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# Review

# Chromatographic separation of cholesterol in foods

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ABSTRACT

Based on the current literature and on experience gained in the laboratory, a simplified procedure using direct saponification (0.4 *M* potassium hydroxide in ethanol and heating at 60°C for 1 h) is the most appropriate method for the determination of total cholesterol in foods. Extraction of the unsaponifiable matter with hexane is efficient and no extra clean-up is required before quantification. An internal standard, 5α-cholestane or epicoprostanol, should be added to the sample prior to saponification and, together with reference standards, carried through the entire procedure to ensure accurate results. A significant improvement in cholesterol methodology has been achieved by decreasing the sample size and performing all the sample preparation steps in a single tube. The method has the advantages of elimination of an initial solvent extraction for total lipids and errors resulting from multiple extractions, transfers, filtration and wash steps after saponification. The resulting hexane extract, which contains a variety of sterols and fat soluble vitamins, requires an efficient capillary column for complete resolution of cholesterol from the other compounds present. The development of fused-silica capillary columns using cross-linked and bonded liquid phases has provided high thermal stability, inertness and separation efficiency and, together with automated cold on-column gas chromatographic injection systems, has resulted in reproducible cholesterol determinations in either underivatized or derivatized form. If free cholesterol and its esters need to be determined separately, they are initially extracted with other lipids with chloroform–methanol followed by their separation by column or thin-layer chromatography and subsequently analysed by gas or liquid chromatography. Although capillary gas chromatography offers superior efficiency in separation, the inherent benefits of liquid chromatography makes it a potential alternative. Isotope dilution mass spectrometry has been widely accepted as a reliable analytical method for highly accurate determination of cholesterol in serum and several definitive methods have been reported. The combination of capillary gas chromatography with mass spectrometry has become an excellent approach for the determination of cholesterol in complex mixtures of sterols and tocopherols, providing high resolution with positive identification. When used to determine cholesterol in multi-component foods, spectrophotometric methods have been documented to overestimate significantly the amount of cholesterol owing to the presence of other interfering substances. A re-evaluation of food products should be undertaken using the more specific chromatographic methods to accumulate data that will more accurately reflect the true cholesterol content.

CONTENTS

## 1. INTRODUCTION

Cholesterol is a 27-carbon steroid which is present in all animal tissues as a major structural component of cellular membranes. It is the precursor of bile acids, provitamin $D_3$ and the steroid hormones. Cholesterol can be present in the free form or esterified at the hydroxyl group with fatty acids of various chain length and saturation.

Public interest in cholesterol has increased owing to awareness and publicity of the relationship of plasma cholesterol levels to the risk of developing coronary artery disease. The relationship between dietary cholesterol and heart disease has been the subject of much research. Owing to public concern, specific recommendations have been made regarding desired changes in the diet [1] and as a result there is a need for more complete labelling for the levels of cholesterol in various food products.

Major sources of cholesterol in the diet are animal products, including eggs, milk and meat. Fish is relatively rich in cholesterol, but in shellfish (e.g., clams, oysters and scallops) other sterols are present in substantial amounts [2,3]. Much of the earlier data on the cholesterol content of food was produced using either spectrophotometric or gravimetric methods of analysis [4]. These data showed a wide variation in the cholesterol content; values often differed among investigators by more than 100%. Various spectrophotometric [5,6] and enzymatic [7] methods, originally developed to determine cholesterol in blood, were found to be unsuitable for the determination of cholesterol in food. Owing to various interferences caused by the presence of a considerable amount of plant or other sterols in food, these methods normally gave higher values for cholesterol content than the newer chromatographic procedures [3,8–11]. Problems with interferences and limitations of spectrophotometric methods have been discussed [4,8,12]. Differences in the cholesterol contents of various foods as reported in the literature, therefore, can be attributed to a great extent to differences in the analytical procedures used. Chromatographic procedures are highly specific and therefore more precise and accurate for the determination of cholesterol in food. These procedures also have the benefit of allowing the simultaneous measurement of plant sterols. Gas chromatographic (GC) procedures have been accepted as the method of choice for the determination of cholesterol in food [4,10,13,14]. However, even among the chromatographic techniques used there is a lack of standardization of analytical conditions which could lead to inaccurate data. This review is concerned with potential problems in analytical procedures associated with sample preparation and in particular with the chromatographic methods used in the determination of cholesterol in food.

## 2. SAMPLE PREPARATION

### 2.1. Lipid extraction

The method of sample preparation can have a significant influence on the results of cholesterol determination. Extraction of total lipids with a solvent has usually been the first step in sample preparation. Numerous extraction procedures involving many different solvent systems have been applied to foods (Table 1). Previous methods for the determination of fatty acids using 4 M HCl digestion followed by diethyl ether extraction were found to be unsuitable for sterol extaction [15]. In a comparison of seven extraction procedures developed for total lipids, fatty acids, cholesterol and other sterols from food products, Hubbard et al. [15] reported that the Folch method [16] [chloroform–methanol (2:1, v/v)] gave slightly higher sterol values than did the method of Bligh and Dyer [17]. The Folch method also gave significantly higher recoveries for cholesterol and other sterols compared with the hydrochloric acid–diethyl ether extraction system. Some workers have compared the Folch method or a modification of it [18] with other solvent systems for cholesterol extraction {e.g., light petroleum–

TABLE 1

LIPID EXTRACTION METHODS

| Sample type | Extraction method | Lipid class | Comments | Ref. |
|---|---|---|---|---|
| Food | (1) 4 M HCl digestion and diethyl ether extraction<br>(2) Folch<br>(3) 6 others tested, unsatisfactory | Fatty acids | 4 M HCl was chosen owing to the speed, simplicity and recovery | 115 |
| Food products | (1) 4 M HCl<br>(2) Folch<br>(3) Bligh and Dyer | Cholesterol and other sterols | Folch was recommended based on superior extraction of cholesterol | 15 |
| Egg yolk | (1) Light petroleum–ethanol<br>(2) Folch | Cholesterol esters and cholesterol | No difference | 19 |
| Food | (1) Chloroform–methanol<br>(2) Hot benzene<br>(3) Acetone<br>(4) Diethyl ether | Total lipids and cholesterol | Chloroform–methanol gave the highest yield for lipid and for cholesterol content | 21 |
| Ready-to-serve food | (1) Light petroleum using Soxhlet apparatus<br>(2) Folch | Total lipids and cholesterol | Folch was better | 20 |
| Raw and cooked beef muscle | (1) Diethyl ether<br>(2) Folch | Total fat | Folch gave 6.2% higher recovery for raw and 13.4% higher for cooked | 116 |

ethanol (2:1, v/v) for egg yolk [19] or Soxhlet extraction with light petroleum for "ready to serve" food [20]} and observed no difference in cholesterol values. Kaneda et al. [21] compared four different solvent systems (Folch method, hot benzene, acetone and diethyl ether) for extracting lipids and cholesterol from food and found the Folch method to give higher percentage yields for both lipid and cholesterol from short-necked clam and top shell fish. Two popular methods for sample preparation include the official AOAC method [22] for multicomponent foods (based on the method of Punwar [13] which includes an initial extraction with chloroform–methanol–water [17]) and the method of Sheppard et al. [14] (based on the Folch method). Both of these methods involve multiple steps requiring homogenization of the sample with large volumes of solvent and quantitative transfer and filtering into a separating funnel. The extract is washed repeatedly with water or salt solution and centrifuged to separate the chloroform and aqueous phases. The chloroform phase is then passed through sodium sulphate and finally evaporated under nitrogen. In an attempt to simplify this lengthy procedure, Washburn and Nix [18] reported no difference in the amount of cholesterol extracted

with or without the use of sodium sulphate or whether water or 0.88% KCl solution was used. Currently, the most suitable and commonly used method for the extraction of lipids and cholesterol from food is the Folch method (including all its modifications). For total lipid extraction, our laboratory uses and recommends a modified Folch method [23] that reduces both the solvent requirements and the number of wash steps.

Autoxidation of lipids can occur easily if they are exposed to air, light and heat, and precautions should be taken during sample preparation to ensure that no degradation or other alteration occurs. Cholesterol, having a double bond, can also undergo autoxidation in the presence of oxygen. To avoid or minimize autoxidation, samples should be stored at low temperature (−20°C or lower) and analysed as soon as possible to prevent enzymatic degradation before solven extraction. An excellent review of sample storage and handling before extraction of lipids has been published [24]. After extraction, samples should be kept in inert solvents under nitrogen in air tight vials and refrigerated to help reduce autoxidation. If fatty acids are of interest, an antioxidant such as 2,6-di-tert.-butyl-p-cresol (BHT) or pyrogallol is often added to the

extraction solvents to prevent oxidative degradation of unsaturated lipids. Many foods contain $\beta$-carotene and tocopherols, which are natural antioxidants that offer some protection to lipid extracts.

Maerker and Unruh [25] studied the autoxidation of cholesterol during saponification by two different methods, a dry column procedure [26] and the official AOAC procedure [22], and compared them with a sample without prior saponification. They reported that regardless of the method of saponification, it contributes to the generation of oxidation products and that even without saponification some oxidation products were present. They also found 0.16 $\mu$g of oxides formed as artifacts per milligram of cholesterol during their HPLC–GC procedure. Addition of 0.3% of BHT as an antioxidant in the dry column saponification of cholesterol did not result in reduction of oxidation products. It was concluded that autoxidation of cholesterol during analysis can be minimized but it cannot be completely eliminated. However, the amount of autoxidation products relative to the amount of cholesterol is very small and should not be significant in the determination of total cholesterol. Oles et al. [27] investigated factors affecting the recovery of cholesterol from baked food products and found that pyrogallol addition did not significantly alter the recovery.

## 2.2. Direct saponification

Recently there has been a trend towards simplifying sample preparation in order to reduce the solvent volume and shorten the analysis time. After extraction of lipids the usual next step is basic hydrolysis (saponification) to separate cholesterol and other unsaponifiable matter from fatty acids, which usually account for most of the extracted lipids. When cholesterol and other sterols are of primary interest, many workers have used direct saponification of the sample in order to eliminate the initial extraction step and thus simplify the procedure. Various workers have compared direct saponification for cholesterol determination to lipid extraction prior to saponification [23,28–33]. Kovacs et al. [31] found the cholesterol content in cod muscle determined using direct saponification to be higher than that determined by the AOCS official method [34]. Van Elswyk et al. [32] also obtained higher values for egg yolk cholesterol using direct saponification than with the AOAC official method [35] and

concluded that direct saponification is more accurate. They obtained a value of 19.1 $\pm$ 0.4 [standard error (S.E.)] mg cholesterol per gram of yolk in the National Institute of Standards and Technology (NIST) reference material [cholesterol in whole egg powder which had a certified concentration of 19.0 $\pm$ 0.2 (S.E.)]. Others [30] have reported comparable or slightly higher cholesterol values in twenty different meat products using direct saponification and a 99.8% recovery of added cholesterol. Al-Hasani et al. [28], using a direct saponification method for cholesterol in frozen foods, found a superior recovery of cholesterol from spiked samples, 100.2% versus 94.9% for the AOAC method, and a good correlation ($r$ = 0.9996) with the AOAC method. In our laboratory we have studied factors affecting the determination of cholesterol in egg yolk [23] and found no significant difference between using direct saponification or chloroform–methanol extraction prior to saponification. Other workers have also reported no difference between the two methods when analysing for total cholesterol in milk [33], egg products [29] and blood plasma [36].

The inconsistency in reported results could be due, in part, to the lack of standardization of conditions used for the saponification–extraction steps (Table 2). Factors contributing to variations in cholesterol determinations include sample size and composition, volume and strength of base, type of alcohol used, temperature and time of hydrolysis, the use of refluxing apparatus or capped tubes, type and volume of extraction solvent and post-extraction manipulations.

For precise results to be obtained for cholesterol determination, certain requirements must be met in the saponification and extraction steps. Cholesterol must be released from lipoprotein complexes, cholesterol esters must be completely hydrolysed and fatty acids held as soaps in the aqueous phase and thus separated during extraction. The extraction solvents should recover cholesterol quantitatively from the aqueous phase without forming emulsions. After a comprehensive review of the methodology for cholesterol determination in serum, De La Huerga and Sherrick [37] concluded that the saponification procedure of Abell et al. [38] was the most suitable. They suggested that the concentration of KOH in the final saponification solution should be between 0.33 and 0.5 $M$ in ethanol and that choles-

TABLE 2

SAPONIFICATION AND EXTRACTION CONDITIONS FOR SAMPLE PREPARATION FOR CHOLESTEROL DETER-MINATION

| Sample matrix | Reagent[a] | Temperature (°C) | Time (min) | Extraction conditions | Ref. |
|---|---|---|---|---|---|
| 0.5 g food (0.2 g oil) | 10 ml EtOH + 2 ml KOH (50%, v/v) | 70 (capped tube) | 8 | 30 ml water + 20 ml hexane–diisopropyl ether (3:1) | 86 |
| Lipid extract | 20 ml 0.5 M KOH in MeOH | Reflux | 20 | 5 ml saturated NaCl + 10 ml n-heptane, repeat twice with light petroleum, wash 5 times with water | 84 |
| 1 g oil | 100 ml 0.8 M KOH in EtOH | 80 | 30 | Twice with 100 ml diethyl ether, wash with water, dry with Na$_2$SO$_4$ | 85 |
| Lipid extract | 2.5 ml 2% (w/v) KOH in EtOH | 37 | 90 | 2.5 ml water + 5 ml light petroleum | 8 |
| Lipid extract | 2 ml 1 M KOH in 93% EtOH | 80 | 240 | 5 ml water + 3 times with 5 ml hexane | 75 |
| 1 g egg noodles | 1 ml 2 M KOH in MeOH | Reflux | 30 | 60 ml 1.7% NaCl + twice with 100 ml diethyl ether–light petroleum (1:1) | 45 |
| 0.5 g food | 1 ml 50% KOH + 4 ml 95% EtOH | Reflux | 60 | 2.5 ml water + 4 times with 5 ml hexane | 31 |
| 1 g milk | 1 ml KOH (80 g per 50 ml) + 4 ml EtOH | 100 | 60 | Solid-phase extraction | 33 |
| 0.5 g egg product | 10 ml 2.4 M KOH in EtOH | 70 | 60 | 5 ml saturated NaCl + 5 ml water, 3 times with 10 ml light petroleum | 29 |
| <0.8 g lipid from meat | 8 ml 50% KOH + 40 ml EtOH–MeOH (95:5) | Reflux | 60 | 100 ml toluene, wash twice with 1 M KOH and 5 times with water, dry with Na$_2$SO$_4$ | 30 |
| 10 g frozen food | 10 ml 60% KOH + 40 ml EtOH | Reflux | 30 | Twice with 50 ml toluene or hexane, wash 3 times with water, dry with Na$_2$SO$_4$ | 28 |
| 1 g egg | 8 ml 50% KOH + 40 ml 95% EtOH | 60 (capped tube) | 60 | 40 ml water, 3 times with 60 ml hexane, wash 5 times with 100 ml water, dry with Na$_2$SO$_4$ | 32 |
| 0.2 g egg | 0.6 ml 33% KOH + 9.4 ml 95% EtOH | 60 (capped tube) | 60 | 5 ml water + 10 ml hexane | 23 |
| 1.5 g food | 2 ml 50% KOH + 15 ml EtOH or iPrOH | (1) Ambient (2) Reflux | 18 h 60 | 10 ml 10% NaCl, 7 ml toluene or hexane–diethyl ether (85:15), wash with 10 ml 10% NaCl, dry with Na$_2$SO$_4$ | 27 |
| Lipid | 1 ml 40% KOH + 20 ml EtOH | 85 | 60 | 20 ml water, 3 times with 20 ml diethyl ether, dry with Na$_2$SO$_4$ | 21 |
| Lipid | 25 ml 1 M NaOH in EtOH | Reflux | 60 | 3 times with diethyl ether, wash with water | 20 |
| 5 g oil | 50 ml 2M KOH in MeOH | Reflux | 60 | 100 ml water, 5 times with diethyl ether, wash 3 times with water, dry with Na$_2$SO$_4$ | 40 |
| 0.2 g egg | 6 ml 2.5% KOH in EtOH + 0.3 ml water | 70 | 90 | 6 ml water, 12 ml light petroleum | 19 |
| Plant lipids | 1 g KOH in 5 ml aqueous EtOH | Reflux | 120 | 1 time with diethyl ether, wash with water, dry with Na$_2$SO$_4$ | 44 |
| 100 mg lipid | 0.5 ml saturated KOH + 8 ml EtOH | 80 (capped tube) | 8 | 12 ml water, twice with 20 ml cyclohexane | 42 |
| Lipid | 8 ml KOH (60 g/40 ml) + 40 ml EtOH–MeOH–iPrOH (90:5:5) | Reflux | 60 | 100 ml benzene, wash once with 200 ml 1 M KOH, once with 40 ml 0.5 M KOH and 3 times with 40 ml water, dry with Na$_2$SO$_4$ | 22 |

[a] MeOH = Methanol; EtOH = ethanol; iPrOH = isopropanol (2-propanol).

terol esters are completely hydrolysed at 37–40°C in 60 min. In order to test the hydrolysis step of cholesterol esters to cholesterol in serum, Lillienberg and Svanborg [36] varied the hydrolysis time (from 5 to 120 min) at three different temperatures (35, 55 and 75°C). At 37°C they obtained higher values with

increasing hydrolysis time. Hydrolysis at 55°C gave significantly higher values up to 30 min, after which no further increase was observed. At 75°C slightly higher values were observed up to 15 and 30 min but the values were lower compared with hydrolysis at 55°C for 30 min. Both investigators used a hydrolysis solution consisting of 94 parts of 95% ethanol and 6 parts of 33% aqueous KOH [38]. This hydrolysis solution is routinely used in our laboratory for egg yolk cholesterol determination. Fresh egg yolk (0.2 g) and 10 ml of hydrolysis solution are heated at 60°C for 1 h in capped 50-ml tubes. The tubes are shaken occasionally during heating to bring into solution any sample adhering to the wall of the tube. It is important that the entire sample is evenly distributed in the hydrolysis solution. These conditions are sufficient for complete hydrolysis of cholesterol esters and also give clean samples without any free fatty acids as determined by GC analysis. In contrast, in our laboratory when studying phytosterols, a much larger sample size is used (2–5 g of dried egg yolk and 100 ml of hydrolysis solution) and free fatty acids consistently appear in the chromatograms in varying concentrations. To minimize contamination, all operations are performed in clean glass tubes with PTFE-lined screwcaps and all solvents are purified by distillation using an efficient fractionating column. If the samples contain very small amounts of cholesterol, contamination of the sample with flakes of skin might be a problem as skin contains relatively large amounts of cholesterol and other sterols [39].

More recently, Lognay et al. [40] studied the efficiency of the saponification extraction step for the extraction of sterols from edible oils using radiolabelled cholesterol and cholesteryl oleate. Using diethyl ether, one of the more commonly used solvents (Table 2), as extraction solvent after saponification, five extractions were required for the quantitative recovery of labelled cholesterol (96% recovery for spiked sunflower oil and 99% recovery for spiked butter oil). Significant differences were observed for the initial extraction recoveries from sunflower oil (60%) and butter oil (75%). Washing the ether phase three times with water accounted for a ca. 1% loss of cholesterol.

Oles et al. [27] studied some of the more significant factors affecting the recovery of cholesterol from various food matrices. Factors examined were: type of alcohol, extraction solvent, use of antioxidants, time and temperature of hydrolysis and spiking level. Hydrolysis conditions had a significant impact on the recovery of cholesterol when isopropanol was used as a solvent for hydrolysis but when ethanol was used there was no substantial effect. The extraction solvents compared were hexane–diethyl ether (85:15) and toluene, with the latter giving significantly higher recoveries.

In our laboratory, hexane was found to be an efficient solvent for the extraction of cholesterol from the saponification mixture of egg yolk. Standard additions from 1.2 to 6 mg of cholesterol yielded a recovery in excess of 98% with a single extraction for egg yolk samples of 0.2 g or less [23]. Similar results were obtained in experiments with [4-$^{14}$C]cholesterol [41], which showed that a single hexane extraction of the hydrolysis mixture extracted about 99% of the labelled cholesterol. Hexane is an excellent solvent because it is less toxic than other solvents commonly used and does not form emulsions as does toluene [30]. Being nonpolar, it is more suited for the efficient partitioning of unsaponifiable matter into the organic phase and eliminates the necessity to dry the solvent with anhydrous sodium sulphate. It also does not form peroxides as does diethyl ether, which could cause the production of degradation products representing as much as 5% of the total sterols [40]. In our laboratory it was also observed that the amount of fat in the sample can affect the extraction of cholesterol. By increasing the amount of soybean oil added to a cholesterol standard (for 0 to 170 mg) the efficiency of the first extraction, as percentage of the total, decreased from 98.2% to 94.1% [23]. Slover et al. [42] also demonstrated that the presence of fat in the saponification mixture affected the extraction of both tocopherols and sterols and that two extractions with cyclohexane were needed for quantitative recovery.

Some workers found it necessary to purify the solvent extract further by either thin-layer chromatography (TLC) [43,44] or column chromatography [27,45]. Tsui [33] isolated cholesterol and an internal standard after saponification by solid-phase extraction on a non-polar adsorbent $C_{18}$ cartridge instead of using a solvent extraction procedure. He described many critical steps in which losses of cholesterol can occur. It was emphasized that the adsor-

bent must be properly conditioned with methanol and then water and not be allowed to dry prior to application of the sample. The transfer of the rinse on to the adsorbent material should be quantitative, overloading of the cartridge should be avoided and the pH must be adjusted to between 2 and 5.

When diethyl ether alone or in combination with other solvents is used as an extraction solvent, some workers found impurities in the unsaponifiable matter [46]. These were primarily residual soaps [40] and extracted lipids (most likely free fatty acids) and they caused rapid contamination of the capillary column [27].

## 3. CHROMATOGRAPHIC ANALYSIS

### 3.1. Gas chromatography

#### 3.1.1. Separation by packed versus capillary columns

Sterols of plant and marine origin are found in the unsaponifiable fraction of lipid extracts from foods and their presence may interfere with analysis for cholesterol by both spectrophotometric and chromatographic methods.

Plant sterols are biologically important cholesterol analogues which differ structurally in the presence of a methyl or ethyl group at the C-24 position or unsaturation of the side-chain. Although campesterol, $\beta$-sitosterol and stigmasterol are plant sterols which make up the major portion of the total sterol fraction of edible plant oils [2], significant amounts of other sterols can be present in various oils (e.g., $\Delta^7$-stigmasterol, brassicasterol and avenasterol).

Tocopherol, tocotrienol and their $\alpha$, $\beta$, $\gamma$ and $\delta$ isomers, which are widely distributed in foods of plant and animal origin, are also present in the unsaponifiable fraction. In foods of animal origin the $\alpha$-tocopherol content is in the range 1–30 $\mu g/g$. The $\alpha$-tocopherol content of plant material (grain, fruits and vegetables) ranges between 1 and 25 $\mu g/g$, with higher values for almonds and filberts, 270 and 210 $\mu g/g$, respectively [47]. In vegetables and seed oils other isomers occur in substantial amounts with the total tocopherol content in the range 40–2600 $\mu g/g$. In addition to sterols and tocopherols, other compounds found in the unsaponifiable matter include saturated hydrocarbons, squalene, aliphatic alcohols, terpene alcohols, triterpene alcohols and steryl esters [48–51].

Analysis for free and esterified sterols and triterpene alcohols (4-monomethyl- and 4,4-dimethylsterols) may be applied to characterize edible oils and to detect possible adulteration [50–54]. For example, the presence of margarine in butter can be detected by analysing for $\beta$-sitosterol, which is present only in vegetable oil. Similarly, substitution of cheaper rapeseed oil for olive oil would result in easily detectable brassicasterol, not found in olive oil, and substitution of animal-derived fat would result in an increase in cholesterol content.

The determination of sterols by GC on packed columns has been reviewed previously [55–57]. Retention characteristics for 50 free sterols and their trimethylsilyl (TMS) derivatives relative to cholesterol on several different stationary phases ranging in polarity from non-polar SE-30 to polar Silar 5-CP have been reported [55]. Retention times for 92 sterols and closely related compounds as steryl acetates were determined on four common stationary phases on packed columns [58]. More recently, Xu et al. [59] studied the chromatographic properties of TMS ethers of 100 sterols and related compounds and determined their retention times relative to cholesterol on an SE-30 packed column. Relative retention times of 168 acetate derivatives of sterols and triterpene alcohols have also been determined on OV-1 and OV-17 support-coated open-tubular (SCOT) glass capillary columns [60].

Although information obtained using packed columns was useful, packed columns have been largely replaced by wall-coated open-tubular (WCOT) fused-silica column technology in most laboratories during the past decade. Packed column instruments can easily be converted for use with 0.53 mm I.D. capillary columns (referred to as "megabore" or "halfmil") with currently available injector and detector adapters. These fused-silica columns offer a number of advantages over packed columns. WCOT columns which are surface bonded and cross-linked offer increased thermal stability and higher operating temperatures with significantly less bleeding. A lower level of column bleeding minimizes detector contamination, extends the lifetime of the column and makes reproducible peak integration easier. The high number of theoretical plates with capillary columns allows faster analysis times and improved resolution. The manufacturer's quality control testing on individual columns en-
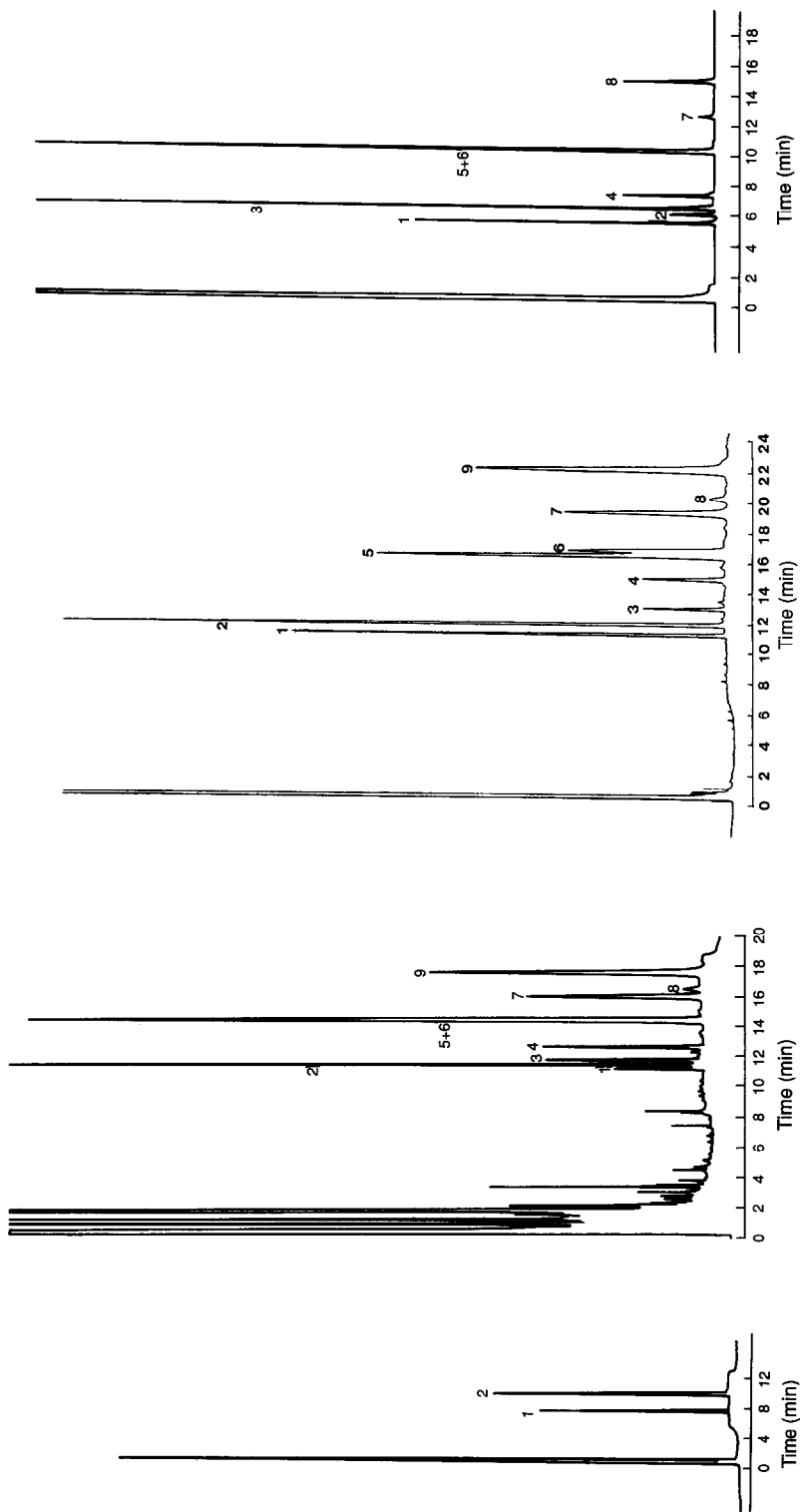
Fig. 1. Gas chromatogram of an egg yolk sample. Peaks: 1 = 5α-cholestane; 2 = cholesterol. Column, DB-5 (J&W Scientific, Folsom, CA, USA), 15 m × 0.53 mm I.D., 1.5 μm $d_f$; temperature, 70°C; on-column injection at 80°C, then increased at 150°C/min to 270°C; detector temperature, 270°C; carrier gas, helium at 70 cm/s.

Fig. 2. Gas chromatogram of TMS derivatives of standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 + 6 = α-tocopherol and cholesterol; 7 = campesterol; 8 = stigmasterol; 9 = sitosterol. Conditions as in Fig. 1.

Fig. 3. Gas chromatogram of underivatized standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = cholesterol; 6 = α-tocopherol; 7 = campesterol; 8 = stigmasterol; 9 = sitosterol. Column, SPB-5 (Supelco, Bellefonte, PA, USA), 15 m × 0.53 mm I.D., 1.5 μm $d_f$. Other conditions as in Fig. 1.

Fig. 4. Gas chromatogram of TMS derivatives of standards. Peaks: 1 = squalene; 2 = δ-tocopherol; 3 = 5α-cholestane; 4 = γ-tocopherol; 5 + 6 = α-tocopherol + cholesterol; 7 = campesterol; 8 = sitosterol. Column, DB-17 (J&W Scientific) 30 m × 0.25 mm I.D., 0.15 μm $d_f$; temperature, 250°C, then increased at 5°C/min to 270°C; injector temperature, 270°C; detector temperature, 270°C; carrier gas, helium at 30 cm/s; splitting ratio, 15:1.

sures higher column to column reproducibility. The greater inertness of fused-silica and more extensive deactivation allow acidic and basic compounds to be analysed on the same column, giving better peak shapes for active compounds. A narrower and more symmetrical peak shape improves quantification by improving the integration accuracy, leading to lower limits of detection.

Although thin-film columns are preferred for the determination of high-molecular-mass, high-boiling-point compounds (to minimize the bleeding from the column), we achieved exceptionally good results on a short, thick-film megabore column [DB-5, 15 m × 0.53 mm I.D. with film thickness $(d_f)$ 1.5 $\mu$m] for the determination of cholesterol in egg yolk. Fig. 1 shows a typical chromatogram of a sample after more than 6000 injections on this column. As can be seen, the cholesterol peak is sharp, without tailing. It shows no adsorption, and this is confirmed by the fact that the response factors have remained constant over a long period of use. Thick films cover the active silanol groups on the surface of the fused-silica, providing high inertness and preventing tailing and adsorption of polar compounds when analysed in the underivatized form. Alternatively, a standard SE-30 capillary column (30 m × 0.25 mm I.D., 0.25 $\mu$m $d_f$) showed increased tailing of the cholesterol peak with column use and a corresponding increase in response factors.

The short, thick-film column gives adequate separation for cholesterol determination in samples where cholesterol makes up most (>98%) of the sterols and tocopherols present (e.g., egg yolk). However, as the resolving power of a column is inversely dependent on film thickness and column diameter [61], longer columns with smaller inner diameter and thinner film thickness are needed for the more complex separations requiring maximum resolution. In analyses of multi-component foods, in which sterols and tocopherols of plant and animal origin may be present in significant amounts together with cholesterol, very efficient capillary columns are required to prevent co-elution of compounds. The separation of cholesterol and cholestanol, which differs from cholesterol only in the absence of a double bond, is difficult to achieve. Noda et al. [44], using gas chromatography–mass spectrometry (GC–MS), showed that plant surface

sterols often contain cholestanol as a minor component that is inseparable from cholesterol on pakced columns (e.g., SE-30, OV-1, OV-101) but separation was possible on an SE-52 capillary column. The resolution between these two sterols was found to be affected by the carrier gas pressure and injection splitting ratio [62] and their order of elution was reversed on a polar capillary column (SP-2330) [63]. Other closely related sterols found in seafood, desmosterol, 22-dehydrocholesterol, 24-methylene-cholesterol and brassicasterol [2,3], have retention times which are very close to that of cholesterol. Sterols of different molecular size are easily separated on non-polar columns but pairs of sterols differing in degree of unsaturation are better separated on polar phases. Although highly efficient capillary columns can improve the separation of critical pairs of sterols, factors such as changes in film thickness, type of phase, temperature programming and derivatization all may have an effect on separation.

Another difficult separation is that of cholesterol and $\alpha$-tocopherol, which co-elute on many GC systems. With packed columns their retention times are nearly identical [14]. Figs. 2–7 show separations of a mixture of standards of cholesterol, 5$\alpha$-cholestane, squalene, phytosterols and $\alpha$-, $\gamma$- and $\delta$-tocopherol using various columns and conditions (author's laboratory, unpublished data). Figs. 8–11 show separations of actual food analysis (pea soup sample was from local cafeteria and the mayonnaise sample was purchased at the supermarket). There is little information in the literature on the determination of cholesterol in the presence of significant amounts of other sterols and tocopherols.

### 3.1.2. Derivatization

Derivatization of cholesterol and other sterols improves the peak shape and may contribute to reduced retention time and improve sensitivity. Although a variety of other derivatives including butyryl esters [14] and acetates [60] have been employed, the formation of trimethylsilyl (TMS) ethers is preferred in the GC of sterols. TMS ethers offer higher thermostability and lower polarity and exhibit less tailing due to lack of adsorption to polar sites on the column. Many different silylating reagents are available for the preparation of TMS derivatives of sterols (Table 3). To optimize the
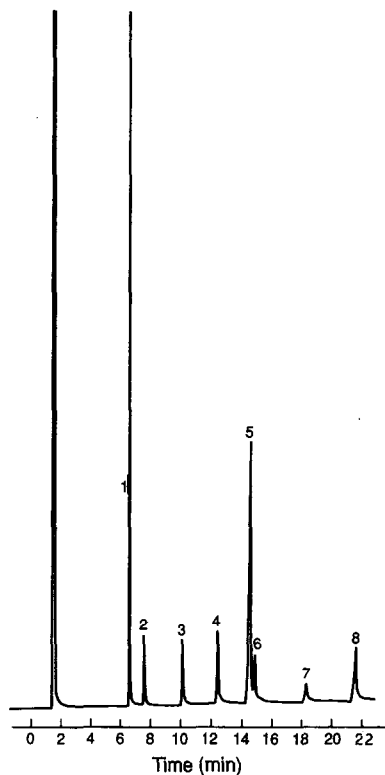
Fig. 5. Gas chromatogram of underivatized standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = α-tocopherol; 6 = cholesterol; 7 = campesterol; 8 = sitosterol. Conditions as in Fig. 4.
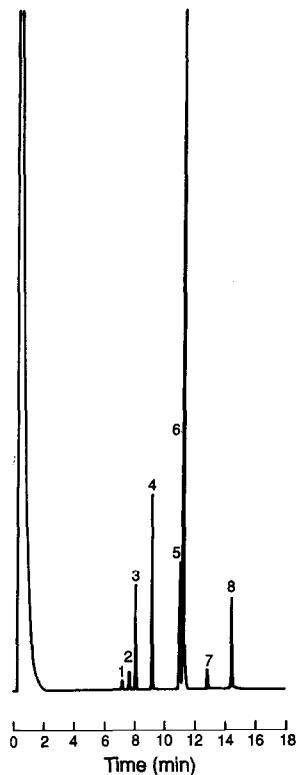
Fig. 6. Gas chromatogram of TMS derivatives of standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = α-tocopherol; 6 = cholesterol; 7 = campesterol; 8 = sitosterol. Column, $RT_x$-1 (Restex, Bellefonte, PA, USA), 30 m × 0.25 mm I.D., 0.25 $\mu$m $d_f$; temperature, 240°C, then increased at 5°C/min to 280°C; injector temperature, 270°C; detector temperature, 270°C; carier gas, helium at 25 cm/s; splitting ratio, 15:1.
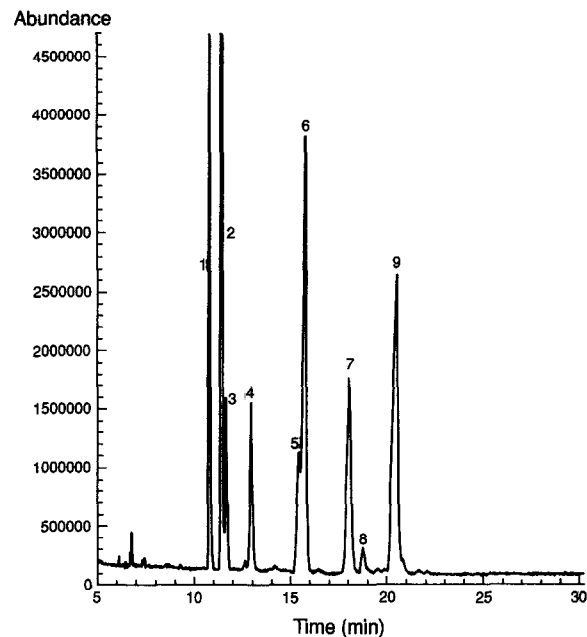
Fig. 7. Separation of TMS derivatives of standards using GC–MS. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = α-tocopherol; 6 = cholesterol; 7 = campesterol; 8 = stigmasterol; 9 = sitosterol. Column, BP-5 (SGE International, Victoria, Australia), 25 m × 0.22 mm I.D., 0.25 $\mu$m $d_f$; temperature, 70°C, then increased at 30°C/min to 270°C; on-column injection at 80°C, then increased at 150°C/min to 270°C; detector temperature, 280°C; carrier gas, helium at 30 cm/s.

silylating conditions for cholesterol and its oxides, Nawar et al. [64] tested three silylating agents, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), BSTFA–1% trimethylchlorosilane (TMCS) and Sylon BTZ [N,O-bis(trimethylsilylacetamide (BSA): TMCS:trimethylsilylimidazole (TSIM) 3:2:3] and different time–temperature relationships. They found BSTFA–1% TMCS to give the best results within 1 h at 80°C. BSTFA is the preferred reagent because it produces hydrofluoric acid in the flame ionization detector, which reacts with the silicone to form the volatile $SiF_4$ preventing excessive build-up of silicon deposits in the detector. As cholesterol is poorly soluble in Sylon BTF [BSTFA–TMCS (99:1)] alone [39], pyridine was added during derivatization and then evaporated to eliminate the excessive tailing of the solvent peak during GC analysis. TMS reagents and derivatives are sensitive to moisture and therefore must be stored in tightly capped tubes and in moisture-free solvent to avoid hydrolysis. Also, the carrier gas must have an efficient moisture trap in-line to prevent hydrolysis of the TMS derivatives at high temperatures. Many silylating reagents are toxic, flammable and corrosive (chlorosilanes release hydrochloric acid on exposure to moisture) resulting in a need for proper ventilation in the sample preparation area and in the GC instrument area to remove vapours from the injector, detector and autosampler.

With improvements in thermal stability of liquid phases and with the development of inert fused-silica columns, many workers have found it unnecessary to prepare derivatives and prefer to determine sterols in the free form [21,23,29,57,65]. TMS derivatization not only adds an extra step in the procedure but could also contribute to increased noise, formation of artifacts, loss of sample and decreased linearity due to silicone deposits in the flame ionization detector [31,57]. Even on packed columns, Kovacs et al. [31] found that silylation did not result in a significant difference in sterol recovery. Van Delden et al. [20] reported good linearity and reproducibility for determination of cholesterol without prior derivatization as long as silylated supports were used. Kaneda et al. [21] obtained a good recovery and separation of cholesterol from other compounds from food without derivatization and, as the results were similar to those obtained using TMS and acetate derivatives, it was concluded

that derivatization was not essential and that the unsaponifiable matter can be directly analysed by GC in order to save time. In our laboratory, we also found no significant difference for cholesterol content in egg yolk with or without derivatization (unpublished data). A mixture of cholesterol and eleven cholesterol oxide standards was analysed using on-column capillary GC with and without prior silylation [25]. The results showed that silylation changed the order of elution and that underivatized oxides were better separated from cholesterol than derivatized oxides on a 5% phenylsilicone column.

### 3.1.3. Quantitative analysis

*3.1.3.1. Injection techniques.* Split/splitless is the most commonly used injection technique for capillary GC but the recently available cool on-column injection systems have definite advantages. A more reproducible quantitative transfer of the sample to the column occurs than with split/splitless injection. Cold sample introduction ensures greater stability of thermally labile compounds and prevents discrimination and activity toward polar and high-boiling compounds as in the hot split/splitless injection. When using split/splitless injection, a significant reduction in peak degradation and tailing can be obtained through the use of silanized injection port inserts when analysing for polar compounds.

The cold on-column injection technique is most reliable for quantitative analysis [66,67] since the loss of sample due to thermal decomposition and irreversible adsorption on active sites in the column is minimized. With on-column injection, the sample is deposited directly into the highly inert fused-silica column at low temperature and then the injector temperature is raised rapidly to volatilize the sample. Normally a 5-m length of wide-bore (0.53 mm I.D.) deactivated fused-silica tubing, called a "retention gap" [68], is connected to the narrow-bore column with a glass butt connector. It traps nonvolatile residues and prevents damage to the liquid phase if dirty samples are injected. It also has the function of refocusing the sample, resulting in increased resolution and decreased peak splitting [66]. An alternative to on-column injection is "direct injection", in which the sample is deposited in a low-volume deactivated glass liner in the injection port. With these injection techniques the problems
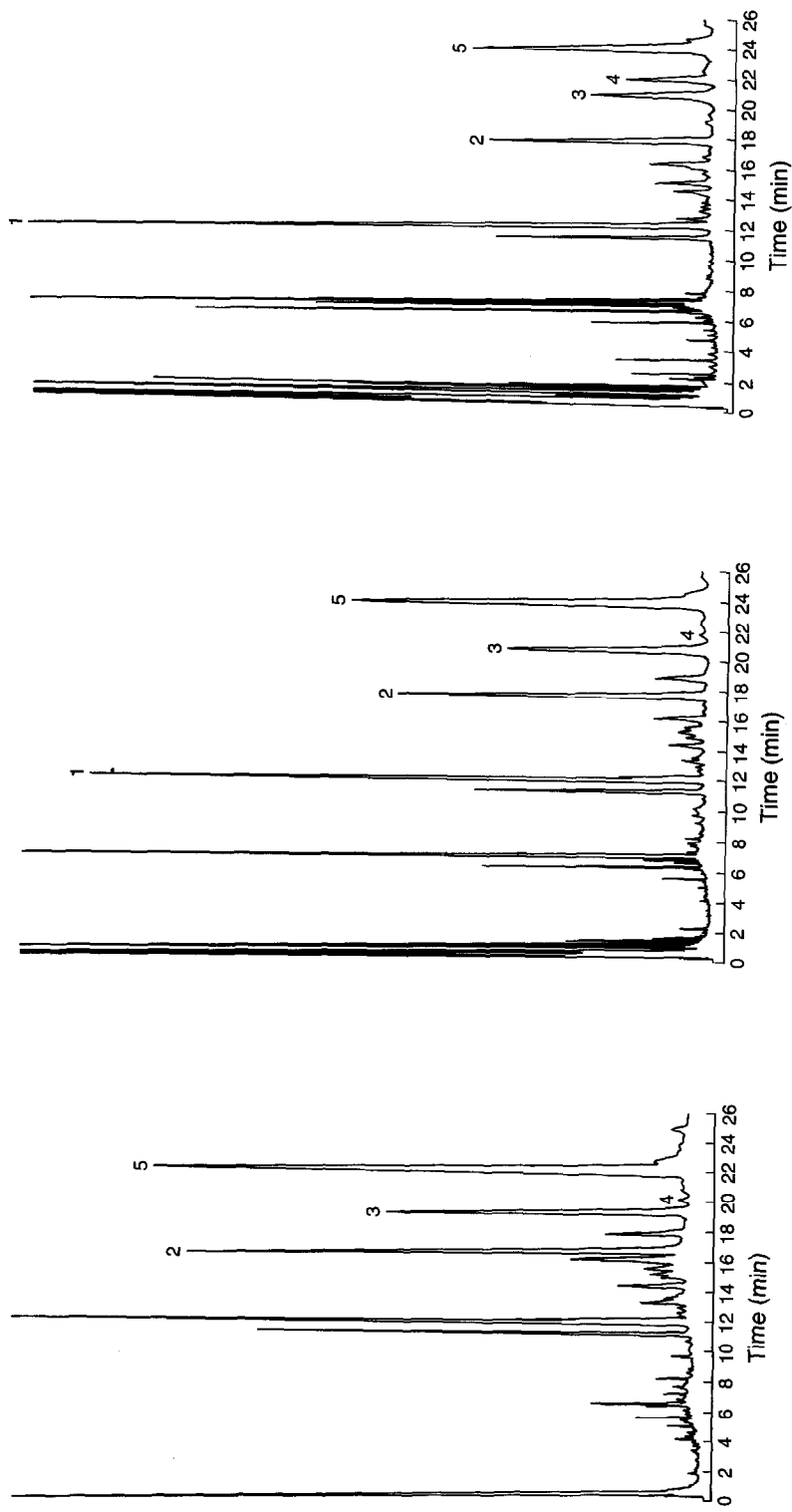
Fig. 8. Gas chromatogram of underivatized sample of split pea soup. Peaks: 1 = 5α-cholestane; 2 = cholesterol; 3 = campesterol; 4 = stigmasterol; 5 = sitosterol. Column, SPB-5 (Supelco, Bellefonte, PA, USA), 15 m × 0.53 mm I.D., 1.5 μm $d_f$; temperature, 70°C, then increased at 30°C/min to 270°C; on-column injection at 80°C, then increased at 150°C/min to 270°C; detector temperature, 270°C; carrier gas, helium at 70 cm/s.

Fig. 9. Gas chromatogram of TMS derivatives of split pea soup sample. Peaks and conditions as in Fig. 8.

Fig. 10. Gas chromatogram of TMS derivatives of mayonnaise sample. Peaks and conditions as in Fig. 8.
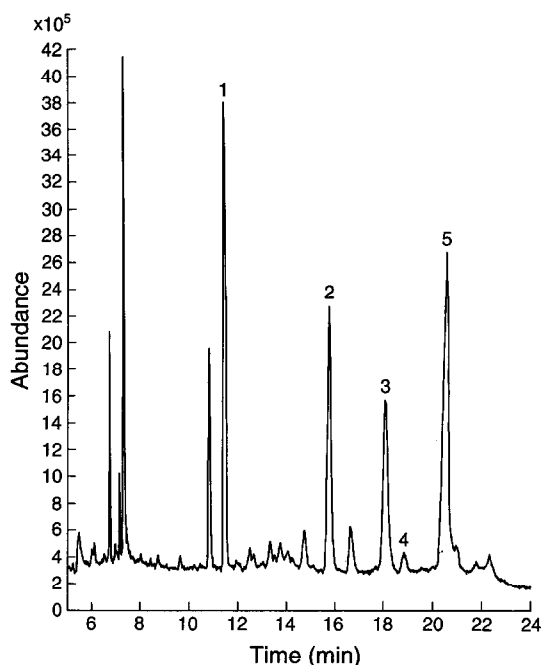
Fig. 11. Separation of TMS derivatives of split pea soup sample with GC-MS. Peaks: 1 = 5α-cholestane; 2 = cholesterol; 3 = campesterol; 4 = stigmasterol; 5 = sitosterol. Conditions as in Fig. 7.

caused by non-volatile sample residues deposited during injection can be easily overcome by either cutting off a section of the retention gap or cleaning the glass insert.

*3.1.3.2. Detection and quantification.* The most frequently used detection technique for cholesterol and other sterols is flame ionization detection (FID). It has good sensitivity and a wide linear range. Using a cooled on-column injector and an efficient capillary column (5% phenylsilicone), the detection limit for a cholesterol standard was determined to be 325 pg [25]. When helium is used as the carrier gas with capillary columns, nitrogen may be supplied as the make-up gas to improve the sensitivity of FID.

The low flow-rate of the carrier gas from the capillary column is an advantage in GC-MS in which the mass-selective detector is directly coupled to the GC column and requires a very low flow through the system. In the last decade, mass spectrometry coupled with GC or high-performance liquid chromatography (HPLC) has become an important and powerful technique for the accurate analysis of extremely complex mixtures in which

compounds of unknown nature may co-elute with cholesterol. It provides high resolution along with positive identification of the individual components. Mass-selective detectors can yield sensitive and highly specific data and in combination with isotope-labelled internal standards provide highly accurate and precise measurements. Retention times in GC provide only tentative identification and the proof of structure is usually based on characteristic fragmentation patterns provided by MS. Isotope dilution mass spectrometry (IDMS) has been recognized as a reliable and highly accurate method for measuring cholesterol in serum [41,62,69,70] and in food matrices [71].

In isotope dilution (ID) analysis, isotopically labelled cholesterol ([3,4-$^{13}$C]cholesterol or cholesterol-d$_7$) is added to the sample to serve as an internal standard and after saponification, extraction and formation of TMS ethers, the ion intensity ratio of the molecular ions are measured by GC–MS. Gambert *et al.* [69] evaluated the accuracy of the GC method in comparison with isotope dilution mass fragmentation (IDMF) in the chemical ionization mode and found the correlation coefficient between the two methods to be 0.997. Takatsu and Nishi [70] determined total cholesterol in serum by electron impact IDMS using HPLC to separate cholesterol. When they compared the results with the conventional GC–IDMS method [41], the mean values obtained by the two methods agreed within 1%. The suitability of MS detection for HPLC has recently been reviewed [72]. High precision (coefficient of variation <0.5%) can be achieved by GC–IDMS if close attention is paid to possible interferences and sources of analytical error [41,62].

The choice of internal standard and the step in the analytical procedure at which it is added to the sample are important for the determination of cholesterol. The internal standard should be added to the sample at the earliest possible step to compensate for losses which occur during extraction, transfers, filtration, evaporations and derivatization. A frequently used internal standard for cholesterol analysis is 5α-cholestane, a non-polar, saturated compound of similar chemical stucture which is easily extracted with non-polar solvents. As it does not have a hydroxyl group it elutes as a sharp peak well before cholesterol in GC. As in other GC analyses, to achieve accurate quantification, experi-

TABLE 3

GC CONDITIONS AND DERIVATIZATION PROCEDURES FOR CHOLESTEROL AND STEROL DETERMINATIONS

| Sample type | Derivative[a] | Internal standard | Column | Temperature (°C) | Ref. |
|---|---|---|---|---|---|
| *Cholesterol* | | | | | |
| Eggs | (1) TMS (2) None | 5α-Cholestane | (1) Packed OV-17 (2) Packed SP-2340 | (1) 250 (2) 160–180 | 19 |
| Serum | TMS (BSA) | [3,4-$^{13}$C]Cholesterol | 25 m × 0.31 mm I.D., 0.25 μm $d_f$ Ultra-1 | 295 | 62 |
| Mixed diets | TMS | Cholestanol | 25 m × 0.2 mm I.D., 0.11 μm $d_f$ 5% phenyl methyl silicone | 260 | 9 |
| Food | TMS (DMF + BSTFA) | External standard | 15 m × 0.32 mm I.D., 1.0 μm $d_f$ DB-5 | 260 | 27 |
| Food | None | Cholesteryl *n*-butyrate | Packed 5% SE-52 | 240 | 20 |
| Food | TMS | External standard | Packed 0.5% Apiezon L | | 13 |
| Food | None | 5α-Cholestane | Packed 5% SE-30 | 260 | 21 |
| Milk | TMS (BSTFA + TMCS) | 5α-Cholestane | 30 m × 0.25 mm I.D., 0.25 μm $d_f$ DB-1 | 245–285 | 33 |
| Meat | TMS (HMDS + TMCS) | 5α-Cholestane | 25 m × 0.32 mm I.D., DB-5 | 190–260 | 30 |
| Food | TMS (HMDS + TMCS) | 5α-Cholestane | Packed 0.5% Apiezon L | 235 | 28 |
| Eggs | TMS (HMDS + TMCS) | 5α-Cholestane | Packed 3% SP-2401 | 230 | 32 |
| Egg products | None | 5α-Cholestane | 15 m × 0.25 mm I.D., 0.1 μm $d_f$ DB-52 | 275 | 29 |
| Egg yolk | None | 5α-Cholestane | 30 m × 0.25 mm I.D., 25 μm $d_f$ SE-30 | 70–300 | 23 |
| Coconut oil | TMS (BSA) | Isotopically labelled cholesterol | Packed 1.5% OV-101 | 230 | 71 |
| Serum | TMS (BSA) | [3,4-$^{13}$C]Cholesterol | 50 m × 0.3 mm I.D., SE-54 | 280 | 70 |
| Serum | TMS (BSA) | Cholesterol-d$_7$ | Packed 1.5% OV-101 | 250–265 | 41 |
| Serum | TMS (BSTFA + TMCS) | (1) Epicoprostanol (2) 5α-Cholestane (3) [3,4-$^{13}$C]Cholesterol | 25 m × 0.22 mm I.D., SE-30 | 250–285 | 69 |
| *Sterols* | | | | | |
| Food | Butyryl esters | 5α-Cholestane C$_{28}$ hydrocarbon C$_{30}$ hydrocarbon C$_{32}$ hydrocarbon | Packed 1% SE-30 | 250–260 | 14 |
| Food | (1) None (2) TMS | 5α-Cholestane | Packed 3% OV-17 | 230 | 31 |
| Fats and oils | TMS (BSTFA + TMCS) | 5,7-Dimethyltocol | 50 m × 0.25 mm I.D. Dexsil 400 | 260 | 42 |
| Soybean hulls | TMS (Sylon BTZ) | 5α-Cholestane | 12 m × 0.25 mm I.D., 0.1 μm $d_f$ DB-1 | 100–265 | 117 |
| Serum and dietary supplements | None | 5α-Cholestane | 10 m × 0.26 mm I.D., CP-Sil 8 | 220–245 | 65 |
| Serum | TMS (BSTFA) | Epicoprostanol | 25 m × 0.3 mm I.D., OV-1 | 240 | 74 |

[a] TMS = Trimethylsilyl; BSA = N,O-bis(trimethylsilyl)acetamide; BSTFA = N,O-bis(trimethylsilyl)trifluoracetamide; DMF = N,N-dimethylformamide; HMDS = hexamethyldisilazane; TMCS = trimethylchlorosilane.

mentally derived response factors are used to compensate for the varying response of the flame ionization detector to different compounds and losses resulting from other manipulations. Reference standards of a known amount of internal standard and cholesterol, preferably a minimum of three varying concentrations bracketing the sample, should be carried through the entire procedure to determine the relative response factor for quantification and to establish linearity of response. By analysing the reference standards regularly the entire analytical process can be monitored to evaluate the errors introduced by differences in reagents and variations in GC performance resulting from irreversible adsorption on the column, accumulation of contaminants in the injector and leaks. Use of procedures which omit internal standardization should be strongly discouraged as there is no common control over each individual sample throughout the procedure. Other compounds suggested as internal standards include 5,7-dimethyltocol [43], cholesteryl n-butyrate [20], cholestanol [9] and betulin [43,75]. Epicoprostanol (a sterol differing from cholesterol only in the lack of a double bond) has been used as an internal standard in the GC determination of cholesterol in serum [69,74]. Its suitability as an internal standard was tested [74] by determining relative recovery of epicoprostanol and cholesterol after saponification, extraction and derivatization steps and was found to be not significantly different from that obtained by direct derivatization of a cholesterol–epicoprostanol mixture.

## 3.2. Liquid chromatography

Although HPLC has become a very useful analytical tool in lipid analysis in general [24,75] and can offer a non-destructive alternative to GC techniques, its specific application to cholesterol determination has been limited because cholesterol does not have a strong absorption peak in the UV region. Cholesterol and related sterols do, however, have an unsaturation centre and a functional group that absorbs in the range 203–214 nm [46] with a maximum at 205 nm for cholesterol [59]. HPLC offers the advantage that many separations can be achieved at ambient temperature and the separated compounds can be recovered from the mobile phase for further analysis by complementary techniques such as GC [51] and MS [70].

Because cholesterol and cholesterol esters may play an important role in the diagnosis of certain diseases, their concentrations need to be determined separately. Many HPLC methods have been published for the determination of free cholesterol, individual cholesterol esters and cholesterol metabolites [76,77] in plasma [78,79] and other biological samples [80,81]. Normal-phase columns have been used to separate triglycerides, diglycerides, sterols, free fatty acids and monoglycerides after removal of phospholipids by column chromatography but reversed-phase separations have generally been preferred in the determination of cholesterol. Vercaemst et al. [82] achieved good HPLC separations of free cholesterol and cholesterol esters from both macrophage cells and human low-density lipoproteins by isocratic elution with acetonitrile–isopropanol (50:50, v/v) on a reversed-phase column (Zorbax ODS) with detection at 210 nm. Lipids were extracted with hexane–isopropanol (3:2, v/v) and cholesteryl heptadecanoate was used as an internal standard. Similar separations were obtained [83] using a linear gradient of water (3 to 0%) in acetonitrile–tetrahydrofuran (65:35, v/v) and UV detection at 213 nm. Earlier reports on the application of reversed-phase HPLC to the determination of free and total cholesterol in serum [79] after isopropanol extraction of lipids resulted in poor separation of cholesterol and cholesterol esters when triglycerides were present. Newkirk and Sheppard [84] also observed that triglycerides, which comprise most of the total lipids in food, interfere with cholesterol detection and quantification and recommended the removal of the fatty acids from initial lipid extracts by saponification as a critical step in the HPLC determination of cholesterol.

To improve the measurement of total cholesterol in foodstuffs, Newkirk and Sheppard [84] converted cholesterol to its benzoate ester by reaction of the non-saponifiable fraction of the lipid extract with benzoyl chloride in pyridine. Amounts as low as 10 ng of cholesteryl benzoate were detected when using a reversed-phase column (μBondapak ODS) with a methanol mobile phase and UV detection at 230 nm. A variety of foods were analysed and the levels determined compared favourably with those obtained by GC [14]. Goh et al. [80] reported a sensitive method for desmosterol, 7-dehydrocholesterol and cholesterol in biological material by oxida-

tive conversion of the sterols with cholesterol oxidase into their conjugated enone forms followed by HPLC determination. Even higher sensitivities (2 pg for cholesterol and 3 pg for cholestanol) were obtained for cholesterol and cholestanol in plasma by converting them into the corresponding fluorescent carbamate esters prior to analysis. Following derivatization with 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide in benzene, the derivatives were separated on a reversed-phase $C_8$ column with acetonitrile–methanol–water (81:9:10, v/v/v) as eluent and detected spectrofluorimetrically with excitation and emission wavelengths of 360 nm and 440 nm, respectively [78].

The total cholesterol content has also been determined in the non-saponifiable lipid fraction of milk [46] and egg noodles [45] by HPLC. After clean-up by use of Sep-Pak silica gel to remove interfering substances, HPLC of total cholesterol was completed using a reversed-phase column (Alltech ODS), a hexane–isopropanol (99.9:0.1, v/v) mobile phase and detection at 205 nm. Beyer and Jensen [8] used HPLC to separate cholesterol from other compounds in the unsaponifiable matter in egg yolk and showed that 17.5% of the total cholesterol content as determined by the spectrophotometric method of Zlatkis *et al.* [5] was due to the presence of non-cholesterol compounds.

Foods may contain a variety of ingredients including plant and vegetable oils that contain phytosterols and tocopherols. The presence of these compounds may interfere with the quantitative separation of cholesterol. Holen [85] investigated the influence of various mobile phases and different column temperatures with reversed-phase $C_8$ and $C_{18}$ columns on the separation of eight structurally closely related sterols, desmosterol, ergosterol, brassicasterol, fucosterol, cholesterol, stigmasterol, campesterol and sitosterol. The optimum column temperature was found to be 30°C and elution with methanol–water (99:1, v/v) gave a superior separation *versus* acetonitrile–water (100:0 to 95:5). Although the $C_{18}$ column was found to be superior to the $C_8$ column for separation of cholesterol from most other sterols, the separation of cholesterol and fucosterol was poor and sensitivity was low (0.4 $\mu$g of cholesterol).

To detect cholesterol, phytosterols and tocopherols simultaneously in food, Indyk [86] used UV

detection at 212 nm to monitor sterols and fluorescence detection (excitation at 295 nm, emission at 330 nm) in series to measure tocopherols. Using a reversed-phase $C_{18}$ column (Rad-Pak) and a methanol mobile phase, cholesterol and the main phytosterols (stigmasterol, campesterol and sitosterol) were resolved (although not to the baseline) and well separated from isomers of tocopherol. With a hexane–isopropanol (99.9:0.1, v/v) mobile phase, phytosterols were eluted as a single peak before cholesterol. Although a satisfactory separation can be obtained at ambient temperature, as above, temperature and polarity of the solvent may be used to improve separations between structurally similar sterols [59]. For example, it was noted that cholesterol and lanosterol did not separate on a reversed-phase $C_{18}$ column at 25°C but when the temperature was increased to 40°C partial separation was achieved.

Products of cholesterol oxidation have been found in food and, because they have been implicated in adverse human health effects [87,88] such as cytotoxicity, mutagenicity, carcinogenicity and angiotoxicity, many papers have been published on this subject in the last decade. Various amounts of cholesterol oxidation products have been found in cholesterol-containing food products that have been processed or stored under oxidizing conditions, including egg yolk powder [89–93], pork muscle [94], heated lard [95], tallow [96], butter, cookies and cakes [97] and infant formulas [98]. The level of oxidation products relative to the cholesterol concentration is very low. For example, in egg yolk powder the level of total cholesterol oxides ranged from undetectable to 311 ppm of total lipids [99] and from 3.6 to 6.2 ppm dry mass in mixed diets [100].

Many techniques have been used to separate and determine cholesterol oxidation products. They involve isolation and prefractionation steps on the extracted lipids using TLC [90,101], silicic acid column chromatography [64,100], semi-preparative HPLC [25,89,99] or saponification [90,95,97]. Quantification has been achieved by HPLC with normal-phase columns using UV detection and various mobile phases [91,92,94,101], capillary GC of TMS derivatives [64,93,97,100,102] or GC–MS [89,93,97, 100].

### 3.3. Other chromatographic methods

### 3.3.1. Complementary liquid column chromatographic procedures

Animal and plant tissues contain complex lipid mixtures of both polar and non-polar nature. The application of chromatographic techniques to the separation of lipids has been comprehensively reviewed [24,103–107]. Preliminary fractionation into polar and non-polar lipid classes is normally done by adsorption column chromatography. The total lipid extract is applied to a short column of silica gel or commercially available prepacked cartridges (e.g., Sep-Pak or Bond Elut) and neutral lipids are eluted with chloroform and polar lipids with methanol [24]. Individual lipid classes can be eluted from these columns with increasing concentration of diethyl ether in hexane. Typically, cholesterol esters would be eluted with 2% diethyl ether in hexane followed by triglycerides with 5%, diglycerides and cholesterol with 15% and monoglycerides with 100% diethyl ether, and phospholipids can then be eluted with methanol [24].

Another approach to the separation of neutral and polar lipids is by the dry column method [26], in which simultaneous lipid extraction and separation of neutral and polar lipid classes are accomplished by sequential extractions from a dry column. In this procedure samples of meat or meat products are ground with anhydrous sodium sulphate and blended with Celite 545 and packed into a glass column on top of the trapping material, which is calcium hydrogenphosphate–Celite 545 (1:9). Neutral lipids are then extracted with dichloromethane followed by dichloromethane–methanol (9:1) to elute the polar lipid fraction.

### 3.3.2. Thin-layer chromatography

TLC is a relatively rapid and powerful method for the separation of individual lipid classes from a total lipid extract, but it is rarely used for quantitative purposes. Cholesterol is present in food as both free cholesterol and its esters and can be separated by TLC to allow individual determination by other chromatographic means. Most lipid class separations are carried out using silica gel G adsorbent layers and hexane–diethyl ether–formic acid (80:20:2, v/v/v) as the solvent system [106]. This results in the separation of cholesterol esters at the

solvent front followed by triglycerides, free fatty acids, free cholesterol, diglycerides, monoglycerides and phospholipids at the origin. Bands are detected and eluted with chloroform–methanol (2:1, v/v) [103] for further quantitative analysis.

TLC or liquid column chromatography has also been used for sample clean-up after saponification and before GC [27,43] or HPLC [45,46,98] in the determination of cholesterol. Kaneda et al. [21], however, found that the procedures used to remove impurities (TLC, column chromatography and precipitation with digitonin) from the unsaponifiable fraction decreased the cholesterol recoveries and should be omitted. Tsui [33] used $C_{18}$ Bond Elut cartridges to isolate cholesterol after saponification instead of solvent extraction.

Other applications of TLC in cholesterol determination include the analysis of intact steryl esters by HPLC after TLC of the Folch extract and the analysis of TMS ethers of cholesterol and cholesterol oxides by capillary GC after their separation on TLC plates from other unsaponifiable matter.

### 3.3.3. Thin-layer chromatography with flame ionization detection

A combination of TLC and FID in the Iatroscan analyser has provided a means for the determination of separated lipid components. This technique has found widespread application in a variety of fields and is particularly suited for the analysis of fats and oils. The TLC–FID system, developed in the early 1970s, has undergone many improvements, including new Chromarods, a new collector, an improved semi-automatic sample spotter and data acquisition [108]. These have resulted in improved quantification, linearity of response and sensitivity [109]. Although it has the advantages of high sample throughput, minimum sample preparation and can determine both free and esterified cholesterol, its application to the determination of cholesterol in various food matrices is limited owing to a lack of resolution between cholesterol and other sterols which are similar in molecular structure. In foods where cholesterol makes up >98% of total sterols or where only total sterols are of interest, TLC–FID was reported to give results that were not significantly different from those obtained by GC [110]. Lipids were extracted from sea food samples, saponified and the non-saponifiable matter extracted with

hexane. Although both internal and external standard methods gave linear calibration graphs for amounts of cholesterol in the range 1–20 $\mu$g applied to the Chromarods, the use of an internal standard was preferred for accurate determination because it minimized the effect of rod-to-rod variation. Comparison between TLC–FID and GC techniques for the determination of total cholesterol, fatty acids and plasmalogens gave very similar data but the reproducibility of the TLC–FID system was lower [111]. The inter-rod reproducibility was found to be too large, requiring determination of a response factor for each lipid component on individual Chromarods, and thus to improve the accuracy and precision each rod was considered as an individual analytical unit. Some workers [112] have reported similar results for the determination of lipid classes by TLC–FID and TLC followed by GC, but others [113] have found the accuracy and reproducibility of TLC–FID to be unacceptable. In applying TLC–FID to the determination of cholesterol in lipid extracts from chicken plasma, we found that the relative response factor varied with the concentration of cholesterol in the standard mixture, which created problems for accurate quantification. Reliable quantification can only be achieved if a suitable internal standard is used and peak areas are corrected using proper response factors. Response factors can be affected by several variables, including sample size, speed of scanning and flow-rate of hydrogen to the detector [114].

A variety of solvent systems can be used for the development of the Chromarods, depending on the specific application, but that most commonly used to separate total lipids into lipid classes is hexane–diethyl ether–acetic acid (or formic acid) (80:20:1, v/v/v). We have found that benzene–chloroform–acetic acid–methanol (50:10:0.3:0.2, v/v/v/v) is also a good solvent system for resolving these lipid classes. A good reference text on TLC–FID principles and applications has recently been published [114].

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