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ORIGINAL PAPER

Rapid Separating and Enrichment of 4,4′-Dimethylsterols of Vegetable Oils by Solid-Phase Extraction

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Abstract Phytosterols are separated into three classes: 4-desmethylsterols, 4-monomethylsterols and 4,4′-dimethylsterols. 4,4′-Dimethylsterols are used to detect vegetable oil adulteration and some compounds from this class can have anti-inflammatory and anticancer properties. There are methods such as thin layer chromatography (TLC) and solid phase extraction (SPE) used to separate phytosterol classes from each other. However, in some cases, separation of all three classes is not required. In addition, TLC has some drawbacks such as low recovery and it is time consuming. An SPE method has previously been used, but it was necessary to use high volume of solvents with this method to avoid coelution of phytosterol classes. In this study, an SPE (silica, 1 g) method was developed to separate and enrich only 4,4′-dimethylsterols from unsaponifiables of vegetable oil samples using 25 mL *n*-hexane and diethyl ether (95:5, v:v). This method was applied to hazelnut and olive oils and results were compared with those of TLC and the previously developed SPE method. Recovery of 4,4′-dimethylsterols was two times higher with the new SPE method compared with the TLC method. The newly developed SPE method generally gave a similar recovery compared with the previously developed SPE method. Moreover, the SPE method developed in this study has the advantage of using a 3.5 times lower volume of solvent than previously developed SPE methods. Because the newly developed SPE method has a single step requiring a low volume of solvents, it is rapid and simple, and can easily be used to detect olive oil adulteration with hazelnut oil and to analyze and quantify effective nutritional compounds in the 4,4′-dimethylsterols class.

Keywords 4,4′-Dimethylsterols · Hazelnut oil · Olive oil · Solid phase extraction · SPE · Phytosterols

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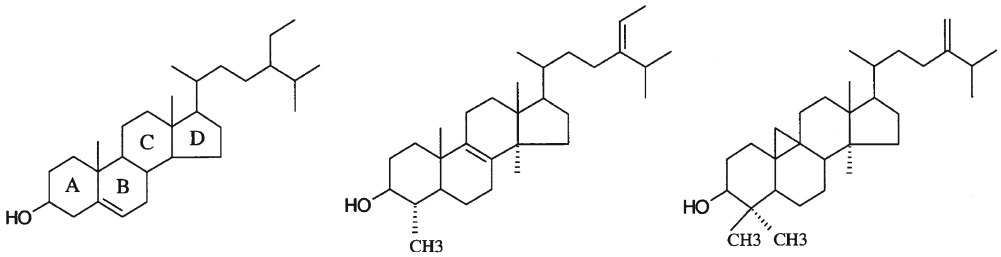
Introduction

Phytosterols (plant sterols) comprise a major portion of the unsaponifiables in vegetable oils [1]. Phytosterols can be separated into three classes based on the presence or absence of methyl groups at the C4 position in the A ring: 4-desmethylsterols (without a methyl group), 4-monomethylsterols (one methyl group) and 4,4′-dimethylsterols (triterpene alcohols, two methyl groups) [1]. These structural differences are shown in Fig. 1. Methylsterols are generally present in much lower amounts in vegetable oils than 4-desmethylsterols. In addition, some of the compounds from different classes of phytosterols can overlap during analysis by gas chromatography (GC) [2]. Therefore, it is necessary to separate and enrich phytosterol classes prior to quantification by GC and GC–mass spectrometry (MS) [2, 3].

Phytosterols are used to characterize vegetable oils [1, 2]. For this purpose, 4,4′-dimethylsterols are better markers since they vary more among vegetable oils [1, 2].



Sitosterol (4-desmethylsterol) Citrostadienol (4-monomethylsterol) 24-Methylenecycloartanol (4,4´-dimethylsterol)

Fig. 1 Chemical structures of the a major 4-desmethylsterol, a 4-monomethylsterol, and a 4,4′-dimethylsterol (triterpene alcohol), showing no, 1 and 2 methyl groups at position 4 in the A ring structure

4,4′-Dimethylsterols are also important from a nutritional point of view [4]. There are some compounds beneficial to health in the 4,4′-dimethylsterol fraction such as lupeol which have anti-inflammatory and anticancer properties [4].

Preparative thin layer chromatography (prep-TLC) is used as a conventional method to separate phytosterol classes [3, 5]. However, different phytosterol classes have close Rf values on TLC which may cause mixing during the scraping of TLC bands [5, 6]. In addition, prep-TLC has also other drawbacks such as low recovery, it is time consuming and laborious [7, 8].

Solid-phase extraction (SPE) is a simple and inexpensive chromatographic method. This chromatographic method has been widely used in the preparation of lipid classes prior to further analyses by GC or HPLC. SPE has been used to extract and purify total sterols from other unsaponifiables [9]. Moreover, SPE has been shown to separate total sterols in some vegetable oils more effectively and conveniently than traditional TLC separation [10]. An SPE method has previously been used to separate three phytosterol classes [7]. In this method, it was necessary to use a less polar solvent mixture to avoid coelution of phytosterol classes particularly 4-desmethylsterols and 4-monomethylsterols [7]. In some cases, 4,4′-dimethylsterols are only needed to be analysed e.g. detection of vegetable oil adulteration [2, 7], evaluate effect of vegetable oil refining [11], to determine composition of this class for nutritional purpose etc. [4].

For the above mentioned reasons, the aim of this study was to develop a rapid and simple SPE method to facilitate and improve the separation and enrichment of 4,4′-dimethylsterols when other phytosterol fraction are not required for further analysis.

Experimental

Samples

Hazelnuts were collected in Iran (Ardabil, Iran). A virgin olive oil and a refined olive oil sample were purchased from a local market (Tabriz, Iran).

Oil Extraction and Saponification

Hazelnut oil was extracted following the method described by Savage et al. [12]. Olive and hazelnut oil samples were saponified according to the method described by Azadmard-Damirchi et al. [2] and unsaponifiables were extracted with hexane.

Separation and Enrichment of Phytosterol Classes by Preparative TLC

Separation of phytosterol classes by TLC was carried out according to Kornfeldt and Croon [5] after slight modification [2]. In brief, the extracted unsaponifiables from the saponified oil sample were applied to the TLC plate (Silica gel 60, 20 cm × 20 cm, 0.25 mm thickness; Merck). To correctly identify and locate the phytosterol classes, a sample containing these classes was also applied by the side of the sample lane on the TLC plate as reference. After developing the plate twice in hexane/diethyl ether/acetic acid (70:30:1), the reference band was exposed to iodine vapor, while the sample area was covered with a glass plate. Three zones (4-desmethyl-, 4-monomethyl-, and 4,4′-dimethylsterols) were marked out and then 4,4′-dimethylsterols scraped and collected in a tube. After adding 20 μg 5α-cholestane as an internal standard in the tube, 4,4′-dimethylsterols was extracted three times with 2 mL dichloromethane and stored at −20 °C until further analysis.

Separation and Enrichment of Phytosterols by the SPE Method

4,4′-Dimethylsterols was separated and enriched by an SPE (1 g silica; IST, Mid Glamorgan, UK), according to the previously described method by Azadmard-Damirchi and Dutta [7]). In brief, the dissolved unsaponifiables in hexane (5 mL) obtained from 0.5 g saponified oil were loaded onto an SPE cartridge previously conditioned with 5 mL *n*-hexane. The cartridge was washed with 40 mL *n*-hexane-diethyl ether (99:1). Thereafter, 4,4′-dimethylsterols were

then eluted with 40 and 10 mL *n*-hexane-diethyl ether (99:1) and (98:2), respectively.

The New SPE Method to Separate and Enrich 4,4′-Dimethylsterols

The dissolved unsaponifiables in hexane (5 mL) obtained from 0.5 g saponified oil were loaded onto an SPE silica cartridge (1 g silica; IST, Mid Glamorgan, UK), previously conditioned with 5 mL *n*-hexane. Thereafter, pure 4,4′-dimethylsterols were eluted with 25 mL hexane:diethyl ether (95:5).

Evaluation of the Developed SPE Method

To evaluate ability of developed SPE method, after elution of 4,4′-dimethylsterols, the purity of this fraction was checked by TLC according to the method described by Azadmard-Damirchi et al. [7]. In brief, the collected fraction containing 4,4′-dimethylsterols from SPE was applied to the TLC plate. Along with that fraction, unsaponifiables extracted from olive oil sample containing phytosterol classes were applied to same TLC plate as a reference to check possible coelution of 4,4′-dimethylsterols with other phytosterol classes. Then the TLC plate was developed with a mobile phase, hexane:diethyl ether:acetic acid (70:30:1, v:v:v). After developing, the TLC plate was dried briefly in air and then sprayed with 10% phosphomolybdic acid in diethyl ether/ethanol (50:50, v:v). The plate was placed in an oven at 120 °C for 10 min for colour development and then evaluated visually for the ability of the developed SPE method to separate and enrich 4,4′-dimethylsterols.

Analysis of 4,4′-Dimethylsterols by GC and GC–MS

Separated and enriched 4,4′-dimethylsterols from oil samples by developed SPE method were analyzed as their trimethylsilyl (TMS) ether derivatives by GC and GC–MS. The method to prepare TMS-ether, GC and GC–MS conditions are described elsewhere [2, 7].

Statistical Analysis

The statistical analyses were carried out with the Minitab 14 (Minitab Inc., PA, USA) on data obtained from triplicate determinations of each of the compounds.

Results and Discussion

Methylsterols are present in low amounts in vegetable oils. In addition, some of the compounds present in different phytosterol classes have a similar retention time on GC and therefore they may overlap during GC analysis [2, 11]. For these reasons, separation and enrichment of the phytosterol classes is a necessary analytical step before their analysis and quantification by GC and GC–MS. For this purpose, TLC is a conventional method, but this method has several drawbacks [5–8]. Therefore, an SPE method has been developed to separate and enrich three phytosterol classes [7]. This newly developed method has a higher recovery rate than the conventional TLC method [7]. In the previously developed SPE method to separate all three phytosterol classes from each other, it was necessary to use a high volume of less polar solvent mixture; otherwise desmethylsterols could coelute with 4-monomethylsterols [7]. In some analyses, there is no need to separate 4-monomethylsterols and desmethylsterols [4, 7]. Therefore, it is possible to use a more polar solvent mixture to separate 4,4′-dimethylsterols without taking in account the problems with separation of other remaining phytosterol classes. For example to detect olive oil adulteration with hazelnut oil, 4,4′-dimethylsterols are only analysed to be used as markers [2, 7]. In addition, there are some compounds beneficial to health only present in the 4,4′-dimethylsterols class, e.g. lupeol [4].

The 4,4′-dimethylsterol class has the lowest polarity among phytosterols. Due to an additional numbers of methyl groups, the polarity of the three sterol classes decreases as 4-desmethylsterols > 4-monomethylsterols > 4,4′-dimethylsterols [2, 7]. Because of these differences in polarity, it is possible to separate 4,4′-dimethylsterols with suitable solvent mixtures from other phytosterol classes [7]. In this study, several solvent combinations were tested to separate and enrich 4,4′-dimethylsterols from unsaponifiables by SPE with a lower amount of solvent compared with a previously published SPE method [7]. The solvent mixture of hexane:diethyl ether (95:5, v:v) in a volume of 25 mL was able to elute and separate pure 4,4′-dimethylsterols from other unsaponifiables material (Fig. 2). TLC of the eluted fraction confirmed that separated and enriched 4,4′-dimethylsterols are pure and no 4-monomethylsterols or 4-desmethylsterols are coeluted with this fraction (Fig. 3). GC–MS analysis of 4,4′-dimethylsterols and their fragmentation pattern was also confirmed that there is no compounds from other phytosterol classes. There was an unknown fraction (D) less polar than 4,4′-dimethylsterols coeluted with this class of phytosterols. It should be noted this unknown fraction is not related to phytosterol classes and it has previously been reported that they have same the retention factor as free fatty acids on TLC [2, 7]. In addition, this fraction does not interfere during analysis of phytosterols by GC or GC–MS [2, 7].

A large portion of diethyl ether in the solvent mixture was tested to increase the polarity of the solvent mixture and reduce the volume of solvent used for separation of

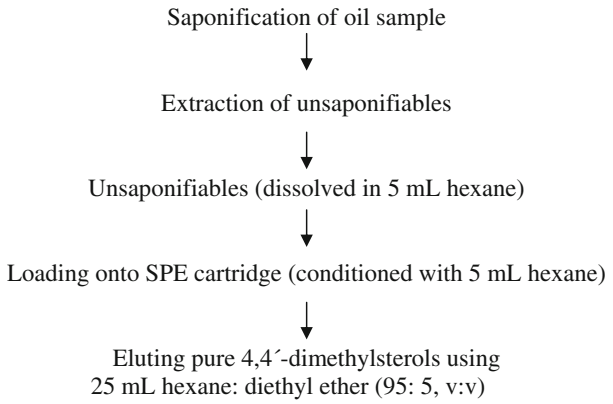


Fig. 2 Work up steps of the SPE method for separation of triterpene alcohols (4,4′-dimethylsterols)

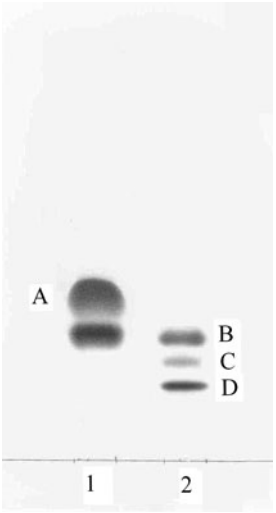


Fig. 3 Purity of 4,4′-dimethylsterols separated by the newly developed SPE method on analytical TLC, developing solvent; *n*-hexane:diethyl ether:acetic acid (70:30:1). For the SPE method and separation conditions see experimental section. *Lane 1* pure 4,4′-dimethylsterols separated by the SPE method, *lane 2* a standard mixture of phytosterol fractions. **a** Unknown fraction, **b** 4,4′-dimethylsterols, **c** 4-monomethylsterols, **d** 4-desmethylsterols. Sitosterol (4-desmethylsterol) Citrostadienol (4-monomethylsterol) 24-Methylenecycloartanol (4,4′-dimethylsterol)

dimethylsterols. However, it caused the elution of monomethylsterols with dimethylsterols. Monomethylsterols are more polar than dimethylsterols, therefore when relatively less polar solvents were used [hexane:diethyl ether (95:5, v:v)], monomethylsterols were retained in SPE and it was possible to get pure dimethylsterols. However, with more polar solvent mixtures, some portion of the monomethylsterols were not retained in the SPE and came out with the dimethylsterols fraction.

The amount of solvent used in this new SPE method (25 mL) is much less than the solvent volume used with the previous SPE method (90 mL) [7]. Because the solvent mixture of hexane:diethyl ether (99:1, v:v) was used in the previous SPE method for separation of 4,4′-dimethylsterols which is less polar than the solvent mixture of hexane:diethyl ether (95:5, v:v) used in the new developed SPE method. The new developed SPE in this study is more rapid than the previously developed SPE [7], because the volume of solvent required is less and consequently time spent for separation and elution is also shorter. It is also obvious that the SPE method for separation of 4,4′-dimethylsterols is much more rapid than TLC because there is no need to spend time scraping silica containing dimethylsterols on TLC and also further extraction of dimethylsterols from the scrapped silica. In addition, at least 2 h are need for developing TLC and subsequent marking phytosterol classes out on the developed TLC.

This newly developed method was applied to separate 4,4′-dimethylsterols of olive and hazelnut oils. These two oil samples were selected because olive oil has a large amount of 4,4′-dimethylsterols while hazelnut oil has a small amount of 4,4′-dimethylsterols [2, 7]. Therefore, it was possible to evaluate the ability of this newly developed SPE method for vegetable oils with small and large amounts of 4,4′-dimethylsterols by analyzing these two oil samples. Compounds detected and quantified from 4,4′-dimethylsterols were δ-amyrin, taraxerol, β-amyrin, cycloartenol, lupeol, Δ^7-sterol and 24-methylenecycloartanol (Table 1). However, there were a few peaks with trace amounts which were not identified. Taraxerol and lupeol were not detected in the hazelnut oil sample and olive oil samples, respectively (Table 1). The results obtained agree with previously published data [2, 7, 11, 13]. It has been reported that lupeol can be used to detect olive oil adulteration of olive oil with hazelnut oil [2, 7, 14]. In hazelnut oil, 24-methylenecycloartanol was the predominant compound followed by lupeol and β-amyrin (Table 1). In olive oil samples, 24-methylenecycloartanol was the predominant compound followed by cycloartenol and taraxerol (Table 1). The analysed hazelnut oil sample had generally 9–10 times lower 4,4′-dimethylsterols content compared with olive oil samples (Table 1). This is in agreement with previously published results [2, 7, 11, 13].

4,4′-Dimethysletrols were separated and enriched with conventional TLC, a previously developed SPE method and a newly developed SPE method in this study (Table 1). Results showed that SPE methods give a higher recovery of 4,4′-dimethylsterols compared with the TLC method [2, 7]. This low recovery can be explained by unavoidable losses during scrapping the stationary silica phase containing phytosterols from TLC and also during extraction of phytosterols from this scraped silica [2, 7]. Recovery of 4,4′-dimethysletrols was two times lower using TLC compared with SPE methods (Table 1). A newly developed SPE method was able to purify and enrich 4,4′-dimethylsterols

Table 1 4,4′-Dimethylsterols content (μg/g oil) in samples of hazelnut and olive oils separated and enriched by TLC and SPE methods

4,4′-dimethylsterol	Hazelnut oil			Olive oil			Refined olive oil		
	TLC	SPE[A]	New SPE[B]	TLC	SPE	New SPE	TLC	SPE	New SPE
δ -Amyrin	2.0[C]b	4.9a	5.0a	11.2b	23.5a	24.1a	10.8b	15.2a	17.0a
Taraxerol	ND	ND	ND	8.1c	14.2b	18.8a	4.7c	10.1b	13.9a
β-Amyrin	5.6c	13.1b	15.3a	27.0b	52.1a	59.2a	16.9b	34.9a	40.1a
Cycloartenol	2.6b	5.3	6.0a	115.7b	204.8a	213.0a	92.1b	165.0a	172.8a
Lupeol	5.0b	14.8a	15.0a	ND	ND	ND	ND	ND	ND
Δ^7-Sterol	1.1b	3.5a	3.2a	4.4b	7.9a	8.0a	2.8b	6.3a	7.0a
24-Methylenecycloartanol	10.1b	17.5a	18.9a	159.0c	270.2b	289.5a	118.6b	224.0a	235.8a
Unknown	8.3c	15.6b	19.5a	69.1b	100.9a	110.4a	70.0b	126.1a	139.0a
Total	34.7b	74.7a	82.9a	394.5b	673.6a	723.0a	315.9b	581.6a	625.6a

Different lowercase letters a–c denote statistically significant differences ($p < 0.05$)

[A] Previously developed SPE method by Azadmard-Damirchi and Dutta [7]

[B] Newly developed SPE method (see materials and methods)

[C] Means of triplicate analyses (CV is generally less than 5%)

comparable to the previously developed SPE method. Even though, the content of individual and total 4,4′-dimethylsterols recovered by the new SPE method were higher compared with the previously developed SPE method, there were generally no significant differences ($p < 0.05$) between these two methods (Table 1). However, this newly developed method needs a comparably lower volume of solvent to separate and enrich 4,4′-dimethylsterols (Fig. 2).

The newly developed SPE method gives a higher recovery of 4,4′-dimethylsterols compared with the traditional TLC method. This SPE method is rapid, needs fewer work up steps and it is possible to purify and enrich 4,4′-dimethylsterols from several samples at the same time. In addition, the developed SPE method needs much less time and a lower volume of solvent and gives a statistically similar recovery as the previously developed SPE method. This method can easily be used to detect olive oil adulteration with hazelnut oil and to analyze and quantify nutritional effective compounds in the 4,4′-dimethylsterols class of vegetable oils.

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