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Article

Comparative Analysis of Eight Esterification Methods in the Quantitative Determination of Vegetable Oil Fatty Acid Methyl Esters (FAME)

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Foram comparados os resultados de determinações quantitativas de ésteres metilícos de ácidos graxos de óleos vegetais (soja, linhaça e dendê) entre oito métodos de esterificação com catálises ácidas e básicas. Os métodos selecionados foram descritos por Metcalfe, 1966 (MET, ref. 17); Bannon, 1982 (BAN, ref. 18); Joseph e Ackman, 1992 (JAC, ref. 3); Hartman e Lago, 1973 (HLA, ref. 19); Jham, 1982 (JHA, ref. 20); ISO 5509, 1978 (ISO, ref. 21); Bannon, 1982 (BBA, ref. 15) e Schuchardt e Lopes, 1988 (SLO, ref. 22). Apesar da grande variação apresentada para o teor de ácidos graxos insaturados, todos os métodos foram eficientes para os ácidos graxos saturados. Os resultados obtidos mostram que a determinação de ácidos graxos pode ser afetada pela composição do óleo e que os métodos JAC, ISO e BBA são os mais eficientes. Os métodos ISO e BBA por apresentarem baixa toxidez e baixo custo são os recomendados para amostras que apresentem baixa acidez. O método JAC deve somente ser recomendado para amostras com alta acidez porque os métodos ISO e BBA são realizados em meio básico e podem não determinar os ácidos graxos livres.

The results of the quantitative determination of fatty acid methyl esters of vegetable oils, (soybean, flaxseed, and palm oils) by eight basic and acid catalysis esterification methods were compared. The selected methods were described by Metcalfe, 1966 (MET, ref. 17); Bannon, 1982 (BAN, ref. 18); Joseph and Ackman, 1992 (JAC, ref. 3); Hartman and Lago, 1973 (HLA, ref. 19); Jham, 1982 (JHA, ref. 20); ISO 5509, 1978 (ISO, ref. 21); Bannon, 1982 (BBA, ref. 15) and Schuchardt and Lopes, 1988 (SLO, ref. 22). Despite the large variation in the determination of unsaturated fatty acids, all the methods were efficient in the analysis of saturated fatty acids. The results obtained show that fatty acid analysis may be affected by oil composition and that JAC, ISO, and BBA methods are more efficient. ISO, and BBA are recommended for low acidity samples due to their low reagent toxicity and cost. The JAC method is recommended only for high acidity samples, as the ISO and BBA methods are carried out in basic medium and cannot analyze the free fatty acids.

Keywords: esterification methods, gas chromatography, fatty acids, vegetable oils

Introduction

Advancements in gas chromatography (GC) have furthered the study of lipids and provided knowledge on fatty acid composition in a short span of time. Esterification, the conversion of fatty acids into methyl esters, is commonly used to analyze fatty acids and to reduce the adsorption of solutes on the support and the surface of the column and improve compound separation.[1,2]

Presently, several researchers investigate esterification procedures in search of an efficient method to obtain fatty acid methyl esters (FAME). These studies have been supported by advances in gas chromatography. The use of capillary column and software has had a large impact on the study of fatty acids as well, mainly due to the resulting easy and more efficient separation, identification, and quantification of FAME.[3,4] Together, these advances have enabled a more accurate investigation of esterification procedures, as for example, in the quantification of fatty acids with an internal standard. Nevertheless, few works in literature compare esterification methods.

The addition of an internal standard has been used in the analysis of fatty acid. This method is less sensitive to errors as the internal standard and the sample are injected together. It also allows expressing fatty acid results in weight.[5-7]

The derivatization methods most commonly used in GC analysis involve the transesterification of acylglycerols

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and the esterification of free fatty acids into FAME. This process is also called methylation.[8-10]

The reagents most used in acid catalysis esterification are hydrochloric acid (HCl), sulfuric acid (H_2SO_4), and boron trifluoride (BF_3) in methanol. They are all used in the esterification of acylglycerols and free fatty acids; however, none at room temperature. Although largely used, BF_3 in methanol is extremely toxic.[11-13]

The reagents most commonly used in the transesterification of acylglycerols by basic catalysis are sodium (NaOH) or potassium (KOH) hydroxide in methanol and sodium methoxide ($NaOCH_3$) in methanol.[14] Transesterification with these reagents may be carried out at room temperature in a very short time. However, a disadvantage is that they do not convert free fatty acids to FAME, which limits their application to highly acid oils.[2,15]

The incomplete conversion of lipids to FAME, changes in fatty acid composition during esterification, and the formation of compounds that may be mistakenly identified as fatty acids may also affect the quantification of FAME directly.[8,9]

It must be pointed out that these methods have been known for over 15 years and are currently used worldwide, at times with some modifications, but following their original principles.[10] Considering the possibility of obtaining distinct FAME results for the same sample submitted to varied esterification methods, the present work sought to investigate the efficiency and applicability of eight esterification methods involving acid and basic catalysis to the quantification of FAME in vegetable oils.

Experimental

Sampling

Refined soybean oil and two raw oils, flaxseed and palm oils, were selected for analysis due to their different concentrations of the main fatty acids, palmitic acid (16:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), and α - linolenic acid (C18:3n-3) (Figure 1). With the exception of palm oil, which was hand-produced in Bahia State, the oils were purchased from local shops in Maringá, PR. The oils were filled into 16 brown flasks (100 mL, two for each method), under N_2 flow and stored under refrigeration during analysis. Analysis was carried out in six repetitions for each method. The acidity index was determined according to Adolfo Lutz.[16]

Esterification methods studied

Method described by Metcalfe et al.,[17] 1966 (MET)

Approximately 150 mg of oil was mixed with 4.0 mL of

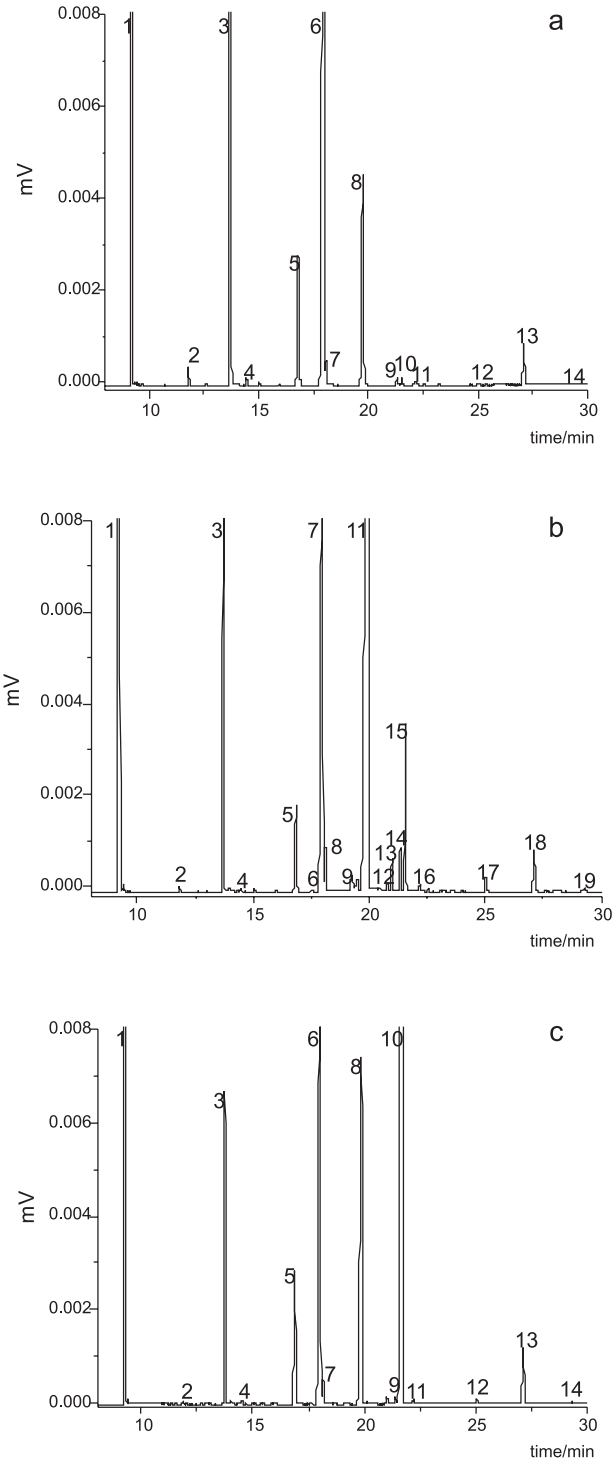

Figure 1. Chromatograms of vegetable oil FAME. (a) Palm oil. 1-Solvent, 2-C14:0, 3-C16:0, 4-C16:1n-9, 5-C18:0, 6-C18:1n-9, 7-C18:1n-7, 8-C18:2n-6, 9-C20:0, 10-C18:3n-3, 11-C20:1n-9, 12-C22:0, 13-C23:0, 14-C24:0; (b) Soybean oil. 1-Solvent, 2-C14:0, 3-C16:0, 4-C16:1n-9, 5-C18:0, 6-C18:1*t*9, 7-C18:1n-9, 8-C18:1n-7, 9-C18:2*c*9*t*12, 10-C18:2*t*9*c*12, 11-C18:2n-6, 12-C18:3*t*9*c*12*t*15, 13-C18:3n-6, 14-C20:0, 15-C18:3n-3, 16-C20:1n-9, 17-C22:0, 18-C23:0, 19-C24:0; (c) Flaxseed oil. 1-Solvent, 2-C14:0, 3-C16:0, 4-C16:1n-9, 5-C18:0, 6-C18:1n-9, 7-C18:1n-7, 8-C18:2n-6, 9-C20:0, 10-C18:3n-3, 11-C20:1n-9, 12-C22:0, 13-C23:0, 14-C24:0.

NaOH in 0.50 mol L^{-1} methanol and heated in a bath at 100 °C until the dissolution of the fat globules (*ca.* 5 min). Next, 5.0 mL BF_3 (12%) in methanol was added and the mixture was heated for another 2 min. After cooling, approximately 5.0 mL of a saturated sodium chloride solution was added. The mixture was transferred to a separation funnel with 20.0 mL of petroleum ether. The funnel was vigorously stirred for 1 min and next left at rest for phase separation. The aqueous phase was discarded and the ether phase was filtered with paper filter into a balloon. The solvent was evaporated in a bath at 60 °C and the residual solvent was removed with nitrogen flow at room temperature. The methyl esters were solubilized in *n*-heptane before injection into the gas chromatographer.

Method described by Bannon et al.,[18] 1982 (BAN)

Approximately 150 mg of oil was weighed and added with 5.0 mL of KOH in 0.50 mol L^{-1} methanol. The mixture was heated under reflux for 3 min. Next, 5.0 mL BF_3 (14%) in methanol was added in and the mixture was heated under reflux for another 3 min. After cooling, 3.0 mL of isooctane and approximately 15.0 mL of saturated sodium chloride were added in and vigorously stirred for 15 s. After phase separation, *ca.* 2.5 µL of the top phase was collected before injection into the gas chromatographer.

Method described by Joseph and Ackman,[3] 1992 (JAC)

Approximately 25 mg (± 0.1 mg) of oil was weighed and added with 1.5 mL of NaOH in 0.50 mol L^{-1} in methanol. The mixture was heated in a bath at 100 °C for *ca.* 5 min and next cooled at room temperature. The mixture was added with 2.0 mL BF_3 (12%) in methanol and heated again in a bath at 100 °C for 30 min. Next, the tube was cooled in running water at room temperature before adding 1 mL of isooctane. It was vigorously stirred for 30 s before adding 5.0 mL of a saturated sodium chloride solution. The esterified sample was placed in a refrigerator and left to rest for better phase separation. After collecting the supernatant, it was added another 1.0 mL of isooctane to the tube and it was stirred. The supernatant was collected and added to the previous fraction. The sample was concentrated to a final volume of 1.0 mL for later injection into the gas chromatographer.

Method described by Hartman and Lago,[19] 1973 (HLA)

An amount of 200-250 mg of oil was added with 5.0 mL of NaOH 0.50 mol L^{-1} in methanol and the mixture was heated under reflux for 5 min. After adding 15.0 mL of the esterification reagent (prepared from a mixture of 2.0 g of ammonia chloride, 60.0 mL of methanol, and 3.0 mL of concentrated sulfuric acid for *ca.* 15 min), the mixture was heated under reflux for another 3 min and next transferred to a separation funnel along with 25.0 mL of petroleum ether and 50.0 mL of deionized water. After agitation and phase separation, the aqueous phase was discarded. To the organic phase, it was added 25.0 mL of deionized water. It was agitated and after phase separation, the aqueous phase was discarded and the procedure was repeated. The organic phase was colleted, the solvent was evaporated in a rotavapor apparatus and the residue was removed under nitrogen flow. The methyl esters were solubilized in *n*-heptane before injection into the gas chromatographer.

Method described by Jham et al.,[20] 1982 (JHA)

An amount of 50 µL of oil was transferred to a tube and added with 1.0 mL of 0.50 mol L^{-1} KOH in methanol and heated in a bath at 100 °C for 5 min. It was added 400 µL of HCL in aqueous methanol (4:1 v/v). The mixture was heated in a bath at 100 °C for 15 min, cooled, and next, added with 2.0 mL of water and 3.0 mL of petroleum ether and stirred. After collecting the supernatant, 3.0 mL of petroleum ether was added another to the tube and stirred. The supernatant was collected and added to the previous fraction. The organic phase was collected and dried using sodium sulfate anhydride. The solvent was evaporated and the esters were dissolved again in 500 µL of chloroform before the injection into the gas chromatographer.

Method 5509 described by ISO,[21] 1978 (ISO)

A mass of *ca.* 1.0 g of oil was weighed and added with 10.0 mL of n-heptane and stirred. Next, 0.50 mL of 2 mol $^{-1}$ NaOH in methanol was added and stirred for 20 s. After phase separation, the supernatant was collected for later gas chromatography analysis.

Method described by Bannon et al.,[15] 1982 (BBA)

A mass of *ca.* 150 mg of oil was weighed and added with 5.0 mL of NaOMe (0.25 mol/L) in methanol:diethyl ether (1:1) and stirred for 2 min. After adding 3.0 mL of isooctane, *ca.* 15.0 mL of saturated sodium chloride was added in. The mixture was vigorously stirred for 15 min, and after phase separation, 2.5 µL of the top phase containing FAME was collected for gas chromatography analysis.

Method described by Schuchardt and Lopes,[22] 1988 (SLO)

A mass of *ca.* 250 mg of oil was weighed and added with 2.0 mL of tetramethylguanidine in methanol (1:4 v/v). The mixture was heated in a bath at 100 °C for 2 min. Next, it was cooled to room temperature and 20.0 mL of a saturated sodium chloride solution was added along with 8.0 mL of petroleum ether. After phase separation, the

organic phase was collected and the solvent was evaporated under nitrogen flow. The methyl esters were solubilized in isooctane before injection into the gas chromatographer.

Chromatographic analysis of FAME

Chromatographic analysis was carried out on a Varian apparatus, model CP 3380, equipped with a flame ionization detector, split/splitless injector, and a fused silica capillary column CP-Select CB-FAME (100% bonded cyanopropyl, 100 m, 0.25 mm i.d. and 0.39 μm stationary phase). The operation parameters were: column temperature of 197 °C for 23 min and 235 °C (20 °C min^{-1}) for 20 min at 40 psi. The injector and detector temperatures were kept at 220 and 245 °C, respectively. The gas flow rates used were 1.4 mL min^{-1} carrier gas (H_2), 30 mL min^{-1} make-up gas (N_2), and 30 mL min^{-1} and 300 mL min^{-1} flame gas, H_2 and synthetic air, respectively. The sample split mode was 1/80. The injections were carried out in duplicate and the injection volume was 1 μL. The peak areas of FAME were determined with software Workstation version 5.0 (Varian). FAME were identified by comparison of retention time of the sample constituents with Sigma FAME standards and by spiking. To evaluate the flame ionization detector response, it was used Sigma FAME standards with known concentrations. The experimental response factor was calculated according to Ackman.[23] FAME were quantified after the esterification of the vegetable oils. It was verified if the agreement of the experimental and theoretical response factors was within the working concentration range. Vegetable oil FAME were quantified in relation to the internal standard, methyl tricosanoate (23:0). The internal standard solution was prepared with a concentration of 1.0 mg mL^{-1} in isooctane. The internal standard (methyl tricosanoate) was added before weighing the oil in the esterification recipient. The amount added was established by keeping an oil and internal standard mass proportion of approximately 200:1. After the addition of the internal standard, the solvent was evaporated under nitrogen flow. The FAME concentration obtained after esterification by the several methods was calculated according to Cantellops *et al*.[24] The results are expressed in mg of FAME *per* gram of oil. The limits of detection and quantification were estimated according to the ACS recommendations[25] with successive dilutions of a standard solution of methyl arachidate considering the signal noise ratios equal to three and ten, respectively. Accuracy was estimated through addition and recovery tests. The triacylglycerol standards (Sigma) used were: tripalmitin (16:0), triolein (TG 18:1n-9), trilinolein (TG 18:2n-6), trilinolenin (TG18:3n-3). These triacylglycerols were chosen because they are the main ones present in the analyzed vegetable oils. Initially, it was determined the concentrations of triacylglycerols tripalmitin, triolein, trilinolein, and trilinolenin in mg of TG g^{-1} of oil according to Cantellops *et al.*[24] in the oils studied. Before weighing and esterifying the oil, it was added with the internal standard and known amounts of triacylglycerols and the solvent was evaporated under nitrogen flow.

Statistical analysis

The results are expressed as mean ± standard deviation. The different methods were compared by variance analysis (ANOVA) at 5% significance using program Statistica 7.0.[26] The mean values were compared by Tukey test.

Results and Discussion

This comparative study evaluated commonly used esterification methods applied to different vegetable oils to determine the most adequate one in relation to analysis frequency, reagent consumption and toxicity, the cost-benefit ratio, and the amount of FAME determined in the analyzed oils.

Determination of acidity index of vegetable oils

The acidity index values obtained for the vegetable oils (Table 1) show that the oils used, both raw (flaxseed and palm) and refined (soybean), are little acid. Due to the low acidity of the vegetable oils analyzed, it is estimated that the fatty acids present are bound to glycerol molecules, predominantly in the form of triacylglycerols, as described in literature.[27]

Table 1. Acidity index of the vegetable oils analyzed[a]

Vegetable Oils	Acidity index/(mg KOH g^{-1})
soybean	0.052 ± 0.003
flaxseed	0.067 ± 0.000
palm	0.104 ± 0.004

[a] Means of six repetitions ± standard deviations.

Quantification of FAME present in vegetable oils

Table 2 presents the experimental response factor values obtained from a FAME standard mixture and the theoretical one for methyl tricosanoate. The experimental factors for saturated FAME were closer to the theoretical factors when compared with the values obtained for FAME: linoleate (C18:2n-6), α- and δ-methyl linolenate (C18:3n-3 and C18:3n-6, respectively). This difference may be due to the

oxidative instability of unsaturated FAME. Thus, saturated FAME was used to verify the analysis optimization. After optimization, it is recommended the use of theoretical factors in quantitative determinations of polyunsaturated fatty acids.[28] Thus, it was used the theoretical response factors in the quantification of FAME.

Table 2. Response factor values for methyl tricosanoate

FAME	Response Factor	
	Experimental[a]	Theoretical
C14:0	1.10 ± 0.125	1.080
C16:0	1.08 ± 0.094	1.055
C18:0	1.09 ± 0.064	1.035
C18:1n-9	1.04 ± 0.067	1.028
C18:2n-6	1.13 ± 0.074	1.021
C18:3n-6	1.25 ± 0.104	1.014
C20:0	1.06 ± 0.029	1.019
C18:3n-3	1.26 ± 0.078	1.014
C22:0	0.979 ± 0.0150	1.006
C24:0	0.957 ± 0.0161	0.9951

[a] Means of six repetitions ± standard deviations.

After verification of the agreement between experimental and theoretical response factors in the concentration range used in the quantification of FAME, the esterification procedures were applied after the addition of the internal standard. This verification allowed establishing a ratio of approximately 200:1 between sample mass and the internal standard without compromising FAME quantification results and avoiding the excessive consumption of internal standard, which is costly and may add to the final cost of chromatographic analysis.

The values of limits of detection and quantification obtained were 0.148 and 0.476 mg g^{-1} of oil, respectively.

Analysis of the influence of the different esterification methods under the main saturated FAME present in the vegetable oils analyzed

The concentration of methyl palmitate obtained in palm oil was lower with the JAC (376 mg g^{-1}) method. Methods HLA (403), ISO (402), and SLO (398 mg g^{-1}) were more efficient. These concentration values were different ($p < 0.05$) from those of the other methods analyzed (MET, BAN, JHA, and BBA), which gave a mean value of 385 mg g^{-1}.

The use of the different esterification methods did not influence the methyl palmitate amount determined in soybean oil, as there was no significant difference ($p > 0.05$).

Methods JAC, HLA, and JHA gave higher and different ($p < 0.05$) methyl palmitate concentrations in flaxseed oil (61.1, 61.4, and 61.0 mg g^{-1}, respectively) in comparison to the other method results (Figure 2a), with a mean concentration of 59.5 mg g^{-1}.

The analysis of methods MET, BAN, and JAC, which use the same reagent (BF_3 in methanol) and differ mainly in the esterification step heating time (2, 3, and 30 min, respectively), reveals that this difference in heating time may be related to the fact that MET and BAN presented a higher methyl palmitate concentration than that given by JAC. Considering that the heating time of 30 min may result in the volatization of methyl palmitate, methods HLA, ISO, and SLO were more efficient in the esterification of methyl palmitate than the other methods studied here when applied to palm oil. The fact that ISO and SLO (basic catalysis) presented concentration values close to those obtained by HLA (acid catalysis) method is directly related to the low acidity of the oils studied (Table 1). However, the HLA method has the disadvantage of the esterification reaction time and number of steps required in comparison to those of the ISO and the SLO methods, as the reaction occurs in two steps (saponification and esterification), which increases the FAME analysis time.

Analysis of the influence of the different esterification methods under the main unsaturated FAME present in the vegetable oils analyzed

The main monounsaturated FAME present in the vegetable oil was methyl oleate (Figure 2b). The concentration results of monounsaturated methyl esters C18:1n-9 and C18:1n-7 were calculated after the summation of their areas due to the difficult separation of these peaks. Thus, the concentration of methyl oleate (C18:1) refers to this summation.

The analysis of the results obtained for palm oil shows that the lowest concentrations of methyl oleate obtained were those of methods JHA (421) and SLO (419 mg g^{-1}), which differed ($p < 0.05$) from those of methods HLA (431), ISO (436), BBA (442), and JAC (443 mg g^{-1}).

When applied to soybean oil, method JHA also presented the lowest concentration of methyl oleate (200 mg g^{-1}), in contrast to the other method results ($p < 0.05$), while methods MET, BAN, HLA, and SLO gave equal ($p > 0.05$) mean concentrations of 210 mg g^{-1}. However, the most efficient esterification methods were JAC, ISO, and BBA, with 218, 219, and 217 mg g^{-1}, respectively.

The methods that gave the best esterification yield for methyl oleate in flaxseed oil were JAC (185 mg g^{-1}), ISO

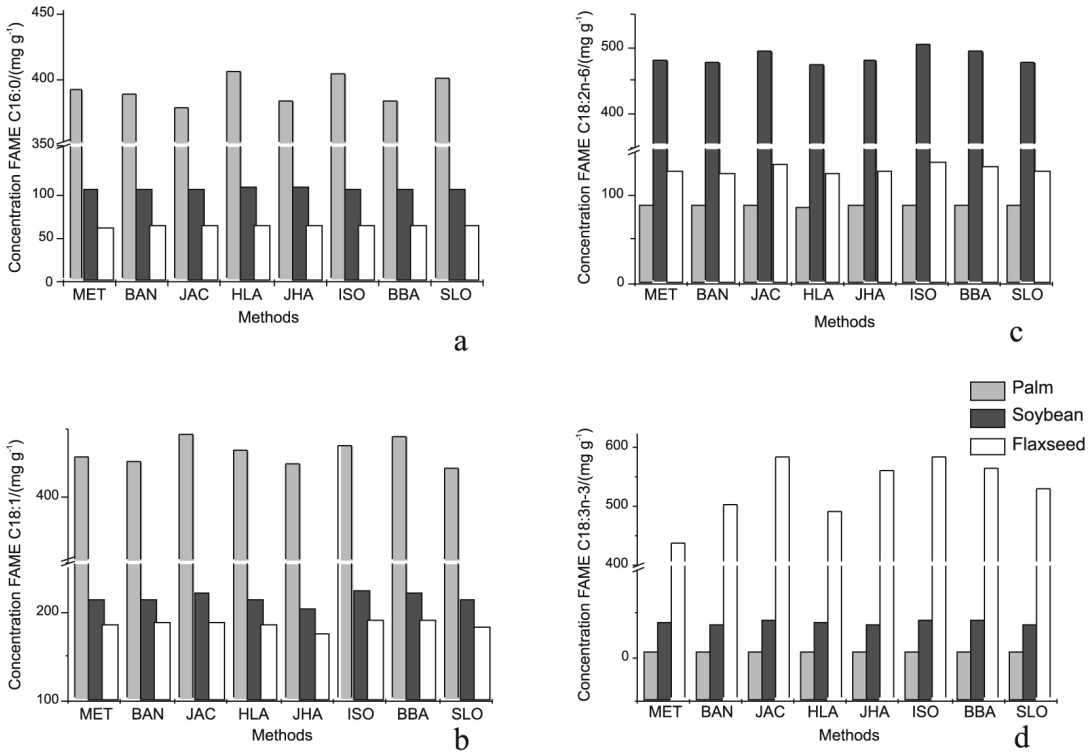

Figure 2. Concentrations of the main FAME (mg g^{-1} oil) in the analyzed vegetable oil. FAME (a) C16:0; (b) C18:1; (c) C18:2n-6 and (d) C18:3n-3.

and BBA (186 mg g^{-1}), and the lowest concentrations were obtained with JHA (171) and, SLO (178 mg g^{-1}). Methods JAC, ISO, and BBA were statistically equal to each other and different ($p < 0.05$) from the other methods.

JHA may have given a low concentration because of the presence of water, as it requires the use of aqueous HCl, which may have provoked hydrolysis. However, it must be considered that the JHA method uses HCl in methanol as a catalyst, which has the advantage of low cost and large availability in relation to those of BF_3 used in other methods.

The concentrations of methyl linoleate in palm oil (Figure 2c) obtained by JAC (86.1), ISO (86.3), and BBA (86.2 mg g^{-1}) were equal ($p > 0.05$), differing only from the concentration given by method HLA (83.6 mg g^{-1}).

The most efficient esterification methods of methyl linoleate in soybean oil were also JAC (491), ISO (501), and BBA (490 mg g^{-1}), with a mean concentration of 474 mg g^{-1} and without significant difference ($p > 0.05$).

The concentrations of methyl linoleate in flaxseed oil after esterification were the highest for methods JAC (132), ISO (133), and BBA (129 mg g^{-1}) without any significant difference ($p > 0.05$). Methods HLA (121) and SLO (124 mg g^{-1}) presented significantly different ($p < 0.05$) low concentrations, while the other methods (MET, BAN, and JHA) presented intermediate mean concentrations of 123 mg g^{-1}.

The concentration of methyl linoleate in palm and flaxseed oils by HLA method was low, probably due to the esterification heating time (3 min) and the preferential position of the unsaturated fatty acids at sn-2[29] in triacylglycerol.

However, even though HLA presented a low concentration of methyl linolenate, it must be considered that the HLA method uses H_2SO_4/NH_4Cl/methanol as a catalyst, which has the advantages of low cost and large availability in relation to those of other methods.

The SLO method also gave a low concentration of methyl linoleate, which may have resulted from steric hindrance due to the reagent molecule size and the distortions of the unsaturated fatty acid chains and the heating time used (2 min), as also observed for methyl oleate.

The most efficient methods for the esterification of α-methyl linolenate in soybean oil were JAC, ISO, and BBA (Figure 2d), with a significantly equal ($p > 0.05$) mean value of 37.3 mg g^{-1}. These values were higher than and different ($p < 0.05$) from those of the other esterification methods.

The concentrations of α - methyl linolenate in flaxseed oil obtained by the most efficient methods were JAC (577) and ISO (576 mg g^{-1}), followed by BBA and JHA (559 and 554 mg g^{-1}), respectively. The least efficient method in the esterification of α-methyl linolenate was MET, with a value of 432 mg g^{-1}.

Methods JAC, ISO, and BBA were the most efficient in the esterification of unsaturated fatty acids of the analyzed

vegetable oils, possibly due to the low acidity (Table 1) of the oils and the method procedural steps.

Method ISO uses *n*-heptane to solubilize the oil, which is then added with methanol. This may have contributed to the results obtained as the oil was dispersed in the solvent and the esterification reaction may have been promoted, as also reported by Glass.[29] The same applies to method BBA, as the catalyst used (sodium methoxide) was in diethyl ether solution.

The comparison of method JAC to methods ISO and BBA shows that the first uses a smaller amount of reagent. Nevertheless, the reagent used (BF_3) is extremely toxic, costly, and has limited useful lifetime.

Myristate (C14:0), estearate (C18:0), eicosanoate (C20:0), docosanoate (C22:0), tetracosanoate (C24:0) and δ-linolenate (C18:3n-6) are other FAME present in small quantities in the analyzed oils.

Soybean oil also presented some *trans* FAME due to its industrial refining.[31] The concentration values of methyl elaidate (C18:1*t*9) of 0.502 mg g^{-1} by MET, a mean value of 0.669 mg g^{-1} by BAN, JAC, HLA, ISO, and SLO, and 0.803 mg g^{-1} by JHA were statistically different ($p < 0.05$). The BBA method did not quantify this methyl ester. *Trans* FAME C18:2*c*9*t*12 and C18:2*t*9*c*12 were also quantified in low and statistically different concentrations ($p < 0.05$) of 5.90 and 5.28 mg g^{-1} by MET, means of 7.09 and 6.74 mg g^{-1}, respectively, for the other methods investigated.

Addition and recovery test

The relative recovery results for the tripalmitin standard (TG 16:0) submitted to different esterification methods are given in Table 3. The evaluation of the recovery of triacylglycerol was carried out by the addition of the internal standard (methyl tricosanoate). This procedure is recommended due to the simultaneous analysis of the analyte and of the internal standard.[24] These results show the efficiency of esterification of tripalmitin. Therefore, a less costly and toxic reagent and a shorter analysis time may be achieved.

Table 4 shows the relative recovery results for methyl tricosanoate for the triolein standard (TG 18:1n-9). The data show that the methods are efficient for the esterification of triolein, with the exception of JHA, which presented a recovery of only 89.5%. A low efficiency was also observed in the quantification of methyl oleate in vegetable oils. The addition test of tripalmitin and triolein was not carried out by SLO due to leak loss during the esterification process.

The relative recovery data obtained for methyl tricosanoate with standards trilionein (TG 18:2n-6) and trilinolenin (TG 18:3n-3) by different methods are given in Tables 5 and 6, respectively. The recovery value of HLA was lower than those of the other methods (85.9%) and so was the concentration determined in the analyzed oils. The other methods presented close values, being higher for JAC and BBA (101%). The trilinolein standard addition test was not carried out for method ISO due to the concentration of this ester in olive oil, which would require the addition of a large amount of standard. The methods that gave the lowest recovery percentuals of trilinolenin were MET (87.0), BAN (85.8 mg g^{-1}), HLA (84.4), and SLO (88.6 mg g^{-1}). These methods also had low yields of methyl linolenate in the vegetable oils analyzed. The results showed the efficiency of methods JAC, ISO, and BBA in the esterification of trilinolenin, as observed with standards trilinolein and triolein.

Table 3. Relative recovery data of methyl tricosanoate for tripalmitin (TG 16:0) after esterification by several methods[a]

Method[b]	Concentration in the Sample	Amount Added	Spike Amount	Recovered Amount	Recovery/ (%)
MET	6.12	3.48	9.04	2.92	95.2
BAN	6.10	3.48	8.82	2.72	93.1
JAC	1.23	1.19	2.29	1.07	96.0
HLA	9.00	5.04	13.5	4.44	96.7
JHA	1.41	0.625	1.94	0.529	96.2
ISO	23.2	12.7	33.3	10.1	93.8
BBA	6.32	6.95	12.4	6.08	94.5
SLO	-	-	-	-	-

[a] Results expressed as mean of triacylglycerol in three repetitions for the addition of internal standard. [b] Metcalfe, 1966 (MET, ref. 17); Bannon, 1982 (BAN, ref. 18); Joseph and Ackman, 1992 (JAC, ref. 3); Hartman and Lago, 1973 (HLA, ref. 19); Jham, 1982 (JHA, ref. 20); ISO 5509, 1978 (ISO, ref. 21); Bannon, 1982 (BBA, ref. 15) and Schuchardt and Lopes, 1988 (SLO, ref. 22). Results given as means of three repetitions injected in duplicate.

Table 4. Relative recovery data of methyl tricosanoate for triolein (TG 18:1n-9) after esterification by different methods[a]

Method[b]	Concentration in the Sample	Amount Added	Spike Amount	Recovered Amount	Recovery/ (%)
MET	26.8	12.6	37.8	11.0	97.0
BAN	29.3	12.6	40.3	11.0	97.2
JAC	6.02	1.98	7.89	1.87	99.7
HLA	37.1	17.0	50.8	13.7	94.9
JHA	6.76	2.54	8.24	1.48	89.5
ISO	92.1	29.3	115	23.1	95.9
BBA	29.1	12.6	41.7	12.6	101
SLO	-	-	-	-	-

[a] Results expressed as mean of triacylglycerol in three repetitions by the addition of internal standard. [b] Metcalfe, 1966 (MET, ref. 17); Bannon, 1982 (BAN, ref. 18); Joseph and Ackman, 1992 (JAC, ref. 3); Hartman and Lago, 1973 (HLA, ref. 19); Jham, 1982 (JHA, ref. 20); ISO 5509, 1978 (ISO, ref. 21); Bannon, 1982 (BBA, ref. 15) and Schuchardt and Lopes, 1988 (SLO, ref. 22). Results given as mean of three repetitions injected in duplicate.

Table 5. Relative recovery data of methyl tricosanoate for trilinolein (TG 18:2n-6) after esterification by different methods[a]

Method[b]	Concentration in the Sample	Amount Added	Spike Amount	Recovered Amount	Recovery/ (%)
MET	10.6	5.02	14.7	4.13	95.3
BAN	11.1	5.02	15.6	4.41	97.2
JAC	2.26	1.02	3.27	1.01	101
HLA	16.9	7.01	20.3	3.45	85.9
JHA	2.89	1.21	3.71	0.815	91.4
ISO	-	-	-	-	-
BBA	12.2	5.02	17.2	4.97	101
SLO	18.9	8.00	25.2	6.30	94.6

[a] Results expressed as means of triacylglycerol in three repetitions by addition of internal standard. [b] Metcalfe, 1966 (MET, ref. 17); Bannon, 1982 (BAN, ref. 18); Joseph and Ackman, 1992 (JAC, ref. 3); Hartman and Lago, 1973 (HLA, ref. 19); Jham, 1982 (JHA, ref. 20); ISO 5509, 1978 (ISO, ref. 21); Bannon, 1982 (BBA, ref. 15) and Schuchardt and Lopes, 1988 (SLO, ref. 22). Results given as means of three repetitions injected in duplicate.

Table 6. Relative recovery data of methyl tricosanoate for trilinolenin (TG 18:3n-3) after esterification by different methods[a]

Method[b]	Concentration in the Sample	Amount Added	Spike Amount	Recovered Amount	Recovery/ (%)
MET	0.848	0.429	1.09	0.241	87.0
BAN	0.858	0.429	1.08	0.224	85.8
JAC	0.185	0.090	0.249	0.064	92.3
HLA	1.38	0.588	1.62	0.249	84.4
JHA	0.243	0.090	0.308	0.065	94.3
ISO	3.14	1.48	4.39	1.25	97.0
BBA	0.961	0.429	1.31	0.353	96.4
SLO	1.45	0.753	1.91	0.464	88.6

[a] Results expressed as mean of triacylglycerols after three repetitions by addition of internal standard. [b] Metcalfe, 1966 (MET, ref. 17); Bannon, 1982 (BAN, ref. 18); Joseph and Ackman, 1992 (JAC, ref. 3); Hartman and Lago, 1973 (HLA, ref. 19); Jham, 1982 (JHA, ref. 20); ISO 5509, 1978 (ISO, ref. 21); Bannon, 1982 (BBA, ref. 15) and Schuchardt and Lopes, 1988 (SLO, ref. 22). Results given as means of three repetitions injected in duplicate.

The addition and relative recovery test of methyl tricosanoate confirmed the accuracy of the esterification methods analyzed and the quantification data of the oils studied.

Conclusion

These results show the efficiency of esterification for the main saturated fatty acid (C16:0). A low cost and toxicity reagent and a short analysis time may be used for the analyzed samples. The three most efficient esterification methods of unsaturated fatty acids are JAC, ISO, and BBA. The comparison of method JAC to methods ISO and BBA shows that it requires less reagent. Nevertheless, the reagent used (BF_3) is extremely toxic, costly, and has limited useful lifetime. Therefore, methods ISO and BBA, which use less toxic and cheaper reagents, may be used in the place of JAC for low acidity samples, as the possibility of formation of salt due to the presence of free fatty acids is minimized. The results obtained also show that the fatty acid analysis method chosen may depend on other factors besides the composition of the oil investigated, as previously mentioned. It should be stressed that if the samples present high acidity, the JAC method must be chosen despite its high toxic reagents to avoid analysis errors due to the presence of free acids.

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