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Review

Chromatographic methods in the analysis of cholesterol and related lipids

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Abstract

Methods using thin-layer chromatography, solid-phase extraction, gas chromatography, high-performance liquid chromatography and supercritical fluid chromatography are described for the analysis of single cholesterol, esterified and sulfated cholesterol, and for cholesterol in the context of other lipid components, like other sterols and lipid classes. In connection with these techniques several clinical applications are mentioned.

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List of abbreviations

ACAT	Acyl-CoA:cholesterol O-acyl transferase (EC 2.3.1.26)
ALBK	Abell–Levy–Brodie–Kendall
FI(D)	Flame ionisation (detection)
GC	Gas chromatography
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
HPLC	High-performance liquid chromatography
LCAT	Lecithin:cholesterol acyl transferase (EC 2.3.1.43)
MS	Mass spectrometry
MTBE	Methyl *tert.*-butyl ether
SFC	Supercritical fluid chromatography
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
UV	Ultraviolet

1. Introduction

Cholesterol is an important lipid in many vital processes. It is a structural constituent of cell membranes, and a substrate for the synthesis of bile acids and steroid hormones. Cholesterol de novo synthesis occurs from acetyl-CoA units in the cytoplasm of virtually all cells. The capacity is greatest in liver, intestine, adrenal cortex, and reproductive tissue, including ovaries, testes, and placenta. An important intermediate is 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is further metabolized to mevalonic acid by HMG-CoA reductase. This is the rate limiting step in cholesterol biosynthesis. From lanosterol, an intermediate at the end of cholesterol biosynthesis, two branches lead to the production of cholesterol, probably using the same enzymes. The major branch includes lathosterol and 7-dehydrocholesterol as intermediates, whereas desmosterol is an intermediate of the minor branch. A combination of abnormally low plasma cholesterol levels with markedly elevated concentrations of 7-dehydrocholesterol is seen in patients with the Smith–Lemli–Opitz syndrome, suggesting a block in cholesterol biosynthesis at the reduction of the C-7 double bond [1,2].

Cholesterol is transported in plasma lipoproteins and stored in the liver in the form of cholesterol esters. At least two enzymes are responsible for cholesterol esterification: acyl-CoA:cholesterol O-acyl transferase (ACAT; EC 2.3.1.26) exerts its function intracellularly for storage and internal secretion, and lecithin: cholesterol acyl transferase (LCAT; EC 2.3.1.43) esterifies cholesterol in plasma for reverse transport of superfluous tissue cholesterol to the liver. Cholesterol esters and triacylglycerols, derived from endocytosis of low-density lipoprotein vesicles, are hydrolyzed intracellularly by a lysosomal acid lipase (EC 3.1.1.13). In normal plasma about 75% of total cholesterol is esterified to long-chain fatty acids. LCAT deficiency causes a high percentage of unesterified cholesterol and a low concentration of lysophosphatidylcholine in plasma. Target-shaped red blood cells contain abnormally high amounts of unesterified cholesterol and phosphatidylcholine, as is the case in patients with severe liver diseases and LCAT deficiency. A modified LCAT in plasma from patients with fish-eye disease results in a moderately lower percentage of esterified cholesterol.

High total serum cholesterol can be found in subjects with familial hypercholesterolemia, coronary heart disease and atherosclerosis. Familial hypercholesterolemia can originate from

a modified apolipoprotein B as well as from a modified LDL receptor, resulting in insufficient removal of cholesterol containing lipoproteins by the liver. Atherosclerosis is caused by inadequate transport of cholesterol, leading to accumulation of cholesterol esters in vessel walls, uptake by macrophages, formation of foam cells and subsequent plaque formation. A high linoleic acid intake is supposed to prevent or diminish the effects of atherosclerosis. Plasma cholesterol ester species are parameters reflecting dietary fat intake. Prescription of drugs lowering plasma (low-density lipoprotein) cholesterol, such as bile acid sequestrants and HMG-CoA reductase inhibitors, has experienced a tremendous rise in the past decade.

In the intestines, sterols other than cholesterol are usually poorly absorbed. Plasma samples from subjects with phytosterolemia contain increased amounts of phytosterols, like β-sitosterol and campesterol, which is likely to be caused by the loss of normal intestinal selectivity for cholesterol absorption [3–6].

Impaired synthesis of bile acids due to mitochondrial 26-hydroxylase deficiency causes accumulation of cholesterol and cholestanol (5α-cholestan-3β-ol) in cerebrum, tendons and liver (cerebrotendinous xanthomatosis) [7]. In normal serum, cholestanol is less than 0.2% of cholesterol [7], but about 3% of cholestanol can be present in serum from patients with cerebrotendinous xanthomatosis [3,7,8] or phytosterolemia and xanthomatosis [3,5,6], and in serum from patients suffering from various liver diseases [7].

Cholesterol sulfate is a minor constituent of various mammalian tissues. Cholesterol sulfate in plasma and erythrocytes is elevated in patients with recessive X-linked ichthyosis, caused by deficiency of steroid sulfatase (EC 3.1.6.2) [9–12].

In this review, methods using thin-layer chromatography (TLC), solid-phase extraction, gas chromatography (GC), high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) are described for the analysis of single cholesterol, esterified and sulfated cholesterol, and for cholesterol in the context of other lipid components, like other sterols and lipid classes.

The analysis of total cholesterol in serum is usually performed by routine colorimetric or enzymatic methods. GC methods have been developed to serve as reference or definitive methods. For the identification of e.g. cholestanol, plant sterols and 7-dehydrocholesterol, chromatographic methods are indispensable, as these sterols are not distinguished from cholesterol in routine procedures.

Cholesterol ester species have been separated by GC, reversed-phase HPLC, as well as SFC. GC on apolar columns separates cholesterol esters only according to their molecular masses, whereas apolar columns permit separations of cholesterol ester species also on the basis of their degree of unsaturation. However, cholesterol esters containing highly unsaturated fatty acid moieties suffer from thermal degradation. HPLC and SFC procedures do not require high temperatures, thereby avoiding underestimation of highly unsaturated cholesterol esters. Using these methods identification is more difficult, because of the coelution of many components due to insufficient separating capacity. When applying an ultraviolet (UV) detector, quantification is almost impossible, as cholesterol ester species show very different absorption properties, depending on the number of double bonds. Argentation HPLC separates intact cholesterol esters on the basis of the number of double bonds in the fatty acid moiety.

Chromatography of total lipid classes, such as acylglycerols, cholesterol esters, cholesterol, and phospholipids, can be performed with GC as well as straight-phase HPLC methods. GC methods are usually employed for plasma-derived total lipids, requiring pretreatment of the sample by phospholipase C and separating lipid classes into their individual species. In straight-phase HPLC methods, lipid classes are usually chromatographed as single peaks, allowing separation and identification of lipid extracts of many different tissues.

2. Extraction of lipids

Isolation of total lipids, including cholesterol and cholesterol esters, from tissue matrices in a

relatively pure state is usually done by solvent extraction. The method as described by Folch et al. [13], using a mixture of chloroform–methanol (2:1, v/v), is widely employed. Together with endogenous water of the tissue this mixture forms a ternary component system, giving rise to proportions of chloroform–methanol–water of 8:4:3 (v/v/v). Radin describes an extraction solvent (isopropanol–hexane, 2:3, v/v) that is less toxic and gives a clear extract [14]. However, the method has not been tested with a sufficiently wide variety of tissues. Other solvents that have been used for lipid extraction are diethyl ether [15], isooctane–chloroform (80:20, v/v) [16], isopropanol [17,18], tetrahydrofuran [19], *n*-octane [20], hexane-methanol (recovery of lipids not optimal) [20], ethyl acetate [21] and isopropanol–water–10 *M* sodium hydroxide (250:125:10, v/v/v) with *n*-octane added afterwards [22]. The latter extraction eliminates most of the triacylglycerols and all phospholipids, leaving mainly cholesterol and cholesterol esters in the upper octane phase. However, some isopropanol-fatty acid esters may contaminate the cholesterol ester fraction [20]. To protect lipids, especially polyunsaturated fatty acids, from autoxidation it is advisable to add an antioxidant (usually butylated hydroxytoluene) as soon as possible and during all following procedures.

3. Thin-layer chromatography

TLC can be performed on glass or quartz Chromarods [23–27] or on plates coated with silica gel. Most methods describing the separation of lipid classes, such as cholesterol esters, triacylglycerols, free fatty acids, diacylglycerols, cholesterol, monoacylglycerols and total phospholipids (origin), are based on a solvent mixture containing hexane (or petroleum ether)–diethyl ether–acetic acid in proportions of about 85:15:2 (v/v/v) [20,24,28–31]. Many variations have been developed, e.g. changing hexane for heptane [21,32], using formic acid instead of acetic acid [27], or leaving out diethyl ether or acetic acid [23–25]. Some methods use a 2-step development, the first step for the chromatography of phospholipids, the second for the separation of neutral lipids [33–36].

Kovács et al. [35] use a 2-step development in which the second solvent system, consisting of *n*-hexane–acetone, is able to separate cholesterol ester fractions with 4 double bonds (18:4 and 20:4), 2 double bonds (18:2 and 20:2), 1 double bond (16:1 and 18:1) and no double bonds (16:0). A method describing the chromatographic separation of cholesterol ester species (cholesteryl stearate, oleate and linoleate) uses an elution mixture of (chilled) chloroform–*n*-heptane (40:60, v/v) at 4°C for 15 min [32].

Cholesterol sulfate has been determined with reversed-phase TLC after purification of sulfated steroids on reversed-phase solid-phase extraction columns [11]. After hydrolysis, the extract is applied to the reversed-phase TLC plate and developed with chloroform–acetonitrile (35:40, v/v).

3.1. Argentation chromatography

Argentation chromatography of several sterols has been performed on plates coated with aluminium oxide G/silver nitrate (94.5/28.8 by weight) and chloroform as solvent [29]. 7-Dehydrocholesterol and desmosterol are retained on the origin (R_F 0.0), whereas cholesterol, cholestanol and lathosterol have R_F values of 0.50, 0.84 and 0.87, respectively. Tint et al. [2] prepare argentation TLC plates by dipping them in a solution of 5% silver nitrate in methanol. Plates are developed in chloroform–acetone (85:15, v/v) at 5°C in the dark. Relative mobilities (R_F) of 7-dehydrocholesterol, a dehydrocholesterol isomer, desmosterol, and cholesterol are 0.29, 0.54, 0.69, and 0.72, respectively.

Argentation chromatography of sterol esters has been achieved on 2% $AgNO_3$-silica gel plates which are developed with hexane–toluene (70:30, v/v) [37]. Bands are detected containing sterol esters with long-chain (C_8–C_{22}) saturated fatty acyl groups (R_F 0.75–0.80), monoenoic acyl groups (R_F 0.65), dienoic acyl groups (R_F 0.55), trienoic acyl groups (R_F 0.35) and tetraenoic acyl groups (R_F 0.20). Saturated short-chain (C_2–C_6)

fatty acyl esters give R_F values in the range of 0.45-0.7, depending upon their chain length.

Silver nitrate impregnated silica gel plates have been developed with *n*-hexane–diethyl ether–methanol (18.8:1.5:1.0, v/v/v) for the resolution of cholesteryl stearate, oleate, linoleate and linolenate [38].

3.2. Detection methods

Qualitative (and quantitative) detection of lipid fractions on TLC can be performed densitometrically after charring, by chemical methods following elution of the material from the plate, or by flame ionisation detection (FID).

Charring has been made more easily by spraying with 10–20% phosphomolybdic acid in ethanol [11,33,35], sulphuric acid [20,29,32,37,39], 0.25% sodium dichromate in 15% sulfuric acid [34], 3% cupric acetate in 8% phosphoric acid [34] or 10% cupric sulfate in 8% phosphoric acid [31,38] prior to heating.

Other ways of visualisation, without destructive charring, allow chemical quantification analyses of isolated TLC fractions. Spots have been visualized by spraying with 10% phosphomolybdic acid in ethanol and colour development at room temperature [28], spraying with Rhodamine 6G solution allowing UV detection [20], spraying with 2,5-bis[5′-*tert.*-butylbenzoxazolyl-(2′)]thiophene in diethyl ether and viewing under UV light [30], spraying with 0.005% berberine in ethanol (UV detection) [37] or by immersion in iodine vapour [21,30,38]. Iodine has to be sublimated and removed before lipid zones can be scraped off [21]. Lipids without double bonds do not develop an intense yellow colour on exposure to iodine.

FID has been performed by passing Chromarods through a commercially available FI detector [23–27]. This detection has the profit of better quantitative results and scraping the components from the TLC plate is not necessary. Calibration dependencies are non-linear, giving typical sigmoid curves [27].

Although TLC procedures have been improved, they are still not suitable for the measurement of low lipid levels and subtle changes. They can, however, be very useful for quick qualitative evaluations.

4. Solid-phase extraction

Due to the complexity of lipid extracts from natural sources it is rarely possible to separate them into all classes in one chromatographic procedure. Often it is preferable to perform a prefractionation to obtain more specific information of different lipid classes. For this purpose solid-phase extraction columns, mostly packed with silica or aminopropyl-silica, are valuable. The use of argentation solid-phase extraction is restricted to separation of a single lipid class.

4.1. Silica gel chromatography

Prefractionation by adsorption chromatography can easily be performed on home-made or pre-packed silica gel columns. Wang and Peter use pre-packed cartridges of silica gel and elute cholesterol esters from a human serum lipid extract with 1.5% diethyl ether in light petroleum [20]. More complete separation of lipid classes on silica gel columns has been obtained by Hamilton and Comai [40]. Combined neutral lipids (cholesterol esters, triacylglycerols, fatty acids and cholesterol), phosphatidylethanolamine and phosphatidylcholine are isolated [40]. Elution of neutral lipids is done with chloroform–acetic acid (100:1, v/v), phosphatidylethanolamine is extracted with methanol–chloroform (2:1, v/v) and phosphatidylcholine, containing some residual phosphatidylethanolamine, with methanol–chloroform–water (2:1:0.8, v/v/v). A follow-up of this study reports the replacement of chloroform by methyl *tert.*-butyl ether (MTBE) for the isolation of separate neutral lipid classes [41]. Combinations of hexane and MTBE are used to progressively elute cholesterol esters (hexane–MTBE, 200:3, v/v) and triacylglycerols (96:4, v/v) from the column. After column acidification, fatty acids are eluted (hexane–MTBE–acetic acid, 100:2:0.2, v/v/v), followed by cholesterol (MTBE–acetic acid, 100:0.2, v/v). Polar lipids can be extracted using

combinations of MTBE–methanol–ammonium acetate (pH 8.6). If present, fatty acid methyl esters and simple alkyl esters coelute with cholesterol esters, whereas monoacylglycerols, 1,2- and 1,3-diacylglycerols emerge in the cholesterol fraction. A combination of hexane with MTBE proves to be superior, compared with combinations of hexane with ethylacetate, chloroform or dichloromethane. MTBE is preferred to diethyl ether because the latter is much less stable than MTBE and many of the added stabilizers interfere with the analysis [41].

4.2. Aminopropyl-bonded silica gel chromatography

Kaluzny et al. describe solid-phase extraction on aminopropyl silica columns, leading to fractionation of seven different lipid classes [31]. Three columns are necessary for the complete separation of neutral lipids (cholesterol esters, triacylglycerols, cholesterol, diacylglycerols and monoacylglycerols), fatty acids and combined phospholipids (phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin and neutral glycolipids [31,36]). Neutral lipids are initially eluted as one fraction with chloroform–isopropanol (2:1, v/v). Consecutively they are transported to a second column, extracted with hexane to elute cholesterol esters and then with diethyl ether–methylene chloride–hexane (1:10:89, v/v/v) to obtain triacylglycerols. In the latter step about 10% of cholesterol coelutes, requiring an additional column to trap the cholesterol. Both columns are then eluted with ethyl acetate–hexane (5:95, v/v) to isolate cholesterol. Part of this extraction procedure has effectively been used for the separation of cholesterol esters and triacylglycerols from plasma total lipid extracts [42]. Others have used the complete fractionation for total lipid extracts from *Escherichia coli* and human spermatozoa [36]. They extend the method of Kaluzny et al. [31] by an isolation step of polar acidic lipids (phosphatidylglycerol, cardiolipin, phosphatidylinositol, phosphatidylserine, seminolipid lipid A and acidic glycosphingolipids) [36].

4.3. Argentation chromatography

Evershed et al. describe a method using medium-pressure liquid argentation chromatography for the separation of sterol ester mixtures [37]. Stepwise elution is performed with dichloromethane, dichloromethane–MTBE (90:10, v/v) and finally with dichloromethane–MTBE (50:50, v/v). Combined fractions contain sterol esters bearing saturated fatty acyl groups (fractions 1–7), monoenoic (8–14), dienoic (15–18), trienoic (19–24) and tetraenoic (25–30) esters. Sterol esters bearing eicosenoate (20:1) and brassidate (22:1) elute in the saturated fraction, although they contain one double bond in the fatty acyl moiety.

Hoving et al. have separated intact plasma cholesterol esters by argentation chromatography on solid-phase extraction columns packed with a bonded sulfonic acid phase [43]. Elution is performed in 7 steps, isolating fractions containing cholesterol esters with zero to six double bonds, starting with dichloromethane and gradually introducing acetone and acetonitrile, respectively. Depending on column sample load, cross-overs between fractions can be considerable. Whether this method is useful for the separation of complex sterol ester mixtures, remains to be established.

4.4. Extraction of cholesterol sulfate

Cholesterol sulfate and other sulfates have been isolated by anion exchange chromatography by applying them to quaternary amine cartridges [9]. After washing the columns with methanol, cholesterol sulfate is eluted with 1 mol/l ammonia in aqueous methanol (water–methanol, 75:25, v/v).

Another method describes the resolution of cholesterol sulfate on a reversed-phase extraction column [11]. Serum lipids are extracted with methanol, and after the addition of potassium chloride to the organic layer, the solution is supplied to a C_{18} reversed-phase column. The column is washed with ether and with 5% acetonitrile in ether, and consecutively sulfated cholesterol is eluted with acetonitrile. Others,

also using C_{18} cartridges, first wash the columns with a 25 mmol/l ammonium acetate solution, then with a methanol–ammonium acetate solution (4:6, v/v), and finally with water [12]. Cholesterol sulfate is eluted with methanol.

Isolation of cholesterol sulfate has also been performed on silica gel cartridges [44]. After sample introduction the column is washed with cyclohexane–chloroform (2:1, v/v), and cholesterol sulfate is eluted with methanol–chloroform (2:1, v/v).

5. Gas chromatography

GC analyses of cholesterol and related compounds have been performed for many different materials. Most commonly they are used for the quantification of cholesterol in serum. Many methods, with FI or mass spectrometric (MS) detection, have been proposed as reference or definitive methods, as GC methods are more accurate than the standard colorimetric or enzymatic procedures. Cholestanol, having approximately the same retention time as cholesterol, can be an interfering component. Cholesterol has been determined in combination with related compounds, like plant sterols in sitosterolemic plasma, and animal and plant sterols and their bacterial conversion products in faeces. Esterified sterols have been analyzed after their separation from other lipid classes. Without prior isolation, they can be chromatographed as part of a total lipid class profile.

GC of lipids has started on apolar packed columns, but nowadays these have been replaced by capillary columns in a broad range of polarity. The apolar stationary phases, usually of the methylsilicone type like OV-1 and SE-30, are still preferable for most lipid analyses. Presently, fused-silica columns have appeared with chemically bonded polarizable stationary phases of the methylphenylsilicone type, like OV-17. Intermediate polar stationary phases, like SP-2401, are composed of a combination of trifluoropropyl and methyl. Sterol esters have been successfully analyzed on highly polar cyanosiloxane-type stationary phases, such as SP-2330 or SP-2340 [5,21,45,46].

5.1. Quantification of serum cholesterol

Most GC fractionations for the determination of serum cholesterol have been performed on apolar columns with FI [47–52] or MS [50,53–60] detection.

For the determination of total serum cholesterol, hydrolysis of cholesterol esters is usually performed according to the procedure as described by Abell–Levy–Brodie–Kendall (ALBK) [61,62], by the addition of ethanolic potassium hydroxide. The hydrolysis step has been of major concern, whatever analytical technique was used, and therefore several authors investigated the efficiency of the conditions of the hydrolysis procedure [47,48,52,55,56,60]. Addition of Triton X-100 to the ethanolic potassium hydroxide solution renders the hydrolysis step less critical, by preventing protein precipitation and disrupting serum lipoprotein complexes, without changing the results [56].

Already in 1973 Blomhoff suggested to use GC analysis of cholesterol as a reference method for the estimation of free and total cholesterol in serum [47], as the routinely used colorimetric and enzymatic methods lack specificity.

The internal standard is normally added to the serum before hydrolysis and silylation. However, 5α-cholestane, which remains underivatized, is added just before GC analysis [47,48]. Therefore internal standards, like 3α-5β-cholestanol [49], epicoprostanol (3α-hydroxy-5β-cholestane) [50,52] and stigmasterol [51], which resemble cholesterol more, are preferred. In some cases 5α-cholestane is used as a secondary standard to check the reproducibility of the extraction yield of the primary internal standard [50,51].

Total cholesterol data of GC methods have been compared with data from colorimetric methods and are normally found to be somewhat lower for samples from controls [48,49] and from patients without liver diseases [47]. This can depend on serum constituents other than cholesterol, like cholesterol metabolites, reacting in the colorimetric reaction. Interferences by other

sterol compounds have been described by Pelletier et al. [56] and Bernert et al. [60]. High total bilirubin levels markedly influence the colorimetric method resulting in higher levels [47]. Comparison of cholesterol data from GC methods with data from enzymatic methods shows equal [49] or lower [48] values for the enzymatic method. The lower values are probably due to incomplete enzymatic hydrolysis of cholesterol esters [48].

Mass detection allows the use of stable isotopes as labelled internal standards. Deuterium-labelled compounds have been produced carrying the label at the cholesterol ring [53,54], or at the side chain [54,55]. Highly labelled cholesterol (with 6 or 7 deuteriums) is less suitable for use on capillary instead of packed columns as it is partially or completely resolved from unlabelled cholesterol [54,58], whereas for high-precision work the labelled and unlabelled analytes should coelute [58]. Instead of deuterium also labelled carbon atoms (^{13}C) have been utilized, as side chain [58–60] or ring [50,56] labelling. One method with MS detection has been described using a nonlabelled internal standard, lathosterol (5α-cholest-7-en-3β-ol), being a structural isomer of cholesterol [57]. It has the same mass as cholesterol, but a different retention behaviour, appearing behind cholesterol [56,58,60]. Very low measurable amounts of endogenous lathosterol are usually detectable in serum (about 0.1% of cholesterol) [60].

Recordings of cholesterol and the internal standard are normally performed with a multiple detection unit. Mass detection is preferably used in the electron-impact ionization mode, which records the full ion of cholesterol at m/z 386 ($[M]^+$, without derivatization) [53], or at m/z 458 ($[M]^+$, trimethylsilyl (TMS) derivatized) [54,55,57,58,60], or cholesterol fragment ions at m/z 368 ($[M - TMS - H_2O]$) [56,59], or at m/z 329 ($[M - (TMS)OC_3H_4]^+$, for confirmatory measurements) [55,58]. The m/z 368 ion gives about 2.5 [54] to 10 [56] times more intensity than the m/z 458 molecular ion of the TMS derivative.

Whereas the electron-impact ionization mode, which leads to multiple fragments, is particularly interesting for structure elucidation studies, chemical ionization is more suitable for fragmentographic methods since it produces preponderantly few high-mass ions. Using methane as the ionizing reagent gas, recordings of cholesterol can be performed at m/z 443.5 (TMS-cholesterol minus a methyl group, $[M - 15]$) [50]. Ammonia chemical ionization has been operated using ion recording at m/z 386 ($[M + NH_4\text{-}(TMS)OH]^+$) [55,58].

The currently accepted reference method for quantification of serum cholesterol is the colorimetric maximal extraction ALBK procedure [61], as established and maintained by the Centers for Disease Control [62]. GC methods have been tested for the allowance of their use as reference methods, with FI [52], or MS detection with a nonlabelled cholesterol isomer as internal standard [57]. Several isotope-dilution–MS determinations have now been proposed as definitive methods [55,56,58]. Requirements that have to be fulfilled to develop an absolute fragmentographic analysis, concerning the use of internal standards and the performance of the GC–MS procedure, have been evaluated extensively by Wolthers et al. [54]. Alternatively, a reference standard method combining HPLC with isotope dilution–MS has been proposed [63–65].

For the estimation of free cholesterol, the hydrolysis step has to be omitted, and lipids are extracted twice with 95% ethanol–diethyl ether (3:1, v/v) [47]. The precision for free cholesterol is not as good as for total cholesterol [47]. Blomhoff mentions deterioration of the packed column after one month of use resulting in lower cholesterol values compared to the internal standard [47]. The author supposes the extensive usage and the high temperature to be the most important factors determining the lifetime of the columns [47]. Retaining of intact lipids on the column, when analyzing free cholesterol in unhydrolyzed serum, can also be a cause of contamination of the stationary phase.

5.2. Cholesterol measurements in biological fluids other than serum

Determinations of cholesterol in other biological fluids than serum, like tear [66], bile [18], or saliva and urine [67], deal with problems as

low cholesterol [66,67] or high bilirubin [18] concentrations. Schwertner et al. have developed a GC procedure with electron-capture detection [67]. Cholesterol and the internal standard, epicoprostanol (5β-cholestan-3α-ol), are derivatized with pentafluorobenzoyl chloride and separated on an apolar stationary phase with methane–argon (5:95, v/v) as the carrier gas. The method claims to be about 500-fold more sensitive (lower detection limit about 100 pg injected) than chromatographic methods involving FID (limit at 20 ng).

5.3. Separation of cholesterol and cholestanol

In apolar packed column GC assays cholestanol is not separated from cholesterol, and thus is a possible source of error [47–49,53–55,68]. On apolar capillary columns cholestanol elutes just behind cholesterol [50,56,60,69,70]. An OV-17 capillary column does not separate cholesterol from cholestanol [70]. Separation on a packed column is possible if an intermediate polar stationary phase is used, resulting in a small cholestanol peak running just behind cholesterol [71,72]. Utilising columns of higher polarity, packed [3] or capillary [5,7], these two compounds are separated in the inverse way, cholestanol eluting just in front of cholesterol. With mass detection it is important to attain full resolution, as the molecular mass of cholestanol is 2 Da greater than that of cholesterol and thus can interfere with labelled internal standards [50,56,60]. However, by using e.g. a $^{13}C_3$-labelled internal standard, this problem is solved [60].

5.4. Separation of sterols

Sterol composition has been measured in diet [5,68,73,74], small intestine (luminal and mucosal samples) [68], plasma [1–5,74] and faeces [69,70,72]. Depending on the investigated material, chromatograms show a complex mixture of different steroids of both animal and plant origin. For the GC separations of sterols packed [3,68,69,71,72] as well capillary columns [4,5,56,60,69,70,73,74] have been used, ranging in polarity from apolar methyl [1,2,4,56,60,69–71,73,74], to polarizable methyl (50%) phenyl (50%) [68,71], to intermediate polar methyl (50%) trifluoropropyl (50%) [71,72] and higher polar polyethylene glycol [1–3,73] or phenyl (10%) cyanopropyl (90%) [5] columns. If an internal standard is required, 5α-cholestane is usually added after lipid extraction and derivatization [3,69–72,74], but it can also be added previously [71]. Lipid extracts are hydrolyzed and sterols can be derivatized to their TMS ethers [1,2,4,56,60,69,70] or to their acetates [5], or they are analyzed without prior derivatization [3,68,71,72,74]. Detection can be performed with FID (most methods) or with full scan MS [56,60].

In plasma from subjects with sitosterolemia and xanthomatosis the sterol composition has been analyzed [3–5]. Samples contain cholestanol, cholesterol, campestanol, campesterol, sitostanol, β-sitosterol [3,5], and small amounts of brassicasterol, stigmasterol, 24-methylenecholesterol, stigmastanol and avenasterol [5].

Plasma and several tissues from patients with the Smith-Lemli-Opitz syndrome contain low levels of cholesterol, and high levels of a dehydrocholesterol isomer (most likely cholest-5,8-diene-3β-ol), an unidentified compound, and 7-dehydrocholesterol [1,2].

Faecal neutral sterols are usually fractionated prior to GC analysis [69,70,72]. Fractionation on TLC results in three fractions containing (I) oxosterols (mainly coprostanone, methyl-coprostanone and ethyl-coprostanone), (II) 3-hydroxylated 5β-sterols (mainly coprostanol, methyl-coprostanol and ethyl-coprostanol) and (III) 3-hydroxylated 5α-sterols (mainly cholestanol, campestanol and β-sitostanol) and 3-hydroxylated Δ5-sterols (mainly cholesterol, campesterol and β-sitosterol) [69,72]. The third fraction can additionally be separated into two fractions by TLC on silica gel–silver nitrate for the resolution of sterols from their 5α-saturated homologues [72]. Korpela uses a prefractionation step on a Lipidex-5000 column in the reversed-phase mode for the separation of free and esterified faecal neutral sterols [70]. Esterified sterols are hydrolyzed, and free hydroxylated sterols are separated from their oxoforms by a straight-phase chromatographic step on a Lipidex-5000 column. The sterol fractions are then analyzed as their TMS derivatives [69,70] or as native compounds

[72], on a packed apolar [69] or intermediate polar [72], or a capillary apolar [69,70] column. Miettinen compares the chromatograms from three separate TLC neutral faecal sterol fractions with a GC run without prior TLC fractionation [69]. Using a 46 m apolar capillary column, the unfractionated mixture can be separated accurately without loosing much of the original information (see Fig. 1).

Oxidative demethylation of lanosterol, a precursor in cholesterol biosynthesis, has been studied by Shafiee et al., using a combination of reversed-phase HPLC and GC–MS techniques [75]. Cholesterol oxidation products, 25-, 7α- and 7β-hydroxycholesterol, 6-ketocholestanol, 7-ketosterol, and α- and β-epoxycholesterol, have been analyzed by Wasilchuk et al. by GC–MS with ammonia chemical ionization [76].

5.5. Separation of (chole)sterol esters

The more standard approach for the determination of the cholesterol ester composition is to measure their fatty acid methyl ester composition after hydrolysation and methylation. This is still the method of choice when cholesterol is the only sterol present, but valuable data can be lost when several sterols are present in the esterified form.

(Chole)sterol esters have been obtained from diverse materials, like rabbit aortic atherosclerotic lesion [46], human bile [21], plasma from a patient with abetalipoproteinemia, showing essential fatty acid deficiency (see Fig. 2) [42], plasma lipoproteins from a sitosterolemic patient [5], and oils [37]. Isolated esterified cholesterol [21,42,45,46] or sterols [5,37] have been subjected to chromatography on, preferably short, apolar [37,46], polarizable (50%) methyl (50%) phenyl [42] and highly polar cyanopropyl [5,21,45,46] columns. Smith [46] compares the chromatographic properties of cholesterol esters on highly polar and apolar capillary columns. Retention on both columns is achieved according to molecular mass and to some extent to the degree of unsaturation. Peak shape is better on the apolar column, but cholesterol esterified with saturated or unsaturated fatty acids with equal chain length

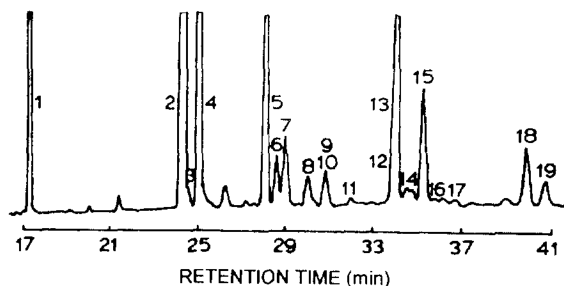

Fig. 1. GC–FID of faecal neutral sterol TMS derivatives on an apolar (SE-30, 46 m) capillary column. Temperature program: 20°C/min from 170°C to 265°C. 1 = 5α-cholestane (internal standard), 2 = coprostanol, 3 = epicoprostanol, 4 = coprostanone, 5 = cholesterol, 6 = cholestanol, 7 = methylcoprostanol, 8 = methylcoprostanone, 9 = ethylcoprostenol, 10 = lathosterol, 11 = ethylcoprostenone, 12 = campesterol, 13 = ethylcoprostanol, 14 = campestanol, 15 = ethylcoprostanone, 16 = stigmasterol, 17 = stigmastenol, 18 = β-sitosterol, 19 = β-sitostanol. Reproduced from Ref. [69] with kind permission from the publisher.

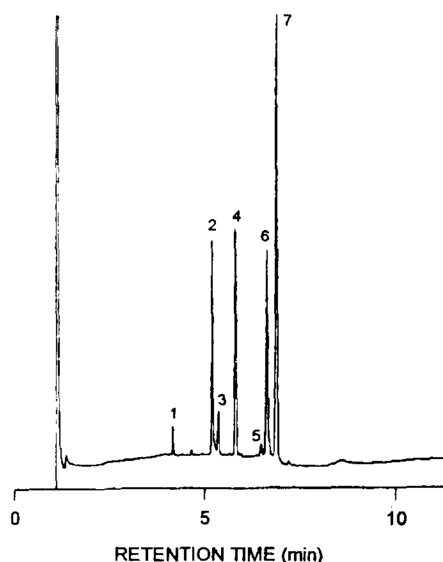

Fig. 2. GC–FID of plasma cholesterol esters from a patient with abetalipoproteinemia. Capillary column (25 m), coated with 50% phenyl–50% methyl and a temperature program of 2°C/min from 330°C to 350°C, hold for 15 min. 1 = cholesteryl myristate, 2 = cholesteryl palmitate, 3 = cholesteryl palmitoleate, 4 = cholesteryl heptadecanoate (internal standard), 5 = cholesteryl stearate, 6 = cholesteryl oleate, 7 = cholesteryl linoleate. Reproduced from Ref. [42] with kind permission from the publisher.

are only partially separated, the unsaturated esters eluting before the saturated. On the polar stationary phases this resolution is baseline (unsaturated cholesterol esters eluting behind the saturated esters), but monoenoic cholesterol esters with a fatty acid chain n carbons in length have a tendency to co-elute with the corresponding odd chain $n+1$ carbon cholesterol esters. On both polar and apolar columns considerable losses have been found for cholesterol esters containing fatty acids with longer chain length [46] and higher unsaturation, such as arachidonic acid [5,42,77,78].

For the chromatography of unhydrolyzed lipids hydrogen is preferably used as the carrier gas. The most important advantage of using this gas, compared to both nitrogen and helium, is the lower elution temperature, leading to shorter analysis time and lower thermal degradation [45]. Unfavourable hydrogenation of unsaturated cholesterol esters has been described using a polar column (SP-2330) and hydrogen as the carrier gas [45]. For this reason helium [42,46] and argon [21,37] have been preferred instead of hydrogen. However, other authors did not observe any reduction of the double bonds in either the sterol or the fatty acid part of the ester employing glass capillary columns coated with SP 2330 and hydrogen as the carrier gas [5,77].

In simple cases, where pure compounds are usually available, for example the common serum cholesterol esters, identification is not a problem. The situation becomes more complex for mixtures of natural origin where a higher number of sterols and fatty acids appear. Two strategies have been followed. The first is the isolation of sterol ester fractions by argentation TLC, grossly resulting in fractions with equal amounts of double bonds in the fatty acid moiety, prior to capillary GC analyses of the sterol moieties [5]. The second approach is the identification of sterol esters by GC–MS [37].

Evershed et al. describe a method for the chromatography of sterol esters with MS detection [37]. The electron-impact mass spectra of sterol esters do not display significant molecular ions or fatty acyl fragment ions. However, it does provide an ion for the sterol moiety, derived by loss of the fatty acyl group. Chemical ionisation has been employed to obtain mass spectra displaying a molecular ion and/or fragment ions corresponding to both sterol and fatty acyl moieties. The mass spectrometer has been operated in the positive ion chemical ionisation mode with methane or ammonia as reagent gas, as well as in the negative-ion scanning mode using ammonia as the chemical ionisation reagent gas. Using the latter mode, a molecular ion is not observed, but instead characteristic ions are seen for the sterol and the fatty acyl moieties [37].

5.6. Separation of lipid classes

A major problem concerning GC analyses of intact lipids is their low volatility, and thermal instability of unsaturated compounds at the required high temperatures. Because of the thermal instability of both the analytes and the stationary phase, the high boiling neutral lipids are usually determined by employing short capillary GC (about 5–15 m length) [4,73,77,79,80] and hydrogen as the carrier gas [4,77–80]. Usually chromatography is performed on apolar columns, which have greater stability at high temperatures than polar columns. Separations on columns with apolar stationary phases are based on the vapour pressure differences between the sample components, so that roughly a molecular mass-distribution chromatogram is obtained. Unsaturation hardly contributes to the retention of the components.

Chromatograms of plasma lipid classes present fatty acids, monoacylglycerols, (chole)sterols, diacylglycerols (from phosphatidylcholines), ceramides (from sphingomyelins), underivatized (chole)sterol esters and triacylglycerols [4,78,79,81]. Plasma samples are prepared by exposure to phospholipase C, and derivatization to TMS esters or ethers. Other studies (without phospholipase C predigestion) show a combination of cholesterol, cholesterol esters and triacylglycerols in their chromatograms [16,73,77,80], with possibly some other lipids. Cholesterol ester and triacylglycerol species tend to overlap, especially when triacylglycerols containing high levels of short-chain fatty acids are present. With this

chromatographic system also phytosterols and some of their esterified C_{18} derivatives have been determined in plasma from a sitosterolemic patient (see Fig. 3) [4].

Some methods have been accomplished for the separation of lipid classes on polarizable columns [77,78]. On these stationary phases, there is a substantial contribution of the double bonds to the retention, resulting in separation according to both carbon number and unsaturation. Due to the separation of unsaturated and saturated species, overlap of lipid classes and the formation of critical pairs can become rather complex. Since polarizable columns, like OV-17, develop significant polarity only above a critical sequence-inversion temperature (somewhere between about 250°C and 300°C), analytes that are recovered below this temperature are solely resolved on the basis of molecular mass [78,82]. Consequently, these stationary phases should merely be used at temperature ranges entirely above or below the inversion temperature, but not combining both regions in one run.

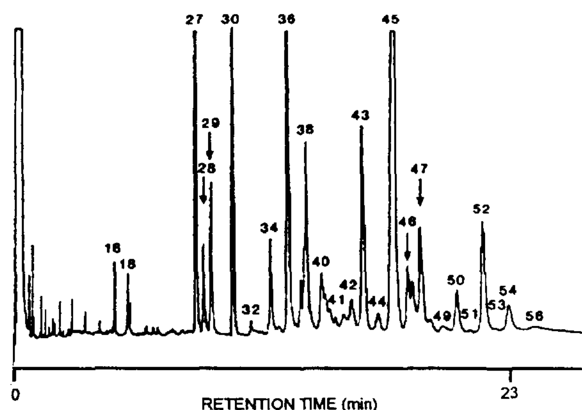

Fig. 3. Plasma total lipid GC–FID (8 m, apolar) profile of a patient with phytosterolemia. Components are chromatographed as their TMS ether or ester derivatives. Peak designations: 16 and 18 = fatty acids with 16 and 18 carbon atoms, 27 = cholesterol, 28 = campesterol, 29 = β-sitosterol, 30 = tridecanoylglycerol (internal standard), 32–42 = diacylglycerol and ceramide moieties with 30–40 fatty chain carbons, 43-49 = sterol esters with 43–49 total carbon atoms (45–47 = C_{18} fatty acid esters of cholesterol, campesterol, and β-sitosterol, respectively), 50–56 = triacylglycerols with 50–56 acyl carbons. Temperature program: from 40°C to 150°C at 30°C/min, then to 230°C at 20°C/min, to 280°C at 10°C/min, and to 340°C at 5°C/min. Reproduced from Ref. [4] with kind permission from the publisher.

5.7. *Analysis of cholesterol sulfate*

Cholesterol sulfate itself is highly polar and thermally instable, and therefore unsuitable for GC analysis. As cholesterol sulfate is only a minor component of plasma (about 0.1%) and erythrocytes compared to cholesterol, it must be carefully isolated before it can be solvolyzed and determined as cholesterol. 5β-Cholestane-3α-ol sulfate [9], heptadeuterated [10] or trideuterated cholesterol sulfate [44] have been added as internal standards. Prior isolation of cholesterol sulfate and its internal standard is done by means of anion-exchange chromatography on a quaternary amine cartridge [9], by TLC [10], or on a silica gel cartridge [44]. Cholesterol sulfate and the internal standard are solvolyzed, trimethylsilylated [9,10] or left underivatized [44], and determined by GC–FID [9] or GC–MS [10,44].

6. High-performance liquid chromatography

6.1. *Detectors*

HPLC detection systems in lipid analyses have been reviewed by Aitzetmüller in 1975 [83], by Shukla in 1988 [84] and by Christie in 1992 [85].

Many different detectors have been used with HPLC, but most of them are not generally specific for lipids. The approach to lipid separation is governed largely by the nature of the available detector(s). It determines which solvents can be used and whether gradient elution is possible. As lipid classes often respond with different efficiencies, careful calibration is necessary. In complex lipid mixtures compounds frequently coelute and form critical pairs, making quantification impossible.

The *UV detector* has been one of the earliest and most popular HPLC detectors used for analyzing lipids. However, most lipids do not have specific UV-absorbing properties but absorb generally in the 190–210 nm range. The only functional groups detectable in this UV region

are the carbonyl groups (C = O) and double bonds in the fatty acid chains (C = C). Because absorption is dependent on the concentration and on the degree of unsaturation of the molecular species, direct quantification of a complex lipid mixture is not possible. However, at the wavelength of 220 nm UV absorption of triacylglycerols is basically due to the ester C = O function of these molecules, and good quantification can be achieved [84]. Absorption of cholesterol is due to its double bond in the steroid nucleus, so cholestanol, which lacks this double bond, will not respond or interfere [17]. Cholesterol and related compounds absorb very little UV radiation, and therefore UV detection is not very sensitive. Traditional lipid chromatographic solvents such as chloroform, ethyl ether and acetone are of limited value because of their strong absorbing properties in the UV region of interest. Solvents such as MTBE, hexane, isopropanol, acetonitrile and methanol have been used successfully in both straight- and reversed-phase HPLC separations of lipids. The most predominant methods that have used UV detection are the separations of non-polar lipids on straight-phase columns [40,86,87], and the separations of cholesterol esters on reversed-phase columns [17,88–93].

UV detection at other wavelengths has been performed after derivatization of the investigated compounds, e.g. cholesterol and cholestanol as their benzoyl derivatives at 228 nm [8]. Cholesterol, converted by the enzyme cholesterol oxidase to Δ4-cholestenone, is detectable at 240 nm [94,95].

The refractive index detector (differential refractometer) requires isocratic elution, is sensitive to minor fluctuations in temperature and is far less sensitive than UV detection. As this detector is not compatible with gradient elution, it is preferably used for the separation of lipids that are closely related, such as sterols [6].

Optical activity detection monitors the optical rotation of the eluate, which is induced by the chiral centres of lipid molecules [19]. It has been used for the detection of individual cholesterol esters [19]. Because the chiral centres are all in the cholesterol part, specific rotations are assumed to be similar. The optical activity detection is selective, hence there is no interference of triacylglycerols, except for those which possess an asymmetric carbon (with different fatty acids on *sn*-1 and *sn*-3, about 40% of total triacylglycerols). However, these only cause insignificant, if any, optical rotation.

Transport flame ionization or moving wire detectors are often termed "universal detectors". The eluents are deposited on a moving wire or belt and the volatile solvents from the solutes are removed in an intermediate furnace region and finally the nonvolatile lipid is carried through a FI detector where it is combusted and detected. In contrast to the limitations in solvent choice with UV detectors, FI detectors allow a much wider variety of solvents to be used in gradient elutions. Nevertheless, there are some restrictions. For instance acetic acid, *n*-propanol and toluene, among others, should not be used because their boiling points are too high for efficient evaporation. Buffers that leave residues following evaporation give reduced detector response, but ammonium buffers are acceptable. The detector response is rectilinearly related to the amount of the eluting lipid. However, the response depends not only on the carbon content of the sample, but also on chromatographic conditions, such as solvent flow-rate and the nature of the eluting solvent. The transport FI detector has been used with the analysis of non-polar [15] or total [96] lipid classes.

The light-scattering (or mass) detector is an uncomplicated easy to operate detector. As with the FI detector, the light-scattering detector is destructive, but it is a simple matter to install a stream splitter to permit further investigations. Comparable with the transport-FI detector, the use of the light-scattering detector imposes two constraints on the elution solvents used, i.e., they must be sufficiently volatile to evaporate under conditions that do not vaporize the solute, and they should not contain inorganic ions, which will not evaporate. Gradient elution is possible and, with some restrictions, allows quantification. The linearity and response of the light-scattering detector may be influenced by the nature of the eluting solvent. Especially in the low mass range,

careful calibration is necessary. Until now, this detector has mainly been used in combination with total lipid class separations [97–99].

Also *fluorescence* labelling has been used in lipid analyses, resulting in high sensitivities during rapid scanning fluorescence detection of HPLC effluents. This detection has proven to be valuable for simultaneous quantification of cholesterol and cholestanol [100,101].

Considerable efforts have been made to produce *HPLC–MS* systems. Three interface types between the HPLC and the mass spectrometer have been investigated: direct liquid introduction, thermospray and the moving belt. The direct liquid inlet HPLC–MS system allows only 1% of the HPLC elution being introduced into the MS, thereby diminishing sensitivity [5,102]. In combination with reversed-phase HPLC, MS has been used for the detection of (chole)sterol esters [5,102], cholesterol and its labelled internal standard [63–65], and cholesterol sulfate and its labelled internal standard [12].

6.2. Straight-phase high-performance liquid chromatography

Traditionally, lipid class separations are carried out by adsorption on silica gel columns, whereby lipid classes are separated as single peaks, although sometimes some species separation occurs as well. Some methods have been developed to separate neutral lipid classes (like cholesterol esters, tri-, di- and monoacylglycerols, cholesterol and fatty acids) [15,40,86,87,103], whereas other studies extend the chromatographic analyses to polar lipid classes (phospholipids) [96-99]. UV (at about 206 nm) [40,86,87,103], light-scattering [97–99] or FI detection [15,96] have been employed. Other applications that use straight-phase HPLC, describe separations of short-chain cholesterol esters [37] and cholesterol and four of its major oxidation products [104]. A method for the measurement of sub-nanomolar quantities of cholesterol in biological fluids has been developed in which cholesterol was converted to Δ^4-cholestenone by the enzyme cholesterol oxidase [94].

6.2.1. Separation of neutral lipid classes

In case of neutral lipid class separations, samples have to be prefractionated to exclude polar lipids from the lipid extract, thereby avoiding contamination of the column with retained polar lipids.

Aitzetmüller and Koch describe a technique for the separation of non-polar sebum lipid classes, using a moving-wire FI detector for identification and quantification [15]. In previous studies they relied on hydrocarbon–ether–alcohol solvent sequences for generating the polarity gradient, but use of chlorinated hydrocarbon solvents proves to result in better resolution between free cholesterol and 1,2-diacylglycerols. The gradient elution starts with a mixture of carbon tetrachloride–isooctane (34:66, v/v), then introduces chloroform–dioxane–*n*-hexane (40:11:49, v/v/v) and finally chloroform–methanol–diisopropyl ether (34:36:30, v/v/v). This gradient is able to separate non-polar lipid classes, ranging in polarity from hydrocarbons to monoacylglycerols. Fatty acids are analyzed as their methyl esters after methylation with diazomethane, and mono-olein diacetate, eluting behind the triacylglycerols, is added as internal standard.

Schlager and Jordi [86] use a linear gradient from 100% hexane to hexane–isopropanol–water (6:8:0.75, v/v/v) for the separation of non-polar lipids (cholesterol, cholesterol esters, fatty acids and triacylglycerols) monitoring absorption at 206 nm. Species of cholesterol esters, fatty acids and triacylglycerols are partly resolved.

Hamilton and Comai [40,87] separate non-polar lipids (cholesterol esters, triacylglycerols, fatty acids and cholesterol) from phospholipids by solid-phase silica columns prior to straight-phase chromatographic analysis. Two isocratic mobile phases have been used that are suitable for UV detection at 206 nm [40]. The first is a mixture of hexane–isopropanol–acetic acid (100:0.5:0.1, v/v/v) for the separation of total fatty acids and cholesterol. Using this mixture in another ratio (100:2:0.02, v/v/v) 1,2-diacylglycerols and 1,3-diacylglycerols can additionally be separated within 10 min [87]. The second mobile phase is a mixture of hexane–*n*-butyl chloride–

acetonitrile–acetic acid (90:10:1.5:0.01, v/v/v/v), resulting in complete separation of cholesterol esters (in the breakthrough volume), triacylglycerols (with some differentiation of triacylglycerol species), fatty acid species (increased retention with increasing carbon number and unsaturation), and cholesterol. The higher retention of unsaturated lipids compared to saturated is probably due to acetonitrile in the mobile phase. The n-butyl chloride in the solvent mixture is used to make a single phase system with hexane and acetonitrile, which are not miscible without this addition. A mobile phase containing hexane–MTBE–acetic acid (100:5:0.02, v/v/v) proves effective in separating cholesterol esters and triacylglycerols in less than 10 min [87].

Straight-phase cyanopropyl HPLC has been performed for the separation and identification of triacylglycerols, cholesterol esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol and retinol [103]. Detection is done with a diode array detector scanning between 190 and 370 nm, or at a fixed wavelength of 210 nm. The isocratic mobile phase consists of 0.1% isopropanol in heptane. With this elution solvent the fraction with the shortest retention time contains a combination of cholesterol esters, retinol esters and tentatively vitamin D esters.

6.2.2. Separation of total lipid classes

When covering the full-scale analysis from neutral to polar lipid classes, gradient elution is always necessary. A ternary gradient solvent system is required to separate the total range of non-polar to polar lipid classes [97–99]. Detection and quantification has been performed with a light-scattering detector. The gradient starts with isooctane [97,99] or hexane [98,99] containing 1% of tetrahydrofuran, then isopropanol–chloroform (4:1, v/v) is introduced to separate each of the simple lipids, and finally a mixture of isopropanol–water (1:1, v/v) is added in a small amount for the elution of phospholipids. A gradient in the reverse direction is generated to remove most of the bound water and to re-equilibrate the column prior to the next analysis. Resolution of the more acidic lipids is improved by addition of small amounts of organic ions to the aqueous component of the eluent (0.5–1 mM serine adjusted to pH 7.5 with ethylamine) [98]. Isopropanol, miscible with hydrocarbons as well as with water, is required to mediate between these elution solvents. The separations do not show any baseline drift in spite of the changes in solvent composition during the elution program, as is shown in Fig. 4. Christie indicates better resolution with a smaller 3 μm particle size column than with 5-μm particles [97]. Theoretically a particle diameter of about 2 μm will be optimal for many separations [84]. Columns packed with smaller particles achieve better separations in less time, require very-low-dead volume HPLC systems and allow quick changeovers between mobile phases.

A silica column treated with ammonium hydroxide has been used to give complete, re-

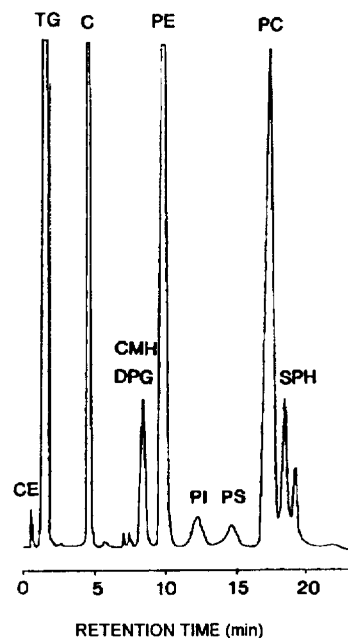

Fig. 4. Straight-phase HPLC with light-scattering detection of a lipid extract from rat kidney. Solvent gradient: see text. Abbreviations: CE = cholesterol esters; TG = triacylglycerols; C = cholesterol; DPG = diphosphatidylglycerol (cardiolipin); CMH = ceramide monohexoside; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; SPH = sphingomyeline (double peak). Reproduced from Ref. [98] with kind permission from the publisher.

producible lipid class separation [96]. With FID, each of the lipid classes (cholesterol, cholesterol esters, triacylglycerols, phosphatidylethanolamine and phosphatidylcholine) gives a linear response over the tested range of 6–200 μg, although response factors vary with lipid classes. The ternary elution gradient starts with dichloromethane–hexane (55:45, v/v), then changes to dichloromethane–chloroform (67:33, v/v) and finally introduces ammonium hydroxide (6% concentrated NH_4OH in methanol) into the mobile phase.

6.2.3. Other separations using straight-phase columns

HPLC has been employed to separate cholesterol esters bearing short-chain (C_2–C_8) fatty acyl moieties, using isocratic elution with hexane containing 0.25% of MTBE and UV detection at 206 nm [37].

An HPLC method for the quantification of cholesterol and four of its major oxidation products, 25-hydroxycholesterol, 7-ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol in muscle and liver tissues has been developed by Csallany et al. [104]. For the analysis of cholesterol and 25-hydroxycholesterol the mobile phase consists of hexane–isopropanol (97:3, v/v), and UV detection is at 206 nm. A more polar mobile phase of 93:7 (v/v) hexane–isopropanol has been used to achieve separation of the other three cholesterol oxidation products, monitoring absorption at 233 nm for detection of 7-ketocholesterol and at 206 nm for the other two compounds.

A sensitive HPLC method for the measurement of sub-nanomolar quantities of cholesterol in biological fluids has been described in which cholesterol is converted to Δ^4-cholestenone by the enzyme cholesterol oxidase [94]. The mobile phase consists of hexane–isopropanol (95:5, v/v) and Δ^4-cholestenone can be detected at 240 nm.

6.3. Reversed-phase high-performance liquid chromatography

Separation of lipid class species is usually done on reversed-phase columns with mostly isocratic mobile phases. Methods are reviewed here that describe chromatographic analyses of cholesterol and cholesterol ester species, cholesterol and cholestanol, sterols and cholesterol sulfate.

6.3.1. Separation of cholesterol ester species

In 1979 Duncan et al. have developed methods for the measurement of total and free cholesterol in serum samples [17]. Many other investigations reporting the analysis of cholesterol ester species have been based on this study [88–93]. An example of such a separation is seen in Fig. 5. Duncan et al. use a C_{18} reversed-phase column packed with 10-μm particles and mobile phases containing isopropanol–acetonitrile (50:50, v/v) or isopropanol–acetonitrile–water (60:30:10, v/

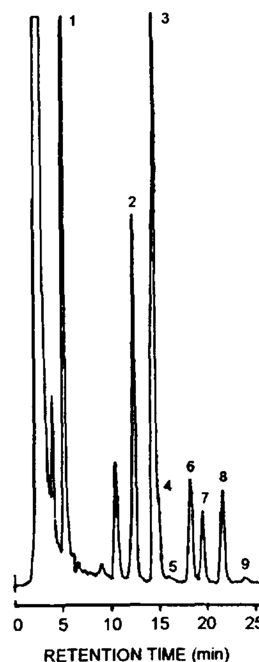

Fig. 5. Reversed-phase HPLC with UV detection (210 nm) of cholesterol and cholesterol esters from human low-density lipoproteins. Elution was isocratic with acetonitrile–isopropanol (1:1, v/v) at a flow-rate of 1.2 ml/min and a temperature of 45°C. Peak numberings: 1 = cholesterol, 2 = cholesteryl arachidonate, 3 = cholesteryl linoleate, 4 = cholesteryl palmitoleate, 5 = cholesteryl myristate, 6 = cholesteryl oleate, 7 = cholesteryl palmitate, 8 = cholesteryl heptadecanoate (internal standard), 9 = cholesteryl stearate. Reproduced from Ref. [90] with kind permission from the publisher.

v/v), both at a flow-rate of 1 ml/min and UV detection at 200 nm [17]. The first solvent system is used for the measurement of serum total cholesterol (in hydrolyzed samples), and for the production of lipid profiles (showing cholesterol, triacylglycerol and cholesterol ester species, in this order) from (unsaponified) serum lipid extracts. Four cholesterol ester species are identified, carrying arachidonic, linoleic, oleic and palmitic acid [17]. As detection is determined by the number of double bonds, cholesteryl arachidonate is overestimated, and cholesteryl stearate is generally not detected [17,89]. The second solvent system is used for the measurement of nonesterified cholesterol in unsaponified serum lipid extracts, because it prevents the interference of triacylglycerols with cholesterol [17].

In the reversed-phase mode of HPLC, lipids elute in the order of increasing "partition number". The integral partition number is calculated as the total number of carbon atoms in the fatty acid chain minus twice the total number of double bonds [88]. Lipids with identical partition numbers, which tend to overlap, are called critical pairs, e.g. cholesteryl linoleate and palmitoleate. Also coelution is observed of cholesterol and cholestanol, but, as cholestanol does not have any absorbing properties in the operated UV region, it does not disturb the measurement of cholesterol. Triacylglycerols with higher molecular mass interfere with cholesterol esters with lower molecular mass. All interferences make this kind of separation less accurate than the traditional transmethylation of cholesterol esters and consecutive chromatography of the fatty acid methyl esters. However, resolution of intact sterol esters is indispensable for the determination of the fatty acid esters of multiple sterols. Some of the overlaps can be avoided if the sterol esters are subjected to argentation chromatography prior to the HPLC run [5,37]. Interference by triacylglycerols can be avoided by isolation of the cholesterol esters from the lipid extract [89,93] or by treatment of the serum samples with lipase prior to lipid extraction and chromatographic separation [88]. Resolution of individual cholesterol esters improves with increasing acetonitrile content (60%), but also increases the retention [93]. The usage of columns packed with smaller spherical particles results in better (less critical pairs) and quicker separations, concomitant with higher sensitivity. Particle sizes of 7 μm [92,93], 5 μm [88–90] and 3 μm [91] have been tested. Bilirubin, vitamin A, vitamin D, phospholipids, monoacylglycerols and diacylglycerols do not interfere with the measurement, because they elute before cholesterol [17]. Interference of some sterols, as lathosterol and 7-dehydrocholesterol, can occur [17]. In the described lipid profiles α-tocopherol can simultaneously be detected at the same wavelength (205 nm), running before cholesterol. Additionally it can be sensitively monitored by fluorescence detection (excitation at 225 nm, emission at 320 nm) [91].

Other solvent mixtures than isopropanol–acetonitrile(–water) for the separation of cholesterol esters have been investigated. The resolution of cholesterol and 15-ketosterol (5α-cholest-8(14)-en-3β-ol-15-one, a potent inhibitor of cholesterol synthesis) and the fatty acid esters of the two sterols has been determined on a 5 μm C_{18} column by elution with a solvent gradient of isopropanol–methanol–water by gradually decreasing the amounts of methanol and water, combined with an increasing flow-rate with UV detection at 210 nm [105]. Better resolution of the palmitate and oleate esters was achieved on a C_6 column with isocratic elution. Evershed et al. [37] describe a method in which the isocratic elution is done by a mixture of acetonitrile–tetrahydrofuran (65:35, v/v) containing up to 2% of water, with UV detection at 206 nm. The use of tetrahydrofuran in the low-UV range is limited, owing to a variable cut-off limit (varying from 212 to 220 nm), resulting in baseline instability when operating a gradient system [84,90].

Kuo and Yeung use a laser-based optical activity detector at 514.5 nm [19]. A lipid extract of human serum has been separated on a 10 μm C_{18} column, with a mobile phase of tetrahydrofuran–water (76:24, v/v) at a flow-rate of 0.5 ml/min. Many coeluting critical pairs of cholesterol esters are observed. Interference by triacylglycerols is negligible, as they hardly give any

response using this detector. In this system cholestanol, 7-dehydrocholesterol and lathosterol possibly interfere with cholesterol.

Kuksis et al. [5] separate an isolated fraction of intact sterol esters by reversed-phase HPLC combined with a mass spectrometer via direct liquid interface. A gradient of propionitrile in acetonitrile is used as the eluting solvent. About 1% of the column effluent is admitted to the quadrupole mass spectrometer, which is operated in the positive chemical ionization mode, with the HPLC effluent serving as the reagent gas. The less extensive fragmentation in the chemical ionization mode compared to the electron-impact mode provides increased sensitivity of detection, which is most important in view of the unfavourable effluent split. It is possible to monitor fragment ions representing the steroid nuclei of cholesterol (m/z 369), campesterol and 22,23-dihydrobrassicasterol (both m/z 383), stigmasterol and avenasterol (both m/z 395), and β-sitosterol (m/z 397). This system also allows an examination of each sterol ester peak for the presence of the corresponding 5α-stanol esters, with fragment ions of two mass units higher than their parent sterols. Certain critical pairs of sterol esters tend to overlap, i.e., cholesteryl linoleate and palmitoleate, and cholesteryl palmitate and campesteryl oleate. These esters are identified by an MS examination of the appropriate sterol ester peaks. Some of the overlaps can be avoided when the sterol esters are subjected to argentation TLC prior to the HPLC–MS run. Glycerol esters show very little response using positive chemical ionization [102]. They can be detected with increased sensitivity after chloride attachment and subsequent negative chemical ionization MS recording their $[M+35]^-$ and $[M+37]^-$ ions [102].

6.3.2. Separation of cholesterol esters by argentation chromatography

Argentation HPLC of intact plasma cholesterol esters has been performed on a column packed with a bonded sulphonic acid phase loaded with silver ions (see Fig. 6) [43]. Loading of the column is done according to Christie [106]. Number and distribution of double bonds are the major determinants influencing chromatographic behaviour. The gradient elution starts with a mixture of dichloroethane–dichloromethane (1:1, v/v) and successively acetone and acetonitrile are introduced. Detection is done by a light-scattering detector. Cholesterol esters from human and sheep plasma with up to six double bonds can be separated and isolated. Some differentiation is seen between cholesterol ester isomers, but this has not been further investigated. This method has not yet been tested for the separation of complex sterol ester mixtures.

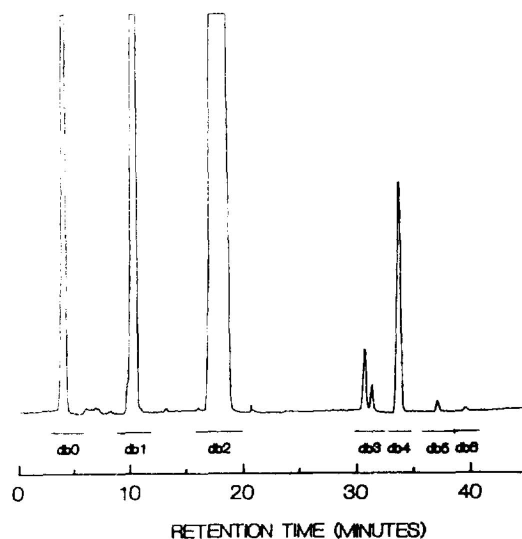

Fig. 6. Argentation HPLC with light-scattering detection of plasma cholesterol esters. An HPLC column with a bonded sulfonic acid phase was loaded with silver ions. The gradient (1 ml/min) starts with 50% dichloroethane–50% dichloromethane, then introduces acetone to 50% acetone–25% dichloroethane–25% dichloromethane in 20 min, changing in a further 20 min to 94% acetone–6% acetonitrile, and finally to 90% acetone–10% acetonitrile in again 20 min. Abbreviations: db0–db6 = fractions containing cholesterol esters with fatty acid moieties having 0–6 double bonds, respectively. Reproduced from Ref. [43] with kind permission from the publisher.

6.3.3. Proposed reference standard method for total serum cholesterol

Isotope dilution MS with HPLC separation has been evaluated as a reference standard method to estimate the plasma total cholesterol content [63–65]. In contrast to GC methods, no deri-

vatization is required. A reversed-phase column has been used, with a mobile phase of acetonitrile–isopropanol (3:1, v/v) [63] or methanol [64,65]. Introduction of the HPLC eluate into the MS is off-line [63], with atmospheric pressure ionization [64], or with discharge-assisted thermospray (plasmaspray) [65]. With acetonitrile–isopropanol as the mobile phase, the electron-impact ionization technique has been used, measuring isotope ratios by multiple ion detection at $[M]^+$ m/z 386 for cholesterol and 388 for the internal standard ([3,4-^{13}C]cholesterol) [63]. Using atmospheric pressure ionization or plasmaspray, the flow solvent acts as a reagent in chemical ionization. Because of smaller proton affinity, methanol should be preferably used as the mobile phase. The dehydrated ions $[MH - H_2O]^+$ at m/z 369 and 371 show the highest intensity [64,65].

6.3.4. Separation of cholesterol and cholestanol

HPLC with UV or fluorimetric detection has been reported for simultaneous detection of cholesterol and cholestanol in human serum. Cholesterol and cholestanol can be converted into their epoxidation derivatives [107], benzoyl derivatives (UV detection of the benzene ring at 228 nm) [8], or carbamic acid ester derivatives (fluorimetric detection at excitation and emission wavelengths of 360 and 440 nm [100], or 312 and 427 nm [101]). Isocratic elution is done with a combination of acetonitrile and water, and additionally acetic acid [8], methanol [100], or ethanol [101].

6.3.5. Separation of sterols

HPLC of sterols can efficiently be performed on a reversed-phase column [6,95,108,109]. The mobilities of cholesterol and some plant sterols (campesterol, β-sitosterol and stigmasterol) have been investigated on a reversed-phase C_{18} silica (12% carbon loading, 10 μm particles) microbore column (50 cm $\times$ 1 mm I.D.) [108] and on a C_{18} 5 μm particles column [109] with methanol as the mobile phase and UV detection at 212 nm. Additionally, tocopherols can be detected by fluorimetry (excitation at 295 nm, emission at 330 nm) [109]. Salen et al. [6] perform their plasma sterol separation on a C_{18} column (5 μm particle size) with a mobile phase containing methanol–water–chloroform and a refractive index detector. To increase the sensitivity of UV detection, Goh et al. convert sterols with cholesterol oxidase (from *Streptomyces*) to their conjugated enones [95]. For the measurement of total instead of free sterols, cholesterol esterase is also included in the reaction mixture. The authors effectively separate cholesterol and its immediate precursors, desmosterol and 7-dehydrocholesterol, on a reversed-phase C_{18} column containing 3-μm particles with a mobile phase of methanol–acetonitrile (1:1, v/v) and detection at 240 nm.

6.3.6. Analysis of cholesterol sulfate

HPLC–MS analysis of plasma cholesterol sulfate detects the intact analyte, not needing hydrolysis [12]. Traces of cholesterol do not disturb in this method, in contrast to GC–MS and GC–FID methods, as cholesterol sulfate is differentiated from cholesterol. After addition of the internal standard, either [$^{13}C_2$]- or [$^2H_6$]-cholesterol sulfate, a single solid-phase extraction reversed-phase C_{18} cartridge is used for plasma extraction. HPLC of cholesterol sulfate is performed on a 5-μm particles C_{18} column which is eluted with a solvent system of methanol–ammonium acetate (0.2 mol/l) (9:1, v/v). Negative-ion thermospray mass spectra of steroid sulfates are dominated by the molecular anion $[M - H]^-$. Selected-ion monitoring is done at m/z 465 for the analyte and 467 or 471 for the internal standards.

6.3.7. Other reversed-phase high-performance liquid chromatographic methods

Edlund [110] combines the detection of cholesterol (UV detection at 215 nm) with the coulometric detection of coenzyme Q_{10} and α-tocopherol. Bhat and Ansari [111] describe a method for the separation of cholesterol and its xenobiotic mono-, di- and tri-chloroacetate analogues.

7. Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is especially useful for the separation of nonvolatile and thermally labile compounds. Two methods have been presented for the separation of cholesterol and cholesterol esters in human serum.

The first method is performed on an inert C_{18} silica gel packed column (250 mm × 4.6 mm I.D., particle size 5 μm) using supercritical carbon dioxide without any modifier as mobile phase [112]. Chromatograms are obtained by monitoring the eluent simultaneously (by splitting) with FI and UV (190 nm) detection (see Fig. 7). The flow-rate of the mobile phase is 750 ml/min (10 ml/min to the FI detector), with a run time of less than 20 min. Residual silanol groups, present on the silica gel surface, interact with polar compounds such as cholesterol by irreversible adsorption and lead to peak tailing or considerable delay of sample elution. Cholesterol esters are easier to elute than cholesterol, because of the absence of polar hydroxyl groups.

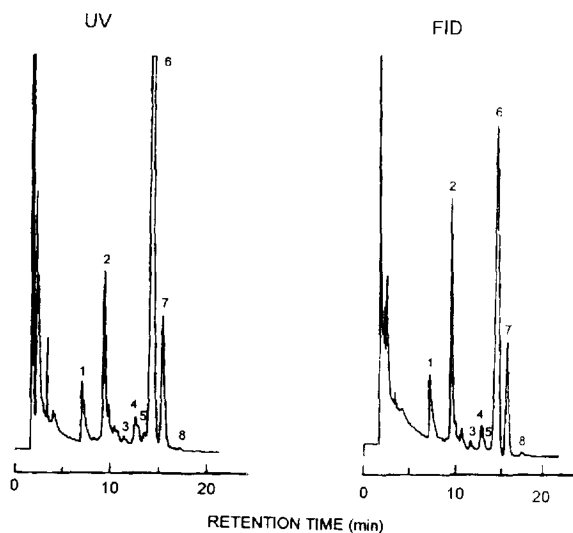

Fig. 7. SFC with UV (190 nm) and FI detection of cholesterol and cholesterol esters from serum reference material. Peak numberings: 1 = cholesterol, 2 = cholesteryl laurate (internal standard), 3 = cholesteryl myristate, 4 = cholesteryl palmitoleate, 5 = cholesteryl linolenate, 6 = cholesteryl palmitate + cholesteryl linoleate + cholesteryl arachidonate, 7 = cholesteryl oleate, 8 = cholesteryl stearate. Chromatographic conditions: a reversed-phase HPLC (250 mm × 4.6 mm I.D., 5 μm) column, column temperature 45°C, CO_2 pressure 200 atm, CO_2 flow-rate 750 ml/min under 760 mmHg. Reproduced from Ref. [112] with kind permission from the publisher and the authors.

As in liquid chromatography, the separation mode is reversed-phase. Peaks of cholesteryl palmitate, linoleate and arachidonate completely overlap, but resolution is good for saturated cholesterol esters as well as for cholesteryl palmitoleate, linolenate, linoleate and oleate. The detection limit is 20 ng for cholesterol and cholesteryl palmitate by both UV and FI detection, using cholesteryl laurate as internal standard.

One of the characteristic advantages of SFC without an organic modifier is that the analytes can be detected by UV detection even at the wavelength of 190 nm, which is usually not possible with HPLC by the opacity of most solvents at this wavelength. Peak areas of all cholesterol esters increased when the wavelength was decreased to 190 nm.

The second method uses a 10 m × 50 μm I.D. column coated with 50% *n*-octyl, 50% methylpolysiloxane, carbon dioxide as the mobile phase and FID [113]. Compared to GC, SFC has two major advantages: there is no need for derivatization of cholesterol, and high-molecular-mass unsaturated cholesterol esters do not suffer from thermal decomposition. The oven temperature is set at 65°C, and carbon dioxide is linearly pressure-programmed from 137.90 to 275.79 bar at 1.03 bar/min, resulting in a run time of nearly 110 min. The order of elution is based on molecular mass and polarity: cholesterol, cholesteryl *n*-valerate (internal standard), triacylglycerols and cholesterol esters. The elution time is lower for higher unsaturated cholesterol esters. Depending on oven temperature and pressure ramping rate, critical pairs are formed by cholesteryl oleate and arachidonate, or by cholesteryl linoleate and oleate. Also triacylglycerol and cholesterol ester species are likely to interfere.

In conclusion, SFC for the separation of cholesterol esters is still suffering from a lack of separating power. Future perspectives would perhaps combine the low temperature operating SFC with the identification power of MS.

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References

[1] G.S. Tint, M. Irons, E. Roy Elias, A.K. Batta, R. Frieden, T.S. Chen and G. Salen, *N. Engl. J. Med.*, 330 (1994) 107.
[2] G.S. Tint, M. Seller, R. Hughes-Benzie, A.K. Batta, S. Shefer, D. Genest, M. Irons, E. Elias and G. Salen, *J. Lipid Res.*, 36 (1995) 89.
[3] G. Salen, P.O. Kwiterovich Jr., S. Shefer, G.S. Tint, I. Horak, V. Shore, B. Dayal and E. Horak, *J. Lipid Res.*, 26 (1985) 203.
[4] A. Kuksis, J.J. Myher, L. Marai, J.A. Little, R.G. McArthur and D.A.K. Roncari, *J. Chromatogr.*, 381 (1986) 1.
[5] A. Kuksis, J.J. Myher, L. Marai, J.A. Little, R.G. McArthur and D.A.K. Roncari, *Lipids*, 21 (1986) 371.
[6] G. Salen, V. Shore, G.S. Tint, T. Forte, S. Shefer, I. Horak, E. Horak, B. Dayal, L. Nguyen, A.K. Batta, F.T. Lindgren and P.O. Kwiterovich Jr., *J. Lipid Res.*, 30 (1989) 1319.
[7] B.J. Koopman, J.C. van der Molen, B.G. Wolthers, A.E.J. de Jager, R.J. Waterreus and C.H. Gips, *Clin. Chim. Acta*, 137 (1984) 305.
[8] T. Kasama, D-S. Byun and Y. Seyama, *J. Chromatogr.*, 400 (1987) 241.
[9] F.A.J. Muskiet, G. Jansen, B.G. Wolthers, A. Marinkovic-Ilsen and P.C. van Voorst Vader, *Clin. Chem.*, 29 (1983) 1404.
[10] I. Björkhem, E. Enocksson and P. Harper, *Scand. J. Clin. Lab. Invest.*, 45 (1985) 83.
[11] S. Serizawa, T. Nagai and Y. Sato, *J. Invest. Dermatol.*, 89 (1987) 580.
[12] C.H.L. Shackleton and S. Reid, *Clin. Chem.*, 35 (1989) 1906.
[13] J. Folch, M. Lees and G.H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
[14] N.S. Radin, *Methods Enzymol.*, 72 (1981) 5.
[15] K. Aitzetmüller and J. Koch, *J. Chromatogr.*, 145 (1978) 195.
[16] P. Mareš, E. Tvrzická and V. Tamchyna, *J. Chromatogr.*, 146 (1978) 241.
[17] I.W. Duncan, P.H. Culbreth and C.A. Burtis, *J. Chromatogr.*, 162 (1979) 281.
[18] C.H. Bolton, T.S. Low-Beer, E.W. Pomare, A.C.B. Wicks, J. Yeates and K.W. Heaton, *Clin. Chim. Acta*, 83 (1978) 177.
[19] J.C. Kuo and E.S. Yeung, *J. Chromatogr.*, 229 (1982) 293.
[20] S.T. Wang and F. Peter, *J. Chromatogr.*, 276 (1983) 249.
[21] P.E. Ross, E. Kouroumalis, A. Clarke, D. Hopwood and I.A.D. Bouchier, *Clin. Chim. Acta*, 144 (1984) 145.
[22] F. Peter and R.G. Reynolds, *J. Chromatogr.*, 143 (1977) 153.
[23] D. Vandamme, G. Vankerckhoven, R. Vercaemst, F. Soetewey, V. Blaton, H. Peeters and M. Rosseneu, *Clin. Chim. Acta*, 89 (1978) 231.
[24] D.M. Bradley, C.R. Rickards and N.S.T. Thomas, *Clin. Chim. Acta*, 92 (1979) 293.
[25] K. Hiramatsu, H. Nozaki and S. Arimori, *J. Chromatogr.*, 182 (1980) 301.
[26] E. Peuchant, G. Covi and R. Jensen, *J. Chromatogr.*, 310 (1984) 297.
[27] E. Tvrzická, P. Mareš, M. Votruba and P. Hrabák, *J. Chromatogr.*, 530 (1990) 424.
[28] J. Gloster and R.F. Fletcher, *Clin. Chim. Acta*, 13 (1966) 235.
[29] J.H. Williams, M. Kuchmak and R.F. Witter, *Clin. Chem.*, 16 (1970) 423.
[30] S.R. Cairns and T.J. Peters, *Clin. Chim. Acta*, 127 (1983) 373.
[31] M.A. Kaluzny, L.A. Duncan, M.V. Merritt and D.E. Epps, *J. Lipid Res.*, 26 (1985) 135.
[32] G. Schmitz and G. Assmann, *J. Chromatogr.*, 307 (1984) 65.
[33] J. Wildgrube, W. Erb and E. Böhle, *Z. Klin. Chem. Klin. Biochem.*, 7 (1969) 514.
[34] L.J. Macala, R.K. Yu and S. Ando, *J. Lipid Res.*, 24 (1983) 1243.
[35] L. Kovács, A. Zalka, R. Dobó and J. Pucsok, *J. Chromatogr.*, 382 (1986) 308.
[36] J.G. Alvarez and J.C. Touchstone, *J. Chromatogr.*, 577 (1992) 142.
[37] R.P. Evershed, V.L. Male and L.J. Goad, *J. Chromatogr.*, 400 (1987) 187.
[38] H.K. Bhat and G.A.S. Ansari, *J. Chromatogr.*, 483 (1989) 369.
[39] K. Li, *J. Chromatogr.*, 532 (1990) 449.
[40] J.G. Hamilton and K. Comai, *J. Lipid Res.*, 25 (1984) 1142.
[41] J.G. Hamilton and K. Comai, *Lipids*, 23 (1988) 1146.
[42] E.B. Hoving, G. Jansen, M. Volmer, J.J. van Doormaal and F.A.J. Muskiet, *J. Chromatogr.*, 434 (1988) 395.
[43] E.B. Hoving, F.A.J. Muskiet and W.W. Christie, *J. Chromatogr.*, 565 (1991) 103.
[44] M.P. Veares, R.P. Evershed, M.C. Prescott and L.J. Goad, *Biomed. Environ. Mass Spectrom.*, 19 (1990) 583.
[45] N.B. Smith, *J. Chromatogr.*, 249 (1982) 57.
[46] N.B. Smith, *J. Chromatogr.*, 254 (1983) 195.
[47] J.P. Blomhoff, *Clin. Chim. Acta*, 43 (1973) 257.
[48] L. Lillienberg and A. Svanborg, *Clin. Chim. Acta*, 68 (1976) 223.
[49] F.R. Hindriks, B.G. Wolthers and A. Groen, *Clin. Chim. Acta*, 74 (1977) 207.

[50] P. Gambert, C. Lallemant, A. Archambault, B.F. Maume and P. Padieu, *J. Chromatogr.*, 162 (1979) 1.
[51] J.W.I. Brunnekreeft, G.J.M. Boerma and B. Leijnse, *Ann. Clin. Biochem.*, 20 (1983) 360.
[52] H.J.G.M. Derks, A. van Heiningen and H.C. Koedam, *Clin. Chem.*, 31 (1985) 691.
[53] I. Björkhem, R. Blomstrand and L. Svensson, *Clin. Chim. Acta*, 54 (1974) 185.
[54] B.G. Wolthers, F.R. Hindriks, F.A.J. Muskiet and A. Groen, *Clin. Chim. Acta*, 103 (1980) 305.
[55] A. Cohen, H.S. Hertz, J. Mandel, R.C. Paule, R. Schaffer, L.T. Sniegoski, T. Sun, M.J. Welch and E. White, *Clin. Chem.*, 26 (1980) 854.
[56] O. Pelletier, L.A. Wright and W.C. Breckenridge, *Clin. Chem.*, 33 (1987) 1403.
[57] M. Kinter, D.A. Herold, J. Hundley, M.R. Wills and J. Savory, *Clin. Chem.*, 34 (1988) 531.
[58] P. Ellerbe, S. Meiselman, L.T. Sniegoski, M.J. Welch and E. White, V., *Anal. Chem.*, 61 (1989) 1718.
[59] J.H. Eckfeldt, L.A. Lewis, J.D. Belcher, J. Singh and I.D. Frantz Jr., *Clin. Chem.*, 37 (1991) 1161.
[60] J.T. Bernert Jr., J.R. Akins, G.R. Cooper, A.K. Poulose, G.L. Myers and E.J. Sampson, *Clin. Chem.*, 37 (1991) 2053.
[61] L.L. Abell, B.B. Levy, B.B. Brodie and F.E. Kendall, *J. Biol. Chem.*, 195 (1952) 357.
[62] I.W. Duncan, A. Mather and G.R. Cooper, *The procedure for the proposed cholesterol Reference Method*, Centers for Disease Control, Atlanta, GA 30333, 1982.
[63] A. Takatsu and S. Nishi, *Clin. Chem.*, 33 (1987) 1113.
[64] A. Takatsu and S. Nishi, *Anal. Chem.*, 60 (1988) 2237.
[65] A. Takatsu and S. Nishi, *Biol. Mass Spectrom.*, 22 (1993) 247.
[66] R. Ruiz and P. Dea, *J. Chromatogr.*, 146 (1978) 321.
[67] H.A. Schwertner, E.R. Johnson and T.E. Lane, *Clin. Chem.*, 36 (1990) 519.
[68] N. McIntyre, K. Kirsch, J.C. Orr and K.J. Isselbacher, *J. Lipid Res.*, 12 (1971) 336.
[69] T.A. Miettinen, *Clin. Chim. Acta*, 124 (1982) 245.
[70] J.T. Korpela, *Scand. J. Clin. Lab. Invest.*, 42 (1982) 529.
[71] S.N. Hooper and R.F. Chandler, *J. Pharm. Sci.*, 67 (1978) 1157.
[72] M. Arca, A. Montali, S. Ciocca, F. Angelico and A. Cantafora, *J. Lipid Res.*, 24 (1983) 332.
[73] G. Lercker, *J. Chromatogr.*, 279 (1983) 543.
[74] E. Tvrzická, P. Mareš, A. Písaříková, J. Novaković and P. Hrabák, *J. Chromatogr.*, 563 (1991) 188.
[75] A. Shafiee, J.M. Trzaskos, Y.K. Paik and J.L. Gaylor, *J. Lipid Res.*, 27 (1986) 1.
[76] B.A. Wasilchuk, P.W. Le Quesne and P. Vouros, *Anal. Chem.*, 64 (1992) 1077.
[77] P. Mareš, *Prog. Lipid Res.*, 27 (1988) 107.
[78] A. Kuksis, J.J. Myher and P. Sandra, *J. Chromatogr.*, 500 (1990) 427.
[79] J.J. Myher and A. Kuksis, *J. Biochem. Biophys. Methods*, 10 (1984) 13.
[80] A. Lohninger, P. Preis, L. Linhart, S.V. Sommoggy, M. Landau and E. Kaiser, *Anal. Biochem.*, 186 (1990) 243.
[81] A. Kuksis, J.J. Myher, K. Geher, A.G.D. Hoffman, W.C. Breckenridge, G.J.L. Jones and J.A. Little, *J. Chromatogr.*, 146 (1978) 393.
[82] H. Traitler, *Prog. Lipid Res.*, 26 (1987) 257.
[83] K. Aitzetmüller, *J. Chromatogr.*, 113 (1975) 231.
[84] V.K.S. Shukla, *Prog. Lipid Res.*, 27 (1988) 5.
[85] W.W. Christie, in W.W. Christie (Editor), *Advances in Lipid Methodology—one*, The Oily Press, Ayr, 1992, Ch. 7, p. 239.
[86] S.I. Schlager and H. Jordi, *Biochim. Biophys. Acta*, 665 (1981) 355.
[87] J.G. Hamilton and K. Comai, *Lipids*, 23 (1988) 1150.
[88] S.L. Smith, M. Novotný, S.A. Moore and D.L. Felten, *J. Chromatogr.*, 221 (1980) 19.
[89] J.T. Bernert Jr., J.R. Akins and D.T. Miller, *Clin. Chem.*, 28 (1982) 676.
[90] R. Vercaemst, A. Union and M. Rosseneu, *J. Chromatogr.*, 494 (1989) 43.
[91] K. Seta, H. Nakamura and T. Okuyama, *J. Chromatogr.*, 515 (1990) 585.
[92] F. Cheillan, H. Lafont, E. Termine, Y. Hamann and G. Lesgards, *Lipids*, 24 (1989) 224.
[93] J. Böswart, P. Kostiuk, J. Vymlátil, T. Schmidt, V. Pacáková and K. Štulík, *J. Chromatogr.*, 571 (1991) 19.
[94] M. Murata and T. Ide, *J. Chromatogr.*, 579 (1992) 329.
[95] E.H. Goh, S.M. Colles and K.D. Otte, *Lipids*, 24 (1989) 652.
[96] R.J. Maxwell, E.H. Nungesser, W.N. Marmer and T.A. Foglia, *LC–GC Int.*, 1 (1987) 57.
[97] W.W. Christie, *J. Lipid Res.*, 26 (1985) 507.
[98] W.W. Christie, *J. Chromatogr.*, 361 (1986) 396.
[99] P.R. Redden and Y.-S. Huang, *J. Chromatogr.*, 567 (1991) 21.
[100] T. Iwata, M. Yamaguchi and M. Nakamura, *J. Chromatogr.*, 421 (1987) 43.
[101] Y. Tsuruta, T. Teranishi, Y. Date and K. Kohashi, *J. Chromatogr.*, 617 (1993) 213.
[102] A. Kuksis, L. Marai and J.J. Myher, *Lipids*, 26 (1991) 240.
[103] M.D. Greenspan, C.-Y.L. Lo, D.P. Hanf and J.B. Yudkovitz, *J. Lipid Res.*, 29 (1988) 971.
[104] A.S. Csallany, S.E. Kindom, P.B. Addis and J.-H. Lee, *Lipids*, 24 (1989) 645.
[105] A. Chu and G.J. Schroepfer Jr., *J. Lipid Res.*, 29 (1988) 235.
[106] W.W. Christie, *J. Chromatogr.*, 454 (1988) 273.
[107] C. Matsuoka, H. Nohta, N. Kuroda and Y. Ohkura, *J. Chromatogr.*, 341 (1985) 432.
[108] P.H. Patrick and W.H. Elliott, *J. Chromatogr.*, 347 (1985) 155.
[109] H.E. Indyk, *Analyst*, 115 (1990) 1525.
[110] P.O. Edlund, *J. Chromatogr.*, 425 (1988) 87.
[111] H.K. Bhat and G.A.S. Ansari, *J. Chromatogr.*, 462 (1989) 467.
[112] A. Nomura, J. Yamada, A. Takatsu, Y. Horimoto and T. Yarita, *Anal. Chem.*, 65 (1993) 1994.
[113] D.H. Kim, K.-J. Lee and G.S. Heo, *J. Chromatogr.*, 655 (1994) 1.