# Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters

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**Abstract** **It has been suggested that phytosterol and phytostanol esters possess similar cholesterol-lowering properties, however, whether mechanisms responsible are identical has not been addressed. To address this question, cholesterol plasma levels, absorption, biosynthesis, and turnover were measured in 15 hypercholesterolemic subjects consuming prepared diets each over 21 d using a crossover design. Diets contained either *i*) margarine (M), *ii*) margarine with phytosterol esters (MSE) (1.84 g/d), or *iii*) margarine with phytostanol esters (MSA) (1.84 g/d). Cholesterol absorption was measured using the ratio of [$^{13}$C]cholesterol$_{oral}$:D$_7$-cholesterol$_{IV}$; biosynthesis using D incorporation from D$_2$O and turnover by D$_7$-cholesterol$_{IV}$ decay rates. Plasma total cholesterol level at d 21/22 was lower ($P < 0.05$) for MSE (13.4%) but not MSA (10.2%) versus M (6.0%) diets. Plasma low density lipoprotein-cholesterol (LDL-C) mean reductions at d 21/22 were larger ($P < 0.05$) for MSE (12.9%) and MSA (7.9%) compared with M (3.9%). Plasma TG and high density lipoprotein-cholesterol (HDL-C) levels did not differ across diets. Cholesterol absorption was reduced ($P < 0.05$) 36.2 and 25.9% at d 21 for MSE and MSA versus M, while cholesterol biosynthesis was reciprocally increased ($P < 0.05$) 53.3 and 37.8% for MSE and MSA versus M, respectively. Cholesterol turnover was not influenced by diet. These data indicate that plant sterol and stanol esters differentially lower circulating total and LDL cholesterol levels by suppression of cholesterol absorption in hypercholesterolemic subjects.**—Jones, P. J., M. Raeini-Sarjaz, F. Y. Ntanios, C. A. Vanstone, J. Y. Feng, and W. E. Parsons. **Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters.** ***J. Lipid Res.*** **2000.** 41: **697–705.**

**Supplementary key words** phytosterols • phytostanols • cholesterol • absorption • synthesis

Phytosterols, including β-sitosterol, campesterol, and stigmasterol, naturally occur in plants in both free and esterified form, as well as conjugated as glycosides (1). Saturation of phytosterols at the 5-alpha position form compounds including sitostanol and campestanol. For several decades it has been appreciated that consumption of plant sterols and stanols led to favorable shifts in circulating lipid levels (1–13). In general terms, plant sterol and stanol consumption in human subjects under a range of experimental circumstances reduces plasma total and low density lipoprotein cholesterol (LDL-C) concentrations within the range of 0.5–26% and 2–33%, respectively (ref. 14 for review). Indeed, addition of plant sterols and stanols to foods for the purpose of lowering plasma cholesterol concentrations presently reflects a major development in the functional foods area in Europe and North America.

There remains controversy, however, over the relative cholesterol-reducing efficacy of plant sterols versus stanols. A recent finding that esters of unsaturated β-sitosterol and campesterol produce the same cholesterol-lowering efficacy as esters of sitostanol and campestanol, when added to margarines (10), is somewhat inconsistent with previous animal data (4, 5, 7, 11). This earlier data from animals suggest that efficacy of cholesterol-lowering action increases with the extent of hydrogenation of the phytosterol mixture.

For this reason, the aim of the present investigation was to re-examine whether margarines containing esters of unsaturated plant sterols possess the same efficacy as those containing esters of saturated plant sterols in the modification of circulating lipoprotein cholesterol levels in hypercholesterolemic subjects. The secondary objective was to determine whether alterations in cholesterol absorption, turnover, or synthesis could account for any relative actions of esters of unsaturated versus saturated plant sterols on circulatory lipid levels. To achieve this aim, the current study examined the effect of feeding esterified plant sterols and stanols in margarine mixtures on sterol metabolism in hyperlipidemic males consuming prepared,

Abbreviations: LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; M, margarine diet; MSE, margarine + phytosterol esters diet; MSA, margarine + phytostanol esters diet; TC, total cholesterol; TG, triglyceride; RBC, red blood cells; FSR, fractional synthesis rate.

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fixed foods diets. The null hypothesis was that feeding the margarine alone, versus that with added plant sterol or stanol esters, to mildly hyperlipidemic subjects for 3 weeks would not influence their plasma lipid profiles, cholesterol absorption, turnover, or synthesis, nor plasma phytosterol levels.

## METHODS

### Human subjects

Hyperlipidemic males (37 to 61 yr) participated in the study. Subjects were screened for plasma total cholesterol (TC) and triglyceride (TG) levels. Criteria for acceptance were fasting plasma TC concentrations in the range of 6.0 to 10.0 mmol/L and TG less than 3.0 mmol/L. Subjects were screened for familial hypercholesterolemia using a family history questionnaire. Individuals reporting a personal history of diabetes, heart disease, or hypothyroidism, or who had been using drug therapy for hypercholesterolemia at any time during the 2 months prior to the start of the study, were excluded. The experimental protocol was approved by the Ethics Committee of the Faculty of Agriculture and Environmental Sciences for the School of Dietetics and Human Nutrition at McGill University. All subjects received a thorough explanation of the protocol and were given the opportunity to discuss any queries prior to signing a consent form.

### Protocol and diet

Using a randomized crossover double-blind design, three coded margarine mixtures were assigned to the three dietary treatments; one contained margarine alone, a second contained margarine with 8% (wt/wt based on free sterol content) plant sterol esters, and a third contained 8% (wt/wt based on free stanol content) plant stanol esters. Plant sterols were derived from vegetable oil and esterified with fatty acids obtained from rapeseed oil. Similarly, plant stanols were derived from vegetable oil, hydrogenated, then esterified with fatty acids from rapeseed oil. Fatty acids esterified to sterol and stanol esters included C18:1n−9 (58.6%), C18:2n−6 (18.9%), and C18:3n−3 (9.9%). Sterol and stanol esters were dissolved in margarine during their formulation. Margarine fatty acid and phytosterol composition is given in **Table 1**. To ensure that the crossover design was balanced, subjects were randomly assigned to one of six predetermined Latin squares, where each square possessed 3 sequenced phases and 3 subjects.

Subjects consumed a fixed intake North American solid foods diet during each of the three treatments. Each phase consisted of a 21-day feeding period followed by a 5-week washout. The diet was formulated to meet Canadian Recommended Nutrient Intakes. Dietary protein, carbohydrate, and fat made up 15, 50, and 35% of ingested energy, respectively. Dietary fat was comprised of 15, 10, and 10% of energy as monounsaturated, saturated, and polyunsaturated fats, respectively, using a blend of olive oil, butter, and a sunflower oil-based margarine. The diet was designed using a 3-day rotating meal cycle to provide variety over feeding periods. Meals were prepared in the Mary Emily Clinical Nutrition Research Unit metabolic kitchen. Subjects were required to consume a minimum of 2 meals per day, including breakfast, at the Nutrition Research Unit under the supervision of the unit's staff. Subjects were repeatedly instructed not to consume any food or beverages other than those provided by the Nutrition Research Unit.

The amount of food consumed by each subject was formulated to maintain individual weight balance, using a predictive equation based on each subject's weight, height, age, and activity level (15). Weight changes were monitored daily and food amounts were adjusted accordingly where necessary.

Margarine mixtures were incorporated into respective diets at a mean level of 23 g margarine per day, corresponding to 1.84 g free sterols or stanols, per day. Daily margarine doses were added to each meal divided into equal portions.

Blood samples were collected from subjects before breakfast on days 0, 8, 15, 18, 19, 20, 21, and 22 of each feeding period. On days 0 and 21 of each phase an additional blood sample was drawn to perform complete blood analyses to monitor the

TABLE 1. Plant sterol and fatty acid composition of spreads

| | Sitostanol Ester Treatment | | β-sitosterol Ester Treatment | | Control | |
|---|---|---|---|---|---|---|
| Composition | mg kg$^{-1}$ | % (w/w) | mg kg$^{-1}$ | % (w/w) | mg kg$^{-1}$ | % (w/w) |
| Total plant sterols | | | | | | |
| Cholesterol | 202 | 0.26 | 470 | 0.56 | 30 | 1.2 |
| Brassicasterol | 173 | 0.23 | 1251 | 1.49 | 240 | 11.3 |
| Campesterol | 1646 | 2.14 | 21611 | 25.80 | 610 | 28.9 |
| Campestanol | 21304 | 27.79 | 770 | 0.92 | 40 | 2.1 |
| Stigmasterol | 743 | 0.97 | 16106 | 19.23 | 40 | 2 |
| β-Sitosterol | 3868 | 5.04 | 38402 | 45.85 | 980 | 46.9 |
| Sitostanol | 46770 | 61.02 | 1610 | 1.92 | 20 | 1.1 |
| d5-Avenasterol | 133 | 0.17 | 838 | 1.00 | 60 | 2.9 |
| Other sterol | 1700 | 2.22 | 2700 | 3.22 | 80 | 3.6 |
| Total | 76639 | 100 | 83757 | 100 | 2100 | 100 |
| Total fat (as glycerides) | g kg$^{-1}$ | % (w/w) | g kg$^{-1}$ | % (w/w) | g kg$^{-1}$ | % (w/w) |
| Lauric (C12:0) | 7.0 | 1.8 | 10 | 2.2 | 10 | 2.3 |
| Myristic (C14:0) | 4.0 | 1.0 | 5 | 1.2 | 5 | 1.2 |
| Palmitic (C16:0) | 43 | 10.5 | 51 | 11.7 | 51 | 11.8 |
| Stearic (C18:0) | 13 | 3.2 | 14 | 3.2 | 13 | 3.1 |
| Oleic (C18:1 9c) | 166 | 40.5 | 174 | 39.7 | 181 | 41.8 |
| Linoleic (C18:2 9c,12c) | 137 | 33.4 | 143 | 32.6 | 130 | 30.0 |
| Linolenic (C18:3 9c,12c, 15c) | 23 | 5.6 | 24 | 5.5 | 25 | 5.9 |
| Other fatty acids | 17 | 4.2 | 17 | 3.9 | 17 | 4.0 |
| Total | 411 | 100 | 439 | 100 | 433 | 100 |

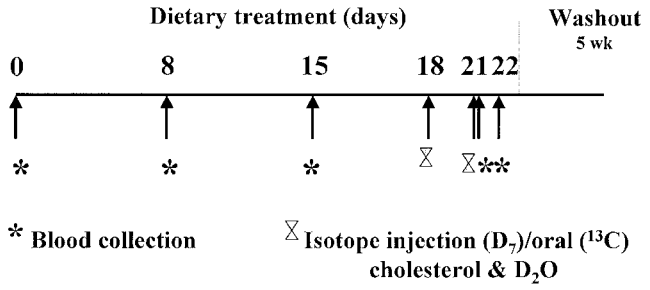


**Fig. 1.** Protocol time-line used in study.

health of the subjects. Subjects also underwent complete physical examinations and urinalyses at the beginning and end of each dietary treatment.

Ninety-six hours before the end of the trial on day 18, subjects were intravenously (iv) injected with 15 mg $D_7$-cholesterol and simultaneously ingested 90 mg [3,4-$^{13}$C]-cholesterol. Five subjects during the first phase did not receive $D_7$-cholesterol. In these cases, the average enrichment from the other two phases was calculated and utilized as a surrogate for the true $D_7$-cholesterol enrichment. The $D_7$-cholesterol isotope was prepared for injection by dissolving it in ethanol at a concentration of 5 mg/ml under sterile conditions. The isotope/ethanol mixture was then added drop-wise to Intralipid™ for a total injectable volume of 9 ml. Blood samples were taken at baseline, 6 h, and 12 h on day 18, as well as fasting samples on days 19, 20, and 21 to monitor isotopic enrichment/decay levels. On day 21 of each feeding period, approximately 25 ml of deuterium oxide was given orally to each subject. The change in deuterium enrichment within red blood cell (RBC) free cholesterol was determined as an index of synthesis over days 21 and 22 (72 and 96 h after initial isotope administration). The time line for the protocol used is provided in **Fig. 1**.

## Lipoprotein lipid analyses

Blood was centrifuged for 15 min at 1,500 rpm within 30 min of phlebotomy to separate plasma from RBCs. Plasma and RBCs were immediately stored at −80°C until further analysis. Plasma TC, high density lipoprotein cholesterol (HDL-C), and TG concentrations were analyzed in quadruplicate with enzymatic kits, standardized reagents, and standards using a VP Autoanalyzer (Abbott Laboratories, North Chicago, IL). The equation of Friedewald, Levy, and Fredrickson (16) was used to calculate LDL-C concentrations.

## Determination of cholesterol absorption

Cholesterol absorption was determined using the dual stable isotope ratio technique of Bosner et al. (17). Free cholesterol extracted from RBCs was used to determine $^{13}$C- and $D_7$-cholesterol enrichment. Red cells contain almost exclusively free cholesterol designated as part of the rapid changeover pool. Briefly, lipid was extracted from RBC in duplicate using a modified Folch, Lees, and Sloane Stanley extraction procedure (18). Thin-layer chromatography (20 × 20 cm, 250 μ, Scientific Adsorbents Inc., Atlanta, GA) was used to separate free cholesterol from cholesteryl ester. The free cholesterol band was scraped from the silica gel plate and saponified with 0.5 m methanolic KOH to eliminate any fatty acid contaminants. Free cholesterol extracts were dried under nitrogen and transferred into 18-cm sealed combustion tubes (Vycor, Corning Glass Works, Corning, NY). Cupric oxide (0.5 g) and a 2-cm length of silver wire were added and tubes were sealed at less than 20 mtorr pressure. Cholesterol samples were then combusted to $^{13}$C-enriched $CO_2$ and D-enriched water for 4 h at 520°C. The generated $CO_2$ was transferred under vacuum into Vycor tubes for measurement of $^{13}$C enrichment, while D-enriched water was vacuum-distilled into sealed tubes containing 60 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington, IN). Tubes containing water and zinc were reduced to D-enriched hydrogen gas at 520°C for 30 min.

Nuclear magnetic resonance was used to verify that the isotopic enrichments of the starting materials [3,4]-$^{13}$C-cholesterol and $D_7$-cholesterol (CDN Isotopes, Point Claire, Quebec) were greater than 99 atom percent excess. The $^{13}$C enrichments of free cholesterol were measured by differential isotope ratio mass spectrometry (IRMS) using an automated dual inlet system (SIRA 12, Isomass, Cheshire, UK). Enrichments were expressed relative to Pee Dee Belemnite (PDB) limestone standard of the National Bureau of Standards (NBS). Linearity and gain of response of the SIRA IRMS instrument were assessed using a reference tank $CO_2$ and NBS standards of known isotopic enrichment. The D enrichments of free cholesterol were measured by differential IRMS using a manually operated dual inlet system with electrical $H^{3+}$ compensation (VG Isomass 903D, Cheshire, UK). For deuterium, enrichments were expressed relative to Standard Mean Ocean Water (SMOW) and a series of NBS standards of known enrichment were analyzed concurrently on each day of measurement to correct for any variations in linearity of gain of response of the 903D IRMS. Precision of measurement expressed as coefficient of variation for replicate $^{13}$C and D enrichment analyses was 0.08 and 2.3 *del* (parts per thousand relative to PDB and SMOW standards), respectively. The $^{13}$C and D enrichments in 48 and 72 h RBC free cholesterol relative to baseline (t = 0) samples were utilized to calculate the percent cholesterol absorption using the ratio of orally ingested $^{13}$C- to intravenously administered D-cholesterol as described by Bosner et al. (17) where:

$$\text{absorption (pool/pool)} = \frac{del^{13}\text{C} \times 7 \times \text{iv dose of D-cholesterol(mg)} \times 0.0112}{del\text{D} \times 2 \times \text{i.g. dose of } ^{13}\text{C-cholesterol(mg)} \times 0.000155} \quad \textit{Eq. 1}$$

where *del* for $^{13}$C and D is the difference between the enriched sample at 48 or 72 h and the baseline abundance (at t = 0) in parts per thousand relative to PDB and SMOW standards, respectively, the factor 7/2 reflects the ratio of labeled atoms per mg of dose, and the constants 0.0112 and 0.000155 represent factors converting the *del* to equivalent atom percent excess for the PDB and SMOW scales, respectively.

## Determination of cholesterol biosynthesis and turnover

Cholesterol biosynthesis was determined as the rate of incorporation of deuterium from body water into RBC membrane free cholesterol over the period between 72 and 96 h at the end of each treatment as described (19). Fractional synthesis rate (FSR) represents that fraction of the cholesterol pool that is synthesized in 24 h and is calculated as per the equation (20, 21):

$$\text{FSR (pools/day)} = (del_{\text{cholesterol}}/del_{\text{plasma}}) \times 0.478 \quad \textit{Eq. 2}$$

where *del* for D enrichment in cholesterol is the difference between enriched free cholesterol and plasma water at 72 and 96 h in parts per thousand relative to a SMOW standard. The factor 0.478 reflects the ratio of labeled H atoms replaced by D ($^{22}/_{46}$) during in vivo biosynthesis (19).

Turnover rates for RBC free cholesterol were determined from the $D_7$-cholesterol enrichment decay curve over 24 to 72 h. Exponential curves were fitted to 24, 48, and 72 h RBC $D_7$ cho-

lesterol enrichments after subtraction of baseline D abundance at 0 h.

### Determination of plasma plant sterol levels

Plant sterols were measured by gas–liquid chromatography (GLC) (HP 5890 Series II, Palo Alto, CA) equipped with flame ionization detection and auto-injector system as described (11, 13). A 30-m SAC-5 column (Sigma-Aldrich Canada Ltd., Oakville, Ont.) was used. Briefly, an internal standard, 5 alpha-cholestane, was added to each plasma sample. Samples were saponified and sterols were extracted, re-suspended in chloroform, and injected into the GLC. The column temperature was 285°C. Isothermal running conditions were maintained for 42 min. The injector and detector were set at 300°C and 310°C, respectively. The carrier gas (helium) flow rate was 1.2 ml/min with the inlet splitter set at 100:1. Individual plant sterol and stanols were identified using authentic standards (Sigma-Aldrich Canada Ltd., Oakville, Ont). Internal standards were used to calculate detector response factors. Margarine mixtures were also analyzed by GLC to assess sterol and stanol, as well as fatty acid, content using similar procedures.

### Analysis of data

Data were expressed as the mean ± standard error mean (SEM). For lipoprotein cholesterol, TG, and phytosterol levels, data at commencement and end of each dietary period were compared using a crossover repeated measures ANOVA model to identify time and treatment effects and their interactions. Endpoints for lipid level data were taken as averages of day 21 and 22 values. For cholesterol absorption, synthesis, and turnover, data at the end of each dietary period were compared using a crossover ANOVA model to identify treatment effects. When treatment effects were identified as significant, Duncan's post-hoc tests were utilized to identify significant effects of diet at particular time-points. Separate comparisons were performed on endpoints for circulating lipid levels using ANOVA followed by Students' paired *t*-tests with Bonferroni adjustment to control the overall alpha level. Tests for associations between variables were also performed using Pearson Correlation Coefficient analyses. Specifically, associations were investigated between circulating sterol levels at the end of each treatment and cholesterol absorption, cholesterol biosynthesis, and plant sterol levels. The data were analyzed using Proc-General Linear Model SAS (version 6.12) software.

## RESULTS

### Subject compliance and drop-out rate

Eighteen subjects commenced the study protocol, with sixteen subjects completing all three treatments. However, data from only 15 subjects were included in final analyses because two subjects left the study at the end of the first phase due to personal reasons and a third was terminated due to poor compliance.

### Subject blood and urine parameters and demographic response to treatment

Complete blood counts (CBC), biochemistry (SMAC), and urinalysis results during the three phases of the trial remained within normal ranges. Regular physical exams revealed no suggestion of any clinical irregularities. No significant mean group weight changes occurred across any of the three treatments. Subjects tolerated the diet without any reported adverse effects, reporting no abnormal or atypical smell, taste, color, or mouth-feel effects across treatments. Subjects were unable to distinguish between dietary treatments.

### Plasma lipid profile in response to treatment

Plasma TC concentrations measured across all treatments of the feeding trial showed large between-subject variation (**Table 2**), while the time by diet interaction was

TABLE 2. Plasma lipid levels at days 0 and mean of days 21 and 22 of each dietary period

| Lipid | β-Sitosterol Ester | Sitostanol Ester | Control |
|---|---|---|---|
| | | *mmol L*$^{-1}$ | |
| Total cholesterol | | | |
| Day 0 | 6.39 ± 0.18 | 6.37 ± 0.18 | 6.47 ± 0.22 |
| Day 21/22 | 5.49 ± 0.15**$^{b}$ | 5.71 ± 0.18**$^{ab}$ | 6.04 ± 0.18*$^{a}$ |
| % Change | −13.4 ± 2.6$^{b}$ | −10.2 ± 2.1$^{ab}$ | −6.0 ± 2.4$^{a}$ |
| % Relative to control | −9.1 | −5.5 | |
| Low density lipoprotein | | | |
| Day 0 | 4.29 ± 0.25 | 4.35 ± 0.23 | 4.46 ± 0.25 |
| Day 21/22 | 3.66 ± 0.15**$^{c}$ | 3.95 ± 0.19*$^{b}$ | 4.22 ± 0.18$^{a}$ |
| % Change | −12.9 ± 3.0$^{b}$ | −7.9 ± 3.7$^{ab}$ | −3.9 ± 3.1$^{a}$ |
| % Relative to control | −13.2 | −6.4 | |
| Triglyceride | | | |
| Day 0 | 2.52 ± 0.21 | 2.39 ± 0.20 | 2.24 ± 0.21 |
| Day 21/22 | 1.98 ± 0.21* | 1.86 ± 0.16** | 1.93 ± 0.15 |
| % Change | −18.9 ± 8.3 | −17.4 ± 5.6 | −5.7 ± 9.3 |
| % Relative to control | 1.0 | 0.9 | |
| High density lipoprotein | | | |
| Day 0 | 0.99 ± 0.06 | 0.92 ± 0.05 | 0.98 ± 0.06 |
| Day 21/22 | 0.93 ± 0.06 | 0.93 ± 0.05 | 0.93 ± 0.06 |
| % Change | −5.6 ± 2.3 | 0.8 ± 2.7 | −4.5 ± 3.2 |
| % Relative to control | 0.0 | 0.0 | |

Values are expressed as mmol/L ± SEM. Values carrying different superscript letters indicate that there is a significant difference between mixtures ($P < 0.05$). Percent change is based on individual data. Percent change relative to control is based on the mean for days 21 and 22.

*$P < 0.05$; ** $P < 0.01$: significant differences within each diet (between day 0 and mean of days 21 and 22).

marginally significant ($P = 0.09$). Main effects of time and diet ($P < 0.0001$ and 0.05, respectively) were significant for TC levels. Mean reductions in total cholesterol over days 21 and 22, relative to day 0, were 13.4 ± 2.6, 10.2 ± 2.1, and 6.0 ± 2.4% for sterol ester, stanol ester, and control treatments, respectively. When means of TC levels at the end of each phase were compared, control values were higher than those after consumption of the sterol ($P < 0.005$), but not stanol ester-containing diets (**Fig. 2**). The decline in TC levels at the end of sterol and stanol ester phases, relative to that at the end of the control phase, were 9.1 and 5.5%, respectively.

As with TC level, between-individual variation in LDL-C concentration responses were substantial (Table 2). While time-by-diet interactions were not significant, main effects of time ($P = 0.0001$) and diet ($P < 0.01$) for LDL-C levels were observed. Mean reductions in LDL-C levels over days 21 and 22, relative to day 0, were 12.9 ± 3.0, 7.9 ± 3.7, and 3.9 ± 3.1% for sterol ester, stanol ester, and control treatments, respectively. Both the sterol and stanol ester-containing margarines decreased ($P < 0.02$) LDL-C concentration relative to control (**Fig. 3**). Moreover, significant differences were identified among all three treatments at days 21/22, with the lowest levels observed with the sterol ester margarine. The declines in LDL-C levels at the end of the sterol and stanol ester phases, relative to control, were 13.4 and 6.4%, respectively.

TG and HDL-C concentrations are listed in Table 2. There were no significant effects of diet on either TG or HDL-C levels with either of the margarines. However, a significant main effect of time ($P < 0.005$) was found for TG and HDL-C, with levels declining over time in each case. Plant sterol and stanol-induced changes in both TG and HDL-C, relative to changes in control, were minimal (Table 2).

### Cholesterol absorption in response to treatment

Cholesterol absorption data, provided as enrichment values for $^{13}$C- and D-labeled cholesterol in RBC free cholesterol are presented in **Fig. 4.** At 48 h after isotope administration, mean cholesterol absorption coefficient (cholesterol oral dose/cholesterol iv dose) was lower ($P < 0.005$) after sterol ester and stanol ester feeding (0.387 ± 0.056 and 0.454 ± 0.087 pool/pool, respectively), relative to control feeding (0.664 ± 0.078 pool/pool). There was,

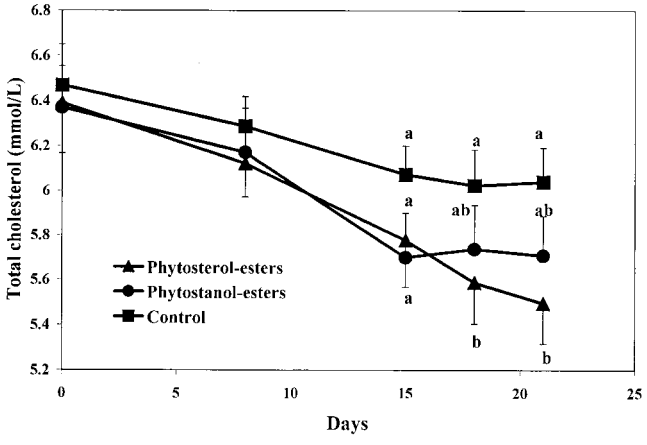


**Fig. 2.** Plasma total cholesterol levels of subjects over each dietary period. Different letters indicate significant differences ($P < 0.02$) among diets. Endpoint data represent means of values obtained on days 21 and 22. Bars represent SEM (standard error of mean).

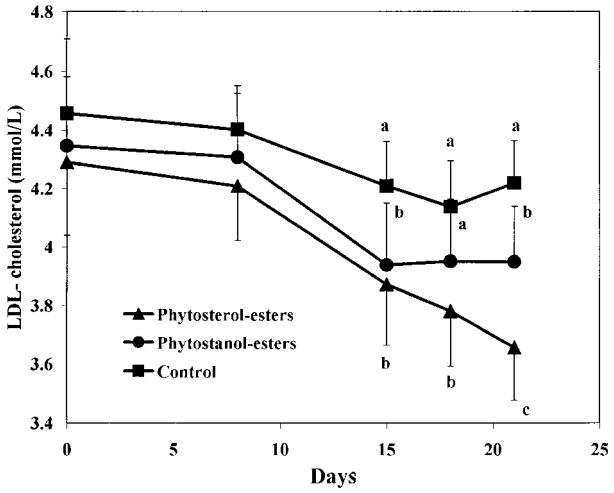


**Fig. 3.** Plasma low density lipoprotein levels of subjects over each dietary period. Different letters indicate significant differences ($P < 0.02$) among diets. Endpoint data represent means of values obtained on days 21 and 22. Bars represent SEM (standard error of mean).

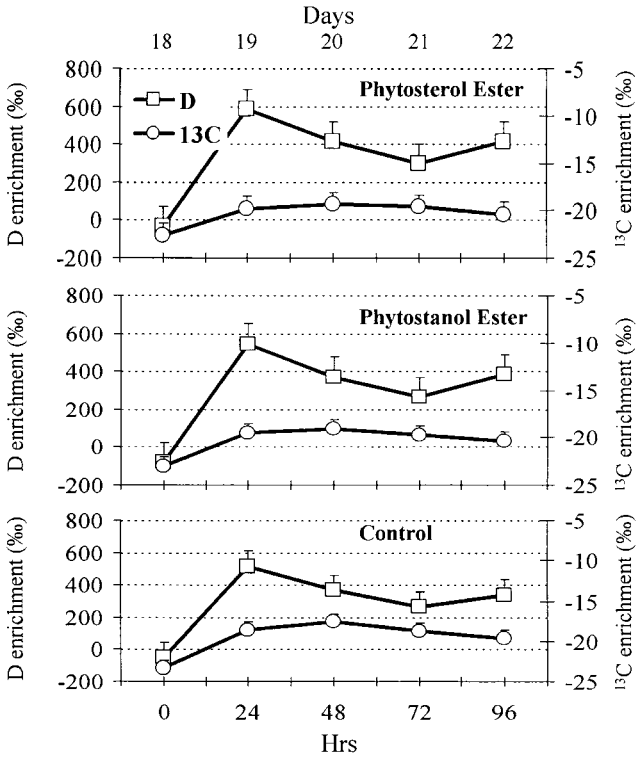


**Fig. 4.** Enrichment of $^{13}$C and D in plasma cholesterol during the final week of each dietary period. Bars represent SEM (standard error of mean).

however, no difference observed in cholesterol absorption between the sterol versus stanol-containing diets. The trend in absorption rate tended to be proportional to the extent of cholesterol-lowering, particularly in the case of LDL-C.

At 72 h, mean cholesterol absorption coefficient was also marginally lower ($P = 0.08$) after sitosterol ester (0.488 ± 0.078 pool/pool), relative to control (0.709 ± 0.092 pool/pool) diets. There was, however, no difference observed in cholesterol absorption between the two diets containing phytosterols, or between the stanol ester (0.550 ± 0.122 pool/pool) and control diets. The trend in absorption here also tended to directly mirror the degree of cholesterol-lowering in that the group with the lowest LDL-C levels in the group displayed the most suppressed cholesterol absorption.

When means of each time-point (i.e., 48 and 72 h) were computed for each subject, cholesterol absorption was lower ($P < 0.01$) after sterol and stanol ester feeding (0.438 ± 0.062 and 0.502 ± 0.103 pool/pool, respectively), relative to control (0.687 ± 0.083 pool/pool) diet. Relative to control, absorption was reduced 36.2 and 25.9% for sterol ester and stanol ester-containing diets, respectively. There was, however, no difference in cholesterol absorption coefficient between the sterol versus stanol-containing diets. At day 21, the group provided with sterol esters displayed both the lowest LDL-C levels and the lowest cholesterol absorption coefficient.

### Cholesterol synthesis in response to treatment

Deuterium enrichments in RBC cholesterol after deuterated water during 72–96 h post-infusion are shown in Fig. 4. Fractional synthesis rates were higher ($P < 0.05$) after sterol and stanol ester feeding (0.0535 ± 0.0069 and 0.0481 ± 0.0054 pools/day, respectively), relative to the control diet (0.0349 ± 0.0043 pools/day). Relative to control, synthesis was increased 53.3 and 37.8% for sterol and stanol ester-containing diets, respectively. There was, however, no statistically significant difference observed in synthesis rates between the sterol and stanol-containing diets. The trend in synthesis rate tended to inversely mirror the degree of cholesterol-lowering and cholesterol absorption coefficient. At day 21, the lowest LDL-C levels in the group provided with sterol esters corresponded with a similar trend towards higher cholesterol biosynthesis within the same group.

### Cholesterol turnover in response to treatment

Turnover rates were obtained from the cholesterol deuterium enrichment data over 24–72 h (Fig. 4) for treatments containing sterol esters, stanol esters, and control margarines. RBC free cholesterol turnover rates were 0.310 ± 0.16, 0.308 ± 0.14, and 0.293 ± 0.21 pools/day, respectively. There were no significant differences observed between diets.

### Plasma plant sterol profile in response to treatment

Plasma phytosterol concentrations and ratios are presented in **Table 3**. Mean plasma concentrations of campesterol and β-sitosterol at day 21 increased ($P < 0.0001$) compared with day 0 with consumption of sterol ester diet by 71.6 and 32.5%, respectively, and were significantly different ($P < 0.05$) from the changes observed with consumption of stanol esters and control diets. The ratio of campesterol:β-sitosterol with sterol ester increased ($P < 0.0001$) over time by 32.7%, which was different ($P < 0.05$) from the changes observed with the stanol ester and control diets. Consumption of the sterol ester-containing diet increased ($P < 0.0001$) both the campesterol:cholesterol and β-sitosterol:cholesterol ratios by 94.2 and 53.5%, respectively, and both ratios differed from those of diets containing stanol esters or the control margarine. Even though consumption of the stanol ester margarine decreased ($P < 0.0001$) the campesterol:cholesterol and β-sitosterol: cholesterol ratios by 19.7 and 13.8%, respectively, neither ratio was significantly different from those after control diets.

### Associations between plasma lipid levels and kinetic measurements

For all study subjects, both plasma campesterol ($r = 0.36$, $P < 0.02$) and β-sitosterol ($r = 0.53$, $P < 0.0005$) levels were found to vary directly with plasma TC concentration. Similarly, β-sitosterol ($r = 0.36$, $P < 0.02$) levels were found to vary directly with the level of LDL-C in plasma. Furthermore, whereas cholesterol absorption coefficient was marginally correlated ($P = 0.07$) with TC concentration, there was a stronger association ($r = -0.45$, $P < 0.002$) between cholesterol synthesis and TC concentrations. Moreover, FSR varied inversely with plasma levels of campesterol ($r = -0.32$, $P < 0.05$) and β-sitosterol ($r = -0.45$, $P < 0.005$) in all subjects. When subjects were analyzed by treatment grouping, an association ($r = 0.53$, $P < 0.05$) between TC concentration and cholesterol absorption coefficient was observed for the sterol ester but not for the stanol ester or control diets.

## DISCUSSION

This study shows for the first time that, under controlled dietary conditions, plant sterols possess improved efficacy in reducing plasma total and LDL-C concentrations compared with stanol esters at the level of intake presently utilized. This reduction occurred as a result of the ability of sterols and stanols to depress cholesterol absorption while partially de-suppressing cholesterol biosynthesis. The fact that the final circulatory TC level was positively associated with the cholesterol absorption, particularly in the group showing the most pronounced cholesterol lowering, indicates inhibition of absorption as a chief mechanism in the cholesterol-modulating effect of these dietary agents. In addition to sterols and stanols, dietary fatty acid composition may have also been partially responsible for the changes in lipids observed. Levels of all lipids measured, except HDL cholesterol, fell across the control dietary period, likely indicative of the beneficial substitution of unsaturated fat for that typically consumed by subjects.

Although moderate reductions in plasma total and LDL-C concentrations have been demonstrated with the use of plant sterols (8, 9, 14, 22), stanols at similar or lower dosages have been suggested as possessing a greater lipid-

TABLE 3. Plant sterol levels at day 0 and 21 of each dietary period

| Phytosterol | β-Sitosterol Ester | Sitostanol Ester | Control |
|---|---|---|---|
| | | *mmol L$^{-1}$* | |
| Campesterol | | | |
| Day 0 | 0.0128 ± 0.0015 | 0.0144 ± 0.0017 | 0.0124 ± 0.0014 |
| Day 21 | 0.0209 ± 0.0024**$^{a}$ | 0.0099 ± 0.0012*$^{b}$ | 0.0119 ± 0.0016$^{b}$ |
| % Change | 71.6 ± 13.1$^{a}$ | −27.9 ± 7.6$^{b}$ | 4.1 ± 12.6$^{b}$ |
| % Relative to control | 75.6 | −16.8 | |
| | | *mmol L$^{-1}$* | |
| β-Sitosterol | | | |
| Day 0 | 0.0086 ± 0.0007 | 0.0100 ± 0.0009 | 0.0085 ± 0.0008 |
| Day 21 | 0.0112 ± 0.0012**$^{a}$ | 0.0076 ± 0.0009**$^{b}$ | 0.0086 ± 0.0010$^{b}$ |
| % Change | 32.5 ± 10.9$^{a}$ | −22.7 ± 7.6$^{b}$ | 7.6 ± 10.9$^{a}$ |
| % Relative to control | 30.2 | −11.6 | |
| | | *mol mol$^{-1}$* | |
| Campesterol:β-sitosterol | | | |
| Day 0 | 1.446 ± 0.078 | 1.408 ± 0.088 | 1.427 ± 0.046 |
| Day 21 | 1.857 ± 0.049**$^{a}$ | 1.308 ± 0.055$^{b}$ | 1.388 ± 0.065$^{b}$ |
| % Change | 32.7 ± 6.8$^{a}$ | −4.7 ± 4.2$^{b}$ | −1.6 ± 5.1$^{b}$ |
| % Relative to control | 33.8 | −5.8 | |
| | | *mmol mol$^{-1}$* | |
| Campesterol:total cholesterol | | | |
| Day 0 | 0.199 ± 0.022 | 0.223 ± 0.024 | 0.185 ± 0.017 |
| Day 21 | 0.371 ± 0.037**$^{a}$ | 0.168 ± 0.018**$^{b}$ | 0.192 ± 0.021$^{b}$ |
| % Change | 94.2 ± 12.3$^{a}$ | −19.7 ± 8.9$^{b}$ | 13.5 ± 14.6$^{b}$ |
| % Relative to control | 93.2 | −11.9 | |
| | | *mmol mol$^{-1}$* | |
| β-Sitosterol:total cholesterol | | | |
| Day 0 | 0.132 ± 0.010$^{ab}$ | 0.157 ± 0.013$^{a}$ | 0.128 ± 0.011$^{b}$ |
| Day 21 | 0.198 ± 0.017**$^{a}$ | 0.128 ± 0.013*$^{b}$ | 0.142 ± 0.014$^{b}$ |
| % Change | 53.5 ± 11.3$^{a}$ | −13.8 ± 9.6$^{a}$ | 19.4 ± 13.7$^{b}$ |
| % Relative to control | 39.4 | −9.8 | |

Values are expressed ± SEM. Different superscript letters indicate significant differences ($P < 0.05$) between diets. Percent change is based on individual data; percent change relative to control diet for day 21.

* $P < 0.05$; ** $P < 0.01$: significant difference within each diet (between day 0 and day 21).

reducing capacity (23–29). However, only one previous study performed a systematic cross-comparison of sterol and stanol esters in adults (10). Here, consumption of esterified sterol and stanol mixtures resulted in reductions in plasma TC concentrations, which were for the greater part indistinguishable (10). Differences between study design including phytosterol dosage or formulation, or dietary composition may be responsible for the variable results observed between the present and previous (10) studies.

Major sub-objectives of the present research were to assess mechanisms of action through which these plant materials act. To meet these objectives, we utilized a novel combination of techniques for measurement of cholesterol absorption, biosynthesis, and turnover using three isotopes provided simultaneously, cholesterol labeled with $^{13}$C and D and deuterated water. Each technique possesses strengths and drawbacks.

The approach used to measure cholesterol absorption presently is that of the accepted procedure described by Bosner et al. (17), which assesses the ratio in the plasma pool of labeled cholesterol given orally versus that provided intravenously. However, Bosner et al.(17) measured enrichments with selected ion monitoring mass spectrometry; our study is the first to use more sensitive isotope ratio mass spectrometry, together with $^{13}$C and D labeled cholesterol tracers. We (11) and others (30) have previously utilized the ratio of $^{18}$O to $^{13}$C cholesterol to determine its coefficient of absorption in animals and in humans. However, both current problems of $^{18}$O isotope availability and the corrections needed when $^{18}$O-labeled cholesterol is combusted with copper oxide resulted in the substitution of polydeuterated cholesterol in the present experiment.

The dual isotope absorption measurement method uses intravenously administered labeled cholesterol as a tracer to monitor of exit rate of both body and dietary cholesterol from the rapid pool relative to orally administered cholesterol labeled with a second tracer to monitor the rate of intestinal absorption. This ratio allows for correction in turnover rate possibly affected by diet or physiological state. Presently we observed that turnover rates of cholesterol were 25–30% per day and did not vary across dietary treatments. However, when the coefficient of absorption was calculated using equation 1, it was demonstrated that absorption rate was reduced significantly with the consumption of the sterol ester-containing diet, and reduced at a level that approached statistical significance with the consumption of the stanol ester diet. The levels

of reduction of cholesterol absorption with sterol and stanol ester diets, relative to control (36.3 and 26.9%, respectively), are not substantially different from those reported in animals for stanol esters (36.1%) at comparable doses (11). In humans using serum cholestanol/cholesterol ratios as an index of cholesterol absorption, stanol ester mixtures provided at approximately 3 g/d for 6 weeks resulted in a decline in absorption of 6 to 21% relative to control diets (31).

The use of deuterium as a marker for cholesterol biosynthesis is based on the methodology developed in animals (19) using radioactive tritiated water. Early work (32) has shown that the equilibration of deuterated cholesterol across plasma and red cell sterol pools is extremely rapid, but that attaining a plateau of enrichment is not achieved even after 100 days of constant deuterium enrichment of body water. These data, taken together with our results demonstrating that deuterium uptake is largely linear over 48 h (20), allow a monoexponential linear model to be fit to the deuterated cholesterol enrichment curve. Application of this model facilitates the interpretation of the deuterium incorporation results.

Although the deuterium uptake methodology has been validated against cholesterol balance (21), plasma mevalonic acid levels (33), and more recently mass isotopomer distribution analysis (M. F. Di Buono, P. J. H. Jones, L. Wykes, and L. Baumier, unpublished results), the technique has not been previously applied in a context where baseline deuterium levels were changing, as in the present experiment. The present application of the method used a modified approach, assessing the baseline enrichment of deuterium at 72 h. It is evident from the 24 and 48 h D enrichment profiles (Fig. 4) that the 72 h D enrichment values likely overestimated the true baseline at 96 h, as the D enrichment continued to decrease over 72 to 96 h. Indeed, mean FSR values were lower than those previously observed in other dietary studies (20, 21, 33). It was contemplated that extrapolation of the true 96 h baseline from the three previous time-points might be a possible means of obtaining a more accurate value for FSR. However, the imprecision created in extrapolating cholesterol deuterium incorporation measure is considered to outweigh any systematic underestimation of FSR by using the true 72 h accessible values. The approach taken did permit almost simultaneous measurement of cholesterol absorption, synthesis, and turnover, as well as circulating lipoprotein cholesterol levels, during the period of investigation. Moreover, demonstration of enhanced cholesterol biosynthesis in response to addition of dietary phytosterols confirms the results of previous reports (34) which identify this compensatory mechanism as a part of the metabolic response to the reduction in cholesterol absorption. The increase in synthesis with the sterol and stanol ester-containing diets seen presently is consistent with previous human (34) and animal (11) data.

Concurrent with measurement of cholesterol synthesis and absorption, changes in plant sterol levels in plasma were also detected. Absolute levels of circulatory β-sitosterol and campesterol found in the present study were not dissimilar to those seen previously (13, 14). As previously, we were unable to detect sitostanol in plasma in any group, although this component has been identified in previous work in animals (27). Data in the present experiment show that changes in levels of campesterol and β-sitosterol were diet-dependent. Particularly, 71.6% and 32.5% increases in plasma campesterol and β-sitosterol levels, respectively, were observed during consumption of the sterol ester but not with the consumption of the other diets. These findings indicate that the non-hydrogenated plant sterols are absorbed to a certain extent. The lower reported absorption efficiency of stanols (14) is likely responsible for the failure to observe any increase in the plasma level of this compound after consumption of a stanol ester-enriched diet for 3 weeks. Indeed, a decrease in both β-sitosterol and campesterol levels was noted in subjects consuming stanol esters. The potential effects of changing circulating plant sterol levels after consumption of phytosterols are not known. It has been suggested that individuals heterozygous for the disorder phytosterolemia, a rare genetic inborn disease, absorb more β-sitosterol than do healthy individuals (35, 36), however, levels of circulatory plant sterols in these individuals are similar to those found in vegetarians (37).

In conclusion, the present study demonstrates that in esterified forms, phytosterol efficacy in total and LDL-cholesterol lowering may be influenced by the saturation state of the plant sterols constituents. Moreover, a mechanism contributing to the action of plant sterols in lowering TC and LDL-C concentrations is through reduction of absorption of dietary cholesterol. Cholesterol biosynthesis is de-suppressed, but not to an extent that plasma cholesterol levels are unaffected. In summary, both esterified β-sitosterol and sitostanol are efficacious in favorably reducing circulating cholesterol concentrations in hyperlipidemic males.

Financial support for this research was received from Lipton Inc. Canada. The diligent effort, skill, and efficiency of Christopher Vanstone, Fenny Ismoyo, Hui Li, Martine Bernard, Cynthia Barrand, Andrea Papamandjaris, Nirupa Matthan, Marie-Pierre St. Onge, and Dayle Eshelby is acknowledged.

*Manuscript received 30 September 1999 and in revised form 8 February 2000.*

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