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Gas chromatographic determination of cholesterol in processed foods

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A precise and accurate gas chromatographic method for the determination of cholesterol in processed foods, without prior total lipid extraction, is described. The accuracy and precision of the method was assessed by using a BCR reference material (anhydrous butter fat). Furthermore, the procedure was compared with an enzymatic assay. Results of the two methods compared favourably, except for foods containing both animal and vegetable fats. The relative standard deviation of the proposed method was 1·78% at an analyte level of 267·8 mg 100 g^{-1} (anhydrous butter fat) and 2·46% at an analyte level of 59·1 mg 100 g^{-1} (Frankfurter sausages, wet weight).

INTRODUCTION

Since the advent of the 'lipid hypothesis' the intake of dietary cholesterol has been linked with the incidence of coronary heart disease (National Diet–Heart Study Research Group, 1968). Although the validity of this hypothesis is widely debated, there is a general need for accurate data concerning the cholesterol content of various foods. The classical methods for the determination of dietary cholesterol are based on the Liebermann–Burchard colour reaction or the Zlatkis procedure (Zak, 1977). Another commonly used method is the enzymatic determination of cholesterol by means of cholesterol oxidase. These methods are not strictly specific and are therefore subject to errors due to interfering substances. To overcome this disadvantage, chromatographic techniques are now preferred for dietary cholesterol analysis.

Theoretically, a protocol for a chromatographic cholesterol assay would include the following steps: (1) extraction of total lipids with an organic solvent or solvent mixture; (2) removal of the solvent; (3) alkaline saponification of the total lipids; (4) extraction of the unsaponfiables with an organic solvent; (5) removal of the solvent; (6) possibly the formation of derivatives of the unsaponifiable matter; and (7) chromatographic estimation of the analyte. These sample pretreatment steps are time-consuming and limit, in consequence, the sample through-put. Tsui (1989) presented a method for the determination of cholesterol in homogenized milk, in which the various extraction steps were eliminated by using solid phase extraction cartridges packed with C18 material. Direct saponification of the food sample followed by extraction of the alkaline media with an organic solvent and subsequent determination of cholesterol by means of gas chromatography (GC) has also been proposed (Schulte, 1988). Unfortunately, in this report only egg-containing foods were examined and the method validation procedure was not given in detail. A similar method was used by Kovacs *et al.* (1979) for the analysis of fish-based food products.

This paper describes and evaluates a rapid method for the determination of cholesterol in selected processed foods, obviating the time-consuming total lipid extraction step. The performance of this method was tested by using a BCR reference material and by comparison with an enzymatic determination.

EXPERIMENTAL

Food samples

Food samples were obtained from local supermarkets. Noodles were ground with a coffee mill to pass a 0·25 mm sieve. Frozen dressed fish sticks and frozen red cabbage were thawed in a household microwave oven (Siemens) and homogenized with an Osterizer. Sausages and mayonnaise were also homogenized. Egg yolk was separated from the egg white and stirred with a glass rod. Emmental cheese was ground in a mortar. The BCR

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reference material CRM 164 (anhydrous milk fat) was purchased from the Community Bureau of Reference (Brussels, Belgium).

Chemicals

Cholesterol and cholestane (both >99% purity) were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade and purchased from E. Merck, Darmstadt, Germany.

Lipid extraction

Total lipids were extracted with chloroform/methanol by a modified Bligh & Dyer technique as described by Daugherty and Lento (1983).

Direct saponification/extraction of food samples (DSE method)

A sample of extracted fat (100 mg) or fatty food (300–500 mg wet weight) was weighed in a Pyrex test tube (22 mm × 200 mm) fitted with a PTFE lined screw cap. Ethanol (8 ml) containing 0·125% (w/v) butylated hydroxy anisole (BHA), internal standard solution (0·25 ml, 200 μg cholestane ml^{-1} *n*-hexane) and aqueous KOH (0·5 ml, 400 g l^{-1}) were added to the sample. In order to remove oxygen from the head space, the test tubes were flushed with nitrogen and capped tightly. The samples were saponified by heating in a water bath, at 80°C for 15 min. Tubes were removed from the bath and shaken after 1, 2 and 4 min. The saponified solution was cooled under tap water and cyclohexane (15 ml), followed by distilled water (12 ml), were added. The tubes were vortexed for 1 min and centrifuged for 5 min at 360*g*. The upper phase was removed with a pipette and the cyclohexane extraction repeated. The combined extracts were concentrated with a rotavapor (RE 121 Büchi Laboratoriums-Technik AG, Flawil, Switzerland) to a few millilitres, transferred into a test tube (16 × 100 mm) and dried in a stream of nitrogen. The residue was redissolved in *n*-hexane (0·25 ml) and 1 μl taken for GC.

Gas chromatography (GC)

A Carlo Erba MEGA 5160 gas chromatograph (Carlo Erba Strumentazione SpA, Milano, Italy) equipped with a split/splitless injector, a flame ionization detector and a fused silica capillary column (30 m × 0·25 mm i.d.) coated with DB-5, film thickness 0·25 μm (J & W Scientific, Folsom, CA, USA) was used. Chromatograms were recorded with an SP 4270 computing integrator (Spectra Physics, San Jose, CA, USA). Hydrogen was the carrier gas and delivered to the column at a head pressure of 0·5 bar. The split vent flow was 47 ml min^{-1}. The temperature of the column was held for 1 min at 260°C and programmed at a rate of 4°C min^{-1} to 300°C. Cholesterol was quantified by means of the internal standard (IS) cholestane.

Gas chromatography/mass spectrometry (GC/MS)

An ion trap detector ITD 800 (Finnigan MAT, San Jose, CA, USA) was connected via an open split interface to the above-mentioned GC system. EI mass spectra of the column effluent were obtained at an ionization energy of 70 eV. The ITD was operated from 50 to 500 amu with one scan per second. Data were acquired and processed with an IBM AT compatible computer using the ITDS software. Cholesterol was identified by matching the unknown spectra with those of an authentic standard.

Enzymatic cholesterol determination

A commercial test kit for cholesterol analysis (Kit No. 139 050, Boehringer Mannheim GmbH, Mannheim, Germany) was used. The working instructions given by the manufacturer for the analysis of liver sausages were applied to all food samples tested.

Statistical analysis

Student's *t*-test, regression analysis and ANOVA were performed as described by Sachs (1984).

RESULTS

The GC separation of a cholestane/cholesterol standard solution (A), the unsaponifiable matter of a chloroform/methanol extract of Emmental cheese (B), and the DSE method applied to the same sample (C) is shown in Fig. 1. Identity of cholesterol was confirmed by matching the mass spectra of the substance leading to a peak with retention time of 7·56 min in (B) and (C) to an authentic cholesterol standard sample.

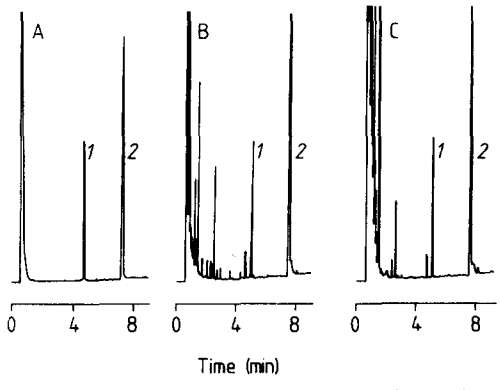

Fig. 1. GC profiles of a cholestane/cholesterol standard solution (A), unsaponifiable matter of Emmental cheese with prior total lipid extraction (B) and without prior total lipid extraction (C). 1, Cholestane (IS); 2, cholesterol.

Table 1. Stability of the cholesterol calibration within a five-week period. The test solution consisted of 100 μg cholesterol and 50 μg cholestane 0·25 ml⁻¹ *n*-hexane and was prepared freshly before each measurement

Cholesterol (μg 0·25 ml⁻¹) at test period (weeks)				
1	2	3	4	5
97·9	101·9	102·7	103·2	98·3
98·8	100·9	100·2	102·7	99·9

For quantification purposes, cholesterol/cholestane mixtures in *n*-hexane were prepared and chromatographed. Test mixtures consisted of a fixed amount of cholestane (50 μg) and varying amounts of cholesterol (10–250 μg) made up to 0·25 ml with *n*-hexane. Peak area ratios of cholesterol/cholestane were plotted against the corresponding cholesterol mass. A straight line was fitted to the data points by using regression analysis. It intercepted the ordinate (area ratios) at −0·0031; the slope of the line was 0·0171 ± 0·0007, and the correlation coefficient 0·995. This calibration function was used for the quantification of cholesterol in subsequent experiments.

To test the long-term stability of the method, solutions containing 50 μg cholestane and 100 μg cholesterol in 0·25 ml *n*-hexane were prepared freshly once a week, the solvent evaporated and carried through the complete DSE procedure. As can be seen from the results summarized in Table 1, the values found were very close to the known composition. Therefore, a frequent recalibration of the procedure was not necessary.

Recovery studies of cholesterol added to vegetable oils yielded quantitative recovery of the analyte. From sunflower oil fortified at a level of 50 mg cholesterol 100 g⁻¹, 102·7 ± 1·8% (n = 3) were recovered. Similar values were found for soya oil (40 mg spike 100 g⁻¹, 100·8 ± 2·1% recovery). In Fig. 2, the GC recorder tracing of

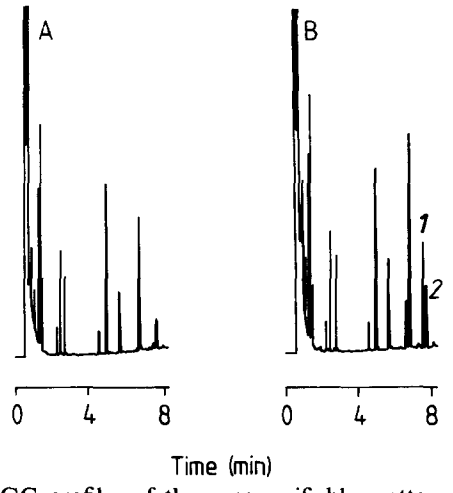

Fig. 2. GC profiles of the unsaponifiable matter of an unfortified soya oil (A) and a soya oil spiked with α-tocopherol and cholesterol (B). 1, Cholesterol; 2, α-tocopherol.

Table 2. Precision and accuracy of the proposed method tested by repeated work-up of the BCR reference material (anhydrous butter fat)

	Cholesterol (mg 100 g⁻¹)		
	Subsample 1	Subsample 2	Subsample 3
Repetitions	263·9	269·2	264·4
	264·2	264·9	265·1
	261·3	271·6	266·6
Mean value	263·1	268·6	266·6
Overall mean	265·7		

the unsaponifiables of both the spiked and the native soya oil are shown. α-Tocopherol eluted close to cholesterol. Nevertheless, in this case the peaks showed base-line separation. Foods of animal origin contain comparatively low levels of α-tocopherol which may explain the small shoulder on the cholesterol peak in the chromatogram shown in Fig. 1.

Precision and accuracy of the method were checked by analysing the CRM 164 sample (anhydrous butter fat). A cholesterol value of 273 ± 39 mg 100 g⁻¹ is indicated for this material. The results of three independent determinations and three replicate injections per subsample are listed in Table 2. Analysis of variance of these data resulted in a within-run standard deviation (SD) of 2·26 mg cholesterol 100 g⁻¹ fat and a between-run SD of 4·73 mg 100 g⁻¹. The overall mean value of 265·7 mg cholesterol 100 g⁻¹ did not differ significantly from the quoted value ($P > 0·05$).

A similar evaluation scheme was applied to test the precision of the method for the case of a multicomponent food. Six subsamples of homogenized Frankfurter sausages were analysed by the DSE method. Statistical analysis showed a between-run SD of 2·10 mg cholesterol 100 g⁻¹ sample (wet weight) and a within-run SD of 1·04 mg 100 g⁻¹. Although the relative SD of the overall mean value (x = 59·1) was only 2·46%, the F-value was significant at $P = 0·05$ but not significant at $P = 0·025$; this means that small inhomogeneities of the sample material may influence the performance of the proposed method.

The accuracy of the results obtained by the DSE method was estimated by comparison with the estimates of an independent method, i.e. the enzymatic determination of cholesterol. With the enzymatic method a cholesterol value of 58·3 ± 3·10 mg 100 g⁻¹ (n = 4) Frankfurter sausage (wet weight) was found. No statistically significant difference was observed between the results of the two methods (t-test, $P > 0·05$). Cholesterol data for a variety of processed foods and egg yolk as obtained by the DSE method, the enzymatic method and the conventional method (lipid extraction with chloroform/methanol followed by saponification) are provided in Table 3. The cholesterol values of most of the food samples are in close agreement, irrespective of

Table 3. Cholesterol content (mg 100 g^{-1} wet weight) in various foods, as obtained by three methods (direct saponification without fat extraction, DSE; fat extraction followed by saponification, FES; enzymatic method, EM). Samples were analysed in duplicates at least

	Cholesterol (mg 100 g^{-1})		
	DSE	FES	EM
Canned liver sausage	117	112	110
Mayonnaise	113	nd[a]	322[b]
	125	nd	348[b]
Frankfurter sausage	49	52	53
	59	61	58
Sausage	96	nd	96
Noodles	70	70	98[b]
Dressed fish sticks	36	43	70[b]
Emmental cheese	89	93	85
Dried soup	33	34	58[b]
Red cabbage meal	27	30	31

[a] Not determined.
[b] Phytosterols detected by GC.

the analysis method used. Samples including vegetable oils (mayonnaise, noodles and dressed fish sticks) showed higher cholesterol values with the enzymatic test than with the DSE method or the conventional method, due to the interference of phytosterols.

DISCUSSION

The debate on the nutritional significance of dietary cholesterol has directed efforts to nationwide food monitoring programmes to establish data on the cholesterol content of various foods. As a prerequisite for these surveys an efficient technique for cholesterol analysis is needed. Moreover, the method should be accurate, precise and cost-effective. The determination of cholesterol by GC after different sample preparation steps, as outlined in the AOAC method (AOAC, 1984) for multicomponent foods, is widely employed for this purpose. The method used in this study is a modification of the protocol elaborated by Slover *et al.* (1983) for the determination of tocopherols and sterols in fatty oils, with the exception that the whole wet food sample is taken for analysis. Furthermore, cholesterol was chromatographed without forming silyl ethers. Omitting the derivatization step had no deleterious effect on the peak shape because of the inert fused silica column used. Nevertheless, a small shoulder on the cholesterol peak appeared during the separation of the unsaponifiables originating from animal foods. This insignificant disturbance was attributed to the presence of traces of α-tocopherol. With solute pairs, which differ greatly in concentration, it is always difficult to achieve complete resolution (Jennings, 1980). Silylation of the unsaponifiable matter changed the elution order (α-tocopherol emerged before cholesterol), but did not improve the resolution of the two substances (data not shown). In contrast, cholesterol in vegetable oils was clearly separated from α-tocopherol (Fig. 2). A closer inspection of the chromatograms provided by Slover *et al.* (1983) also revealed a small shoulder on the α-tocopherol peak of some samples, which might be due to interfering cholesterol. Cholesterol was neither identified nor quantified in that article, although it is known that almost any vegetable oil contains small but significant amounts of cholesterol (Seher, 1986). However, the imperfect resolution of cholesterol in the free OH form had virtually no negative effect on the quantification of cholesterol, as shown by analysis of the certified BCR butter fat (Table 2) as well as the recovery experiments with spiked vegetable oils. Several conclusions may be drawn from the proven accuracy of the method. First, the saponification of cholesterol esters in the butter fat was complete. Secondly, cholesterol oxidation was effectively prevented by the BHA present in the saponification media and by nitrogen flushing of the head space. Thirdly, the formation of soap micelles did not hinder the quantitative extraction of the analyte and the IS.

Several variations of the saponification step in the methodology for chromatographic cholesterol assays have been described in the literature. For example, Kovacs *et al.* (1979) and Tsui (1989) boiled the sample for 1 hour in the absence of an antioxidant, Schulte (1988) saponified the sample for 3 h without antioxidant protection in an oven at 120°C, Beyer and Jensen (1989) used 37°C for 1·5 h, Homberg (1987) 15 min under reflux conditions, and Slover *et al.* (1983) 8 min at 70°C. In light of our findings, 15 min at 80°C is a good compromise, which ensures that cholesterol esters are completely hydrolysed, and the time spent for saponification is minimised. The protective effect of BHA in the saponification media was not separately tested, but from the quantitative results of the recovery experiments with vegetable oils we conclude that this lipid-soluble antioxidant adequately prevented the formation of cholesterol oxidation products. Bohac *et al.* (1988) used a much higher concentration of BHT (12·5% in the media for saponification of meat lipids), and they stated that 0·53% BHT was not completely effective in obviating the formation of artefacts. These unknown substances increased the apparent cholesterol values of meat samples determined by a colorimetric assay. In the present study, cholesterol was chromatographically separated from possible by-products, thereby avoiding interferences during the quantitation process.

In general, the cholesterol values obtained by GC were equivalent, regardless of the different sample pretreatment steps. Neither a statistically significant difference between the quantitative results of the two methods tested nor additional substances, coeluting with the cholesterol peak, were observed. The enzymatic determination of sterols is not strictly specific for cholesterol.

Sterols with a 3β-OH group, including phytosterols, also react with the enzyme. Thus, the enzymatic method applied to foods containing both animal and vegetable fats overestimates the true cholesterol content (Table 3). The cholesterol concentration in foods of animal origin determined by the enzymatic method agreed favourably with the values estimated chromatographically. Summing up all peaks attributable to sterols in the chromatograms of multicomponent foods and converting this value to mass-% cholesterol did not yield the same result as the enzymatic method. In principle, the summing-up of cholesterol and other sterols, in order to obtain the total sterol content, is admissible because it is reasonable to assume that the response factors of phytosterols relative to cholesterol are close to unity (ISO, 1989). This discrepancy is probably caused by different reaction rates of individual sterols with the cholesterol oxidase (Homberg & Bielefeld, 1987).

The cholesterol assay presented fulfils the requirements of accuracy, precision, selectivity and speed of analysis and is thus well suited for routine use in food labelling studies. In particular, the selectivity of the method is a valuable advantage, since colorimetric or enzymatic methods may overestimate the true cholesterol content.

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