Separation of Wax Esters from Olive Oils by High-Performance Liquid Chromatography

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To detect adulteration of olive oil with solvent-extracted oils, the determination of the wax ester content has become more important during recent years. Hence, a greater number of wax ester analyses need to be performed by quality control laboratories. The most common method in use requires a liquid chromatographic (LC) separation of the less polar fraction, which contains the wax esters, from the glyceride matter on a hand-filled silica gel column. The aim of this project was to verify the possibility of replacing LC with high-performance liquid chromatography by taking advantage of the greater reliability and repeatability of this technique, as well as of the possibility of making the separation automatic. The paper describes how to perform the analysis and the statistical test that was carried out; furthermore, a comparison has been made with the usual method and results are in good agreement.

KEY WORDS: Crude pomace oil, detection of adulteration in olive oils, extravirgin olive oil, high-performance liquid chromatography (HPLC), high-resolution gas chromatography (HRGC), liquid chromatography (LC), refined olive oil, separation of wax esters, wax esters.

Wax esters of analytical interest in vegetable oils are esters of fatty acids with aliphatic or diterpenic alcohols, with a number of carbon atoms between 36 and 46. In olives, wax esters are mainly located on the epicarp of the drupe, and during the extraction process a fraction of these esters is transferred into the oil. The extraction of pomace oil by solvent causes a greater quantity of wax esters to be transferred and, therefore, the concentration of wax esters is much higher in pomace oils than in cold-pressed oils.

To detect adulteration with solvent-extracted oils, wax ester analysis has become increasingly important during the last few years. Quantitation of alkanols in the unsaponifiable matter is normally performed for the same purpose, even if it is no longer suitable. In fact, dewaxing processes by crystallization from solvent (*i.e.*, acetone) at low temperature greatly reduce the wax content of oils, and high, free alkanol levels could lead to wrong evaluations.

In the waxy fraction, C40–C46 esters are the least affected by dewaxing processes, which, for technological and economical reasons, are not worthwhile. This is why the wax analysis is normally carried out, taking into account only the wax esters with an even number of carbon atoms from C40–C46. From a legal point of view, the recent European Economic Community (EC) Regulation No. 183/93 requires the quantitation of wax esters, and a previous convention among the members of the International Olive Oil Council about the marketing of olive oils with the United States requires the content to be known. Liquid chromatography (LC) followed by gas chromatography (GC) is one of the most common methods currently in use.

Henon (1) suggested separating wax esters from sunflower oil on a mixed column of silica and silica impregnated with silver nitrate. In various papers Mariani *et al.* (2–5) described the isolation of wax esters on columns filled with silica gel and investigated the possibility of revealing the adulteration of cold-pressed olive oil with solvent-extracted oils. Moreover, they studied the effects of the refining process on different types of seed oils.

Kiosseoglou *et al.* (6) studied the fatty acids in wax esters of several types of seed oils, separating the polar fraction by means of a column of silica gel and $AgNO_3$-thin-layer chromatography (TLC). On the other hand, Kawanishi *et al.* (7) optimized the TLC separation of free primary alcohols from oil and wax esters of different fatty matters. Using the Chromarod-Iatroscan system, Ohshima *et al.* (8) investigated the behavior of free fatty acids, triglycerides, sterol esters and wax esters by using four different developing solvents.

Because wax esters cause turbidity when undewaxed seed oils are chilled, the winterization process removes them from such oils. To have a rapid procedure available for the control of winterization of sunflowerseed oil, Moulton (9) optimized a turbidimetric method to determine wax ester contents and tested its good repeatability. Recently Grob *et al.* (10–12) developed the powerful "on-line" coupled LC–GC technique, which is useful for simultaneous analyzation of many free and combined minor components contained in olive oil.

Finally, Grob *et al.* (13) and Mariani *et al.* (14,15) optimized a gas-chromatographic method to simultaneously determine sterols, alkanols, triterpenic alcohols, squalene, tocopherols and wax esters, as present in the fatty matter, and they demonstrated the utility and reliability of the method. The collection of Norme Italiane per il controllo dei Grassi e Derivati (NGD) (16) includes a method for the determination of wax esters (NGD C80-1989), which is substantially the same method included in EC Regulation No. 183/93 and is essentially described by Mariani *et al.* (2). It separates the low-polarity components from triglycerides by LC on a column filled with hydrated silica and subsequent high-resolution gas chromatography (HRGC). Unfortunately, this procedure is rather time-consuming and requires substantial manual operations, mainly for the preparation of the silica gel column. The aim of this work was to take advantage of high-performance liquid chromatography (HPLC) to perform automatic and quicker separations of wax ester from olive oils and to allow more samples to be analyzed daily. We used four types of olive oils (extravirgin oil, crude olive oil, refined oil and crude pomace oil) and made 25 repeated analyses for each type. Moreover, some comparisons were made with results obtained by means of NGD C80–1989.

MATERIALS AND METHODS

Samples. Four different types of olive oils were used: Extra virgin oil (obtained by pressing the olives, without refining or other treatments except of filtration; acidity < 1%); crude olive oil (obtained by pressing the olives, with acidity > 3.3% and/or unpleasant flavor, which is sold only after refining); refined oil (obtained by refining crude olive oil); and crude pomace oil (obtained by solvent

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extraction of olive pomace and which is sold only after refining).

Reagents. HPLC-grade reagents were used. *n*-Hexane, *n*-heptane and diethyl ether were obtained from Fluka (Buchs, Switzerland). Lauryl arachidate (obtained from Sigma Chemical Co., St. Louis, MO) 0.1% wt/vol in *n*-hexane was used as the C32 wax ester internal standard.

Apparatus and materials. For HPLC separations, the following conditions were used: gradient pump, LDC Analytical CM 4000 (LDC Analytical, Riviera Beach, FL); ultraviolet detector, Milton Roy (Rochester, NY) SpectroMonitor 3100; column, Supelcosil LC-Si, 15 cm × 4.6 mm i.d., 5 μ (Supelco, Bellefonte, PA); flow rate, 1.00 mL/min; detection wavelength, 203.5 nm; range, 0.10 absorbance units full-scale; response time, 0.10 s; loop, 20 μL. Elution gradient with *n*-hexane/diethyl ether: 0 min, 100:0; 1–2.5 min, 92:8; 13.5 min, 0:100; 13.6–25 min, 100:0; 25 min, ready for next run.

Sample injection was performed by the automatic sampler SpectraSystem AS1000 (Spectra Physics, San Jose, CA), and collection of the fraction containing wax esters was carried out with an FC 203 (Gilson Medical Electronics, Inc., Middleton, WI) fraction collector.

For the HRGC separations, the following equipment was used: gas chromatograph Carlo Erba (Milan, Italy) Mega Series HRGC 5160; capillary column, SPB-5 (5% diphenyl/94% dimethyl/1% vinylpolysiloxane), fused silica, 7 m × 0.25 mm i.d., 0.25 μm film thickness (Supelco). Oven temperature was programmed from 100 to 140°C at the maximum rate, then to 290°C at 5.5°C/min, then to 345°C at 3.0°C/min and a further 5 min at 345°C; detector (flame-ionization) temperature, 370°C; carrier gas, hydrogen at 1.3 mL/min; injection volume (on-column), 0.5–1/μL. Computing was performed with Maxima 820 software (Water Dynamic Solution, Millipore, Milford, MA), installed on an IBM PS2/H21 personal computer (Princeton, NJ).

Experimental procedures. For an olive oil analysis, weigh the sample exactly and add about 0.02% (w/w) of internal standard (C32). For example, 0.1 mg for 500 mg of oil (for pomace oil, add 0.25–0.50 mg for 500 mg of oil), and dilute with the same quantity of *n*-hexane, then mix well. If an automatic sampler is available, transfer a suitable volume of oil solution into the vial; otherwise, perform a manual injection. It is advisable that the first injection be made to set the correct collecting window for the fraction collector. If this device is not available, the fraction could be collected by hand. Evaporate the solvent to dryness, then add a suitable volume of *n*-heptane (*e.g.*, if the sample weighed 500 mg, add 200 μL of *n*-heptane). The solution is now ready to be analyzed by HRGC (Fig. 1).

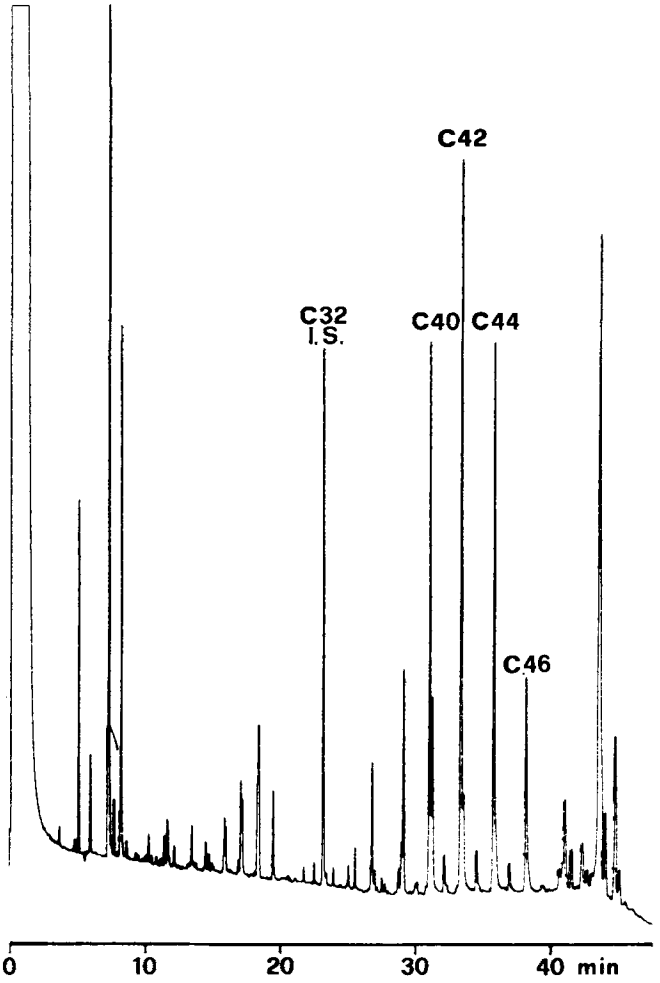

FIG. 1. Gas chromatogram showing wax esters separation; I.S., internal standard.

RESULTS AND DISCUSSION

Under the HPLC conditions described above, the collecting window starts at 4.5 min and ends at 8.0 min (Fig. 2). The sampling window was extended from 3.5 min to 9.0 min to verify the complete recovery of the wax esters, and the presence of the analytes was checked in the first and in the last minute. The result was that the wax esters are totally eluted in the narrower window, as shown in Figure 2. Furthermore, to verify any selective losses of wax esters, we compared the percentages of single wax esters in a standard solution (determined by direct HRGC analysis) with the percentages obtained after the HPLC separation of the same solution. Because the C46 wax ester was not available, the comparison was performed only for C36, C38, C40, C42 and C44 esters. As shown in Table 1, the data are in good agreement. After these tests were performed, a series of 25 analyses of each type of oil (100 in all) was made to evaluate the method's repeatability, and a comparison with NGD C80-1989 was made by performing five analyses for each type of oil. For each series of determinations, the mean value, the SD, the relative SD and the repeatability [according to Cozzoli *et al.* (17)] were calculated, and no statistical tests for anomalous data were applied (Figs. 3 and 4, Tables 2 and 3). It should be noted that the NGD repeatability value is merely approximate because of the few analyses performed.

The comparison between the mean values (Fig. 3 and Table 2) shows that the results from HPLC are nearly always lower than values from NGD, even if they are close. We suspected that this was caused by an overestimate of the internal standard due to the presence of interfering substances with the same retention time. Hence, we made some analyses without the internal standard but no interfering substances were detected. The reason for the different results remains to be explained.

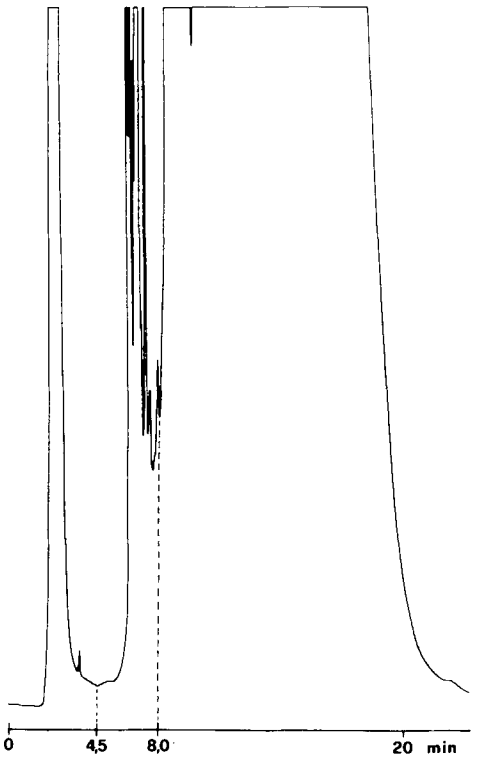

FIG. 2. High-performance liquid chromatography separation and collecting window.

As expected, comparisons among relative standard deviations (Fig. 4) show that HPLC separation almost always provides more precise values (sometimes 2 or 3 times more precise). Exceptions are pomace oil (for which relative SD values are low for both methods and C42 wax ester in extravirgin oil and for C46 in refined oil.

TABLE 1

Determination of Wax Ester Contents in a Standard Solution

	%				
	C36	C38	C40	C42	C44
By direct HRGC[a] separation	20.7	20.8	19.5	21.2	17.8
By previous HPLC[b] separation	20.5	21.2	19.2	21.0	18.1

[a]High-resolution gas chromatography.
[b]High-performance liquid chromatography.

TABLE 2

Mean Wax Ester Values for Crude Pomace Oil as Determined by HPLC and NGD Methods[a]

	ppm				
	C40	C42	C44	C46	Total
HPLC	1516	1863	1387	590	5356
NGD	1549	1906	1428	606	5489

[a]HPLC, high-performance liquid chromatography; NGD, Norme Italiane per il controllo dei Grassi e Derivati; (Milan, Italy).

We think that a more extensive investigation and a suitable statistical analysis should confirm the greater precision of the HPLC method, which is apparent from this work. Moreover, as the number of carbon atoms increases, corresponding to lower concentrations, the relative SD also increases. This is due to changes in the baseline drift, which mostly affects those quantitations, as Grob *et al.* (11) have noted.

Table 3 shows a comparison between the repeatabilities of the two methods but, as we mentioned above, the NGD method is provided as an example only. The repeatability obviously reflects the relative SD pattern.

In conclusion, replacing LC separation with HPLC allows us to benefit from all the advantages of this

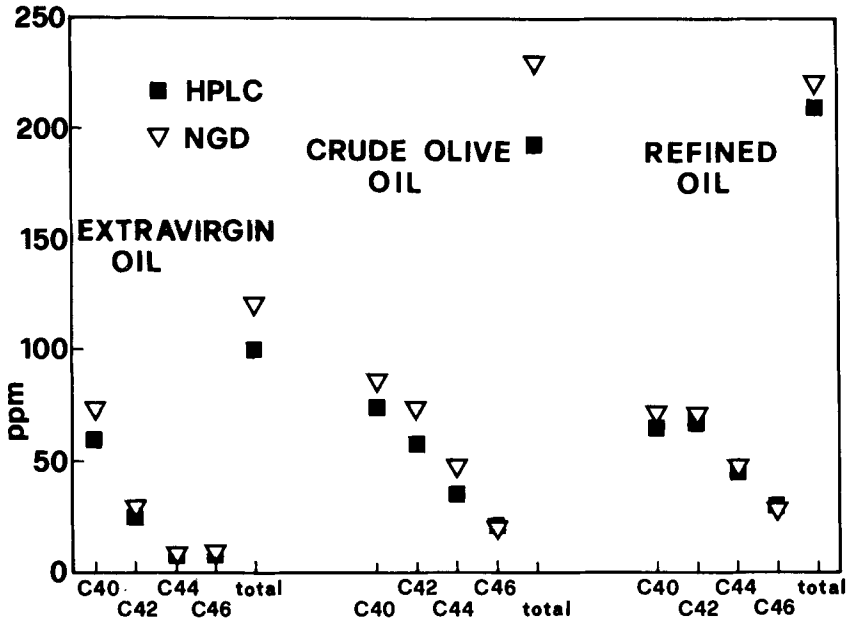

FIG. 3. Mean values for each type of olive oil. HPLC, high-performance liquid chromatography; NGD, Norme Italiane per il controllo dei Grassi e Derivati.

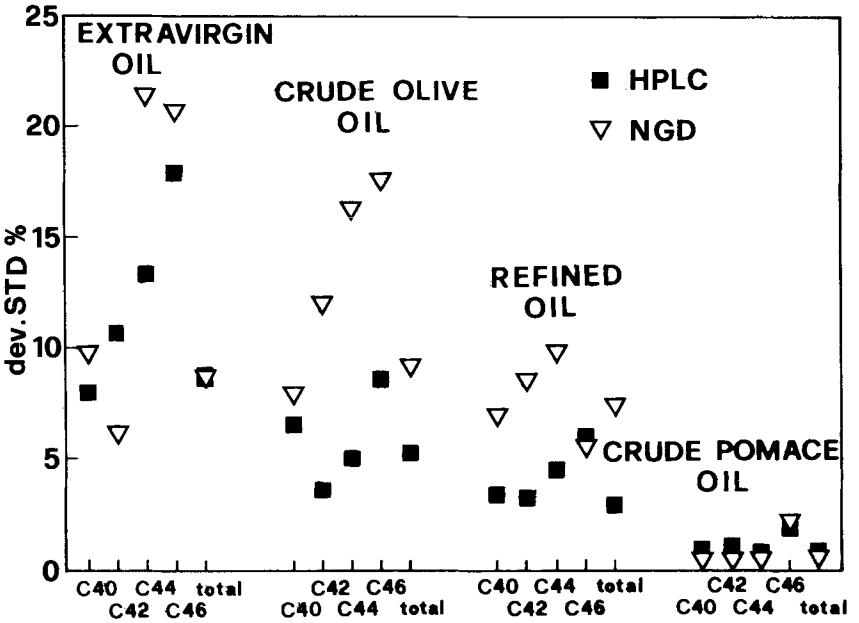

FIG. 4. Relative SD (%) for each group of analyses. Abbreviations as in Figure 3.

TABLE 3

Comparison of the Repeatability for Each Type of Oil

	ppm									
	C40[a]		C42		C44		C46		Total	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Extravirgin oil	14.1	28.8	7.8	7.3	2.7	6.8	4.2	7.2	25.1	41.8
Crude olive oil	14.4	27.1	6.3	35.2	5.3	31.2	5.7	14.2	29.5	83.6
Refined olive oil	6.5	22.9	6.4	27.3	6.0	21.6	5.4	7.4	18.0	74.4
Crude pomace oil	46.9	38.7	64.7	42.1	36.6	35.1	32.7	52.5	147.9	154.8

[a](a), This work; (b), NGD methods. Abbreviations as in Table 2.

separation technique, mainly pertaining to the repeatability and standardization of the analytical condition (*i.e.*, silica gel columns do not have to be prepared). Furthermore, a quality control laboratory has to perform large numbers of analyses daily, for which it would be useful to have automatic procedures. For this purpose, an automatic sampler for GC on-column injections and suitable software to process sequences of analyses should make them more reliable and less time-consuming.

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