Separation of Stigmasta-3,5-diene, Squalene Isomers, and Wax Esters from Olive Oils by Single High-Performance Liquid Chromatography Run

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ABSTRACT: To ascertain the authenticity of olive oils, the European Community Regulation requires the stigmasta-3,5-diene and wax ester contents to be determined. The official methods are time-consuming and not suitable for many daily analyses, as quality-control laboratories need. A method is presented here that allows single high-performance liquid chromatography separation of stigmasta-3,5-diene and wax esters, as well as of the squalene isomers, which give further information on the oil's authenticity. For stigmasta-3,5-diene, the comparison with results obtained with the official method is good. Also for wax esters, the agreement was good, even if they were compared with results obtained from a quicker method as reliable as the official one. The possibility of separating the squalene isomers also at the same time makes the proposed method more advantageous. On the whole, the method, which is suggested for routine and quick screening but not for the exact evaluation of the analyte contents, seems to be a convenient choice for ascertaining on a daily basis the samples' legal compliance (i.e., whether the analyte content is or is not below the legal value).
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KEY WORDS: EC regulations, HPLC, HRGC, olive oils, squalene isomers, stigmasta-3,5-diene, wax esters.

Nowadays, olive oil quality control is ever more important, especially in those years when the price is higher than usual. Furthermore, owing to its easy availability on international markets, i.e., Europe, North America and Australia, suitable laws are required to preserve its authenticity and quality.

The European Community (EC) has promulgated a regulation to set the characteristics of olive oil and the necessary analytical methods (Regulation 2568/91, July 11, 1991) (1). In recent years, this regulation has been updated and widened to take into account new knowledge on olive oils and to vary the concentration limits for many components to be analyzed. On March 28, 1995, Regulation 656/95 (2) was promulgated to introduce the stigmastadienes content determination and to set their legal limits for virgin olive oils (obtained by pressing the olives, without refining or other treatments). Stigmastadienes (the most important is stigmasta-3,5-diene) are dehydration compounds of sitosterol and belong to the sterenes that are of the wider family of dehydration compounds from sterols. They are produced during the refining process, mainly during bleaching and deodorizing steps. This is why they must not be present in virgin olive oils. Thus, their presence in declared virgin oils means a fraudulent blend with refined oils. In 1989, Lanzon *et al.* (3) suggested the stigmastadiene determination, besides other dehydration compounds, in virgin olive oil to reveal this type of fraud. Since that time, the method has been widely debated, tested, improved, and is still under detailed investigation. The method released by the EC is suitable for only those vegetable oils with stigmastadiene contents between 0.01 and 4.0 mg/kg. The procedure requires the unsaponifiables to be prepared, followed by a liquid chromatography (LC) separation on a silica-gel column. The collected fraction of interest undergoes further analysis by capillary gas chromatography (GC) for quantitation.

A similar way of analyzing stigmasta-3,5-dienes differs from the official method by isolating them directly from the oil without saponification. This method is quicker than the official one and gives closely concordant results, even though it requires only 0.5 g of oil instead of 20 g for the official method.

Other nonofficial analyses have been proposed to obtain more information about the presence of refined oils in virgin olive oils. For example, Mariani *et al.* (4) and Grob *et al.* (5) investigated the presence of squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexaene) isomers in oils that had been treated with maleic anhydride to disguise the fact that they had been subjected to illegal refining processes. Such isomers are not present in virgin oils. The authors showed that the maleic anhydride treatment changes neither the squalene isomers nor the stigmasta-3,5-diene content, while it is effective on conjugated fatty acid dienes and trienes to which the spectrophotometric characteristics of oil are related. The method requires the separation of squalene isomers and stigmasta-3,5-diene from the oil. For this purpose, the authors took advantage of an on-line LC–GC system, equipped with suitable chromatographic columns.

A different kind of fraud is the blend of olive oil with solvent-extracted pomace oil. To reveal this fraud, the EC promulgated Regulation 183/93, January 29, 1993, which describes the wax ester determination and sets the allowed con-

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centration. Wax esters of analytical interest have a carbon atom number between 40 and 46. Wax esters are mainly located on the epicarp of the olive drupe and, owing to their solubility, are more abundant in solvent-extracted oils, while virgin oils contain a lower concentration. This method requires wax esters to be separated from the oil by LC on a silica-gel column, followed by GC separation on a capillary column. Both of those determinations, i.e., stigmasta-3,5-diene and wax esters, require a silica-gel column to be prepared. This time-consuming step could be avoided by replacing it with a more efficent high-performance liquid chromatography (HPLC) separation, which recently led to new interesting results about sterenes (6,7).

Laboratories that routinely perform quality control on olive oils must carry out an increasing number of analyses. They need quicker reliable methods to ascertain legal compliance. Biedermann *et al.* (8) developed a method with two HPLC columns: the first one separates the hydrocarbon fraction (from paraffins to just before squalene) from the oil without prior saponification, while the second one quantitates the sterenes by ultraviolet (UV) detection (235 nm). Meanwhile, the first column is backflushed to remove the more polar fraction. This method requires an expensive system that is equipped with one pump and three valves or two pumps and two valves, which are not common in olive oil quality-control laboratories. Furthermore, the analyst needs to determine the wax ester content, which requires further analysis. A method is described here that permits the separation of sterenes, in particular stigmasta-3,5-diene, squalene isomers, and wax esters by HPLC in a single run, adapting a previous work (9) that described a method of separating wax esters by HPLC. The single fractions could be collected and then analyzed by high-resolution gas chromatography (HRGC) for quantitation. The present method is proposed for quick screening of large numbers of samples with the only aim of verifying whether the analytes' contents are sharply lower or higher than the legal limits. In case of doubt, the EC method has to be performed.

EXPERIMENTAL PROCEDURES

HPLC separation. The following conditions were used: gradient pump, LDC Analytical CM4000 (LDC Analytical, Riviera Beach, FL); UV detector, Milton Roy (Rochester, NY) Spectromonitor 3100; column, Supelcosil LC-Si, 15 cm × 4.6 mm i.d., 5 μ (Supelco, Bellefonte, PA); detection wavelength, 217.6 nm; range, 0.10 absorbance units full-scale; response time, 0.10 s; loop, 100 μL. The gradient was the following: *n*-hexane/diethyl ether: 0–17 min, 100:0; 17–18 min to 92:8 (linear gradient); 18–19.5 min, 92:8; 19.5–30.5 min, to 0:100 (linear gradient); 30.5–30.6 min to 100:0 (linear gradient); 30.6–54 min, 100:0; 54 min ready for next run; flow rates (every change is instantaneous): 0–17 min, 0.2 mL/min; 17–30.6 min, 1.0 mL/min; 30.6–53.9 min, 2.5 mL/min; 53.9 min, 0.2 mL/min. Sample injection was performed by an automatic sampler, SpectraSystem AS1000 (Spectra Physics, San Jose, CA), and collection of the fractions containing sterenes, squalene, and wax esters was carried out with an FC203 (Gilson Medical Electronics, Inc., Middleton, WI) fraction collector.

HRGC separation. For wax esters, the following conditions were 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 120 to 140°C at 7°C/min, then to 290°C at 5.5°C/min, then to 345° at 3°C/min and a further 5 min at 345°C; detector (flame ionization) temperature, 370°C; carrier gas, hydrogen at 68.6 cm/s; injection volume (on-column), 1 μL.

For squalene isomers and stigmastadienes, the following conditions were used: gas chromatograph, Fisons Instrument (Milan, Italy) GC8160; capillary column, SPB-5 (5% diphenyl/94% dimethyl/1% vinylpolysiloxane), fused silica, 30 m × 0.25 mm i.d., 0.25 μm film thickness (Supelco). Oven temperature was programmed from 140 to 225°C at the maximal rate (about 18–20°C/min) and a further 7 min at 225°C, then to 285°C at 2°C/min; detector (flame ionization) temperature, 320°C ; carrier gas, helium at 26.6 cm/s; injector temperature, 300°C; injection (5 μL) for both sterenes and squalene isomers, splitless for 50 s after injection.

Solvents. Isooctane (2,2,4-trimethylpentane) was of UV grade (Merck, Darmstadt, Germany), while all other solvents were of HPLC grade and obtained from Fluka (Buchs, Switzerland).

Reagents. Cholesta-3,5-diene, lauryl arachidate, and *n*-nonacosane were obtained from Sigma Chemical Co. (St. Louis, MO); *n*-dotriacontane was purchased from Fluka.

Solutions. *n*-Dotriacontane (*n*-$C_{32}H_{66}$): dissolve 0.25 mg of *n*-dotriacontane into 50 mL of isooctane (~5 ppm). *n*-Nonacosane (*n*-$C_{29}H_{60}$): dissolve 10 mg into 100 mL of *n*-hexane (~100 ppm). Standard 1 (cholesta-3,5-diene): Solution A, dissolve 10 mg cholesta-3,5-diene into 50 mL isooctane (~200 ppm); Solution B, make up 2.5 mL of Solution A to 25 mL with isooctane (~20 mg/kg), then dilute 6.5 mL of Solution B to 25 mL with isooctane (~5 ppm). Standard 2 (lauryl arachidate, 0.1% wt/vol): dissolve 0.1 g lauryl arachidate into 100 mL *n*-hexane.

Samples. Four different types of olive oils were used: one extra-virgin oil (obtained by pressing the olives, with acidity <1.0%); one crude olive oil (obtained by pressing the olives, with acidity >3.3% and/or unpleasant flavor, which is sold only after refining); two refined oils (obtained by refining crude olive oil) with stigmasta-3,5-diene contents of about 2–3 and of about 8–10 mg/kg, respectively.

Sample preparation. In a 2-mL screw-top vial, weigh exactly about 0.25 g of oil (or 0.125 g for higher sterene content samples), add 50 μL of Standard 1 (100 μL for higher sterene content samples), add 50 μL of Standard 2, then dilute with 730 μL *n*-hexane and mix well. After the HPLC separation, the collected fractions are dried with a gentle nitrogen stream (no heating for sterenes). Then, sterenes are diluted with 10

μL of *n*-dotriacontane solution, wax esters with 70 μL *n*-heptane, and squalene isomers with 50 μL isooctane. These solutions are then ready for HRGC analysis.

RESULTS AND DISCUSSION

The possibility of isolating sterenes directly from oil offered the opportunity of setting up a suitable method of separating stigmastadienes, squalene isomers, and wax esters in a single run, while the official method for sterenes requires a saponification step, which would cleave wax esters. Furthermore, one of the present work targets was to verify the proposed method's repeatability. To achieve it, 30 repeated separations of each type of olive oil were performed. Because reference method repeatability and reproducibility data are not available, only rough comparisons were performed. In particular, for stigmasta-3,5-diene, the comparison was carried out with three repetitions performed according to EC Regulation 656/95, Annexe XVII (2). For wax esters, 30 repetitions were performed according to the method described in Reference 9. For squalene isomers, the appropriate separation and window were simply set up, and the analytes present in "stressed" refined olive oil (it was submitted to high temperature, 180°C, and a large quantity of bleaching earth, 5%, for about 40 min) were available for collection. Figure 1 shows an HPLC chromatogram where the collection windows for each analyte are shown. Stigmastadiene collection is the most critical of all, because the *n*-alkanes, which do not give UV responses, and squalene, with its isomers, are eluted closely. An analytical HPLC column with a higher theoretical plate number should improve the resolution without a significant increase of the total run time. Even if *n*-alkanes do not interfere with the GC separation [see note 7 of the official method (2)], it is advisable to reduce their content in the collected fraction to improve the reliability of identification and quantitation of the analytes. The addition of *n*-dotriacontane is useful to compute more suitable relative retention times (RRT) in the GC separation. In fact, they could be referred to as the shorter interval (10 min, instead of about 40 min) comprised between the elution of cholestadiene (RT ~ 29 min) and *n*-dotriacontane (RT ~ 39 min) in which sterenes are eluted. Optimization of the gas-chromatographic conditions was performed according to the quoted EC method (separation of cholestadiene from *n*-nonacosane). Figure 2 shows an HRGC separation and peak identification. In setting up the whole LC separation process, fraction collection, and HRGC analysis, the optimal choice of solvents is important. This is particularly true for this hydrocarbon fraction because it can suffer important interferences, more so than other fractions. In fact, the collected volume (about 130 μL) has to be evaporated and diluted with 10 μL of *n*-dotriacontane solution. This means that impurities are concentrated 13 times. When a queue of samples is to be analyzed, and the collected fractions have to wait for a long time before the next steps (e.g., overnight separations), to avoid the analytes drying out owing to the relative volatility of the solvent (*n*-hexane), it is advisable to add about 200 μL of a solvent with a higher boiling point. Among the tested solvents, we chose isooctane (UV grade) because of its low level of impurities.

Moreover, owing to the small quantity of collected analytes (e.g., for an oil containing 0.05 mg/kg of stigmasta-3,5-diene, the corresponding quantity injected in the HPLC is 0.005 μg), it is advisable to perform GC injection in the splitless mode. Wax ester separation was performed in a similar way to that previously described (9). We verified only that the collection window was correct and compared GC results to those obtained according to Reference 9. Finally, for squalene isomers, quantitative comparisons were not carried out because only a qualitative ascertainment is generally required. Based on the results obtained (Table 1), the following remarks can be made: For stigmasta-3,5-diene, the agreement between the mean values from the proposed and the official methods

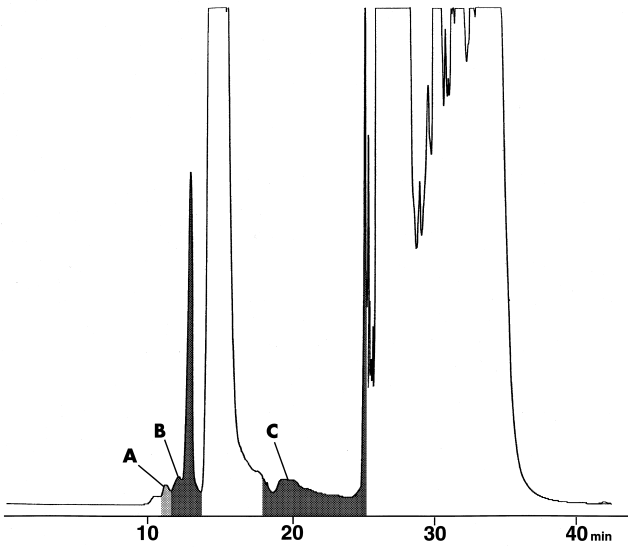

FIG. 1. High-performance liquid chromatography separation and collection windows. A = stigmasta-3,5-diene; B = squalene isomers; C = wax esters.

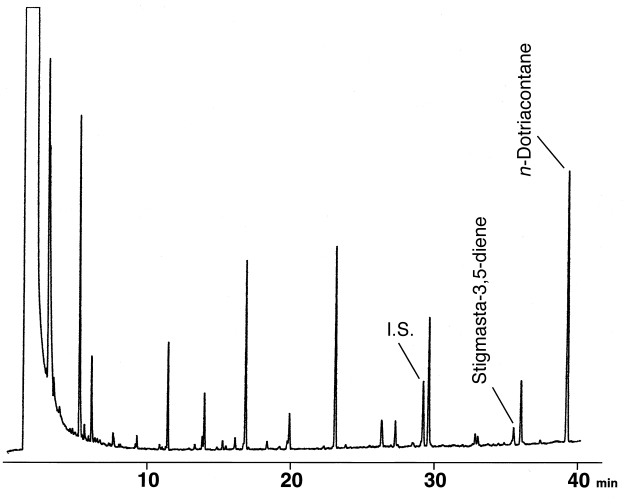

FIG. 2. High-resolution gas chromatography separation of stigmasta-3,5-diene. I.S. = internal standard.

TABLE 1
Mean Values (*x*), Standard Deviation (SD), and Repeatability (*r*) for Stigmasta-3,5-diene and Wax Esters

	Extra-virgin olive oil		Crude olive oil		Refined olive oil (lower sterene content)		Refined olive oil (higher sterene content)	
	1[a]	2[b]	1	2	1	2	1	2
Stigmasta-3,5-diene								
x	<0.01	<0.01	0.32	0.34	2.5	2.6	8.7	10.6
SD	—	—	0.00	—	0.1	—	0.6	—
r	0.01	—	0.03	—	1.60	—	0.22	—
Wax esters								
x	69	95	226	221	199	201	207	213
SD	5.6	1.8	9.8	8.0	8.8	4.9	9.1	5.6
r	15.9	5.2	27.8	22.6	25.8	15.8	24.8	13.9

[a]Proposed method.
[b]Reference method.

is good, even if the discrepancy is greater for the refined olive oil with a higher sterene content. It should be remembered that the official method is suitable for oils with a content of such hydrocarbons not more than 4.0 mg/kg, whereas we carried out the method for a refined oil with about 10 mg/kg. Standard deviation and repeatability were not calculated for data obtained from the official method because only three repetitions were performed. Also for wax esters, the agreement is good. For extra-virgin olive oil, the discrepancy was due to not having set the right collection window; in fact, it was rectified for the other samples, and the agreement improved.

In conclusion, for routine analyses the whole method allows the analyst to save time and solvents (about 75 mL per run only). The required equipment is usually available in common olive oil quality-control laboratories, and the method is also suitable to automate the HPLC and HRGC separations by using autosamplers and a fraction collector. We believe that it could be a shortcut to performing the increasing number of routine analyses to test only legal compliance.

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