Determination of the Wax Ester Content in Olive Oils. Improvement in the Method Proposed by EEC Regulation 183/93

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A simpler and faster procedure than the official one described in document IV of European Economic Community Regulation 183/93 is proposed. The wax ester fraction is isolated from triglycerides using a commercially available silica gel column and carbon tetrachloride as eluent. The recovered wax ester fraction, with the addition of a suitable internal standard solution, is analyzed by gas chromatography. A column with a 65% phenyl methyl silicone stationary phase allows a satisfying separation of wax ester fraction in comparison with both a steryl ester and a light fraction eluted before the internal standard. Furthermore, also the single components of the wax ester fraction are suitably separated.

Keywords: *GC; olive oil; solid-phase extraction; steryl esters; wax esters*

INTRODUCTION

The European Economic Community classifies olive oils in nine categories and provides for the destination of human consumption only the following: extra-virgin olive oil, virgin olive oil, olive oil, and "sansa" olive oil (EEC, 1991). Pressure oils are characterized by a low content in wax esters, while "sansa" olive oils are characterized by a high content in wax esters.

The EEC Commission, on January 29, 1993, under Regulation 183/93, which modifies Regulation 2568/91 regarding the characteristics of olive oil and "sansa" olive oil, introduced the wax ester parameter to distinguish pressure oils from solvent-extracted oils and to detect fraudulent additions of "sansa" olive oil to pressure oils (EEC, 1993). The analysis of wax esters must be carried out according to the method described in document IV of the modification of EEC Regulation 2568/91 (EEC, 1991). Even though this method is accurate, it requires a great amount of time and staff to be carried out. Various attempts have been made to reduce the required time and to simplify the analysis procedure. Among these methods, the most interesting one, which was proposed by Grob, separates the wax ester fraction by means of a high-performance liquid chromatography (HPLC) and automatically transfers it to a gas chromatograph for a definitive analysis (Grob et al., 1989, 1990). Unfortunately, this elegant procedure requires sophisticated tools that are not easily found, even in specialized laboratories.

In terms of wax esters, we were able to determine that the ECC method provides accurate results if the procedure is carried out in a scrupulous way, a finding that was recently the subject of a published study (Imperato, 1996).

In our study, starting from the principles of the official method (EEC, 1993), we developed a reliable procedure that simplifies the operation regarding the isolation and recovery of the wax ester fraction of the oil.

MATERIALS AND METHODS

Principles of the Method. The fatty sample, with the addition of the internal standard, was fractioned by solid-phase extraction using a commercial silica gel column; the wax ester fraction was then eluted and analyzed by gas chromatography (GC).

Reagents and Chemicals. *n*-Hexane and carbon tetrachloride analytical grade were purchased from Fluka Chemie (Buchs, Switzerland). Lauryl arachidate (C32), palmityl oleate (C34), stearyl oleate (C36), arachidyl oleate (C38), behenyl oleate (C40), behenyl arachidate (C42), behenyl behenate (C44), and behenyl lignocerate (C46) came from Larodan (Malmo, Sweden).

Analytical Procedure. A SPE Chromabond 1000 mg silica gel column (Macherey-Nagel, Duren, Germany) was rinsed with 6.0 mL of carbon tetrachloride. A standard solution of lauryl arachidate (400 mg/L in *n*-hexane) was prepared (solution A), and by diluting it, a standard solution of lauryl arachidate of 40 mg/L was prepared (solution B). Then 20 mg of oil was weighed in a test tube, and 1.0 mL of the internal standard solution B was added; the solvent was removed with nitrogen flow. The dried sample was transferred into the silica gel column rinsed with carbon tetrachloride; the test tube was washed three times with 100 μL of *n*-hexane, and these solutions were transferred into the rinsed silica gel column. The sample was eluted with 6.0 mL of carbon tetrachloride; the eluate was dried by using a rotating evaporator and recovered with 100 μL of *n*-hexane. Finally, 1 μL of the solution was injected into the gas chromatograph.

Gas Chromatography. The instrument used was a Dani model 86.10 HT equipped with a programmed temperature vaporizer injector, a flame ionization detector (Dani, Milan, Italy), and an electronic integrator HP 3394 (Hewlett-Packard, Palo Alto, CA). The operating conditions were as follows: carrier gas, helium; carrier linear velocity, 33 cm/s; oven temperature program, 2 min at 270 °C, raised at 5 °C/min to 360 °C, and 7 min at 360 °C; PTV injector temperature

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Table 1. Comparison of the CEE Method and the Proposed Method

analytic characteristics	official EEC method	optimized method
LC column	prepare	commercially available column
quantity of silica gel to utilize	15 g	1 g
stability of eluent	low	high
volume of solvent to utilize	200 mL of *n*-hexane:diethyl ether (99:1)	10 mL of carbon tetrachloride
quantity of the sample	500 mg	20 mg
column flow	critical	not critical
GC analysis capillary column	5% phenyl methyl silicone	65% phenyl methyl silicone
total analysis time	3 h (with great attention by operator)	40 min (with little attention by operator)

program, 10 s at 60 °C, raised at 200 °C /min to 370 °C, and 3 min at 370 °C; split ratio, 1:80; FID temperature, 370 °C; capillary column RTX-65 TG (Restek Corp., Bellefonte), 30 m × 0.25 mm i.d.; film thickness 0.10 μm.

Calculation of a Single Wax Ester:

$$\text{wax ester (mg/kg)} = [A(\text{W})\ C(\text{IS})\ 1000]/[A(\text{IS})\ P]$$

where A(W) is the peak area of wax ester (C40−C46), C(IS) represents the concentration of the solution B (mg/L), A(IS) is the peak area of the internal standard, and P is the weight of the sample (mg).

Evaluation of the Total Wax Ester Content:

$$\text{wax esters (mg/kg)} = \sum[\text{wax ester } C_{40}\ (\text{mg/kg}) - \text{wax ester } C_{46}\ (\text{mg/kg})]$$

Summary of the Procedure for the Determination of Wax Esters in Olive Oils, According to EEC Regulation 183/93. The most important details related to the time and staff commitment are reported.

Silica Gel Preparation. Silica gel is put in a muffle furnace at 500 °C for 4 h. After cooling, it is hydrated at 2%. To homogenize the mass, it is stirred vigorosly. Before it is used, it must be kept in the dark for at least 12 h.

Packing the Chromatographic Column for the Separation of Wax Esters Fraction. Silica gel (15 g) hydratated at 2% is put in suspension in anhydrous *n*-hexane and then transferred into the column. After the silica gel has settled down spontaneously, arrangement of the chromatographic bed is made more homogeneous by means of an electric shaker. The obtained silica gel column is 25 cm high at least. To purify the column, 30 mL of *n*-hexane is eluted. The sample is loaded into the column, and it is eluted with a *n*-hexane/diethyl ether (99:1) mixture with a flow of 2.1 mL/min. A volume of 140 mL of eluate is collected and dried to a volume of about 2.0 mL by means of rotating evaporator. The sample is transferred and completely dried with a nitrogen flow. In our laboratory, the above-reported steps require at least 3.0 h, with careful undertaking by the operator.

RESULTS AND DISCUSSION

Critical Evaluation of the EEC Method for the Determination of Wax Esters. In Table 1 the salient phases of the study are reported, regarding the EEC procedure for the determination of wax esters. As one can see, the EEC procedure is laborious and calls for a great amount of time to both prepare the chromatographic silica gel column and elute the sample; the described phases require 3 h in order to be carried out and much attention by the operator. It also becomes evident that this procedure requires a large quantity of silica gel and *n*-hexane for each determination. Finally, the elution with 140 mL of the 99:1 *n*-hexane/diethyl ether mixture is not always able to separate the wax ester fraction completely from the triglycerides. In fact, the quantity of diethyl ether contained in the mixture is critical: even a slightly greater quantity of diethyl ether than the required one can cause an incomplete separation between the wax ester fraction and the triglyceride fraction, while a smaller quantity

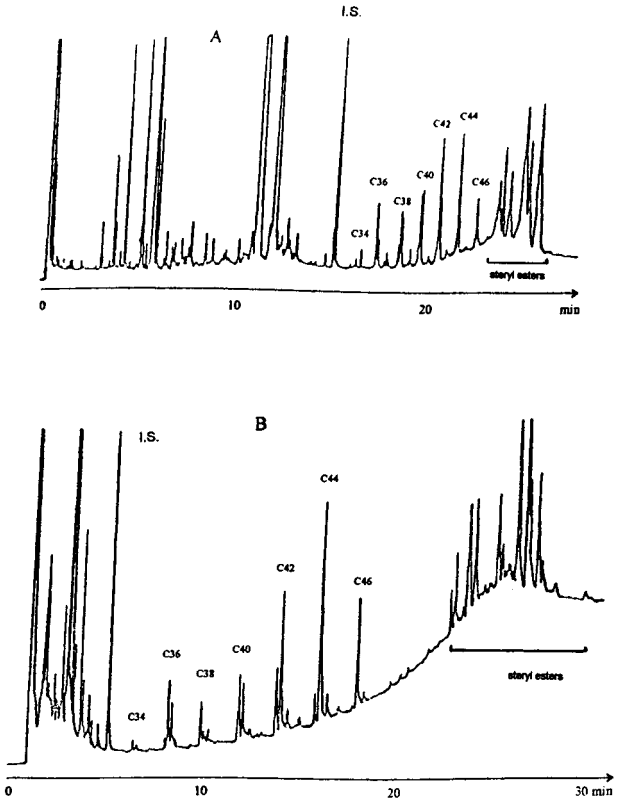

Figure 1. Gas chromatogram of the apolar fraction of a "lampante" olive oil obtained according to the official method in a 5% phenyl methyl silicone column (A) and in a 65% phenyl methyl silicone column (B).

does not allow the complete recovery of the wax esters in the estimated volume of eluent. In addition, the low boiling point of diethyl ether makes it difficult to do an accurate preparation of the eluant mixture. We remind the reader that the EEC method, regarded as the reference analysis method, allows one to obtain accurate results only if the procedure is scrupulously carried out.

The above-mentioned procedure we developed calls for the use of commercially available 1000 mg silica gel columns with a 1.0 cm high stationary phase and 6.0 mL of eluant. The official method requires an approximately 25 cm high silica gel column packed by the operator and 140 mL of eluant. The sample loading into the silica gel column must accurately be weighed; a greater than 25 mg quantity of sample saturates the silica gel column and prevents the wax ester fraction from completely separating from the triglyceride fraction. The optimal quantity of the sample is 20 mg of oil, which means that the amount of materials necessary for each determination is quite modest.

The required eluent, just 12 mL, consists of a single solvent and therefore avoids the changes in polarity over time, eliminating all the chromatographic risks regarding eluant composition variations.

Table 2. Ratio Values between the Areas of the Single Wax Esters (C34−C46) and the Area of the Internal Standard (C32) of a Solution Analyzed Five Times before and after Being Passed through Five Different Silica Gel Columns[a]

C34/C32	C36/C32	C38/C32	C40/C32	C42/C32	C44/C32	C46/C32
Solution before Being Passed through Silica Gel Column						
0.60	0.91	1.05	1.00	0.85	0.51	0.46
0.61	0.92	1.06	0.99	0.86	0.51	0.48
0.59	0.90	1.05	1.00	0.86	0.50	0.49
0.59	0.91	1.05	1.01	0.85	0.52	0.47
0.60	0.91	1.06	0.99	0.85	0.51	0.46
$M = 0.60$	$M = 0.91$	$M = 1.05$	$M = 1.00$	$M = 0.85$	$M = 0.51$	$M = 0.47$
$S = 1.7$	$S = 1.1$	$S = 1.0$	$S = 1.0$	$S = 1.2$	$S = 2.0$	$S = 4.3$
Solution after Being Passed through Silica Gel Column						
0.60	0.92	1.04	1.00	0.84	0.50	0.49
0.62	0.91	1.07	1.00	0.86	0.52	0.48
0.61	0.91	1.06	0.99	0.85	0.50	0.49
0.61	0.89	1.06	0.99	0.85	0.51	0.46
0.60	0.90	1.07	1.00	0.85	0.52	0.46
$M = 0.61$	$M = 0.91$	$M = 1.06$	$M = 1.00$	$M = 0.85$	$M = 0.51$	$M = 0.51$
$S = 1.6$	$S = 2.2$	$S = 1.9$	$S = 1.0$	$S = 1.2$	$S = 2.0$	$S = 4.2$

[a] M = average value. S = maximum percentage deviation from average value (%).

Table 3. Total Wax Esters Content According to the Official Method and to the Proposed Method for Olive Oil Samples Belonging to the Nine EEC Categories

olive oil category	total wax esters (mg/kg) according to the proposed method[a]	percentage deviation observed (%)	total wax esters (mg/kg) according to the official method[b]	percentage deviation between the average values obtained with the two procedures
extra-virgin	94	5.7	97	3.0
virgin	130	3.4	136	4.4
current virgin	207	3.0	203	1.9
virgin "lampante"[a] (acidity 4.4%)	447	3.1	450	0.6
refined	385	3.0	381	1.0
olive oil	533	3.0	540	1.3
crude residue oil	4018	2.7	4072	1.3
refined residue oil	3396	2.1	3390	0.2
residue oil	5289	2.0	5297	0.2
virgin "lampante" (acidity 10.3%)	681	5.2	693	1.7

[a] Average value of a single sample of oil analyzed 10 times. [b] Average value of a single sample of olive oil analyzed three times.

The speed of elution ranges from 0.50 mL/min to 1.50 mL/min; the time required for the procedure, up to the collection of the wax ester fraction, is about 20 min.

Evaluation of the Proposed Procedure. In the interest of checking the amount of recovery of the wax esters from the silica gel columns, a solution of wax esters (C34−C46) ranging from 250 to 500 mg/L was prepared; as an internal standard, an appropriate quantity of a 500 mg/L lauryl arachidate (C32) solution was added to the same solution. Five 300 μL aliquots of this solution were analyzed by passing them through five columns and eluting them with 6.0 mL of carbon tetrachloride, according to the proposed procedure. The eluates were analyzed by gas chromatography. The same solution, without being passed through the column, was concentrated three times and analyzed five times. In Table 2 the ratio values between the areas of the single wax ester and the area of the internal standard are reported as well as the percentage of standard deviation for each ratio compared to the average. The analysis of the results leads to the conclusion that the difference between the values of the analyzed solutions before the passage through the column and the values of the solution after the passage through the column is within the limits of experimental errors; therefore, it can be asserted that the passage through silica gel does not bring irreversible adsorption. In Table 3 the repeatability values of wax esters content obtained for the nine categories of olive oils with the proposed procedure are reported. In the same table, the results of wax esters content obtained with the EEC method for the same samples of oils are shown as well; the EEC method was regarded as a reference method. As it can be seen, the highest percentage deviation for the various categories of olive oils does not exceed 3.6%. Extra-virgin olive oils are an exception because they are characterized by a low wax esters content. The percentage deviation observed in the analyzed samples does not exceed 5.7%. The results showed for "lampante" oils are remarkable: a free acidity, at least up to 10%, does not deactivate silica gel and so does not produce matrix effects. The related gas chromatograms have a trend similar to those of the other categories of oils. The results obtained with the proposed procedure are in line with those obtained with the EEC method, regarded as the reference analysis method.

Gas Chromatographic Conditions. Because we are interested in the determination of both the single wax ester and the total wax ester, particular attention was paid to the choice of the gas chromatographic column and to the operating conditions. Satisfying results were obtained using a more polar capillary column than that advised by the EEC procedure. To illustrate this point, in Figure 1 the gas chromatograms for the same sample of wax ester are reported, one obtained with a 5% phenyl methyl silicone capillary column as suggested in the EEC method (A) and the other with a 65% phenyl methyl silicone capillary column that we proposed (B).

In the less polar column, the cereous compounds are separated principally as a function of the number of carbon atoms, and as a consequence the separation of

isomers is quite low; the same considerations are valid for steryl esters. For this class of compounds, better results were obtained with the use of the more polar 65% phenyl methyl silicone column.

CONCLUSIONS

The method proposed in this study finds results similar to those obtained with the EEC procedure, but the proposed method is easier to use, quicker, and accurate at the same time. Moreover, it is particularly convenient when a great number of samples have to be analyzed in a short time.

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