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Review

Chromatographic analysis of plant sterols in foods and vegetable oils

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Abstract

This paper reviews recently published chromatographic methods for the analysis of plant sterols in various sample matrices with emphasis on vegetable oils. An overview of structural complexities and biological/nutritional aspects including hypocholesterolemic activities of phytosterols is provided in the Section 1. The principal themes of the review highlight the development and application of chromatographic techniques for the isolation, purification, separation and detection of the title compounds. Pertinent gas chromatographic and high-performance liquid chromatographic methods from the literature are tabulated to illustrate common trends and methodological variability. The review also covers specific analyses of natural/synthetic standard mixtures to shed light on potential applicability in plant sample assays. Examples of combined chromatographic techniques linked in tandem for the analysis of complex samples are included. Elution characteristics of sterol components are discussed in the context of analyte substituent effects, structural factors and stationary/mobile phase considerations. Published by Elsevier Science B.V.

Keywords: Reviews; Vegetable oils; Food analysis; Photosterols; Sterols

Contents

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1. Introduction

Sterols are a group of naturally occurring substances derived from hydroxylated polycyclic isopentenoids having a 1,2-cyclopentanophenthrene structure (Fig. 1). These compounds contain a total of 27–30 carbon atoms (the number of carbon atoms in the biosynthetic precursor squalene oxide) in which a side chain with carbon atoms ≥7 is attached at the carbon 17 position (C-17). Their structures are closely related and varied depending on the extent of modifications of the ring system and side chain variations [1]. Thus, the number and position of double bonds in both the polycyclic and side chain systems of sterols can be different. In addition to the reduction of the A ring size to a pentacyclic variant as in A-nor sterol, the side chains can also be broadened, lengthened, or shortened at certain carbon positions beyond C-22. In general, the sterols can be categorized into three subclasses (Fig. 1): (I) 4,4-desmethylsterols (Table 1); (II) 4α-methylsterols (Table 2); and (III) 4,4-dimethylsterols (Table 2).

Sterols are known to have a wide range of biological activities and physical properties. Plant sterols (i.e. phytosterols), in particular, are important agricultural products for health and nutrition industries. They are useful emulsifiers for cosmetic manufacturers and supply the majority of steroidal intermediates and precursors for the production of hormone pharmaceuticals [2]. A number of plant sterol with specific structures are known to inhibit oxidative deterioration of oils serving as potential antipolymerization agents for frying oils. Hypocholesterolemic activities of some phytosterols (e.g. soy sterols, vegetable oil components and sitosterol) have been documented [3–6]. The saturated analogues of phytosterols and their esters have been suggested as effective cholesterol-lowering agents

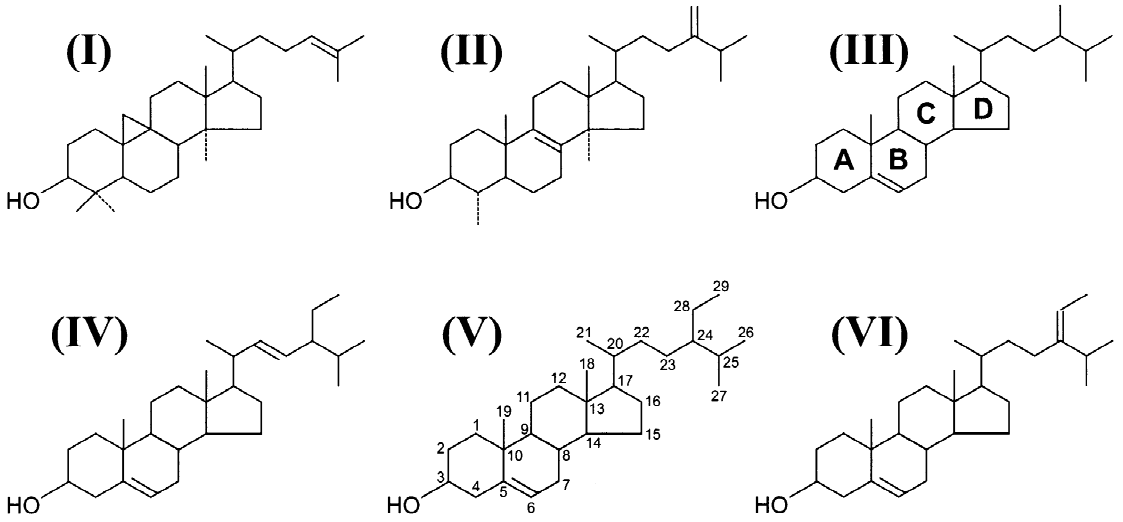

Fig. 1. Examples of ubiquitous plant sterol structures: (I) 4,4-dimethylsterol, cycloartenol; (II) 4-methylsterol, obtusifoliol; (III) 4-desmethylsterol, campesterol; (IV) stigmasterol; (V) sitosterol; (VI) Δ5-avenasterol.

Table 1
Selected natural occurring 4,4-desmethylsterols

Desmethylsterol	
5-Avenasterol (5AV)	7-Avenasterol (7AV)
Brassicasterol (BR)	22,23-Dihydro-BR (2223HBR)
Campesterol (CA)	Chondrillasterol (CD)
25-Dehydro-CD (DCD)	Campestanol (CAa)
7-Campesterol (7CA)	7-Campestenol (7CAe)
Cholestanol (CHa)	Cholesterol (CH)
Clerosterol (CL)	Clionasterol (CLI)
Crinosterol (CR)	22,24-Dihydrobrassicasterol (2224HBR)
Desmosterol (DE)	Ergostanol (EGa)
Ergosterol (EG)	Ergostatetraenol (EGT)
Fucosterol (FU)	Fungisterol (FN)
Isofucosterol (IFU)	24-Methylencholesterol (MCH)
Poriferasterol (PO)	22,23-Dihydro-PO (HPO)
Poriferastenol (POe)	Schotenol (SC)
Sitosterol (SI)	Sitostanol (SIa)
α-Spinasterol (SP)	25-Dehydro-SP (DSP)
5,23-Stigmastadienol (523STD)	5,24-Stigmastadienol (524STD)
Stigmastanol (STa)	7-Stigmastenol (7STe)
22,7-Stigmastenol (227STe)	Stigmasterol (ST)
7,22,25-Stigmastatrienol (72225STT)	7,25-Stigmastadienol (725STD)
Vernosterol (VE)	Zymostenol (ZYe)
Zymosterol (ZY)	

Abbreviations in parentheses. Some sterol standards can be obtained from Sigma (St. Louis, MO, USA), Matreya (Pleasant Gap, PA, USA) and Spectrum Chemical Manufacturing Corp., Gardena, CA, USA). Some standard materials of steryl *p*-coumarates and ferulates can be obtained from Tsuno Rice Fine Chemicals (Wakayama, Japan) and CTC Organics (Atlanta, GA, USA). Other detailed source information on standards can be obtained from references cited in this review.

Table 2
Selected natural occurring 4-methylsterols and 4,4′-dimethylsterols

4-Methylsterol	4,4′-Dimethylsterol
Citrostadienol (CI)	α-Amyrin (αAM)
Cycloeucalenol (CE)	β-Amyrin (βAM)
Gramisterol (GR)	Butyrospermol (BU)
Lophenol (LO)	Cycloartanol (CYa)
4α-Methylzymostenol (MZYe)	Cycloartenol (CYe)
4α-Methylzymosterol (MZM)	Cyclobranol (CB)
31-Norcycloartenol (NCYe)	Cyclolaudenol (CYL)
31-Norlanostenol (NLAe)	Lanostenol (LAe)
Obtusifoliol (OB)	Lupeol (LU)
Methylvernosterol (MVE)	24-Methylencycloartanol (MCYa)
Ethyllophenol (ELO)	24-Methylenlanostenol (MLAe)
31-Norlanosterol (NLA)	Parkeol (PA)
	4,4′,14-Timethyl-24-methylencholesterol (MMCH)
	Tirucalladienol (TI)
	Dihydrolanosterol (HLA)
	Erythrodiol (ER)
	Uvaol (UVA)
	Euphol (EU)

Abbreviations in parentheses. For sources of standards, see footnote to Table 1.

offering cardiologic health benefits [4]. Commercial margarines formulated with certain levels of phytosterols are currently available in several countries.

While cholesterol is present in animals in relatively high abundance, plants, with very few few exceptions, produce negligible amounts of this compound. Reported phytosterol data [7] for some plant foods and vegetable oils have shown that nuts and oils contain higher levels ($\geq$1%) of sterols than fruits and vegetables ($<$0.05%) (Table 3). It is noteworthy that the compositional distributions of phytosterols in certain vegetable oils have been used for their identification, despite their presence in the lipids as minor constituents [8–13]. Hence, phytosterols and other non-saponifiable compounds in oils are often used as markers for the assessment of adulterated oils [14–29].

To evaluate phytosterols mixed with a diversity of other non-saponifiable components in food lipids of complex sample matrices is a formidable task and requires reliable analytical techniques for the extraction, isolation, separation, purification, detection and quantitative data analyses [140]. Isolation and enrichment of sterols from plant tissues or oilseeds entails initial solvent extraction, supercritical fluid extraction (SFE), or supercritical fluid fractionation (SFF) followed by various clean-up and chromatographic procedures. For subsequent characterization and quantification of sterol compounds, the crude isolate can be purified and separated by a wide variety of chromatographic techniques including column chromatography (CC), gas chromatography (GC), thin-layer chromatography (TLC), normal-phase high-performance liquid chromatography (HPLC), reversed-phase HPLC and capillary electrochromatography (CEC). The sterols can be detected with flame ionization detection (FID), UV detection (UV), evaporative light scattering detection (ELSD), infrared detection (IR), nuclear magnetic resonance detection (NMR) and mass spectrometry (MS). With the advent of sophisticated column technologies, complex mixtures of sterols can be efficiently separated.

Table 3
Some reported sterol concentrations in selected foods and vegetable oils (mg/100 g) [7]

Food	Phytosterol
Potato	5
Tomato	7
Pear	8
Lettuce	10
Carrot	12
Apple	12
Onion	15
Banana	16
Fig	31
Garbanzo bean	35
Kidney bean	127
Soybean	161
Pecan	108
Almond	143
Cashew nut	158
Peanut	220
Sesame seed	714
Peanut oil	207
Olive oil	221
Soybean oil	250
Cottonseed oil	324
Safflower oil	444
Sesame oil	865
Corn oil	968
Rice bran oil	1190

A cursory literature survey revealed that most investigators have preferred capillary GC techniques as the methods of choice for the analysis of sterols and related compounds. In some cases, GC appears to provide greater selectivity for certain isomers than HPLC. The increasing public interest in the cholesterol-reducing capacity of phytosterols has provided impetus to review existing chromatographic methods for the analysis of sterols in plant samples with emphasis on vegetable oils.

2. Isolation and enrichment procedures

Isolation techniques depend largely on the nature of the sample source and vary among solid and liquid samples. Plant-derived sterols in tissues and oilseeds can be isolated by solvent extraction with chloroform–methanol [94], hexane [35,76,109], methylene chloride [114,116] or acetone [108] followed by saponification and chromatographic purification for obtaining enriched total sterols. Mixing of solvent with homogenized materials can be achieved by shaking the mixtures in heated sealed tubes for 1–18 h [30] or by refluxing with a Soxhlet extractor [31]. Alternatively, the plant samples can be ex-

tracted by SFE [38–40,42] with supercritical carbon dioxide (CO_2) to obtain total lipid extracts from which sterols can be enriched and isolated after sponification or SFF along with, if necessary, additional sample clean-up using various chromatographic techniques. As compared to solvent extraction, SFE is an environmentally more acceptable new technique which provides a more convenient way for sample extraction with reduced loss of sterol analytes. Either continuous solvent extraction with solvents of suitable polarity or repetitive SFE of oilseeds can afford sterol extracts in quantitative yields (95–100%) with minimum sample losses. Recovery studies must be incorporated into sample extraction procedures to determine extraction efficiences.

2.1. Solvent extraction and saponification

In a typical procedure for vegetable oilseeds [35], a sample is homogenized in a coffee bean grinder (or any commercially available grain mill) and extracted with absolute ethanol in a Soxhlet apparatus overnight in a steam bath. Water and petroleum ether are added to the cooled extract and shaken in a separatory funnel. Evaporation of the top organic layer under water aspirator pressure leaves the total lipid extract. For saponification of the lipid extract or crude/refined vegetable oils [32–35], an aliquot of the oil sample is stirred overnight at room temperature with 1 *M* ethanolic potassium hydroxide. The mixture is diluted with water and extracted with three portions of diethyl ether. The combined ether extract is saponified again with ethanolic potassium hydroxide, washed with several batches of distilled water until neutral to pH paper and then dried sequentially with short columns of anhydrous sodium sulfate, deactivated alumina and anhydrous sodium sulfate. Removal of solvent yields an unsaponifiable residue suitable for chromatographic quantification of sterols.

2.2. Supercritical fluid extraction and fractionation

As in solvent extraction, the choice of a SFE method is determined by the type of plant under study [30,38–43]. Experimental parameters such as the CO_2 flow-rate, pressure, temperature and extraction time vary with sample sizes, analytical/pilot scales, analyte levels and instrument varieties requiring optimization to achieved maximal extraction efficiency. It is essential to maintain adequate flow and pressure for obtaining rapid and complete SFE. In most cases, the efficiency of SFE is comparable to that of solvent extraction. Generally, a sample (2–10 g) of ground or flaked oilseeds including homogenized plant materials is extracted in a commercial automated SFE extractor with supercritical CO_2. The CO_2 flow-rate, pressure, extraction temperature and extraction times are set at 2–2500 ml/min, 5000–12 000 p.s.i., 40–80°C and 10–130 min, respectively (1 p.s.i.=6894.76 Pa). Repetitive extractions are often needed to obtain sufficient materials. The collected total lipid extracts are pooled and dissolved in hexane and stored in a freezer for later enrichment of sterols by saponification or SFF. When using SFE, it is important to do recovery studies on specific matrix being assayed.

To avoid saponification, sterols and sterol esters in total lipid extracts or vegetable oils can be fortified by coupled SFE–SFF [40,43]. Since differential solute molecular mass/chemical property factors govern the success of fractionation, it is difficult to isolate pure sterol fractions without co-extraction of other components (e.g. triglycerides). Optimization with various packed columns of normal/reversed-phase sorbents provides variable degrees of sterol enrichments. SFE–SFF is an ideal technique for the direct concentration of sterol esters, which are vulnerable to saponification.

3. Purification techniques

Of all chromatographic isolation/separation techniques, CC and TLC procedures [44] employ the most accessible and affordable equipments and instruments notwithstanding their obvious inadequacy in analytical precision. Complex and/or large-quantity samples of more than 200 mg can be conventionally evaluated by CC, whereas relatively simple and/or small-quantity samples of less than 200 mg can be assessed by TLC. Both CC and TLC techniques are suitable for sample clean-up, purification, qualitative assays and preliminary estimates of the sterols in test samples. Often qualitative and quantitative analyses

of sterols in samples of vegetable oils, oil hydrolysates, or SFF fractions are carried out directly bypassing CC and TLC purification steps.

3.1. Column chromatography

For initial sample clean-up and isolation, a crude lipid extract (1 g) is loaded onto a column of silicic acid (60 g) and eluted with pure and mixed solvents. Co-eluting with some other lipid components, steryl esters, sterols (4,4-dimethylsterols/4α-methylsterols/4,4-desmethylsterols) and sterylglycosides are found in fractions of hexane–diethyl ether (1:1), diethyl ether and methanol, respectively [45,46]. In other situations, an aliquot (1 g) sample of crude lipid extracts, non-saponifiable fractions, or SFF enriched fractions can be chromatographed in a column packed with deactivated alumina (3% water) (100 g) for subclass separations. Solvent gradient elution of the sample affords subclasses of steryl esters, 4,4-dimethylsterols/4α-methylsterols, 4,4-desmethylsterols and sterylglycosides in fractions of 10–30% ether in hexane, 40–50% ether in hexane, 60–70% ether in hexane and ether–methanol, respectively [31,47–49]. If necessary, the subclass fractions can be purified by reversed-phase CC on Sephadex LH-20 or Lipidex 5000 [50]. To quantify and characterize individual sterol components, each CC subclass fraction that may contain non-sterol contaminants or unresolved sterols can be further purified by small-scale adsorption CC, reversed-phase CC, or argentation CC on silver nitrate-impregnated silica gel or alumina. Generally, the selection of these CC methods is dictated by solute polarity, sizes and olefinic structures [47].

3.2. Thin-layer chromatography

Subclass fractions of small amounts (<200 mg) of lipid extracts or non-saponifiable oil extracts can be preliminary separated by silica gel TLC with suitable developing solvents. Visualization of TLC spots or bands is normally carried out with a UV lamp on a silica gel plate impregnated with a fluorescence indicator (e.g. dichlorofluorescein) or by spraying the plate with 50% sulfuric acid in ethanol (or 80% potassium dichromate in sulfuric acid) followed by destructive carbonization. Preferably, a TLC lane of standard reference compounds sprayed with a non-destructive ethanolic solution of rhodamine or fluorescein is used to identify the sample bands, which are then scraped off from the plate and extracted with diethyl ether. Separations are significantly improved by two-dimensional TLC with multiple solvent development. Depending on the sample load and plate thickness, TLC can be performed in the analytical and preparative modes.

It has been reported [51] that TLC of an unsaponifiable matter in a refined oil with hexane–diethyl ether (7:3) leads to distinctly separated spots of three sterol subclasses in an ascending order (4,4-desmethylsterol→4-methylsterol→4,4-dimethylsterol), which is consistent with decreasing solute polarity. Other mobile phases (e.g. benzene–ether, 9:1) have been used for the separation of sterol subclasses and their esters in various sample matrices [31,52,53]. Crude TLC subclass fractions can be further purified by reversed-phase CC prior to the resolution of individual sterols from each subclass using small-scale (<10 mg) adsorption, reversed-phase, or argentation TLC techniques [54,55]. It must be stressed that in spite of the operational convenience and simplicity, the TLC resolution of individual sterol components is less efficient than CC or HPLC.

Commercial HPTLC plates can also be used in the lipid analysis [56]. With the recent introduction of an automated multiple development (AMD) technique utilizing computerized gradient optimization [57], lipid extracts with complex analyte components of different polarity have been separated directly, without alkaline treatment, by AMD of HPTLC plates in conjunction with 25-step mobile phase gradient of methanol, diethyl ether and hexane [58]. After visualization with a solution of cupric sulfate–phosphoric acid in aqueous methanol, the plates are inspected with a TLC scanning densitometer. The densitometric chromatogram peaks due to sterols, sterol esters and other endogenous compounds are quantified. A preparative TLC method for the fractionation of phytosterols in plant oils has been described [59]. In the procedure, an unsaponified oil extract is streaked on an activated tapered silica gel preparative plate and developed with light petroleum diethyl ether (70:30). The phytosterol bands are extracted with chloroform–diethyl ether (80:20)

followed by the high-resolution GC analysis of the sterol analytes as their acetates.

3.3. Solid-phase extraction

It is well recognized that CC or TLC procedures for purification of oil extracts or non-saponifiable fractions that contain a host of compound classes (e.g. hydrocarbons, carotenes, tocopherols, tocotrienols, linear fatty alcohols, triterpenic alcohols and sterols) are very time-consuming and tedious. A solid-phase extraction (SPE) technique has proved to be a viable alternative for vegetable oil analyses. SPE is in some respect advantageous over CC or TLC because it can be done in a short time and uses only small volume of solvent. In an optimized reversed-phase mode [60], a sample of the unsaponifiable extract (pH adjusted to 3.5) is loaded onto an octadecylsilica SPE cartridge pre-conditioned sequentially with methanol and water. Elution with chloroform–methanol (95:5) affords the purified sterol fraction. After evaporation of the solvent, the material is ready for GC quantification. On the other hand, in an optimized normal-phase mode [61,62], a vegetable oil sample, without saponification, is directly treated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide in pyridine to give the trimethylsilyl (TMS) derivatives of all the oil components that have the hydroxyl functionality. The derivatized material is then applied to a silica SPE cartridge pre-conditioned with hexane–*tert*.-butyl methyl ether (99:1) followed by elution with the same solvent system to yield an eluate analyzable for hydrocarbons, tocopherols, sterols and sterol esters by GC.

4. Gas chromatography

4.1. General considerations

GC is the most frequently use technique for the analysis of sterols [47,63–65]. Consistent with the progress in column technologies, GC column systems have evolved from packed columns (3–8 mm I.D.) to capillary columns (0.1–0.3 mm I.D.). Glass- and fused-silica capillary columns contain respective coated and chemically bonded stationary phases of variable polarity. The latter fused-silica capillary column has deposed the former and has been widely accepted as the column of choice by virtue of its durability and flexibility. When high-temperature capillary GC columns (e.g. phenylmethylsilicone) are used, sterol sample assays can be achieved with high degrees of detection sensitivity and component resolution. In a typical analysis, GC is interfaced with FID to monitor analytes in the column effluents or to MS for structural identification and quantitation by single-ion monitoring (SIM) or multiple-ion monitoring (MIM). As FID systems lack detection selectivity and specificity, GC–FID analyses are often preceded by CC and/or TLC for clean-up and pretreatment of unsaponifiable samples. In addition, due to destruction of column effluents by FID, a splitter is needed to be inserted between the packed column outlet and the FID system to collect analyte peak components for structural characterization and confirmation. Other detectors (e.g. electron capture, thermal conductivity detectors) are less commonly used because FID has the best GC features in terms of detection sensitivity, response linearity and response generality.

Table 4 compiles some recently published methods for the analysis of sterols in vegetable oils, oilseeds and various plants. For purposes of volatilizing the hydroxy-containing GC detectants, enhancing component resolution and stabilizing thermally labile unsaturated sterols, the title compounds are most commonly analyzed as their TMS and acetate derivatives. As compared to the acetates, the TMS derivatives are more suitable for the GC–MS characterization and quantitation of sterols. In view of the procedural simplicity and facile reaction duration, undoubtedly many research groups have incorporated a TMS derivatization step in their GC analyses of plant sterols (Table 4). An example of the derivatization procedure is described as follows: a dried non-saponifiable material of a vegetable oil sample (0.1 g) dissolved in dry pyridine (0.25 ml) is placed in a septum-capped vial and treated with bis-(trimethylsilyl)-trifluoroacetamide (0.25 ml). The mixture is heated in a heating block for 15 min at 60°C and then left standing at room temperature overnight. The content of the vial is diluted to a exact volume with methylene chloride and the aliquots are analyzed by GC. Depending on sample

Table 4
GC analysis of plant sterols

Method detection	Stationary phase (supplier)	Sterol investigated (derivative)	Plant matrix	Ref.
(1) FID	SAC-5, d_f=0.25 μm 30 m×0.25 mm (Supelco)	BR, CA, SI, ST (TMS)	Vegetable oils	[67]
(2) SPE, FID	SAC-5, d_f=0.25 μm 30 m×0.25 mm	5AV, 7AV, CA, SI, ST, 7ST (TMS)	Vegetable oils	[68]
(3) FID, MS	NB-17, d_f=0.25 μm 25 m×0.32 mm (Nordion, Finland)	5AV, CA, SI, ST (TMS)	Wheat flour	[69]
(4) FID, MS	CP-Sil-5CB, d_f=0.13 μm 25 m×0.25 mm (Chrompack)	5AV, CA, SI, ST, OB (TMS)	Olive–hazelnut	[70]
(5) SPE, FID	DB-17HT, d_f=0.15 μm 13 m×0.32 mm (J & W)	5AV, 7AV, BR, CA, SI, ST, 7ST (TMS)	Vegetable oils	[62]
(6) LC, FID	DB-5, d_f=0.1 μm 10 m×0.32 mm (J & W)	5AV, 7AV, BR, CA, SI, ST, 7ST (TMS)	New seed oils	[71]
(7) FID	PTE-5, d_f=0.25 μm 30 m×0.25 mm (Supelco)	BR, CA, SI (TMS)	Canola oild	[35]
(8) FID, MS	RTx-5, d_f=0.1 μm 60 m×0.25 mm (Restek)	AV, BR, CA, CAa,CH, SI, SIa, ST (TMS)	Diet foods	[72]
(9) TLC, FID	CP-Sil-8CB, d_f=0.12 μm 25 m×0.25 mm (Chrompack)	5AV, 7AV, BR, CA, 7CA CAa, CH, MCH, CL, ER, SI, SIa, ST, 7STe, UVA (Acetate)	Vegetable oils	[73]
(10) FID, MS	DB-5MS, d_f=0.25 μm 30 m×0.25 mm (J & W)	5AV, 7AV, CA, CH, SI, ST (TMS)	Oat cultivars	[74]
(11) FID	OV-17, d_f=0.25 μm 25 m×0.25 mm (Ohio Valley)	5AV, 7AV, CA, CH SI, ST, 7ST (TMS)	Plant seed oils	[75]
(12) FID	OV-17-diatomite C 2.8 m×4 mm (Ohio Valley)	5AV, 7AV, CA, SI, ST, 7ST	Sesame oils	[76]
(13) SPE, FID, MS	NB-17, d_f=0.25 μm 25 m×0.32 mm	5AV, BR, CA, CH, SI (TMS)	Edible oils	[60]
(14) MS	DB-5MS, d_f=0.33 μm 12.5 m×0.20 mm (J & W)	CA, CH, EGa, IFU, SI, ST, STa	Fruit juices	[77]
(15) LC, FID	DP–DM (5:95), d_f=0.25 μm 30 m×0.25 mm (Sugelabor, Spain)	7AV, BR, CA, SI	Rapeseed oil	[78]

Table 4. Continued

Method detection	Stationary phase (supplier)	Sterol investigated (derivative)	Plant matrix	Ref.
(16) MS–MS	DB-5, d_f=0.25 μm 10 m×0.25 mm (J & W)	CA, SI, ST	Tobacco	[79]
(17) FID	TAP, d_f=0.10 μm 25 m×0.25 mm (Chrompack)	5AV, HBR, CA, CH SI, ST (TMS)	Coffees	[80]
(18) FID	PTE-5, d_f=0.25 μm 30 m×0.25 mm	CA, SI, ST (TMS)	Soybean oils	[81]
(19) FID, MS	OV-1, d_f=0.52 μm 25 m×0.32 mm (Ohio Valley)	5AV, 7AV, CA, SI STa,ST,7ST (TMS)	Hydrogenated oils	[82]
(20) TLC, FID	SPB-5, d_f=0.25 μm 30 m×0.25 mm (Supelco)	5AV, 7AV, CA, CH, CL SI, SIa, ST, 7STe 523STD, 524STD (TMS)	Olive oils	[83]
(21) FID, MS	Lipolysis TAP-CB, d_f=0.25 μm 30 m×0.25 mm (Chrompack)	5AVL, 5AVO, CAL CAO, CAS, SIL SIO, SIS, STL, STO, STS (Steryl esters)	Sunflower oil	[37]
(22) FID	TAP, d_f=0.10 μm 25 m×0.25 mm	5AV, CA, CI, CYe, MCYa SI, ST (TMS)	Coffees	[84]
(23) FID	TAP, d_f=0.10 μm 25 m×0.25 mm	5AV, CA, CI, CYe, MCYa SI, ST (TMS)	Vegetable oils	[18]
(24) LC–LC, MS	CP-SIL-5-CB, d_f=0.12 μm 10 m×0.25 mm (Chrompack)	CD, EG, EGT, FN SC, ST, ZY	Alga and yeast	[85]
(25) TLC, FID, IR, MS	SPB-5, d_f=0.25 μm 30 m×0.25 mm (Supelco)	5AV, 7AV, BR, CA, CH, SI, ST	Tropical seed oil	[86]
(26) FID	DB-1701, d_f=0.25 μm 30 m×0.25 mm (J & W)	CA, SI, ST (TMS)	Buckwheat	[87]
(27) TLC, FID	SE-52, d_f=0.25 μm 25 m×0.32 mm (General Electric)	5AV, 7AV, BR, CA, CAa, 7CAa CH, CHa, MCH, CL, SI, ST STe, 7STe, 32STD (TMS)	Tobacco seeds	[88]
(28) TLC, FID	CP-SIL-5-CB, d_f=0.12 μm 10 m×0.26 mm (Chrompack)	BR, CA, CH, SI, ST	Diet samples	[89]
(29) FID	SE-30 1.5 m×4 mm (General Electric)	7AV, CA, CH, SI, ST, 7STe (TMS/acetate)	Pine, plants	[90]
(30) FID	DB-1, d_f=0.10 μm 12 m×0.25 mm (J & W)	CA, SI, ST (TMS)	Soybean hulls	[91]
(31) FID	SE-52, d_f=0.32 μm 30 m×0.32 mm	OB, GR, CE, CI (TMS)	Corn oil	[92]

Table 4. Continued

Method detection	Stationary phase (supplier)	Sterol investigated (derivative)	Plant matrix	Ref.
(32) FID	SE-52, d_f=0.2 μm 30 m×0.32 mm	αAM, βAM, CYe, MCYa (TMS)	Corn oil	[93]
(33) TLC, LC, FID, MS	BP-1, d_f=0.10 μm 12 m×0.22 mm (SGE)	BRB, BRL, BRO, BRP, CAB CAE, CAL, CAO, CAP, CAS CHS, SIB, SIE, SIL, SIO, SIP SIS (Steryl esters)	Rapeseed oil	[94]
(34) TLC, FID	OV-17 SCOT 30 m×0.3 mm (Ohio Valley)	SI, ECHT, ECHD, IFU SC (Acetate)	Cucurbitaceae seed	[95]
(35) FID	SE-52, d_f=0.1–0.15 μm 25 m×0.32 mm	βAM, CYe, MCYa	Linseed oil	[96]
(36) TLC, FID, MS	SE-54, d_f=0.25 μm 12 m×0.2 mm (General Electric)	CA, CH, SI, ST	Citrus roots	[97]
(37) TLC, FID	OV-17 d_f=0.25 μm 16 m×0.3 mm	5AV, 7AV, BR, CA, CH MCYa, SI, ST, 7ST	Edible oils	[98]
(38) TLC, FID, MS	3% OV-17 1.8 m×3 mm	5AV, CA, SI, ST, CYe, CE MLAe, MCYa, CI, OB, ELO LO, GR, MMCH, NLAe, NLA	Cocoa butter	[99]
(38) LC, TLC, FID	3% OV-17 1.8 m×4 mm	5AV, CA, SI, ST	Peanut- and corn oils	[100]
(39) FID	SE-30, d_f=0.1–0.15 μm 1 m×0.32 mm (General Electric)	5AV, BR, CA, CH, MCH, SI, ST, 7STe (TMS)	Tomato seed	[101]
(40) TLC, FID	2% OV-17 SCOT 50 m×3 mm (Ohio Valley)	CA, CH, MCH, SI, ST (TMS)	Plants	[102]
(41) TLC, FID, MS	1.5% OV-17 2 m×3 mm	BR, CA, SI, ST, 7STe	Vegetable oils	[103]

For compound abbreviation, see Tables 1 and 2. Other abbreviations: 5AVL=5AV linoleate, 5AVO=5AV oleate, BRB=BR brassidate, BRL=BR linoleate, BRO=BR oleate, BRP=BR palmitate, CAB=CA brassidate, CAE=CA eicosenoate, CAL=CA linoleate, CAO=CA oleate, CAP=CA palmitate, CAS=CA stearate, SIB=SI brassidate, SIE=SI eicosenoate, SIL=SI linoleate, SIO=SI oleate, SIP=SI palmitate, SIS=SI stearate, CHS=CH stearate, STL-ST linoleate, STO=ST oleate, STS=ST stearate. ECHT=24-ethylcholesta-8,22,25-trienol, ECHD=24-ethylcholesta-5,25-dienol. DP-DM=diphenylpolysiloxane-dimethylpolysiloxane.

matrices, the number of analyte species in samples and specific application, GC analyses of plant sterols preferably in the form of TMS derivatives can be run isothermally or by temperature programming at various gas flow-rates to bring the peak of interest within reasonable retention times without peak overlapping.

4.2. Columns and detection techniques

The polarity and molecular volatility of a steroidal compound play a pivotal role in the GC separation. During the chromatographic separation processes, GC partition between sterol solutes and the stationary liquid phase is influenced by the polarity of both interacting partners. Component resolution of a structurally similar sterol mixture can be dramatically improved by increasing the polarity of the stationary phase used in capillary GC at the expense of increases in solute retention times. McReynolds constants (χ') [66] are generally used to measure the column polarity. A higher χ' value means a more

polar liquid phase for a GC column. On the other hand, the analyte molecular volatility that is related to molecular mass, size and volume can be enhanced by chemical derivatization of sterols via timethylsilylation or acetylation.

Examination of the methods list in Table 4 reveals that the majority of laboratories have employed polysiloxane phases for sterol assays. These modifications of alkylsilicones with χ' values ranging from 16 to 319 are coded with manufacturer's labels for specific GC applications. Other stationary phases used in sterol analyses include materials made of polyesters (χ', 272–345), polycarboranesiloxane ($\chi'=47$) and poly-*m*-phenylether ($\chi'=257$). For the analysis of sterols in vegetable oils, various capillary GC stationary phases such as SAX-5 or PTE-5 [35,67,68,81], DB-17 or OV-17 [62,75,98], CP-Sil-5CB, OV-1, or BP-1 [70,82,94], CP-Sil-8CB, SPB-5, or SE-52 [73,83,92,93,96], TAP [18,37] and NB-17 [60] have been used (Table 4). In particular, the SAC-5 column, essentially an SE-54 type phase, has been specially developed and tested for reproducible analyses of sterols. A number of laboratories have adopted different columns (e.g. DB-5, SPB-5, RTx-5, SE-52 and SE-54) of the same type of packing materials for the analysis of sterols in various plant samples [71,72,79,86,88,97]. Another example is found in studies [85,90,91] where CP-Sil-5CB, SE-30 and DB-1 all belonging to the same stationary phase type were used to analyze sterols in alga, pine and soybean hulls, respectively. Free sterols and other oil constituents in edible oils have been simultaneously analyzed (Fig. 2) by direct on-line HPLC–GC with a diphenylpolysiloxane (DP)–dimethylpolysiloxane (DM) (5:95) phase [78].

Table 4 shows very few laboratories have used packed GC columns for the analysis of plant sterols. In general, it is possible to detect individual sterols in the low nanogram range by capillary GC–FID, which is about 20 times more sensitive than GC with a packed column. Additional advantages of capillary GC techniques over packed GC methods include reduction in analysis times and peak interferences, improvement in component resolution and high thermal stability. Further, improved resolution of sterol components (Fig. 3) can be obtained by using a polar capillary column of high thermal stability as opposed to non-polar columns [18]. Disregading the non-uniformity in GC column selection, nearly all

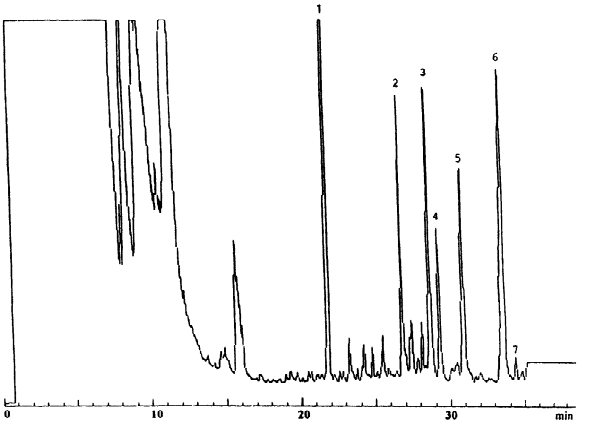

Fig. 2. On-line (HPLC)–GC–FID detection of a rapeseed oil. Capillary column, diphenyl–dimethylpolysiloxane (DP–DM) (5:95), $d_f=0.25$ μm, 30 m×0.25 mm. Relevant sterol peaks: 4, brassicasterol; 5, campesterol; 6, sitosterol; 7, 7-avenasterol (from [78] with permission). GC analysis of sterol fractions from on-line HPLC.

the methods listed in Table 4 have used FID for sterol detection. In conjunction with GC–FID, GC–MS analyses of sterols in vegetable oils [37,60,70,72,82,94,99,103] and plant samples [69,74,77,79,85,86,97] have been conducted in numerous laboratories (Table 4). In light of the

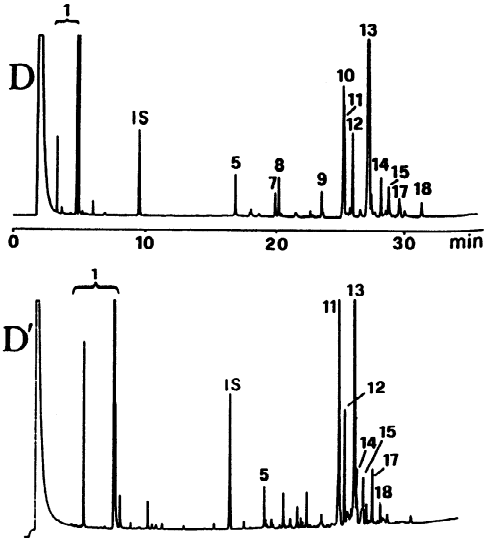

Fig. 3. GC–FID of a corn oil. Column, Compack TAP (D), General Electric SE 52 (D′). Relevant sterol peaks:11,campesterol; 12, stigmasterol; 13, sitosterol, 14, Δ5-avenasterol; 15, cycloartenol; 17, 24-methylenecycloartanol; 18, citrostadienol; 19, oleanolic acid (from [18] with permission).

increased detection sensitivity and analyte specificity of MS detection, plant samples containing trace levels of sterols can be analyzed by GC–MS-SIM or GC–MS-MIM [104]. A highly selective tandem GC–MS–MS detection system (method 16, Table 4) implemented in the selected reaction mode has been applied to rapid direct measurement of free phytosterols in tobacco samples [79]. In this method, no sample clean-up is required. However, about half of the compiled methods in Table 4 have employed LC, SPE, or TLC procedures for purification of samples prior to GC analysis.

4.3. Structure effects on elution characteristics

As in any chromatographic practice, the GC peaks of sterols are represented by their retention times relative to a reference standard or an internal standard (RRTs) to eliminate analytical errors due to fluctuations of instrument operation conditions as well as other experimental variables. Thereby, it is possible to use the RRT data not only to identify sterol components in test samples by comparisons with standards but also to predict the unknown molecular features of unknown components in sterol samples [47].

GC of the some of the phytosterols found in vegetable oils using a SE-30 column yields the following elution sequence of increasing retention times CH<CHa<BR<MCH<CA<CAa<ST<7CA<523STD<OB<CL<SI<SIa<5AV<524STD<7STe<CYe<7AV<MCYa (Tables 1 and 2) with RRT values ranging from 1.00 to 2.21. Their TMS and acetate derivatives have RRT values comparable but slightly different from the parent compounds. Cholesterol (CH) and its derivatives are the most frequently used reference compounds to obtain RRT data for sterols. Analysis of structural factors affecting the retention behavior of the above series of sterols indicates that saturation of 5- or 22-olefinic moiety has tendency to increase the analyte retention time (CH vs. CHa; CA vs. CAa; SI vs. SIa; BR vs. CA; ST vs. SI). Also, sterols with a double bond at the 7-position tend to have longer retention times than those having the olefinic bond at the 5-position (CA vs. 7CA; 5AV vs. 7AV; ST vs. 7STe). Interestingly, the elution of 523STD before 524STD seems to suggest that the latter 24-double bond analogue is more polar than the 23-double bond counterpart. Further, extension of the carbon side chain or addition of methylene/methyl/ethyl groups to a lower-carbon number sterol structure results in an increase in analyte retention (CH vs. MCH; CYe vs. MCYa; BR vs. ST; CA vs. SI; CH vs. SI). The observed structure–retention relationships are in consonance with GC adsorption rationale based on molecular mass and analyte polarity.

For quantitative assessment of structure–retention relationships, the relation between various sterol substituents and sterol retention times can be expressed in terms of separation factors. Based on published retention data for sterols, substituent effects on sterol retention time can be treated with separation factors according to ring double bond-, alkyl substituent- and side-chain double bond-structural features. Thereby, the separation factors are used to compute and estimate the relative retention time of a sterol structure. The calculated retention value may serve as a useful clue for the prediction of an unknown sterol structure or may aid in the corroboration of a sterol structure established by other means. In addition, ΔR_{Ac} values, the ratios of the RRTs of steryl acetates to that of the parent sterols, have been used to differentiate desmethylsterols, 4-methylsterols and 4,4′-dimethylsterols. For example, capillary GC with an OV-17 packed column at 260°C yields ΔR_{Ac} ranges of 1.32–1.36, 1.26–1.32 and 1.18–1.23 for desmethylsterols, 4-methylsterols and 4,4′-dimethylsterols, respectively [105].

In connection with discussion of structural effects on sterol separations, it is worthwhile to examine the GC characteristics of closely related epimeric sterol compounds. C-24 epimers of some major sterols of vegetable oils have been separated by capillary GC of the TMS derivatives on SP-2340 at 195°C [106]. Thus, nine epimeric steryl TMS ether pairs EGa-CAa, HBR-CA, HPO-SI, CR-BR, ST-PO, 7EGe-7CAe, 7POe-7STe, SP-CD and DSP-DCD (Table 1) were resolved with variable degrees of separations. Their RRT values relative to CH-TMS ranged from 1.119 to 2.046 at 195°C, but increased to 1.133–2.119 at lower temperature 185°C. Correlation of the sterol structures in the epimeric series with RRT values shows that the presence of a 25-double bond significantly increases the retention times of the

compounds evaluated (e.g. SP<DSP; CD<DCD), but an opposite effect of a 22-double bond on the analyte retention time is demonstrated (e.g. CD< 7POe; SP<7STe). Of the epimers studied, base-line resolution was achieved for the four pairs EGa-CAa, HBR-CA, 7EGe-7CAe and DSP-DCD, while other pairs remained partially or poorly resolved. The investigated individual pairs eluted in the order of increasing retention times: EGa<CAa, HBR<CA, HPO<SI, CR<BR, ST<PO, 7EGe<7CAe, 7POe< 7STe, SP<CD, DSP<DCD. Evidently, the 24β-epimers of 24-methyl- and 24-ethyl analogues with saturated side chains (i.e. EGa, HBR, HPO, 7EGe and 7POe) were the early-eluting isomers. On the other hand, the presence of an alkenyl bond at the 22-position in the proximity of the 24-epimeric center caused the reversal of the elution sequence. Thus, the 24α-epimers CR, ST, SP and DSP eluted before their corresponding 24β-isomers. Except for BR which has a 24β configuration, the three other sterols CA, SI and ST commonly found in vegetable oils all have 24α stereochemistry. While ST was observed to elute faster than its β-isomer, the remaining major seed oil sterols BR, CA and SI were found to be the later-eluting isomers of the corresponding epimers.

4.4. Sterols and steryl esters in vegetable oils

Apparently, the sterols occurring in vegetable oils are mainly desmethylsterols. In other words, the edible oils contain much smaller number of 4-methylsterols and 4.4′-diemthylsterols in relatively low abundance. The most abundant sterol components present in the sterol fractions of commodity vegetable oils (i.e. coconut, canola, cocoa butter, corn, cottonseed, linseed, olive, palm, peanut, rice bran, safflower, sesame, soybean, sunflower oils) are campesterol (CA) (2.6–38.6%) and sitosterol (SI) (40.2–92.3%). These are followed by stigmasterol (ST) and 5-avenasterol (5AV) in abundance. The percent compositions of ST and 5AV in these oils range 0.0–31.0% and 1.5–29.0%, respectively [68,103]. In fact, the high percentage of 5AV is found only in the unique case of coconut oil. The levels of 5AV in the rest of oils are somewhat lower (1.5–18.8%). In comparison with the oils just described, pumpkin seed oil has a rather unusual composition of its major sterols: 7,22,25-stigmastatrienol (72225STT) (29%), spinasterol (SP) 27% and 7,25-stigmastadienol (725STD) (22%) [68].

As sterol esters are sensitive to saponification with strongly basic reagents, these compounds along with free sterols or by themselves are commonly isolated from lipid extracts or vegetable oils by CC [110], CC/TLC [113], lipolysis [36,37], TLC [112,124], preparative TLC/HPLC [94], HPLC [85,109,111, 121,123,126] or SPE [140]. In a recently published lipolysis procedure [37], a mixture of an oil sample and porcine pancreatic lipase is stirred with 1 *M* tris(hydroxymethyl)methylamine (Tris) (pH 8), 22% calcium chloride in water and 1% aqueous bile salt for 1 h at 45°C. Vacuum filtration of the diethyl ether extracts is followed by concentration of the ethereal solution to give the lipolized material. Flash chromatography of the lipolysis extract onto a short octadecylsilica column (15×2 cm I.D.) removes thoroughly the non-sterol components (e.g. fatty acids, monoglycerides and diglycerides) in the first methanol fraction and retains most of the sterol esters (>90%) in the column until elution with hexane. The later eluate is evaporated to a residue to be analyzed by GC.

For the investigation of steryl esters, GC is believed to be more sensitive analytical procedure than HPLC. There are two methods 21 and 33 in Table 4 focusing on the analysis of steryl esters in oils or plant tissues [37,94]. In these procedures where the traditional saponification step must be avoided, lipid samples are either selectively lipolyzed (method 21) or subjected to extensive purification by preparative TLC/HPLC (method 33) preceding the GC–MS quantification of intact steryl esters. Using the lipolysis technique [37], the major steryl esters found in sunflower oil were SI linoleate (L), 5AV linoleate (L), SI oleate (O), ST palmitate (P) and 5AV oleate (O) at concentrations 0.103–0.534 mg/g oil, whereas the major species detected in rapeseed oil were CA L, SI L, CAO, SIO and BRO at concentrations 0.274–1.780 mg/g oil. The analysis was carried out on a wall-coated open tubuler column of fused-silica coated with a TAP CB liquid phase and the GC instrument was operated with temperature programming: initially 50°C for 0.5 min, gradient rate 30°C min^{-1}, 255°C for 5 min, gradient rate 5°C min^{-1} and finally 350°C for 15

min. In another approach (method 33) to the steryl ester assays, a sample of rapeseed oil was analyzed via multiple-steps of sample clean-up procedures without going through saponification. A BP-1 fused-silica column was used with temperature programming from 50 to 330°C at 8 or 12 min. By way of the elaborate analytical scheme, seventeen steryl esters derived from BR, CA and SI were found to be present in rapeseed oil and were identified by GC–MS with negative ion chemical ionization [94].

5. High-performance liquid chromatography

5.1. General considerations

With the advent of the HPLC technology in recent years, many investigators have used both analytical and preparative scale HPLC methods for the analysis of sterols. HPLC has surpassed GC in one area that it operates under milder column temperature and non-destructive detection conditions. Therefore, the technique is ideally suited for the analysis of thermally unstable sterols. In some cases where lipid extract samples are simple and homogeneous such as certain seed oils, direct HPLC analysis of sterols can be performed with little sample purification to prevent from unwanted sample losses. Otherwise, samples of complex matrices must go through CC, TLC or other chromatographic pretreatment to eliminate interfering endogenous matters. Hence, working HPLC procedures are much constrained by the complexity of sample matrices, the type and concentration of sterol components, routine or none-routine assays and analytical or preparative scale separations.

Since the pioneering HPLC separations of sterols in 1976 [107], normal-phase and reversed-phase HPLC techniques have been widely used for the analysis of lipid classes and individual sterols in various sample matrices. Direct fractionation of total lipid extracts and non-saponifiable matters has often done by normal-phase HPLC with a silica column and has met with reasonable success despite obvious shortcomings of long equilibration times inherent with the normal-phase systems and the employment of hazardous volatile organic solvents. In contrast, reversed-phase HPLC employs less volatile polar organic solvents in water and offers ready equilibration of a bonded silica stationary phase with the mobile phase solvents. The demonstrated selectivity of bonded-silica stationary phases for individual sterols differing in molecular size and the number of double bonds has important bearing on the proliferation of reversed-phase HPLC applications in sterol analyses. As in any other chromatographic techniques, optimization of HPLC experimental parameters (e.g. stationary phases, mobile phases, flow-rates, isocratic/gradient elution etc) is crucial for the accurate quantification of the title compounds. In general, HPLC analyses of sterol components in purified sterol fractions of non-saponifiable lipids have more often been carried out in the reversed-phase mode. Because most phytosterols of cardiologic value are ubiquitous substances occurring in vegetable oilseeds at concentrations higher than other plants (Table 3), separations and analyses of the compounds in commodity oils are the main points of discussion.

5.2. Normal-phase HPLC

Table 5 summarizes selected published HPLC methods for the analysis of sterols. A total of 33 methods are listed in a chronological order beginning with the most recent research. Nearly one third of listed methods employed normal-phase HPLC with different silica columns mostly for separations of lipid classes. In normal-phase HPLC, analytes participate in adsorptive interactions with the surface of silica in a solvent system consisting of a non-polar solvent and a polar modifier. As shown in method 2 of Table 5, LiChrosorb DIOL (5 μm) has been used successfully to separate steryl ferulates (F) (hypocholesterolemic substances), steryl fatty acid esters and phytosterols from other lipid classes in corn fiber [109]. Christie and his co-workers have also achieved separations of sterols, steryl esters and steryl glycosides along with other potato lipids on cyanopropylsilica (Spherisorb S3CN, 3 μm) and polyvinyl alcohol-bonded silica (YMC PVA-Sil, 5 μm) [111]. Several reports [85,119,121,123,126] described the use of silica phases SGX Si, Spherisorb S5W, LiChrosorb Si, μPorasil and LiChrospher Si having variable particle size 5–10 μm for the separation of sterol, steryl ester and/or steryl conjugate fractions in vegetable oils and plant lipids.

Table 5
HPLC analysis of plant sterols

Method detection	Stationary mobile phase (Supplier)	Sterol investigated (Derivative)	Plant matrix	Ref.
(1) ELSD	Alltech C_{18}, 5 μm 250×4.6 mm A = MeCN–AcOH (90:10:0.1); B = MeCN–MeOH–CH_2Cl_2 (60:8:40); gradient elution	CA, EG, SI	Standards and wood	[108]
(2) UV–ELSD	LiChrosorb DIOL, 5 μm 100×3 mm A = HX–AcOH (1000:1); B = IPA; gradient elution (Chrompack)	Steryl ferulates stterols, steryl esters	Corn fiber	[109]
(3) CC, DAD	Deltabond ODS, 5 μm 250×4.6 mm MeCN–BuOH–AcOH–water (82:3:2:13) (Keystone)	CAF, CAaF, CBF, CYeF CYaF, MCYaF, SIC, SIaF CYaC[steryl ferultes(F) and coumarates(C)]	Corn and rice	[110]
(4) ELSD	Spherisorb S3CN, 3 μm 100×3.2 mm YMC PVA-Sil, 5 μm 250×4.6 mm A = Isohexane–MTBE (98:2); B = IPA–$CHCl_3$–AcOH (82:20:0.01); C = IPA–water–Et_3N (47:47:6); gradient elution (Hichrom, UK)	Steryl esters, sterols, steryl glycosides	Potato lipids	[111]
(5) TLC, UV	Nucleosil-300, C_{18}, 7 μm 250×4 mm A = MeOH–water (80:2); B = MeCN–MeOH (60:40); C = MeCN–THF (99.5:0.5); D = IPA–MeCN (99:1); gradient elution (Analysentechnik, Greece)	CHL, CHP, CH, DE EG, SI, ST	Plant and soybean oil	[112]
(6) CC, TLC, DAD	Supelcosil LC_{18}, 5 μm 250×4.6 mm MeCN–BuOH–AcOH–water (93:4:2:1) (Supelco)	7CAeC, 7CAeF, CAC, CAF, CAaC, CAaF, SIF SIaC, SIaF, STF,7 STeF [Ferulates (F) and coumarates(C)]	Corn bran	[113]
(7) TLC, UV, MS, NMR	Ultrasphere ODS, 5 μm 250×10 mm MeOH (Beckman)	αAM, βAM, BU, CYa CYe, LU, MCYa (4,4-dimethylsterol fractions) (acetate)	Leguminosae seeds	[114]

Table 5. Continued

Method detection	Stationary mobile phase (Supplier)	Sterol investigated (Derivative)	Plant matrix	Ref.
(8) DAD	Hypersil ODS, 5 μm 200×2.1 mm A=MeCN–MeOH–IPA–water (45:45:4:5); B=MeCN–MeOH–IPA (50:45:5); gradient elution (Hewlett-Packard)	CAF, CYeF, CYaF, SIF [Steryl ferulates (F)]	Rice bran oil	[115]
(9) TLC, UV, MS, NMR	Ultrasphere ODS, 5 μm 250×10 mm MeOH (Beckman)	CA, ECHa, CL, FU IFU, CL, SI, ST (desmethylsterol fractions) (acetate)	Leguminosae seeds	[116]
(10) TLC, UV, MS, NMR	Ultrasphere ODS, 5 μm 250×10 mm MeOH (Beckman)	αAM, βAM, AV, CE, CI MCYa, GR, IFU, LU OB, SI, ST, VE, MVE (acetate)	Vernonia seeds	[117]
(11) UV	SGX Si, 7 μm 250×8 mm A=propylnitrile, B=MTBE; gradient elution (Tessek)	Sterol- and steryl ester fractions	Alga and yeast	[85]
(12) LC, UV	SGX C_{18}, 5 μm 250×4 mm MeCN–THF–MeOH; gradient elution (Tessek)	EG, EGT, FN, SC ST, ZY and steryl esters	Alga and yeast	[85]
(13) UV	TSK-Gel ODS, 5 μm 250×4.6 mm MeOH–IPA (4:1) (Toyosoda, Japan)	BR, CA, SI, SP, ST and epimers	Epimers	[118]
(14) LC, GC–FID	Spherisorb S-5-W, 5 μm 100×2 mm HX–0.5% MTBE (Hichrom)	CA, CYe, MCYe ER, SI, SIS, ST from sterol fraction (acetate)	Olive oils	[119]
(15) UV	Rad-PAK C_{18}, 5 μm MeOH (Waters)	CA, CH, SI, ST	Foods	[120]
(16) FID	LiChrosorb Si, 7 μm 100×3 mm A=Isooctane–THF (99:1); B=IPA; C=water; gradient elution (Chrompack)	Stero esters, sterols sterol glycosides	Plant lipids	[121]

Table 5. Continued

Method detection	Stationary mobile phase (Supplier)	Sterol investigated (Derivative)	Plant matrix	Ref.
(17) UV	TSK-Gel ODS, 5 μm 250×4.6 mm HX–IPA–MeCN (1:3:16) (Toyosoda, Japan)	BR, HBR, CA, CLI CR, SI, ST, PO (benzoate)	Epimers	[122]
(18) LC, RI, NMR	μPorasil 10 μm 300×3.9 mm Heptane–butyl acetate (99.55:0.45) (Waters)	Steryl/triterpenyl ester fractions	Vegetable oils	[123]
(19) TLC, UV	Spherisorb S3 ODS, 3 μm 150×4.6 mm MeCN–MeOH (1:1) (Phase Separations, UK)	CAF, CAaF, CYeF MCYaF SIF, STaF [Steryl ferulates(F)] (acetate)	Oil seeds	[124]
(20) UV	Partisil C_{18}, 5 μm 110×4.7 mm MeOH–water (94:6) (Whatman)	Sterols, steroids and triterpenoids	Test compounds	[125]
(21) FID	LiChrospher Si-100, 10 μm 250×4.6 mm HX–CH_2Cl_2–$CHCl_3$–NH_3; gradient elution (Bodman)	Sterol, steryl ester fractions	Lipid classes	[126]
(22) UV	Spherisorb S3 ODS, 3 μm 150×4.6 mm MeCN–THF–water (65:35:1.5) (Phase Separations)	CA, CH, SI, ST (fatty acid esters)	Plant and animal tissues	[94]
(23) UV	Seibersdorf RP8, 7 μm 250×5 mm MeCN–IPA–water (50:25:25)	BR, CA, CH, EG, SI, ST and sterol esters	Standards	[127]
(24) TLC, UV	LiChrosorb RP-8, 5 μm 250×4.6 mm MeCN–water (90:1) (E. Merck)	αAM, βAM, 5AV, 7AV, BR, CA, 7CAe, CH, MCYa, EG, HLA, SI, ST (acetate)	Standards and sunflower oil	[128]
(25) UV	Brownlee RP18, 5 μm 250×4.6 mm MeOH–water (99:1)	BR, CA, CH, DE, EG, FU, SI, ST	Oil, fat, plant	[129]
(26) UV	μBondapak C_{18}, 10 μm 300×3.9 mm MeCN (Waters)	Sterols	Test compounds	[130]

Table 5. Continued

Method detection	Stationary mobile phase (Supplier)	Sterol investigated (Derivative)	Plant matrix	Ref.
(27) UV, RI	μPorasil 300×3.9 mm HX–IPA (100:3) (Waters)	Oxygenated CH's and related compounds	Standards	[131]
(28) UV	Zorbax BP-SIL, 7–8 μm 250×4 mm HX–EtOH (DuPont)	Triterpenoids	Standards	[132]
(29) UV	Zorbax BP-ODS, 7–8 μm 250×4 mm MeOH–water + formic acid (DuPont)	Triterpenoids	Standards	[132]
(30) UV	μPorasil 300×3.9 mm HX–$CHCl_3$ (6:4) (Waters)	C-27 precursors of CH (acetate)	Standards	[133]
(31) UV	μBondapak C_{18} 300×3.9 mm MeOH–MeCN (4:1) (Waters)	C-27 precursors of CH (acetate)	Standards	[133]
(32) UV, RI	Pyrocarbon Si, 10 μm 150×4.6 mm $CHCl_3$–MeOH(6:4)	BR, CA, CH, EG LA, SI, ST	Standards and Colza	[134]
(33) UV	Bondapak C_{18}–Porasil B, 75 μm 16 ft×1/8 in (1 ft = 30.48 cm; 1 in = 2.54 cm) HX–IPA (99.5:0.5) (Waters)	CA, CH, EG, SI, ST	Standards	[135]

For compound abbreviations, see Tables 1 and 2. F = ferulate. C = *p*-coumarate. ECHa = ethylcholestanol. SIS = SI stearate. CHL = CH linoleate. CHP = CH palmitate.

Both methods 27 and 30 employ μPorasil for the analysis of oxygenated cholesterols (CH) [131] and CH precursors [133], respectively. HPLC separations of triterpenoids on Zorbax BP-SIL (7–8 μm) (method 28) have been described [132].

Although silica column specifications vary among manufacturers, normal-phase HPLC with stationary phases of small particle size 3–5 μm allows efficient separations of sterol classes. For preparative HPLC, a column packed with silica of particle size grater than 10 μm suffices the separation of sterol fractions from other lipids. In typical assays, standard column dimensions are 250×4.6 mm I.D. and 250×10 mm I.D. for respective analytical and preparative separations of lipid extracts. Alternatively, short columns (100 mm) of smaller I.D. (2.0–3.2 mm) with 3–5 μm silica packings often save analysis times/solvents and can be used for lipid class separations [109,111,119,121]. A fully automated on-line normal-phase HPLC–GC method (method 14) for the analysis of sterols in olive oils on a short (100 mm) and small I.D. column (2 mm) has been reported [119].

With a few exceptions, binary systems of a polar organic modifier in hexane are commonly use in normal-phase HPLC of sterol fractions. The organic

modifiers can be alcohol, ether, ester, acid and/or base. A survey of the selected normal-phase methods (Table 5) indicates that few investigators chose the same or similar mobile phase systems used by others. Some of the methods listed in Table 5 employed heptane, isohexane, or isooctane instead of hexane as the non-polar mobile phase solvent [111,121,123]. Ether modifiers such as methy-*tert*.-butyl ether (MTBE) and tetrahydrofuran (THF) were used by three laboratories [85,111,119]. Four published methods 2, 4, 16 and 27 (Table 5) incorporate isopropanol in mobile phases to facilitate adequate separations. In some cases, acetic acid, butyl acetate, triethylamine were added to mobile phases to improve HPLC peak separations [109,111,123]. Except for methods 27, 28 and 30 dealing with the analysis of individual sterols, the rest of the selected published normal-phase HPLC methods compiled in Table 5 were developed for the separation of lipid classes.

Individual sterols have been analyzed by normal-phase HPLC under isocratic elution [131–133], whereas sterol fractions were more frequently separated from other lipids by normal-phase HPLC under gradient elution [85,109,111,121,126]. However, mobile phases used in both the methods 14 and 18 were operated under isocratic elution to fractionate lipid extracts for the subsequent GC analysis of isolated sterol fractions [119,123]. In one study [85], sterol and steryl ester fractions of yeast lipids were separated by gradient semi-preparative normal-phase HPLC followed by reversed-phase HPLC of each sterol fraction for the identification of individual compounds. In practice, gradient elution usually starts out with solvents of low polarity and gradually changes to solvents of higher polarity and solvent strength. It requires painstaking optimization experiments to achieve workable HPLC conditions. Mobile phase gradient elution can be simple or complex, depending on analyts of interest, study objectives and most importantly sample matrices. Gradient HPLC techniques can be applied to special cases to handle particular analyte species in complex plant samples.

Sample assays are normally conducted under optimal conditions to achieve rapid separations with maximal analyte resolution. The unique feature of normal-phase HPLC in lipid analysis is the direct isolation of sterol fractions from lipid extracts avoiding saponification so that intact sterol esters/conjugates are obtained for characterization. It has been reported that, in corn fiber oil, steryl fatty acid esters (SEs) elute before free sterols (Ss) followed by steryl ferulates (SFs) (in 30 min) in the order of increasing polarity [109]. In another study on potato lipids, normal-phase elution order of SEs$<$Ss$<$steryl glycosides (SGs) with increasing retention times was observed [111]. Further, earlier HPLC analyses of plant lipids showed that SEs, Ss, acylated SGs and SG had retention times of 1.16, 14.11, 17.83 and 27.68 min, respectively [121].

The aforementioned literature reports clearly demonstrate the wide applicability of normal-phase methods for the isolation of sterol fractions from lipid classes. In can be envisioned that the limited separability among closely related sterol molecular species of similar polarity (e.g. those found in vegetable oils) prohibits differential adsorptive interactions between analyte solutes and the stationary phase under the normal-phase conditions employed. On the other hand, if there is sufficient polarity difference between individual sterol structures, adequate separations can be achieved as demonstrated in the cases of cholesterol derivatives and triterpenoids [131–133]. Various oxygenated cholesterols have been separated in the normal-phase mode to give peak components whose RRT values (0.05–50.0) increased with the number of hydroxy groups or oxygenated moieties [131]. Successful normal-phase separations of β-amyrin analogues, dihydroxy/monohydroxy monocarboxy triterpenoids and monketonic/monohydroxyterpenoids have been described [132]. In this study (method 28 in Table 5), polar substituent effects on analyte elution order were clearly demonstrated. The elution order was much influenced by the number and location of hydroxyl groups. It also depended on whether carboxyl groups in triterpenoid structures are exposed or shielded. Thus, the more polar eburicoic acid with an open carboxyl group eluted after the less polar oleanolic acid with shielded carboxyl group [132]. Using procedures in method 30, acetate derivatives of C_{27} sterol precursors of cholesterol differing in the number and position of double bonds have been separated by normal-phase HPLC [133]. As frequently observed in normal-phase HPLC, decreasing

the polarity of a mobile phase solvent mixture can enhance resolution of sterol components of interest [132,133].

5.3. Reversed-phase HPLC

As gathered from Table 5, reversed-phase HPLC techniques have been more widely used in the analysis of individual sterols than normal-phase HPLC. This is partly due to some practical advantages of the former for its convenient operations with mobile phases of low volatility, reproducible chromatographic peak characteristics and good column selectivity for homologues and unsaturated-analogues. Hydrophobic interactions between analyte solutes and a stationary phase increase with increasing molecular sizes and decreasing number of double bonds in sterol molecules.

Approximately two third of the selected HPLC methods listed in Table 5 pertains to the reversed-phase HPLC analysis of plant sterols. Applications of alkylsilica phases in sterol assays overwhelmingly outnumber those of other stationary phases. Most reversed-phase methods (20 out of 23 methods) utilize octadecylsilica (ODS) columns. An Ultrasphere ODS phase (5 μm) has been used in three methods shown in Table 5 [114,116,117]. Also shown in the table, there are two methods in which a Spherisorb S3 ODS (3 μm) has been used for the analysis of sterols in oil seeds and plants [94,124]. The use of a TSK-Gel ODS (5 μm) column for the separation of epimers has been reported in two studies [118,122]. Other reversed-phase columns used by various laboratories for the analysis of sterols in vegetable oils include Deltabond ODS (5 μm) [110], Nucleosil-300 C_{18} (7 μm) [112], Supelcosil LC18 (5 μm) [115], Hypersil ODS (5 μm) [115], LiChrosorb RP-8 (5 μm) [128], Brownlee RP18 (5 μm) [129] and Pyrocarbon Si (10 μm) [134]. Food samples containing vegetable oils have been analyzed for sterols by reversed-phase HPLC with a Rad-Pak C_{18} (5 μm) column [120]. Chromatographic properties of numerous plant sterols including triterpenoids on a Partisil C_{18} (5 μm) phase have been evaluated [125]. A μBondapak C_{18} column was used by two laboratories for independent studies of sterols and C_{27} precursors of cholesterol [130,133]. Closely related sterols have been separated and isolated by preparative HPLC in a Bondapak C_{18}–Porasil B (37–75 μm) column system. In general, stationary phase particle sizes, column dimensions and other specific considerations concerning reversed-phase HPLC column selection are similar to those discussed earlier for normal-phase HPLC.

Mobile phases for reversed-phase HPLC of sterols employ polar organic solvents acetonitrile [130], methanol [114,116,117,120], or their aqueous solutions [125,128,129] depending on analyte structures and sample matrices. A large number of laboratories (Table 5) have used mixed organic solvents with small percentages of water for the separation of sterols. Occasional addition of an acid (acetic acid or formic acid) to mobile phase eluents improves peak characteristics and component resolution [108,110,113,132]. Many investigators have used non-aqueous mobile phases for their assays in light of highly hydrophobic properties of sterols [85,114,116–118,120,122,124,130,133–135]. A variety of less polar organic modifiers such as isopropanol, butanol, dichloromethane, chloroform, THF and hexane have been added to either aqueous or non-aqueous reversed-phase mobile phase systems to meet optimal separation requirements for specific analytical applications as indicated in 13 of the 23 reversed-phase methods listed (Table 5). With regard to elution modes, isocratic elution procedures have been the predominant methods (19 methods in Table 5) of choice for the reversed-phase separation of sterols. By contrast, fewer laboratories (methods 1, 5, 8 and 12) analyzed sterols by reversed-phase HPLC under gradient elution [85,108,112,115]. HPLC of plant samples containing a wide range of endogenous compounds usually call for gradient elution to include all structural types within reasonable run times despite obvious methodological simplicity of isocratic elution.

In an ODS (or octylsilica) system, the elution of plant sterol components follows the order of increasing analyte hydrophobicity. In principle, C_{27}, C_{28}, C_{29} sterols and sterols having different degrees of unsaturation can be readily separated especially as their acetates. However, reversed-phase HPLC selectivity for sterols differing in the position of unsaturation is somewhat inferior to argentation TLC. Under conditions specified in method 1 of Table 5, three

sterols in a mixture containing fatty acids and triglycerides are reportedly to elute in the order EG→CA→SI [108]. The presence of a 24-ethyl group in SI make it more hydrophobic than its 24-methyl homologue, CA, which in turn is less polar than the highly unsaturated trienol EG. In a HPLC study [112] on plant lipids and soybean oils, simultaneous separations of main neutral lipids into classes and subclass species have been achieved by gradient HPLC to give an elution order EG→CH→ST→SI→CHL→CHP (method 5, Table 5) in the sterol and steryl ester regions of chromatograms. CH contains one double bond without a 24-ethyl group and is expected to elute after the polar EG and before SI, which is more hydrophobic than its 22-olefinic analogue ST. In spite of the two double bond structure, ST has a longer retention time than CH because of the extra 24-ethyl group in the former. However, the saturated 16:0 palmitate of CH (CHP) appears to have a higher degree of hydrophobicity than its 18:2 linoleate (CHL).

Akihisa and associates have investigated HPLC separations of 4,4-dimethylsterol fractions obtained from preparative TLC and argentation preparative TLC of leguminosae seed extracts and observed the major components among others eluting in the sequence: LU→BU→TI→βAM→CYe→αAM (acetates) [114]. They also separated and isolated individual sterols from desmethylsterol fractions of leguminosae seeds the major components of which were found to elute in the manner CL→FU→IFU→ST→CA→SI (acetates) [116]. Further, from exotic plant seeds, they separated the sterol constituents by HPLC (method 10, Table 5) noting an elution order VE→LU→MVE→OB→GR→CE+βAM→AV+IFU→αAM→MCYa→ST→CI→SI (acetates) for the major species [117].

HPLC properties of about 100 sterols have been evaluated in the context of stereochemistry and substituent effects on the analyte chromatographic behavior [125]. In the study, the plant sterols commonly found in vegetable oils and oil seeds eluted as follows: EG→BR→OB→CL→CH→FU+βAM→IFU→CA+ST→αAM→LO→CYe→SI, where CA and ST co-eluted under conditions shown in method 20, Table 5. It is apparent that acetylation (method 9) appears to facilitate resolution of the CA–ST pair. In a separate study [127], HPLC of a sterol test mixture under isocratic elution with a mobile phase other than aqueous methanol (method 23) yielded well-resolved components emerging from an octylsilica column in the following order BR→EG→CH→CA→ST→SI. Clearly, the CA–ST pair was separated but the elution was opposite to that obtained with aqueous methanol. With an aqueous acetonitrile mobile phase, a standard mixture of sterols found in sunflower seed oil has been resolved into components: EG→CH→CYe→βAM→αAM→CA→ST→MCYa→SI→HLA (acetates), but the sterols in the oil sample has been determined by GC/GC–MS of each of five HPLC fractions obtained from a TLC sterol zone [128]. A rapid reversed-phase HPLC separation of free sterols eluting in the order EG→BR→FU→CH→ST→CA→SI has been described in a study with oil, fat and plant samples [129]. The chromatographic behavior of these sterols was affected by changing the column temperature (22–50°C) with shorter retention times at higher temperature and the optimal temperature for separation was found to be 30°C.

Nes et al. have determined σ values (defined as contributions that individual molecular features made to retentions α_c relative to cholesterol) for numerous sterols and triterpenoids in which a few selected compounds relevant to plant sterols had the elution order LU→EG→βAM→CYe→αAM→BR→CH+FU+IFU→CA→ST→SP→SI→CHa [130]. All the HPLC results [116,120,125,127–130] discussed so far indicate that the counteracting effect of the 24-ethyl group and 22-double bond moiety in ST in relation to CA on the elution behavior is much sensitive to changes in HPLC conditions. Thus, the order of elution ST→CA was observed in all studies [116,120,129] with methanol in mobile phases, whereas a reversed order CA→ST was observed in all studies [127,128,130] with acetonitrile in mobile phases.

Some selected triterpenoids with different polar function groups and ring structures have been separated by reversed-phase HPLC with elution order not exactly in the reversed order of that in normal-phase HPLC notwithstanding the complementary separation results [132]. As expected, analyte retention decreases with the number of polar groups and with increasing polarity of the function groups: soyasapogenol→oleanolic acid→Erythrodiol; LU→

EU→LA→CYe→LO→HLA. Some reversed-phase HPLC separations of acetates of C_{27} precursors of cholesterol based on the degree of unsaturation have been published [133]. Nearly two decades ago, Guiochon's group demonstrated the separation of free sterols on a pyrocarbon modified silica gel column (method 32, Table 5) with which an elution LA→SI→CH→ST→BR→EG (conventional normal-phase order) of a test mixture was obtained [134]. The observed elution order was interpreted by geometrical considerations. Due to incomplete coverage of silica surface, the reversed-phase packing material Bondapak C_{18}–Porosil B (method 33) can also be used in normal-phase mode if normal-phase solvents are used. Using such a column and a hexane–isopropanol mobile phase, a preparative HPLC procedure has been developed for the separation and isolation of C_{27}, C_{28} and C_{29} sterols with following normal-phase elution order SI→ST→CA→CH→EG [135].

Several laboratories (methods 3, 6, 8, 12, 126 and 96, Table 5) have addressed the analysis of individual sterol conjugates of nutritional significance by reversed-phase HPLC. Norton analyzed samples of corn bran, rice bran and their oils for steryl ferulates (F) and *p*-coumarates (C) (Fig. 4) which eluted in the order CyeF→MCYaF→CBF→CAF→CAaC→CAaF→SIC→CYaF→SIaF [110]. In an earlier study, the same investigator elaborated reversed-phase separations of steryl esters in corn bran and evaluated their RRT values of the steryl ferulates and *p*-coumarates having an elution order 7CAeC→7CAeF→CAF→STF→CAC→7STeF→CAa→SIF→CAaF→CAaC→SIa→SIaF→SIaC [113]. The elution patterns from the above two studies [110,113] appear to be illustrative of the polar nature of the ferulates relative to the coumarates but exceptions exist in the cases of the two pairs 7CAeC→7CAeF and CAaC→CAaF. Hence, the elution order of the steryl ferulates and coumarates was found very sensitive to variation in HPLC stationary phase- and mobile phase conditions. The elution of CAF before STF is in the same trend described earlier as the parent free sterols separated with an acetonitrile mobile phase (method 6).

Rogers et al. have analyzed samples of rice ban oil for γ-oryzanols (ferulic acid esters of sterols and triterpene alcohols) and identified the ferulate components with an elution order CYeF→MCYaF→CAF→SIF+CYaF [115]. In fact, Goad's research group first isolated and characterized intact steryl ferulates from seeds [124]. From a maize seed sample, they identified two major species campestanyl- (CAaF) and stigmastanyl- (STaF) ferulates which eluted in the following order along with other compounds studied: CYeF→MCYaF→CAF→SIF→CAaF→STaF (as 4′-acetates). In general, the elution of γ-oryzanol components on ODS is predictably in the order of increasing molecular hydrophobicity.

On the other hand, steryl fatty acid esters in

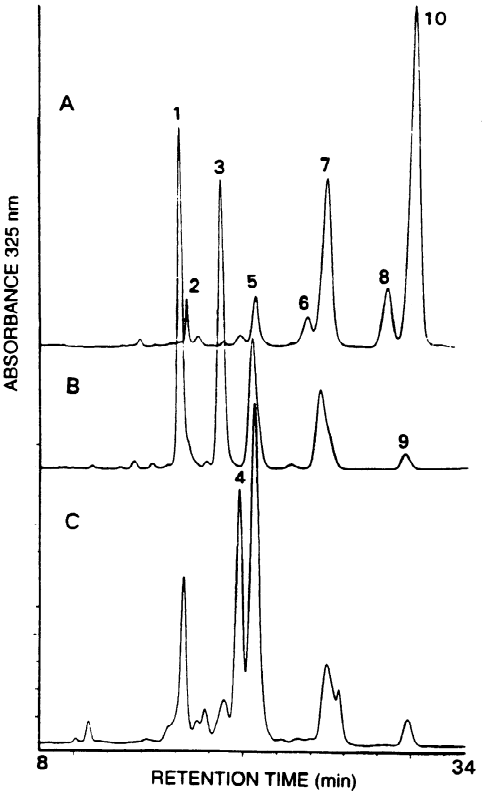

Fig. 4. Reversed-phase HPLC–DAD of corn and rice samples with an octadecylsilica phase. Mobile phase, MeCN–butanol–acetic acid–water (82:3:2:13). Samples (A) ultra high fiber corn bran; (B) rice bran; (C) commercial rice bran oil. Peaks: 1, cycloartenyl ferulate; 3, 24-methylenecycloartanyl ferulate; 4, cyclobranyl ferulate; 5, campesteryl ferulate; 6, campestanyl *p*-coumarate; 7, campestanyl ferulate; 8, sitostanyl *p*-coumarate; 9, cycloartanyl ferulate; 10, sitostanyl ferulate (from [110] with permission).

some plant samples have been separated into individual species [85]. The elution of EGT-myristate (EGTM) (14:0)→EG-palmitoleate (EGPO) (16:1)→ EGO(18:1) → ZYO(18:1) → EGP(16:0) → EGS(18:0) observed in HPLC of a yeast sample is reflective of an interplay of hydrophobicity contributed from both the sterol- and fatty acid structures. Since EGTM contains four double bonds in the sterol moiety and myristic acid (M) of the shortest fatty acid carbon chain, it seems justified to be the earliest elution component. Steryl esters of unsaturated fatty acids EGPO, EGO and ZYO elute before the saturated fatty acid esters EGP and EGS. Evershed et al. developed sequential preparative TLC, preparative argentation medium-pressure LC and reversed-phase HPLC procedures for the analysis of steryl esters in plants and vegetable oils [94] They measured the RRT values (relative to cholesterol palmitate) of the steryl fatty acid esters detected in rapeseed oil: CAL → SIL → CAO → SIO → CAP → SIP → CHS → CAS → SIS. This elution order is virtually in line with the increasing solvophobicity of fatty acid acids linoleic acid (L) (18:2)→oleic acid (O) (18:1)→palmitic acid (P) (16:0)→stearic acid (S) (18:0).

Reversed-phase HPLC separations of the epimers of C-24 alkylsterols of biological importance have been documented [118,122]. HPLC with an ODS column thermostated at 12°C (method 13) led to the resolution of epimeric sterols with 24 β-isomers eluting later [118]. RRT values relative to cholesterol ranged from 0.52 to 1.16. Sitosterol (SI) failed to separate from its 24 β-isomer under the conditions employed. Baseline resolution was achieved for four C_{28} epimeric pairs CA→HBR, 8CA→8EG, CR→BR and 8CA→8EG. Most of C_{29} sterols each having a 24-ethyl group were only partially resolved. Apparently the presence of 24-methyl groups in sterols seems to enhance hydrophobic differentiation of epimers in the reversed-phase HPLC separation processes. In another approach (method 17), HPLC of benzoates of 24-epimers with the same column as above but in a different solvent system resulted in variable degrees of resolution [122] with elution characteristics analogous to those observed in the separation of free sterol epimers [118]. It is of note that the SI–CL pair was partially resolved in this instance despite the observed inseparability of the parent epimeric pair.

5.4. Detection techniques

The commercially available HPLC detection methods (Table 5) for the detection of sterols include ELSD, UV, photodiode array detection (DAD), FID and refractive index detection (RI) systems. Of these, UV (200–210 nm settings) and RI detectors are the most useful for sterol assays with the former providing better detection sensitivity. Because the number and location of double bonds in sterols differ widely, HPLC quantification of sterols with UV detection is less than straightforward owing to their variable UV molar absorption coefficients. Therefore, calibration plots of UV detector responses against analyte standard concentrations must be constructed prior to HPLC sample quantitation. In HPLC–DAD system, analytes emerging from column effluents are monitored with multiple-wavelength UV diode array detector scanning from 200 to 400 nm suitable for the selective analysis of sterols with various conjugated olefinic structures. In recent years, ELSD methodology has been widely utilized in lipid analysis. Normally, ELSD systems are compatible with gradient elution of mobile phases. However, ELSD is less sensitive than UV and, like UV, requires calibration for quanitification due to its dependency on the analyte molecular mass. FID has recently found its popularity in the analysis of lipid classes. Although plant sterols in vegetable oils are rarely analyzed by HPLC–fluorescence (FL) detection, the FL detection technique can be valuable in trace analyses of fluorescence-labeled sterols with enhanced detection sensitivity and specificity.

The UV detection technique has been used in most (about two third) of the selected published methods compiled in Table 5. Two laboratories (methods 2 and 4) analyzed corn fiber and potato lipids for sterol- and steryl ester fractions by normal-phase HPLC–ELSD under gradient elution [109,111]. A gradient reversed-phase HPLC–ELSD method for the analysis of wood samples has been described [108]. Two groups of investigators (methods 16 and 21) have developed gradient normal-phase HPLC–FID methods for the separation and isolation of lipid classes [121,126]. Both ELSD and FID are capable of detecting analytes transparent to UV. There are three methods 3, 6 and 8 in Table 5 that utilize a DAD system for the reversed-phase analysis of

sterols and steryl conjugates of ferulic acid and coumaric acid [110,113,115]. In these instances, detection of multiple-component analytes inherent with various UV-absorbing chromophores mandates the use of the multiple scanning absorbance detection DAD system to facilitate measurement of each compound at λ_{max} for achieving maximal detection sensitivity and selectivity.

Coupling HPLC with MS or NMR not only will allow simultaneous separations and quantitation but also will provide a useful means for structural elucidation and confirmation of sterol structures. However, few publications on the practical application of HPLC–MS and HPLC–NMR techniques in sterol analysis have appeared in the literature. In methods 7, 9, 10 and 18, the sterol fractions obtained from HPLC were characterized by GC–MS or the individual sterols isolated from HPLC were analyzed by MS or NMR.

6. Quantitative analysis

GC–FID is most commonly used to quantitate sterols in various sample matrices by virtue of a large linear mass range of response of the FID system. For quantitative measurement of sterols, it is imperative to determine the linear range of response for each GC–FID system to a sterol standard. All quantitation requires statistical method validation in the context of reproducible retention times, precision, recovery studies with spike samples and absolute response factors [59]. Losses of sterol analytes during isolation and separation must be corrected for the final results of analyte quantification by using internal standardization with standards not present in samples or radioisotopes [102]. Evaluation of the repeatability of the best extraction efficiency can further reduce analytical errors in estimating sterols in assay samples. As compared to GC, quantitative analysis of sterols by HPLC is somewhat limited. Using dose–response calibration curves, HPLC–PDA is useful for quantitation of specific sterols [47,53].

7. Other chromatographic techniques

CEC is first introduced in 1974 by Pretorius et al. [136] and is a relatively new technique in its infancy for the analysis of lipids and sterols. CEC features high efficiency-, high resolution- and high speed micro-scale separations with minimal solvent consumption. It is a combination of capillary electrophoresis and HPLC. In comparison with electrophoretic techniques including CEC, the column efficiency in HPLC is low because of the need of an external pressure to pump analytes with the mobile phase through the column. In contrast, the chromatographic flow in CEC is driven by electroosmotic force (EOF) to transport solutes through a packed capillary column. Thereby, the high column efficiency of CEC is its primary benefit derived from the flat flow profile of the EOF relative to the parabolic flow from HPLC. As in HPLC, CEC separations are influenced by operational parameters: percentage of mobile phase organic modifier, buffer pH/concentration, type of stationary phase. In general, organic modifiers with low viscosity and high dielectric constant such as acetonitrile are best suite for CEC. Low conductivity buffers (e.g. Tris) are recommended to avoid excessive heat generation. Lowest possible buffer concentrations are normally maintained in CEC systems to provide high EOF and low current. The optimum pH range for neutral compounds is 4–8 and pH 2.5 should be used for acidic compounds. Stationary phases with high residual silanols or charged groups produce fastest EOF suitable for the separation of uncharged or weakly ionizable compounds in the reversed-phase mode. The CEC reversed-phase elution patterns are comparable with HPLC.

Most recently, CEC of cholesterol and its ester derivatives has been reported [137]. Under optimized conditions with UV detection at 200 nm, cholesterol and twelve fatty acid esters were completely separated on a fused-silica capillary (20 cm$\times$100 μm) packed with 3 μm ODS. In a mobile phase system of THF–MeCN–Tris buffer (35:60:5), the compounds eluted as follows: acetate(2:0) → butyrate(4:0) → valerate(5:0) → hexanoate(6:0) → heptanoate(7:0) → CH → octanoate(8:0) → non-anoate(9:0) → decylate(10:0) → laurate(11:0) → linoleate(18:2) → oleate(18:1) → palmitate(16:0). The short chain acid esters with the carbon number equal or smaller than seven emerged from the column faster than CH, while the long chain fatty acid esters were retained on the stationary phase longer than CH. Evidently,

the palmitate derived from the 16 carbon chain saturated acid is more retentive than the eighteen carbon chain linoleate and oleate containing respective two and one double bond in the fatty acids. In addition, dramatic effects of CEC experimental variables including acid modifiers, mobile phase composition, buffer concentration and added pseudo-stationary phase (Fig. 5) on component separations were demonstrated. Application of the method to the analysis of crude extracts from atherosclerotic plaque of a human aorta showed the presence of CH, CH-oleate (CHO) and CH-palmitate (CHL).

CEC studies [138,139,141] on some steroids and isomers have also been described. In light of the demonstrated feasibility of CEC as a rapid and high efficiency separation technique, CEC with isocratic elution can substitute gradient elution HPLC and provides potential utility in the future analysis of plant sterols in various samples.

Supercritical fluid chromatography (SFC) employs inert supercritical carbon dioxide as mobile phase eluent. The technique is suitable for the analysis of phytosterols in vegetable oils. Coupling supercritical fluid extraction (SFE) with SFC enables sample extraction, preconcentration, preparative fractionation and chromatographic quantitation in a single operation. Snyder et al. used SFE to enrich sterols from vegetable oils before analysis by SFC [39]. SFC–UV (or FID) of cholesterol and its acyl esters on ODS yielded partial separations of the test components [140]. However, a base-line separation of these components have been achieved by CEC–UV [137] and by HPLC [140].

Apparently, the separation power of CEC is greater than HPLC which, in turn, is much greater than SFC. GC appears to have greater ability (or comparable) to resolve complex mixtures than CEC despite superior analyte selectivity (and suitability for thermally labile analytes) of the latter. Analytical precision and sensitivity of the various techniques currently available for sterol assays seems to follow the order GC>HPLC>SFC>CEC. The sensitivity

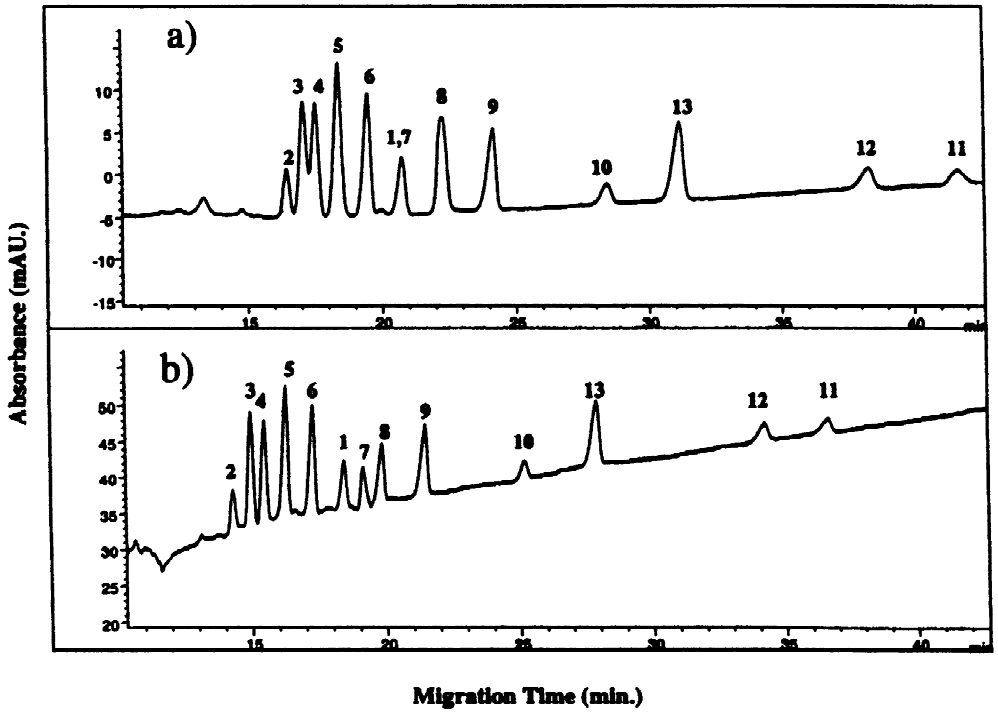

Fig. 5. CEC–UV detection of cholesterol and its ester derivatives on a capillary column packed with octadecylsilica. Mobile phases: (a) Tris buffer (25 m*M*/pH 8)–THF–MeCN (5:35:60). (b) Tris buffer (25 m*M*/pH 8)–THF–MeCN (5:35:60) with added 5 m*M* poly sodium *N*-undecanoylglycinate. Peaks:1, acetate; 2, butyrate; 3, valerate; 4, hexanoate; 5, heptanoate; 6, cholesterol; 7, octanoate; 8, non-anoate; 9, decylate; 10, laurate; 11, linoleate; 12, oleate; 13, palmitate (from [137] with permission).

order may varies depending on sterol structures (e.g. fluorescent labels) and detectors coupled to chromatographic instruments. While GC, HPLC, SFC and CEC (in its developmental phase) are individually meritorious for specific applications, GC–FID (or MS) is considered to be the method of choice for practical analyses of plant sterols in foods and vegetable oils.

8. Nomenclature

AM	Amyrin
AV	Avenasterol
BR	Brassicasterol
BU	Butyrospermol
CA	Campesterol
CAa	Campestanol
CAe	Campestenol
CB	Cyclobranol
CC	Column chromatography
CD	Chondrillasterol
CE	Cycloeucalenol
CEC	Capillary electrochromatography
CH	Cholesterol
CHa	Cholestanol
CI	Citrostadienol
CL	Clerosterol
CLI	Clionasterol
CR	Crinosterol
CYa	Cycloartanol
CYe	Cycloartenol
DAD	Photodiode array detection
DCD	Dehydro-CD
DE	Desmosterol
DSP	Dehydro-SP
EG	Ergosterol
EGa	Ergostanol
EGT	Ergostatetraenol
ELO	Ethyllophenol
ELSD	Evaporative light scattering detection
ER	Erythrodiol
EU	Euphol
FID	Flame ionization detection
FN	Fungisterol
FU	Fucosterol
GC	Gas chromatography
GR	Gramisterol
HBR	Dihydrobrassicasterol
HLA	Dihydrolanosterol
HPLC	High-performance liquid chromatography
HPO	Dihydro-PO
HX	Hexane
IFU	Isofucosterol
IPA	Isopropanol
IR	Infrared detection
LAe	Lanostenol
LO	Lophenol
LU	Lupeol
MCH	Methylencholesterol
MCYa	Methylencycloartanol
MIM	Multiple-ion monitoring
MLAe	Methylenlanostenol
MMCH	Trimethyl-24-methylencholesterol
MS	Mass spectrometry
MTBE	Methyl *tert*.-butyl ether
MVE	Methylvernosterol
MZM	4α-Methylzymosterol
MZYe	Methylzymostenol
NCYe	Norcycloartenol
NLA	Norlanosterol
NLAe	Norlanostenol
NMR	Nuclear magnetic resonance detection
OB	Obtusifoliol
ODS	Octadecylsilica
PA	Parkeol
PO	Poriferasterol
POe	Poriferastenol
SC	Schotenol
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SFF	Supercritical fluid fractionation
SI	Sitosterol
SIa	Sitostanol
SIM	Single-ion monitoring
SP	α-Spinasterol
SPE	Solid-phase extraction
ST	Stigmasterol
STa	Stigmastanol
STe	Stigmastenol
STD	Stigmastadienol
STT	Stigmastatrienol
THF	Tetrahydrofuran
TI	Tirucalladienol
TLC	Thin-layer chromatography

TMS	Trimethylsilyl
UV	UV absorbance detection
UVA	Uvaol
VE	Vernosterol
ZY	Zymosterol
ZYe	Zymostenol

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