans*/*cis* isomers and it was possible to quantify them. Note that the peak corresponding to 18:1 *cis* in the GC analysis was approximately the sum of the 18:1, *cis* and 18:1, *trans* obtained by HPLC (65.6 *ca.* 38.7 + 32.4).

Conclusions

In summary, we have developed two HPLC-UV analytical methods using UV detection at 205 nm suitable for simultaneous determination of total amounts of triacylglycerides, diacylglycerides, monoacylglycerides and fatty acid methyl esters in alcoholysis of different oil samples (method A) and to determine the fatty acid composition of oils analyzing their methyl ester derivatives (method B). These methods presented satisfactory repeatability (RSD < 3%), linearity ($r^2 > 0.99$) and sensitivity (limit of quantification). Method B was compared with an official AOCS method by GC-FID for FAME determination in biodiesel real samples.

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