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Short Communication

Determination of sterol content in different food samples by capillary gas chromatography

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Abstract

An accurate method for the determination of sterols by capillary gas chromatography was developed and applied to the analysis of food. The procedure includes the following steps: dichloromethane–methanol (2:1, v/v) lipid extraction, saponification at 80°C and separation of the unsaponifiable matter with cyclohexane, derivatization to form trimethylsilyl ethers and gas chromatography using 5α-cholestane as the internal standard. The method shows good accuracy, precision and sensitivity and is suitable for the determination of sterols in food.

1. Introduction

The accurate determination of the sterol content in food is of great importance, not only because adulteration can thus be detected, but also owing to the increasing interest in the possible link between arteriosclerotic and coronary diseases and cholesterol [1,2]. A rapid method is therefore needed to assist in research towards low-cholesterol or cholesterol-free diets. Moreover, owing to the relationship with coronary heart disease, the food industry has been focusing on the possibility of developing new products with low cholesterol content. Therefore, it is necessary to develop a rapid and accurate method to determine sterols in food.

Many methods have been published for the determination of cholesterol, including spectrophotometric, enzymatic, gas chromatographic and high-performance liquid chromatographic [3] techniques. Spectrophotometric and enzymatic methods are not strictly specific and are therefore subject to errors due to interfering substances, and they may overestimate the cholesterol content [4,5]. Nowadays, gas chromatography is the preferred method. Several groups [6–9] followed a direct saponification method. Using gas chromatography, some workers have avoided derivatization by dissolving the residue obtained from the unsaponifiable extraction in *n*-hexane [10]. Tsui [11] developed a gas chromatographic method in which, after saponification, the isolation was carried out by solid-phase

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extraction on a non-polar adsorbent, such as C_{18} packed in disposable polypropylene tubes. Fenton and Sim [12] reported an on-column capillary gas chromatographic method, also avoiding lipid extraction and derivatization.

Little information is available in the literature on the cholesterol value of multi-component food products.

This paper describes a rapid method for the determination of the sterol content in prepared food by means of capillary gas chromatography. The method was used to establish the sterol content of the diet in an old people's home, so that the daily cholesterol consumption could be evaluated. The procedure includes lipid extraction, separation of the unsaponifiable fraction, derivatization to form trimethylsilyl ethers and gas chromatography.

2. Experimental

2.1. Reagents and chemicals

5α-Cholestane, cholesterol, squalene, stigmasterol, campesterol, β-sitosterol and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) were purchased from Sigma (St. Louis, MO, USA), 3-*tert.*-butyl-4-hydroxyanisole (BHA) from Fluka (Buchs, Switzerland), dried pyridine and *n*-hexane from Merck (Darnstadt, Germany), dichloromethane, methanol and anhydrous granulated Na_2SO_4 from Probus (Badalona, Spain), diethyl ether from SDS (Peypin, France) and cyclohexane and KOH pellets from Panreac (Barcelona, Spain).

2.2. Samples

The samples consisted of different cooked dishes on the menu in an old peoples' home. Each sample was analysed after homogenizing all the ingredients. The menu was representative of the mediterranean diet; vegetable oil was used for cooking and dressing the dishes. Some of the samples analysed were vegetable soup, hamburger with green salad, roast chicken and potatoes, fried fish, spaghetti bolognese, paella, turkey and eggs with tomato sauce.

2.3. Sample preparation and lipid extraction

Food samples were ground and homogenized individually. The samples were then thoroughly mixed with anhydrous granulated Na_2SO_4 to improve lipid extraction and kept at −20°C until further analysis.

First a quantitative fat determination of the sample was performed by means of acid hydrolysis followed by Soxhlet fat extraction.

The fat for the sterol determination was extracted using 225 ml of dichloromethane–methanol (2:1, v/v) with a magnetic stirrer, according to a modification of Folch *et al.*'s procedure [13]. Dichloromethane was chosen instead of chloroform owing to its lower toxicity and equal extraction capacity [14,15]. The extract was filtered into a separating funnel, 20 ml of distilled water were added and the funnel was shaken and left for the phases to separate. The organic phase was filtered through anhydrous granulated Na_2SO_4 into a dried flask and evaporated. The residue was dissolved in diethyl ether, in order to eliminate the non-lipidic substances retained by methanol, filtered and evaporated in a rotatory evaporator. Fat was kept in a dark vial. The vials were flushed with nitrogen, capped tightly and stored at −20°C until analysis.

2.4. Separation of the unsaponified fraction

The method proposed by Slover *et al.* [16] was used and modified as follows. About 100 mg of sample were placed in a 20 × 120 mm screw-capped test-tube (Trallero, Barcelona, Spain) and 8 ml of ethanolic BHA solution (115 mg of BHA in 100 ml of ethanol) and 0.25 ml of internal standard solution (200 μg/ml of 5-α-cholestane in *n*-hexane) were added, followed by 0.5 ml of 0.4 mg/ml KOH solution. The tubes were flushed with nitrogen and tightly closed. The samples were saponified by incubation in a water-bath at 80°C for 15 min with periodic shaking. The saponified solution was cooled

under tap water and 15 ml of cyclohexane were added, followed by 12 ml of distilled water. The tubes were shaken and centrifuged for 5 min at 360 *g*. The upper phase was carefully removed with a pipette and the cyclohexane extraction was repeated. The combined extracts were concentrated to a few millilitres in a rotatory evaporator and then transferred into a 10 × 100 mm screw-capped tube (Corning, New York, USA). The remaining solvent was removed using a stream of nitrogen. The sample was then ready for derivatization.

2.5. Derivatization

Sylon HTP (Supelco, Bellefonte, PA, USA) and BSTFA containing 1% of TMCS and dry pyridine were tested as derivatization reagents and the latter was chosen. Pure, dry pyridine (50 μl) and of BSTFA containing 1% of TMCS (50 μl) were added and the tubes were securely stoppered with PTFE-lined caps and mixed thoroughly. The samples were held at room temperature for at least 15 min before analysis.

2.6. Gas chromatography

A Shimadzu GC-14A gas chromatography, equipped with a flame ionization detector and a 30 m × 0.25 mm I.D. fused-silica capillary column coated with SPB-5 (Supelco) with a 0.25-μm film thickness was used under the following conditions: oven temperature, constant at 280°C; injector temperature, 265°C; helium carrier gas pressure, 15 p.s.i. (1 p.s.i. = 6894.76 Pa); helium flow-rate, 1 ml/min; air and hydrogen flow-rates, adjusted to give the maximum detector response; detector temperature, 290°C; injection volume, 0.3 μl; and injection, split mode with a splitting ratio of 1:100. A Shimadzu (Kyoto, Japan) C-R6A Chromatopac integrator was used.

3. Results and discussion

Many solvents were tested for their ability to extract the unsaponifiable matter. Cyclohexane was selected because it is less toxic, does not form peroxides as does diethyl ether and, above all, because it does not form stable emulsions during the procedure.

In the capillary gas chromatographic analysis of sterols, the derivatization process has the advantages of providing sharp peaks and improving their quantification. Two derivatizating reagents were tried. The first, Sylon HTP, gave turbidity and poor reproducibility. BSTFA containing 1% of TMCS with pyridine was tested. The latter gave high levels of all sterols and also better precision and recovery. Fig. 1 shows a typical chromatogram for a food sample. The resolution was satisfactory.

A plot of the ratio of peak area of cholesterol to that of 5α-cholestane *versus* amount of cholesterol (mg) gave a linear response in the range 0.05–0.5 mg of cholesterol ($r > 0.99$) (Fig. 2). Good linearity of the method for squalene and stigmasterol was also found, with $r > 0.99$ in both instances.

The precision of the method was tested by analysing ten aliquots of the same sample. The relative standard deviation for cholesterol, squalene and stigmasterol varied from 2.48 to 3.21%. This intra-laboratory precision is within the limits of acceptable variability in methods of analysis proposed of Horwitz [17] for analyte concentrations of the order of 0.2 mg/g sample.

The standard addition method was used to test the recovery of the analysis. Five levels of standard concentrations were added to a known mass of sample, and then they were carried through the entire procedure until the trimethylsilyl ethers of sterols were obtained, and then injected into the column. Recoveries between 100.63 and 80.33% were obtained. Details are given in Table 1.

The detection and quantification limits found were of the order of nanograms (Table 2). These limits show that the method has excellent sensitivity [18,19].

The method was applied for the analysis of 35 food samples, always with good results, and it may therefore be considered suitable for the routine determination of sterols in food. Moreover, it is rapid without the need to use separating funnels to obtain the unsaponifiable matter,

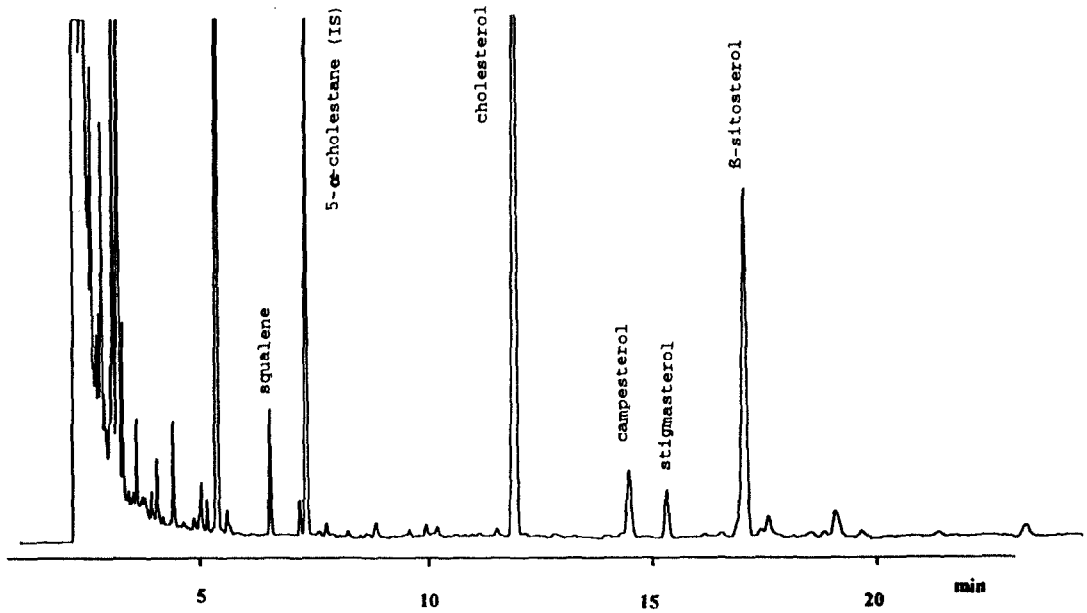

Fig. 1. Chromatogram of non-saponifiables isolated from a food sample (canned sardines with green salad).

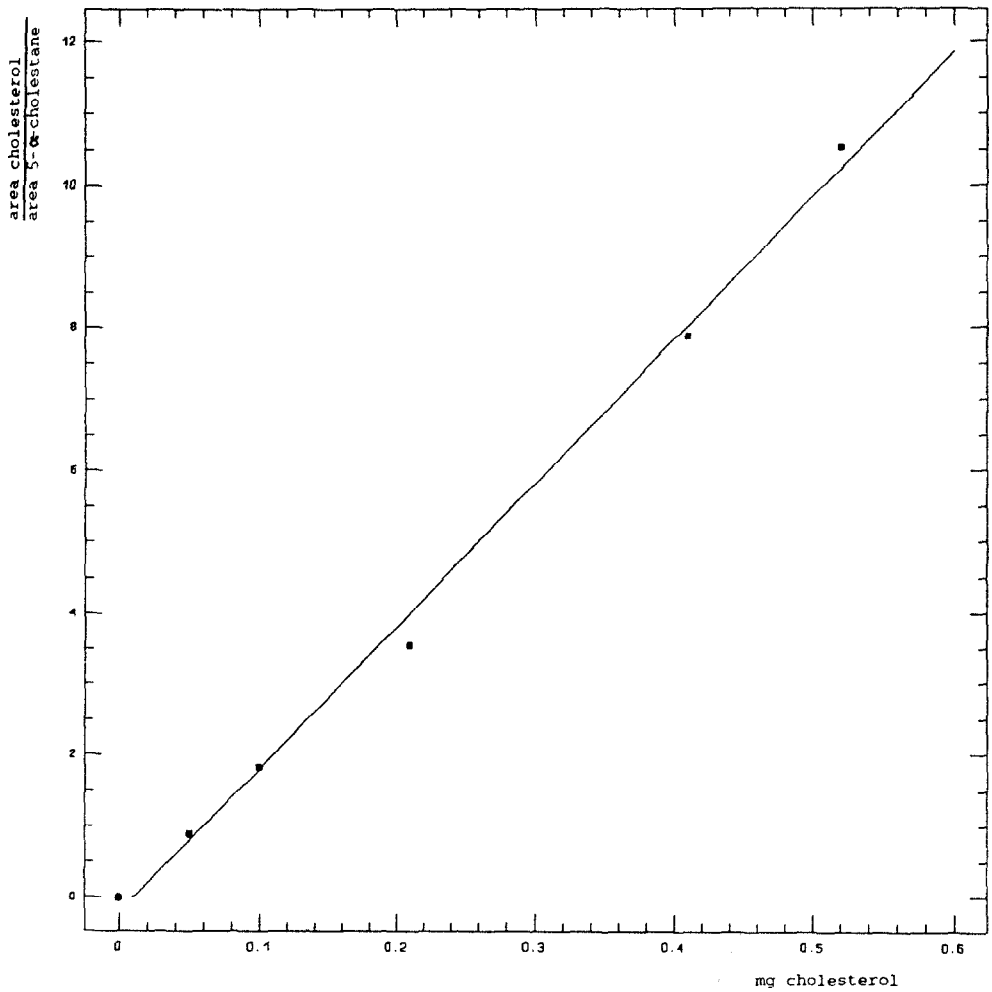

Fig. 2. Linearity of detector response for 0.05–0.5 mg of cholesterol: $y = 20.18x - 0.22$; $r > 0.99$.

Table 1
Recovery of the method

Cholesterol level (mg)	Recovery ($n = 2$) (%)	Squalene level (mg)	Recovery ($n = 2$) (%)	Stigmasterol level (mg)	Recovery ($n = 2$) (%)
1.1	95.65, 97.97	4.05	96.20, 96.95	1.67	99.18, 102.08
0.55	99.73, 100.27	2.02	94.62, 96.68	0.83	93.21, 93.75
0.22	93.04, 96.32	0.81	98.86, 100.12	0.33	86.26, 88.40
0.11	84.91, 87.27	0.40	94.31, 96.81	0.17	79.35, 81.31
0.02	92.41, 93.05	0.08	98.50, 98.78	0.03	93.10, 93.72
Mean	94.06 ± 5.2	Mean	97.18 ± 1.8	Mean	91.04 ± 7.6

Table 2
Limits of detection and quantification

Sterol	Detection limit[a] (ng)	Quantification limit[a] (ng)
Squalene	1.35	3.87
Cholesterol	1.08	3.09
Stigmasterol	1.12	3.18

[a] Signal-to-noise ratio = 3.
[b] Signal-to-noise ratio = 10.

Table 3
Sterol contents of some food samples (mg sterol per 100 g of sample)

Food	Squalene	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol
Fried fish	1.7	40.3	2.4	1.9	15.1
Spaghetti bolognese	0.6	5.4	1.8	0.8	9.2
Paella	0.4	7.2	0.8	0.7	4.0
Turkey	1.6	22.4	3.0	1.2	1.83
Eggs with tomato sauce	1.4	242.0	0.1	1.2	7.0

and also avoids the necessity for thin-layer chromatography [20]. Table 3 gives the results obtained with different samples.

4. Acknowledgements

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