Available online at www.sciencedirect.com
SCIENCE DIRECT

ELSEVIER

Journal of Chromatography A, 985 (2003) 211–220

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Solid-phase extraction–thin-layer chromatography–gas chromatography method for the detection of hazelnut oil in olive oils by determination of esterified sterols

Luisito Cercaci, Maria Teresa Rodriguez-Estrada*, Giovanni Lercker

Department of Food Science, University of Bologna, Viale Fanin 40, 40127 Bologna, Italy

Abstract

The sterol composition of extra virgin olive oil is very characteristic and, thus, has become a helpful tool to detect adulterations with other vegetable oils. Special attention has been addressed to the separate determination of the free and esterified sterol fractions, since both have different compositions and can thus provide more precise information about the actual origin of the olive oil. In the case of admixtures with small amounts of hazelnut oil, this approach can be extremely useful, because the similarity between the fatty acid compositions of both oils hampers the detection of the fraud. A hyphenated chromatographic method was developed for a sensitive and precise determination of esterified sterols in olive oils. The oil was subjected to silica solid-phase extraction (SPE) fractionation, cold saponification of the collected fraction and purification on silica TLC. The sterol band was then injected into an SPB-5 (30 m×0.25 mm I.D., 0.25 μm film thickness) and the ratio [% campesterol×(% 7-stigmastenol)^2]/(% 7-avenasterol) was calculated. The method was tested on extra virgin olive oil; good sterol recoveries and repeatability were obtained. The results were compared with another method, which has a different sample preparation sequence (silica column chromatography, hot saponification and silica TLC). Similar results were achieved with both methods; however, the SPE–cold saponification–TLC–capillary GC was faster, required less solvent and prevented sterol decomposition. The SPE-method was applied to an admixture with 10% of hazelnut oil and to a screening of 11 oils (husk oil, virgin and refined olive oils) from different Mediterranean countries.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Oils; Olive oil; Food analysis; Sterols

1. Introduction

Adulteration of virgin olive oil with different seed oils has always represented a problem for the Mediterranean countries that produce and import olive oil. In particular, detection of admixtures of virgin olive oils with hazelnut oil is difficult [1–8], since these oils have similar triacylglycerol, total sterol and fatty acid compositions and, when mixed in certain proportions, it is impossible to evince the presence of hazelnut oil. This type of adulteration is even more frequent in refined olive oils and husk oils, because higher amounts of hazelnut oil can be added therein and there are not many reliable methods for detection of such admixtures. New methods that employ new extraction systems, such as steam distillation-solvent extraction and supercritical fluid extraction, cannot be used for the detection of these admixtures, due to the elimination of volatiles

*Corresponding author. Tel.: +39-051-209-6011; fax: +39-051-209-6017.
E-mail address: estrada@sun.economia.unibo.it (M.T. Rodriguez-Estrada).

0021-9673/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(02)01397-3

used as markers of hazelnut oil (such as (*E*)-5-methyl-hepta-2-en-4-one) [9,10]. Hyphenated analytical techniques, such as liquid chromatography–gas chromatography, differential thermal analysis [11], Fourier transform infrared spectroscopy (FT-IR), and ^{13}C- and ^{1}H-nuclear magnetic resonance (NMR) [9], have been applied for the study of olive oils and different admixtures; however, their sensitivity level is not enough for detecting low amounts of hazelnut oil. Likewise, some composition parameters and ratios, such as the amount of triacylglycerols with 50 carbon atoms [11], the ratio of some triacylglycerols esterified with palmitic (P), oleic (O), linoleic (L) and linolenic (Ln) acids (LLO+OLnO/LLP+LnOP) [12] and the amount of waxes with 37 and 39 carbon atoms [11], that have been suggested as detection methods for these admixtures, are not able to claim adulterations, without uncertainties, at low percentages of hazelnut oil.

On the other hand, the oil's tocopherol composition determined by reversed-phase high-performance liquid chromatography and the ratios between the different classes of tocopherols (γ/β and β/δ), have proved to be good indicators of the presence of hazelnut oil in olive oils; however, it is advisable to perform other analysis so as to avoid claiming false adulterations due to the large variability of the oil tocopherol composition and to tocopherol degradation during refining [11].

The analysis of the unsaponifiable fraction is usually a helpful tool to detect adulterations of olive oils with other vegetable oils; however, it is not efficient in the case of admixtures with hazelnut oil. The amount and composition of total sterols, linear and triterpenic alcohols cannot evince levels of hazelnut oil below 30%. Nevertheless, the separate determination of the free and esterified sterol fractions seem to be an option for the detection of such admixtures, since both fractions have different compositions and can thus provide more precise information about the actual origin of the olive oil [11,13]. In fact, Mariani et al. [11] have recently set up a chromatographic method for the determination of esterified sterols in olive oils, which allows the detection of such admixtures by calculating the ratio [% campesterol$\times$(% 7-stigmastenol)^2]/(% 7-avenasterol) in the esterified sterol fraction; they observed that this ratio was always lower or equal to 1 for non-adulterated olive oils.

Considering the aforementioned information and the fact that there are no European official methods for the detection of 10% (or less) hazelnut oil in refined olive oils, it is evident the need to develop reliable, repeatable and fast methods for this scope. The objective of this study was to develop a partially automated and inexpensive chromatographic method for the detection of such admixtures and to compare it with the method suggested by Mariani et al. [11]. The new method includes silica solid-phase extraction (SPE), cold saponification of the collected fraction, purification on silica thin-layer chromatography (TLC) and injection of the sterol TLC band in a gas chromatographic instrument coupled to a non-polar capillary gas chromatography (cGC) column. The two methods were tested on virgin olive oil and an admixture with 10% of hazelnut oil. A final screening of 11 oils (husk oil, virgin and refined olive oils) from different Mediterranean countries, was performed.

2. Experimental

2.1. Reagents and solvents

Chloroform, *n*-hexane, diethyl ether (stabilized with butylated hydroxytoluene; BHT), light petroleum, methanol, potassium hydroxide and anhydrous sodium sulfate, were purchased from J.T. Baker (Deventer, The Netherlands). Silylating agents (pyridine, hexamethyldisilazane and trimethylchlorosilane) were supplied by Merck (Darmstadt, Germany). Stearic acid and β-sitosterol (60% β-sitosterol and 40% campesterol) were purchased from Sigma (St. Louis, MO, USA), whereas 5α-cholestan-3β-ol (>99% purity) and trilinolein were supplied by Fluka (Buchs, Switzerland) and Nu Check (Elsyan, MN, USA), respectively. Prepacked Bond-Elut LRC silica SPE cartridges (500 mg) were purchased from Varian (Harbor City, CA, USA). Large silica TLC plates (20 cm$\times$20 cm$\times$0.25 mm of film thickness) were supplied by Merck. Small silica TLC plates (5 cm$\times$20 cm$\times$0.25 mm of film thickness) and 2′,7′-dichlorofluorescein (sodium salt) were purchased from Farmitalia Carlo Erba (Milan, Italy).

2.2. Synthesis of the internal standard (stearyl cholestanol)

Stearyl cholestanol was synthesized in the laboratory, by using stearic acid and 5α-cholestan-3β-ol as precursors. The synthesis was performed according to the method described by Fieser and Fieser [14]; the reactions are schematized as follows:

$$\mathrm{R{-}COOH + SOCl_2 \rightarrow R{-}COCl}$$

$$\mathrm{R{-}COCl + R'{-}OH \rightarrow R{-}CO{-}OR'}$$

where R–COOH is stearic acid, $SOCl_2$ is thionyl chloride, R–COCl is stearyl chloride, R′–OH is 5α-cholestan-3β-ol and R–CO–OR′ is stearyl cholestanol.

One gram of stearic acid, 10 ml of toluene and thionyl chloride (in excess of 30%) were placed in a round-bottom flask fitted with a reflux condenser and drying tube and mounted in a hood; the whole system was under vacuum and subjected to heating for 45 min. The extra amount of thionyl chloride was eliminated by distilling under vacuum and by adding 20 ml of toluene 3–4 times. An amount of 5α-cholestan-3β-ol dissolved in toluene, lower than that required from the stoichiometric standpoint, was added; pyridine was added right after to neutralize the formation of HCl. Once the reaction has been completed, the solvent was removed in a rotavapor under vacuum. The final residue was redissolved in diethyl ether and subjected to several acid and basic rinses with diluted solutions of HCl and $KHCO_3$, respectively.

After different purification stages, the purity degree of the standard was controlled by cGC, ^{1}H-NMR and FT-IR [15].

2.3. cGC and cGC–ITDMS instruments and analytical conditions

cGC was performed with a Fisons HRGC 8560 (Rodano, Milan, Italy), equipped with a split-splitless injector and a flame-ionization detector, and coupled to a Fisons DP 800 integrator. The column used was a fused-silica SPB-5 (30 m×0.25 mm I.D., 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA), coated with 5% diphenyl–95% dimethyl-polysiloxane. Analyses were performed under isothermal conditions. Oven, injector and detector temperatures were set at 270, 290 and 310 °C, respectively. Helium was used as carrier gas at a flow-rate of 1.6 ml/min. All injections were performed in the split system, using different splitting ratios; a 1:50 ratio was employed for the determination of esterified sterols after column chromatography, whereas a 1:10 ratio was set for the sterols' determination after SPE fractionation.

cGC–ITDMS analyses for sterol identification were performed using a Varian (Walnut Creek, CA, USA) model 3300/3400 gas chromatograph equipped with a split-splitless injector and coupled to a Finningan MAT model ITS40 (San Jose, CA, USA) mass spectrometric detector. The analyses were performed by electron impact ionization (EI) and a low bleed fused-silica capillary column (30 m×0.25 mm I.D., film thickness 0.25 μm) coated with poly-(5% diphenyl–95% dimethyl-siloxane) (Supelco) was used. The temperature was programmed from 250 to 320 °C at a gradient of 3 °C/min; the injector, transfer line and manifold temperatures were set at 325, 325 and 220 °C, respectively, and helium was the carrier gas. The filament emission current was 10 μA and the electron energy was 70 eV.

2.4. Samples

The extra virgin olive oil used for the method set-up and statistical analysis was obtained from the Marche Region (Italy) during the 1999–2000 harvest.

The hazelnut oil was produced in the laboratory, by Soxhlet extraction [16] of dried, crushed hazelnuts. The extraction was performed using light petroleum (b.p. 40–60 °C).

Eleven olive oils from different Mediterranean countries were sampled from large containers transported by foreign boats or from hundreds of commercial bottles, following the ISO 5555 protocol [17].

To reduce data variability, an oil solution was prepared for each oil tested. All oils were homogenized and filtered before preparing the solutions. These solutions were made by placing 25 g of oil and 1 ml of a 0.1% stearyl cholestanol solution (w/v in *n*-hexane) (internal standard) in a 50-ml volumetric flask, which were then taken to volume with *n*-hexane.

The solution of the 10% (w/w) admixture with hazelnut oil was prepared by placing 22.5 g of the Marche extra virgin olive oil, 2.5 g of the hazelnut oil and 1 ml of a 0.1% stearyl cholestanol solution (w/v in *n*-hexane) (internal standard) in a 50-ml volumetric flask, which was then taken to volume with *n*-hexane.

2.5. Methods

2.5.1. Determination of esterified sterols according to Mariani et al. [11] (method 1)

This method is based on separation of esterified sterols by silica column chromatography. Ten ml of the oil solution (5 g of oil) were loaded into a column containing 25 g of silica gel, previously conditioned with 30 ml of *n*-hexane–diethyl ether, and eluted with 150 ml of *n*-hexane–diethyl ether (87:13, v/v). The solvent of the collected fraction was eliminated with a rotavapor; the fraction was taken to dryness under nitrogen stream and then subjected to hot saponification, according to the official method for the determination of total sterols in olive oil [18]. The unsaponifiable fraction was dried under nitrogen flow, dissolved in chloroform (10% solution, w/v) and loaded on 5 cm of a silica TLC plate (about 10 μl of chloroformic solution/cm); a spot containing sterol standards (β-sitosterol and campesterol) was loaded on the same TLC plate, so as to correctly identify the sterol band. The mobile phase was a mixture of *n*-hexane–diethyl ether (65:35, v/v). The sterol TLC band was visualized under UV light (254 nm), after being sprayed with a 0.2% ethanolic solution of 2′,7′-dichlorofluorescein sodium salt. The sterol band was then scraped off, extracted twice with 5 ml of diethyl ether and the solvent was evaporated under nitrogen flow at room temperature. The solution containing the sterol TLC band was then silylated with 200 μl of a pyridine–hexamethyldisilazane–trimethylchlorosilane (9:3:1, v/v) mixture; after 15–20 min at room temperature, the sample was evaporated to dryness under nitrogen flow and dissolved in 50 μl of *n*-hexane. One microliter of this solution was then injected into the cGC system under the aforementioned conditions.

The Mariani ratio (R_{MAR}) of the esterified sterols fraction was calculated as follows:

$$R_{MAR} = \frac{\% \text{ campesterol} \times (\% \text{ 7-stigmastenol})^2}{\% \text{ 7-avenasterol}}$$

Method repeatability and sterol recoveries were calculated for extra virgin olive oil. Six independent replicates were run and each replicate was injected three times.

2.5.2. Determination of esterified sterols by SPE (method 2)

This determination was performed by silica SPE fractionation according to Bortolomeazzi et al. [19]. One ml of the oil solution (0.5 g of oil) was loaded onto an SPE silica cartridge, previously conditioned with 3 ml of *n*-hexane, and was eluted with 3 ml of *n*-hexane and then with 3 ml of *n*-hexane–diethyl ether (8:2, v/v). Both eluates were combined, dried under nitrogen stream, weighed and subjected to cold saponification [20]. The unsaponifiable fraction was fractionated on silica TLC, as described in method 1. The sterol band was then extracted, silylated and injected into the cGC, following the same analytical conditions of method 1.

Method repeatability and sterol recoveries were calculated for extra virgin olive oil. Six independent replicates were run and each replicate was injected three times. The esterified sterols were quantified and the R_{MAR} of this fraction was calculated.

The SPE method was applied to the admixture with 10% of hazelnut oil and 11 olive oils (husk oil, virgin and refined olive oils) from different Mediterranean countries.

2.6. Sterol identification and quantitation

Sterol identification was performed by comparing the peak retention times with those of the sterol standards, as well as by injecting into cGC–ITDMS.

The internal standard method was used for quantitation of sterols. Since the synthesized standard contained 94% esterified cholestanol (see Results), it was considered for calculation purposes that 56% of the weighed standard actually corresponded to the 5α-cholestan-3β-ol. The cGC response coefficient was considered equal to 1.

3. Results and discussion

The efficiency of the synthesis of the stearyl cholestanol (internal standard) was controlled by cGC, ^{1}H-NMR and FT-IR. The resulting product was subjected to hot saponification, extraction with diethyl ether and silylation, giving mainly two cGC peaks with the same retention times as those of the precursors (stearic acid and 5α-cholestan-3β-ol). The composition of the purified synthesized standard was determined by cGC, giving 81% of stearyl cholestanol, 13% of cholestanol esterified with heptadecanoic acid (due to a 13% initial impurity in the stearic acid standard) and 6% of various reagent impurities that do not overlap with the compounds of interest. In addition, when the ^{1}H-NMR spectra of the 5α-cholestan-3β-ol and the synthesized standard were compared, a noticeable increase of a resonance peak was evident in the latter, which can be attributed to aliphatic methylenic groups. Regarding the FT-IR analysis, the spectrum of the stearyl cholestanol displayed a specific absorbance that corresponded to the asymmetric stretching of the ester group, which was not present in that of 5α-cholestan-3β-ol; in addition, the characteristic absorbance of the hydroxyl group completely disappeared in the stearyl cholestanol spectrum.

Fig. 1 shows a cGC trace of the trimethyl silyl derivatives of sterols of extra virgin olive oil, after saponification. Their elution order under the cGC conditions tested, is listed as follows: cholesterol, 5α-cholestanol (internal standard), 24-methylencholesterol, campesterol, campestanol, stigmasterol, 7-campesterol, clerosterol, sitosterol, sitostanol, 5-avenasterol, 5,24-stigmastadienol, 7-stigmastenol, 7-avenasterol, erythrodiol and uvaol.

As observed in a previous study [21], it was confirmed that different analytical steps included in the determination of the esterified sterol fraction of extra virgin olive oils and admixtures could generate a series of impurities that can hamper a correct determination of these compounds. Main interferences are due to steroids that are naturally present in the silica TLC plate and are co-extracted with the oil sterols. In addition, BHT used as stabilizer for diethyl ether, gives two cGC peaks that seem to arise from BHT polymerization. Another two interfering compounds derive from 2′,7′-dichlorofluorescein,

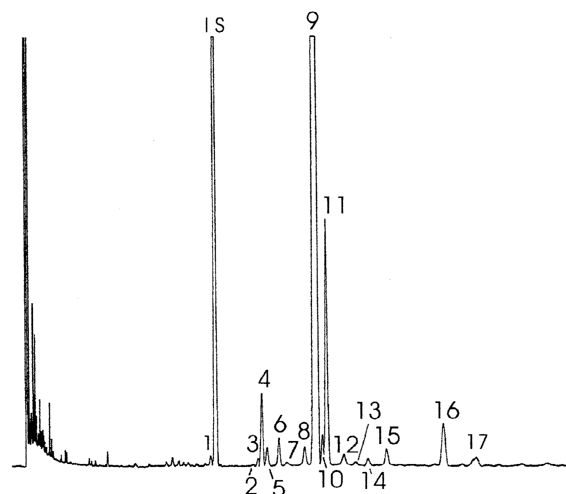

Fig. 1. cGC trace of the trimethyl silyl derivatives of sterols of extra virgin olive oil, after saponification. For cGC conditions, refer to Experimental. Peak identification: 1, cholesterol; I.S., 5α-cholestanol (internal standard); 2, unknown; 3, 24-methylencholesterol; 4, campesterol; 5, campestanol; 6, stigmasterol; 7, 7-campesterol; 8, clerosterol; 9, sitosterol; 10, sitostanol; 11, 5-avenasterol; 12, 5,24-stigmastadienol; 13, 7-stigmastenol; 14, 7-avenasterol; 15, diethyl ether impurity; 16, erythrodiol; 17, uvaol + unknown.

one of which has a slightly superior retention time than that of 7-avenasterol. It should be also pointed out that if the TLC band of sterols is not correctly scrapped off, triterpenic alcohols can be co-extracted with the sterols band; this could represent a great problem, since cycloartanol displays the same retention time as 7-stigmastenol (fundamental for evincing the presence of hazelnut oil). In addition, incomplete silylation of triterpenic alcohols generates peaks that can be identified as sterols.

Table 1 shows the percent composition of the extra virgin olive oil esterified sterols and the R_{MAR} obtained with methods 1 and 2, as well as the corresponding statistical parameters [22]. Six independent replicates were run and each value given in this table is the mean of three injections. As shown in Table 1, good repeatability of the amount of single and total esterified sterols and the R_{MAR}, were obtained with both methods. Some sterols, such as 5,24-stigmastadienol and 7-sterols, displayed a large percent relative standard deviation (RSD), which might be due to their relatively low concentrations. In any case, these results are reliable according to the

Table 1
Comparison between percent composition of the extra virgin olive oil esterified sterols and the R_{MAR} obtained with methods 1 and 2

Sterol (%)	Method 1 (Mariani et al.) [11]			Method 2 (SPE)			F-test[d] ($P=0.05\%$)	t-test[e] ($P=0.05\%$)
	Mean[a]	SD[b]	RSD (%)[c]	Mean[a]	SD[b]	RSD (%)[c]		
Cholesterol	0.45	0.09	20.0	1.52	0.26	17.1	8.35	2.25
24-Methylencholesterol	0.10	0.08	80.0	0.12	0.09	75.0	0.79	0.10
Campesterol	3.20	0.17	5.3	3.14	0.25	8.0	2.16	0.11
Campestanol	0.75	0.06	8.0	0.80	0.08	10.0	1.78	0.29
Stigmasterol	0.41	0.03	7.3	0.40	0.04	10.0	1.78	0.12
7-Campesterol	0.15	0.06	40.0	0.08	0.03	37.5	4.00	0.60
Clerosterol	0.94	0.16	17.0	0.91	0.10	11.0	2.56	0.09
Sitosterol	79.40	0.69	0.9	79.00	0.30	0.4	5.29	0.31
Sitostanol	3.26	0.35	10.7	4.14	0.22	5.3	2.53	1.23
5-Avenasterol	8.50	0.40	4.7	7.61	0.28	3.7	2.04	1.05
5,24-Stigmastadienol	0.43	0.11	25.6	0.32	0.09	28.1	1.49	0.45
7-Stigmastenol	0.59	0.07	11.9	0.58	0.05	8.6	1.96	0.07
7-Avenasterol	1.83	0.21	11.5	1.40	0.17	12.1	0.66	0.92
Total esterified sterols (mg/kg)	255	27	10.6	270	30	11.3	0.79	0.21
Mariani ratio	0.62	0.25	40.3	0.75	0.14	18.7	3.19	0.26

[a] Mean value of six independent replicates; each replicate was injected three times in the GC system.
[b] Standard deviation.
[c] Percent relative standard deviation.
[d] F-test for the comparison of the SD obtained with methods 1 and 2; $F_{5,5}=7.15$ ($P=0.05\%$).
[e] t-Test for the comparison of the mean values of each sterol, obtained with methods 1 and 2; $t_{10}=2.23$ ($P=0.05\%$).

sterol variability range reported by the official method for the determination of sterols [18], which demonstrates that these sterols show RSD higher than 20% [23].

No significant differences were found between the percentage of the single sterols, the total sterol content and the R_{MAR} obtained with the two methods. This underlines the fact that, although olive oil has a low content of esterified sterols, both methods can accurately detect and quantify this type of compound. Moreover, it should be pointed out that the SPE method displayed a good repeatability and sensitivity despite the small amount of sample used.

Table 2 reports the limit values of several parameters used for the quality control of extra virgin olive oil suggested by the European Union (EU) [18] and the International Olive Oil Council (IOOC) [24], and the corresponding values found in the extra virgin olive oil utilized for the SPE method set-up and the 10% admixture with hazelnut oil. This table also includes the percent total and esterified sterols determined by the official method [18] and method 2, respectively, in the extra virgin olive oil and the 10% admixture with hazelnut oil. If the total sterol content and the other official parameters are considered, the 10% admixture with hazelnut oil appears as a normal extra virgin olive oil. However, the adulteration is evident from the R_{MAR} and the percent compositions of the esterified sterols of the extra virgin olive oil and the admixture. In fact, the R_{MAR} of the 10% admixture (1.78) was markedly higher than the limit suggested by Mariani et al. [11] for non-adulterated olive oils ($R_{MAR} \leq 1$), whereas the extra virgin olive oil had an R_{MAR} (0.75) far below such limit. Considering these results, it might be possible that even a 5% admixture with hazelnut oil could be detected by using this sterol ratio; however, such addition is unlikely to be performed, since it yields little profit.

As shown in Table 2, stigmasterol is mainly present in its non-esterified form in the extra virgin olive oil and the 10% admixture with hazelnut oil, whereas campesterol and the 7-sterols (7-stigmastenol and 7-avenasterol) are mostly esterified sterols. Nevertheless, the percent compositions of esterified and total sterols of both oils are signifi-

Table 2
Some quality parameters of the extra virgin olive oil and its 10% admixture with hazelnut oil and their limit values suggested by the European Union (EU), the International Olive Oil Council (IOOC) and the Norme Grassi e Derivati (NGD). This table also includes the percent total and esterified sterols composition of the extra virgin olive oil and its 10% admixture with hazelnut oil

Sterols (%)	Extra virgin olive oil			Extra virgin[a] olive oil (Italy)		Extra virgin[a] +hazelnut oil 10% (w/w)	
	Limit EU [18]	Limit IOOC [24]	Value range NGD [29]	Ester	Total	Ester	Total
Cholesterol	≤0.5	≤0.5	Max 0.5	1.70	0.24	1.85	0.12
Brassicasterol	≤0.1	≤0.1	Max 0.1	nd[b]	nd	nd	nd
24-Methylencholesterol			Max 0.3	0.13	0.16	0.08	0.09
Campesterol	≤4.0	≤4.0	2.5–4.0	3.14	2.62	3.68	2.86
Campestanol			Max 1.0	0.78	0.14	0.41	0.11
Stigmasterol	<campe	<campe	<campe	0.41	0.82	0.68	0.85
7-Campesterol			Max 0.3	0.07	nd	0.11	0.04
Clerosterol			0.5–1.5	0.91	0.86	1.05	0.85
Sitosterol			75.0–87.0	78.3	79.5	77.5	80.6
Sitostanol			0.3–1.2	4.09	0.55	3.16	0.61
5-Avenasterol			5.0–15.0[d]	7.50	12.60	8.36	12.0
5,24-Stigmastadienol			Max 0.8	0.32	0.62	0.45	0.66
7-Stigmastenol	≤0.5	≤0.5	Max 0.5	0.52	0.32	0.91	0.40
7-Avenasterol			Max 1.0	1.33	0.62	1.69	0.68
Content of sterols (mg/kg)	≥1000	≥1000		275	1856	377	1926
Mariani ratio (%)	–	–	–	0.75		1.78	
Other parameters							
Acidity (% C18:1)	≤1.0	≤1.0	Max 0.1	0.28		0.35	
Peroxide value (mequiv. O_2/kg)	≤20	≤20	Max 20	8.5			
K 270	≤0.20	≤0.25	≤0.20	0.11		0.10	
K 232	≤2.50			2.03		2.10	
Delta K	≤0.01	≤0.01	≤0.01	<0.01		<0.01	
C16:0 pos. 2 of TG (%)	≤1.3	≤1.5		0.7		0.7	
Total alcanols (mg/kg)			≤300	184		180	
Stigmastadienes (mg/kg)	≤0.15	≤0.15		<0.01		<0.01	
Delta ECN 42	≤0.2	≤0.2		0.06		0.03	
Myristic acid (%)	≤0.05	≤0.05	Max 0.1	<0.01		<0.01	
Linolenic acid (%)	≤0.9[c]	≤1.0	Max 1.5	0.63		0.60	
Eicosanoic acid (%)	≤0.6	≤0.6	Max 0.7	0.39		0.37	
Eicosenoic acid (%)	≤0.4	≤0.4	Max 0.5	0.30		0.25	
Behenic acid (%)	≤0.2	≤0.2	Max 0.3	0.10		0.10	
Lignoceric acid (%)	≤0.2	≤0.2	Max 0.5	0.05		0.06	
Trans oleic isomers (%)	≤0.05	≤0.05		<0.01		<0.01	
Trans linoleic isomers (%) plus *Trans* linolenic isomers (%)	≤0.05	≤0.05		<0.01		<0.01	

[a] Extra virgin olive oil from the Marche Region (Italy).
[b] nd, not detected.
[c] 1.0% for oil from Morocco until 31/10/2003 (Reg. CE 2042/2001).
[d] More than 15.0% of 5-avenasterol can be found in some Greek oils.

cantly different and, therefore, they can be helpful for evincing admixtures with 10% hazelnut oil. This confirms the importance of the study of the composition of the various sterol fractions (esterified, non-esterified and total), because they provide useful information for detection of adulterations, as previously reported by several authors [6,11,21,25–28]. For instance, the presence of refined oil can be detected by calculating the composition of the sterol fractions, because free sterols are removed and this

Table 3
Mariani ratios calculated for different types of oils from various countries, as well as for the 10% admixture with hazelnut oil

	Type of oil												
	Husk oil	Olive oil									Extra virgin olive oil		Admixture (extra virgin olive oil +10% hazelnut oil)
	Morocco	Italy			Malta	Turkey					Tunisia	Italy	
Mariani ratio[a]	1.89	0.71	2.16	1.74	1.47	0.70	1.38	1.42	2.16	1.39	0.77	0.75	1.78

[a] Mean value of three independent replicates.

leads to a decrease in the total sterol content; however, the esterified sterol fraction remains practically unaltered.

Regarding the R_{MAR} of the 11 commercial olive oils (Table 3), the extra virgin olive oil and two olive oils displayed a value lower than 1 (limit suggested for non-adulterated olive oils [11]), whereas the other oils (70% of all samples) had an R_{MAR} higher than 1, half of which were above 1.5. These ''atypical'' oils, however, appear normal if only the legal analytical parameters for the corresponding oil categories [18] are taken into account. Considering these results, it is evident that the R_{MAR} provides further information about possible admixtures with hazelnut oil, which cannot be detected with the other analytical parameters, and thus its inclusion among the legal parameters for identification of adulterated olive oils could be extremely helpful.

Table 4 compares the percent total and esterified sterols of 13 oils (husk oil, refined and extra virgin olive oil and 10% admixture with hazelnut oil). The percent esterified sterols was quantified by using the SPE method, whereas the total sterol content was determined by using the official method [18]. Only the extra virgin olive oil and the 10% admixture with hazelnut oil registered less than 20% esterified sterols, being the lowest value among the monitored oils. Six oils had more than 50% of esterified sterols, two refined Turkish oils being those with the higher content (almost 80%). Since extra virgin olive oils and non-refined olive oils usually do not present more than 20–30% esterified sterols [21], regardless of the origin and type of cultivar, it could be assumed that the quantity of total sterols present in olive oils containing an elevated amount of esterified sterols (>1000 mg/kg), might have been 4–5 times higher before refining. It should be noticed that such a high content of total sterols could be only found in seed oils and, rarely, in some husk oils. Since the interesterification induced by the elevated acidity of the oils could not have generated such a high level of esterified sterols, it would be advisable to study in more depth the composition of such oils so as to verify their genuineness.

Table 4
Esterified and total sterols (expressed in mg/kg oil and as percentage) of different types of oils from various countries, as well as of the 10% admixture with hazelnut oil. All values are the mean of three independent replicates

	Type of oil												
	Husk oil	Olive oil									Extra virgin olive oil		Admixture (extra virgin olive oil +10% hazelnut oil)
	Morocco	Italy			Malta	Turkey					Tunisia	Italy	
Esterified sterols (mg/kg oil)	1364	653	928	469	927	834	975	942	1159	832	383	275	377
Total sterols (mg/kg oil)	2681	1525	1712	1229	2482	2147	1653	1186	1521	1473	2089	1856	1856
Esterified sterols (%)	51%	43%	54%	38%	37%	39%	59%	79%	76%	56%	18%	15%	20%

4. Conclusions

The scope of this study was to develop a hyphenated chromatographic method for a sensitive and precise determination of esterified sterols in olive oils, that would be able to detect adulterations with hazelnut oil. The method included silica SPE fractionation, cold saponification of the collected fraction, purification on silica TLC and cGC injection of the trimethyl silyl derivatized sterol band. The method was initially tested on extra virgin olive oil. An oil solution in *n*-hexane and an esterified internal standard were used in order to reduce data variability; in fact, good data repeatability and sterol recoveries were obtained. The SPE method was compared with another method [11] that had a different sample preparation sequence, both giving similar results; however, the SPE method was faster, required less solvent (about 1/10) and prevented sterol decomposition. In any case, attention should be paid to interferences due to the silica TLC, reagents and solvents, which can mislead in sterol identification and quantitation.

The R_{MAR} ratio of the esterified fraction [11] was 0.75 and 1.78 for the extra virgin olive oil and the 10% admixture with hazelnut oil, respectively. These results confirmed the validity of this ratio to evince adulterations with such oil. It must be noticed that legal analytical parameters for the control of olive oil genuineness, such as the total amount of sterols and their percent composition, were not able to detect the presence of the 10% hazelnut oil, whereas important information can be obtained from the percent composition of the esterified sterols.

Screening of 11 commercial oils by using the SPE method, showed that 80% of these oils had an R_{MAR} higher than 1 (limit for non-adulterated olive oils) [11], whereas all the extra virgin olive oils displayed a value below 0.8. It was confirmed that the amount of esterified sterols of the extra virgin olive oils was around 20%, whereas the other oils showed a much higher content of such fraction. In addition, the percent composition of total and esterified sterols of the oils were clearly different. Considering that the esterified sterol fraction remains practically unchanged after refining, it can be used as fingerprints of the oil origin.

The results presented in this work confirm the importance of the study of the different sterol fractions (esterified, non-esterified and total) of the olive oils, which could be a useful source of information for detection of adulterations in this sector.

References

[1] V. Paganuzzi, Riv. Ital. Sostanze Grasse 56 (1979) 431.
[2] L. Camera, F. Angerosa, Riv. Ital. Sostanze Grasse 55 (1978) 138.
[3] V. Paganuzzi, Riv. Ital. Sostanze Grasse 57 (1980) 145.
[4] A. Cert, A. Lanzon, A. Carelli, T. Albi, Food Chem. 49 (1988) 1.
[5] A. Cert, Grasas y Aceites 46 (1995) 175.
[6] O. Meneghetti, G. Amelotti, A. Griffini, Riv. Ital. Sostanze Grasse 64 (1987) 461.
[7] G. Lercker, M.T. Rodriguez-Estrada, J. Chromatogr. A 881 (2000) 105.
[8] M.H. Gordon, C. Covell, N. Kirsch, J. Am. Oil Chem. Soc. 78 (2001) 621.
[9] J. Mannina, M. Patumi, P. Fiordiponti, M.C. Emanuele, A.L. Segre, Ital. J. Food Sci. 2 (1999) 139.
[10] P.G. Blanch, M. Caja, M.L. Ruiz del Castillo, M. Herraiz, J. Agric. Food Chem. 46 (1998) 3153.
[11] C. Mariani, G. Bellan, G. Morchio, A. Pellegrino, Riv. Ital. Sostanze Grasse 76 (1999) 297.
[12] J. Parcerisa, I. Casal, J. Boatella, R. Codony, M. Rafecas, J. Chromatogr. A 881 (2000) 149.
[13] S. Vichi, L. Pizzale, E. Toffano, R. Bortolomeazzi, L. Conte, J. AOAC Int. 84 (2001) 1534.
[14] L.F. Fieser, M. Fieser, Reagents for Organic Synthesis, John Wiley, New York, 1967.
[15] L. Cercaci, Specialization Thesis in "Chemical Methodologies for Control and Analysis", Sciences Faculty MM.FF.NN, University of Bologna, 2000.
[16] W. Horwitz, in: 17th ed, Official Methods of Analysis AOAC International, Vol. 31, AOAC International, Gaithersburg, MD, 2000, p. 10.
[17] CE Regulations no. 379/99 of 19th February 1999 and no. 455/2001 of 6th March 2001, which modify CE Regulation 2568/91.
[18] Regulation 2568/91 and modifications, European Commission, 11th July 1991.
[19] R. Bortolomeazzi, N. Frega, G. Lercker, Ital. J. Food Sci. 4 (1990) 265.
[20] D.B. Sander, P.B. Addis, S.W. Park, D.E. Smith, J. Food Protect. 52 (1989) 109.
[21] L. Cercaci, G. Lercker, Boll. Chim. Igenisti 51 (2000) 25.
[22] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, Ellis Horwood, New York, 1988.
[23] W. Moreda, M.C. Perez Camino, A. Cert, Grasas y Aceites 46 (1995) 279.
[24] International Olive Oil Council, 08/11/2001, COI/T.15/NC no. 2/Rev. 10, Trade Standard Applying to Olive Oil and Olive-Pomace Oil.

[25] G. Morchio, R. De Andreis, Riv. Ital. Sostanze Grasse 64 (1987) 185.
[26] A.M. Leone, V. Liuzzi, E. La Notte, M. Santoro, Riv. Ital. Sostanze Grasse 61 (1984) 69.
[27] C. Mariani, S. Venturini, E. Fedeli, Riv. Ital. Sostanze Grasse 67 (1990) 611.
[28] T. Verleyen, M. Forcades, R. Verhe, K. Dewettinck, A. Huyghebaert, W. De Greyt, J. Am. Oil Chem. Soc. 79 (2002) 117.
[29] Norme Grassi e Derivati (NGD Ia 008-1989 e NGD 1984), Milan.