

# Influence of the esterification method on the quantification of olive oil fatty acids

## Influência do método de esterificação na quantificação de ácidos graxos em óleo de oliva

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### Abstract

To analyze fatty acids by gas chromatography, it is necessary to apply esterification procedures to convert fatty acids into more volatile compounds, such as fatty acid methyl esters (FAME). Esterification methods are usually subdivided into two categories: acidic catalysis and basic catalysis. Due to the possibility of obtaining different concentrations of fatty acids for the same sample as a function of the esterification method used, the efficiency of eight different esterification methods that involve acidic and basic catalysis in the quantitative determination of FAME in olive oil was verified. The selected methods were described by Metcalfe, 1966 (MET); Bannon, 1982 (BAN); Joseph and Ackman, 1992 (JAC); Hartman and Lago, 1973 (HLA); Jham, 1982 (JHA); ISO 5509, 1978 (ISO); Bannon, 1982 (BBA) and Schuchardt and Lopes, 1988 (SLO). The results showed the efficiency of the esterification methods for the main saturated fatty acids that were present in the olive oil analyzed. The most efficient methods for the esterification of unsaturated fatty acids in the oils analyzed were JAC, ISO, and BBA. Nevertheless, the reagent BF<sub>3</sub> in methanol, used in the JAC method, is extremely toxic. Thus, when the oil to be analyzed has low acidity, the basic catalysis methods ISO and BBA can be used instead, since they use inexpensive reagents of low toxicity. The results obtained showed that the choice of a method for the analysis of fatty acids also depends on the composition of the oil to be studied.

**Keywords:** Esterification Methods; Fatty Acids; Olive Oil; Gas Chromatography.

### Resumo

Para a análise de ácidos graxos por cromatografia a gás, é necessário aplicar procedimentos de esterificação para converter os ácidos graxos em compostos mais voláteis, tais como ésteres metílicos de ácidos graxos (EMAG). Métodos de esterificação normalmente se subdividem em categorias: Catálises ácidas e catálises básicas. Devido a possibilidade de obtenção de diferentes concentrações de ácidos graxos para a mesma amostra em função do método de esterificação utilizado, a eficiência de de oito diferentes métodos de esterificação que envolvem catálises ácidas e básicas na determinação quantitativa de EMAGs em óleo de oliva foi avaliada. Os métodos selecionados foram descritos por Metcalfe, 1966 (MET); Bannon, 1982 (BAN); Joseph e Ackman, 1992 (JAC); Hartman e Lago, 1973 (HLA);

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Jham, 1982 (JHA); ISO 5509, 1978 (ISO); Bannon, 1982 (BBA) e Schuchardt eLopes, 1988 (SLO). Os resultados mostram a eficiência dos métodos de esterificações para os principais ácidos graxos saturados presentes no óleo de oliva analisado. Os mais eficientes métodos para a esterificação de ácidos graxos insaturados nos óleos analisados foram JAC, ISO, e BBA. Porém, o reagente BF<sub>3</sub> em metanol, usado no método JAC é extremamente tóxico. Assim, quando o óleo a ser analisado tem baixa acidez, os métodos de catálise básica ISO and BBA podem ser usados no lugar dele, uma vez que são mais baratos e menos tóxicos. Os resultados obtidos mostram que a escolha do método para a análise dos ácidos graxos depende também da composição do óleo estudado.

**Palavras-chave:** Métodos de esterificação; ácidos graxos; óleo de oliva; cromatografia a gás.

## Introdução

Gas chromatography (GC) stands out among other methods of analysis of fatty acids because it allows their easy separation and quantification. However, to analyze fatty acids by gas chromatography, it is necessary to increase fatty acid volatility by converting them into methyl ester derivatives (EDER, 1995; ULBERTH; SCHRAMMEL, 1995; JEYASHOKE; KRISNANGKURA; CHEN, 1998; SEPPÄNEN LAAKSO; LAAKSO; HILTUNEN, 2002). FAME are used mostly due to the large number of conversion procedures available in literature.

Esterification methods are normally divided into two categories: acidic catalysis and basic catalysis. The reagents most used in acidic catalysis esterification are hydrochloric acid (HCl), sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), and boron trifluoride (BF<sub>3</sub>) in methanol (CH<sub>3</sub>OH) (METCALFE; WANG, 1981; MEHER; SAGAR; NAIK, 2006). They are all used in the esterification of acylglycerols and free fatty acids. However, all these reagents require heating. Among these reagents, BF<sub>3</sub>/CH<sub>3</sub>OH is the most used in the esterification of all lipid classes (BANNON et al., 1982a).

The reagents most commonly used in the esterification of fatty acids by basic catalysis are methanolic solutions of (NaOCH<sub>3</sub>) sodium (KOH) and potassium hydroxide and sodium methoxide (NaOH). The basic catalysis esterification methods are fast and may be carried out at room temperature, reducing the risk of decomposition of polyunsaturated fatty acids and

avoiding the use of an antioxidizing agent. One of the disadvantages is that these reagents do not convert free fatty acids into methyl esters of fatty acids (BANNON et al., 1982b; GLASS, 1971; GUTNIKOV, 1995). The reagent guanidine and its methanol alkylated derivatives are also used to convert the compounds present in oils and fats into methyl esters. (SCHUCHARDT; LOPES, 1988; EDER, 1995).

As previously stated, the esterification procedures used may affect the quantitative results directly due to the incomplete conversion of lipids into FAME, changes in fatty acid composition during esterification, the contamination of the chromatographic column with traces of the esterification agent, incomplete extraction of FAME, and the loss of short-chain methyl esters and volatiles. (SHANTA; NAPOLITANO, 1992; BRONDZ, 2002).

The addition of an internal standard is much used in the analysis of fatty acids because it allows expressing the results in weight. This method is less sensitive to errors as the internal standard and the sample are injected together, and also because it is possible to express the results of fatty acids in weight rather than in FAME through the use of correction factors. (ACKMAN; SIPOS, 1964; VISENTAINER; FRANCO, 2006).

Given the possibility of obtaining different fatty acid quantification results for the same sample depending on the esterification method used, the objective of the present work is to investigate the efficiency of the determination of

olive oil fatty acids and the applicability of eight esterification methods involving acidic and basic catalysis.

## Experimental

### *Sampling*

Olive oil was used as purchased from shops in Maringá, Paraná State. The oil was filled into a flask under N<sub>2</sub> flow and stored under refrigeration during analysis. The analysis was carried out in five repetitions. The oil acidity was determined according to Adolfo Lutz (1985).

### *Esterification Methods*

#### *Method described by Metcalfe, Schmitz e Pelka, 1966 (MET)*

Approximately 150 mg of oil was mixed with 4.0 mL of NaOH in 0.50 mol L<sup>-1</sup> methanol and heated in a bath at 100 °C until the dissolution of the fat globules (ca. 5 min). Next, 5.0 mL BF<sub>3</sub> (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 then left at rest for phase separation. The aqueous phase was discarded and the ether phase was filtered with a 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., 1982a (BAN)*

Approximately 150 mg of oil was weighed and 5.0 mL of KOH in 0.50 mol L<sup>-1</sup> methanol was

added. The mixture was heated under reflux for 3 min. Next, 5.0 mL BF<sub>3</sub> (14%) in methanol was added 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 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, 1992 (JAC)*

Approximately 25 mg (± 0.1 mg) of oil was weighed and 1.5 mL of NaOH in 0.50 mol L<sup>-1</sup> in methanol was added. The mixture was heated in a bath at 100 °C for ca. 5 min and next cooled to room temperature. The mixture was added with 2.0 mL BF<sub>3</sub> (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 the addition of 1 mL of isooctane. It was vigorously stirred for 30 s before the addition of 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, another 1.0 mL of isooctane was added and the tube 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, 1973 (HLA)*

To 200 250 mg of oil was added 5.0 mL of NaOH 0.50 mol L<sup>-1</sup> 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 then 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 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 collected, 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, Teles e Campos, 1982 (JHA)*

An amount of 50  $\mu\text{L}$  of oil was transferred to a tube and 1.0 mL of 0.50 mol  $\text{L}^{-1}$  KOH in methanol was added to it and heated in a bath at 100 °C for 5 min. Then 400  $\mu\text{L}$  of HCL in aqueous methanol (4:1 v/v) was added. The mixture was heated in a bath at 100 °C for 15 min, cooled, and then 2.0 mL of water and 3.0 mL of petroleum ether were added and stirred. After collecting the supernatant, 3.0 mL of petroleum ether was added to another tube and stirred for 5 min. 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  $\mu\text{L}$  of chloroform before the injection into the gas chromatographer.

*Method 5509 described by ISO, 1978 (ISO)*

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

*Method described by Bannon et al., 1982b (BBA)*

A mass of ca. 150 mg of oil was weighed and 5.0 mL of NaOMe (0.25 mol. $\text{L}^{-1}$ ) in methanol:diethyl ether (1:1) was added 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  $\mu\text{L}$  of the top phase containing FAME was collected for gas chromatography analysis.

*Method described by Schuchardt and Lopes, 1988 (SLO)*

A mass of ca. 250 mg of oil was weighed and 2.0 mL of tetramethylguanidine in methanol (1:4 v/v) was added. 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.

*Apparatus and 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 7420 from Varian (USA) (100% bonded cyanopropyl, dimensions: 100 m, 0.25 mm, and 0.39  $\mu\text{m}$  i.d.). The operation parameters used were: column temperature of 197 °C for 23 min and 235 °C (20 °C/min) for 20 min at 40 psi. The injector and detector were kept at 220 °C and 245 °C, respectively. The gas flows used were 1.4 mL/min carrier gas ( $\text{H}_2$ ), 30 mL/min make up gas ( $\text{N}_2$ ), 30 mL/min hydrogen, and 300 mL/min flame synthetic air. The splitter

used was 1/80. Injections of 1  $\mu\text{L}$  sample were carried out in duplicate. The FAME peak areas were determined with the software Workstation version 5.0 (Varian).

### *Identification*

The FAME were identified by comparison of the retention times of the sample constituents with those of Sigma standards (USA), with the addition of standard and by ECL (Equivalent Chain Length) according to Visentainer and Franco (2006). The peak areas of FAME were determined with the software Workstation version 5.0 (Varian).

Evaluation of the flame ionization detector response: The answer factor for each FAME was determined according to Ackman (1972) to verify the agreement between theoretical and experimental response factors.

Limits of detection and quantification: The limits of detection and quantification were estimated according to the ACS recommendations with successive dilutions of a standard solution of methyl arachidate, considering the signal noise ratios equal to 3 and 10, respectively.

### *FAME quantification*

FAME were quantified in relation to the internal standard, methyl tricosanoate (23:0) from Sigma. The internal standard solution was prepared with a concentration of 1.0  $\text{mg mL}^{-1}$  in isooctane. The internal standard (methyl tricosanoate) was added before weighing the oil

in the esterification recipient. The concentrations of olive oil fatty acids were calculated according to Cantellops et al. (1999). The results are expressed in mg of FAME per gram of oil.

### *Statistical Analysis*

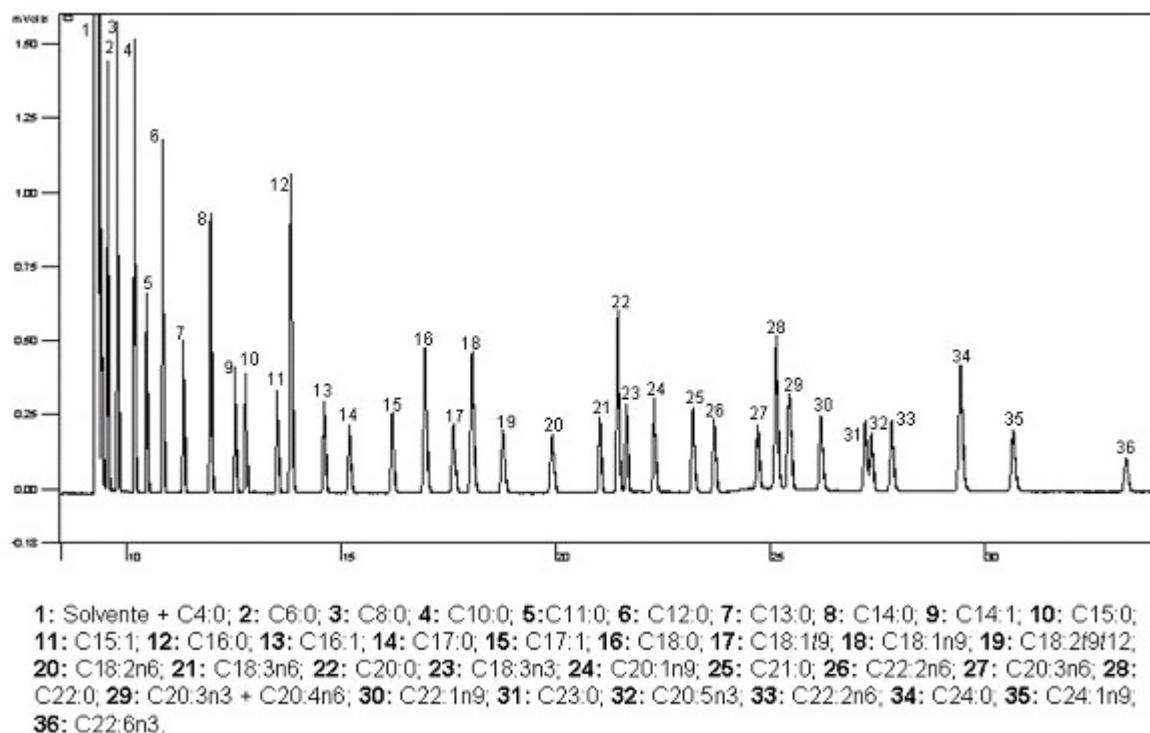
The results are expressed as mean  $\pm$  standard deviation. The different methods were compared by variance analysis (ANOVA) at 5% significance using the program Statistica 7.0. The mean values were compared by Tukey's test.

## **Results and Discussion**

This study evaluated eight methods of esterification applied to olive oil aiming at the determination of the most adequate method for the quantitative analysis of the study sample and considering the toxicity of the methods and the cost-benefit relationship.

The acidity index obtained for olive oil was  $0.078 \pm 0.002^*$ , (\*mean of six repetitions plus standard deviation), which indicates that the study oil has low acidity.

The FAMES were identified by spiking with standard, comparison with standards (Figure 1), and by ECL, which allowed for the confirmation of the constituent fatty acids of olive oil. The use of ECL together with the other tools mentioned allowed the confirmation of the fatty acids in a simple way at low cost and with high efficacy. This procedure was essential for the quantification of FAME.

**Figure 1.** Chromatogram of FAME standards Sigma

Before the quantitative analysis of FAME, it was necessary to evaluate the response of the flame ionization detector of the gas chromatograph. This was carried out by verifying the experimental response factors obtained with a mixture of FAME standards and the theoretical response to methyl tricosanoate. The experimental factors of saturated FAME were closer to the theoretical values when compared with the values obtained for unsaturated FAME. This difference may have been due to the oxidative instability of unsaturated FAME; therefore, saturated FAME was used to verify the equipment optimization. After optimization, theoretical factors were used in the quantitative determination of polyunsaturated fatty acids in accordance with Bannon et al., 1982a.

After verifying the agreement between the experimental and theoretical response factors in the concentration range used in the quantification of FAME with dilutions of standard solutions of FAME C18:1n-9, C18:2n-6, C18:3n-3, C20:0 in relation to methyl tricosanoate and the addition of the

internal standard, the esterification procedures were employed.

The limits of detection and quantification were estimated from successive dilutions of a standard solution of methyl arachidate, giving values of 0.148 and 0.476 mg/g of oil, respectively.

The results of FAME in olive oil obtained by the different esterification methods are presented in Table 1. Eight FAME were quantified in olive oil; the major ones (Figure 2) were esters methyl palmitate (C16:0), methyl oleate (C18:1n-9), and methyl linoleate (C18:2n-6).

The methyl palmitate concentration found after esterification of olive oil differed ( $p < 0.05$ ) from those of the methods BAN (100 mg/g) and JHA (105 mg/g), and from the results of the other investigated methods, which gave intermediate values.

Among the analyzed methods, the concentration found for methyl estearate was not significantly different ( $p > 0.05$ ), with a mean value of 29.3 mg/g.

The esterification methods JHA and SLO had the lowest esterification yield of methyl oleate (741 and 745 mg/g, respectively), followed by methods MET, BAN, and HLA. In contrast, the esterification yields of methods JAC, ISO, and BBA stood out, with a mean value of 776 mg/g, which were different from the other ( $p < 0.05$ ) methods.

The concentrations found for methyl linoleate by methods JAC, ISO, and BBA were higher (80.4, 80.5, and 80.8 mg/g, respectively), and different ( $p < 0.05$ ) from the other methods. Methods MET, BAN, and JHA gave close and statistically equal ( $p > 0.05$ ) concentrations. The method that gave the lowest yield for this FAME was HLA, with 71.1 mg/g.

**Table 1.** Concentrations of the fatty acid methyl esters (FAME) (mg g<sup>-1</sup> oil) in the analyzed olive oil after esterification by different methods.

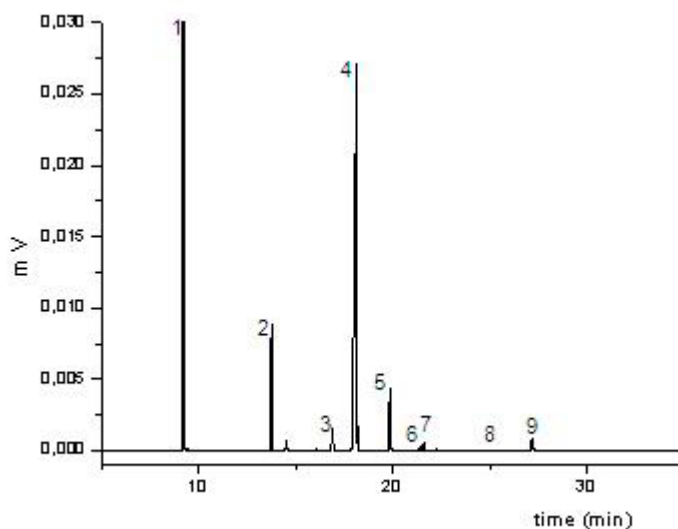
FAME (mg g <sup>-1</sup> oil)	Methods*				
	Acid catalysis				
	MET	BAN	JAC	HLA	JHA
C16:0	101 ± 1.9 AB	100 ± 1.6 A	100 ± 1.5 AB	103 ± 1.1 BC	105 ± 2.5 C
C18:0	29.1 ± 0.14	29.5 ± 0.13	29.4 ± 0.37	29.3 ± 0.31	29.5 ± 0.27
C18:1**	756 ± 4.0 B	753 ± 2.0 B	779 ± 4.7 C	759 ± 3.6 B	741 ± 3.1 A
C18:2n-6	72.9 ± 0.32 B	73.8 ± 0.83 B	80.4 ± 0.44 D	71.1 ± 0.76 A	73.2 ± 1.17 B
C20:0	3.84 ± 0.092 BC	3.85 ± 0.031 BC	3.99 ± 0.050 C	3.62 ± 0.111 AB	3.86 ± 0.130 BC
C18:3n-3	5.85 ± 0.131 A	5.68 ± 0.053 A	6.57 ± 0.190 C	5.79 ± 0.090 A	6.15 ± 0.151 B
C22:0	1.11 ± 0.070 B	1.05 ± 0.020 AB	1.11 ± 0.050 B	1.05 ± 0.053 AB	1.06 ± 0.051 AB
C24:0	0.543 ± 0.0512	0.502 ± 0.0210	0.531 ± 0.0512	0.492 ± 0.0322	-
Summation	970 ± 6.2	967 ± 4.8	1001 ± 6.8	973 ± 5.5	960 ± 6.8

FAME (mg g <sup>-1</sup> oil)	Methods*		
	Basic catalysis		
	ISO	BBA	SLO
C16:0	102 ± 1.0 ABC	100 ± 0.7 AB	100 ± 0.2 AB
C18:0	29.3 ± 0.06	29.3 ± 0.36	29.3 ± 0.31
C18:1**	775 ± 5.0 C	773 ± 3.1 C	745 ± 2.5 A
C18:2n-6	80.5 ± 0.57 D	80.8 ± 0.17 D	76.1 ± 0.80 C
C20:0	3.83 ± 0.033 BC	3.50 ± 0.050 A	3.74 ± 0.073 B
C18:3n-3	6.13 ± 0.060 B	6.38 ± 0.074 BC	5.83 ± 0.101 A
C22:0	1.05 ± 0.022 AB	1.07 ± 0.041 AB	1.02 ± 0.030 A
C24:0	0.491 ± 0.0330	-	0.482 ± 0.0312
Summation	998 ± 6.6	994 ± 4.2	961 ± 3.7

\*Metcalf e al., 1966 (MET); Bannan et al., 1982 (BAN), Joseph e Ackman, 1992 (JAC); Hartman e Lago, 1973 (HLA); Jham et al., 1982 (JHA); ISO 5509, 1978 (ISO); Bannan et al., 1982 (BBA); Schuchardt e Lopes, 1988 (SLO). \*\*Summation of FAMEs C18:1n-9 e C18:1n-7. Results given as means of five repetitions injected in duplicate. Different letters in the same line indicate significant difference by Tukey's test at 5% level.

**Figure 2.** Chromatogram of olive oil FAME. 1- Solvent, 2-C16:0, 3-C18:0, 4-C18:1n-9 and C18:1n-7, 5-C18:2n-6, 6-C20:0, 7-C18:3n-3, 8-C22:0, 9-C23:0.



For methyl alpha-linolenate, methods MET, BAN, and HLA, and SLO gave a mean value of 5.79 mg/g, which was statistically equal ( $p > 0.05$ ). This value was lower than those of the other esterification methods.

We could observe that the concentration of methyl palmitate in olive oil had little influence on its quantification.

Methods HLA, JHA, and ISO were more efficient in the esterification of methyl palmitate than the other studied methods. The fact that the ISO (basic catalysis) values were close to those obtained by acid catalysis (HLA and JHA) is directly related to the low acidity of olive oil that was analyzed.

However, the HLA and JHA methods had disadvantages in comparison to the ISO method in terms of time of analysis and number of steps in the esterification reaction, as the latter requires two steps, which increases the time of analysis of FAME.

Among the three methods considered the most efficient, method ISO requires the fewest number of steps and does not require heating. In relation to the quantification with an internal standard, method ISO

requires a great amount of internal standard, since a certain ratio of sample mass and internal standard mass is necessary. As this method employs 1.0 g of sample, it increases the cost of analysis.

The esterification methods used did not influence the quantification of methyl estearate in olive oil, which had a lower concentration than methyl palmitate. Probably, the position of the saturated acids in the triacylglycerol (*sn-1 and sn-3*) can be attributed to this result, as the nucleophilic attack of the carbonyl carbon can occur more easily.

The main monounsaturated FAME present in olive oil is methyl oleate. Methods JHA and SLO gave lower concentrations. In method JHA, this fact can be related to the presence of water, as the method requires the use of aqueous HCl, which may have led to hydrolysis. However, it must be considered that the JHA method used HCl in methanol as a catalyst, which has the advantage of low cost and availability of the reagent in relation to the methods that use  $\text{BF}_3$ .

The lower concentration of methyl oleate for the SLO method may be correlated to the esterification reagent used (tetramethylguanidine in methanol)



and a heating time of only 2 min. These two factors may be attributed to the low concentration of this FAME, probably due to steric hindrance resulting from the size of the tetramethylguanidine molecule and the distortions of the unsaturated fatty acid chain, as well as the heating time used, which may have been insufficient for the complete esterification of the methyl oleate.

Methods MET, BAN, and HLA gave close values of concentrations of methyl oleate in olive oil; however, these values were lower than those of methods JAC, ISO, and BBA

As previously described, methods MET and BAN employ the same reagents; however, their heating times are shorter than that of the JAC method. This may have contributed to the lower yield of methyl oleate, as the total esterification time may have been insufficient. It must also be considered that BAN has been proposed and investigated in the esterification of short-chain fatty acids.

The investigated olive oil had a lower concentration of methyl linoleate with method HLA, which was probably due to the heating time used in the esterification step and the preferential position of the fatty acids in the triacylglycerol at *sn*-2.

However, even though the HLA method gave a lower concentration of methyl linoleate, it must be considered that it uses  $\text{H}_2\text{SO}_4$  -  $\text{NH}_4\text{Cl}$  in methanol, which has the advantage of low cost and reagent availability in relation to the methods that use  $\text{BF}_3$ .

As observed for methyl alpha-linolenate, the methods that were least efficient were MET, BAN, HLA, and SLO, probably due to the insufficient heating time of the esterification step, as previously described.

The most efficient methods for unsaturated fatty acids of olive oil were JAC, ISO, and BBA, which may be related to the low acidity of the oil and the steps of the methods.

In the ISO method, first the solvent (n-heptane)

is used to solubilize the oil, and next the base in methanol is added. This may have contributed to the results obtained, as the olive oil was dispersed in the solvent and this may have made the esterification reaction easier, as this method does not require heating and uses only agitation after the addition of the catalyst. This fact was also reported by Glass (1971) and is also valid for the BBA method, as the catalyst used (sodium methoxide) is in a diethyl ether solution.

As compared to methods ISO and BBA, method JAC, which uses  $\text{BF}_3$  in methanol and a long heating time, consumes less reagent, but the reagent,  $\text{BF}_3$ , is extremely toxic, costly, and has a short useful life. Therefore, methods ISO and BBA, which use less toxic and inexpensive reagents, can be employed instead of the JAC method. The results obtained by Milinsk et al. (2008) 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.

## Conclusion

Among the methods studied for the analysis of olive oil, methods ISO and BBA are recommended for their low toxicity and cost in relation to method JAC, which uses  $\text{BF}_3$  in methanol and requires a long heating time.

The present results show that the choice of a method for the analysis of fatty acids may depend on the oil composition, as well as on other factors already mentioned.

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*Recebido em 8 Abril 2001- Received on April 8, 2011.*

*Aceito em 25 Maio, 2011 - Accepted on May 25, 2011.*

