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Free and Esterified 4,4′-dimethylsterols in Hazelnut Oil and their Retention During Refining Processes

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Abstract Free and esterified forms of sterols provide detailed information on the identity and the quality of vegetable oils. In this study, 4,4′-dimethylsterols in free and esterified forms were investigated in hazelnut and virgin olive oils. Moreover, a sample of solvent-extracted hazelnut oil was refined at the laboratory to monitor the effects of processing on the levels of 4,4′-dimethylsterols. Generally, the level of total 4,4′-dimethyslterols was higher in the esterified form (49–68%) compared with that in free form (32–51%) of these compounds in the hazelnut oil. In virgin olive oil samples, cycloartenol and 24-methylenecycloartanol were present in higher amounts in free forms (70–80%) than in esterified forms (20–30%). Among the refining processes, degumming, deodorization, neutralization and bleaching, only neutralization and bleaching considerably reduced 4,4′-dimethylsterols. In fully refined hazelnut oil, 18 and 37% of lupeol and an unknown compound X in the esterified form were lost, respectively. The loss of these two compounds in the free form was considerably higher, 26 and 72%, respectively. GC–MS analysis showed that adulteration of olive oil with a sample of fully refined hazelnut oil could be detected at a level as low as 2% by tracing lupeol in total or only in esterified forms of 4,4′-dimethylsterols. Further studies on the levels of free and esterified 4,4′-dimethylsterols and their retention during refining processes are anticipated in hazelnut cultivars from different origins.

Keywords Adulteration · Esterified 4,4′-dimethylsterols · Free 4,4′-dimethylsterols · Esterified sterols · Free sterols · Hazelnut oil · Olive oil · Triterpene alcohols · Vegetable oil refining

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Introduction

Phytosterols are divided into three classes: 4-desmethylsterols, 4-monomethylsterols, and 4,4′-dimethylsterols (triterpene alcohols) [1]. These sterols can occur in free and esterified forms in vegetable oils [2]. The conventional method for total sterol analysis is saponification of the oil sample followed by extraction of the unsaponifiables with an organic solvent. On the other hand, separate determination of sterols in free and esterified forms provides detailed information of their distribution and stability [3]. Data on total 4,4′-dimethylsterols of hazelnut oil have been published [4–6] but information on these sterols in free and esterified forms is lacking. It has been demonstrated that during refining processes total sterols, as well as esterified and free sterols, are reduced to various levels [7]. There is no report on the effects of refining processes on the levels of 4,4′-dimethylsterols in hazelnut oil.

Differences among the 4,4′-dimethylsterols provide a basis on which to detect virgin olive adulteration with hazelnut oil [4, 6]. Lupeol and an unknown compound

X (containing a lupane skeleton) are present only in 4,4′-dimethylsterols fraction of hazelnut oil, and have been used to detect virgin olive oil adulteration with hazelnut oil at low levels [4, 6]. Adulteration of virgin olive oil with refined vegetable oils is generally detected by determining the contents of *trans* fatty acids and stigmasta-3,5-diene [8]. These compounds cannot be used to detect adulteration of refined olive oil with refined hazelnut oil because they are present in both oils. It has also been suggested that adulteration of virgin olive oil with crude hazelnut oil could be detected by tracing volatile filbertone (5-methylhept-2-en-4one), the flavor-impact component of hazelnut oil [9]. However, this cannot be done effectively because filbertone can be removed during refining processes, particularly during the deodorization step [9, 10].

Since there is no method for the detection of refined olive oil adulterated with refined hazelnut oil, it is of interest to investigate the effects of refining processes on 4,4′-dimethylsterols of hazelnut oil, especially on the marker compounds that have been used to detect virgin olive adulteration with hazelnut oil [4, 6].

In this study, free and esterified 4,4′-dimethylsterols in some selected samples of hazelnut and virgin olive oils from different countries were determined. Moreover, the effects of the refining process of vegetable oil were studied on the levels of 4,4′-dimethylsterols in a sample of solvent-extracted hazelnut oil followed by the detection of olive oil adulteration with this fully refined hazelnut oil. A simple solid phase extraction (SPE) method to separate free and esterified sterols was developed. Furthermore, a combination of two columns with different polarity, DB5-MS and DB17-MS, was also investigated to improve the separation of 4,4′-dimethylsterols by GC.

Materials and Methods

Samples A sample of hazelnuts was collected from Iran (Rodsar, Iran). Another hazelnut sample from Germany (Atco Haselnusskerne, Hamburg, Germany) was purchased from a local market (Uppsala, Sweden). A sample of refined hazelnut oil from Italy (Lazeo, Italy), a refined and winterized hazelnut oil sample from Turkey (Ordu Soya Industries, Inc., Ordu, Turkey), and a commercial hazelnut oil from France (Philippe Vigean, France) were collected. Virgin olive oil samples from Italy (Bertolli, Italy) and Spain (Sierra de Genave, Genave-Jaen, Spain) were obtained from a local market (Uppsala, Sweden).

Oil Extraction Oil was extracted from the hazelnuts originated from Germany and Iran, following the method described by Azadmard-Damirchi et al. [6].

Laboratory Scale Oil Refining

Degumming Procedure Degumming of the solvent-extracted oil from the nuts (Atco Haselnusskerne, Hamburg, Germany) was performed according to the method described by Vereleyen et al. [11]. In brief, the crude oil (200 g) was heated to 70 °C under gentle agitation and 6 mL water was mixed with the oil at 250 rpm for 30 min on a magnetic stirrer. The oil and water mixture was centrifuged (10 min at 1,700×*g*) (Sorvall products, Wilmington, Delaware, USA), and the supernatant oil layer was collected.

Neutralization Procedure Neutralization of the degummed oil was performed according to the method described by Vereleyen et al. [11] with slight modification. After heating the degummed oil (150 g) to 55 °C, 7.6 mL of NaOH (10% in water) was added under agitation at 250 rpm according to Hodgson [12], followed by continued agitation at 175 rpm for 40 min. Thereafter, the oil was maintained at 75 °C for 15 min without mixing. The mixture was centrifuged for 10 min at 1,700×*g* (Sorvall products, Wilmington, Delaware, USA). Then, the oil phase was separated and washed twice with water (10% w/w) under gentle mixing at 175 rpm.

Bleaching Procedure Bleaching of the neutralized oil sample was done according to the method described by Bortolomeazzi et al. [13] with slight modification. In brief, bleaching earth (1% w/w, Tonsil 210 optimum, Südchemie, Germany) was added to a round-bottomed flask containing 100 g of oil. The flask was then connected to a water pump and the mixture was warmed to 85 °C by a water bath. Bleaching process was carried out for 1 h at this temperature, under vacuum and vigorous stirring. After cooling, the mixture was immediately filtered on a Büchner funnel to remove the earth.

Deodorization Procedure Deodorization of the bleached oil sample was done according to the method described by Bortolomeazzi et al. [13]. A round-bottomed flask containing 10 g of the oil sample was connected to a pump (KNF Neuberger-LABOPORT, Best Lab Deals Inc, USA) and the mixture was heated to 180 °C in a glycerol bath. Deodorization was performed at 180 °C under a pressure of less than 2 mbar and stirring for 1 h at 180 rpm.

Separation of Total Free and Esterified Sterols from Oil by SPE Total free and esterified sterols were separated from oil according to a newly developed method at our laboratory. The oil sample (ca. 0.5 g) was

dissolved in 1 mL hexane and was loaded onto the SPE cartridge (500 mg silica; IST, Mid Glamorgan, UK) previously conditioned with 3 mL hexane. Esterified sterols fraction was eluted with 9 mL hexane, thereafter free sterols fraction was eluted with 6 mL hexane: diethyl ether (4:6). These separations were checked by analytical TLC (Silica gel 60. 20 × 20 cm, 0.25 mm thickness; Merk, Darmstadt, Germany) in the solvent system hexane:diethyl ether:acetic acid (85:15:1).

Saponification of Oil Sample and Total Free and Esterified Sterols Saponification of oil samples (ca. 0.5 g) and separated total free and esterified sterols was carried out according to the method described by Azadmard-Damirchi et al. [6].

Separation of 4,4′-dimethylsterols by SPE 4,4′-Dimethylsterols from unsaponifiables of oil samples, and free and esterified sterols from the above saponified samples were separated according to an SPE method previously published by Azadmard-Damirchi et al. [14].

Preparation of Trimethylsilyl (TMS) Ether Derivatives of Sterols TMS ether derivatives of sterols were prepared prior to gas chromatography (GC) and GC mass spectrometry (GC–MS) analyses according to the method described by Azadmard-Damirchi et al. [6].

Analysis of Sterols by GC GC analyses were performed with a Varian Star 3400 Cx instrument (Varian, Palo Alto, CA, USA). The GC was equipped with a flame ionization detector and split/splitless injector. A combination of two columns, DB5-MS (10 × 0.18 mm, 0.18 μm (J&W Scientific, Folsom, CA, USA) and DB17-MS (10 m × 0.18 mm, 0.18 μm) (J&W Scientific), which were joined together by a universal press-fit connector (NTK Kemi, Sweden), was used for sterol analysis. Injector and detector temperatures were 260 and 310 °C, respectively. Oven conditions were 60 °C for 1 min and raised to 260 °C at rate of 50 °C/min and maintained for 5 min and then raised to 280 °C at rate of 1 °C/min and maintained for another 10 min. The TMS ether derivatives of sterols were injected in a splitless mode of injection. Helium was used as the carrier gas at 18 psi and nitrogen as the make-up gas at a flow rate of 30 mL/min. The peak areas were computed with Star Chromatography Software version 4.01 (Varian, Palo Alto, CA, USA) and quantification was done relative to the cholesterol as an internal standard. All samples were analyzed in triplicate, and mean results are reported.

Analysis of Sterols by GC–MS For identification of 4,4′-dimethylsterols, the GC–MS analysis was performed on a GC8000 Top Series gas chromatograph (CE Instruments, Milan, Italy) coupled to a Voyager mass spectrometer operated with MassLab data system version 1.4 V (Finnigan, Manchester, UK). The column combination and conditions were the same as used for GC analysis. The full scan mass spectra were recorded in EI^+ mode at an electron energy of 70 eV and ion-source temperature of 200 °C. Sterols were identified by comparing the mass spectra with published data [6, 15]. We also used a fused-silica capillary column DB5-MS 30 m × 0.25 mm, 0.50 μm (J&W Scientific) for GC–MS analyses to separate sterols to compare with our new column combination.

Detection of Olive Oil Adulteration with Refined Hazelnut Oil An olive oil sample was mixed with 2% fully refined hazelnut oil for this purpose (for detail see extraction and refining sections in Materials and Methods). Total and esterified 4,4′-dimethylsterols were separated and analyzed by GC–MS following the sample work-up procedures as described earlier.

Solvents and Reagents All chemicals and solvents used in this study were of analytical grade and were purchased from VWR (Stockholm, Sweden) unless otherwise stated.

Results and Discussion

We developed an improved SPE (Silica) method to separate total esterified and free sterols from oil samples. Since esterified sterols are less polar than free sterols, total esterified sterols were eluted with hexane followed by eluting total free sterols by a mixture of hexane:diethyl ether. The esterified sterol fraction contained the major portion of the triacylglycerols (TAG), trace amounts of free fatty acids and tocopherols. The free sterols fraction also contained trace amounts of TAG, the major portion of free fatty acids and tocopherols. This SPE method was tested on several occasions with olive oils, hazelnut oils, hazelnut oils at different refining stages, and with corn oil at least in triplicate, for the reproducibility of the method (results not shown). We also have checked that the maximum amount of 0.5 g oil could be used for effective separation of free and esterified sterols. The total esterified and free sterols separated by the SPE method were regularly checked by analytical thin-layer chromatography (TLC). Since TAG was also co-eluted with both the total esterified and free sterols, saponification of these fractions was necessary for further analysis.

In our previous study, we observed that in GC analysis α-amyrin was co-eluted with cycloartenol and lupeol from hazelnut oil (Fig. 1) [3, 6]. Similarly, tirucalla-7,24-dienol was co-eluted with cycloartenol in

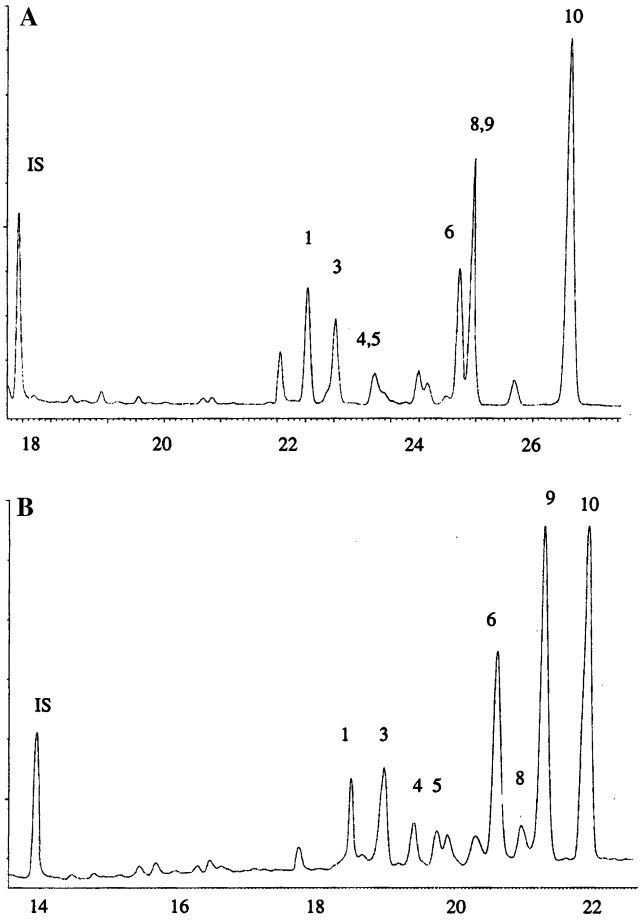

Fig. 1 GC–MS total ion chromatograms of 4,4′-dimethylsterols from hazelnut oil obtained with **a**, nonpolar DB5-MS column, **b** combined nonpolar DB5-MS and mid polar DB17-MS (for details see Materials and Methods). IS, internal standard (cholesterol), *1* compound X, *3* δ-amyrin, *4* β-amyrin, *5* butyrospermol, *6* cycloartenol, *8* α-amyrin, *9* lupeol, *10* 24-methylenecycloartanol

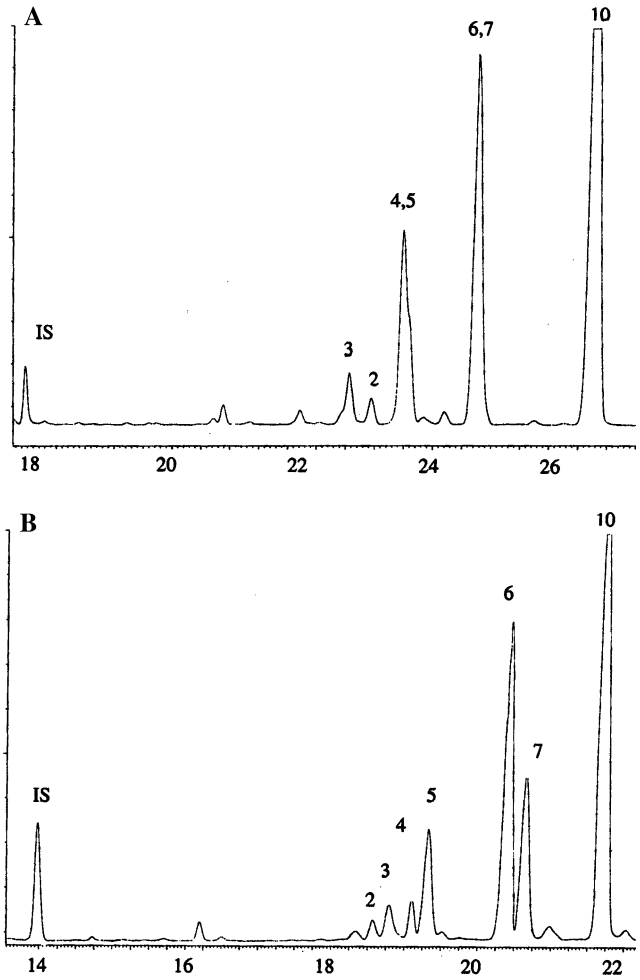

Fig. 2 GC–MS total ion chromatograms of 4,4′-dimethylsterols from virgin olive oil obtained with **a** nonpolar DB5-MS column, **b** combined nonpolar DB5-MS and mid polar DB17-MS (for details see Materials and Methods). *IS* internal standard (cholesterol), *2* taraxerol, *3* δ-amyrin, *4* β-amyrin, *5* butyrospermol, *6* cycloartenol, *7* Tirucalla-7,24-dienol, *10* 24-methylenecycloartanol

virgin olive oil (Fig. 2) [3, 6]. Resolution of sterols by capillary column GC depends on many factors, e.g., length, internal diameter, film thickness, polarity of columns [16]. In this study, with GC–MS by combining a nonpolar DB5-MS column (10 m × 0.18 mm, 0.18 μm) and a mid-polar DB17-MS column(10 m × 0.18 mm, 0.18 μm), thesee 4,4′-dimethylsterols were separated effectively compared with the single DB5-MS column (30 m × 0.25 mm, 0.50 μm) used previously (Table 1; Figs. 1 ,2.).

Sterols profile can be used as a means for differentiating between vegetable oils or detecting their authenticity [18]. It is known that 4,4′-dimethylsterols are more variable in composition than 4-desmethylsterols, and therefore that they are more effective for detecting vegetable oil adulteration [4, 6, 18]. Commercial hazelnut oil samples from France, Italy, and Turkey, and solvent-extracted oils from hazelnut samples from Germany and Iran were analyzed for their 4,4′-dimethylsterols profile. Since there are very few reports on free and esterified 4,4′-dimethylsterols composition of olive oil [17], we also included two virgin olive oil samples in this study. Total 4,4′-dimethylsterol content of hazelnut and virgin olive oil samples are shown in Table 2. Free and esterified 4,4′-dimethylsterols of analyzed hazelnut and virgin olive oils are also given in Table 3. The amounts of total 4,4′-dimethylsterols show slightly higher amounts (2–8%) than those obtained by summing free and esterified 4,4′-dimethylsterols (Tables 2, 3). This can be explained by some minor losses during separation of free and esterified sterols by SPE prior to determination of 4,4′-dimethylsterols content.

Table 1 Relative retention times (RRT) of TMS ether derivatives of 4,4′-dimethylsterols separated on two different GC column systems

Sterol	DB5-MS RRT	DB17-MS/DB5-MS[a] RRT
Compound X	1.25	1.33
Taraxerol	1.26	1.35
δ-Amyrin	1.28	1.37
β-Amyrin	1.30	1.39
Butyrospermol	–[b]	1.41
Cycloartenol	1.39	1.47
Tirucalla-7,24-dienol	–[c]	1.49
α-Amyrin	–[c]	1.50
Lupeol	1.40	1.53
24-Methylencycloartanol	1.49	1.57

[a] Combination of two columns: DB5-MS and DB17, joined together by a universal press-fit connector (for details see Materials and Methods)

[b] Overlapping with β-amyrin

[c] Overlapping with cycloartenol and lupeol

Total 4,4′-dimethylsterols in hazelnut oil ranged from 44 to 84 ppm (Table 2). Hazelnut oil from Turkey contained the highest amount of 4,4′-dimethylsterols (84 ppm) among the samples analyzed. Among the 4,4′-dimethylsterols of the hazelnut oils, the content of lupeol, as a marker for detect olive oil adulteration, was the highest (13–27 ppm), followed by 24-methylenecycloartanol (11–21 ppm) (Table 2). Another marker compound, compound X, was present at lower levels (2–10 ppm) than lupeol in all the hazelnut oil samples (Table 2). The results of the present study generally concur with previously published data [4, 6, 14]. Levels of total 4,4′-dimethylsterols were higher in the esterified form (23–53 ppm) compared with those in the free form of these compounds (13–38 ppm) in the hazelnut oil, as shown in Table 3. Generally, α-amyrin was present in higher amounts in the esterified form than in the free form in hazelnut oil samples, ranging from 2 to 15 ppm: the sample from Italy had the highest amount among all the samples (Table 3). Lupeol was present at 6–16 ppm in the esterified form and at 6–10 ppm in the free form: and for compound X, 1–5 and 0.8–5 ppm (Table 3).

In the analyzed virgin olive oils, 24-methylenecycloartanol was predominant, followed by cycloartenol and butyrospermol in total 4,4′-dimethylsterols (Table 2). Levels of 4,4′-dimethylsterols were higher in the esterified form compared with those in the free form, except for cycloartenol and 24-methylenecycloartanol (Table 3). The total content of 4,4′-dimethylsterols in the free form was higher (293–448 ppm) than that in the esterified form (180–315 ppm) (Table 3). Tirucalla-7,24-dienol, and taraxerol are present only in virgin olive oils and were not detected in the analyzed hazelnut oils (Tables 2, 3).

We were able to identify α-amyrin by comparing published GC–MS data in the olive oil samples in both the free and esterified forms in trace amounts. Reports on the presence of α-amyrin in olive oil are controversial. It has been reported that α-amyrin was not detected in selected virgin olive oil samples from France, Spain and Tunisia. This compound has been used as a marker to detect virgin olive adulteration with hazelnut oil [4]. On the other hand, the presence of α-amyrin has been reported in virgin olive oil in published reports [1, 19]. The quantification of

Table 2 Content and distribution of total 4,4′-dimethylsterols in hazelnut and virgin olive oils

Sterol	Oil extracted from commercial hazelnuts μg/g oil (%)		Commercial hazelnut oils μg/g oil (%)			Virgin olive oils μg/g oil (%)	
	Germany	Iran	France	Italy	Turkey	Italy	Spain
Compound X[a]	4.4 (9.2)	3.0 (4.7)	1.9 (4.3)	3.8 (4.6)	10.4 (12.3)	Nd[b]	Nd
Taraxerol	Nd	Nd	Nd	Nd	Nd	9.2 (1.1)	3.6 (0.7)
δ-Amyrin	4.6 (9.6)	5.0 (7.9)	5.2 (11.9)	5.3 (6.4)	5.0 (5.9)	21.5 (2.7)	14.0 (2.7)
β-Amyrin	1.4 (2.9)	0.5 (0.8)	1.7 (3.9)	3.0 (3.6)	3.5 (4.1)	15.0 (1.9)	7.8 (1.5)
Butyrospermol	Tr[c]	3.6 (5.7)	2.7 (6.2)	10.0 (15.1)	3.7 (4.4)	67.0 (8.3)	59.0 (11.4)
Cycloartenol	6.2 (13.0)	3.5 (5.5)	3.5 (8.0)	4.3 (5.2)	12.0 (14.2)	257.3 (32.0)	90.2 (17.5)
Tirucalla-7,24-dienol	Nd	Nd	Nd	Nd	Nd	60.3 (7.5)	50.0 (9.7)
α-Amyrin	1.6 (3.3)	2.4 (3.8)	2.2 (5.0)	16.7 (20.3)	5.0 (5.9)	Tr	Tr
Lupeol	12.5 (26.3)	24.0 (38.0)	15.6 (35.6)	25.1 (30.5)	26.7 (31.6)	Nd	Nd
24-Methylencycloartanol	16.9 (35.6)	21.1 (33.4)	11.0 (25.1)	14.2 (17.2)	18.1 (21.4)	373.9 (46.5)	290.8 (56.4)
Total	47.5	63.1	43.8	82.4	84.4	804.2	515.4

Each value is a mean of triplicate analyses and the CV is generally less than 5%

[a] An unknown compound

[b] Not detected

[c] Trace amount (<0.1 μg/g oil)

Table 3 Content and distribution of 4,4′-dimethylsterols in free and esterified forms in hazelnut and virgin olive oils

Sterol	Oil extracted from commercial hazelnuts µg/g oil (%)				Commercial hazelnut oils µg/g oil (%)						Virgin olive oil µg/g oil (%)			
	Germany		Iran		France		Italy		Turkey		Italy		Spain	
	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified
Compound X[a]	2.5 (56.8)	1.9 (43.2)	1.3 (46.4)	1.5 (53.6)	0.8 (44.5)	1.0 (55.5)	1.6 (45.7)	1.8 (54.3)	4.9 (46.3)	5.1 (53.7)	Nd[b]	Nd	Nd	Nd
Taraxerol	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	1.2 (13.3)	7.8 (86.7)	1.0 (31.2)	2.2 (68.7)
δ-Amyrin	1.5 (33.3)	3.0 (66.7)	0.6 (11.8)	4.5 (88.2)	0.7 (14.0)	4.3 (86.0)	1.4 (26.9)	3.8 (73.1)	1.0 (19.6)	4.1 (80.4)	2.0 (9.6)	18.9 (90.4)	1.0 (7.7)	12.0 (92.3)
β-Amyrin	0.5 (38.5)	0.8 (61.5)	0.1 (33.3)	0.2 (66.7)	0.6 (37.5)	1.0 (62.5)	1.3 (46.4)	1.5 (53.6)	2.0 (55.5)	1.6 (44.4)	1.0 (7.8)	11.8 (92.2)	0.6 (9.2)	5.9 (90.8)
Butyrospermol	Tr[c]	Tr	0.5 (15.1)	2.8 (84.8)	0.4 (16.7)	2.0 (83.3)	1.2 (12.8)	8.2 (87.2)	2.0 (58.8)	1.4 (41.2)	10.3 (17.1)	53.0 (82.9)	4.5 (9.9)	40.7 (90.0)
Cycloartenol	3.0 (50.0)	3.0 (50.0)	0.7 (18.9)	3.0 (81.1)	0.5 (16.1)	2.6 (83.9)	1.0 (23.8)	3.2 (76.2)	6.8 (56.2)	5.3 (43.8)	167.2 (69.0)	75.2 (31.0)	68.2 (79.9)	17.2 (20.1)
Tirucalla-7,24-dienol	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	2.1 (3.8)	52.8 (96.2)	1.1 (2.6)	41.3 (97.4)
α-Amyrin	0.5 (33.3)	1.0 (66.7)	0.4 (17.4)	1.9 (82.6)	0.6 (30.0)	1.4 (60.0)	3.0 (19.9)	12.1 (80.1)	3.1 (68.9)	1.4 (31.1)	Tr	Tr	Tr	Tr
Lupeol	6.1 (50.4)	6.0 (49.6)	7.6 (35.0)	14.1 (65.0)	7.8 (52.0)	7.2 (48.0)	8.8 (37.0)	15.0 (63.0)	10.1 (38.5)	16.1 (61.1)	Nd	Nd	Nd	Nd
24-Methylencycloartanol	9.6 (57.8)	7.0 (42.2)	8.2 (40.2)	12.2 (59.8)	2.0 (21.7)	7.2 (78.3)	6.8 (48.9)	7.1 (51.1)	8.5 (49.7)	8.6 (50.3)	264.0 (73.4)	95.4 (26.5)	217.0 (78.2)	60.5 (21.8)
Total	23.7 (51.1)	22.7 (48.9)	19.4 (32.5)	40.2 (67.4)	13.4 (33.4)	26.7 (66.6)	25.1 (32.3)	52.7 (67.7)	38.4 (47.7)	43.6 (52.3)	447.8 (58.7)	315.0 (41.3)	293.4 (58.9)	179.8 (41.1)

Each value is a mean of triplicate analyses and the CV is generally less than 5%

[a] An unknown compound

[b] Not detected

[c] Trace amount (<0.1 µg/g oil)

α-amyrin in virgin olive oils from different origins and varieties needs further study.

Solvent-extracted crude vegetable oil must be refined for human consumption [20]. Refining processes generally comprise of various steps: degumming, neutralization, bleaching and deodorization [20]. Each step can cause specific changes in oil properties, particularly in minor constituents such as sterols and tocopherols [7, 11, 13]. In order to study the effects of the refining processes, oil was extracted from a sample of hazelnuts (Atco Haselnusskerne, Hamburg, Germany) with solvent and was refined on a laboratory scale by degumming, neutralization, bleaching and deodorization. The total amount of 4,4′-dimethylsterols in the refined hazelnut oil was determined after each refining process (Table 4). To study the effects of each refining step on free and esterified 4,4′-dimethylsetrols, they were separately analyzed. Levels of 4,4′-dimethylsterols in the esterified and free forms of hazelnut oil after each refining step are shown in Table 5.

The objective of the degumming step is to remove the phosphatides in the oils after mixing with water via centrifugal separation [20]. Generally, the degumming process did not alter qualitatively or quantitatively the 4,4′-dimethylsterol composition (Tables 4, 5).

Neutralization is done to remove, e.g., free fatty acids, gums, and color pigments from degummed oil via centrifugal separation, after reaction with alkaline, followed by washing with water and separation of residual soap [20]. Total 4,4′-dimethylsterols were reduced 22% during neutralization (Table 4). The compound X, cycloartenol and 24-methylencycloartanol showed the highest reduction among the 4,4′-dimethylsterols (Table 4). In this refining process, the free and esterified forms of 4,4′-dimethylsterols were reduced by 23 and 17%, respectively (Table 5). It is known that, during naturalization, free sterols can be reduced by transfer to the soapstock formed [11]. The slight reduction in the esterified forms of 4,4′-dimethylsterols was probably due to hydrolysis of the esterified sterols to free forms during the neutralization process. This has been demonstrated previously by the level of sterol losses during neutralization, which was up to 9–22% [21].

During the bleaching process, color pigments, oxidized components and residual gums are removed via absorption by bleaching clay, followed by separation of the spent bleaching clay [20]. The total amount of 4,4′-dimethylsterols were reduced by about 24% during bleaching of hazelnut oil (Table 4). Both free and esterified 4,4′-dimethylsterols were affected by the bleaching process (Table 5). Esterified sterols loss (29%) was greater than free sterols loss (18%) during this refining step. 24-Methylencycloartanol had the

Table 4 Effects of refining processes on the levels and distribution of total 4,4′-dimethylsterols in hazelnut oil

Sterol[a]	Crude μg/g oil (%)	Degummed μg/g oil (%)	Neutralized μg/g oil (%)	Bleached μg/g oil (%)	Deodorized μg/g oil (%)
Compound X	4.4 (9.2)	4.5 (9.4)	2.3 (6.1)	2.0 (7.0)	1.9 (7.7)
δ-Amyrin	4.6 (9.7)	4.6 (9.6)	3.2 (8.6)	2.6 (9.1)	2.2 (8.9)
β-Amyrin	1.3 (2.7)	1.4 (2.9)	1.0 (2.7)	0.8 (2.8)	0.6 (2.4)
Cycloartenol	6.2 (13.0)	6.5 (13.5)	4.2 (11.2)	3.8 (13.3)	2.6 (10.5)
α-Amyrin	1.6 (3.3)	1.7 (3.5)	1.2 (3.2)	1.0 (3.5)	1.0 (4.0)
Lupeol	12.5 (26.3)	12.3 (25.6)	11.5 (30.7)	10.2 (35.8)	9.4 (38.0)
24-Methylencycloartanol	16.9 (35.6)	17.0 (35.4)	14.0 (37.4)	8.1 (28.4)	7.0 (28.3)
Total	47.5	48.0	37.4	28.5	24.7

Each value is a mean of triplicate analyses and the CV is generally less than 5%, oil was extracted in the laboratory from a hazelnut sample from Germany

[a] Butyrospermol was present in trace amounts (<0.1 μg/g oil)

highest reduction in both forms (40%), followed by esterified δ-amyrin (30%) and esterified cycloartenol (28%) (Table 5). The reduction in the esterified forms of sterols during bleaching has been explained by acid-catalyzed hydrolysis of the esterified sterols on the acid-activated bleaching earth [11]. Sterols dehydration during bleaching can also cause reduction in sterols content [11].

During the deodorization process residual volatile impurities and free fatty acids are removed from vegetable oils to produce an odorless product [20]. Total 4,4′-dimethylsterols were reduced by 13% during the deodorization process (Table 4). Among the free and esterified forms of 4,4′-dimethylsterols, the free form was affected considerably (21%, Table 5). In contrast, esterified 4,4′-dimethylsterols was generally not affected by deodorization. The free form of cycloartenol had highest reduction (44%) followed by 24-methylenecycloartanol (23%) and lupeol (12%) during this refining process (Table 5). Although no directly comparable result has been published, our results are generally comparable with published results on the effects of deodorization on free and esterified 4-desmethylsterols [11, 22].

In fully refined hazelnut oil, total 4,4′-dimethylsterols were reduced to a level of 48% compared with crude hazelnut oil (Table 4). Free and esterified 4,4′-dimethylsterols were lost to levels of 52 and 41%, respectively, compared with their levels in crude hazelnut oil (Table 5). Among the individual 4,4′-dimethylsterols in fully refined hazelnut oil, 18 and 37% of lupeol and the compound X in the esterified form, respectively, were lost. The losses of these two compounds in the free form were 26 and 72%, respectively. To our knowledge, there is no published data on the effects of vegetable oil refining on free and esterified 4,4′-dimethylsterols to compare with our results. Nevertheless, our results on the retention of 4,4′-dimethylsterols during refining are generally comparable with published results on free and esterified 4-desmethylsterols. A reduction in free 4-desmethylsterols in refined vegetable oils has been reported previously [11]. It has been shown that esterified 4-desmethylsterols in vegetable oils were decreased slightly after complete refining [22]. Different condi-

Table 5 Effects of refining process on the levels and distribution of 4,4′-dimethylsterols in the free and esterified forms in hazelnut oil

Sterol[a]	Crude μg/g oil (%)		Degummed μg/g oil (%)		Neutralized μg/g oil (%)		Bleached μg/g oil (%)		Deodorized μg/g oil (%)	
	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified
Compound X	2.5 (56.8)	1.9 (43.2)	2.4 (54.5)	2.0 (45.5)	1.0 (43.5)	1.3 (56.5)	0.8 (40.0)	1.2 (60.0)	0.7 (36.8)	1.2 (63.2)
δ-Amyrin	1.5 (33.3)	3.0 (66.7)	1.3 (30.2)	3.0 (69.8)	0.8 (25.8)	2.3 (74.2)	1.0 (38.5)	1.6 (61.5)	0.6 (28.6)	1.5 (71.4)
β-Amyrin	0.5 (38.5)	0.8 (61.5)	0.5 (38.5)	0.8 (61.5)	0.4 (44.4)	0.5 (55.6)	0.4 (57.1)	0.3 (42.9)	0.3 (50.0)	0.3 (50.0)
Cycloartenol	3.0 (50.0)	3.0 (50.0)	3.0 (46.1)	3.5 (53.8)	1.6 (39.0)	2.5 (61.0)	1.8 (50.0)	1.8 (50.0)	1.0 (37.0)	1.7 (63.0)
α-Amyrin	0.5 (33.3)	1.0 (66.7)	0.4 (30.8)	0.9 (69.2)	0.6 (60.0)	0.4 (40.0)	0.7 (80.0)	0.3 (20.0)	0.7 (70.0)	0.3 (30.0)
Lupeol	6.1 (50.4)	6.0 (49.6)	5.9 (49.2)	6.1 (50.7)	5.4 (48.2)	5.8 (51.8)	5.1 (51.0)	4.9 (49.0)	4.5 (47.9)	4.9 (52.1)
24-Methylen-cycloartanol	9.6 (57.8)	7.0 (42.2)	9.0 (54.5)	7.5 (45.4)	7.5 (54.0)	6.4 (46.0)	4.4 (55.7)	3.5 (44.3)	3.4 (49.3)	3.5 (50.7)
Total	23.7 (51.1)	22.7 (48.9)	22.5 (48.6)	23.8 (51.4)	17.3 (47.4)	19.2 (52.6)	14.2 (51.1)	13.6 (48.9)	11.2 (45.5)	13.4 (54.5)

Each value is a mean of triplicate analyses and the CV is generally less than 5%, oil was extracted at the laboratory from hazelnuts sample from Germany

[a] Butyrospermol was present in trace amounts (<0.1 μg/g oil)

tions during refining processes may cause different levels of losses in 4,4′-dimethylsterols in hazelnut oil, particularly in the free forms of these compounds, and this need further study. In addition, different cultivars and species of hazelnuts from various origins can be of interest to study the levels and retention of 4,4′-dimethylsterols during refining processes.

In order to investigate the possibility of detecting adulterated olive oil with fully refined hazelnut oil, a sample of virgin olive oil was mixed with 2% refined hazelnut oil. Total and esterified 4,4′-dimethylsterols of the adulterated olive oil sample were analyzed and the marker compounds were traced by GC–MS. We were able to detect lupeol at this level in adulterated olive oil under the present analytical conditions. In this study, we showed that the esterified form of 4,4′-dimethylsterols has higher retention during refining in hazelnut oil. Therefore, tracing the esterified fraction of 4,4′-dimethylsterols alone can be used to detect adulteration of olive oil with refined hazelnut oil.

To our knowledge, this is the first report on the free and esterified forms of 4,4′-dimethysletrols in hazelnut oil and their fate during refining processes. Generally, the content of esterified 4,4′-dimethylsterols were higher compared with their free forms in hazelnut oil samples collected from several countries. In the few virgin olive oils analyzed, cycloartenol and 24-methylenecycloartanol were present in higher amounts in the free form compared with the esterified form. Among the refining processes, degumming generally caused no effects, deodorization caused a minor decrease, and neutralization and bleaching caused a considerable loss of 4,4′-dimethylsterols in a sample of hazelnut oil. After full refining, the esterified form of 4,4′-dimethylsterols was retained at a higher level (59%) compared with their free form (48%). The esterified 4,4′-dimethylsterols from an admixed olive oil with 2% refined hazelnut oil was effectively enriched by a new one-step SPE method and separated on a new capillary column combination in order to detect olive oil adulteration at a level as low as 2%. It may be possible to lower this detection limit further by enrichment of the 4,4′-dimethylsterols fraction with larger amounts of adulterated olive oil.

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