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Identification of Metabolites of Phytosterols in Rat Feces Using GC/MS

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β-Sitosterol, campesterol and stigmasterol have been known to the phytosterols the most frequently found in plants. Metabolism of phytosterols was investigated using rat feces and liver microsomes. Feces were collected after phytosterols (a well characterized mixture of β-sitosterol 40%, campesterol 30% and dihydrobrasicasterol) were administered orally (0.5 g/kg) to rats. Metabolites of phytosterols were identified using GC/MS. Three peaks were eluted at 12.47, 12.65, 12.87 min and had characteristic molecular ions *m/z* 428, 430, 432, respectively. Three fecal metabolites were identified as androstadienedione, androstenedione, and androstanedione. No metabolites could be detected in the rat liver microsomal reaction mixture. The results suggest that the metabolites of phytosterols in rat feces are formed by oxidation at 3- position, saturation at 5- and 6- position, and 17- side chain cleavage in the rat large intestine.

Key words: Phytosterols, β-Sitosterol, Metabolites, Rat feces, Rat microsomes, GC/MS

INTRODUCTION

Plant-derived sterols have a cycopenteno-phenanthrene ring with a 3-beta hydroxy substitution and a 5-6 double bond. The most frequently found phytosterols include β-sitosterol, campesterol and stigmasterol (Ling *et al.*, 1995). Phytosterols are structurally related to cholesterol, but differ only in a side-chain substitution of an ethyl group (sitosterol), methyl group (campesterol) at position 24 or an additional double bond at position 22 (stigmasterol) next to an ethyl group at position 24 from cholesterol (Fig. 1). The main sources of phytosterols in the diet are cooking oils and margarines. Total adult intake has been estimated to be about 250 mg/day for non-vegetarians and about 500 mg/day for vegetarians (Ling *et al.*, 1995). It has been known that phytosterols reduce blood cholesterol levels (Mattson *et al.*, 1982; Westrate *et al.*, 1998) and have effects on the reproductive system. In particular, they have been shown to possess estrogenic activity (El Samannoudy *et al*, 1980; Elghamry *et al.*, 1969; Malini *et al.*, 1991). However, Baker *et al.* (1999) showed that phyto-

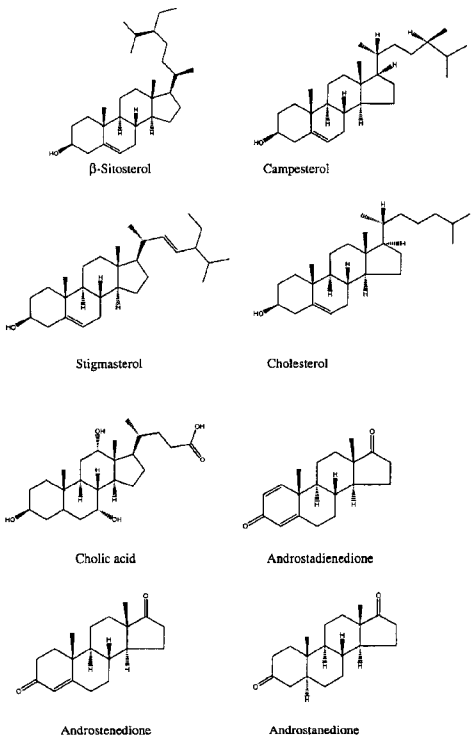

Fig. 1. Chemical structures of phytosterols, cholesterol, cholic acid, androstadienedione, androstenedione, and androstanedione

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sterols did not either bind to the estrogen receptor (ER) or stimulate transcriptional activity of the human ER in a recombinant yeast strain. In addition, uterotrophic assay showed no indication of estrogenicity when the mixture was administered by oral gavage to immature female rats. Cleavage of the carbonaceous 17-side chain of steroids is well known to be a frequent microbial steroid transformation, which has been demonstrated in many aerobic species of fungi and bacteria. A few anaerobic transformations of this kind have also been known (Funken *et al.*, 1972). Naturally occurring steroids and their derivatives because of the specific hormonal and clinical values, are of commercial significance (Djerassi *et al.*, 1976; Kieslich *et al.*, 1986). However, few studies have been reported about the fecal metabolism in animal intestine filled with microflora. The introduction of spreads enriched in vegetable oil sterols in the food supply increase the exposure of the intestinal tract to phytosterols in consumers of such spreads. The large majority of phytosterols fed are not absorbed in the small intestine. Consequently, they are concentrated in the large intestine before being excreted in the feces. In addition, β-sitosterol was shown to be converted into polar compounds in the bile acid fraction of rats (Skrede *et al.*, 1985). The aim of this work was to study *in vivo* metabolism of phytosterols (a well characterized mixture of β-sitosterol 40%, campesterol 30% and dihydrobrasicasterol) in rat feces and *in vitro* metabolism β-sitosterol in rat liver microsomes by identifying metabolites of phytosterols using GC/MS.

MATERIALS AND METHODS

Chemicals

Phytosterols (a well characterized mixture of β-sitosterol 40%, campesterol 30% and dihydrobrasicasterol), β-sitosterol, campesterol, stigmasterol, cholesterol, cholic acid, androstadienedione, androstenedione, and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Their stock solutions in methanol or in ethanol were stored at 4°C. All other chemicals were of analytical grade.

Animals

Male Sprague-Dawley rats (220-280 g) were purchased from Dae Han Laboratory Animal Research Center Co., Ltd. (Eumsung, Korea). Tap water and food (Samyang Co., Korea) were provided *ad libitum* during the accommodation period for a week. The animals were kept in Nalgene metabolism cages.

Treatment

Phytosterols were given orally (0.5 g/kg) to the rats. Phytosterols 0.25 g was suspended in 1 ml of polyethylene glycol 200 and 50% ethanol. Major phytosterols in the test phytosterols were β-sitosterol (40%), campesterol (30 %). Feces were collected for 48 hr after oral administration of phytosterols.

Sample preparation

Fresh fecal samples were collected into sterile bags held within a rigid plastic container. Each sample was homogenized with 9 volume of anaerobic dilution solution (Byrant *et al.*, 1953) and stored at -20°C in polypropylene 50 ml centrifuge tubes until analysis. Aliquots (0.5 ml) of 10% (w/w) fecal solution were hydrolyzed with 1 ml of 2 M KOH at 80°C for 30 min. Immediately after hydrolysis, the supernatant was obtained by refrigerated centrifugation at 2500 rpm for 5 min and then 1 ml of distilled water was added. Neutral sterols were extracted with 5 ml of n-hexane. The hexane extract was evaporated to dryness. After acidification of water phase to pH 1.0 with hydrochloric acid, the solution was extracted with 5 ml of ethyl acetate. The ethyl acetate extracts were transferred to the hexane extract-evaporated tubes, and combined extracts were evaporated to dryness. Derivatization to trimethylsilyl (TMS) ethers was performed by addition of 50 μl of MSTFA with 5% NH_4I and incubation at 60°C for 30 min.

In vitro metabolism using rat microsomes

Rat liver microsomes were prepared according to the previously reported method (Guengerich *et al.*, 1986). A microsomal pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA, and stored at -80°C until used. Protein concentration was determined by the method of Lowry *et al.* (1951) method. β-Sitosterol was dissolved in ethanol. The solvent of β-sitosterol in ethanol was evaporated under stream of nitrogen gas, and 0.2 mg of microsomal protein in 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.4) was added. After preincubation at 37°C for 5 min, the reaction was initiated by the addition of the NADPH-generating system (10 mM glucose-6-phosphate, 0.67 mM $NADP^+$ and 1 unit of glucose-6-phosphate dehydrogenase). The final concentration of β-sitosterol in reaction mixture was 1 μg/ml. The reaction was stopped by the addition of 1 ml of 2 M KOH. Following hydrolysis, reaction mixture was processed as described below for analysis of the meta-bolites using GC/MS.

Instrumental conditions

Gas chromatography/mass spectrometry (GC/EIMS, 5890 A/5970B Model, Hewlett- Packard, CA, USA) was used. Separation of the standard mixture (androstadienedione, androstenedione, cholesterol, campesterol, stigmasterol, cholic acid, and β-sitosterol) was performed using a methyl

silicone capillary column (ultra 1, length 25 m, inner diameter 0.2 mm, film thickness 0.3 mm). The carrier gas was helium and the flow rate was adjusted to 0.78 ml/min. Injector temperature was 280°C, and detector temperature 300°C. Starting oven temperature was 200°C, which was increased by 10°C/min to 270°C and then by 5°C/min to a final temperature of 300°C. The injection mode was splitless. The mass selective detector was operated in the electron impact-selected ion-monitoring (EI-SIM) mode with the ionizing voltage set at 70 eV. Standard androstadienedione, androstenedione, cholesterol, campesterol, stigmasterol, cholic acid, and β-sitosterol were provisionally identified by their retention times. Their identities were confirmed using GC/MS. Although standard androstanedione was not used in TIC, peak at 12.87 min was identified as androstanedione by retention time and fragmentation patterns. Androstadienedione, androstenedione, androstanedione, cholesterol, campesterol, cholic acid, and β-sitosterol from rat feces were identified by their fragmentation pattern while scanning the mass range m/z 50-700 and retention times.

RESULTS

Metabolites of phytosterols in rat feces

After phytosterols were administered orally (0.5 g/kg) to rats, feces were collected for 48 h to identify metabolites using GC/MS. Total ion chromatogram (TIC) of standard androstadienedione, androstenedione, androstanedione, cholesterol, cholic acid, and phytosterols including β-sitosterol is shown in Fig. 2A. β-Sitosterol and cholesterol were separated at 24.56, 19.62 min, respectively. Androstadienedione, androstenedione, and androstanedione were detected at 12.47, 12.65, and 12.87 min, respectively. The chromatogram for rat blank feces is shown in Fig. 2B. The total ion chromatogram obtained from rat feces after oral administration of phytosterols is shown in Fig. 2C. In the TIC, several peaks derived from phytosterols were observed. Characteristic mass ions of β-sitosterol include molecular ion of *m/z* 486 (M^+), base peak *m/z* 396 (M^+-OTMS), and significant ion *m/z* 471 (M^+-15) for determination of β-sitosterol in feces (Table I). Androstadienedione, androstenedione, and androstanedione had characteristic molecular ions *m/z* 428, 430, 432, respectively (Table I). Unchanged form of β-sitosterol had the retention time of 24.56 min and its scan mass spectrum is shown in Fig.

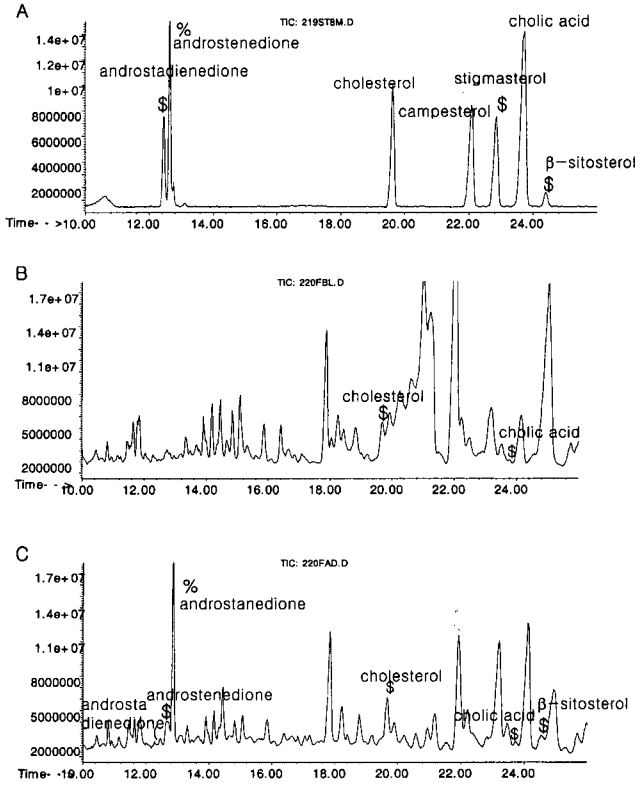

Fig. 2. Total ion chromatograms of GC/MS of standard phytosterols, cholesterol, cholic acid, androstadienedione, androstenedione, and androstanedione (A), and rat blank feces (B), and feces after oral administration of phytosterols (C) (a well characterized mixture of β-sitosterol 40%, campesterol 30% and dihydrobrasicasterol, 0.5 g/kg) to rats.

Table I. Retention times and fragmentation patterns of TMS-derivatized phytosterols, cholesterol, cholic acid, androstadienedione, androstenedione, and androstanedione

Compound	Retention time (min)	M	M-15	M-90	M-105
β-Sitosterol	24.56	486	471	396	381
Campesterol	22.09	472	457	382	367
Stigmasterol	22.87	484	469	394	379
Cholesterol	19.62	458	-	368	353
Cholic acid	23.68	-	682	-	592
Androstadienedione	12.47	428	413	338	323
Androstenedione	12.65	430	415	340	325
Androstanedione	12.87	432	417	342	327

M = molecular ion, M-15 = molecular ion-15 *m/z* etc.

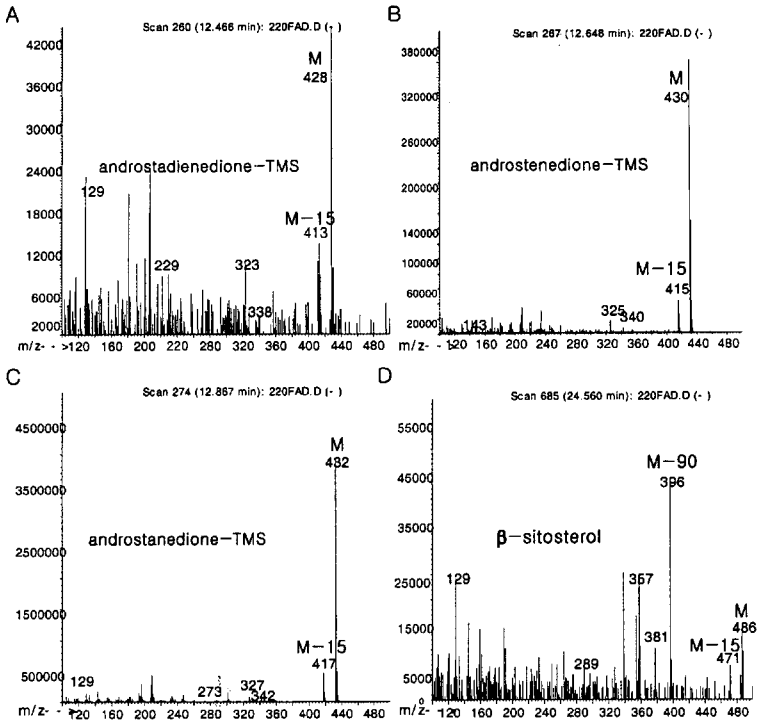

Fig. 3. Scan mass spectra of TMS-metabolites of androstadienedione (A), androstenedione (B), androstanedione (C), and TMS-β-sitosterol (D) in rat feces after oral administration of phytosterols (a well characterized mixture of β-sitosterol 40%, campesterol 30% and dihydrobrasicasterol, 0.5 g/kg) to rat.

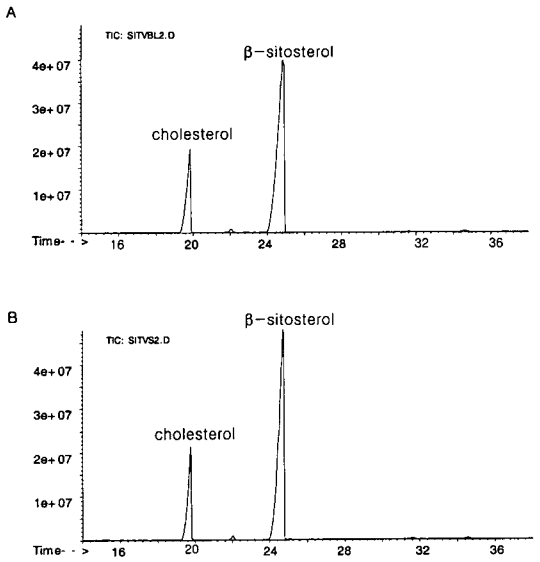

Fig. 4. Total ion chromatograms of extracts after incubation of β-sitosterol (1 mg/kg) with rat liver microsomes without (A), with (B) the NADPH-generating system.

3D. Three peaks at 12.47, 12.65, and 12.87 min were identified to be metabolites of phytosterols (Fig. 2C, Table 1). The first peaks having retention time of 12.47 min had molecular ion *m/z* 428 (M^+) and significant ion *m/z* 413 (M^+-15). Based on the scan mass spectrum and retention time data of standard androstadienedione, it was identified to be androstadienedione (Fig. 3A). The second peak having retention time of 12.65 min was identified to be androstenedione (Fig. 3B). The third peak having retention time of 12.87 min from feces was identified to be androstanedione based on its scan mass spectrum and retention time although standard androstanedione was not available (Fig. 3C). Three metabolites including androstanedione as a major metabolite, androstadienedione, and androstenedione were found to be produced from phytosterols in rat feces.

In vitro metabolism using rat liver microsomes

In order to identify the *in vitro* metabolites produced by rat liver microsomes, β-sitosterol (1 μg/ml) was incubated with rat liver microsomal protein in the NADPH-generating system. After incubation, the reaction mixtures were extracted and TIC is shown in Fig. 4B. Compared with TIC obtained in the absence of the NADPH-generating system (Fig. 4A), there was no difference between them, indicating that b-sitosterol was not metabolized by the rat liver microsomal system.

DISCUSSION

Although cleavage of the carbonaceous 17-side chain of phytosterols is well known to be a frequent microbial

steroid transformation, metabolism by intestinal bacteria in the rat gut has not been reported. The present study identified three metabolites including androstadienedione, androstenedione, and androstanedione in rat feces after oral administration of phytosterols to rats. Roy *et al.* (1991) reported transformation of β-sitosterol to androstadienedione by the side-chain cleavage activity of immobilized *Mycobacterium* species NRRL-B 3683. Oxidation-reduction reaction at the 3-hydroxyl group and hydrogenation of the Δ^5 double bond of cholesterol and other Δ^5 sterols have been known (Eyssen *et al.*, 1988). Weststrate *et al.* (1999) also suggested that the major sterol metabolites excreted formed by, predominantly, oxidation at the 3-positiona and metabolites saturated at the 5-and 6-position in a β-configuration. So, metabolites of phytosterols from human feces include hydrogenated phytosterols or stanols and 3-ketosterols. In addition, 4-cholestene-3-one, putative mutagenic substance, excretion was increased slightly in the human consuming the sterol-enriched margarines (Bartram *et al.*, 1996). Androstadienedione, androstenedione, and androstanedione detected in rat feces are suggested to be formed by 17-side chain cleavage, saturation at 5- and 6- position and oxidation at 3-position of phytosterols by intestinal bacteria in rats. In general, phytosterols and their metabolites have negligible absorption rates, thus not contributing to the body burden of these compounds. 7-Hydroxylation and 5,6-epoxide of β-sitosterol in rat liver microsomes were much less than those of cholesterol (Aringer *et al.*, 1973, 1974). Westrate *et al.* (1999) reported no evidence for the formation of so-called oxysterols, sterol oxide, from phytosterol in the gut. The present *in vitro* metabolism study of β-sitosterol using rat liver microsomes did not show any hepatic conversion of β-sitosterol, indicating resistance to hepatic metabolism. Identification of the metabolites formed is difficult due to the presence of a great excess of metabolites from cholesterol and phytosterols in foodstuffs. Therefore, it is suggested that using radiolabeled phytosterols in metabolism and pharmacokinetic study of phytosterols is recommended for better results. In conclusion, orally administered phytosterols were transformed to one major metabolite, androstanedione, and two minor metabolites, androstadienedione and androstenedione by oxidation at 3-position, saturation at 5- and 6-position, and 17-side chain cleavage in the rat intestine.

REFERENCES

Aringer, L. and Eneroth, P., Studies on the formation of C7-oxygenated cholesterol and beta- sitosterol metabolites in cell-free preparations of rat liver. *J. Lipid Res.*, 14, 563-572 (1973).

Aringer, L. and Eneroth, P., Formation and metabolism in vitro of 5,6-epoxides of cholesterol and beta-sitosterol. *J. Lipid Res.*, 15, 389-398 (1974).

Baker, V. A., Hepburn, P. A., Kennedy, S. J., Jones, P. A., Lea, L. J., Sumpter, J. P. and Ashby, J., Safety evaluation of phytosterol esters. Part 1. Assessment of oestrogenicity using a combination of in vivo and in vitro assays. *Food Chem. Toxicol.*, 37, 13-22 (1999).

Bartram, H. P., Gostner, A., Kelber, E., Dusel, G., Weimer, A., Scheppach, W. and Kasper, H., Effects of fish oil and fecal bacterial enzymes and steroid excretion in healthy volunteers: implications for colon cancer prevention. *Nutrition and Cancer., 25,* 71-78 (1996).

Byrant, M. B., Burkey, L.A., Cultural methods and some characteristics of the more numerous groups of bacterial in the bovine rumen. *J. Diary Sci.*, 36, 205-17 (1953).

Djerassi, C., The manufacture of steroidal contraceptives: technical versus political aspects. *Proc. R. Soc. Lond B Biol. Sci.*, 195, 175-186 (1976).

El Samannoudy, F. A., Shareha, A. M., Ghannudi, S. A., Gillaly, G. A. and El Mougy, S. A., Adverse effects of phytoestrogens-7. Effect of beta-sitosterol treatment on follicular development, ovarian structure and uterus in the immature female sheep. *Cell Mol. Biol.*, 26, 255-266 (1980).

Elghamry, M. I. and Hansel, R., Activity and isolated phytoestrogen of shrub palmetto fruits (Serenoa repens Small), a new estrogenic plant. *Experientia.*, 25, 828-829 (1969).

Eyssen, H and Caenepeel, P. H., Metabolism of fats, bile acids and steroids. I.R. Rownland, Academic Press. London, 1988.

Funken, G. S. and Johnsons, R. A., Chemical oxidations with microorganisms: Chap 6- Microbiological Baeyer-villiger Oxidation. Dekker, New York, 1972.

Guengerich, F. P., Martin, M. V., Beaune, P. H., Kremers, P., Wolff, T., and Waxman, D. J., Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.*, 261, 5051-5060 (1986).

Kieslich, K., Production of drugs by microbial biosynthesis and biotransformation. Possibilities, limits and future developments (1st communication): *Arzneimittelforschung.*, 36, 774-778 (1986).

Ling, W. H. and Jones, P. J., Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sci.*, 57, 195-206 (1995).

Lorwy, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275 (1951).

Malini, T. and Vanithakumari, G., Antifertility effects of beta-sitosterol in male albino rats. *J.Ethnopharmacol.*, 35, 149-153 (1991).

Mattson, F. H., Grundy, S. M., and Crouse, J. R., Optimizing the effect of plant sterols on cholesterol absorption in man. *Am. J. Clin. Nutr.*, 35, 697-700 (1982).

Mellanen, P., Petanen, T., Lehtimaki, J., Makela, S., Bylund, G., Holmbohn, B., Mannila, E., Oikari, A., and Santti,

R., Wood-derived estrogens: studies in vitro with breast cancer cell lines and in vivo in trout. *Toxicol. Appl. Pharmacol.,* 136, 381-388 (1996).

Roy, P. K., Khan, A. W., and Basu, S. K., Transformation of sitosterol to androsta-1, 4-diene-3, 17-dione by immobilized Mycobacterium cells. *Indian J. Biochem. Biophys.,* 28, 150-154 (1991).

Skrede, B., Bjorkhem, I., Bergesen, O., Kayden, H. J., and Skrede, S., The presence of 5 alpha-sitostanol in the serum of a patient with phytosterolemia, and its biosynthesis from plant steroids in rats with bile fistula. *Biochim. Biophys. Acta.,* 836, 368-375 (1985).

Weststrate, J. A. and Meijer, G. W., Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.,* 52, 334-343 (1998).

Weststrate, J. A., Ayesh, R., Bauer-Plank, C., and Drewitt, P. N., Safety evaluation of phytosterol esters. Part 4. Faecal concentrations of bile acids and neutral sterols in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester- enriched margarine. *Food. Chem. Toxicol.,* 37, 1063-1071 (1999).