

ORIGINAL COMMUNICATION

Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal

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Objective: To measure the relative effects of each of four phytosterol ester-enriched low-fat foods (bread, breakfast cereal, milk and yoghurt) on serum lipids, plasma phytosterols and carotenoids.

Design: Three research centres undertook a randomised, incomplete crossover, single-blind study consisting of four treatment periods of 3 weeks each, one of which was a control period. Each sterol-enriched test food provided 1.6 g/day of phytosterols as sterol esters.

Setting: General Community.

Subjects: In all 58, free-living men and women with mean age (s.d.) 54 (8) y, moderately elevated plasma total cholesterol 6.2 (0.7) mmol/l and body mass index 26.2 (3.0) kg/m².

Main outcome measures: Serum lipids, plasma phytosterols and carotenoids.

Results: Serum total and LDL cholesterol levels were significantly lowered by consumption of phytosterol-enriched foods: milk (8.7 and 15.9%) and yoghurt (5.6 and 8.6%). Serum LDL cholesterol levels fell significantly by 6.5% with bread and 5.4% with cereal. They were both significantly less efficacious than sterol-enriched milk ($P < 0.001$). Plasma sitosterol increased by 17–23% and campesterol by 48–52% with phytosterol-enriched milk and bread. Lipid-adjusted β -carotene was lowered by 5–10% by sterols in bread and milk, respectively.

Conclusions: This is the first study to demonstrate that cholesterol-lowering effects of plant sterol esters may differ according to the food matrix. Plant sterols in low-fat milk was almost three times more effective than in bread and cereal. Despite phytosterol-enriched cereal products resulting in lower serum cholesterol reductions compared to sterol-enriched milk, the detection of similar changes in plasma phytosterols demonstrated that such products still delivered and released phytosterols to the gut. *European Journal of Clinical Nutrition* (2004) 58, 503–509. doi:10.1038/sj.ejcn.1601837

Keywords: LDL cholesterol; carotenoids; sitosterol; campesterol

Background

There are extensive data confirming the effectiveness of esterified phytosterols in margarines with LDL cholesterol-lowering of 10–15% with a dose of 1.6–2.4 g/day of sterol (Ling & Jones, 1995; Weststrate and Meijer, 1998; Hendriks *et al*, 1999; Noakes *et al*, 2002). There are no published data on the use of plant sterols in low-fat foods, such as bread and

cereal although the use of a combination of sterols in these products in addition to sterols in margarine, suggest that they are equally efficacious in these foods (Nestel *et al*, 2001). There are two studies showing that 1–2 g/day of sterols or stanols in low-fat yoghurts are effective at lowering LDL cholesterol in patients with moderate primary hypercholesterolemia (Volpe *et al*, 2001; Mensink *et al*, 2002) but no studies have used low-fat milks. It is possible that the milk fat globule membrane which has been altered by acid and/or microbial action in yoghurts may adsorb sterols differently to a native membrane. No study has directly compared different food products to determine if the food matrix alters the effectiveness of phytosterols. The failure of sitostanol in a capsule formulation to lower cholesterol suggests that the

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environment in which the sterol is delivered is important (Denke, 1995). The aim of this study was to demonstrate in a randomised, single-blind, incomplete crossover over design the effect of an equivalent 1.6 g/day free sterol consumed as an ester in bread, breakfast cereal, milk and yoghurt on plasma lipids and plasma carotenoids in mildly hypercholesterolaemic subjects.

Methods

Subjects

Mildly hypercholesterolaemic men and women (20–25 in each centre) were recruited by public advertisement in each of the three clinical research centres. Subjects were screened on the basis of the following inclusion criteria: age 20–75 y, body mass index (BMI) <31 kg/m², total serum cholesterol >5.0 mmol/l and <7.5 mmol/l, no lipid lowering medication, no diabetes, normal thyroid status and no metabolic disorder other than hyperlipidaemia, not taking medications likely to affect lipid metabolism and no requirement for such medication, serum triglycerides <4.5 mmol/l, no strong aversion and no known allergies/intolerances to the foods involved. The study was approved by the CSIRO Human Experimentation Ethics Committee (site 1), the Baker Medical Centre Ethics Committee (site 2) and the Royal Prince Alfred Hospital Ethics Committee (site 3) and all subjects gave informed consent. There were no significant differences between subjects in the three centres which were all located in major cities. The study was a joint initiative of the three research centres hence the multicentre design.

Study design

There were four intervention periods each of 3 weeks duration. During each period, one sterol-rich food was eaten. The foods eaten in each period are outlined in Table 1. The order of test foods was randomised separately for each centre.

Although all four foods were eaten during each period, only one phytosterol-enriched food was tested in each of

three active intervention periods and one period was the control period. There was no washout. During the control period none of the four foods was enriched with phytosterol esters. The study was conducted simultaneously with all subjects in the three centres and food was supplied in one package for each 3-week period.

Food requirements

Serve sizes per day were yoghurt 200 g, bread (white) two slices, cereal (muesli style) 45 g, milk (2% fat, extended shelf-life) 500 ml. Each serve of phytosterol-enriched food contained 1.6 g of phytosterols. Subjects were requested to consume one serve of each food per day spread across at least two meals. The composition of the food products is shown in Table 2. The sterols were predominantly of soybean oil origin and consisted of 50% sitosterol, 20% stigmasterol and 20% campesterol. They were 94% esterified with fatty acids from soybean oil.

Measurements

The following measurements were made during the study:

- Dietary intakes were monitored using food frequency questionnaires (Hodge *et al*, 2000) during each intervention to determine compliance and assess micronutrient intakes. This was carried out in two centres only. A daily record of the consumption of the supplied foods was also used to assess compliance.
- Weight and height of subjects were determined at entry to the study. Subsequently, subject weights were measured at each visit to the clinic. Subjects were provided the opportunity to report adverse events, if any, at each visit.
- Subjects were requested to complete daily a checklist of foods consumed during interventions.
- Serum lipids (total cholesterol, HDL cholesterol, triglycerides) were determined on two consecutive days at the end of each period (weeks 2, 5, 8, 11 and 14). LDL cholesterol levels were calculated (see later).
- Plasma phytosterols and carotenoids were measured in one centre at the end of each period for the control, bread and milk treatments.

Table 1 Study design according to site and interventions by period

	Site 1 (n=22)	Site 2 (n=18)	Site 3 (n=18)
Baseline (2 weeks)		Each individual's baseline (usual) diet	
Period 1 (3 weeks)	Sterol—yoghurt Control—cereal, milk, bread	Sterol—milk Control—cereal, bread, yoghurt	Sterol—yoghurt Control—cereal, bread, milk
Period 2 (3 weeks)	Control—all four foods	Sterol—bread Control—milk, cereal, yoghurt	Sterol—bread Control—cereal, yoghurt, milk
Period 3 (3 weeks)	Sterol—cereal Control—milk, yoghurt, bread	Sterol—cereal Control—milk, bread, yoghurt	Sterol—cereal Control—yoghurt, bread, milk
Period 4 (3 weeks)	Sterol—milk Control—cereal, yoghurt, bread	Control—all four foods	Control—all four foods

Table 2 Approximate nutrient composition of control and test food products

Dietary component ^a	Control milk		Test milk		Control yoghurt		Test yoghurt	
	Per 100 ml	Per day ^b	Per 100 ml	Per day ^b	Per 100 g	Per day ^b	Per 100 g	Per day ^b
Energy (kJ)	212	1060	224	1120	292	584	359	718
Protein (g)	3.9	19.5	4.0	20.0	3.1	6.2	5.0	10.0
Fat total (g)	1.4	7.0	1.4	7.0	0.8	1.6	1.6	3.2
Saturated (g)	0.9	4.5	NA	NA	0.6	1.2	0.42	0.84
Monounsaturated (g)	NA	NA	NA	NA	NA	NA	0.17	0.34
Polyunsaturated (g)	NA	NA	NA	NA	NA	NA	0.01	0.02
Phytosterols ^c (g)	0	0	0.32	1.6	0	0	0.8	1.6
Carbohydrate total (g)	5.5	27.5	6.0	30.0	12.2	24.2	14.7	29.4
Sugars (g)	5.5	27.5	6.0	30.0	11.8	23.6	14.4	28.8
Dietary fibre (g)	0	0	0	0	NA	NA	NA	NA
Cholesterol (mg)	NA	NA	NA	NA	NA	NA	3.0	6.0
Calcium (mg)	134	670	141	705	106	212	154	308
Sodium (mg)	57	285	60	300	46	92	65	130
Potassium (mg)	NA	NA	183	915	NA	NA	211	422
Dietary component ^a	Control bread		Test bread		Control cereal		Test cereal	
	Per 100 g	Per day ^b	Per 100 g	Per day ^b	Per 100 g	Per day ^b	Per 100 g	Per day ^b
Energy (kJ)	1000	789	1000	789	1803	811	1788	805
Protein (g)	7.5	5.9	7.2	5.7	9.5	4.3	8.9	4.0
Fat total (g)	2.8	2.2	4.3	3.4	16.0	7.2	17.5	7.9
Saturated (g)	1.3	1.0	1.4	1.1	4.3	1.9	4.1	1.8
Monounsaturated (g)	NA	NA	NA	NA	NA	NA	NA	NA
Polyunsaturated (g)	NA	NA	NA	NA	NA	NA	NA	NA
Phytosterols ^c (g)	0	0	2.4	1.6	0	0	3.6	1.6
Carbohydrate total (g)	42.2	32.9	40.5	31.6	58.0	26.1	54.5	24.5
Sugars (g)	2.6	2.0	2.5	1.9	20.6	9.3	19.4	8.7
Dietary fibre (g)	6.0	4.7	5.8	4.5	8.1	3.6	7.6	3.4
Cholesterol (mg)	NA	NA	NA	NA	NA	NA	NA	NA
Calcium (mg)	NA	NA	NA	NA	NA	NA	NA	NA
Sodium (mg)	425	332	408	319	165	74	155	70
Potassium (mg)	NA	NA	NA	NA	365	164	343	154

^aNutrient composition determined either by analysis (control foods), or calculation (most test foods) or other sources of available data. Values expressed are generally only approximations.

^b'Per day' refers to the quantity of that food required to be consumed every day according to the dietary intervention plan.

^cMajor sterols, in descending order of predominance; β -sitosterol, campesterol and stigmasterol. All test foods were enriched with phytosterol esters. Levels shown are expressed as the free sterol equivalent.

NA indicates that analytical data are unavailable.

Analyses

Serum lipids. Serum lipids were measured locally in each centre. Venous blood samples (20 ml) were taken into plain tubes after subjects fasted overnight (12 h). Serum was separated by low-speed centrifugation at $600 \times g$ for 10 min at 5°C (GS-6R centrifuge; Beckman, Fullerton, CA, USA) and frozen at -20°C . At the end of the study, all samples from each subject were analysed within the same analytic run (to reduce instrumental variation). Total cholesterol and triacylglycerols were measured on a Cobas-Bio centrifugal analyzer (Roche Diagnostica, Basel, Switzerland) by using enzymatic kits (Hofmann-La Roche Diagnostica, Basel, Switzerland) and standard control sera. Plasma HDL cholesterol concentrations were measured after precipitation of apoB-containing lipoproteins by PEG 6000. The following modification of the Friedewald equation for molar concen-

trations was used to calculate LDL cholesterol in mmol/l: $\text{LDL cholesterol} = \text{total cholesterol} - (\text{triacylglycerol}/2.18) - \text{HDL cholesterol}$. No plasma triglyceride exceeded the cutoff of 4.5 mmol/l.

Plasma phytosterols. Plasma phytosterols were determined by gas chromatography based on a modification of the method described by Wolthers *et al* (1991). Briefly, 400 μl of plasma sample was saponified with 400 μl of 33% KOH at 60°C for 30 min, cooled and extracted with hexane. The extract was evaporated to dryness with a stream of nitrogen and the phytosterols were derivatised by treatment with 150 μl SyLON BTZ (Supelco) for 30 min at 80°C . The silyl derivatives of the phytosterols were extracted into hexane, concentrated with a stream of nitrogen to 50 μl and a 1 μl aliquot was injected onto the GC column (split ratio 1:10).

The gas chromatograph consisted of a DANI 6500 instrument equipped with a split/splitless injector, flame ionisation detector coupled to a DELTA computerised chromatography data system. The injector, detector and oven temperatures were set at 275, 275 and 280°C, respectively. The capillary column used was a 60 m × 0.22 mm BPX5 (SGE Australia P/L). Plasma phytosterol concentrations were calculated from the standard curves using the ratio of the phytosterol peak area to the peak area of the internal standard (5 β -cholestan-3 α -o1). The pure internal standard, lathosterol, campesterol and sitosterol reference samples were obtained from Sigma Chemicals Co (St Louis, USA).

Plasma carotenoids and vitamins A and E. After subjects fasted overnight, blood samples were collected using EDTA as an anticoagulant. The plasma was separated by low-speed centrifugation and frozen immediately in liquid nitrogen and then stored at -80°C until analysis. Plasma extractions and HPLC chromatography were performed according to the method of Yang and Lee (1987). Minor modifications to this method were derived from Khachik *et al* (1992).

Sample preparation and analysis. Only a small number of samples were processed at any one time to minimise the exposure to laboratory conditions. The lighting was minimal throughout sample preparation and amber vials were used for the final extract storage. Samples had the internal standard added and an equal volume of ethanol. Vitamins and carotenoids were extracted with hexane and the extract was evaporated to dryness under nitrogen. Extracts were then stored at -20°C. Mobile phase was used to redissolve the samples ready for HPLC analysis. All samples from each volunteer were extracted in duplicate and analysed in one run on the HPLC to minimise the effect of day-to-day variation.

Quality control. A standard reference material (National Institute of Standards and Technology product 968b) was initially tested after preparation of the standards. All vitamins and carotenoids at the high, medium and low levels fell within the certified ranges. A quality control (QC) plasma was prepared for this study by pooling ~20 ml plasma which was mixed thoroughly and 500 μ l aliquots were transferred into storage vials and run with each batch of samples. QC plasma was stored at -80°C.

A Shimadzu LC 10 HPLC fitted with a refrigerated autosampler and a SPD-M10Avp photodiode array detector with a class LC 10 chromatography work station was used for analysis of the prepared samples. Isocratic separations of the fat-soluble vitamins and carotenoids were carried out on a Rainin (4.6 mm ID × 250 mm length) C18 (5 μ m spherical particles) reverse-phase column. The mobile phase was a mixture of acetonitrile (55%), methanol (22%), hexane (11.5%) and dichloromethane (11.5%) at a flow rate of

1.0 ml/min. Ammonium acetate (0.01% w/v) was added to the mobile phase for stabilisation of the carotenoids. Wavelengths of 292 nm (α -tocopherol and α -tocopherol acetate), 325 nm (retinol), 450 and 472 nm (carotenoids) were monitored throughout each run.

Standards (*trans* α - and β -carotene, lycopene, lutein, retinol, α -tocopherol and α -tocopherol acetate) were obtained from Sigma Chemical Co., St Louis MO, USA. Solvents, (hexane, methanol, acetonitrile and dichloromethane) were all analytical HPLC grade while the ethanol was 99.5% Univar absolute ethanol.

Statistical analysis. Repeated measures analysis of variance was calculated with treatment period as the within-subject factor and with centre and gender as the between-subject factor. Age, baseline LDL cholesterol and BMI and change in weight between periods were inserted into the model as covariates. Baseline carotenoid levels were inserted into the model examining the effects of phytosterols on plasma carotenoids. Carotenoids were adjusted by dividing by the total cholesterol level at the time of measurement of the carotenoid. Where there was a significant treatment effect detected by repeated measures, *post hoc* tests were used to locate differences using a Bonferroni correction to make allowance for the large number of tests performed. Time effects were examined by analysing changes from baseline to each period. Analyses were performed with SPSS 10.0 for WINDOWS (SPSS Inc., Chicago). Significance was set at $P < 0.05$. The study was powered such that 40 subjects across two centres would be sufficient to see a 5% fall in LDL cholesterol.

Results

Subjects

A total of 58 subjects (35 women and 23 men) completed the trial. Five subjects (four in site 3 and one in site 2) failed to complete the trial because of time commitments. They had an average age of 54 y, weighed 74 kg (BMI 26.2 kg/m²) and gained an average of 0.9 kg ($P < 0.01$) over the 12 weeks. The largest weight difference between control and phytosterol-enriched periods was 2 kg. Baseline total cholesterol was 6.24 mmol/l, HDL cholesterol 1.5 mmol/l and triglyceride 1.58 mmol/l. There were no differences between centres in volunteer demographics.

Compliance

Dietary compliance from the food checklist in all three centres was excellent, averaging 96%. There was no variation in compliance across foods or across centres and compliance did not explain differences between foods nor differences in individual results.

Dietary data

There were no changes in reported intakes of energy, fat, carbohydrate or protein intakes across any of the phases or between the centres (data not shown).

Serum lipids

Serum total cholesterol levels (Table 3) were lowered by phytosterol consumption in milk by 0.66 mmol/l (95% CI 0.45–0.74 mmol/l) or 9.7% and in yoghurt by 0.36 mmol/l (95% CI 0.19–0.54 mmol/l), or 5.6%. Similarly, LDL cholesterol levels were lowered by phytosterol consumption in milk by 0.72 mmol/l (95% CI 0.58–0.85 mmol/l), or 15.9%, and in yoghurt by 0.36 mmol/l (95% CI 0.22–0.50 mmol/l), or 8.6% (Table 3). In the 22 subjects who consumed both yoghurt and milk, the significance of the difference between the two foods was $P=0.04$ with a 95% CI of 0.02–0.56 mmol/l for LDL. Baseline LDL cholesterol was unrelated to the response to sterol-enriched foods and there were no statistical differences between centres. There were no time effects. The baseline LDL cholesterol was lower than the control LDL cholesterol probably because of the extra fat in the cereal.

The changes in serum lipids when phytosterol-containing cereal foods were consumed were similar or lower, with LDL cholesterol levels falling 6.5% for bread and 5.4% for breakfast cereal. In this study bread and breakfast cereals were less effective vehicles for cholesterol-lowering with phytosterols than milk ($P<0.001$) with 95% CI of the difference between the effect of phytosterol-enriched milk on serum LDL cholesterol and between phytosterol-enriched bread of 0.39–0.82 mmol/l ($n=18$), and 0.32–0.58 mmol/l for phytosterol-enriched breakfast cereals ($n=40$) in those subjects who ate both. Neither of these two forms of phytosterol-enriched food were significantly different in efficacy from phytosterol-enriched yoghurt.

Serum HDL cholesterol levels fell from baseline to the control period by 0.05 mmol/l ($P=0.01$), which is probably related to the small weight gain seen from baseline (0.9 kg). HDL cholesterol levels rose significantly by 5% in the phytosterol-enriched bread period only compared with control periods. This was probably a chance finding only as it did not occur with other phytosterol-enriched foods. Serum triglyceride levels did not change during the trial.

Plasma phytosterols

Table 4 shows the results for plasma phytosterols and lathosterol (as an indicator of cholesterol synthesis) for control, phytosterol-enriched milk and bread periods in all three centres combined. Measurement of plasma phytosterols indicated that the availability for absorption of the phytosterols may be quite separate from their effects on serum lipids, as sterol-enriched bread elevated plasma sterols as much as sterol-enriched milk although it had much smaller effects on serum LDL cholesterol.

Plasma lathosterol levels did not change but levels of both campesterol and sitosterol increased by 27–52%. Adjusted lathosterol was elevated by about 20% with milk but was not changed by bread. There was no relationship between the change in cholesterol levels and the change in levels of sitosterol or campesterol. The change in cholesterol levels was not predicted by baseline levels of lathosterol, sitosterol or campesterol (either adjusted by dividing by total cholesterol or unadjusted values) or the ratio between plasma phytosterols and plasma lathosterol levels. Thus, subjects who absorbed phytosterols well unexpectedly did not appear to have a better response to phytosterols than those who absorbed phytosterols poorly and/or had high cholesterol synthesis (as assessed by lathosterol levels) (Table 4).

Plasma carotenoids

Plasma carotenoids were measured during the control phase and after sterol-enriched milk and bread. With sterol-enriched milk only adjusted β -carotene levels were significantly lowered (–10%) while total cholesterol levels fell by 9.7%. The correlation between the fall in β -carotene and the fall in cholesterol was weak ($r=0.34$, $P<0.05$), while there was no correlation between falls in *adjusted* β -carotene levels and falls in cholesterol. With sterol-enriched bread the β -carotene reduction was significant (a 4% fall) after log transformation of adjusted data ($P=0.021$). Total cholesterol fell by 5% with sterol-enriched bread. In the 18 subjects who ate both sterol-enriched milk and bread, the fall in unadjusted β -carotene was twice as great with sterol-enriched milk as with sterol-enriched bread, while the other carotenoids were not different.

Table 3 Effect of diets containing 1.6 g/day of phytosterols in four different food vehicles

	Baseline (n=58)	Control (n=58)	Bread (n=36)	Milk (n=40)	Cereal (n=58)	Yoghurt (n=40)
Total cholesterol	6.24±0.74	6.43±0.71	6.08±0.79*	5.90±0.71*	6.23±0.71*	6.04±0.65*
LDL cholesterol	4.03±0.71	4.27±0.73	3.85±0.74*	3.74±0.69*	4.03±0.66*	3.85±0.61*
HDL cholesterol	1.50±0.41*	1.46±0.37	1.50±0.40*	1.43±0.32	1.44±0.42	1.46±0.41
Triglycerides	1.58±0.73	1.64±0.77	1.63±0.87	1.60±0.63	1.58±0.85	1.62±0.83

Expressed as mean ± s.d., mmol/l.

* $P<0.05$ compared with control period.

Table 4 Plasma lathosterol, campesterol and sitosterol after ingestion of control foods and sterol-enriched foods

	Control (n=58)	Bread (n=36)	Milk (n=40)
Lathosterol ($\mu\text{g/ml}$)	$3.08^1 \pm 0.87$	$3.12^1 \pm 1.00$	$2.96^1 \pm 0.74$
Adjusted lathosterol ($\mu\text{g}/\mu\text{mol}$)	$0.44 \text{ (M)}^1 \pm 0.12$		
	$0.49 \text{ (B)}^1 \pm 0.16$	$0.52^1 \pm 0.17$	$0.51^2 \pm 0.12$
Campesterol ($\mu\text{g/ml}$)	$3.72^1 \pm 1.61$	$5.36^2 \pm 2.22$	$5.68^2 \pm 2.19$
Sitosterol ($\mu\text{g/ml}$)	$3.54^1 \pm 1.84$	$4.66^2 \pm 2.74$	$4.51^2 \pm 2.12$

Values with different superscripts are significantly different ($P < 0.05$) from each other. Adjusted lathosterol computed separately for the milk (M) and control pairs ($n=40$) and the bread (B) and control pairs ($n=36$); mean \pm s.d., $\mu\text{g/ml}$.

Discussion

This is the first study to directly compare the efficacy of individual foods fortified with plant sterol. Although all phytosterol-enriched food forms significantly lowered LDL cholesterol levels, the greatest lowering of LDL cholesterol concentration was seen with the low-fat milk, possibly due to the nature of the vehicle. Phytosterols may be incorporated into the milk globule membrane and be readily available for transfer into the micellar membrane, while in the other low-fat foods they may be trapped in the centre of the lipid droplets and not available until the fat is digested. The fall in LDL cholesterol of 16% is greater than usually observed with 1.6 g/day of plant sterols in margarines (Table 5). This fall in LDL cholesterol is very similar to that observed with intakes of 3.2 g/day of phytosterol or phyto- stanol esters consumed in margarines (Weststrate & Meijer, 1998). There may be some benefit in increasing intakes from 1.6 to 3.2 g/day when the food vehicle is bread and cereal, as this food matrix appears to influence phytosterol effectiveness. In considering the effect on blood lipid levels, it should be noted that there is a wide range of reported effects on LDL cholesterol with falls as little as 6.5 and 7.9% with 1.6 and 3.2 g/day phytosterols (delivered as the ester) in margarine respectively (with no difference between these two intakes) (Hendriks *et al*, 1995) and up to 14% cholesterol lowering with 2.6 g/day of stanol ester margarine (Miettinen *et al*, 1995). Clearly, however, low-fat foods can be just as effective as high-fat foods.

Consistent with other studies, very low levels of phytosterols were detected in plasma. These were significantly different from the control period. The detection of phytosterols in plasma demonstrated that despite phytosterol ester-enriched cereal products resulting in lower serum cholesterol reductions (compared to enriched milk), such products still delivered and released phytosterols to the gut which were available for absorption. The elevation in plasma sitosterol and campesterol with phytosterol ingestion is about 50% lower compared with the 39 and 71% increase seen by Weststrate and Meijer (1998); however, the dose used in that study (3.3 g/day) was about twice the dose used in this study. Campesterol appears to be absorbed to a greater degree than sitosterol as the amount in the phytosterol margarine is about half of the sitosterol level, while the increase in plasma is about twice. However, altered hepatic clearance may also account for this difference (Sudhop *et al*, 2002).

Although there were some minor falls in lycopene and α -tocopherol and α -carotene before adjustment the effects on β -carotene levels are the most consistent. The magnitude of the change is in the order of 10% after adjustment regardless of the change in LDL cholesterol with the food. This is negligible compared with seasonal changes of 70% for α - and β -carotene (Maskarinec *et al*, 1999). Dietary advice to eat five serves/day of fruit and vegetables (including one that is carotenoid-rich) can increase α -carotene by 32% in 4 weeks (Noakes *et al*, 2002).

Table 5 Effect of diets containing 1.6 g/day of phytosterols on absolute and adjusted plasma carotenoids and fat-soluble vitamins

Period	Lutein	Retinol	α -Tocopherol	Lycopene	α -Carotene	β -Carotene
Control	0.44 ± 0.22	2.38 ± 0.47	37.2 ± 9.9	0.67 ± 0.34	0.13 ± 0.09	0.55 ± 0.44
Milk	0.41 ± 0.21	2.35 ± 0.39	$34.5^{**} \pm 5.9$	0.62 ± 0.37	$0.11^{**} \pm 0.06$	$0.45^{**} \pm 0.30$
Bread	0.42 ± 0.20	2.35 ± 0.50	$35.8^{**} \pm 9.2$	$0.61^* \pm 0.35$	0.13 ± 0.10	$0.48^{**} \pm 0.42$
Adjusted control	0.067 ± 0.035	0.37 ± 0.093	5.77 ± 1.42	0.10 ± 0.05	0.020 ± 0.012	0.084 ± 0.066
Adjusted milk	0.070 ± 0.037	0.40 ± 0.080	5.88 ± 0.99	0.10 ± 0.06	0.019 ± 0.010	$0.076^* \pm 0.053$
Adjusted bread	0.070 ± 0.034	0.39 ± 0.084	5.73 ± 1.42	0.10 ± 0.05	0.022 ± 0.019	$0.081^* \pm 0.072$

Adjustment was performed by dividing the carotenoid value by the total cholesterol (mean \pm s.d., $\mu\text{mol/l}$ and $\mu\text{mol}/\text{mmol}$).

* $P < 0.05$, ** $P < 0.01$ vs control foods.

The lowering of plasma carotenoid concentrations by spreads containing phytosterols has been reported previously. Weststrate and Meijer (1998) compared a phytostanol-ester spread (Benecol) with esterified sterols from soybean, sheanut or ricebran and found that all reduced lipid standardised carotenoids but to a variable extent (–9 to –43%), and this was not related to the magnitude of lipid lowering. Benecol and the soybean ester margarine (contributing 2.7–3.3 g/day phytosterols) both significantly lowered plasma α - and β -carotene levels by 19%. There was a similar fall in lycopene but it was not significant. Gylling *et al* (1999) also reported a fall of 25% in lipid standardised β -carotene (but not α -carotene) with 2.6 g/day phytosterols from fortified spread. Furthermore, lipid standardised plasma α - plus β -carotene concentrations were decreased by 8, 5 and 15% and lycopene nonsignificantly by 7–10% by daily consumption of 0.83, 1.61 and 3.24 g phytosterol equivalent in spread, respectively (Hendriks *et al*, 1999). The difference between the two highest intakes was significant for α - and β -carotene combined (unadjusted, $P < 0.05$). Interestingly, the α - and β -carotene levels in the Dutch studies (Weststrate & Meijer, 1998; Hendriks *et al*, 1999) are similar to those reported here (the levels are about 20% greater in Australia), while the plasma lycopene in Holland varies from 26 to 60% of the Australian level. Thus, the public health risk of consuming phytosterol-enriched margarine spreads in terms of carotenoid-lowering is minimal as the changes are within the differences seen between countries.

In conclusion, we have demonstrated that phytosterols in all food forms tested in this study lower serum LDL cholesterol with low-fat milk being the most effective vehicle with a 16% lowering with 1.6 g/day of phytosterols.

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References

Denke MA (1995): Lack of efficacy of low-dose sitostanol therapy as an adjunct to a cholesterol-lowering diet in men with moderate hypercholesterolemia. *Am. J. Clin. Nutr.* **61**, 392–396.
Gylling H, Puska P, Vartiainen E & Miettinen TA (1999): Retinol, vitamin D, carotenes and alpha-tocopherol in serum of a moderately hypercholesterolemic population consuming sitostanol ester margarine. *Atherosclerosis* **145**, 279–285.

Hendriks HF, Weststrate JA, van Vliet T & Meijer GW (1999): Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* **53**, 319–327.
Hodge A, Patterson AJ, Brown WJ, Ireland P & Giles G (2000): The Anti Cancer Council of Victoria FFQ: relative validity of nutrient intakes compared with weighed food records in young to middle-aged women in a study of iron supplementation. *ANZ. J. Pub. Health* **24**, 576–584.
Khachik F, Beecher GR, Goli MB, Lusby WR & Smith Jr. JC (1992): Separation and identification of carotenoids and their oxidation products in the extracts of human plasma. *Anal. Chem.* **64**, 2111–2122.
Ling WH & Jones PJH (1995): Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sci.* **57**, 195–206.
Maskarinec G, Chan CL, Meng L, Franke AA & Cooney RV (1999): Exploring the feasibility and effects of a high-fruit and -vegetable diet in healthy women. *Cancer Epidemiol. Biomarkers Prev.* **8**, 919–924.
Mensink RP, Ebbing S, Lindhout M, Plat J & van Heugten MM (2002): Effects of plant stanol esters supplied in low-fat yoghurt on serum lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations. *Atherosclerosis* **160**, 205–213.
Miettinen TA, Puska P, Gylling H, Vanhanen H & Vartiainen E (1995): Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *N. Engl. J. Med.* **333**, 1308–1312.
Nestel P, Cehun M, Pomeroy S, Abbey M & Weldon G (2001): Cholesterol-lowering effects of plant sterol esters and non-esterified stanols in margarine, butter and low-fat foods. *Eur. J. Clin. Nutr.* **55**, 1084–1090.
Noakes M, Clifton P, Ntanos F, Shrapnel W, Record I & McInerney J (2002): An increase in dietary carotenoids when consuming plant sterols or stanols is effective in maintaining plasma carotenoid concentrations. *Am. J. Clin. Nutr.* **75**, 79–86.
Sudhop T, Sahin Y, Lindenthal B, Hahn C, Luers C, Berthold HK & von Bergmann K (2002): Comparison of the hepatic clearances of campesterol, sitosterol, and cholesterol in healthy subjects suggests that efflux transporters controlling intestinal sterol absorption also regulate biliary secretion. *Gut* **51**, 860–863.
Volpe R, Niittynen L, Korpela R, Sirtori C, Bucci A, Fraone N & Pazzucconi F (2001): Effects of yoghurt enriched with plant sterols on serum lipids in patients with moderate hypercholesterolaemia. *Br. J. Nutr.* **86**, 233–239.
Weststrate JA & Meijer GW (1998): 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.
Wolthers BG, Walrecht HT, van der Molen JC, Nagel GT, Van Doormaal JJ & Wijnandts PN (1991): Use of determinations of 7-lathosterol (5 α -cholest-7-en-3 β -o1) and other cholesterol precursors in serum in the study and treatment of disturbances of sterol metabolism, particularly cerebrotendinous xanthomatosis. *J. Lipid Res.* **32**, 603–612.
Yang CS & Lee MJ (1987): Methodology of plasma retinol, tocopherol and carotenoid assays in cancer prevention studies. *J. Nutr. Growth Cancer* **4**, 19–27.