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Review

Analysis of steroidal lipids by gas and liquid chromatography

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Abstract

This article describes the most commonly used procedures and recent laboratory methodologies using gas and liquid chromatography developed for separation and quantitation of non-saponifiable steroidal lipids from clinical (human) studies, edible fats and oils or fatty foods.

Keywords: Reviews; Sample handling; Oils; Fats; Food analysis; Lipids; Steroids; Sterols; Alcohols; Hydrocarbons

Contents

1. Introduction

Cholesterol is the predominant sterol in all membrane cells and a vital constituent of cell membranes. Oxysterols (oxidized derivatives of cholesterol) formed from cholesterol by autoxidation, by action of specific cytochrome P450s, or enter the circulation as contaminants of cholesterol-containing food, are variously metabolized to esters, bile acids, steroid hormones, cholesterol, and other sterols [5,49,54,65,143]. They are found in serum associated with lipoproteins or albumin.

The majority of the known oxysterols are biologically active as cytotoxic, atherogenic, mutagenic, carcinogenic or enzyme inhibitory substances [144]. A few investigators have recognized their potential as therapeutic agents [2,145].

Several high-performance liquid chromatographic (HPLC) [1–16] and capillary gas chromatographic (GC) [2,17–51] methods have been used for the analysis of steroids (sterols and their oxygenated derivatives, triterpene alcohols, methyl sterols, steroidal hydrocarbons) from the non-saponifiable portion of lipid extracts from biological samples and

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foodstuffs. A combination of liquid column chromatography (LC) or preparative thin-layer chromatography (TLC) prior to HPLC or GC or on-line coupled HPLC–GC [61,62,170–172] is often used to achieve fractionation of the compounds [2,52–60,170–172].

The importance of the huge number of studies on the lipid constituents found in biological samples and especially in plasma [38] cannot be underestimated due to their incidence in cardiovascular diseases [63,64].

Oxidation of low density lipoproteins is commonly implicated as an initiator of atherosclerosis [59,76]. Lipid peroxidation leads to the formation of an array of different products with diverse and powerful biological activities [2,22,65–70,124,144,156]. Among them are a variety of different oxysterols [2,65], epoxy-, keto- [66] and aldehydes [77]. In addition to being intermediates in cholesterol degradation, oxysterols have a broad spectrum of biological effects, including modulation of the activity of key proteins involved in cholesterol homeostasis [66,146]. These and other possible roles of oxysterols as modulators of cholesterol and cellular metabolism are included in a review by Schroepfer [2].

The major oxysterols in human circulation are 7α-, 27-, (24*S*)-hydroxycholesterol and (24*S*), 25-epoxycholesterol [49,268]. 27-Hydroxycholesterol is thought to originate in the lungs. It is the major oxysterol in human atherosclerotic lesions [78], followed by 7-ketocholesterol [2,59,79]. Most of the (24*S*)-hydroxycholesterol present in the circulation is thought to be derived from the brain and most of the elimination of the oxysterol occurs in the liver [50]. It has been observed that there are slightly but significantly increased levels of circulating (24*S*)-hydroxycholesterol in a specific population of Alzheimer patients [51,80].

The demonstration that some cholesterol oxides also present in foodstuffs and human tissues possess biological activity, has attracted interest in the food science [43,71–75,81–83,89] and medical fields [2,5,34,65]. Oxysterols have been quantified in cholesterol-rich processed foods [56,105], and it has been under study whether oxidized cholesterol products in arterial lesions [48,63,64] are partly derived from dietary sources [22,276]. The presence of oxysterols in tissues and foods concerning their physiological relevance has been reviewed [65].

Oils, margarines, cereal products, fruits and vegetables play a significant role in human nutrition [87–89]. The most frequent sterol present in edible plant-derived fats and oils is β-sitosterol (~90%) [90,91]. Other minor sterols present in the oils are α-cholestanol, campesterol, campestanol, stigmasterol, Δ7-campesterol, clerosterol, sitostanol, Δ5-24-stigmasterol, Δ7-stigmasterol, Δ7-avenasterol and cholesterol, which is the most important sterol found in animal fats [91]. Sitosterol, campesterol and stigmasterol heated in air oxidize to their 7α- and 7β-alcohol, 7-keto, 5,6-epoxide derivatives, and sitosterol and campesterol appear to be more easily oxidized than cholesterol [92].

The determination of minor components in vegetable oils is of great value in establishing oil quality and identity [93–96]. It has been noticed that the non-saponifiable components furnish a fingerprint useful for the identification of oils and the analysis of non-saponifiables may be helpful in detection of foreign mixtures in an investigated oil [97–100].

The phytonutritients [47] have been categorized into 10 classes of compounds or biologic activities [148]. While structure–activity relationships have not been identified for many of the phytonutritient–biological activities, research activity in this field is continuing to elucidate more of these relationships [148]. Currently, the analysis of phytosterol content in foods is of interest because of their cholesterol-lowering properties ([44,102–104,149–151, see Ref. [101] for review). The addition of plant sterols to foods with the intended effect of lowering plasma cholesterol levels is currently a major development of the food industry in Europe [104] and North America [106,107].

Chromatographic techniques concerning the analysis of the non-saponifiable matter have been recently thoroughly reviewed [2,108,135,142,165]. The present review provides an update of the methods using GC and HPLC for analysis of non-saponifiable steroidal lipids from clinical (human) studies, edible fats and oils or fatty foods.

2. Sterol synthesis

Natural sterols have an oxygen function (usually a hydroxyl group) at C-3 and a hydrocarbon chain at C-17. They differ from each other in the way rings A

and B are fused, in the presence and location of double bonds, and in the presence, nature, and orientation of the alkyl group at C-24 [1]. The side-chains of naturally occurring steroids vary in structure. Oxidation can occur in the A and B rings and also in the sterol side chain [123,152]. Chemical structures (and their trivial and IUPAC names) of the main desmethylsterols (sterols), triterpenic dialcohols, 4-methylsterols, and triterpenic alcohols (4,4′-dimethylsterols) can be found in Ref. [142].

Organisms obtain cholesterol through biosynthesis and diet. Sterols in all living organisms are synthesized from acetic acid via mevalonic acid and squalene (as key intermediates). Lanosterol is the first cyclization product in mammals and fungi, while plants produce cycloartenol instead of lanosterol. The final sterol products are cholesterol in mammals, ergosterol in fungi and phytosterols in plants [45].

In mammals, bile alcohols have been considered to be intermediates in the pathway for the formation of bile acids from cholesterol and are not usually found in considerable amounts under normal conditions [36]. Increased amounts of bile alcohols and cholestanol have been observed in serum, urine, and bile in some patients [7,267]. Une et al. [269] demonstrated the presence of (24*S*,25*R*)-27-nor-5β-cholestane-3α,7α,12α,24,25–pentol (90%) along with (24*R*,25*R*)-isomer (10%) as a minor constituent in urine of healthy humans. Bile alcohols are known to be the major bile constituents of lower vertebrates such as amphibians and some fishes [110].

Ergosterol is the principal sterol in most fungi, and is an indispensable component in membrane structures. Recently, the use of ergosterol as a general indicator of fungal contamination has been reported [3,6].

Dietary fats and oils of plant origin contain small amounts of the phytosterols. The most common plant sterols are β-sitosterol, campesterol, stigmasterol and brassicasterol [122]. When humans eat plant foods phytosterols are ingested and are found in the serum and tissue of healthy individuals, but at concentrations orders of magnitude lower than endogenous cholesterol. Patients with phytosterolemia have increased amounts of phytosterols such as sitosterol and campesterol as well as the corresponding 5α-stanols in blood, plasma, erythrocytes, and different tissues. Elevated levels of cholesterol and cholestanol are also found in the circulation system of many patients [35,119,120].

In plants, steroid derivatives are synthesized by degradation of steroids; e.g. cholesterol to pregnenolone, sitosterol to progesterone, tomatine to allopregnenolone, ecdysone to poststerone, among other steroids [113]. Predective models for the microbial hydroxylation of steroids have been based on the results of the transformation of a large number of steroids by relatively few organisms [114]. The microbial hydroxylation of steroids provides a useful mild synthetic method for obtaining access to rare steroids [115–118].

Cholesterol and its precursor, cycloartenol are the starting materials for the biosynthesis of all the other plant steroids [113]. Structurally these plant sterols are 24-methyl and 24-ethyl derivatives of cholesterol. Saturated plant sterols, referred to as stanols, have no double bond in the ring system. A remarkable feature of these hormones is that they retain the side chains of their biosynthetic precursors, i.e., cholesterol, fucosterol, and campesterol. This is in contrast to adrenocortical and sex hormones where the side chain is completely or almost completely missing. The stereochemistry of the side chain is important for biological activity [121,122].

3. Extraction and isolation of non-saponifiable steroidal lipids

Quantitative extraction of liposoluble components from biological samples involves certain difficulties because of their high water content. Furthermore, extraction methods normally involve procedure which brings the glycerides into close contact with lipolytic enzymes, with the resulting risk of hydrolysis when the contact time is protracted, especially if temperature rises during sample treatment. Lipid extraction is quite simple for certain samples of biological material e.g., red blood cells, serum and plasma. On the other hand, the homogenization step needed for lipid extraction from various other animal and human tissues may complicate the procedure considerably. Isolation of lipids, including cholesterol and cholesterol esters, from tissue matrices in a relatively pure state is usually done by solvent extraction. The most exhaustive and widely cited study is the method of Folch et al. [236], in which

almost total extraction of lipids and pigments is described. This procedure uses a chloroform–methanol (2:1) mixture and has been modified by others [237–245].

Liquid–liquid, or liquid–solid extractions, as well as TLC, are classical techniques for separating lipid classes found in biological fluids, edible fats and oils. In contrast to edible oil studies, the analysis of lipids in food firstly needs an extraction of the food matrix in order to obtain a fraction that is free of non-lipidic components. The lipid fraction can then be further separated into lipid classes [91].

To perform the analysis of the total sterol oxidation products (i.e., the free plus the esterified components), extracted lipids (or oil) are subjected to saponification and separation by either silica or aminopropyl solid-phase extraction (SPE) reviewed recently by Ruiz-Gutiérrez et al. [91]. It has been used effectively to enrich oxysterols from cholesterol and other lipids and to avoid the formation of artifacts and/or breakdown of oxysterols during saponification [85,141].

Saponification serves to remove the triglycerides, and to saponify esterified sterols, thus allowing determination of free and esterified oxysterols. An alternative to the saponification involves alkali-catalyzed transesterification with KOH–methanol. The method was compared with saponification for sterol analysis, giving similar results [99].

Oxysterol formation is influenced by heat (especially for long exposure times), oxygen, light and UV, water activity, and the presence of unsaturated fatty acids. The rate of oxidation increases as the temperature rises. Operations avoiding air and light with added antioxidant (e.g., nitrite, ascorbic acid, phenols or α-tocopherol) and no storage of the sample are necessary to avoid artifactual by-products [25,39,72,105]. The drawback of hot saponification is the potential loss of labile compounds, such as the 5,6-epoxycholestanols [246], whereas cold saponification shows no artifact formation [247], however, requires long reaction times.

A milder, enzymatic procedure for the release of esterified cholesterol oxides was introduced by Nourooz-Zadeh [248]. However, that procedure involves several enrichment steps by TLC and solid-phase extraction, and therefore is time-consuming and hardly applicable for routine analysis. Some authors describe methods with only chromatographic separation of the free polar oxysterols from interfering apolar lipids without prior liberation of esterified sterols [249].

The widely used procedure described by Ishikawa et al. [250] for sample preparation and GC quantitation, consists of saponification with tetramethyl ammonium hydroxide in isopropanol followed by extraction with a mixture of tetrachloroethylene and methyl butyrate. To avoid artifacts, antioxidant is added and operations avoid air and light [2,43]. A modification of this procedure using only tetrachloroethylene for extraction after saponification, reports no formation of by-products [47].

Transesterification is known to be a good alternative to saponification as a means of liberating alcohols. Biedermann et al. [251] successfully used and described in detail an on-line LC–GC method for the determination of the sum of free and esterified sterols in edible oils.

Schmarr et al. [30] studied oxysterols in lipids from foodstuffs with a method involving Soxhlet lipid extraction, transesterification, SPE and GC analysis. The conditions for transesterification are mild compared with saponification, and triglycerides are converted to fatty acid methyl esters within minutes at room temperature (sterol esters need a slightly longer period of 1–2 h) [252].

Regarding the presence of oxysterols in milk powders, Dionisi et al. [32] compared four of the most frequently used methods for oxysterol analysis to identify the best. A method with direct saponification of the sample was compared with three methods involving preliminary fat extraction (Folch's, Radin's and Maxwell's methods) which differ by the use of different solvent mixtures: chloroform–methanol for Folch's method [236], 2-propanol–hexane for Radin's method [253] and dichloromethane–methanol for Maxwell's method [254]. After saponification, the samples were enriched by SPE on aminopropyl cartridges and analyzed by GC–MS. Labeled cholesterol was used to monitor artifact formation. The four methods (direct, Maxwell, Folch and Radin) were applied to the analysis of three milk powders containing different levels (low, medium, and high content) of oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, and 5α-cholestane-3β, 5α, 6β-triol). The

four methods were statistically compared in terms of standard deviation, coefficient of variation, artifact formation and accuracy. The direct saponification of the milk powder, without preliminary fat extraction, followed by aminopropyl–SPE and GC–MS was demonstrated to offer a good repeatability, an acceptable accuracy and the lowest cholesterol autoxidation (total artifact $<0.05\%$) compared to the three other methods.

Batta et al. [270] described a simple direct derivatization method for simultaneous GC quantitation of fecal bile acids and sterols where bile acids are subjected to *n*-butyl ester derivatization, without prior isolation from stool, followed by trimethylsilylation of the sterols and bile acids. The method was compared (fecal bile acids and sterols in five healthy individuals) with Soxhlet extraction (18 h with ammoniacal ethanol), Folch extraction method (chloroform–methanol, 2:1) and NaOH extraction method (1 *M* NaOH at 90°C for 1 h). The recoveries of bile acids and sterols on repeat analysis of fecal samples were found to be similar (within 5% range) to those obtained by methods where rigorous solvent extractions are used.

While the use of SPE for separating different classes of lipids from biological matter has been widely applied for a long time, its use is not very common in the analysis of edible fats and oils [25]. Amelio et al. [55] used NH_4^+ phase cartridges for isolating sterols, erythrodiol, uvaol and alcohols in olive oils. Nourooz-Zadeh and Appelqvist [133] analyzed sterols and sterol oxides from edible oils using the combined Lipidex-5000 and NH_2 cartridge system, resulting in a recovery of 85–88% depending on the compounds.

Recently, fractionation of lipids (including cholesterol and its oxidation products) by liquid–solid chromatography has been reviewed [91].

Currently, the need for easier and quicker analyses has prompted the development of new techniques [255]. Among them, the more outstanding ones have been the use of microwaves [256] and supercritical fluids [257,258]. Both procedures avoid the use of large volumes of solvent and provide rapid lipid isolations. Results from various types of biological samples obtained by microwave-assisted extraction were quantitatively and qualitatively compared to the Folch et al. method in feeds and powdered rat for all classes of lipids [271]. These were also similar to a solvent extraction method with a previous hydrolysis in milk and egg powders and meat samples [256].

Several researchers have compared supercritical fluid extraction (SFE) with conventional extraction methods [272]. Extractions that use only S-CO_2 usually yield good recoveries of nonpolar lipids [257]. To improve the extraction of nonpolar lipids, the polarity of S-CO_2 can be varied by using solvents such as methanol, ethanol, or even water. The presence of water dissolved in the supercritical fluid also increases the solubility of polar compounds, and it has been used successfully to analyze several dairy products [273]. SFE has recently been included in the recommended methods from the Association of Official Analytical Chemists (AOAC) to extract fat from oilseed [259].

Recently, solid-phase microextraction (SPME) has been introduced for the extraction of a broad field of environmental, food, biological and pharmaceutical samples. SPME has advantages such as simplicity, low cost, compatibility with analytical systems, automation and the solvent-free extraction. It has been combined with liquid chromatography, capillary electrophoresis, and gas chromatography [263].

4. Analysis of steroidal components in non-saponifiable matter by HPLC and GC or by combined HPLC–GC

Analysis of the free and esterified compounds of the non-saponifiable matter is possible by HPLC or GC after alkaline hydrolysis (saponification) of biological samples, plants or whole foods or their lipid extracts. Analysis of steroidal compounds of plants or whole foods or their lipid extracts in glycosylated forms requires hydrolysis of the glycoside bonds. Plant sterols may also be bound together with other lipids, to the carbohydrate matrix of the cereal which makes them unavailable for straight extraction with lipophilic solvents [231]. Thus, hydrolysis of the biological sample, may also be needed to release starch-bound lipids including sterols.

For quantitation it is necessary to measure sterols at the nanogram level [125]. Liquid chromatography with ultraviolet light absorption [3–8,13,15,16], flame ionization [19,25,27,31,154], differential index

detection are standard methods, but oxysterol light-absorbing esters [14] and oxysterol Δ^4-3-keto derivatives formed by action of cholesterol oxidase [15] may also be used. However, it is GC of sterol silyl ethers (or acetate derivatives [267]) coupled with electron impact or chemical ionization mass spectrometry that provides the most effective resolution, identification, and quantitation (by selected ion monitoring) [2,22,30,32].

Traditionally, the lipid group separations are carried out by adsorption on silica gel, followed by stepwise or gradient elution with a number of solvents. Steroids are usually present within a large excess of other lipids including mixtures of closely related analogs. Therefore chromatographic systems must be designed to perform correctly in the polarity range of the class of steroids being fractionated and also provide the capacity and sensitivity needed with biological extracts [92].

Chromatography of total lipid classes, such as acylglycerols, cholesterol esters, cholesterol, and phospholipids, can be performed with GC as normal-phase HPLC methods. Large capacity allows HPLC to be used in the preparation of adequate amounts of material for further tests. Chromatographic systems suitable for preparative HPLC may be derived from preliminary tests by TLC.

In normal-phase HPLC methods, lipid classes are chromatographed as single peaks, allowing separation and identification of lipid extracts of different tissues. For analytical purposes, a polar solvent has been used to displace several classes of lipids from the adsorption column. Reversed-phase partition has been used for class separations. For the separation of structurally related individual lipids within a class, gradients are often necessary.

The application of a dynamic chromatographic system, particularly reversed-phase HPLC, for the assessment of molecular hydrophobicity of structurally related compounds has been extensively investigated [8,126].

Quantitation of the steroids requires attention to recoveries in extractions, completeness of derivatization reactions, and the stability of the compounds to reactions used in the isolation and analytical procedures. Although there are chromatographic techniques available for separating and quantifying compounds, the lack of readily available standard compounds has limited the widespread use of these analytical tools [187].

The amount of sterols formed during analysis procedures have been estimated by adding isotopically labeled cholesterol as a control [33]. Labeled cholesterol is added at the beginning of the preparation, and mass spectrometric analysis is used to simultaneously monitor labeled and unlabeled oxidation products. Improved specificity and accuracy of measurement is gained by addition of isotopically labeled individual oxysterols [18,130,131].

Recently, Li et al. [274] described syntheses of deuterium- and fluorine-substituted analogs of a number of oxysterols of high current biomedical interest, including the 4β-hydroxy, 7α-hydroxy, 7β-hydroxy, 7-keto-, and 19-hydroxy derivatives of cholesterol and their analogs with 25,26,26,26,27,27,27-heptafluoro (F_7) and 26,26,26,27,27,27-heksadeuterio (d_6) substitution. The 7α-hydroxy, 7β-hydroxy, and 7-keto- derivatives of (25*R*)-cholest-5-ene-3β,26-diol and their 16,16-dideuterio analogs were also prepared. The A^5–3β,7α,26-and Δ^5–3β,7β,26-triols were regioselectively oxidized/isomerized to the corresponding Δ^4-3-ketosteroids with cholesterol oxidase. Also described were 5,6α-epoxy-5α-cholestan-3α-ol, its 5β,6β-isomer, cholestane-3β,5α,6β-triol, their F_7 and d_6 derivatives, and d_3-25-hydroxycholesterol. The 43 oxysterols and most synthetic intermediates were isolated in high purity and characterized by chromatographic and spectroscopic methods.

Zhang et al. described a method for determination of (24*S*),25-epoxycholesterol, (24*S*)-hydroxycholesterol, 27-hydroxycholesterol and 25-hydroxycholesterol in rat and mouse liver by derivatization to the Δ^4-3-ketones followed by HPLC [268]. To ensure that enones observed by HPLC analysis had not arisen from oxysterols produced by autoxidation during sample preparation, a chiral column was used under conditions that separated (*S*)- and (*R*)-epimers. Autoxidation would produce an essentially 1:1 mixture of these epimers, whereas only the (*S*)-forms are produced enzymatically.

Batta et al. [270] described a simple method for GC quantitation of human fecal bile acids and sterols where bile acids are subjected to *n*-butyl ester derivatization, without prior isolation from the stool, followed by trimethylsilylation of the sterols and bile

acids. Each class of compounds was resolved from the others. Bile acid derivatives are well resolved from each other and from the trimethylsilyl ether derivatives of fecal sterols and no overlap occurs. Cholesterol and coprostanol were the major fecal sterols. Significant amounts of the plant sterols, campesterol and sitosterol, were also present.

The fast screening and tentative identification of steroids can be carried out using the retention index method, developed originally for GC [127]. The incorporation of standards in HPLC analyses has been reported [128]. Retention indices have been widely adopted for recording and comparing retentions, for identifications [129] and as the basis of prediction methods for connection studies between chromatographic (GC, HPLC) data and biological data [155].

A technique has been developed recently that combines HPLC and GC to achieve in one process the isolation of a fraction, its transfer through an interface to the GC, and the GC analysis. This approach allows high separation efficiency and high sensitivity [61,62,170–172]. At present, most analytical methods for oxysterols are based on GC–MS with selected ion monitoring [2].

Recently, Sandhoff et al. [275] presented a method for determination of cholesterol at the low picomole level by nano-electrospray ionization tandem mass spectrometry (nanoESI-MS–MS). The method is based on a simple one-step chemical derivatization of cholesterol to cholesterol-3-sulfate by a sulfur trioxide–pyridine complex. [$^{13}C_2$]Cholesterol is added as an internal standard. The determination of free cholesterol is demonstrated in about 250 cells of a chinese hamster ovary (CHO) cell line. In order to determine inter-assay variability, the cholesterol concentration in CHO cells was also quantified by an enzymatic fluorescence assay with cholesterol oxidase. With the nanoESI-MS–MS assay, the concentration of free cholesterol was determined to be 22.1±1.3 fmol/cell (±5.9% SD) and with the fluorescence assay to 19.3±4.1 fmol/cell (±21.5% SD). This derivatization method can be used to measure sterol mixtures as they are given by biological samples. With the nanoESI-MS–MS, free cholesterol could be quantified in biological membranes down to approximately 5 pmol of total free cholesterol. This corresponds to a sensitivity about 10-fold higher than an enzymatic fluorescence method.

5. Sterols and their oxygenated derivatives

The most studied fraction of the non-saponifiable matter is the sterol one, which has been analyzed frequently by both HPLC and GC [2]. Oxidation products indicative of hydrogen abstraction at the 17-, 20-, 22-, 23-, 24-, 25-, 26-, and 27-positions of cholesterol have been detected [2]. However, the analytical methods to determine cholesterol oxidation have until recently focused on the products of the primary hydrogen abstraction site, the allylic 7-position such as 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, along with selected other products of hydrogen abstraction at the 5- and 6-positions such as 5α,6α- and 5β,6β-epoxycholesterol, and 5α-cholestane-3β,5α,6β-triol [71–74,86].

There are several methods for oxysterol analysis: methods involving a preliminary fat extraction followed by a saponification [34,53,137,138] and direct saponification or transesterification of samples [30,32,33]. Vatassery et al. [153] have described a procedure which involves saponification followed by a single solvent extraction step and determination of the hydroxy and keto cholesterols by GC–MS.

Methods involving a fat extraction differ by the use of different solvent mixtures. More recently, other methodologies have tried to improve those extraction methods proposed previously [32,140].

Silver ion HPLC has been successfully used for the determination of several C-27 sterols differing in the number and location of olefinic double bonds [9]. A number of workers have separated C-27–C-29 sterols by carbon number, using reversed-phase partition liquid chromatography. Cholesteryl esters have been separated by chain length and by degree of unsaturation [126].

The analysis of sterols and oxysterols in most recent studies [157] observed that sterols that cannot be separated by other capillary columns are efficiently separated on a polar column (65% phenyldimethylpolysiloxane).

Methods for the simultaneous analysis of cholesterol and cholestanol include GC [158,159], GC–

MS [160] and HPLC with fluorescence detection [161] and UV detection [162].

Recently, Phillips et al. [29] reported precise quantitative determination of phytosterols, stanols, and cholesterol metabolites in human serum by GC. Quantitation of serum sterols and stanols (5,6-dihydrosterols) is complicated by the presence of numerous other lipids and low concentrations (~0.2–13 μg/ml) of the analytes relative to cholesterol (~1500–30 000 μg/ml).

Cholesterol in food can be oxidized leading to the formation of more than 30 oxysterols [72–74]. Substantial levels of oxysterols in food products have been reported [72,73]. In most preparations, the major oxysterols were 7-oxygenated sterols (7β-OH-, 7α-OH-, and 7-ketocholesterol) and 5,6-oxygenated sterols (5α,6α-epoxy- and 5β,6β-epoxycholesterol, 5α,6β-diOH-cholesterol).

Paniamgvait et al. [72] summarized levels of various types of oxysterols in foods of animal origin. Seven species of oxysterols were identified in processed meats and meat products, although fresh meat had none or only trace amounts of them. Recently, Osada et al. [27] examined cholesterol oxidation in meat products (7α-, 7β-, and 25-hydroxycholesterols, 5α- and 5β-epoxycholesterols, 3,5-cholestadien-7-one, cholestanetriol, and 7-ketocholesterol) and studied the application of apple polyphenols as possible antioxidants for meat lipids, especially cholesterol.

Schmarr et al. [30] evaluated a method allowing quick and reliable analysis of polar oxysterols. Detection of oxysterols is in the low ppm range (mg/kg fat), close to the detection limit of this method (0.1–0.5 ppm). After Soxhlet-extraction, the fat was transesterified under mild conditions, thereby minimizing degradation and allowing determination of the free and esterified cholesterol oxides. Sample fractionation was achieved with aminopropyl solid-phase extraction cartridges and a stepwise elution with hexane, hexane–methyl *tert*.-butyl ether, and acetone to separate polar cholesterol oxides from cholesterol and other lipid products. Further analysis of the trimethylsilyl derivatives was performed by GC–flame ionization detection (FID) or GC–MS. A phytosterol oxide such as sitosterol α-epoxide (24-ethyl-5α,6α-epoxycholestan-3β-ol) was employed for the first time as an internal standard for the quantification of cholesterol oxides in foodstuffs of animal origin.

6. Other steroidal alcohols

Sterols, triterpenic alcohols, methylsterols, and triterpenic dialcohols are important components in vegetable oils [19,25,111,112]. The composition of the free and esterified forms of sterols, alcohols and triterpenic alcohols are not identical, and different extraction procedures or refining methods have different effects on free and esterified constituents. In addition, the ratio of free/esterified alcohols is related to the oil quality [166].

Conventional methods for quantifying sterols, aliphatic and triterpenic alcohols as trimethylsilyl derivatives involves isolation by TLC or semipreparative HPLC followed by capillary GC analysis, with low-polar stationary phase (5% phenyl–methylsilicone) and FID of the fraction [136,167]. More polar phases (50% phenylmethylsilicone) has been used to detect peaks usually not resolved with non-polar columns [169].

Official methods for isolation of the total sterols and alcohols from olive oils includes saponification of the oil, extraction of the non-saponifiable matter with diethyl ether and washing of the extract with water. The extract is fractionated by TLC on silica gel plates yielding four separated bands containing; aliphatic alcohols, together with triterpenic alcohols, methylsterols together with oleanolic aldehyde, sterols and triterpenic dialcohols [136]. Recently, HPLC separation using a silica gel column and refractive index detection has been carried out with better results compared with the official TLC method [167].

With on-line coupled HPLC–GC, free erythrodiol and uvaol, two triterpenic dialcohols has been analyzed without saponification [170,171].

Lechner et al. [25] determined tocopherols and sterols in vegetable oils after removing the bulk lipid material by solid-phase extraction for the quantitative separation of the minor components. By subsequent injection to GC analysis, qualitative and quantitative information about tocopherols, free sterols and sterol

esters in vegetable oils are provided in a single analytical run. The method requires short analysis time and provides the possibility of complete automatization.

The amount of methylsterols (4-methylsterols) found in food matrices is relatively low as compared to that of other components of the non-saponifiable matter [163]. Similarly to triterpenic alcohols, isomerization of methylsterols after refining or hydrogenation allow distinction of illegal addition of refined oils to virgin olive oils [164].

The triterpenic alcohols, also known as 4,4-dimethylsterols, have a steroid structure and are present in all vegetable lipids. They are more polar than tocopherols and are often poorly separated in silica TLC but may be analyzed by GC. Triterpenic alcohols and 4-methylsterols show similar chromatographic properties to sterols. They can strongly influence in some cases the exact determination of sterols by GC if no preceeding group separation of groups is done [25].

Van Boven et al. [168] isolated and identified the free sterols (4-demethylsterols, 4-methylsterols, 4,4-dimethylsterols) and free fatty alcohols in jojoba oil by means of GC–MS of the free alcohols. The method consists in the separation of these compounds from wax esters in the oil by means of an aluminum oxide column followed by further fractionation of the minor components by column chromatography on silica gel.

The cholesterol-lowering effect of rice bran oil (RBO) has been reported by several investigators [60,110]. A number of studies have suggested that the non-saponifiables in the RBO may play a very important role in the cholesterol-lowering effect of this oil which, unlike most vegetable oils, contains a relatively high proportion of non-saponifiable lipids. The nonsaponifiables of RBO contain sterols (43%), triterpene alcohols (28%), 4-methyl sterols (10%), and less polar components (19%) [110].

Kawanishi et al. [173] used TLC with reversed-phase plates to separate alcohols from other interfering material in seed oils. A further development in the isolation of the sterol and alcohol fractions is to pass the non-saponifiable matter through an aluminum oxide column [174] to avoid the extraction and washing steps. This method has been standardized by the International Standard Office (ISO) [175].

7. Steroidal hydrocarbons

Phytosterols can undergo transformation during refining of oils to form isomers and dehydrated products [23]. It is likely that free and esterified sterols behave differently under processing and the content of free and esterified sterols in the oil is therefore of interest [173]. High temperature may cause sterols to isomerize with a shift of a double bond, and dehydrate with the formation of steroidal hydrocarbons (sterenes or steradienes) [31]. 3,5-Steradienes are the main dehydration products of Δ5-sterols, but other isomers such as 2,5-, 4,6-steradienes [174], 3,5-cyclo-6-enes and 2,4,6-trienes [175] are formed together with degradation products of Δ7-sterols, methylsterols and triterpenic alcohols.

In virgin olive oil, the major hydrocarbon is squalene (2,6,10,15,19,23-hexamethyl-2,6, 10,14,18,20-tetracosahexane). During refining, squalene isomerizes [176] yielding a number of components with molecular mass 410. In the same way isoprenoid alkenes of molecular mass 408 have been characterized [177,178] and are attributed to dehydration products of oxidized squalene [179].

Significant amounts of hydrocarbons with steroidal skeleton are formed in vegetable oils during thermal treatment, including stigmasta-3,5-diene which is abundant in all refined vegetable oils since it derives from sitosterol by dehydration [180]. Some oxidation products of β-sitosterol and stigmasterol have been studied because of their possible toxicity similar to the corresponding oxidation products of cholesterol [133,134].

The composition of steroidal hydrocarbon fraction can identify the origin of the oil, because their composition reflects that of the sterols [181].

For quantitative determination of stigmastadienes in crude vegetable oils, the hydrocarbon fraction is analyzed by GC on a fused-silica capillary column coated with 5% phenyl methylsilicone. The method has been adopted by the European Union [182] and the International Standard Office [183].

The determination of sterenes can detect the

addition of refined oil to extra virgin olive oil to as low as the 1% level. This analysis is now a European Union Official analytical method for the control of extra virgin olive oil (Regulation EC/656/95) [182].

8. Other plant steroids

Sterols are known to occur in different quantities in various parts of plants, and different sterols predominate in different parts during the life cycle of some plants. However, little is known about the role of sterols in plants other than the well-known role as a component of cell membranes [144]. However, in the field of human nutrition, it is known that some phytonutrients are consistent with reduced risk of cancer and other chronic diseases [147,149,184,185]. In recent years, an extensive set of qualitative and initially semi-quantitative, then more quantitative dietary epidemiologic data has been developed [148]. Although it is uncontroversial that food composition databases used to evaluate the relationships between diet and diseases need to be as accurate and reliable as possible, data on plant sterols has not usually been included in food composition databases [88,89]. Very few validation data about plant sterols [267] have been presented by most published methods [73].

Although strict structure–activity relationships are not apparent across all phytonutrient classes, phytosterols and saponins, both of which have cyclic structures similar to cholesterol, are known to possess the ability to bind cholesterol and bile salts [150].

Brassinosteroids are potent plant growth regulators. They are polyhydroxylated steroids differing in their functionalities and the stereochemistry present in the A and B rings and the side chain. Structure– activity relationships of brassinosteroids have been conducted to identify key features that are required for biological activity [186,188]. Brassinosteroids have the capability of improving crop yield and quality, minimize environmental stress and herbicidal injury, and control pathogenic diseases [188].

Akihisa et al. [189] have reported the isolation and structure elucidation of a novel cycloartane triterpenoid from *Bryonia dioica* along with eight novel sterols [190] and four novel triterpenoid alcohols [191]. The novel triterpenoid was extracted with chloroform from air-dried and ground aerial part of *B. dioica*. The non-saponifiable lipid, obtained from the extract by alkaline hydrolysis, was subjected to column chromatography which yielded fractions of triterpenoid alcohols (fraction 1), oxygenated triterpenoid alcohols and 4-methylsterols (fraction 2), sterols (fraction 3), and dihydroxy triterpenoids (fraction 4). The fractions were then acetylated and the resulting acetates were subjected to preparative reversed-phase HPLC for isolation of individual compounds.

Many triterpenoids have been isolated from *Ficus fistulosa* as part of a biodiversity study [192]. Anjaneyulu et al. [193] reported that the neutral lipid fraction of *Mangifera indica* yielded two new triterpenoids besides several known triterpenoids namely cycloartenone, 24-methylene cycloartanone, friedelin, taraxerone, friedelan-3β-ol, α-amyrin, β-amyrin, cycloartenol, 24-methylene cycloartanol, sitosterol, 11α,12α-oxido-taraxerol, 6β-hydroxy-stigmast-4-en-3-one, 24-methylene cycloartane-3β,26-diol and a C-24-epimeric mixture of cycloartane-3β,24,25-triol. The acidic fraction of the same extract on column chromatography gave three new triterpenoids.

Fujimoto et al. [194] found that biosynthesis of sterol and ecdysteroids in *Ajuga* hairy roots produce 20-clerosterol, 22-dehydrocholesterol, and cholesterol as sterol constituents, and 20-hydroxyecdysone, cyasterone, isocyasterone, and 29-norcyasterone as ecdysteroid constituents.

Several biologically active triterpenes and sterols have been isolated from mushroom *Canoderma lucidum* and proved effective as cytotoxic, antiviral and anti-inflammatory agents [195]. New 24-methylene tetracyclic triterpenes that possess the lanostane skeleton have been isolated from the stem barks of *Polyalthia lamcilimba* [196].

An investigation of the extracts from bulbs of *Allium porrum L.* led to the isolation of many spirostanol saponins [197]. Mimaki et al. isolated many new steroidal saponins [198] and Ohtsu et al. many new tetracyclic triterpenes [199]. Recently, a new steroidal saponin, dumoside, characterized as (20*S*)-3β, 16β-dihydroxypregn-5-ene-22-carboxylic acid (22,16)-lactone-3-*O*-β-chacotrioside, was isolated from the whole plant of *Aspargus dumosus*

Baker and the structure was deduced from spectral data [200].

Recently, many bioactive compounds such as withanolides [201–203], phytoecdysteroids [204,205], triterpenes [206–212] and sterols [213–221], glycosides [222–230], and a number of brassinosteroids [231–235] have been studied and their structures elucidated.

9. Conclusions

Despite extensive development and abundant literature on the analytical methods suitable for sterol quantification, there is still controversy about the reliability of presented data [2]. A large variety of methods have been described, but they are difficult to compare and lead to different results. The quantitative relationships between various sterols is important, but data on the quantity of total sterols on a mass or per-cell basis is lacking in many publications, as is the use of recovery standards and controls for artifact formation during procedures.

Reliable routine analysis is still a difficult and lengthy procedure. Most oxidation products are present at a low ppm or ppb level, their polarities vary from apolar to very polar, oxidation products may be present in free or esterified form, and components are likely to oxidize or otherwise degrade during sample preparation. An internal standard is required for reliable quantification, and peak assignment verification should be supported by a MS method providing additional information about peak purity [22,27,30,32,49,84]. High-purity reagents and solvents, good collection techniques and highly sensitive analysis are required in order to accurately identify and quantify non-saponifiable components in lipids [142].

Some authors have compared their results with other well established methods. Further development is needed since each biological system is unique. Conditions must be investigated and improved in order to optimize yields for each sample type. The standardization of laboratory analyses using faster procedures with lower detection limits [262], definition, mode of expression and units, has been undertaken by international authorities, and should be promoted.

The metabolic distinction between dietary and endogenously formed oxysterols is of importance. Little is known of the absorption of dietary oxysterols from foods [74,276]. However, efforts are in progress to elucidate the physiological process as well as the storage conditions that can control the content of phytonutritients in foods [81,260,261].

Although some direct derivatization GC methods have turned out to be comparable with methods where rigorous solvent extractions are used, some new sample preparation methods such as SPME combined with HPLC might provide the method of choice for the more polar and temperature sensitive sterols. SPME should provide increased sample loads, decreased labor expenses, and less exposure to chemicals, enhanced productivity and quality of data with increasing regulatory constraints, and integration of information management systems concerning also an integrated database system for nutritional studies [88,264–266].

10. Further reading

[109]; [132]; [139]

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