Determination of Sterols by Capillary Column Gas Chromatography. Differentiation Among Different Types of Olive Oil: Virgin, Refined, and Solvent-Extracted

Oroncio Jiménez de Blas[a,*] and Amando del Valle González[b]

[a]Department of Analytical Chemistry, Nutrition and Food Science, Faculty of Chemical Sciences, University of Salamanca, 37008 Salamanca, Spain, and [b]Central Laboratory of the Spanish Customs House, Madrid, Spain

ABSTRACT: Compositional analysis of the sterol fraction of olive oil can be used to assess the degree of purity of the oil and the absence of admixture with other plant oils. This determination also permits characterization of the type of olive oil in question: virgin, refined, or solvent-extracted. In the present work, 130 samples of olive oil were analyzed, the sterol fractions were separated from the unsaponifiable fraction by silica gel plate chromatography, and later they were analyzed as the trimethylsilyl ether derivatives by capillary column gas chromatography. From the results obtained, it was concluded that this methodology is able to differentiate among virgin, refined, and solvent-extracted olive oils. Stigmasterol, clerosterol, Δ5-avenasterol, Δ7-stigmasterol, and Δ7-avenasterol permit the differentiation of the three types of oil from one another. Campesterol, Δ5,23-stigmastadienol, β-sitosterol, and Δ5,24-stigmastadienol permit the differentiation of only two oils from each other but confirm the conclusions obtained for other sterols. Correlations between the different sterols of virgin, refined, and solvent-extracted olive oil also have been obtained.
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KEY WORDS: Differentiation of oil types, olive oil, refined, solvent-extracted, sterol, virgin.

Sterols are natural components that appear in many plant and animal species. They are also essential for human and animal dietary requirements (1).

The sterol fraction is considered to be important for evaluation of the purity of oil samples (2), hence the limitations in the contents of certain components as established by legislation of several countries and as regulated by article 2568/91 of the European Union (EU) to avoid adulteration.

Modifications in the sterol fraction of olive oils have been studied in detail. In this respect, important studies have been carried out on different varieties of olives, addressing the alterations that occur as the result of maturation (3,4) or of poor conservation of the olives (5) or of the oils themselves. Studies also have assessed the alterations that arise due to refining processes of olive oils (6–8).

*To whom correspondence should be addressed at Plaza de la Merced, s/n. 37008 Salamanca, Spain.

The sterol fraction has also been used to typify olive oils from certain geographic zones (9) and to control their authenticity (10).

Leone *et al.* (7) studied the effect of different phases in refining processes, both physical and alkaline, on the qualitative and quantitative composition of the sterol fraction of nine samples of olive oil. They concluded that the refining process gave rise to a decrease in the total content of sterols in the oil (6). With the use of gas chromatography (GC) with different stationary phases, it was later established that the percentage composition of this fraction did not differ appreciably from that of the respective starting crude olive oil. Recently, with the advent of more selective stationary phases, it has been reported that neutralization with alkali did not cause significant alterations in the percentage sterol composition, although, by contrast, bleaching did produce more or less important alterations related to the amount of bleaching earth employed (11).

The use of GC with capillary columns for the determination of sterols in virgin and refined olive oils affords more information on the effect of refining on the composition of these products because a series of peaks is resolved, some of them unidentified, which shed further light on the changes occurring.

The method most frequently used in the determination of sterols, proposed as the official method by the aforementioned EU legislation, is based on isolation of the unsaponifiable fraction from the oil and later separation of the sterol fraction by basic silica gel plate chromatography. The sterols recovered from the silica gel are transformed into trimethylsilyl ethers and are analyzed by capillary column GC. This method was used in the present study, although it has the drawback of being time-consuming and involving numerous sample manipulations. Proposals have been made of "on-line" high-performance liquid chromatography (HPLC)–GC hybridization (12), which allows the determination of free and bound sterols and also the combination of HPLC and GC for use on the unsaponifiable fraction (13).

In the present work, we determined sterols in a large number of samples of virgin, refined, and solvent-extracted olive oils.

A statistical study also was carried out on the results obtained to draw conclusions regarding the composition of the three types of olive oil—virgin, refined, and solvent-extracted—to be able to characterize a given olive oil from among these types by analysis of its sterols, and to be able to detect possible adulteration of such oils.

MATERIALS AND METHODS

Reagents. The reagents used were of chromatography grade when required and of analytical grade otherwise.

Materials. The samples analyzed consisted of 50 virgin olive oils, 50 refined olive oils, and 30 solvent-extracted olive oils, all from the Central Laboratory of the Customs House in Madrid, Spain. The samples used, coming from the South of Spain, were bottled olive oils for human consumption. The refined olive oils were obtained by chemical and/or physical procedures.

Isolation of unsaponifiable fraction. The unsaponifiable matter was isolated according to the official method of the EU (CEE 2568/91 Annexe V) (14) similar to the method NGD C72 (15).

Isolation of sterol fraction. The sterol fraction was separated by thin-layer chromatography (TLC) according to the official method of the EU (14) on 0.25-mm thick silica gel plates previously activated with a 0.2 N ethanolic solution of potassium hydroxide. The sample was applied as a band across the plate and developed with a mixture (95:5, vol/vol) of benzene–acetone. The sterols were revealed with a 0.2% solution of 2,7-dichlorofluorescein. Chloroform was used to extract the sterols from the silica and then it was evaporated.

GC. The sterol fraction was silanized by adding 250 μL of a freshly prepared pyridine–hexamethyldisilazane–trimethylchlorosilane (9:3:1, vol/vol/vol) mixture and letting it stand for 15 min. An aliquot was taken from the clear solution and injected into the gas chromatograph. The sample should be protected from environmental moisture.

A Hewlett-Packard gas chromatograph (Palo Alto, CA), model 5890 series II, equipped with a split/splitless injector, fitted with a glass insert filled with stationary phase and silanized glass wool, and a flame-ionization detector, was used. The output signal of the detector was processed with a Hewlett-Packard 3396 series II integrator–recorder.

A capillary column of fused silica of 30 m long, 0.25 mm i.d., 0.25 μm of film thickness, with a stationary phase of SPB-5 (Supelco, Inc. Bellefonte, PA) was used.

The chromatographic conditions employed were: injection in the split mode with a split flow of 25 mL/min; pressure at column head, 200 kPa; carrier gas, helium; injector temperature, 295°C; detector temperature, 300°C; initial oven temperature, 265°C; initial time, 35 min; ramp, 20°C/min, final temperature; 300°C; final time, 5 min; injection volume, 1.0 μL.

RESULTS AND DISCUSSION

Care should be taken in the identification of the sterol spot to avoid the loss of sterols on the plate because they are not uniformly spread all over the fluorescent band.

Peak identification was effected by GC–mass spectrometry (MS). Where standards were commercially availabe, identification was confirmed by a comparison of GC retention times.

Typical chromatograms of the sterols obtained for virgin, refined, and solvent-extracted olive oil samples with the order of elution of the silanized derivatives are shown in Figure 1 (16).

To minimize the effect on absolute retention times of small variations under chromatographic conditions, retention times relative to that of campesterol were calculated. Campesterol eluted at approximately 17 min as a well-resolved peak. The relative retention times of the peaks of interest are shown in Table 1.

Quantitation of the sterols was performed by area normalization without taking into account response factors because the same sterols were to be compared among the different types of oils. The precision of the method is 5% as coefficient of variation. The percentages of sterols obtained for the dif-

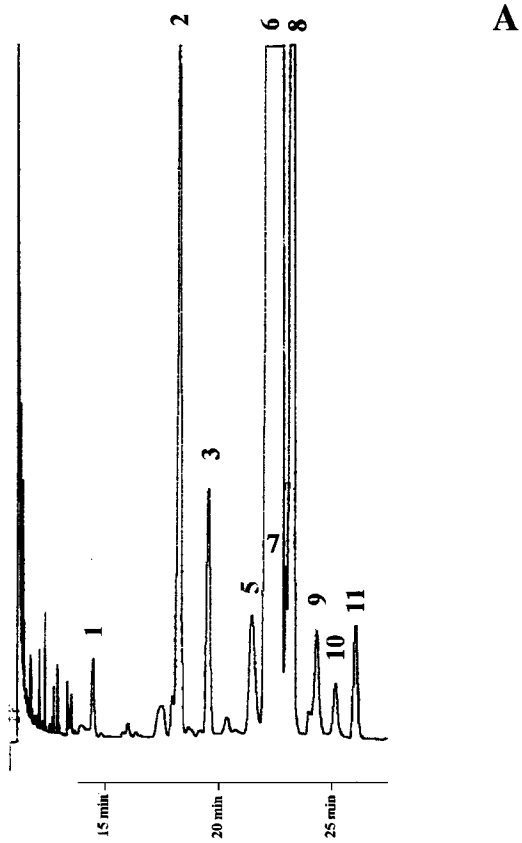

FIG. 1. Chromatographic plots of the trimethylsilyl derivatives of sterols from (A) virgin, (B) refined, and (C) solvent-extracted olive oils, analyzed by gas chromatography. (1) Cholesterol; (2) campesterol; (3) stigmasterol; (4) Δ5,23-stigmastadienol; (5) clerosterol; (6) β-sitosterol; (7) sitostanol; (8) Δ5-avenasterol, (9) Δ5,24-stigmastadienol; (10) Δ7-stigmastenol; (11) Δ7-avenasterol.

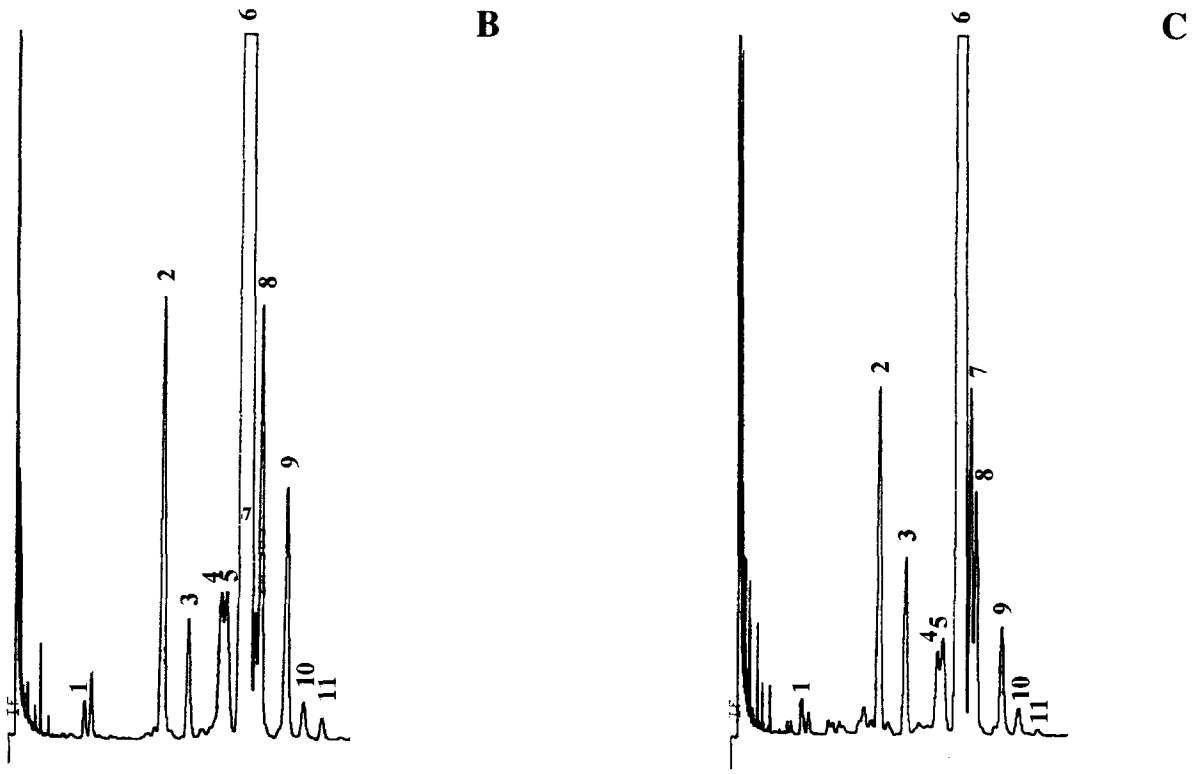

FIG. 1. (continued)

ferent types of oil studied are shown in Table 2. The results were processed statistically to obtain an estimate of the mean interval for a confidence level of 95%, applying the following expression (17):

$$X - t(\alpha/2, n-1) \times \sqrt{(V/n)} < \mu < X + t(\alpha/2, n-1) \times \sqrt{(V/n)} \quad [1]$$

where t is the distribution function for a level of significance α and $n - 1$ degrees of freedom, V is variance, n is the sample size, X is the sample mean, and μ is the mean of the population.

The results obtained showed a similar repeatibility to that obtained in a collaborative study of 26 laboratories (18).

From the results obtained (Table 3), a significant differentiation was seen as regards the content in cholesterol from virgin olive oil, refined olive oil, and solvent-extracted olive oil. This content can be used to differentiate between the three types of oil.

Campesterol was at the same level in the virgin and refined olive oils, but was present at lower proportions in solvent-extracted oil. With respect to stigmasterol, a significant differentiation was seen among the three types of oil.

TABLE 1
Relative Retention Time Averages and Standard Deviations for the Trimethylsilyl Derivatives of Sterols from Olive Oil, Analyzed by Gas Chromatography

Peak number	Sterol	Number of samples	Average	SD
1	Cholesterol	24	0.800	0.005
2	Campesterol	25	1.000	—
3	Stigmasterol	25	1.066	0.001
4	Δ5,23-Stigmastadienol	20	1.150	0.002
5	Clerosterol	25	1.164	0.002
6	β-Sitosterol	25	1.224	0.008
7	Sitostanol	25	1.237	0.004
8	Δ5-Avenasterol	25	1.252	0.005
9	Δ5,24-Stigmastadienol	25	1.315	0.003
10	Δ7-Stigmastenol	25	1.359	0.003
11	Δ7-Avenasterol	25	1.406	0.003

TABLE 2
Average Percentages and Standard Deviations of the Trimethylsilyl Derivatives of Sterols from Different Types of Olive Oil: Virgin, Refined and Solvent-Extracted, Analyzed by Gas Chromatography

Peak number	Sterol	Virgin	Refined	Extracted
1	Cholesterol	0.10 ± 0.05	0.13 ± 0.05	0.23 ± 0.16
2	Campesterol	3.2 ± 0.2	3.4 ± 0.2	3.19 ± 0.19
3	Stigmasterol	0.59 ± 0.09	0.9 ± 0.3	1.3 ± 0.3
4	Δ5,23-Stigmastadienol	0.08 ± 0.08	0.8 ± 0.4	0.8 ± 0.3
5	Clerosterol	0.98 ± 0.08	1.27 ± 0.17	1.3 ± 0.2
6	β-Sitosterol	84.9 ± 1.9	84.9 ± 1.4	81 ± 4
7	Sitostanol	0.43 ± 0.07	0.53 ± 0.12	2.3 ± 0.7
8	Δ5-Avenasterol	7.8 ± 1.7	5.0 ± 1.5	2.7 ± 0.8
9	Δ5,24-Stigmastadienol	0.41 ± 0.10	1.1 ± 0.5	1.2 ± 0.5
10	Δ7-Stigmastenol	0.25 ± 0.05	0.36 ± 0.09	0.55 ± 0.13
11	Δ7-Avenasterol	0.37 ± 0.11	0.30 ± 0.11	0.26 ± 0.07

TABLE 3
Mean Interval Estimation for a 95% Confidence Level of the Trimethylsilyl Derivatives of Olive Oil Sterols, Analyzed by Gas Chromatography

Peak number	Sterol	Virgin	Refined	Extracted
1	Cholesterol	0.09–0.11	0.12–0.14	0.17–0.29
2	Campesterol	3.2–3.4	3.3–3.5	3.12–3.27
3	Stigmasterol	0.56–0.62	0.8–1.0	1.2–1.4
4	Δ5,23-Stigmastadienol	0.06–0.10	0.7–1.0	0.7–0.9
5	Clerosterol	0.96–1.01	1.22–1.31	1.3–1.4
6	β-Sitosterol	84.4–85.4	84.5–85.3	79.5–82.2
7	Sitostanol	0.41–0.45	0.50–0.57	2.0–2.6
8	Δ5-Avenasterol	7.3–8.3	4.6–5.4	2.4–3.0
9	Δ5,24-Stigmastadienol	0.38–0.44	0.96–1.24	1.0–1.4
10	Δ7-Stigmastenol	0.24–0.26	0.33–0.39	0.51–0.60
11	Δ7-Avenasterol	0.34–0.40	0.27–0.33	0.23–0.28

Δ5,23-Stigmastadienol was found in different proportions in virgin oil than in refined and solvent-extracted oils, but not between the latter types. The proportion in virgin olive oil was very small.

Clerosterol, sitostanol, and Δ5-avenasterol also showed marked differences among the three types of olive oil. For sitostanol, however, it is necessary to consider this conclusion carefully because of the poor resolution obtained for this peak under the current chromatographic conditions.

The β-sitosterol contents of virgin and refined oils were similar but significantly different from those of solvent-extracted oils. The Δ5,24-stigmastadienol contents of refined and solvent-extracted oils were similar, but significantly different from those of virgin oils.

Finally, Δ7-stigmastenol and Δ7-avenasterol contents were markedly different among the three types of oil.

To conclude, it can be stated that stigmasterol, clerosterol, Δ5-avenasterol, Δ7-stigmastenol, and Δ7-avenasterol permitted the differentiation of the three types of oil from one another. Campesterol, Δ5,23-stigmastadienol, β-sitosterol, and Δ5,24-stigmastadienol permitted the differentiation of only two oils from each other, but they confirm the conclusions obtained for other sterols.

Correlations also were made among the different sterols for the different types of oil; it was observed that, for virgin olive oil, there was a clear negative correlation between β-sitosterol and Δ5-avenasterol with a correlation coefficient of −0.9603, and there was a poor correlation between Δ7-avenasterol on one hand, and Δ5,24-stigmasterol and Δ7-stigmastenol on the other, with correlation coefficients of 0.6181 and 0.6727.

For refined olive oils, correlations were seen between Δ5,23-stigmastadienol on one hand, and clerosterol (0.7839), Δ5-avenasterol (−0.8506), and Δ5,24-stigmasterol (0.8082) on the other. There was also a correlation between clerosterol on one hand, and Δ5-avenasterol (−0.7142) and Δ5,24-stigmastadienol (0.7784) on the other. Likewise, a correlation was seen between Δ5-avenasterol and Δ5,24-stigmastadienol (−0.7215).

For solvent-extracted oils, the most significant correlation was between stigmasterol and sitostanol (0.8683).

From the results obtained, it may be deduced that sterols can be used to distinguish between different types of olive oil on the basis of the different percentages of the sterols present.

The correlations found among the different sterols are generally quite weak and only allow clear conclusions to be drawn in some of the situations addressed: between β-sitostanol and Δ5-avenasterol for vigin olive oil.

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