Olive Oil

Phytosterols, Tracing of Adulteration with Hazelnut Oil and Chemical Interesterification

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Abstract

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Analyses of phytosterol classes of olive and hazelnut oils collected from different countries by TLC, GC and GC-MS revealed considerable quantitative differences. The composition of 4-desmethyl- and 4-monomethylsterols was similar in both oils, but 4,4'-dimethylsterols composition differed. Lupeol and an unknown (lupane skeleton) compound were exclusively present in hazelnut oil 4,4´-dimethylsterols and could be used as markers to detect virgin olive oil adulteration with hazelnut oil at levels below 4%. Conventional TLC to separate phytosterol classes has a low recovery rate and is time-consuming. A new SPE method to separate phytosterol classes was developed with stepwise elution by increasing the polarity of the *n*-hexane:diethyl ether solvent mixture. Comparison of the results obtained for hazelnut and virgin olive oils with those of TLC revealed that the SPE method was faster and gave higher sterol recovery rates. Free and esterified forms of sterols provide detailed information on the identity and quality of vegetable oils, and therefore 4,4´-dimethylsterols were investigated in hazelnut oil and virgin olive oil. A sample of solvent-extracted hazelnut oil was refined to monitor the effects of processing on 4,4´-dimethylsterol levels and on specific marker compounds. Of the refining processes tested, only neutralisation and bleaching considerably reduced 4,4´-dimethylsterols. In fully-refined hazelnut oil, losses of marker compounds in free form were higher than losses in their esterified form. GC-MS analysis showed that adulteration of olive oil with fully-refined hazelnut oil could be detected at levels of 2% by tracing lupeol in total/esterified forms of 4,4´-dimethylsterols. Olive oil has many applications in the food industry, *e.g.* blended with oils such as palm stearin to produce margarine or shortening by chemical interesterification. Investigation on lipid and minor lipid components of an olive oil-palm stearin blend during chemical interesterification showed that sterols were esterified with fatty acids at a higher level at 120 °C (7%) than at 90 °C (4%). Despite heat treatment and several steps to produce an interesterified product, there were minor losses in phytosterol and tocopherol contents and no significant increases in phytosterol oxidation.

Keywords: adulteration, chemical interesterification, 4-desmethylsterols, 4,4´-dimethylsterols, hazelnut oil, 4-monomethylsterols, olive oil, phytosterols, Solid Phase Extraction, SPE.

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Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Azadmard-Damirchi, S., Savage, G.P. & Dutta, P.C. 2005. Sterol fractions in hazelnut and virgin olive oils and 4,4′-dimethylsterols as possible markers for detection of adulteration of virgin olive oil. *Journal of the American Oil Chemists' Society* 82, 717-725.

II. Azadmard-Damirchi, S. & Dutta, P.C. 2006. Novel solid-phase extraction method to separate 4-desmethyl-, 4-monomethyl-, and 4,4′-dimethylsterols in vegetable oils. *Journal of Chromatography A* 1108, 183-187.*

III. Azadmard-Damirchi, S. & Dutta, P.C. 2007. Free and esterified 4,4′-dimethylsterols in hazelnut oil and their retention during refining processes. *Journal of the American Oil Chemists' Society* 84, 297-304.

IV. Azadmard-Damirchi, S. & Dutta, P.C. 2007. Effects of chemical interesterification of an olive oil and palm stearin blend on lipids and minor lipid components. (Submitted)

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Authors' contributions to the papers

Planning, analysis, evaluation of the results and writing of **Papers I-IV** were carried out by S. Azadmard-Damirchi under the supervision of P.C. Dutta. In **Paper I**, G.P. Savage contributed by providing samples, English correction and partial evaluation of the results.

* Sodeif Azadmard-Damirchi was winner of the 2006 Analytical Division Student's Award, American Oil Chemists' Society. This award was given for the method published in **Paper II**. An oral presentation was delivered by Sodeif Azadmard-Damirchi during the 97th Am. Oil Chem. Soc. Annual Meeting & Expo, 2006, St. Louis, Missouri, USA.

List of abbreviations

APPI	Atmospheric Pressure Photospray Ionisation Source
EOO	2,3-dioleyl-1-eicosenoilglycerol
ESI	Electrospray Ionisation
FAME	Fatty Acid Methyl Ester
FID	Flame Ionisation Detector
FT-IR	Fourier Transform Infrared
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LLL	1,2,3-trilinoleylglycerol;
LnLO	1-linolenyl-2-linoleil-3-oleylglycerol;
LLO	1,2-dilinoleyl-3-oleylglycerol;
LLP	1,2-dilinoleyl-3-palmitoylglycerol;
LnOP	1-linolenyl-2-oleyl-3-palmitoylglycerol;
LOO	2,3-dioleoyl-1-linoleylglycerol;
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PLO	2-linoleyl-3-oleyl-1-palmitoylglycerol;
PLP	2-linoleyl-1,3-palmitoylglycerol;
OLnO	1,3-dioleyl-2-linoleylglycerol
OOO	1,2,3-trioleylglycerol;
POO	2,3-dioleyl-1-palmitoylglycerol;
POP	1,3-dipalmitoyl-2-oleylglycerol;
POPs	Phytosterol Oxidation Products
SFC	Solid Fat Content
SPE	Solid Phase Extraction
SOO	2,3-dioleyl-1-stearoylglycerol;
SOP	2-oleyl-3-palmitoyl-1-stearoylglycerol
TAG	Triacylglycerols
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl

Introduction

Virgin olive oil is obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alteration in the oil (IOOC, 2003). Pressing, centrifugation and percolation are usual methods for extraction of olive oil. Properly extracted olive oil from fruits with good quality can be consumed in crude form, conserving the healthy components of the fruit (Petrakis, 2006; Visioli, 2006).

World-wide production of olive oil during the last 20 years increased by almost 70% (from 1.7 to 2.8 million tons) (Zampounis, 2006). Olive oils makes up a small proportion (<3.5%) of the volume in the world vegetable oil market. However, in terms of product value, olive oil has a 15% share of world trade (Luchetti, 2000). The price of olive oil can be two to five times higher than that of other vegetable oils depending on the country, category of the oil, and year (Luchetti, 2000). Spain is the primary world producer of olive oil, followed by Italy, Greece, Tunisia and Turkey. World consumption generally follows a parallel path to the production rate.

The authenticity of olive oil is an important issue from a commercial and health point of view. Virgin olive oil is highly valued because it is traditionally obtained from olives without the use of heat and is regarded as better tasting and nutritionally favourable. Adulteration of olive oil can occur by mislabelling of less expensive products or by adding less expensive oils to in crease the volume and increase profits. Detection of olive oil adulteration with most other vegetable oils is not very difficult because of the differences in the fatty acid, triacylglycerol, or sterol composition of these oils (Aparicio, 2000). However, the adulteration of olive oil with hazelnut oil is difficult to detect with conventional methods at levels below 20% (Bøwadt & Aparicio, 2003). This is due to the similar chemical composition of the major and some minor components found in hazelnut and olive oils (Benitez-Sánchez *et al.,* 2003). Therefore a new detection method which will provide simple, fast and inexpensive identification of such adulteration is required.

Olive oil has large areas of applications in food preparation, *e.g.* salad oil, in cooking, frying, pasta sauces, as a dip for bread and *etc*. Olive oil can be used for production of margarines and shortenings by hydrogenation or interesterification (Gavriilidou & Boskou, 1991; Alpaslan & Karaali, 1998). Chemical interesterification is an established method in the edible oil industry to improve plasticity, crystallisation habit or functional properties of fats and oils (Rozendaal, 1992; O´Brien, 2004). The effects of this process on physical and chemical properties of the end product have been widely studied (Ledóchowska & Wilczyńska, 1998; Zhang *et al.*, 2005; Daniels *et al.*, 2006; Zhang *et al.*, 2006). However, there are few studies on the effects of this process on minor compounds of the vegetable oils, *e.g.* phytosterols and their oxidation products.

Olive oil

Designations and definitions of virgin olive oils (IOOC, 2003)

Virgin olive oil is obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions that do not lead to alterations in the oil, and the oil does not undergo any treatment other than washing, decantation, centrifugation and filtration. Virgin olive oil fit for direct consumption includes:

(i) Extra virgin olive oil: Virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other characteristics of which correspond to those fixed for this category in this standard.

(ii) Virgin olive oil: Virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 2 grams per 100 grams and the other characteristics of which correspond to those fixed for this category in this standard.

(iii) Ordinary virgin olive oil: Virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 3.3 grams per 100 grams and the other characteristics of which correspond to those fixed for this category in this standard.

Virgin olive oil not fit for direct consumption, designated lampante virgin olive oil, is virgin olive oil which has a free acidity, expressed as oleic acid, of more than 3.3 grams per 100 grams and/or the organoleptic characteristics and other characteristics of which correspond to those fixed for this category in this standard. It is intended for refining or for technical use.

Olive oil composition

Olive oil is a complex mixture consisting of two main groups of substances: (a) saponifiables, which represent nearly 98% of the chemical composition, such as triacylglycerols (TAG), partial glycerides, esters of fatty acids or free fatty acids and phosphatides; and (b) unsaponifiables, which represent only ~ 2% of all olive oil composition, such as phytosterols, tocopherols, hydrocarbons, pigments, phenols, flavonoids or volatile compounds (Aparicio & Aparicio-Ruíz, 2000).

Triacylglycerols and fatty acids

The TAG composition of olive oil is: OOO (40-59%), POO (12-20%), OOL (12.5-20%), POL (5.5-7%), SOO (3-7%) and smaller amounts of POP, POS, OLnL, LOL, OLnO, PLL, PLnO and LLL, LnLO, LnOP, PLP, SOP, EOO (Parcerisa *et al.*, 2000; Boskou *et al.*, 2006). The fatty acid composition of olive oil is: myristic acid (C14:0): ≤ 0.05%, palmitic acid (C16:0): 7.5-20%, palmitoleic acid (C16:1): 0.3-3.5%, heptadecanoic acid (C17:0): ≤0.3%, stearic acid (C18:0): 0.5-5%, oleic acid (C18:1): 55-83%, linoleic acid (C18:2): 3.5-21%, linolenic acid (C18:3): 3.5-21%, eicosanoic acid (C20:0): ≤0.6%, gadoleic acid (C20:1):

≤0.4%, behenic acid (C22:0): ≤0.2%, lignoceric acid (C24:0): ≤0.2% (IOOC, 2003).

Phenolic compounds

The phenolic compounds present in olive oil can be classified into a lipophilic group and a hydrophilic group (Boskou, 2000). Olive oil is a source of at least 30 phenolic compounds belonging to the hydrophilic group (Tuck & Hayball, 2002). The total polyphenolic content of olive oil ranges from 50 to 1000 ppm ((Boskou *et al.*, 2006). The levels of total phenols and individual phenols in olive oil depend on agronomic factors, maturity of the olives, processing, packaging and storage (Boskou *et al.*, 2005). The major phenolic compounds in olive oil are gallic, caffeic, vanillic, p-coumaric, syringic, ferulic, homovanillic, p-hydroxybenzoic and protocatecuic acids, tyrosol and hydroxytyrosol (Montedoro *et al.*, 1992; Mannino *et al.*, 1993). The phenolic compounds present in olive oil are strong antioxidants and radical scavengers. There are several reports showing good correlation of total polar phenol content with the stability of the olive oil (Blekas *et al.*, 2002). It has been demonstrated that phenolic compounds are more effective than tocopherols in enhancing the stability of olive oil toward oxidation (Baldioli *et al.*, 1996). Phenolic compounds, especially secoiridoids and o-diphenols, play an important role in the flavour of olive oil. They are also among the components most responsible for the nutritional and multiple pharmacological effects (Visioli & Galli, 1998; Yang *et al.*, 2007).

Tocopherols

Tocopherols and tocoterienols, which belong to the lipophilic group, are derivatives of 2-methyl-6-chromanol with a side chain of three terpene units attached at C2. They are distinguished by their side chains. The terpenoid side chain occurs in saturated form in tocopherols and in the unsaturated form in tocoterienols, with double bonds in positions 3′, 7′ and 11′. Tocopherols and tocotrienols are further separated into individual compounds designated by the Greek letter prefixes α, β, γ, δ depending on the number and position of methyl substitution on the chromanol ring (Gregory, 1996). α-tocopherol is traditionally considered to be the major antioxidant of olive oil and its concentration varies from a few ppm up to 300 ppm (Dionisi *et al*., 1995; Blekas *et al*., 2002). β-, γ- and δ-tocopherols concentrations have also been reported to range from trace to 25 ppm (Dionisi *et al*., 1995; Boskou *et al.*, 2006; Cunha *et al.*, 2006). α-, β-, and γ-tocotrienols have also been reported in olive oils at concentrations from non-detectable to 3.1, 0.7, and 4.7 ppm, respectively (Benitez-Sánchez *et al*., 2003).

Alcohols

One of the major series of compounds in the unsaponifiables is the alcohols. Aliphatic alcohols can have an even or odd number of carbon atoms. The linear aliphatic alcohols in olive oil are hexacosanol (major), docosanol (approx. 35%), tetracosanol and octacosanol (Benitez-Sánchez. *et al.*, 2003). Tricosanol, pentacosanol, and heptacosanol, aliphatic alcohols with an odd number of carbon atoms, may be found in trace amounts (Boskou *et al*., 2006).

Wax esters

Wax esters occurring in vegetable oils are a group of compounds formed by esterification of high molecular mass alcohols with fatty acids. If the alcoholic group is a long chain aliphatic alcohol, it results in aliphatic waxes of 34–46 carbon atoms (Pérez-Camino *et al.*, 2003). Wax values in virgin olive oil, olive oil and refined olive oil are a maximum 250 and 350 mg/kg (EU Commission Regulation, 1993). Virgin olive oil has a higher content of C36 and C38 waxes than C40, C42, C44, and C46 waxes, whereas the reverse is true in olive pomace oil and refined olive oils (Morales & León-Camacho, 2000). This fact is used to distinguish virgin olive oil from olive pomace oil and refined olive oil.

Hydrocarbons

Squalene is an important hydrocarbon in olive oils (2500-9250 ppm) and makes up more than 90% of the hydrocarbon fraction. This hydrocarbon is a precursor of sterols in vegetable oils. Other hydrocarbons have also been found in virgin olive oil, such as 6, 10-dimethyl-1-undecene, various sesquitterpenes, the series of *n*-alkanes from C14 to C35, n-heptadecene and *n*-9-alkenes (Lanzon *et al*., 1994).

Pigments

Olive oil also contains pigments, chlorophylls and carotenoids. Chlorophylls are encountered as pheophytin. Pheophytin α concentration in olive oil range from 3.3 to 40 ppm, while pheophytin *b* and chlorophyll *b* are present in trace amounts and chlorophyll *a* has not been detected (Psomiadou & Tsimidou, 1998). The main carotenoids present in olive oil are β-carotene (0.3-4.4 ppm) and lutein (trace-1.4 ppm) (Psomiadou & Tsimidou, 1998).

Volatile and aromatic compounds

Olive oil compared with other vegetable oils has a characteristic aroma and flavour. These sensory characteristics, together with nutritional aspects, are the main reasons for the increment of virgin olive oil consumption in recent years (IOOC, 2003). A balanced flavour of green and fruity sensory characteristics of high quality olive oil has a profile of volatile compounds, mainly comprising aldehydes, esters, alcohols and ketones (Aparicio & Morales, 1998). Volatile components can be used to check the quality of olive oil (Angerosa, 2002), to detect an adulteration (Lorenzo *et al.*, 2002), to detect a possible off-flavours (Morales *et al*., 1997) or to determine the variety of olive (Lorenzo *et al*., 2002).

Phytosterols

Phytosterols comprise a major proportion of the unsaponifiables in vegetable oils. Total sterols content in olive oil varies between 1000 and 2300 ppm (Benitez-Sánchez *et al*., 2003; IOOC, 2003). Sterol composition and content of olive oil are affected by cultivar, crop year, degree of fruit ripeness, storage time of fruits before oil extraction and method of oil extraction (Boskou *et al.*, 2006). Since the research presented in this thesis concentrates mainly on phytosterols, more details on these compounds are presented below.

Chemistry and occurrence of phytosterols

Plant sterols, also called phytosterols comprise a major proportion of the unsaponifiables in vegetable oils. They are biosynthetically derived from squalene and form a group of triterpenes (Goodwin, 1980). They are important components of plant cells in controlling membrane fluidity and permeability, although some have a specific function in signal transduction events and the activity of membrane-bound enzymes (Piironen *et al*., 2000). Phytosterols are derivatives of a tetracyclic perhydro-cyclopentano-phenanthrene ring system with a flexible side chain at the C-17 atom and 3β-monohydroxy compounds (Hartmann, 1998). Most phytosterols contain 28 or 29 carbons and one or two carbon–carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain (Moreau, 2005). According to the IUPAC recommendations from 1989, sterol molecules consist of four rings marked as A, B, C and D with standard carbon numbering (Figure 1) (Moss, 1989). Three rings, A, B and C, have 6 carbon atoms in a nonlinear structure and they are fused to one 5 carbon atoms ring (D). The various phytosterols found in plants differ in number of carbon atoms in the side chain and the position and number of the double bonds in the ring and in the side chain.

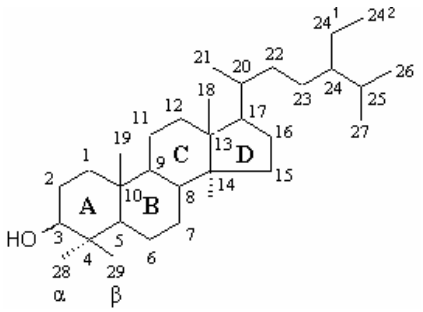


Figure 1. Basic structure of a sterol with standard carbon numbering according to the IUPAC (Moss, 1989).

Phytosterol classes

Phytosterols can be classified into three classes based on the presence or absence of methyl groups at the C4 position in the A ring: 4-desmethylsterols (without methyl group), 4-monomethylsterols (one methyl group) and 4,4′-dimethylsterols (triterpene alcohols, two methyl groups) (Akihisa *et al*., 1991). However, there is another group of compounds present in unsaponifiables called triterpene dialcohols, which are co-chromatographed with 4-desmethylsterols (Boskou *et al.*, 2006). Erythrodiol and uvaol are the main triterpene dialcohols present in olive

oils (IOOC, 2003). The structural formulae of sitosterol (a 4-desmethylsterol), citrostadienol (a 4-monomethylsterol), and 24-methylenecycloartanol (a 4,4´-dimethylsterol) are shown in Figure 2.

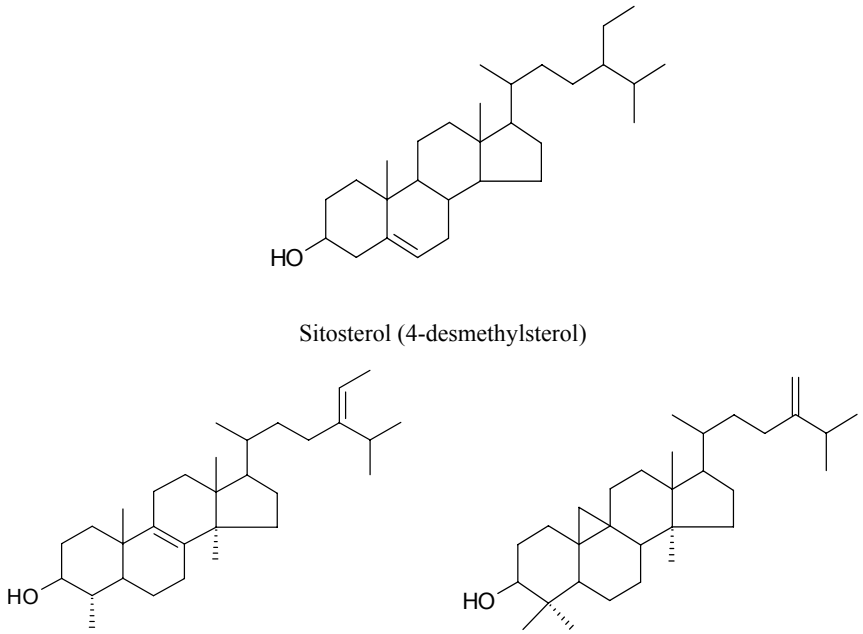


Figure 2. The chemical structure of phytosterol classes.

According to the position and number of double bonds in the B ring, 4-desmethylsterols can be classified into Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$-sterols (Moreau *et al*., 2002). 4-desmethylsterols include all of the common phytosterols with a 28- or 29-carbon skeleton, but also cholesterol with a 27-carbon skeleton (Moreau *et al*., 2002). Cholesterol occurs as a major sterol in animal cells, although only as a few percent in plant cells (Heupel, 1989). Chemically, it is an analogue to the phytosterols, differing only in the side chain. Some common sterols from each class are given in Table 1.

Table 1. *Some common sterols from each class of phytosterols*

Common name	IUPAC name
4-desmethylsterol	
Sitosterol	5α-Stigmast-5-ene 3β-ol
Campesterol	Ergost-5-ene 3β-ol
Stigmasterol	5α-Stigmasta-5,22-diene 3β-ol
Δ^5-Avenasterol	5α-Stigmasta-5,24(28)-diene 3β-ol
4-monomethylsterol	
Citrostadienol	4α-Methyl-24-ethylidene-5α-cholest-7-ene 3β-ol
Obtusifoliol	4α,14α-Dimethyl-24-methylene-9β,19-cyclo-5α-cholest-8-ene 3β-ol
Gramisterol	4α-Methyl-24-methylene-5α-cholest-7-ene 3β-ol
Cycloeucalenol	4α,14α-Dimethyl-9β,19-cyclo-24-methylene-5α-cholestane 3β-ol
4,4'-dimethylsterol	
24-Methylenecycloartanol	24-Methylen-9β,19-cyclo-5α-lanost-24-ene 3β-ol
Cycloartenol	9β,19-Cyclo-5α-lanost-24-ene 3β-ol
α- Amyrin	5α-Urs-12-ene 3β-ol
β-Amyrin	5α -Olean-12-ene 3β-ol

Methylsterols (4-monomethyl- and 4,4'-dimethylsterols) are synthesised at an early stage in the biosynthetic pathway and they are precursors of 4-desmethylsterols (Hartmann, 1998).

Analysis of phytosterols

Methylsterols usually occur in relatively smaller amounts compared with 4-desmethylsterols in vegetable oils and therefore it is necessary to separate and enrich them before quantification. Table 2 shows different methods used to separate sterol classes and also total sterols from unsaponifiables.

Table 2. *Methods currently used to separate and enrich total sterols and sterol classes in vegetable oils*

Method	Stationary phase	Solvent system	Reference
Total sterols			
TLC	Silica	Hexane: diethyl ether: acetic acid (85:15:1)	Morales & León-Camacho, 2000
HPLC	Silica	Hexane: diethyl ether	Amelio *et al.*, 1992
SPE	C_{18}	Methanol: chloroform	Toivo *et al.*, 1998
SPE	C_{18}	Acetonitrile: toluene	Ham *et al.*, 2000
Sterol classes			
TLC	Silica	Hexane: diethyl ether: acetic acid (70:30:1)	Kornfeldt & Croon., 1981
HPLC	Silica	Petroleum ether: ethyl acetate	Li *et al.*, 2001

Thin-layer chromatography is the conventional method to separate and enrich phytosterol classes. However, this method has some drawbacks. Different sterol fractions have close Rf values in TLC, which may cause mixing during scraping of

TLC bands (Kornfeldt & Croon, 1981; Kamal-Eldin *et al*., 1992, Morales & León-Camacho, 2000). In addition, preparative TLC is also disadvantageous because it has a low recovery rate and is time-consuming and laborious (Bello, 1992; Bohacenko & Kopicova, 2001). Preparative HPLC has also been used to separate sterol fractions of vegetable oils (Li *et al*., 2001). However, unlike TLC, use of HPLC may require a high solvent volume and a also higher cost. After separation and enrichment, phytosterol classes are generally derivatised to their trimethylsilyl (TMS) ether derivatives and analysed by GC and GC-MS (Kuksis, 2004).

Level of phytosterols in olive oil

Table 3 shows the 4-desmethylsterol levels according to International Olive Oil Council trade standards (IOOC, 2003). Phytosterol composition can differ in virgin olive oil by cultivar, crop year, ripening, storage time, extraction methods, *etc*. Virgin oil shows a very good correlation between stability and concentration of total sterols, β-sitosterol and Δ^5-avenasterol (Gutiérrez *et al.*, 1999). 4-desmethylsterols level does not vary substantially during ripening of olive fruits, except for a reduction in total sterols and β-sitosterol, and an increase in Δ^5-avenasterol level. The explanation for the decrease in total sterols is that sterols form in the first phases of ripening; as the oil content increases during this period, the sterols are diluted. The decrease in β-sitosterol is exactly the same as the increase in Δ^5-avenasterol, suggesting the presence of a desaturase enzyme that transforms β-sitosterol into Δ^5-avenasterol (Gutiérrez *et al.*, 1999). The influence of storage temperature of olive fruits on sterol composition is more important than the influence of storage time. The total sterol content increases gradually with olive storage time. The increase is greater for olive fruits stored at ambient temperature than those stored at low temperature (5 °C) (Gutiérrez *et al*., 2000). Stigmasterol is related to various parameters of the quality of virgin olive oil. High levels of this compound correlate with high acidity and low organoleptic quality (Gutiérrez *et al*., 2000).

Table 3. *4-desmethylsterol composition (% total sterols) of olive oil according to International Olive Oil Council trade standards (IOOC, 2003)*

Sterol	**Limit**
Cholesterol	< 0.5
Brassicasterol	<0.1
Campesterol	< 4.0
Stigmasterol	< campesterol
Δ^7-Stigmastenol	< 0.5
Apparent β-sitosterol	≥ 93.0% [a]

[a]Apparent β-sitosterol comprises: β-sitosterol, Δ^5-avenasterol, $\Delta^{5,23}$-stigmastadienol, clerosterol, sitostanol, $\Delta^{5,24}$-stigmastadienol

Ntsourankoua *et al.* (1994) have determined the 4,4′-dimethylsterol content of olive oil. Identification of compounds was carried out using GC-MS and authentic

samples of α-amyrin, β-amyrin, lupeol, and also extracted cycloartenol, 24-methylenecycloartanol from sunflower oil. The compounds present include butyrospermol (4.1%), β-amyrin (2.1%), cycloartenol (9.7%), 7,24-tirucallalladienol (4.9%), 28-nor Δ17,18-oleanen-3β-ol (trace), 24-methylenecycloartanol (74.3%) and some unknown compounds in virgin olive oil. Moreover, lupeol was not detected in any sample of olive oil.

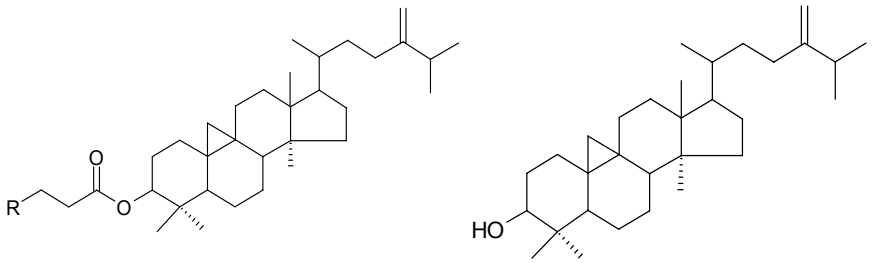
Phytosterol classes of olive oil from different countries have been studied in detail (Paganuzzi & Leoni, 1979; Itoh *et al.*, 1981; Leone *et al.*, 1984, Benitez-Sánchez *et al*., 2003). In these reports, the 4-desmethylsterols class generally includes sitosterol, Δ^5-avenasterol, campesterol, stigmasterol and 24-methylenecholesterol, the 4-monomethylsterols class includes citrostadienol, cycloeucalenol, gramisterol, obtusifoliol and cyclobranol and the 4,4′-dimethylsterols class includes β-amyrin, butyrospermol, cycloartenol, tirucalla-7,24-dienol and 24-methylenecycloartanol, with other minor compounds.

Ranalli *et al.* (2002) have compared the phytosterol classes of seed, pulp and whole olive fruit oil. Seed oil was found to have higher content of total 4-desmethylsterols (2.3-fold higher), sitosterol, campesterol, chlerosterol, Δ^{5-24}-stigmastadienol, Δ^7-stigmastenol and Δ^7-avenasterol compared with the other extracted oil. Pulp and whole olive fruit oil generally had the same amounts of 4-desmethylsterols. In 4,4′-dimethylsterols, β-amyrin, butyrospermol; cycloartenol and 24-methylenecycloartanol were determined. Seed oil had a lower amount of total 4,4'-dimethylsterols and cycloartenol, 24-methylenecycloartanol and higher amount of β-amyrin, butyrospermol (not well separated) compared with other extracted oils. Pulp and whole olive fruit oil generally had similar levels of 4,4′-dimethylsterols. It was concluded that seed oil did not change the phytosterol classes of the whole fruit oil (mixture of seed and pulp oil).

Different processing methods can also affect the levels of phytosterols in olive oils. Oils extracted from olive pastes by the direct centrifugation mode have been compared with the oils produced by the indirect centrifugation (after percolation) mode (Ranalli *et al.*, 2000). The directly centrifuged oils were often higher in total sterols and moreover exhibited higher values of the qualitative campesterol/stigmasterol ratio. However, 4,4′-dimethylsterol content was changed in different ways for different cultivars. Ranalli *et al*. (1999) investigated the effect of using an enzyme processing aid (Cytolase 0) during extraction of olive oil on phytosterol composition. The enzyme processing aid did not seem to influence the content of individual and total 4-desmethylsterols in the olive oil. The values of total sterols and 4-desmethylsterols were within the limits set by the official normal standard (EC. Regulation no. 2568, 1991). However, 4,4′-dimethylsterols were higher for the oils resulting from the enzyme-aided processing system compared with the control sample.

Free and esterified phytosterols

Phytosterols occur in free and esterified forms, *i.e.* as fatty acid esters, steryl glycosides or acylated steryl glycosides (Moreau *et al*., 2002). In free form, the hydroxyl group at the C3 in the A ring is underivatised**,** whereas in esterified form, the hydroxyl group is covalently bound to other constituents (Figure 3) (Moreau, 2005).



A B

Figure 3. Chemical structure of 24-methylenecycloartanol (a 4,4'-dimethylsterol) in esterified (A) and free form (B).

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 on their distribution and stability (Phillips *et al.*, 2002).

The levels of free and esterified sterols in olive oil have been studied in detail (Grob *et al*., 1990). The concentration of free campesterol in pressed olive oil is below 40 ppm. In high quality extra virgin olive oil, the concentration of free stigmasterol is below 10 ppm. Higher concentrations are an indicator of low quality olives (overripe or spoiled fruits). Raw lampante olive oil contains more free stigmasterol than extra virgin olive oil, which is also reflected by a lower campesterol/stigmasterol ratio. After refining, lampante olive oil contains free campesterol and stigmasterol at concentrations not very different from those in extra virgin olive oil. However, as both components are removed during refining at a similar ratio, the campesterol/stigmasterol ratio remains low (Grob *et al*., 1990).

The concentration of sitosterol-C18-esters in high quality extra virgin olive oil is below 200 ppm, but up to 400 ppm must be considered acceptable. As refined solvent-extracted oil contains approximately 2500 ppm sitosterol-C18-esters, the addition of 10% such oil increases the sitosterol ester concentration by about 250 ppm in extra virgin olive oil. The percentage of free sitosterol is a key parameter for assessing the quality of the olive oil. In high quality extra virgin olive oils, the percentage of free sitosterol exceeds 90%. The acceptable limit is around 80%.

Lower relative concentrations indicate the use of low quality olives or forced extraction procedures. This parameter might be useful for setting a limit between extra virgin and lampante olive oil, particularly for those oils that appear to be extra virgin olive oil after gentle neutralisation (Grob *et al*., 1990).

Chryssafidis *et al.* (1992) have reported the amount of free and esterified 4-monomethylsterols and 4,4′-dimethylsterols in virgin olive oil. Obtusifoliol, gramisterol, cycloeucalenol and citrostadienol were identified in both free and esterified forms of 4-monomethylsterols in which citrostadienol was the main sterol in this fraction mostly occurring in esterified form. β-Amyrin, butyrospermol, cycloeucalenol and 24-methylenecycloartanol were the sterols identified in the 4,4′-dimethylsterol class, in which 24-methylenecycloartanol was the main sterol in this fraction and occurring mostly in free form.

Authentication

Olive oil adulteration

Because of the high price of virgin olive oil, there is a great temptation to adulterate it with oils with similar fatty acid and sterol profiles (Aparicio, 2000). Olive oil adulteration with most vegetable oils can be detected by conventional methods. For example, fatty acid composition is useful for the detection of adulteration of olive oil with the following vegetable oils: soybean, walnut, canola, rapeseed, peanut and mustard, even at levels of adulteration below 5% (Christopoulou *et al.*, 2004). ΔECN42 (calculated from the difference between the theoretical and experimental equivalent carbon number 42 in triacylglycerols) can also be used to detect olive oil adulteration with the following vegetable oils: sunflower, soybean, cotton, corn, walnut, sesame, safflower, canola and rapeseed at levels as low as 1% (Christopoulou *et al.*, 2004). Olive oil adulteration with sunflower, soybean, cotton, corn, walnut, sesame, safflower and canola oils can also be detected based on the differences in triglyceride and fatty acid composition between the olive oil and these vegetable oils (Christopoulou *et al.*, 2004).

Hazelnut oil has been used to adulterate olive oil due to its similar composition of triacylglycerols, fatty acids and major sterols (Cercaci *et al*., 2003; Christopoulou et al., 2004) It is estimated that in the European Union, 4 million Euros per year are lost because of this adulteration (European Union Research Committee, 2001). It is difficult to detect olive oil adulteration with hazelnut oil at levels below 20% using conventional methods for detecting the adulteration with other vegetable oils (Bøwadt & Aparicio, 2003; Christopoulou et al., 2004). Table 4 shows the composition of hazelnut and olive oil.

Table 4. *Major and some minor lipid components present in hazelnut and olive oils*[1]

Compound	**Virgin olive oil**	**Hazelnut oil**
TAG by carbon number (CN) (%)		
CN50	Tr-10	0.7-0.9
CN52	17-53	16-20
CN54	30-91	70-84
CN56	Tr-1	0.2-0.6
Fatty acids (%)		
Palmitic acid (C16:0)	7.5-20	5-7
Stearic acid (C18:0)	0.5-5	1-3
Oleic acid (C18:1)	55-83	70-82
Linoleic acid (C18:2)	5-21	8-17
Linolenic acid (C18:3)	0.0-0.9	0.1
Tocopherols (ppm)		
α-Tocopherol	33-219	329-448
β- Tocopherol	0.6-4.0	2-6
γ-Tocopherol	0.1-11.9	5-47
δ-Tocopherol	ND[2]-0.7	0.3-4.5
Phytosterol classes (ppm)		
4-desmethylsterols		
Sitosterol	≥750	1050-1700
Campesterol	≤40	50-95
Stigmasterol	<campesterol	10-18
Δ^5-Avenasterol	40-140	20-80
Total	≥1000	1200-2000
4-monomethylsterols		
Obtusifoliol	ND-59	Tr[3]-18
Gramisterol	ND-48	Tr-17
citrostadienol	17-576	17-122
4,4'-dimetylsterols		
β-Amyrin	8-108	12-192
Butyrospermol	6-104	Tr-27
Cycloartenol	36-856	Tr-96
24-Methylenecycloartanol	203-2190	Tr-72
Wax esters (ppm)		
C36	37-74	42-186
C38	19-55	21-97
C40	3-53	18-80
C42	Tr-76	Tr
C44	13-133	1-16
C46	7-96	3-17
Aliphatic alcohols (ppm)		
C23	ND-11	ND-20
C24	11-204	4-34
C25	4-36	6-34
C26	9-256	5-59
C27	2-18	ND-12

[1] Data reported from IOOC (2003); Benitez-Sánchez *et al*. (2003).
[2] Not detected. [3]Trace.

Detection of olive oil adulteration with hazelnut oil

Different methods have been proposed to detect this adulteration (Bøwadt & Aparicio, 2003). The sterol profile can be used as a means of differentiating between vegetable oils or detecting their authenticity (Itoh *et al.*, 1973b). 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 (Ollivier *et al.*, 1999; Itoh *et al.*, 1973a). Some esterified 4-desmethylsterols (campesterol, Δ^7-stigmastenol and Δ^7-avenasterol) have been used to detect olive oil adulteration with hazelnut oil using the Mariani ratio (R_{MAR}) (Mariani *et al.*, 1999):

R_{MAR} = (% campesterol X (% Δ^7-stigmastenol)^2)/ % Δ^7-avenasterol

For non-adulterated olive oil, R_{MAR} is not more than 1. This method can be used to detect adulteration at a level of 10% (Cercaci *et al.*, 2003). However, 70% of non-adulterated olive oil samples tested had R_{MAR} values higher than 1. It has been concluded that along with this parameter, other analytical parameters should be tested to check the authenticity of these types of olive oil (Cercaci *et al.*, 2003).
It has also been reported that using empirical mathematical models with variables based on the amounts of the three 4-desmethylseterols mentioned as free and esterified forms can give false positives, which confuses the analysis particularly when oil from roasted hazelnuts or adulteration of less than 5% occurs (Bøwadt & Aparicio, 2003).

Mariani *et al.* (2006) have also proposed a method based on some free and esterified 4-desmethylstrerols in a new equation:

R_2 = Free Δ^7-stigmastenol (mg/kg) × (Δ^7-stigmastenol free (%)/Δ^7-stigmastenol ester (%)

It was concluded that the proposed method is more accurate than the previously introduced method (using R_{MAR}) and the adulteration could be detected at level of 6-8%. Changes in the equation could reduce the number of false positives (Mariani *et al.*, 2006).

4-desmethylsterols have been used to detect olive oil adulteration with vegetable oils at levels as low as 5% (Bohačenko & Kopicova, 2001). Δ^7-stigmastenol and campesterol have been used to detect olive oil adulteration with sunflower and soybean oil. Brassicasterol has also been used to detect olive oil adulteration with rapeseed oil. Different types of olive oil (virgin, refined and solvent-extracted) could be classified by using some 4-desmethylsterols (stigmasterol, clerosterol, Δ^5-avenasterol, Δ^7-stigmasterol and Δ^7-avenasterol) as differentiating factors (Jiménez de Blas & Valle-González, 1996).

4,4′-dimethylsterols have been used to detect virgin olive oil adulteration with pomace olive oil at levels as low as 5% (Ntsourankoua *et al.*,1994). Lupeol and α-amyrin have also been used to detect olive oil adulteration with almond hazelnut

oils at levels as low as 5%, analysed by GC (Ollivier *et al*., 1999). However, detailed reports on methylsterols in hazelnut oil were lacking.

Polar components have been studied for tracing olive oil adulteration with hazelnut oil (Zabaras & Gordon, 2004). In pressed hazelnut oil, two unknown polar compounds have been found that are not present in olive oil. However, hazelnut oil samples from Turkey, the USA and France [three sources] had the lowest and highest level of these unknown polar compounds; 0.37-0.71 and 6.23 ppm, respectively. Due to the large variability in these polar components in pressed hazelnut oil from different origins, this method could not be used for the quantitative determination of the level of adulteration.

TAG and unsaponifiables (tocopherols and sterols) have been determined in hazelnut and olive oil and different admixtures of the both oils (Parcerisa *et al.*, 2000). Discriminant analysis showed that TAG can be used to classify hazelnut and olive oil and admixtures of hazelnut oil in olive oil at levels as low as 10%. However, because of similarities in the tocopherol and sterol composition of both oils, these compounds could not be used for this purpose. In another study, it has been shown that TAG composition could not be used to detect olive oil adulteration with hazelnut oil at levels lower than or equal to 5% (Christopoulou *et al*., 2004).

Vichi *et al.* (2001) used a combination of data obtained from free Δ^7-sterols (Δ^7-stigmastenol and Δ^7-avenasterol) and ΔECN42 to detect adulteration at a level of 10%. Data obtained from analysis of free Δ^7-sterols or ΔECN42 were not sufficient alone for this purpose.

A spectrofluorimetric method combined with multivariate analysis has been used to assess the genuineness of olive oil in admixtures with hazelnut oil (Sayago *et al*., 2004). Stepwise linear discriminant analysis applied to each admixture showed that this method can be used to detect the hazelnut oil at levels higher than 5%. More work is needed to validate the method and to evaluate the possibilities of other excitation frequencies. Raman spectroscopy, together with chemometrics, has also been employed to detect olive oil adulteration with hazelnut oil (López-Díez *et al*., 2003). It was concluded that further work should be done to accurately determine the lowest concentration of hazelnut oil that can be detected by Raman spectroscopy in adulterated olive oil.

Olive oil adulteration with hazelnut oil could be detected using Fourier transform infrared (FT-IR) spectroscopy at levels of 25% and higher (Ozen & Mauer, 2002). In another study, spectroscopic analysis (FT-Raman and FT-MIR) was used with the entire oil and also with its unsaponifiables to detect olive oil adulteration with hazelnut oil (Baeten *et al*., 2005). The best results were obtained with the FT-MIR spectra of the unsaponifiable matter samples. However in some case the method gave false positives. The limit of detection was 8% for blends obtained by mixing Turkish hazelnut oil and Turkish olive oil. The limits of detection were not satisfactory for blends with non-Turkish edible oils and blends of Turkish hazelnut oil and European olive oil (15%).

Direct infusion electrospray ionisation (ESI) and atmospheric pressure photospray ionisation source (APPI), coupled to quadruple time-of-flight (QqTOF), have been used to check olive oil adulteration with other cheaper vegetable oils (Gómez-Ariza *et al*., 2006). Mixture of an olive oil sample with a hazelnut oil sample at level of 10% could be distinguished using principal component analysis (PCA) with both ESI-MS and APPI-MS spectra (Gómez-Ariza *et al*., 2006). Differences in TAG composition of the oil samples were used for this purpose. LLL was not detected in the olive oil sample analysed in that study, while it was present in the hazelnut oil sample. It should be noted that LLL have been previously reported in olive oil samples elsewhere in minor amounts (Boskou, 1996; Morales & León-Camacho, 2000).

Peña *et al.* (2005) suggested direct coupling of headspace with mass spectrometry to detection of adulteration of olive oil with hazelnut oil. The system was applied to analysis of the volatile fraction, which can be used for detection of crude hazelnut oil in olive oil. It was concluded that the proposed method was rapid and reliable but disadvantages included the need for multivariate statistical techniques for data treatment. The minimum adulteration levels detected by this method were 7 and 15% of crude hazelnut oil in adulterated refined and virgin olive oil, respectively. It was also noted that adulteration with refined hazelnut oil was not possible to detect with this method, since refined hazelnut oil contains no volatile components.

García-González *et al.* (2004) used ^{1}H and ^{13}C nuclear magnetic resonance (NMR) techniques to detect olive oil adulteration with hazelnut oil. The detection of olive oil adulteration by NMR is based on the qualitative and quantitative chemical information obtained from resonance data. ^{1}H NMR spectra provide information on major compounds such as fatty acids and also on minor compounds such as aldehydes, terpenes and sterols. ^{13}C NMR is a technique that is capable of characterising vegetable oils according to the acyl positional distribution in the glycerol moiety. An artificial neural network based on ^{1}H- and ^{13}C-NMR data could be used to detect olive oil adulteration with hazelnut oil at a level of 8%, with some limitations (García-González *et al.*, 2004).

(*E*)-5-methylhept-2-en-4-one (filbertone) has been identified as the flavour impact component of hazelnuts. There are many studies on using this compound as a marker to detect olive oil adulteration with hazelnut oil. Filbertone could be used as a chiral marker to detect olive oil adulteration with hazelnut oil at levels higher than 10% by direct reversed-phase (RP)-LC-GC analysis under the conditions proposed in the study by Ruiz del Castillo *et al*. (1998). It has also been reported that identification of olive oil, hazelnut oil and mixtures of both oils (85:15) may be possible on the basis of the determination of the presence or absence of filbertone.

On-line coupled reversed phase HPLC and GC have also been used to determine filbertone in hazelnut oil and in olive oil adulterated with hazelnut oil (Flores *et al.*, 2006). This method could be used to detect adulteration of olive oil with some crude and refined hazelnut oil samples at levels of 5% and 12%, respectively. It

should be mentioned that the level of detection in this method was dependent on the level of filbertone in the hazelnut oil sample and when hazelnut oil with a low level of filbertone was used for adulteration, it was difficult to confirm the adulteration by this method.

The presence or absence of filbertone in 21 admixtures of olive oil with crude and refined hazelnut oil (more discussion below) obtained using various processing techniques from different varieties and geographical origins has been evaluated by solid phase microextraction and multidimensional gas chromatography (SPME-MDGC) (Flores *et al*., 2006). The presence of filbertone could occasionally be detected in olive oil adulterated with 7% and 10-20% of crude and refined hazelnut oil, respectively. However, in some cases, this method was not able to detect adulteration at ranges of 5 to 15% due to extremely low levels of filbertone in the hazelnut oil, probably obtained from unroasted nuts.

Detection of olive oil adulteration with refined hazelnut oil

All crude oils obtained after solvent extraction contain variable amounts of non-triglyceride components such as fatty acids, mono- and diglycerides, phosphatides, and *etc*. The amount of the non-triglycerides varies with the oil source, extraction process, season and geographical source. Removal of non-triglyceride constituents from the oil with the least possible damage to the triglycerides and minimal loss of desirable constituents is the objective of the refining process. Low quality vegetable oils are also refined to produce suitable products for edible purposes. Refining processes generally comprise various steps: degumming, neutralization, bleaching and deodorisation (O´Brien, 2004). Table 5 shows the various steps of the refining process and the undesirable compounds removed at each step. Refining can affect minor components present in the unsaponifiable fraction of vegetable oils. During refining processes, particularly during deodorisation and bleaching, *trans* fatty acids and steradienes are also formed (Ferrari *et al.*, 1996).

Table 5. *Substances removed during the vegetable oil refining process*[1]

Refining step	**Substances removed**
Degumming	Phospholipids and gums
Neutralisation	Free fatty acids, residual phospholipids and metals
Bleaching	Pigments, residual soaps and phospholipids
Deodorisation	Volatile oxidation products and other contaminants

[1]Adapted from Čmolík & Pokorny (2000).

Virgin olive oil adulteration with refined vegetable oils can be detected using *trans* fatty acid or steradienes as markers (Lanzón *et al*., 1989; Grob & Bronz 1994). Refined olive oil can also be adulterated with refined hazelnut oil, which is much cheaper. Detection of this adulteration is much more difficult because, *trans* fatty acid and steradienes are present in both kinds of oil. In addition, other marker compounds used to detect the adulteration of olive oil with crude hazelnut oil,

such as filbertone or other volatile compounds, can also be lost mainly during deodorisation process (Flores *et al.*, 2006). These losses depend on how drastic the refining conditions are. It has also been reported that other chemical structures can be altered during the refining processes, so that significant interference from other compounds can give very dirty chromatograms and make the tracing of the filbertone much more difficult and complicated (Flores *et al*., 2006). It has been noted that filbertone may be easily removed upon gentle deodorisation of the oil (Blanch *et al*., 1998). Nevertheless, no reliable method is known to detect adulteration of refined olive oil with refined hazelnut oil.

Industrial applications of olive oil

Chemical interesterification

Most native vegetable oils have limited applications in their original form due to their specific chemical composition. Vegetable oils can be modified to widen their commercial use, either physically by fractionation or blending, or chemically by hydrogenation or interesterification (Hauman, 1994; Anderson, 1996). Blending does not result in chemical modification of the TAG composition. In addition, if the blended oils have very different physical properties, this can result in phase separation during storage. Fats can also be modified by hydrogenation. However, during partial hydrogenation, some *cis* double bonds are isomerised into their *trans* forms. In the past few years, several nutritional studies have suggested a direct relationship between *trans* fatty acids and increased risk for coronary heart disease (Lichtenstein, 1993; Enig, 1996).

Chemical interesterification is an established process to improve plasticity, crystallisation habit or functional properties of fats and oils (Rozendaal, 1992). During interesterification, fatty acids are exchanged within (intraesterification) and among (interesterification) TAGs until a thermodynamic equilibrium is reached. Fatty acids are distributed in a random manner among the TAG molecules and degree of unsaturation or isomeric state of the fatty acid does not change during this process (Noor Lida *et al*., 2002).

Chemical interesterification of olive oil with vegetable oils

There are several reports on interesterification of olive oil blended with other vegetable oils. Chemical interesterification has been used as an alternative to hydrogenation to obtain zero *trans* olive oil products. This is done by chemical interesterification of refined olive oil and tristearin blends (Gavriilidou & Boskou, 1991). Olive oil has been interesterified with distilled fatty acids from waste soapstock and changes in melting and crystallisation properties of the blends before and after interesterification have been evaluated (Sessa *et al.*, 1996). Refined olive oil and palm oil blends have been interesterified to produce plastic fats similar in composition and properties to soft and package type margarine (Alpaslan & Karaali, 1998). In another study, olive oil, as the source of oleic acid, has been interesterified with completely hydrogenated high erucic rapeseed oil, as the source of behenic acid, to prepare low-calorie structured lipids (Tynek & Ledochowska, 2005).

Minor lipid components and chemical interesterification

There are many reports on effects of chemical interesterification on physical and chemical properties of the end product. Chemically interesterified fat/oil blends have been studied regarding melting properties (Rousseau *et al*., 1996; Norizzah *et al*., 2004; Karabulut *et al*., 2004; Mat Dian *et al*., 2006) oxidative stability (Ledóchowska & Wilczyńska, 1998, Daniels *et al*., 2006), storage stability (Zhang

et al., 2005; Zhang *et al*., 2006), triacylglycerol modification (positional distribution of fatty acid in TAGs) (Zeitoun, 1993; Rousseau *et al*., 1996, Norizzah *et al*., 2004), crystallisation (Zeitoun, 1993), and nutritional properties (Ray & Bhattacharyya, 1995). However, there are few reports on effects of chemical interesterification on sterols (Ferrari *et al*., 1997). In addition, there are no reports on the effects of this process on phytosterol oxidation.

Reports on sterol changes during interesterification have mainly focused on alteration of the esterified sterol content of vegetable oils (Ferrari *et al*., 1997). Phytosterols are important from a nutritional point of view because they contribute to lowering serum cholesterol levels in humans (Moreau, 2004). These compounds can be also oxidised like other unsaturated lipids and produce phytosterol oxidation products (POPs) when exposed to air, heat, light or catalysts (Dutta, 2004). Recently, the POPs content of oils and foods with higher amounts of fats has gained interest due to their possible negative biological effects (Adcox, *et al*., 2001; Dutta *et al*., 2007). The effects of sterol structure, temperature, lipid medium, fat and oil refining on POPs have also been studied (Bortolomeazzi *et al*., 2003; Dutta *et al*., 2007). However, scientific literature on the effects of chemical interesterification on phytosterol and POPs content is lacking.

Objectives

This project was initiated in order to develop a rapid and simple way to detect adulteration of olive oil with hazelnut oil. The other objective was to study the effects of chemical interesterification of olive oil with other fats/oils on lipids and minor lipid components.

Specific objectives for the present work were to investigate:

- Phytosterol classes in hazelnut and olive oil collected from different countries
- Utilisation of characteristic sterols as markers to detect olive oil adulteration with hazelnut oil
- Development of a rapid and reliable SPE method to separate phytosterol classes in vegetable oils
- Free and esterified 4,4′-dimethylsterols in hazelnut and olive oil oils
- Effects of vegetable oil refining processes on 4,4′-dimethylsterols in hazelnut oil
- Effects of chemical interesterification of an olive oil/palm stearin blend on lipids and minor lipid components

Materials and methods

This section gives a short description of the materials and methods used in the present study (Table 6). Further details are given in **Papers I-IV**.

Materials

A sample of hazelnuts was collected from Iran (Rodsar, Iran). Other hazelnut samples from Germany (Atco Haselnusskerne, Hamburg, Germany) and Italy (Besana, Italy) were purchased from local supermarkets (Uppsala, Sweden). A sample of refined hazelnut oil from Italy (Lazeo, Italy), a refined and winterised hazelnut oil sample from Turkey (Ordu Soya Industries, Inc., Ordu, Turkey), a commercial hazelnut oil from France (Philippe Vigean, France), a cold pressed hazelnut oil sample (Bayoils Co, Blenheim, New Zealand) and a hazelnut oil made from fresh roasted hazelnuts (Hazelwood Hazelnuts, Amberley, New Zealand) were used in this study. Virgin olive oil samples from Italy (Bertolli, Italy) and Spain (Sierra de Genave, Genave-Jaen, Spain) were obtained from local supermarkets (Uppsala, Sweden). Another virgin olive oil sample was obtained from Norwood olive oil, New Zealand. Refined olive oil and palm stearin were obtained from AarhusKarlshamns Sweden AB.

Authentic samples of 4-desmethylsterols were obtained from Research Plus, Inc. (Bayonne, NJ, USA). Before GC and GC-MS analysis, sterols were silylated using Tri-Sil reagent (Pierce Chemical Co., Rockford, USA). The capillary GC columns were purchased from J&W Scientific (Folsom, CA). Silica SPE cartridges were from IST (Mid-Glamorgan, UK). All other chemicals and solvents used in this study were of analytical grade and purchased from VWR International AB (Stockholm, Sweden) unless otherwise stated. Table 6 shows a summary of the materials, methods, aims and analyses carried out in this thesis.

Oil extraction

Oil samples were extracted from hazelnuts according to the method described by Savage *et al.* (1997).

Table 6. Overall s*ummary of the Papers I-IV*

	Paper I	**Paper II**	**Paper III**	**Paper IV**
Aim	To study phytosterol classes and detection of olive oil adulteration	To develop a rapid SPE method to separate phytosterol classes	To monitor effects of refining processes on 4,4'-dimethylsterols	To investigate effect of chemical interesterification on lipid components
Sample	Hazelnut and virgin olive oils	Hazelnut and virgin olive oils	Hazelnut and virgin olive oils	Refined olive oil and palm stearin
Treatment	Saponification, separation of phytosterol classes by TLC	Saponification, rapid separation of phytosterol classes by SPE	Refining of solvent extracted hazelnut oil	Chemical interesterification
Analysis & achievement	Phytosterol classes by GC and GC-MS; detection of adulteration ~ 3.5%	Phytosterol classes by GC and GC-MS	4,4'-dimethylsterols by GC and GC-MS; detection of adulteration ~ 2%	TAG by GC; fatty acid, sterols, POPs by GC & GC-MS; tocopherols by HPLC

Separation and enrichment of phytosterol classes with TLC (Paper I)

Sterol classes of hazelnut and olive oil samples collected from different countries were separated by TLC according to the method described by Kornfeldt & Croon (1981). For this purpose, oil samples were saponified and unsaponifiables were applied on TLC and developed in hexane/diethyl ether/acetic acid (70: 30: 1). The zones of 4-desmethyl-, 4,-monomethyl-, and 4,4′-dimethylsterols on the TLC were then scraped off and extracted with solvent. Each fraction was derivatised to TMS ether and analysed by GC and GC-MS. The recovery of 4-desmethylsterols was evaluated to determine the level of sterol loss during the work-up procedure.

Detection of olive oil adulteration with hazelnut oil (Paper I)

Mixtures of hazelnut and virgin olive oils were prepared at two levels. A hazelnut oil sample was mixed with a virgin olive oil sample at 3.5% and 5% (w/w). A few 4,4′-dimethylsterols were used as markers to detect olive oil adulteration with hazelnut oil at levels as low as 3.5% by GC-MS.

Separation and enrichment of phytosterol classes by a new SPE method (Paper II)

A new SPE method was developed to separate and enrich phytosterol classes of vegetable oils. This was done with stepwise elution by increasing the polarity of the hexane:diethyl ether solvent mixture (Figure 4). The dissolved unsaponifiables in hexane (5 mL) obtained from 0.5 g saponified oil were loaded onto an SPE silica cartridge (1 g silica), previously conditioned with 5 mL *n*-hexane. The SPE cartridge was washed with 40 mL hexane:diethyl ether (99:1). Pure 4,4´-dimethylsterols were then eluted with 40 mL and 10 mL hexane:diethyl ether (99:1) and (98:2), respectively. After 4,4´-dimethylsterol elution, the cartridge was washed with 10 mL hexane:diethyl ether (98:2). Pure 4-monomethylsterols were eluted with 20 mL hexane: diethyl ether (98:2). After washing the cartridge with 5 mL hexane:diethyl ether (98:2), pure 4-desmethylsterols were eluted with 10 mL hexane:diethyl ether (60:40). The method was applied to samples of hazelnut and olive oils and the results obtained were compared with those of preparative-TLC method. Recovery of 4-desmethylsterols was also tested to investigate the efficiency of the new method.

Free and esterified 4,4´-dimethylsterols in hazelnut and olive oils (Paper III)

Total free and esterified sterols of hazelnut and olive oils were separated by the SPE (500 mg silica) method developed. The oil sample (approx. 0.5 g) was dissolved in 1 mL hexane and loaded onto the SPE cartridge (500 mg silica) previously conditioned with 3 mL hexane. The esterified sterol fraction was eluted with 9 mL hexane, and then free sterol fraction was eluted with 6 mL hexane:diethyl ether (4:6). Oil samples and separated total free and esterified sterols were saponified. 4,4´-dimethylsterols from these saponified samples were separated according to the previously developed SPE method (**Paper II**), and analysed by GC and GC-MS.

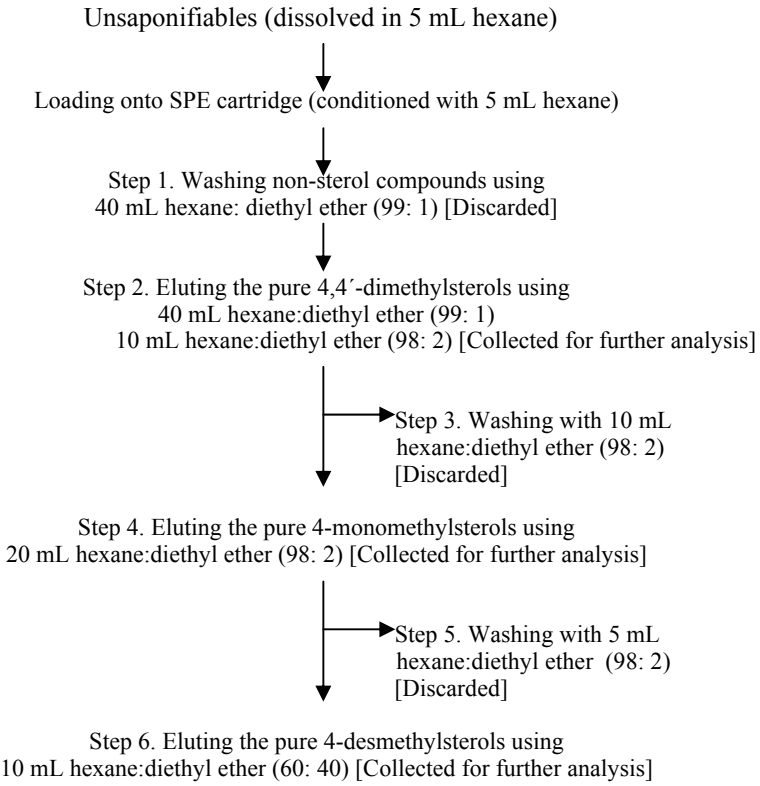


Figure 4. Flow chart of the SPE method to separate phytosterol classes in vegetable oils for further analysis by GC and GC-MS.

Refining of hazelnut oil (Paper III)

Solvent-extracted hazelnut oil was subjected to vegetable oil refining processes (degumming, neutralisation, bleaching and deodorisation) at laboratory scale. The effects of refining processes on total, free and esterified 4,4′-dimethylsterols were determined. GC-MS analysis was used to detect adulteration of olive oil with the sample of fully refined hazelnut oil at levels as low as 2% by tracing lupeol in total or in esterified forms of 4,4´-dimethylsterols.

Chemical interesterification of olive oil and palm stearin (Paper IV)

An olive oil and palm stearin blend (1:1) was interesterified using sodium methoxide as a catalyst (0.5%) at two different temperatures: 90 and 120 °C for 1 hour.

The following parameters were determined before and after chemical interesterification of the blend:

Triacylglycerol profiles were determined by GC according to the method described by Farmani *et al*. (2006). Fatty acid composition of fat blends was determined by GC. Esterified sterols of fat blends were separated by TLC and after scraping off and extraction of esterified sterols from TLC, they were methylated and FAMEs were analysed by GC. Tocopherols and tocoterienols were

analysed by HPLC according to the method described by Dutta *et al.* (1994) with slight modification. Free and esterified sterols of oil samples were determined according to the method developed in **Paper III.**

To determine POPs content, fat blends were transesterified according to the method described by Schmarr *et al*. (1996) after slight modification. POPs were separated and enriched according to the SPE method newly developed at our laboratory. Figure 5 shows the work-up steps of the SPE method for separation and enrichment of POPs. After enrichment, POPs were derivatised to TMS-ether and analysed by GC and GC-MS.

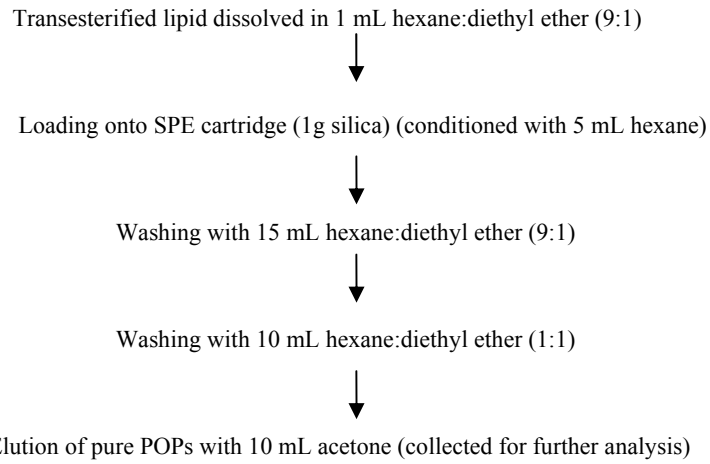


Figure 5. Work-up steps of the SPE method to separate and enrich POPs for further analysis by GC and GC-MS.

GC and GC-MS analysis of phytosterols and POPs (Papers I-IV)

After separation and enrichment of phytosterol classes by TLC and SPE, they were derivatised to TMS-ether and analysed by GC and GC-MS. In **Papers I and II,** a fused-silica capillary column DB-5MS (30 m x 0.25 mm, 0.50 μm) was used. In **Paper III**, the columns used were combination of DB5-MS (10 x 0.18 mm, 0.18 μm) and DB17-MS (10 m x 0.18 mm, 0.18 μm) to improve the separation of sterols compared with single DB-5MS column. In **Paper IV**, TMS-ether derivatives of total, free and esterified sterols and POPs were also analysed using another combination of DB-5MS (15 x 0.18 mm, 0.18 μm) and DB-35MS (10 m x 0.2 mm, 0.33 μm). In all GC analyses, helium and nitrogen were used as carrier and make up gases, respectively. The GC-MS analyses were performed on a GC8000 Top Series gas chromatograph (Thermo Quest Italia S. P. A., Rodano, Italy) coupled to a Voyager mass spectrometer with MassLab data system version 1.4V (Finnigan, Manchester, UK). The full scan mass spectra were recorded at EI^+ mode at electron energy of 70 eV and ion source temperature of 200 ºC. The column and conditions for the analysis were the same as used for GC analysis. More details on the GC and GC-MS analysis are given in the **Papers I-IV.**

Results and discussion

Phytosterol classes in hazelnut and olive oils (Paper I)

Phytosterol classes of hazelnut oil from Iran, Italy, New Zealand and Turkey and virgin olive oil from Italy, New Zealand and Spain were separated and enriched by TLC and determined by GC and GC-MS. Considerable quantitative differences were observed in the relative proportions of sterol fractions for both types of oils (Table 7).

In hazelnut oil, 4-desmethylsterols had the highest proportion (ranging from 86 to 91%), while 4-monomethyl and 4,4´-dimethylsterols showed lower amounts (ranging from 4 to 8% and 3 to 8%, respectively) of total sterols. Olive oil showed the lowest amount of 4-monomethylsterols (ranging from 9 to 11%) while 4-desmethyl and 4,4´-dimethylsterols ranged from 51 to 57% and 32 to 40%, respectively, of total sterols. Generally, the 4-desmethylsterol content of hazelnut oil was qualitatively and quantitatively rather similar to that of virgin olive oils. 4-monomethylsterols were similar in composition in both kind of oil but differed in content (2-3 times lower in hazelnut oil than in olive oil) (Table 7). These results concur with literature data showing the level of 4-desmethyl-, 4-monomethyl-, and 4,4-dimethylsterols content (Benitez-Sánchez *et al*., 2003).

In **Paper I** we used traditional TLC methods to separate and enrich phytosterol classes of oil samples. In order to evaluate the efficiency of the method and check the possible loss of sterols during enrichment, a recovery test was performed under similar conditions used to fractionate the total unsaponifiable matters of oils. The recoveries were 61%, 61%, and 65% for campesterol, stigmasterol and sitosterol, respectively. Losses may have occurred during the extraction of unsaponifiable matters from saponified oil sample, and recovery of sterol fractions in silica gel scraped from TLC plates.

Table 7. *Contents of phytosterols (ppm) in hazelnut and virgin olive oils*[1]

Sterol	**Hazelnut oil (n=5)**	**Virgin olive oil (n=3)**
4-desmethylsterol		
Cholesterol	4-7	4-8
Campesterol	34-41	18-30
Campestanol	2-4	1-2
Stigmasterol	4-12	4-5
Sitosterol	476-785	414-757
Δ^5-Avenasterol	25-75	31-67
$\Delta^{5,24}$-Stigmastadienol	3-11	2-3
Δ^7-Stigmastenol	1-5	1
Δ^7-Avenasterol	3-7	1
Unknown	14-53	8-17
Total	593-984	506-884
4-monomethylsterols		
Obtusifoliol	3-11	9-21
Δ^7-Sterol	1	3-5
Gramisterol	1-4	4-8
Cycloeucalenol	Tr[3]	13-16
Δ^7& Δ^8-Sterol	1-5	2-3
$\Delta^{7,22}$-Sterol	1-2	2-4
Citrostadienol	11-30	40-81
Unknown	9-13	18-20
Total	29-66	91-152
4,4′-dimethylsterols		
X	1-6	ND
δ -Amyrin	1-7	10-20
Taraxerol	ND[4]	7-24
β-Amyrin	1-2	26-58
Cycloartenol	2-5	75-152
Lupeol	3-9	ND
Δ^7-Sterol	1-2	2-3
24-Methylenecycloartanol	6-18	168-303
Unknown	13-19	29-105
Total	31-67	329-695

[1] Data are from **Paper I.** [2]An unknown compound X detected exclusively in hazelnut oil. [3]Not detected. [4]Trace amount (<0.1µg/g oil).

4,4′-dimethylsterols were different in composition and in content in hazelnut and olive oil. The total amount of these compounds was approx. 10 times lower in hazelnut oil than in olive oil. At least two compounds, lupeol and an unknown compound X (containing a lupane skeleton), were present exclusively in hazelnut oil. Figure 6 shows the mass spectra of these two compounds. Taraxerol was also present only in olive oil samples. β-Amyrin, δ-amyrin, cycloartenol, Δ^7- sterol and 24-methylenecycloartanol were detected in both oils (Table 7).

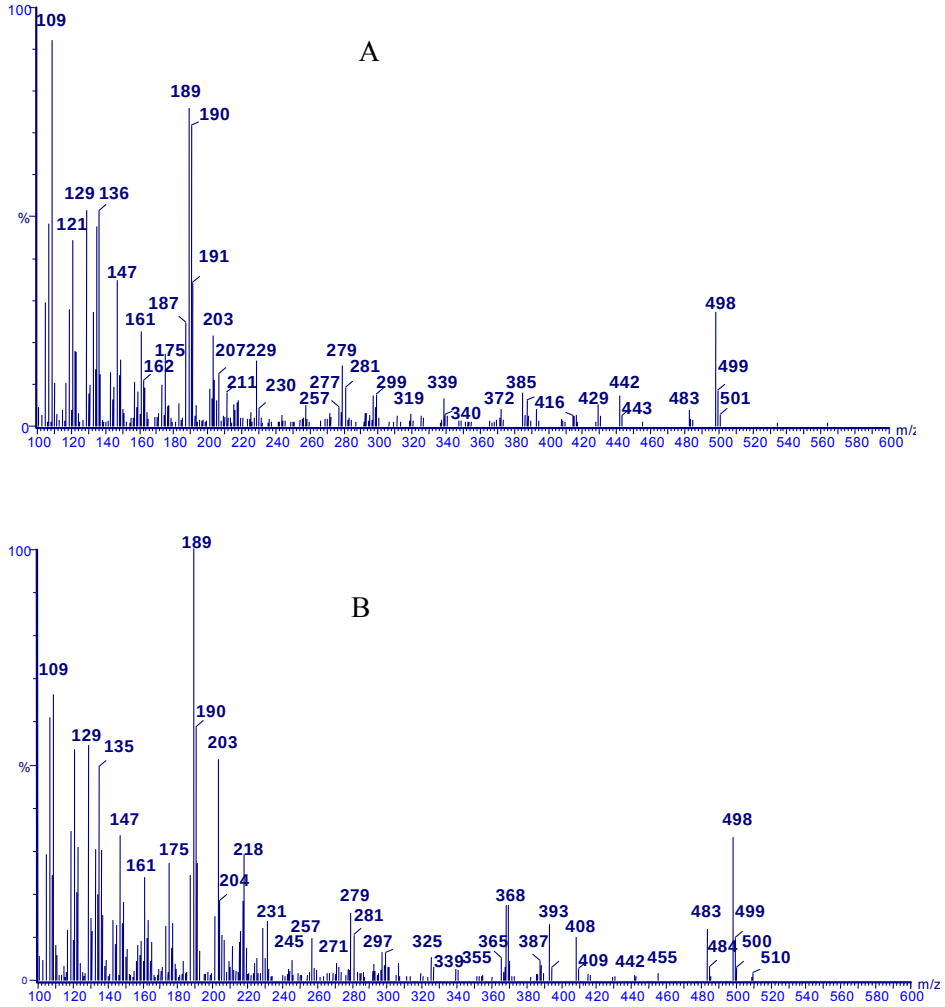


Figure 6. Full scan mass spectra of two 4,4´-dimethylsterols present exclusively in hazelnut oil. A) compound X; B) lupeol.

Detection of olive oil adulteration with hazelnut oil (Paper I)

Marker compounds, lupeol and the unknown compound X were used to detect the adulteration of olive oil with hazelnut oil. The 4,4´-dimethylsterol fraction of olive oil adulterated with hazelnut oil at different levels was enriched and separated with TLC and analysed by GC-MS to trace the marker compounds. It was possible to detect hazelnut oil at levels as low as 3.5% in adulterated virgin olive oil by GC-MS.

New SPE method to separate and enrich phytosterol classes (Paper II)

Our main aim was to develop a rapid and reliable SPE method to separate and enrich sterol classes, particularly 4,4´-dimethylsterols in vegetable oils. Previously, we used prep-TLC method to separate and enrich the sterol classes from unsaponifiables of hazelnut and olive oils but this method has several sample preparation sequences, with many possible sources of error. Sterol classes have close RF values on TLC, which may cause mixing during scraping of TLC bands (Kornfeldt & Croon, 1981). Additional drawbacks of prep-TLC are that it has low recovery rates and is time-consuming and laborious. The new method was tested on hazelnut and virgin olive oil for composition and quantification of their sterol classes.

Due to additional numbers of methyl groups, the polarity of the three sterol classes decreases in the order 4-desmethylsterols > 4-monomethylsterols > 4,4´-dimethylsterols. Because of the polarity of sterol fractions, 4,4´-dimethylsterols are a weakly retained isolate, followed by 4-monomethylsterols. 4-desmethylsterols are retained more strongly than methylsterols on the silica sorbent and were eluted as a last sterol class in this method. Samples of hazelnut and virgin olive oil collected from different countries were separated for their sterol classes using this new SPE method and the results were compared with a previously published prep-TLC method.

The total 4,4´-dimethylsterol content in the oil samples analysed was higher ($p < 0.05$) when quantified with the SPE method compared with prep-TLC (Table 8). All 4,4´-dimethylsterols identified were recovered in higher amounts by the SPE method in hazelnut and olive oil than by the TLC method. Lupeol and the compound X, potential markers to detect virgin olive oil adulteration with hazelnut oil, were obtained at approx. twofold higher rates with this new SPE method than with TLC. Total 4-monomethylsterol content was higher ($p<0.05$) with SPE compared with TLC. Values of individual 4-monomethylsterols in hazelnut oil samples were higher with SPE compared with TLC. Values were generally similar with SPE and TLC in virgin olive oil samples. In general, the results for total and individual 4-desmethylsterols obtained with SPE were also significantly higher ($p<0.05$) compared with prep-TLC (Table 8).

Table 8. *Content of total phytosterols in hazelnut and olive oils obtained with TLC and SPE methods*

Total sterol	**Hazelnut oil (n=2)**		**Virgin olive oil (n=2)**	
	TLC	SPE	TLC	SPE
4-desmethylsterols	731-984[b]	1229-1306[a]	506-585[b]	824-866[a]
4-monomethylsterols	48-57[b]	65-93[a]	91-107[b]	121-126[a]
4,4'-dimethylsterols	33-72[b]	89-146[a]	329-403[b]	589-765[a]

[a-b] Denotes statistically significant differences ($p<0.05$).

A recovery test was performed under similar conditions to fractionate the total unsaponifiable matter of oils by prep-TLC and SPE methods. Recoveries for campesterol, stigmasterol and sitosterol were 61%, 61%, and 65%, and 91%, 94%, and 96%, respectively with prep-TLC and SPE methods.

Free and esterified 4,4´-dimethylsterols in hazelnut and olive oils (Paper III)

4,4′-dimethylsterols like other sterol classes can occur as free and esterified forms in vegetable oils. 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 on their distribution and stability (Phillips *et al.*, 2002). Therefore, in this study (**Paper III**), we determined free and esterified 4,4′-dimethylsterols in hazelnut and olive oil samples from different countries. Furthermore, a combination of two columns with different polarity, DB-5MS and DB-17MS, was also investigated to improve the separation of 4,4´-dimethylsterols by GC.

In GC analysis using the DB-5MS column, α-amyrin was co-eluted with cycloartenol and lupeol from hazelnut oil (Table 9). Similarly, tirucalla-7,24-dienol was co-eluted with cycloartenol in virgin olive oil (Table 9). In this study, a combination of two columns: a non-polar DB-5MS column (10 m x 0.18 mm, 0.18 µm) and a mid-polar DB-17MS column (10 m x 0.18 mm, 0.18 µm) was used in GC and GC-MS analysis. These separated 4,4´-dimethylsterols more effectively compared with the single DB-5MS column (30 m x 0.25 mm, 0.50 µm) previously used (Table 9).

Table 9. *Relative retention times (RRT) of TMS ether derivatives of 4,4´-dimethylsterols separated on two different GC column systems*

Sterol	**RRT**	**RRT**
	DB-5MS	DB-17MS/DB-5MS[a]
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 : DB-5MS and DB-17MS, joined together by a universal press-fit connector. [b]Overlapping with β-Amyrin. [c]Overlapping with cycloartenol and lupeol.

In hazelnut oil samples, levels of total 4,4′-dimethylsterols were higher in the esterified form (23-53 ppm) compared with those in free form of these compounds (13-38 ppm). Of the marker compounds, lupeol was 6-16 ppm in esterified form and 6-10 ppm in free form, while compound X was 1-5 ppm in esterified form and 0.8-5 ppm in free form (Table 10).

In olive oil samples, levels of 4,4′-dimethylsterols were higher in esterified form compared with those in free form, except for cycloartenol and 24-methylenecycloartanol (Table 10). Total content of 4,4′-dimethylsterols in free form was higher (293-448 ppm) than that in esterified form (180-315 ppm). We could also detect two compounds, tirucalla-7,24-dienol, and taraxerol, in 4,4′-dimethylsterols of olive oil samples that were not present in hazelnut oil samples (Table 10). To our knowledge, this is the first report on free and esterified forms of 4,4′-dimethysletrols in hazelnut oil.

Table 10. *Content of free and esterified 4,4'-dimethylsterols in hazelnut and virgin olive oils*[1]

Sterol	**Hazelnut oil (n= 5)**		**Virgin olive oil (n=2)**	
	Free	Esterified	Free	Esterified
Compound X[2]	1-5	1-5	ND	ND
Taraxerol	ND[3]	ND	1	2-8
δ-Amyrin	1-2	3-5	1-2	12-19
β-Amyrin	0.1-2	0.2-2	1	6-12
Butyrospermol	Tr[4]-2	Tr- 8	5-10	41-53
Cycloartenol	1-7	3-5	68-167	17-75
Tirucalla-7,24-dienol	ND	ND	1-2	41-53
α-Amyrin	1-3	1-12	Tr	Tr
Lupeol	6-10	6-16	ND	ND
24-methylencycloartanol	2-10	7-12	217-264	61-95
Total	13-38	23-53	293-448	180-315

[1]Data are from **Paper III.** [2]An unknown compound X detected exclusively in hazelnut oil. [3]Not detected. [4]Trace amount (<0.1µg/g oil).

Effects of refining processes on 4,4′-dimethylsterols in hazelnut oil (Paper III)

Refining processes generally comprise various steps: degumming, neutralisation, bleaching and deodorisation (O´Brien, 2004). Each step can cause specific changes in oil properties, particularly in minor constituents such as sterols and tocopherols (Table 11) (Verleyen *et al.*, 2002; Bortolomeazzi *et al.*, 2003; Dutta *et al.*, 2007). In order to study the effects of the refining processes, a sample of hazelnuts was extracted for oil with solvent and was refined at laboratory scale by degumming, neutralisation, bleaching and deodorisation. Total 4,4′-dimethylsterols as well as free and esterified 4,4′-dimethylsterols of refined hazelnut oil were determined after each refining process.

Generally the degumming process did not alter qualitatively or quantitatively alter the 4,4´-dimethylsterol composition in this study. Total 4,4´-dimethylsterols were reduced by 22% during neutralisation. In this refining process, free and esterified forms of 4,4´-dimethylsterols were reduced by 23 and 17%, respectively, during neutralisation. It is known that free sterols can be reduced by transferring to the soapstock formed (Verleyen *et al*., 2002). The slight reduction in esterified forms of 4,4´-dimethylsterols was probably due to hydrolysis of the esterified sterols to free forms during the neutralisation process. This has been demonstrated previously on the level of sterol losses during neutralisation, which was up to 9-22% of sterols (Leone *et al*., 1976).

During bleaching, total 4,4´-dimethylsterols were reduced by approx. 24% in hazelnut oil. Both free and esterified 4,4´-dimethylsterols were affected by the bleaching process. The loss of esterified sterols (29%) was greater than the loss of free sterols (18%) during this refining step (Table 11). The reduction in esterified forms of sterols during bleaching has been explained by acid-catalysed hydrolysis of the esterified sterols on the acid-activated bleaching earth (Verleyen *et al*., 2002).

During deodorisation, total 4,4´-dimethylsterols were reduced by 13%. Among free and esterified forms of 4,4´-dimethylsterols, the free form was affected considerably (21%). In contrast, esterified 4,4´-dimethylsterols were generally not affected by deodorisation (Table 11). In fully refined hazelnut oil, total 4,4´-dimethylsterols were reduced by 48% compared with crude hazelnut oil. Free and esterified 4,4´-dimethylsterols losses were 52 and 41%, respectively, compared with their levels in crude hazelnut oil. These results demonstrate that esterified 4,4′-dimethylsterols are more stable than free forms during refining. To our knowledge there is no published data on free and esterified 4,4'-dimethylsterols to compare with our results.

Table 11. *Losses of free and esterified 4,4'-dimethylsterols in a sample of hazelnut oil during refining processes*

Process/Condition	**Loss of sterols (%)**[1]	
	Free	Esterified
Degumming/ Treatment of crude oil with warm water followed by centrifugation to separate gums	Generally no effect	Generally no effect
Neutralisation/ Reaction of degummed oil with alkaline, followed by water washing and separation of residual soap by centrifugal separation	23	17
Bleaching/ Treatment of neutralised oil with bleaching clay, followed by separation of the spent bleaching clay by filtration	18	29
Deodorisation/ Treatment of bleached oil at high temperature under vacuum	21	Generally no effect

[1]Each value is the mean of triplicate analyses.

Detection of olive oil adulteration with refined hazelnut oil (Paper III)

Lupeol and the compound X belonging to the 4,4'-dimethylsterols were lost by 18 and 37% in the esterified form, respectively. The losses of these two compounds in the free form were 26 and 72%, respectively. To our knowledge, there are no published data on the effects of vegetable oil refining on free and esterified 4,4´-dimethylsterols. Nevertheless, our results on the retention of 4,4´-dimethylsterols during refining are generally comparable with published results on free and esterified 4-desmethylsterols. It has been shown that esterified 4-desmethylsterols in vegetable oils decrease by minor levels compared with free 4-desmethylsterols after complete refining (Ferrari *et al*., 1996; Verleyen *et al*., 2002). In order to investigate the possibility of detecting olive oil adulteration 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 analysed 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. Therefore, tracing the esterified fraction of 4,4´-dimethylsterols alone can be used to detect adulteration of olive oil with refined hazelnut oil. This achievement was for the first time showing that adulteration of olive oils with refined hazelnut oil can be detected as low as 2%.

Chemical interesterification of olive oil with palm stearin (Paper IV)

Paper IV monitored the effects of chemical interesterification on minor lipid components, *i.e.* phytosterols and POPs. In addition, changes in tocopherols and tocoterienols and fatty acid composition of esterified sterols were investigated during this process. Interesterification altered the TAG profiles of the starting oil blend but no changes were observed in the fatty acid composition of the oil blend. There was a slight reduction in the sterol content of the oil blend after interesterification of 3.2 and 5.5% at 90 and 120 °C, respectively (Table 12). However, the distribution of sterols remained unchanged. Interesterification caused an increase in esterified sterol content of the oil blend of 4.0 and 6.6% at 90 and 120 ºC, respectively.

In the esterified sterols of the starting oil blend, oleic acid was the dominant fatty acid, followed by palmitic acid and eicosanoic acid. Myristic, linolenic and gadoleic acid were not present in the esterified sterols. During interesterification, palmitic, oleic and linoleic acid content increased in the esterified sterols, whereas eicosanoic, behenic and lignoceric acid content decreased.

POPs were enriched and separated by single-step SPE and analysed by GC and GC-MS. The POPs identified were 24-hydroxycampesterol; 7α-hydroxysitosterol; 6β-hydroxycampestanol; 24-hydroxysitosterol; 6β-hydroxysitostanol; 24-methylcholest-4-ene-6α-ol-3-one; 25-hydroxystigmasterol; 25-hydroxysitosterol; and 7-ketositosterol. However, there were some peaks that we could not identify. Among the POPs identified, 6β-hydroxysitostanol dominated, followed by 6β-hydroxycamestanol, and generally their levels were not changed during chemical

interesterification (Table 12). The total amount of POPs was rather low, possibly due to the low phytosterol content in the starting oil blend.

Table 12. *Effects of chemical interesterification of an olive oil and palm stearin blend on minor lipid components (ppm)*[1]

Oil/Fat	Total tocopherols	Total tocoterienols	Total sterols	Total POPs
Starting blend	211	133	509	4.3
Interesterified at 90 °C	210	130	493	4.6
Interesterified at 120 °C	205	122	481	4.6

[1]Each value is the mean of triplicate analyses.

Tocopherols and tocoterienols have antioxidant properties. These compounds can prevent lipid oxidation and phytosterol oxidation (Rudzińska *et al.*, 2004). Only two tocopherols were quantified, of which α-tocopherol dominated (91%), followed by γ-tocopherol (9%). Among the tocoterienols, γ-tocoterienol dominated (52.4%), followed by α-tocoterienol (27%), δ-tocoterienol (14%) and β-tocoterienol (7%). Interesterification caused a slight reduction in tocopherol and tocoterienol content (Table 12). Thus, the interesterification conditions used in this study caused some losses of tocopherols and negligible oxidation of phytosterols.

Conclusions

To achieve the main aims of this study, determination of the authenticity and industrial applications of olive oil, we developed rapid and simple SPE methods to separate sterol classes, free and esterified sterols, and enrichment of POPs. Sterol classes and their free and esterified forms are important parameters not only in vegetable oils but also in cereals and other foods containing these compounds because of their potential nutritional benefits. POPs have also gained interest during recent years because of their possible negative health effects. Therefore, the methods developed here can facilitate identification of these compounds in foods and food ingredients.

The findings of the present thesis can be summarised as follows:

a) Qualitative and quantitative differences were found among the three phytosterol classes of hazelnut and olive oil. At least two 4,4′-dimethylsterols, lupeol and an unknown compound X, were detected in the hazelnut oil which were absent in the olive oil. These two compounds were utilised as markers to detect olive oil adulteration with hazelnut oil by GC-MS.

b) A novel SPE method was developed to separate and enrich phytosterol classes. This new SPE method is advantageous in comparison with traditional prep-TLC for isolating and enriching sterol classes. The SPE method proved to be simple, rapid and gave higher recovery of sterols than the TLC method. In this SPE method, 4,4′-dimethylsterols were eluted prior to 4-monomethyl- and 4-desmethylsterols. This is an advantage when other sterol classes are not required for further analysis.

c) A simple and rapid SPE method was developed to separate free and esterified sterols in vegetable oils. GC separation of 4,4′-dimethylsterols was improved by using a combination of a non-polar DB-5MS column (10 m x 0.18 mm, 0.18 µm) and a mid-polar DB-17MS column (10 m x 0.18 mm, 0.18 µm) compared with the single DB-5MS column (30 m x 0.25 mm, 050 µm).

d) Free and esterified forms of 4,4′-dimethylsterols in hazelnut oil and their retention during refining processes were studied. Among the refining processes, degumming generally caused no effects, deodorisation caused minor decreases, and neutralisation and bleaching caused a considerable loss of 4,4′-dimethylsterols in a sample of hazelnut oil. In fully refined hazelnut oil, total 4,4′-dimethylsterols were reduced to a level 48% compared with crude hazelnut oil.

e) Esterified 4,4′-dimethylsterols were retained to a greater extent compared with free forms of these compounds during refining of hazelnut oil. Therefore, for the first time, lupeol in the esterified fraction alone was used to detect olive oil adulteration with fully refined hazelnut oil at levels as low as 2%.

f) Despite several treatments to produce an interesterified product (heat, catalysis, citric acid treatment, washing and filtration), there were minor losses in phytosterol and tocopherol contents. The levels of POPs in starting oil blends were not changed considerably in the interesterified product.

Future prospects

Based upon the research in this thesis, the following future research is planned:

a) Studies of the detailed sterol composition of hazelnut oil from different origins, species and varieties.

b) Studies of changes in phytosterol content in hazelnuts grown in selected geographical locations over time.

c) Studies of changes in the 4,4′-dimethylsterols in different maturation stages of hazelnut.

d) Studies of the effects of roasting and storage on 4,4′-dimethylsterols in hazelnut oil.

e) Studies of different parameters in the refining process for hazelnut oil, in order to monitor the changes in phytosterol classes.

f) Studies of the structure of compound X, one of the marker compounds used to detect olive oil adulteration with hazelnut oil.

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