NUTRITION REPORTS INTERNATIONAL

COMBINED DETERMINATION OF FREE, ESTERIFIED AND GLYCOSILATED PLANT STEROLS IN FOODS

Diana Jonker[1], Gerard D. van der Hoek[1], Jan F.C. Glatz[1], Chris Homan[1], Maarten A. Posthumus[2] and Martijn B. Katan[1,*]

[1]Department of Human Nutrition and [2]Laboratory of Organic Chemistry, Agricultural University, De Dreijen 12, 6703 BC Wageningen, The Netherlands.

ABSTRACT

An improved method for determination of plant sterols in foods is described. It comprises successively acid and alkaline hydrolysis, extraction, and quantification by gas-liquid chromatography. The recovery of added sitosterol was 88-93% and the combined coefficient of variation within and between runs 5% or less. After introduction of acid hydrolysis sterol contents found in mixed natural diet samples increased by 2-4% for cholesterol, 13-23% for campesterol, 9-39% for stigmasterol and 22-42% for sitosterol. The amount of plant sterol recovered from individual foodstuffs was also increased. The increases are probably due to liberation of free plant sterols from (acylated) steryl glycosides by cleavage of the acid-labile acetal bond. In duplicate diet samples we identified the major sterols mentioned above, and the minor sterols brassicasterol, Δ^7-sitosterol and (iso)fucosterol. The concentration of these minor plant sterols in some foodstuffs is also presented. We conclude that plant sterol consumption as studied in e.g. balance studies is underestimated by the common methods that do not use acid pretreatment. In addition, the conversion by intestinal micro-organisms of Δ^7-sitosterol to sitostanol and of (iso)fucosterol to sitosterol could cause errors in sitosterol balance determinations.

INTRODUCTION

Knowledge of the plant sterol content of foods is important when these sterols are used in cholesterol balance studies as an internal standard to correct for unknown losses of cholesterol during passage through the intestinal tract (1). In addition, the lack of accurate data on the plant sterol contents of many foodstuffs may give rise to difficulties in the dietary treatment of patients with sitosterolemia.

* To whom all correspondence should be addressed.

Trivial names: cholesterol, cholest-5-en-3β-ol; campesterol, 24α-methylcholest-5-en-3β-ol; stigmasterol, 24α-ethylcholesta-5,22-dien-3β-ol; sitosterol, 24α-ethylcholest-5-en-3β-ol; Δ^7-sitosterol, 24α-ethyl-5α-cholest-7-en-3β-ol; brassicasterol, 24β-methylcholesta-5,22-dien-3β-ol; fucosterol, [24E]-24-ethylidene-cholest-5-en-3β-ol; isofucosterol, [24Z]-24-ethylidene-cholest-5-en-3β-ol.

This rare disorder is characterized by accumulation of plant sterols, principally sitosterol, in blood and tissues (2, 3). The accumulation may be due to an increased absorption of plant sterols from the gut coupled with defective excretion. In healthy humans the absorption of dietary plant sterols is limited to less than 5% of the daily intake (4, 5).

For determination of plant sterols in dietary and faecal samples the method described by Miettinen et al. (6) is frequently used (1, 5, 7-9). In this method samples are saponified in 1 M NaOH in 90% ethanol, followed by extraction of the unsaponifiable matter with petroleum ether, thin-layer chromatography of the petroleum ether extracts, and gas-liquid chromatography. When we used this procedure for measurement of the plant sterol balance in a dietary trial in man, we found that the daily excretion of sitosterol and of total plant sterols exceeded the intake in all subjects, suggesting underestimation of the intake.

In foods, plant sterols occur as free sterols, sterolesters, sterylglycosides and acylated sterylglycosides (10, 11) (Fig. 1). The polar (acylated) sterylglycosides are not extractable with petroleum ether and, because the acetal bond between the sugar and the 3-OH group of the sterol is stable towards alkali (12), the liberation of free sterols from the glycosides requires acid hydrolysis. It can therefore be expected that the method of Miettinen et al. (6) will not yield the total amount of plant sterols in foods. Methods have been described in which the several sterol classes are individually determined, but these are complicated and not feasible for analysis of large numbers of dietary samples.

The percentage of (acylated) sterylglycosides in total plant sterols in a mixed natural solid diet is unknown. However, published figures for the (acylated) sterylglycoside content of several foodstuffs (13-17) suggest that the amount of sterylglycosides in such samples may not be neglectable. For example, in potatoes, cauliflower, peas, cherries, cucumbers, radish and wax beans these percentages are 74, 10, 15, 25, 51, 11 and 22, respectively (13-17).

In this paper we describe a simple method for the determination of the total amount of plant sterols in foods. In this method an acid hydrolysis step precedes the alkaline hydrolysis. We applied the method, both with and without acid pretreatment, to three total diet pools and to some individual foodstuffs, and indeed found markedly higher figures for several plant sterols after acid pretreatment of samples.

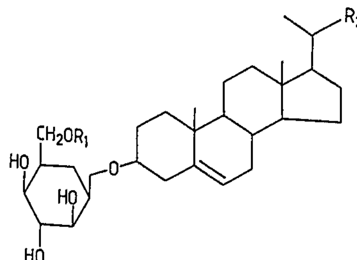

Fig. 1. Structure of a Δ^5-sterylglycoside (R_1 = H) or acylated sterylglycoside R_1 = acyl residue). The side chain R_2 varies between the major plant sterols.

MATERIALS AND METHODS

Samples

Duplicate portions of 3 different diets, containing the daily breakfast, lunch, dinner and snacks, were collected daily during cholesterol balance studies over a period of 3-4 weeks as described (18). The diets were composed of natural foodstuffs. They contained about 13 energy% protein, 44 energy% fat and 43 energy% carbohydrate. They resembled an average Dutch diet (19) except for their fatty acid composition or cholesterol content. Diet A was rich in vegetable oils; it had a polyunsaturated/saturated fatty acids (P/S) ratio of 1.74. Diet B was high in animal fat, especially dairy fat and had a P/S ratio of 0.23. Diet C was characterized by a low cholesterol content (6 mg/100 g homogenized diet sample) and a high content of saturated plant fats. The P/S ratio was 0.36. The duplicate diets were homogenized and freeze dried (18) before sterol analysis.

Foodstuffs were purchased from a local supermarket. We analyzed the raw edible parts of cauliflower (Brassica oleracea L., 2 cabbages), lettuce (Lactuca sativa L., 2 heads), wax beans (Phaseolus vulgaris L., 150 g), potatoes (Solanum tuberosum L., 400 g), cucumber (Cucumis sativus L., two, unpeeled), apple (Malus pumila Mill., five, unpeeled, uncored), banana (Musa sapientum L., three, peeled). They were homogenized to a smooth mass and freeze dried before sterol analysis. Peanut (Arachis hypogaea L., roasted and pooled, 250 g) and dark ryebread (40 g) were also homogenized and freeze dried, and whole wheat bread (158 g), light ryebread (400 g) and whole wheat biscuits (40 g) were homogenized only.

Reagents

The reference sterols 5α-cholestane, cholesterol, stigmasterol and sitosterol were purchased from Serva Feinbiochemica, Heidelberg, FRG; campesterol from Applied Science, Oud-Beijerland, The Netherlands. Dimethylformamide and bis-silyl-trifluoracetamide were obtained from Chrompack, Middelburg, The Netherlands. All other reagents used were of analytical grade.

Experimental method

In a 250-ml glass stoppered flask 3 ml 5α-cholestane solution (0.1 mg/ml in 96% ethanol; internal standard) was evaporated to dryness. One to two g of sample was weighed into the flask, 15 ml 6M HCl was added and the flask was heated for 30 min in a boiling water bath. To determine the optimal hydrolysis time the duration of acid hydrolysis was varied between 10 and 60 min. After 10 min the plant sterol content had already reached a maximum value that remained constant at prolonged hydrolysis. The acid hydrolysate was cooled on ice, 6.72 g KOH pellets and 100 ml 2M KOH in 96% ethanol were added, and the mixture was refluxed for 30 min in a boiling water bath.

For determinations without acid hydrolysis, the sample was refluxed straight away as described above, except that the KOH pellets were omitted.

After the alkaline saponification 100 ml of toluene was added. The mixture was shaken thoroughly and transferred to a 500-ml separa-

ting funnel which contained 100 ml 2M KOH. The two phases were carefully mixed and allowed to separate, 100 ml of water was added, and after shaking carefully again, the aqueous phase was discarded, thus removing the fatty acids.
The toluene phase was washed with 100-ml portions of water until the aqueous phase reacted neutrally with pH-paper, and then dehydrated by adding filter paper until the toluene extract was clear. This extract was evaporated to dryness in a round-bottom flask rotating in a 50°C water bath. The residue was dissolved in 0.5 ml toluene, transferred to a 0.6-ml glass vial and evaporated to dryness at 70°C under a stream of nitrogen.

For preparation of trimethylsilylethers 0.6 ml silylating mixture (dimethylformamide/bis-silyl-trifluoracetamide, 2:1, v/v) was added, the vial was closed and kept for 30 min at 70°C.

We estimated the recovery of sterols throughout the entire experimental procedure by adding various known amounts of campesterol, stigmasterol and sitosterol to a homogenized duplicate diet sample before freeze drying. The recoveries amounted to 85 $\pm$ 2, 98 $\pm$11 and 101$\pm$6%, respectively (mean $\pm$ S.D. for 8 determinations). No differences were observed for the methods with and without an initial acid hydrolysis step, indicating that the extent of acid-induced dehydration at C-3 was negligible. Repeated determinations on diet sample C revealed coefficients of variation for the major sterols of 1-2% within one run and of 2-5% between runs. The method appeared suitable for measuring amounts of sterol down to 1 µg.

Gas-liquid chromatography

The silylated sterol mixtures were analysed on a Varian Aerograph Series 2700 gas chromatograph with a hydrogen flame ionization detector and a column (1.80 m x 2 mm i.d.) packed with 3% OV 17 on Chromosorb WHP 100-120 mesh. The on-column injection temperature was 300°C, the column temperature 275°C and the detector temperature 300°C. Nitrogen was used as carrier gas at a flow rate of 30 ml/min. Peak areas were measured with a Spectra Physics 4100 computing integrator and compared with those of standard solutions of the reference sterols. For brassicasterol, Δ^7-sitosterol and (iso)fucosterol we used a response factor of 1.0. The gas chromatogram of diet sample C is given in Fig. 2.

Identification of plant sterols by gas-liquid chromatography-mass spectrometry

The silylated sterol mixture of diet sample C was injected into a Pye 204 gas chromatograph with a column (1.50 m x 2 mm i.d.) packed with 3% OV 17 on Chromosorb WHP 100-120 mesh. The on-column injection temperature was 250°C, the column temperature 220°C, programmed to 260°C at a rate of 4°/min, and the detector temperature 250°C. The carrier gas was helium at a flow rate of 20 ml/min. The column was connected via a jet separator to a VG MM70-70F mass spectrometer. Electron impact ioonization was used (70 eV).

Campesterol, stigmasterol and sitosterol in foods were identified on the basis of mass spectra and the retention times of the authentic compounds. Published spectra were used to identify Δ^7-sitosterol (20) and (iso)fucosterol (21). Fucosterol and isofucosterol are geometric

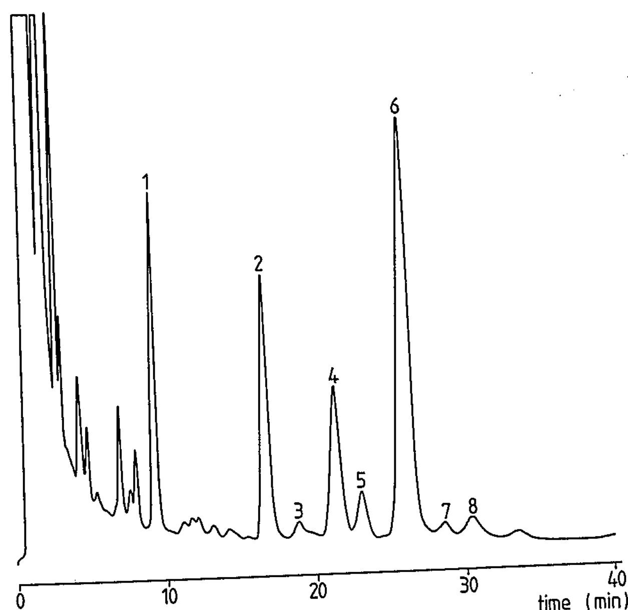

Fig. 2. Gas-liquid chromatography (on 3% OV 17 at 275°C, isothermal) of the sterols in duplicate diet sample C. Peak identification: 1, 5α-cholestane (internal standard); 2, cholesterol; 3, brassicasterol; 4, campesterol; 5, stigmasterol; 6, sitosterol; 7, (iso)fucosterol; 8, Δ^7-sitosterol. Combined gas-liquid chromatography-mass spectrometry revealed that each of the peaks represented one essentially pure compound.

isomers that are indistinguishable by mass spectrometry. Our samples will, however, most likely contain only isofucosterol, as fucosterol has as yet been found only in brown algae and oomycetous fungi (22). Brassicasterol could be identified by the M^+ and M-90 (loss of the trimethylsilyl-residue) peaks that were 14 mass-units lower than the corresponding peaks of stigmasterol, which only differs from brassicasterol in having an ethyl instead of a methyl group at C24, while the spectra of both compounds showed, with similar intensity, the peak due to the loss of TMSOH and of the side-chain at m/z 255. Furthermore, the difference in retention index between brassicasterol and stigmasterol was the same as that between campesterol and sitosterol. Both pairs differ in a methyl- or ethylgroup at C24 only.

RESULTS AND DISCUSSION

Duplicate diet samples

With all three duplicate diet samples the introduction of a preliminary acid hydrolysis step yielded increased contents of individual and total sterols: in diets A, B and C the respective increases were 3, 4 and 2% for cholesterol, 13, 23 and 17% for campesterol, 15, 39 and 9% for stigmasterol, 22, 42 and 30% for sitosterol and 20, 38 and 26% for the total sterol content (calculated from the mean of 5-6 determinations with and 3-6 determinations without acid hydrolysis,

and expressed relative to the latter). All increases in sterol content were statistically significant ($p<0.05$), but they were not the same for the three diet samples examined, most likely because the diets contained different types of fat which may have contained different proportions of (acylated) sterylglycosides. If the observed increases can be completely attributed to the liberation of (acylated) sterylglycosides, the results indicate that for sitosterol, the most common plant sterol encountered in human diets, the percentage of glycosilation is considerable, ranging from 18 to 30% in the three diets that we analyed. The small increases in cholesterol content are probably due to a better release of this compound, since to our knowledge animal sterols are not present as glycosides.

The AOAC-method for the determination of sterols in cereal foods (23) also includes acid hydrolysis in 6 M HCl, but requires many more steps. The extraction procedure in this method also differs from ours in that ether is used as extraction solvent. Using a duplicate diet sample, we compared our toluene extraction with the AOAC-ether extraction procedure, and found no significant differences (the contents of cholesterol were 21.0 ± 0.4 (n=3) and 21.2 ± 0.6 (n=6) mg/g and of total plant sterols 75.0 ± 0.9 (n=3) and 77.6 ± 3.9 (n=6) mg/g, respectively). We prefer to use toluene as it is less flammable.

Plant sterol content of foodstuffs

Various individual foodstuffs were also analyzed for their plant sterol content and the results were compared with data obtained from a literature search (Table I). Application of acid pretreatment resulted in most cases in higher values for the contents of each of the sterols. The differences between our values and those found in the literature can originate in several factors beside the analytical method, e.g. effect of variety, maturity, and geographic origin of the food (10). For 6 out of 8 products our values were similar to or higher than published ones.

We applied the methods with and without a preliminary acid hydrolysis step for the determination of the sitosterol balance in man in a dietary trial, as carried out at our department (25,26). During a period of low cholesterol intake the fecal recovery of dietary sitosterol and its metabolites, expressed as a percentage of daily intake, was 144 ± 65% when the diets were analysed without a preceding acid hydrolysis step, but amounted to 106 ± 30% when this step was included (mean $\pm$ S.D. for 6 subjects). During a period of high cholesterol intake, these percentages were 138 ± 50 and 115 ± 24, respectively (mean $\pm$ S.D. for 6 subjects). In both cases the level of intake of sitosterol ranged from 155 to 320 mg/day.

The presence of Δ^7-sitosterol and (iso)fucosterol in foodstuffs and diet samples is also relevant for sterol balance studies. During passage through the intestinal tract, saturation of the double bonds of Δ^7-sitosterol by intestinal micro-organisms could theoretically result in the formation of sitostanol, (the 5α-isomer of ethylcoprostanol), whereas saturation of (iso)fucosterol could produce sitosterol, and, from this, ethylcoprostanol or sitostanol. Ethylcoprostanol and sitostanol are known microbial metabolites of sitosterol.

Because of its 24β-methyl group, brassicasterol is not likely to give microbial conversion products that are identical to sitosterol or its metabolites.

Table I. Plant sterol content of foodstuffs obtained with the present method and according to literature values.

Food	Source of data	Acid hydrolysis	Plant sterol content (mg/100 g fresh weight)				
			Campesterol	Stigmasterol	Sitosterol	Minor sterols[a]	Total
Cauliflower	Our method	+	7.4	1.7	21.6	0.8	31.4
		−	6.4	1.4	17.3	0.9	26.0
	Ref. 10	Not stated	3	2	12		17
Wax beans	Our method	+	1.3	5.1	8.9	1.8	17.0
		−	1.1	4.2	7.1	2.9	15.2
	Ref. 15[b]	+			8.9		
Lettuce	Our method	+	1.1	4.5	10.6	1.2	17.4
		−	1.0	4.3	7.9	0.8	14.0
	Ref. 10	Not stated	1	4	5		10
Cucumber	Our method	+	<0.1	0.8	2.8	2.9	6.4
		−	<0.1	0.1	2.4	4.0	6.5
	Ref. 17	+	<0.1	0.5	4.0		4.5
Apple	Our method	+	0.6	<0.1	15.0	3.0	18.6
	Ref. 10	Not stated	1		11		12
Banana	Our method	+	1.3	1.7	12.0	1.1	16.1
	Ref 10	Not stated	2	3	11		16
Potato	Our method	+	<0.1	0.5	4.5	2.3	7.3
		−	<0.1	0.2	0.9	0.2	1.3
	Ref. 15[b]	+			2.3		
Peanuts	Our method	+	19.6	14.6	69.1	33.3	136.6
	Ref. 10	Not stated	24	23	142		189
Light rye-bread	Our method	+	21.2	2.2	37.7	2.9	64.0
		−	13.0	1.5	23.7	3.0	41.2
Dark rye-bread[c]	Our method	+	14.3	1.7	32.7	8.5	57.2
Whole wheat bread	Our method	+	12.5	0.8	28.9	3.0	45.2
Whole wheat[c]	Our method	+	12.1	2.4	28.8	1.4	44.7

Data were obtained from the literature and from analyses with and without prior acid hydrolysis. Values from our analyses represent the means of duplicate determinations. Open spaces indicate that no literature data were available.
[a] Sum of brassicasterol, Δ^7-sitosterol and (iso)fucosterol. [b] Original paper gives sterol content per dry weight. We recalculated this value assuming moisture percentages of 89 (wax beans) and 77% (potato) (24).
[c] No literature data found.

Concluding remarks

In conclusion, our results establish that plant sterol contents in foods are underestimated by the method of Miettinen et al. (6), which is often used in dietary trials, and that better results are obtained when an acid hydrolysis step is included in the procedure.

The presence of Δ^7-sitosterol and (iso)fucosterol in the diet could result in overestimation of the fecal excretion of sitosterol and its metabolites. We do not yet know, however, to what extent these minor plant sterols are converted to sitosterol or its metabolites in the human gut.

ACKNOWLEDGEMENTS

We thank Dr. C.E. West for alerting us to the problems studied in this paper and to their possible solution, and P. van de Bovenkamp for expert advice. Part of this study was supported by the Netherlands Heart Foundation, grant no. 78.093.

REFERENCES

1. Grundy, S.M., Ahrens, E.H. and Salen, G. Dietary β-sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies. J. Lipid Res. 9, 374 (1968).
2. Bhattacharyya, A.K., and Connor, W.E. β-Sitosterolemia and xanthomatosis. A newly described lipid storage disease in two sisters. J. Clin. Invest. 53, 1033 (1974).
3. Salen, G., Shefer, S. and Berginer, V.M. In: The Metabolic Basis of Inherited Disease, 5th edn. (Stanbury, J.B., Frederickson, D.S., Goldstein, J.L. and Brown, M.S., eds.). McGraw-Hill, New York, 1983, p.724.
4. Gould, R.G., Jones, R.J., LeRoy, G.V., Wissler, R.W. and Taylor, C.B. Absorbability of β-sitosterol in humans. Metabolism 18, 652 (1969).
5. Salen, G., Ahrens, E.H. Jr. and Grundy, S.M. Metabolism of β -sitosterol in man. J. Clin. Invest. 49, 952 (1970).
6. Miettinen, T.A., Ahrens, E.H. and Grundy, S.M. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. J. Lipid Res. 6, 411 (1965).
7. Connor, W.E., Witiak, D.T., Stone, D.B. and Armstrong, M.L. Cholesterol balance and fecal neutral steroid and bile acid excretion in normal men fed dietary fats of different fatty acid composition. J. Clin. Invest. 48, 1363 (1969).
8. Denbesten, L., Connor, W.E., Kent, T.H. and Lin, D. Effect of cellulose in the diet on the recovery of dietary plant sterols from the feces. J. Lipid Res. 11, 341 (1970).
9. Nair, P.P., Turjman, N., Kessie, G., Calkins, B., Goodman, G.T., Davidovitz, H. and Nimmagadda, G. Diet, nutrition intake, and metabolism in populations at high and low risk for colon cancer. Am. J. Clin. Nutr. 40, 927 (1984).
10. Weihrauch, J.L. and Gardner, J.M. Sterol content of foods of plant origin. J. Am. Diet. Assoc. 73, 39 (1978).
11. Dupéron, P. and Dupéron R. Evolution des differentes catégories de composés steroliques au cours de la germination du tubercule de pomme de terre. Physiol. Veg. 11, 487 (1973).

12. Morrison, R.T. and Boyd R.N. Organic Chemistry, 3rd edn., Allyn and Bacon, Inc., Boston, 1973, p.642.
13. Miyazawa, T., Ito, S. and Fumino, Y. Sterol lipids isolated from pea seeds. Cereal Chem. 51, 623 (1974).
14. Davis, D.L. and Poneleit, C.G. Sterol accumulation and composition in developing zea mays L. Kernels. Plant Physiol. 54, 794 (1974).
15. Dupéron, R., Meance, J., Dupéron, P. and Thiersault, M. Les composés steroliques présent dans les mitochondries et les microsomes de cellules végétales. C.R. Acad. Sci., serie D, 280, 605 (1975).
16. Bishop, D.G. and Wade, N.L. Lipid composition of sweet cherries. Phytochem. 16, 67 (1977).
17. Fishwick, M.J., Wright, A.J. and Galliard, T. Quantitative composition of the lipids of cucumber fruit. J. Sci. Food Agric. 28, 394 (1977).
18. Stasse-Wolthuis,,M., Albers, H.F.F., Van Jeveren, J.G.C., De Jong, J.W., Hautvast, J.G.A.J., Hermus, R.J.J., Katan, M.B., Brydon, W.G. and Eastwood, M.A. Influence of dietary fiber from vegetables and fruits, bran or citrus pectin on serum lipids, fecal lipids and colonic function. Am. J. Clin. Nutr. 33, 1745 (1980).
19. Van Staveren, W.A., Hautvast, J.G.A.J., Katan, M.B., Van Montfort, M.A.J. and Van Oosten-Van der Goes, H.G.C. Dietary fiber consumption in an adult Dutch population. J. Am. Dietetic Ass. 80, 324 (1982).
20. Brooks, C.J.W., Henderson, W. and Steel, G. The use of trimethylsilyl ethers in the characterization of natural sterols and steroid diols by gas chromatography-mass spectrometry. Biochim. Biophys. Acta 296, 431 (1973).
21. Knights, B.A. Identification of plant sterols using combined GLC/mass spectrometry. J. Gas Chromatogr. 5, 273 (1967).
22. Myant, N.B. The biology of cholesterol and related steroids, W. Heinemann Ltd., London, 1981, p.152.
23. Horwitz, W. (ed.). Official Methods of Analysis of the Association of Official Analytical Chemists, 13th edn., AOAC, Washington, 1980, p.129.
24. Katan, M.B. and Van de Bovenkamp, P. Analyse van het totale voedingsvezelgehalte en van het pectine-aandeel hierin in Nederlandse voedingen. Voeding 43, 153 (1982).
25. Brussaard, J.H., Katan, M.B., and Hautvast, J.G.A.J. Fecal excretion of bile acids and neutral steroids on diets differing in type and amount of dietary fat in young healthy persons. Eur. J. Clin. Invest. 13, 115 (1983).
26. Katan, M.B., Beynen, A.C., De Vries, J.H.M. and Nobels, A. Existence of consistent hyper- and hyporesponders to dietary cholesterol in man. Am. J. Epid., submitted.

Accepted for publication: July 21, 1985.