FOOD COMPOSITION AND ADDITIVES

Detection of Hazelnut Oil in Virgin Olive Oil by Assessment of Free Sterols and Triacylglycerols

STEFANIA VICHI, LORENA PIZZALE, EMILIO TOFFANO, RENZO BORTOLOMEAZZI, and LANFRANCO CONTE[1]
Udine University, Food Science Department, via Marangoni 97, 33100 Udine, Italy

Free sterols were evaluated as factors for discriminating between genuine virgin olive oil and hazelnut-mixed virgin olive oil. Numeric analyses of the results amplified the differences between groups. The application of this method to virgin olive oil samples and their mixtures with 10% hazelnut oil distinguished between genuine and nongenuine virgin olive oil with statistical certainty. Triacylglycerol analysis was tested for the same purpose by using parameter ΔECN42, but although it possessed a discriminating capacity, it alone could not distinguish the aforementioned groups with sufficient certainty. Free Δ7-sterols data were combined with ΔECN42 data into a single discriminating function to improve differentiation and bring more ruggedness, and for detection of low amounts (10%) of hazelnut oil in virgin olive oil. In fact, the values obtained by addition of Δ7-sterol data and ΔECN42 data showed a higher discriminating capacity than single parameters. In a single operation the method produced all the oil fractions necessary for analysis of free sterols and triacylglycerols with ECN42. Solid-phase extraction was applied in substitution of traditional chromatography on a silica column.

The recent development of new analytical methods for control of genuine olive oil has been directed toward the detection of hazelnut oils in blends with olive oil. Ruiz del Castillo et al. (1) suggested E–5–methylhepta–2–en–4–one (Filbertone) as a blending marker, as Filbertone is a peculiar volatile hazelnut compound not present in olive oil. Mannina et al. (2) proposed detection of volatile aldehydes of hazelnut by nuclear magnetic resonance (NMR). However, these methods only detect blends of hazelnut oil with virgin olive oil, because volatile compounds are easily removed during deodorization.

Fedeli et al. (3) and Cert and Moreda (4) investigated the use of triacylglycerol analysis for evaluation of genuine olive oil. Fedeli et al. applied ΔECN42 and ΔECN42%, pointing out that these parameters have some limits when applied to blends of olive oils of different fatty acid composition. Cert and Moreda developed a method based on comparison of the values of algorithms (functions of values of ΔECN44, LLL, OLL, and linoleic acid) and a database of experimental values obtained from genuine virgin olive oils.

Mariani et al. (5) and Morchio et al. (6) examined the γ–tocopherol/β–tocopherol ratio and the amounts of δ–tocopherol, which both seem to increase in the presence of hazelnut oil. The γ–tocopherol/β–tocopherol ratio is generally higher than 10 in hazelnut oil and lower than 5 in olive oil. However, the tocopherol fraction undergoes a degradation process during storage and could become less effective in detecting hazelnut oil after storage.

The esterified sterol fraction was studied by Mariani et al. (7) for detection of differences between hazelnut oil and olive oil in the amounts of esterified campesterol and Δ7-sterols. Some of the above-mentioned methods are now submitted to ring tests; however, no method is able to detect the presence of hazelnut oil in low concentration with sufficient certainty.

We evaluated free sterols as a discriminating factor between hazelnut oil and virgin olive oil together with the parameter ΔECN42, which is usually adopted as a marker of the presence of seed oil in olive oil, but is unsuitable for detection of low concentrations of hazelnut oil in olive oil. Our objective was to develop a rapid method which would provide, in a single operation, all the fractions necessary for the analyses, the results of which could be considered as variables in a single discriminating function. This would allow a limit to be fixed above which the oil could not be considered genuine.

Experimental

Samples

Extra virgin olive oils of different origin and mixtures of them were analyzed as the genuine oil group: Two virgin hazelnut oils and one refined hazelnut oil were used to prepare samples of blends of olive oil and 10% hazelnut oil, analyzed as the nongenuine group (Table 1).

Solid-Phase Extraction (SPE)

Sample (3 g) was added to 70 μL 2 mg/mL solution of 5α-cholestanol and diluted to 10 mL with hexane; 5 mL of this sample solution was loaded onto a silica cartridge (Mega Bond Elut 5 g; Varian, CA) previously conditioned with 15 mL methanol followed by 30 mL hexane. Two fractions were separated: the first fraction, eluted with 15 mL hexane and 30 mL hexane–diethylether (95 + 5, v/v) mix-

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[1] Author to whom correspondence should be addressed.

Table 1. List of samples

Code	Sample
N–1	Virgin hazelnut oil (French)
N–2	Virgin hazelnut oil (Turkey)
N–3	Refined hazelnut oil (Turkey)
I–OC	Virgin commercial olive oil (Italy)
I–A	Virgin olive oil (Abruzzo, Italy)
I–P	Virgin olive oil (Puglia, Italy)
I–M	Virgin olive oil (Liguria, Italy)
I–G1	Virgin olive oil (Garda Lake, Italy)
I–G2	Virgin olive oil (Garda Lake, Italy)
I–R	Virgin olive oil (Romagna, Italy)
T–1	Virgin olive oil (Tunisia)
T–2	Virgin olive oil (Sitia, Tunisia)
G–1	Virgin olive oil (Greece)
G–2	Virgin olive oil (Kritza, Greece)
G–3	Virgin olive oil (Kolimbari, Greece)
S–1	Virgin olive oil (North of Spain)
S–J	Virgin olive oil (Jaèn, Spain)
TUR–OC	Virgin commercial olive oil (Turkey)
TUR	Virgin olive oil (Izmir, Turkey)
MIX1	Blend (Abruzzo/Greece 50:50)[a]
MIX2	Blend (Tunisia/Spain 50:50)[a]
MIX3	Blend (Spain/Abruzzo 50:50)[a]
MIX4	Blend (Spain/Puglia 50:50)[a]
MIX5	Blend (Greece/Spain 50:50)[a]
MIX6	Blend (Abruzzo/Greece/Tunisia)
MIX7	Blend (Spain/Puglia/Tunisia 33:33:33)[a]
I–G1/N–1	Mixture I–G1/N–1 90:10
I–G2/N–1	Mixture I–G2/N–1 90:10
I–OC/N–1	Mixture I–OC/N–1 90:10
G–1/N–1	Mixture G–1/N–1 90:10
I–G1/N–2	Mixture I–G1/N–2 90:10
I–G2/N–2	Mixture I–G2/N–2 90:10
I–OC/N–2	Mixture I–OC/N–2 90:10
G–1/N–2	Mixture G–1/N–2 90:10
I–G1/N–3	Mixture I–G1/N–3 90:10
I–G2/N–3	Mixture I–G2/N–3 90:10
I–OC/N–3	Mixture I–OC/N–3 90:10
G–1/N–3	Mixture G–1/N–3 90:10

[a] Blend of virgin olive oils.

ture, and then spiked with 150 μL 5α-cholestanol (0.2 mg/mL) was called F1; the second fraction, eluted with 30 mL diethylether, was called F2. Both fractions were dried in a low-pressure rotary evaporator.

Table 2. Repeatability results obtained for free sterols determination

	Repeatability		
Free sterols	Mean, ppm	RSD_r	RSD_r, %
Campesterol	39.7	1.38	3.5
Stigmasterol	9.6	0.51	5.3
β-sitosterol	913.4	42.20	4.6
Δ5-avenasterol	144.4	8.94	6.2
Δ5,24-stigmastadienol	4.7	0.99	21.3
Δ7-stigmastenol	1.9	0.67	35.1
Δ7-avenasterol	3.0	0.63	21.3
Total	1115.5	51.90	4.7

Alkaline Catalyzed Transmethylation

The fraction designated F1 (250 mg) underwent transmethylation to determine fatty acids composition. Transmethylation was performed as follows: sample F1 was dissolved in 5 mL *n*-hexane; then 250 μL 2M methanolic a KOHsolution was added. The solution was stirred for 30 s, and then gently centrifuged (3000 rpm) to obtain a clear solution.

The entire fraction designated F2 was dissolved with 3 mL *n*-hexane-diethylether (1 + 1, v/v); then it underwent transmethylation as described above for F1.

Thin-Layer Chromatography (TLC)

After transmethylation, F2 was dried, redissolved in chloroform, and applied onto a TLC plate (silica gel 60, Merck, Darmstadt, Germany). The plates were previously made alkaline by immersion in an ethanolic solution of potassium hydroxide according to the Official Method of the European Community for determination of total sterols (8). The plate was developed with a mixture of hexane–diethylether (60 + 40, v/v), and then sprayed with an ethanolic solution of 2,7-dichlorofluorescein, and the band of free sterols was marked under a UV lamp.

Gas Chromatographic (GC) Analysis of Fatty Acids

The analysis was performed on a Carlo Erba HRGC 5160 gas chromatograph fitted with a flame ionization detector and equipped with a 60 m × 0.32 mm id, 0.2 μm film thickness, SP2330 capillary column (Supelco, Bellefonte, PA). The flow rate of the carrier gas (helium) was 1.5 mL/min, the split ratio was 1:80, and the amount of injected sample was 1 μL. Injector and detector were set at 220°C; the oven temperature was 165°C for 10 min; then it was raised to 210°C at a rate of 5°C/min, and held at 210°C for 8 min.

Gas Chromatographic Analysis of Free Sterols

Free sterols were analyzed as trimethylsilylether derivatives using the same gas chromatograph as that used for analysis of fatty acids. The separation was performed with a

Table 3. Assessment of free sterols (expressed in ppm) of virgin olive oils, hazelnut oils, and their blends

Sample	Campesterol	Stigmasterol	β-sitosterol	Δ5-avenasterol	Δ5,24-stigmastad	Δ7-stigmastenol	Δ7-avenasterol	Total
N–1	57.8	13,9	915,3	44,8	6,8	12,0	5.0	1055.7
N–2	53.4	7.8	973.4	52.5	5.9	17.0	6.0	1116.1
N–3	61.0	10.4	1012.0	51.2	6.1	19.3	7.9	1167.8
I–OC	39.7	9.6	913.4	144.4	4.7	1.9	3.0	1116.6
I–A	33.4	8.5	899.6	116.4	6.7	1.4	3.0	1069.0
I–P	30.0	5.6	789.0	98.0	3.9	1.3	1.9	929.6
I–M	29.4	8.7	936.7	102.1	6.1	1.3	2.7	1086.9
I–G1	29.6	6.2	932.2	119.6	1.7	2.3	5.5	1097.2
I–G2	40.0	6.3	807.3	228.7	5.3	1.1	2.7	1091.3
I–R	42.9	9.0	1135.9	72.3	2.1	1.7	2.6	1266.6
T–1	38.2	8.9	949.0	134.3	8.8	2.4	5.0	1146.5
T–2	31.7	15.5	951.5	255.9	7.4	1.0	4.1	1267.1
G–1	36.5	5.3	728.5	256.8	5.8	1.0	2.3	1036.1
G–2	37.8	5.1	819.3	233.7	5.1	1.4	2.7	1105.0
G–3	36.6	5.8	824.1	244.5	5.1	1.1	2.5	1119.6
S–1	40.7	9.6	819.3	173.8	7.6	1.3	3.8	1056.2
S–J	40.1	9.1	1054.3	60.9	1.3	1.6	1.8	1169.1
TUR–OC	36.9	7.3	1080.2	152.2	12.6	2.7	6.3	1298.1
TUR	36.9	8.4	1040.3	153.3	11.3	2.0	6.1	1258.3
MIX1	38.2	5.9	854.7	174.2	3.1	1.3	2.2	1079.6
MIX2	43.6	8.8	1049.0	161.2	7.1	2.1	4.8	1276.5
MIX3	39.7	8.8	1027.5	98.2	4.3	1.0	2.4	1181.8
MIX4	37.8	8.4	926.2	135.7	4.8	1.4	2.9	1117.1
MIX5	36.2	7.5	911.5	159.5	2.4	0.8	2.0	1120.0
MIX6	38.7	7.9	950.8	143.7	5.6	1.3	2.4	1150.4
MIX7	37.9	9.1	1034.4	162.3	7.0	1.8	4.7	1257.2
I–G1/N–1	31.6	7.9	1043.1	134.7	4.5	3.3	6.3	1231.4
I–G2/N–1	33.5	16.3	975.3	241.6	8.0	2.4	4.8	1282.1
I–OC/N–1	43.0	9.1	920.5	145.7	3.5	2.1	2.2	1126.1
G–1/N–1	37.2	6.8	699.0	236.6	3.5	2.2	2.4	987.8
I–G1/N–2	39.0	7.3	1081.3	144.5	4.3	3.4	6.5	1286.3
I–G2/N–2	36.8	13.8	1026.3	245.6	6.3	3.3	4.6	1336.7
I–OC/N–2	34.5	6.2	702.0	131.4	2.7	3.0	3.5	883.3
G–1/N–2	39.2	5.8	783.6	244.0	3.7	2.0	2.8	1081.1
I–G1/N–3	39.6	6.9	1102.6	150.8	4.4	3.3	6.3	1313.8
I–G2/N–3	38.1	14.9	958.8	226.9	5.9	2.9	4.6	1252.0
I–OC/N–3	46.9	7.5	915.5	141.2	3.0	2.7	2.8	1119.6
G–1/N–3	45.7	5.0	858.5	255.9	4.7	3.1	3.0	1175.9

30 m × 0.32 mm id, 0.1 μm film thickness, SPB5 capillary column (Supelco). Carrier gas flow rate, split ratio, and volume of injected sample were as above. Injector and detector had a temperature of 300°C; oven temperature was 270°C (isotherm).

Liquid Chromatographic (LC) Analysis of Triacylglycerols

The sample, an aliquot of F1, was analyzed according to the Official Method of the European Community (9). In brief, triglycerides were separated on an ODS (octadecilsilane)

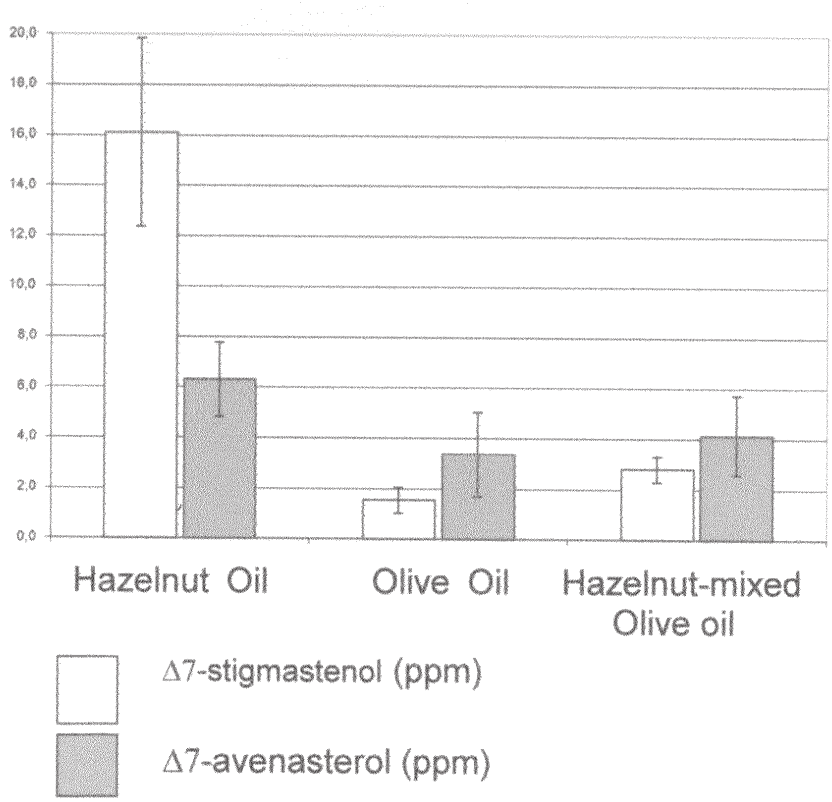

Figure 1. Comparison between average contents of free Δ7–stigmastenol and Δ7–avenasterol of hazelnut oils, virgin olive oils, and their blends.

chromatographic column (250 × 46 mm id, 5 μm particle size), with acetone–acetonitrile (1 + 1) as mobile phase, flushed at 1.5 mL/min, with detection performed by means of a refraction index detector.

Results and Discussion

The applicability of the method based on determination of free Δ7-sterols and triacylglycerols (ECN42) was evaluated on extra virgin oils from 5 different places of origin and blends of oils with different composition characteristics, because they could show a different behavior from single oils. Fedeli et al. (3) noted a limit of applicability of the method for determination of triglycerides with ECN42 in blends of oils with very different fatty acid composition. The experimental value of ECN42 deviated from the theoretical value calculated from the fatty acid composition.

Mixtures of virgin olive oils and 3 different hazelnut oils (2 obtained by pressing seeds and one refined oil) were examined in order to include all types of hazelnut oil used in fraudulent mixing. Some hazelnut oil was added to virgin olive oils to obtain a concentration of 10% hazelnut oil, which was considered a satisfactory limit of detection because mixtures with a lower percentage would be unprofitable.

Determination of Free Sterols

The most polar fraction obtained by SPE (F2), containing free sterols, was transmethylated to eliminate mono- and

Table 4. Application of *t*-test to numeric elaboration of free sterols results[a]

Formula	*F*	ν	*t*	*t* 99.5%	Δ*t*
(I) Δ7-stigmastenol^3/Δ7-avenasterol	5.98	15	7.38	3.29	4.09
(II) Δ7-stigmastenol^3%/Δ7-avenasterol%	12.17	14	5.20	3.33	1.87
(III) Δ7-stigmastenol^2/Δ7-avenasterol	3.05	18	7.50	3.22	4.28
(IV) Campesterol X (Δ7-stigmastenol^2/Δ7-avenasterol)	5.02	15	5.94	3.29	2.65
(V) Campesterol% X (Δ7-stigmastenol%^{2}/Δ7-avenasterol%)	8.40	14	4.55	3.33	1.22

[a] F=*F*-test results; ν = degrees of freedom; *t* =*t* of Student; *t* 99.5%: *t* critic corresponding to a confidence level >99.5%; Δ*t*:= *t* calculated – *t* 99.5%.

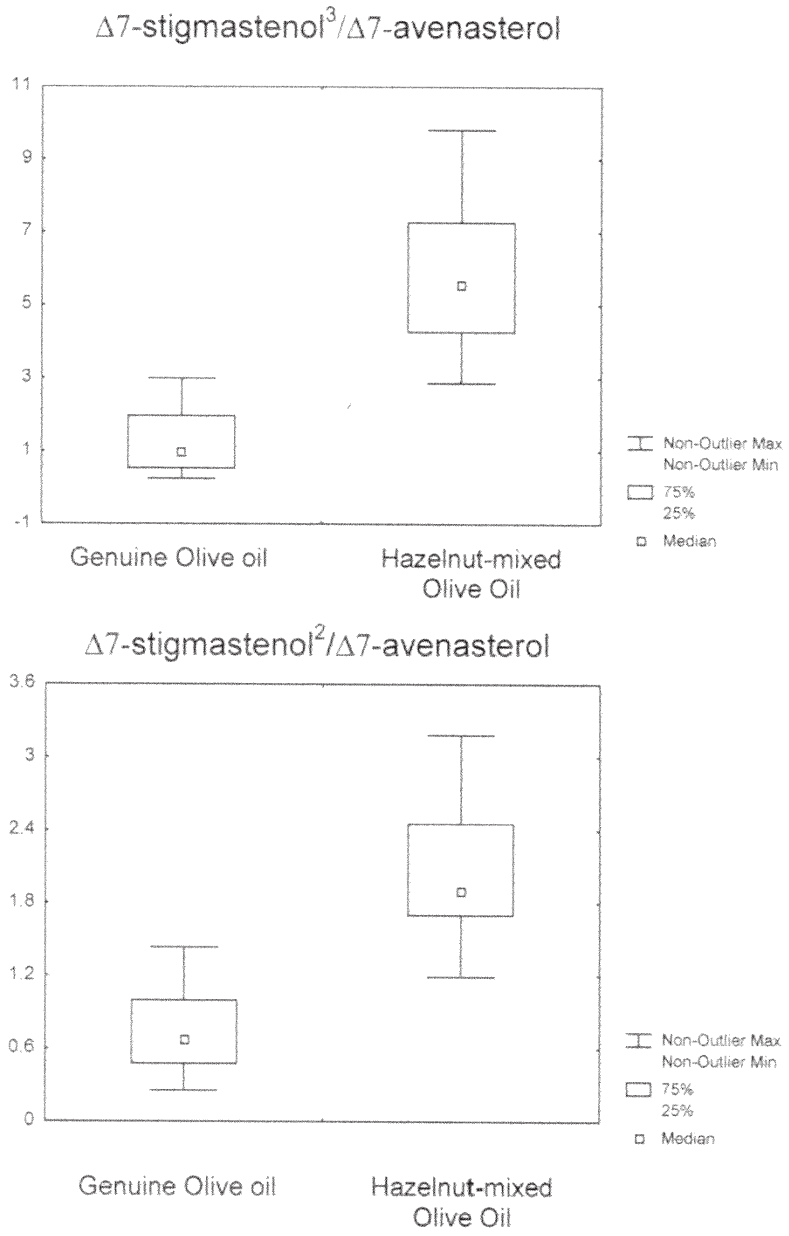

Figure 2. Graphic representation of values obtained by formulas (I) and (III) for genuine virgin olive oils and their blends with 10% hazelnut oil.

diglycerides which could interfere with separation of the sterol band by TLC and subsequently with the GC analysis. Transmethylation of the mono- and diglycerides produced fatty acid methyl esters. After this, the sterol fraction was isolated by TLC. The use of alkaline plates was required to avoid interference with acidic compounds and improve the separation from aliphatic and triterpenic alcohols and methylsterols. Free sterols were then analyzed as trimethylsilylethers by GC and quantitatively determined with the internal standard method.

The repeatability of the method was verified by SPE, TLC, and subsequent GC analysis of 8 samples of the same oil. Mean, standard deviation of the repeatability, and repeatability coefficient of variation were calculated (Table 2). The highest percentage standard deviations were found for Δ5,24-stigmastadienol and Δ7-sterols, probably due to their low concentrations. Nevertheless, these results were considered satisfactory because they were comparable to those obtained by the Official Method of the European Community for the determination of total sterols (8), which reach values of percentage standard deviation around 20%, for the same compounds (10). Table 3 lists the assessment of free sterols in extra virgin olive oils, hazelnut oils, and mixtures of virgin olive

Table 5. Results of analyses of triglycerides with ECN42

Sample	Theoretical ECN42	LC ECN42	|ΔECN42|
I–OC	0.22	0.28	0.06
I–A	0.39	0.41	0.02
I–P	0.30	0.27	0.03
I–M	0.34	0.38	0.04
I–G1	0.34	0.39	0.04
I–G2	0.25	0.30	0.05
I–R	0.27	0.34	0.07
T–1	1.00	1.11	0.11
T–2	0.40	0.36	0.05
G–1	0.26	0.30	0.04
G–2	0.27	0.30	0.03
G–3	0.26	0.34	0.08
S–1	0.50	0.49	0.01
S–2	0.26	0.36	0.10
TUR–OC	0.36	0.40	0.04
TUR	0.34	0.38	0.04
MIX1	0.29	0.36	0.06
MIX2	0.83	0.87	0.04
MIX3	0.32	0.38	0.06
MIX4	0.38	0.41	0.03
MIX5	0.24	0.34	0.09
MIX6	0.31	0.34	0.03
MIX7	0.61	0.71	0.10
I–G1/N1	0.33	0.55	0.22
I–G2/N1	0.21	0.52	0.30
I–OC/N1	0.46	0.26	0.20
G–1/N1	0.26	0.55	0.29
I–G1/N2	0.31	0.42	0.11
I–G2/N2	0.25	0.32	0.07
I–OC/N2	0.24	0.37	0.14
G–1/N2	0.24	0.37	0.13
I–G1/N3	0.26	0.49	0.22
I–G2/N3	0.46	0.26	0.20
I–OC/N3	0.24	0.42	0.17
G–1/N3	0.25	0.56	0.31

Table 6. Application of *t*-test to ΔECN42 parameter

	F	ν	*t*	*t* 99.5%	Δ*t*
ΔECN42	7.97	15	6.22	3.29	2.93

oil with 10% hazelnut oil. The data are expressed as ppm of each sterol.

The free sterols fraction shows a considerable variability of the amount of each sterol in both hazelnut and olive oils, which could be due to the different origin of the oils. The individual sterol contents did not appear to be a discriminating factor between genuine olive oils and olive oil mixed with hazelnut oil. The contents of some sterols differed in hazelnut oil, virgin olive oil, and mixtures of them. Although the amounts of campesterol are higher in hazelnut oil than in genuine virgin olive oil, this difference was insufficient to cause a significant increase in the mixtures with 10% hazelnut oil. In the same way, the low amount of Δ5-avenasterol found in hazelnut oil compared with olive oil could not be used as a discriminating factor, because the reduction of the amount in the mix with only 10% hazelnut oil was not considerable. The addition of virgin or refined hazelnut oil leads to an increase in amount of Δ7-sterols, and increases the Δ7-stigmastenol/Δ7-avenasterol ratio because of the considerably higher content of Δ7-stigmastenol in hazelnut oil. Figure 1 shows the means of Δ7-stigmastenol and Δ7-avenasterol in each group of samples: hazelnut oils, genuine virgin olive oil, and mixtures of olive oil and hazelnut oil.

To highlight the compositional variations caused by addition of hazelnut oil, the data were elaborated to amplify the increase of either the Δ7-sterols or the Δ7-stigmastenol/Δ7-avenasterol ratio (Table 4).

The capacity of each formula to discriminate between a genuine virgin olive oil group and a hazelnut-mixed olive oil group was evaluated with the Student's *t*-test. As shown in Table 4, every analysis of the data distinguished the genuine virgin olive oil group from the hazelnut-mixed olive oil group, with a significance level >99.5%. Because all the formulas could distinguish at this significance level, the discriminating capacity of each was evaluated insofar as the *t* calculated exceeded the critical *t* corresponding to a significance level >99.5% (Δ*t*). On this

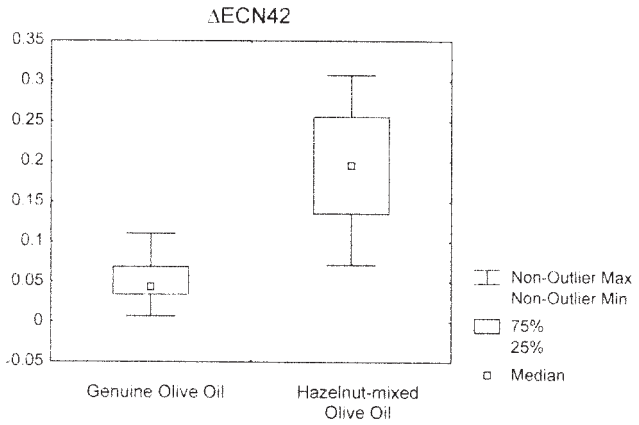

Figure 3. Graphic representation of values of ΔECN42 for genuine virgin olive oils and their blends with 10% hazelnut oil.

Table 7. Application of *t*-test to values obtained by functions with 2 variables[a]

Formula	*F*	ν	*t*	*t* 99.5%	Δ*t*
(VI) Δ7-stigmastenol^3/Δ7-avenasterol + ΔECN42 x 10	4.74	16	9.51	3.25	6.26
(VII) Δ7-stigmastenol^2/Δ7-avenasterol + ΔECN42 x 10	3.89	17	9.46	3.22	6.24

[a] F = *F*-test results; ν = degrees of freedom; *t*:= *t* of Student; *t* 99.5%: *t* critic corresponding to a confidence level >99.5%; Δ*t*:= *t* calculated – *t* 99.5%.

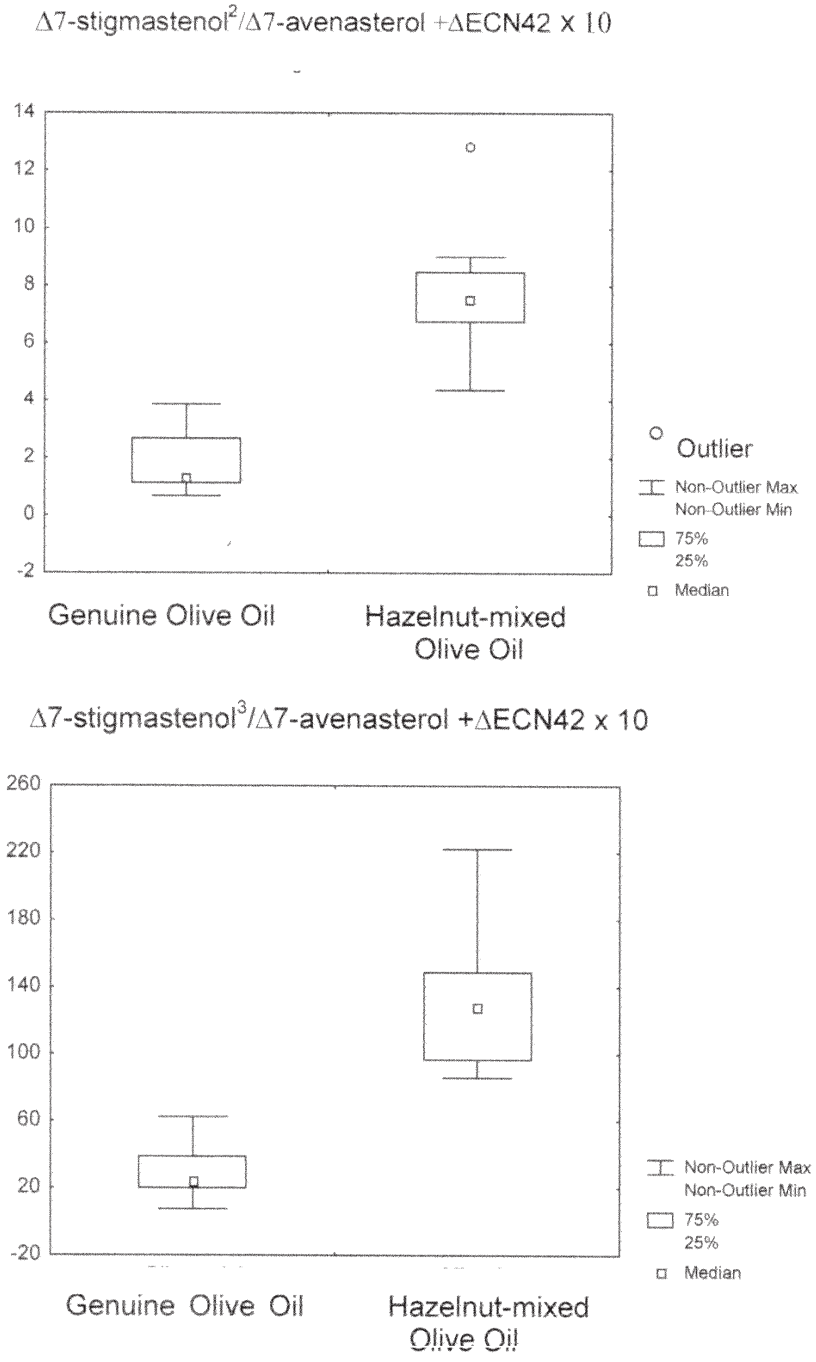

Figure 4. Graphic representation of values obtained by formulas (VI) and (VII) for genuine virgin olive oils and their blends with 10% hazelnut oil.

basis, the more effective formulas were (I) and (III). Their capacity to distinguish the different oil groups is clear from the box plot graphic representation in Figure 2.

Triacylglycerol Analysis Results

The ECN42 method was tested as a possible combination with free sterol analysis as it is usually used to evaluate the genuineness of olive oil. It is indeed a valid method to detect the presence of seed oil in olive oil. The results were at first considered separately to evaluate the discriminant capacity of the method when applied to mixtures of olive oil with relatively low percentages of hazelnut oil. Table 5 reports the results obtained by theoretical calculation from the fatty acid composition (theoretical ECN42), the experimental results achieved by LC analysis (LC ECN42), and the difference between those values (ΔECN42). Within the framework of Community Regulation (EC) 2472/97, the maximum difference admitted between LC data and theoretical data is 0.2 for extra virgin oils. The parameter ΔECN42 was evaluated as a discriminating factor by application of the *t*-test (Table 6) and graphic representation by means of box plot (Figure 3). The difference between theoretic and experimental ECN42 values allowed genuine and adulterated olive oil groups to be distinguished with a significance level >99.5%, even if it there was not a clear-cut division of the values of each group. Figure 3 shows an overlap of values of both groups, which prevents establishment of an analytical limit.

Search for a Discriminating Factor Based on Different Variables

The contribution of more independent variables could give a higher discriminating capacity between genuine and hazelnut-mixed olive oil and at the same time decrease the possibility of incorrect classification of oils with particular compositional characteristics, i.e., it should bring more ruggedness to the method. On the basis of the above results, a search was made for a discriminating function which would correlate both Δ7-sterols and ΔECN42 data and give a single value to include a sample in one of 2 groups: the genuine olive oil group and the hazelnut-mixed olive oil, where the hazelnut oil percentage is at least 10%. For this purpose, only the most effective numerical elaborations of the free sterol data (I and II) were combined with ΔECN42 data. To combine both parameters with the same weight, they were added after being converted to the same magnitude. The following functions were then calculated:

$$\Delta 7\text{–stigmastenol}^3/\Delta 7\text{–avenasterol} + \Delta ECN42 \times 10 \quad \text{(VI)}$$

$$\Delta 7\text{–stigmastenol}^2/\Delta 7\text{–avenasterol} + \Delta ECN42 \times 10 \quad \text{(VII)}$$

These equations were then evaluated using a *t*-test, whose results confirmed the effectiveness of the proposed functions (Table 7). Indeed, each showed a higher discriminating capacity (*t* and Δ*t*) than those with single variables. As shown in Figure 4, the functions allowed the grouping of all genuine olive oil samples and hazelnut-mixed olive oil samples in 2 distinct and not-overlapping ranges of values.

An analytical limit could be then proposed, above which a virgin olive oil has to be considered as nongenuine, corresponding to the higher value of the range which contains 100% of the genuine virgin olive oil sample values.

In summary, the method proposed in this work, based on the combination of free Δ7–sterol amounts and the ΔECN42 parameter into a single discriminating function, represents a rapid and easy analytical approach to detect the presence of relatively low percentages (10%) of hazelnut oil in virgin olive oil.

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