Upsala J Med Sci 85: 35–44, 1980

Determination of the Sterol Composition of Diets used in Dietary Management of Hyperlipoproteinemia

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ABSTRACT

A simplified rapid quantitative method for determination of sterols in food is described. The lipids were extracted in chloroform-methanol and saponified. The unpolar components, containing the sterols, were extracted with petroleum-ether (PE)and prepared for the gas liquid chromatography (GLC). Progesterone (4-pregnene-3,20-dione) was used as an internal standard for the GLC analysis. The recovery of the procedure was studied by adding cholesterol-7-α-^{3}H before extracting the lipids. Average recovery of the method was 87.4 $\pm$ 8.5%. The analytical errors for determination of the sterol content of three different diets were 10, 20, 25 and 15% for cholesterol, campesterol, stigmasterol and β-sitosterol respectively.

INTRODUCTION

Dietary treatment of patients with hyperlipoproteinaemia (HLP) and cardiovascular disease is widely used. As both cholesterol content and plant sterols have been shown to have effects on lipoprotein metabolism a detailed knowledge of the content of such component is pertinent. Few data are available in the literature on the cholesterol content of the food components and no data at all on the more complete sterol composition.

In order to get more exact data on the cholesterol content and data on the content of the other sterols of the diets used in dietary treatment of HLP, the method described below was developed.

Quantitative methods to determinate the sterol content of food by means of GLC have been described earlier (2,8). As these are rather time-consuming involving both thin layer chromatography (TLC) and gas liquid chromatography (GLC) a modification of the earlier methods was developed. The principal steps of the method are

1) Extraction of the lipids
2) Saponification
3) Reextraction of the nonpolar lipids

4) GLC and counting of the radioactivity

Three different diets have been studied used in metabolic ward studies. Each diet includes seven different menus, one for each day of the week. Three analyses have been performed of the food of each day.

CHEMICALS

Solvents. Petroleum ether (boiling point 40-60°C), methanol, heptane and chloroform were redistilled in all glass apparatus before use. Ethanol (96 % v/v) analytical grade was used without purification. 0.2 M NaH_2PO_4 was used for extraction and 1 M NaOH in 90% ethanol for saponification.

Isotopes. Cholesterol-7- α -^{3}H was a kind gift received from the Department of Physiological chemistry, University of Lund and was added to a carrier tripalmitin (5 mg to $2.5 \cdot 10^{-3}$ µC in 10 ml heptane).

Scintillation solution. Toluene containing 0.5 g POPOP and 5.0 g PPO per l from Packard instrument Company Inc was used as scintillation solution.

Reference standard. Sterol standards with high purity (cholesterol, campesterol, β-sitosterol and stigmasterol) and progesterone (4 pregnene-3.20-dione) were bought from Applied Science Laboratories Inc. The standards were dissolved in chloroform:heptane(1:1).The purity of the standards was tested on the GLC and was accepted since each sterol showed a single peak.

GENERAL PROCEDURE

Homogenization. The whole menu of one day was collected in a pre-weighed metal can. Sufficient amount of water was added to permit a fluid homogenate and the can was weighed again. The food was homogenized at 40°C for 2-3 min in a mixer (Stephan - 15 l, 3000 v/min). A sample of the homogenate was taken and kept in a freezer at -20°C until analysing.

Extraction of the lipids. The extraction of the lipids was carried out with methanol and chloroform (1) in the following way: 100 µl cholesterol-7-α-^{3}H with a radioactivity around 20 000 counts per minute (CPM) was added to exact 10 g of the homogenate estimated to contain 0.5 - 5 mg of total sterols in a retort. 50 ml (not critical) of methanol was added while the retort was swirled. Exactly 100 ml of chloroform was added and followed by addition of 150 ml 0.2 M sodium dihydrogenphosphate (NaH_2PO_4). The extract was left until the next day to separate into two phases. Exactly 50 ml of the chloroform was pipetted into a retort for rotary evaporation into dryness.

Saponification. 20 ml 1 M NaOH in 90% ethanol was added to the dry lipid extract. After addition of a few boiling drips the content of the retort was refluxed for 1 h.

Extraction of the sterols. The alkaline extract was quantitatively transferred into a separator funnel with 10 ml of water (1 ml + 3 x 3 ml portions) and with 50 ml petroleum ether (PE) (5 x 10 ml portions). The funnel was shaked carefully and the PE phase was collected. Another two extractions with 50 ml PE wereperformed. 3 ml progesterone solution with the concentration 0.1 mg progesterone per ml PE were added to the collected PE. The PE-phase was evaporated into dryness in a rotary evaporator.

The yellow extract was then dissolved in chloroform and quantitatively brought into a test tube. The chloroform phase was evaporated and the extract was then dissolved in exactly 3 ml chloroform: heptane (1:1).

The addition of cholesterol-7-α-^{3}H to the food homogenate permitted determination of the recovery at the end of the entire procedure and after each step of the procedure, where losses might be suspected. 1 ml of the chloroform phase of the first lipid extraction, 1 ml of the PE-phase after the sterol extraction and 1 ml of the chloroform:heptanesolution at the end of the procedure were pipetted into plastic vials containing 10 ml scintillation solution. The radioactivity of the samples was counted within 24 hours in a Packard Tri-Carb Liquid scintillation spectrometer (Packard Instrument Co). Quenching and self absorption was corrected for by adding the internal standard cholesterol-7-α-^{3}H in heptanesolution with a radioactivity around 20 000 cpm (1).

All samples were counted twice for 10 min and after addition of the internal standard twice for 1 min.

The gas liquid chromatography analyses (see Fig. 1) An aliquot of the sample dissolved in chloroform and heptane (1:1) was injected in a Pye-Unicam 104 gas liquid chromatograph, equipped with a 1.5 M 3% SE column with the internal diameter 4 mm. The temperature of the oven was 240°C and detector temperature 270°C. Carrier gas was nitrogen at a flow rate of 30 ml/min. A flame ionization detector was used and worked together with hydrogen at the same flow rate. Air flow rate was between 450-500 ml/min. The sterols were separated isothermally. The different sterols and their retention times were calculated by a Vidar 6300 digital integrator. The peaks were identified according to retention time in relation to known standards. The amount of sterols was calculated from the known amount of progesterone added after the PE extraction of the analysis. The response factors of the different sterols in relation to progesterone were tested on the GLC according to studies by Blomhoff (3). Solutions with all the sterols and progesterone at different concentrations (50-300 µg/ml) were injected into the GLC column and the response factors were calculated.

Calculations. For calculation of the cholesterol content of the diets the Agricultural Handbook No 8, Composition of Foods (10), the paper by Feeley

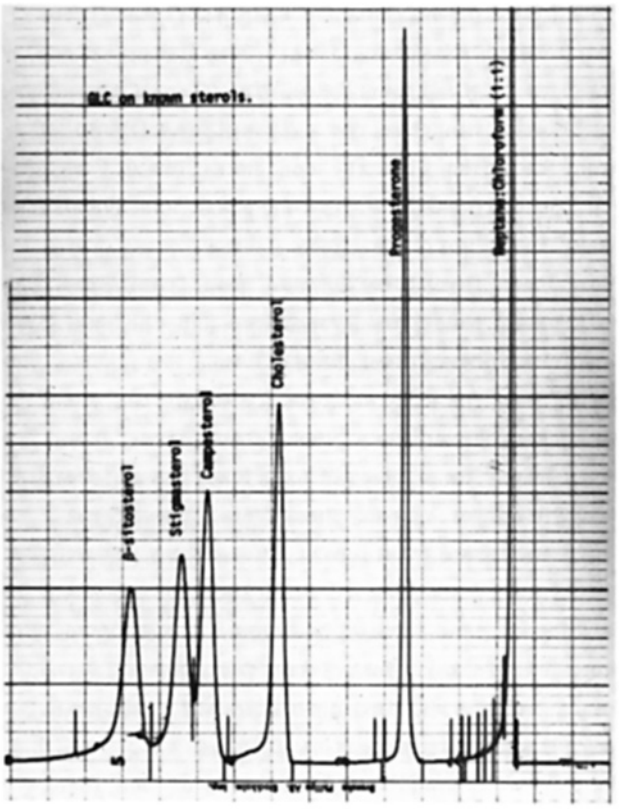

Fig. 1. Gas liquid chromatogram on a mixture of known sterols and progesterone dissolved in heptane:chloroform.

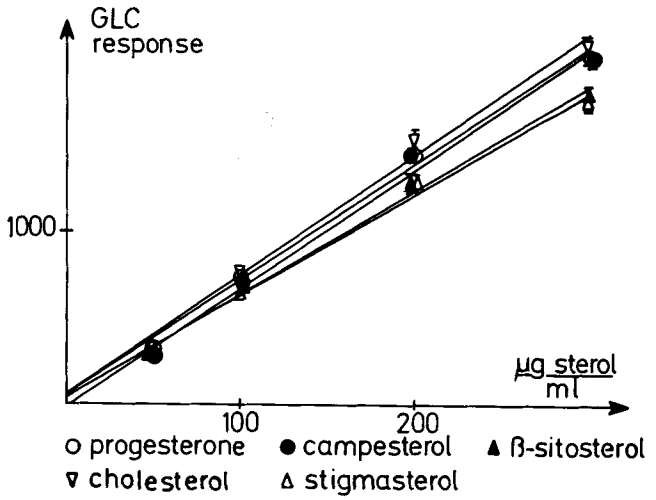

Fig. 2. The response of different sterols and progesterone on the gas liquid chromatograph.

et al (4) and, for diary products, information from the Organisation of the Swedish Milk producers have been used.

The searched sterol was calculated from the following formula.

Searched sterol (g) =

$$\frac{\text{Amount of progesterone} \cdot \text{g} \cdot \text{ml}^{-1} \cdot \text{The peak of the searched sterol} \cdot \text{Response factor} \cdot \text{Total weight of food} \cdot \text{g} \cdot \text{The end volume of the sterol extract} \cdot \text{ml}}{\text{The peak of progesterone} \cdot 0.5 \cdot \text{food sample weighed out (g) for analysing}}$$

The response factors for the GLC analyses were calculated from the equation of the straight line $Y = kx + 1$ and were obtained through the following calculations:

$$\text{Response factors} = \frac{\text{The slope for progesterone}}{\text{The slope for the actual sterol}}$$ see also Fig. 2.

The calculations of the analytical error. The error of determination of the different kinds of sterols was calculated by the formula:

$$\text{S.D.} = \sqrt{\frac{\Sigma d^2}{2n}}$$. (d = difference between two measurements of the same sample, n = number of the duplicates).

DIETS

The diets were used in metabolic ward studies. The composition of the control diet was planned to be very similar to the fat reduced diet. Only the amount of fat differed significantly. The content of fat in the control diet was 40 % of the energy (E%) while the fat content of the fat reduced diet was 20 E%. The P/S-ratio of these diets was 0.24. The calculated cholesterol content was 355 $\pm$ 63 mg and 279 $\pm$ 52 mg per 10 MJ in the control and the fat reduced diet respectively. The protein content of the diets was 12 E% and 14 E% of the control and the fat reduced diet respectively. The II b diet differed in several ways from the others. The fat content and cholesterol content were lower 35 E% and 213 $\pm$ 17 mg respectively. The protein content and P/S-ratio was higher 20 E% and 2.0 respectively while the sugar content of II b diet was reduced compared to the control and the fat reduced diet.

RESULTS

The recovery studies. The internal standard (progesterone) could not be added to the original homogenate of the food because of instability by the alkaline treatment. Two critical steps before the addition of the progesterone became apparent. The first was the chloroform extraction of the lipids. The recovery in this step was (mean $\pm$ SD) 104.9 $\pm$ 2.3 %. The quenching was

10.8 ± 1.4 % (Table 1). The second step of suspected losses was after the PE extraction. The recovery in this step was 102.5 ± 2.9% and the quenching 13.5 ± 5.20. The recovery of the entire procedure was 87.4 ± 8.5 %. The quenching in this last step of the analysis procedure was 66.3 ± 27.8%.

The error of the determination of the cholesterol content was 8.5, 11.9 and 10.4% in the control diet, the II b diet and in the fat reduced diet respectively (Table 2). Determination of the campesterol content had a higher analytical error 18.1, 18.1 and 37% for the control diet, the II b diet and the fat reduced diet respectively probably explained by the small amounts of campesterol in the diets. Similarly for stigmasterol the analytical error was 25.3, 29.7 and 22.6% for the three diets respectively. The determination of the β-sitosterol content resulted in an analytical error of 9.3, 11.1 and 17% for control, II b and the fat reduced diets respectively.

The response factors. The response factors of the different sterols (Fig. 2) in relation to progesterone were studied every month, in order to check the condition of the GLC column. The response factors calculated for seven different occasions were 0.95 ± 0.03, 1.01 ± 0.07, 1.12 ± 0.09 and 1.10 ± 0.10 for cholesterol, campesterol, stigmasterol and β-sitosterol respectively.

The calculated and analysed cholesterol content of the control, II b and the fat reduced diets. The cholesterol content of the diets was calculated by using Food Composition Tables (4, 10). The calculated and the analysed amounts differed significantly ($p < 0.05$) in the control and in the fat reduced diets. The differences were -14.9 ± 5.4, +9.4 ± 5.6 and -20.9 ± 6.0% in the control diet, the II b diet and in the fat reduced diet respectively (Table 3).

The sterol composition of the control, II b and the fat reduced diets. The analysed content of cholesterol, campesterol, stigmasterol and β-sitosterol of the control diet was 292 ± 44, 37 ± 3, 14 ± 2 and 117 ± 11 mg per 10 MJ respectively (Table 4). Similarly for the II b diet was 228 ± 9, 63 ± 3, 28 ± 1 and 285 ± 16 mg for the content of cholesterol, campesterol, stigmasterol and β-sitosterol respectively. The fat reduced diet was analysed to contain 214 ± 41, 34 ± 5, 18 ± 2, 91 ± 10 mg of cholesterol, campesterol, stigmasterol and β-sitosterol respectively.

Table 1. Recovery studies.

Critical step	Number of the samples studied	The recovery % $\bar{x} \pm$ SD	The quenching % $\bar{x} \pm$ SD
Extraction of the lipid with chloroform	30	104.9 ± 2.3	10.8 ± 1.4
Extraction of the sterols with petroleumether	6	102.5 ± 2.9	13.5 ± 5.2
After the entire analyse procedure	42	87.4 ± 8.5	66.3 ± 27.8

Table 2. Calculation of the analytical error of the sterol method.

Diets	Cholesterol		Campesterol		Stigmasterol		β-sitosterol	
	n*	%	n	%	n	%	n	%
Control	21	8.5	22	18.1	20	25.3	20	9.3
II b	21	11.9	21	18.1	19	29.7	21	11.1
Fat reduced	21	10.4	20	37.0	19	22.6	21	17.0

* number of duplicate samples

Table 3. The cholesterol content calculated and analysed in control, II b and fat reduced diet per 10 MJ (2400 kcal). One week's menu.

Diets	Calculated mg	Analysed mg	Difference %	P-value
Control Average of mean for each day ± SEM	355 ± 63	292 ± 44	-14.9 ± 5.4	<0.05
IIb Average of mean for each day ± SEM	213 ± 17	228 ± 9	+ 9.4 ± 5.6	>0.05
Fat reduced diet Average of mean for each day ± SEM	279 ± 52	214 ± 41	-20.9 ± 6.0	<0.05

The statistical significance was tested with the paired Student t-test.

Table 4. The analysed sterol composition in control, II b and fat reduced diet per 10 MJ (2400 kcal). One week's menu.

Diets	Cholesterol mg	Campesterol mg	Stigmasterol mg	β-sitosterol mg
Control Average of mean for each day ± SEM	292 ± 44	37 ± 3	14 ± 2	117 ± 11
IIb Average of mean for each day ± SEM	228 ± 9	63 ± 3	28 ± 1	285 ± 16
Fat reduced diet Average of mean for each day ± SEM	214 ± 41	34 ± 5	18 ± 2	91 ± 10

DISCUSSION AND COMMENTS

The aim was to determine the content of cholesterol and the more detailed sterol composition in our diets. In the present study a method was worked out, which was less time-consuming than earlier described methods involving GLC. The use of progesterone as an internal standard made it possible to exclude the thin layer chromatography. The progesterone showed a single peak apart from other components of the diet without preceding separation on TLC. It should, however, be noticed that progesterone was not resistant to the alkaline treatment and had to be added after saponification. The recovery of the added isotope before adding the progesterone had therefore been studied and no losses were found.

The sterol composition of the three diets differed. There was a higher content of campesterol, stigmasterol and β-sitosterol in the II b diet, which contains more vegetables than the other diets. These sterols were also typical plant sterols. The control diet and the fat reduced diet were very similarly planned besides that the control diet contained 40 E% fat and the fat reduced diet only 20 E% and were supposed to have a similar sterol composition. The very low content of campesterol and stigmasterol influenced on the analytical error which became higher for these sterols. There were probably small amounts of other sterols in our diets, but they could not be detected by our packed column. A GLC equipped with a capillar column would separate all different sterols of the diets.

The differences between the calculated and the analysed cholesterol data might be explained by the following fact. Our calculations were based on data from Handbook no 8 (10) and a report by Feeley et al (4) and information from

the organisation of the Swedish Milk Producers. These data were based on determinations made according to Liebermann-Buchard's colorimetric reaction (9). The colorimetric methods have been reported (3,7) to give higher cholesterol levels than the GLC method. This was the probable reason for the difference between the calculated values and those analysed in the control and in the fat reduced diets.

The II b diet contains only low fat foods, e.g. low fat milk, lean meat and low fat cheese. The data on dairy products used for calculation of the cholesterol content was 2.5 mg cholesterol/g fat for all dairy products. LaCroix et al (6,7) has demonstrated that all dairy products having a fat value higher than whole milk has a cholesterol content of 3 mg cholesterol per gram fat and that the ratio between cholesterol and fat in food with lower fat content was higher (11.3 mg/g fat for skim milk). This is the probable reason why the II b diet had higher analysed values than the calculated ones.

There was a further difference between the diets. The calculated and the analysed values differed in the control diet (14.9%) and in fat reduced diet (20.9%). This fact might be explained by the fact that the control diet contained more dairy products than the fat reduced diet. The former contained at least 60 g fat derived from milk fat. An underestimation of 0.5 mg cholesterol per gram fat in the control diet would make a difference of 30 mg cholesterol a day.

Large amounts of plant sterols were reported to have a decreasing effect on the serum cholesterol concentrations due to a reduced absorption of both exogenous and endogenous cholesterol (5). The content of plant sterol in our diets differed. Most plant sterols were found in the II b diet (376 mg). The amounts showing a therapeutic effect in earlier studies were 5-10 g/day, why the amounts of plant sterols in the II b diet probably are too small to have any effect on the serum cholesterol concentration.

ACKNOWLEDGEMENTS

Laboratory assistant Ingrid Nilsson is gratefully acknowledged for her great help with the laboratory work and Lars-Börje Croon at the National Food Administration for valuable suggestions and discussions. This study was supported by grants from the Swedish Nutrition Foundation and ARLA.

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Accepted September 18, 1979

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