Forbes Medi-Tech INC.

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Food and Drug Administration
Room 1061
5630 Fishers Lane
Rockville, MD 20852

RE: Food Labeling: Health Claims; Plant Sterol/
Stanol Esters and Coronary Heart Disease
Docket Nos. 00P-1275 and 00P-1276
66 Fed. Reg. 50824 (October 5, 2001)

This letter is submitted by Forbes Medi-Tech Inc, Dr. Peter Jones, Dr. William E. Connor and Dr. J. Frohlich in response to the Food and Drug Administration's ("FDA") request in the October 5, 2001 Federal Register, (66 FR 50824-6) for additional comments on its interim final rule permitting health claims stating that plant sterol/stanol esters may reduce the risk of coronary heart disease. Forbes Medi-Tech Inc manufactures the phytosterol ingredient which is being incorporated into food products manufactured by Quaker/Altus.

We have reviewed the comments submitted by Altus, Quaker and Novartis and recognize that their comments adequately address the labeling and safety issues raised. We are separately addressing in more detail questions from the FDA which relate to the lower qualifying limit for the health claim and the equivalence of phytosterols and phytosterol esters as well as the equivalence of sterols, stanols and mixtures thereof. In our comments, phytosterol ingredient comparisons are done on the basis of the sterol or stanol content of esters to avoid confusion since about 40% of the weight of the stanol/sterol esters is fatty acids. The term "phytosterol" is commonly used to include both sterols and stanols and their esters and that definition is adhered to in these comments. It has been recognized by the FDA, that the active form of the phytosterol ester is the free phytosterol which is rapidly liberated from the fatty acid moiety in the small intestine by the action of digestive enzymes. On this basis, it is our view that the free phytosterols should be considered as equivalent to the esterified forms. Certainly, the available human data on activity of non-esterified and esterified phytosterols indicate that on the basis of phytosterol content, there exist no clinically significant difference in cholesterol lowering activity between these forms.

An important issue is the equivalent bioactivity of sterols and stanols particularly at low dosages. In the Interim Final Rule, the FDA set the qualifying dose at 0.8 g/day for sterols (1.3 g/day sterol esters) and 2.0 g/day for stanols (3.4g/day stanol esters). At the time of the ruling, only 4 studies were available to the FDA which had a direct bearing on this issue. In two studies (Table 1), stanol esters and sterol esters were compared directly at the same dosages and conditions. In the study by Westrate and Meijer(1998), cholesterol lowering activity was compared at the relatively high dose of 3 g per day. No significant differences were observed between sterol esters and stanol esters in the lowering of LDL cholesterol. In the study by Jones et al (2000) an intermediate dosage of 1.8 g per day was tested. The reduction in LDL cholesterol was less in the stanol ester group (-6.4%) than in the sterol ester group (-13.2%). The FDA also evaluated two available dose response studies (Table 2); Hendriks et al., 1999 (sterol esters) and Hallikainen et al., 2000 (stanol esters). At the lowest dose, 0.8g/day, the sterol esters significantly lowered LDL

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cholesterol whereas the stanol esters did not. Taken together, these four studies would suggest that although sterols and stanols are equivalent at a high (3g/day) dosage, they are not equivalent at lower dosages. The stanol esters appeared to be ineffective at the low end (0.8g/day) of the dosage range.

We can provide additional data on the comparative effectiveness of non-esterified stanols and sterols in the intermediate (1.8 g/day) dosage range. In a study by Vanstone et al. (2002) which has been accepted for publication in the Am. J. Clin. Nutr., non-esterified stanols were compared with sterols and a 50:50 mixture of stanols and sterols in a butter matrix over an interval of 21 days. Stanols, sterols and their mixture were similarly effective in lowering LDL cholesterol. See Table 1 for summary. This new data shows that stanols and sterols are equivalent in the mid-dose range.

The protein component of LDL, apolipoprotein B, was also measured in the stanol ester study by Hallikainen et al., 2000. At the lowest dose (0.8g/day), the reduction in apo B(-8.7%), was statistically significant. The FDA has asked for comments on the use of serum apo B as a valid predictor of CHD risk and on the relative utilities of apo B and LDL cholesterol in predicting CHD risk. There is substantial evidence to show that apo B is actually a better marker of CHD risk and treatment outcomes than LDL cholesterol (Lamarche et al., 1995; Moss et al., 1999; Gotto et al., 2000; van Lenep et al., 2000).

Related to the dose response characteristics of sterols and stanols, additional data was provided to the FDA in the form of a final clinical study report ( Beer et al, 2000). A non-esterified mixture of stanols and sterols (31% stanols, balance as sterols) was compared at doses of 0.9, 1.8, and 3.6 g per day over a 28 day interval. The reduction in LDL cholesterol at the 0.9 g/day dose (-7.4%) was comparable to the reduction with sterol esters at the 0.83 g/day dose (-6.2%) (Hendriks et al., 1999). These data suggest that the stanol component in the mixture did not reduce its efficacy in cholesterol lowering over the entire dosage range.

In Table 2, the dose response data from the above study are compared with dose response data for sterol esters and stanol esters. If apo B is considered as a valid surrogate risk marker for CHD, the dose response characteristics are similar, if not identical for sterols, stanols and mixtures of sterols and stanols. All show efficacy in the range of the 0.8 g/day limiting dose set by the FDA for sterols (1.3 g/day sterol esters) and show a dose related effect.

The totality of the evidence available is that stanols, sterols, mixtures and esters thereof are equivalent and should be considered as a single class of molecules which by common usage are called phytosterols. At present, the evidence does not support maintaining a distinction between components within that class. The lower qualifying daily dosage value therefore should be applied to all phytosterols. We are in agreement with the FDA's limitation of the claim to phytosterols derived from either the vegetable oil source (virtually all from soy) or from products of the kraft pulping process. The efficacy of phytosterols from other sources is presently unsubstantiated.

As noted above, we are in complete accordance with the comments of Altus and Novartis on safety and labeling. Additionally, we would emphasize that ingredient contents listed on packaging of products containing esterified phytosterols be based on the phytosterol content and not weight of the phytosterol esters to avoid consumer confusion.

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References

1. 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.

2. Jones PJ, Raeini-Sarjaz, Ntanios FY, Vanstone CA, Feng JY, Parsons WE, 2000. Modulation of plasma lipid levels and cholesterol kinetics by phytosterols versus phytostanol esters. J of Lipid Research, 41:697-705.

3. Hendriks HFJ, Westrate 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.

4. Hallikainen MA, Sarkkinen ES, Gylling H, Erkkila AT, Uusitupa MI, 2000. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. Eur J Clin Nutr 54:715-25.

5. Vanstone, CA, Raeini-Sarjaz, M, Parson, WE, Jones, PJH, 2001. Unesterified plant sterols and stanols lower low-density (LDL) cholesterol independent of the degree of hydrogenation in hypercholesterolemics. Accepted for publication in 2002 by the American Journal of Clinical Nutrition.

6. Lamarche B, Tchenof A, Moorjani S, Cantin B, Dagenais GR, Moorjani S, Lupien PH, 1996. Small, dense low-density lipoprotein particles as a predictor of the risk of ischemic heart disease in men. Prospective results from the Quebec Cardiovascular Study. Circulation 95:69-75.

7. Moss AJ, Goldstein Re, Marder VJ et al. Thrombogenic factors and recurrent coronary events. Circulation 1999;99:2517-2522.

8. Gotto AM Jr, Whitney E, Stein EA, Shapiro DR et al. Relation between baseline and on-treatment lipid parameters and first acute major coronary events in the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS). Circulation 2000;101:477-484.

9. van Lennep JE, Westerveld HT, van Lennep HW, Zwinderman AH, Erkelens DW, van Der Wall EE Apolipoprotein concentrations during treatment and recurrent coronary artery events. Arterioscler Thromb Vasc Biol 2000; 20:2408-13.

10. Beer MU, Pritchard H, Belsey, EM, Davidson, M, 2000. Effect of a milk drink enriched with increasing doses of free tall oil phytosterols on plasma lipid levels of mildly hypercholesteroleamic subjects. Novartis Consumer Health unpublished. Full study report provided to FDA in November 21, 2000 in Novartis Consumer Health/ Forbes-Medi-Tech Inc. comments on the Interim Rule.

Table 1: Comparison of Sterols and Stanols on Effectiveness in Lowering LDL Cholesterol

| Reference | No. per group | Phytosterol | Matrix | Dosage g/day[1] | Duration (days) | LDL-C |
|---|---|---|---|---|---|---|
| Vanstone et al., 2001 | 15 | Sterols | Butter | 1.8 | 21 | -10.1%* |
| | 15 | Sterols/Stanols mixture | Butter | 1.8 | 21 | -15.4%* |
| | 15 | Stanols | Butter | 1.8 | 21 | -13.8%* |
| Jones, *et al.*, 2000. | 15 | Sterol esters | Margarine | 1.8 | 22 | -13.2%* |
| | 15 | Stanol esters | Margarine | 1.8 | 22 | -6.4%* |
| Weststrate and Meijer, 1998. | 95 | Sterol esters | Margarine | 3 | 24-25 | -13.1%* |
| | 95 | Stanol esters | Margarine | 3 | 24-25 | -11.9%* |

1. Calculated on basis of phytosterols content
* Difference from placebo is statistically significant

Table 2: Dose Response Studies with Sterol Esters, Stanol esters and a sterol-stanol mixture.

| Reference | No. per group | Phytosterol | Food Matrix | Dosage g/day[1] | Duration (days) | LDL-C | Apo B |
|---|---|---|---|---|---|---|---|
| Hendriks et al., 1999. | 100 | Plant sterol esters | Margarine suspension | 0.83<br>1.61<br>3.24 | 25<br>25<br>25 | -6.2%*<br>-9.2%*<br>-9.8%* | |
| Beer et al., 2000 | 23<br>28<br>23 | Sterol/stanol mixture | Milk Drink | 0.9<br>1.8<br>3.6 | 28<br>28<br>28 | -7.4%*<br>-8.6%*<br>-13. 2%* | |
| Hallikainen *et al.*, 2000 | 22 | Stanol esters | Margarine suspension | 0.8<br>1.6<br>2.4<br>3.2 | 28<br>28<br>28<br>28 | -1.7%<br>-5.6%*<br>-9.7%*<br>-10.4%* | -8.7%*<br>-9.2%*<br>-10.1%*<br>-13.7%* |

1. Calculated on basis of phytosterols content
* Difference from placebo is statistically significant

# Unesterified Plant Sterols and Stanols Lower Low-density Lipoprotein Cholesterol Levels Equivalently in Hypercholesterolemic Individuals

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Running head: phytosterols and lipid metabolism in humans

## ABSTRACT

**Background:** Plant sterols, in various forms, have been shown to reduce total and LDL cholesterol concentrations. Particularly controversial at present is the effect of degree of hydrogenation of plant sterols on cholesterol-lowering efficacy and the responsible mechanisms.

**Objective:** To examine the effect of unesterified plant sterol and stanol supplementation on plasma lipid and phytosterol concentrations, as well as cholesterol absorption, synthesis, and turnover.

**Design:** Fifteen otherwise healthy hypercholesterolemic subjects consumed each of four dietary treatments in a randomized crossover design. Unesterified sterols and stanols were blended into the butter component of the diet at a dose of 1.8 g/day. Diets contained (i) plant sterols (NS), (ii) plant stanols (SS), (iii) a 50:50 mixture sterols and stanols (NSS), or (iv) a control diet (control).

**Results:** Plasma total cholesterol concentration was decreased ($p<0.01$) by 7.8%, 11.9%, and 13.1% in NS, SS, and NSS groups, respectively, versus control. LDL cholesterol was decreased ($p<0.03$) by 11.3%, 13.4%, and 16.0% in NS, SS, and NSS groups, respectively, compared to control. Plasma TAGs and HDL cholesterol levels did not differ across diets. Cholesterol absorption efficiency was reduced ($p<0.001$) by 56.0, 34.4, and 48.9%, while fractional synthesis rates were reciprocally increased ($p<0.03$) by 34.1, and 45.5% for SS, and NSS, respectively, relative to control. Plasma campesterol and sitosterol levels were increased ($p<0.01$) with NS and sitosterol was decreased ($p<0.01$) with SS.

**Conclusions:** These data indicate that in their free, unesterified form, sterols and stanols lower plasma LDL cholesterol equivalently in hypercholesterolemics by suppressing cholesterol absorption while increasing cholesterol synthesis.

23 **INTRODUCTION**

24 Plant sterols and stanols, structural analogs of cholesterol, have been shown to
25 substantially reduce total and low-density lipoprotein (LDL) cholesterol concentrations under a
26 variety of study conditions. Several researchers have claimed that consumption of stanol-
27 containing mixtures is more effective in reducing circulating cholesterol level, compared to
28 sterols (1-8). Recently, however, the paradigm has shifted to a position where sterol and stanol
29 esters are viewed as comparable plasma cholesterol modulators. It was observed that circulating
30 total and LDL cholesterol levels were equally reduced by 8-13% with both the sitosterol-ester
31 and sitostanol-ester margarines at doses of 1.5 to 3.3 g/day (9). Similar lowering of total and
32 LDL cholesterol concentrations was reported with ingestion of sitosterol-esters compared to
33 sitostanol-esters (10).

34 This controversy raises another important question as to whether unesterified (free) plant
35 sterol and stanol mixtures, would possess the same cholesterol-lowering efficacy regardless of
36 their degree of hydrogenation, or whether esterification and solubilization of plant sterol mixtures
37 are responsible for their equal effectiveness. Comparison of free sitosterol and sitostanol in
38 pastil form given to children with severe hypercholesterolemia showed that hydrogenation
39 improved the LDL cholesterol lowering by increasing fecal neutral sterol output to a greater
40 degree, compared to the sitosterol supplemented group (11).

41 Plasma cholesterol lowering efficacy of phytosterols varies according to the composition
42 and dose of the phytosterol mix and the vehicle in which they are given. It has been suggested
43 that high intakes of saturated fat and cholesterol may improve the efficacy of phytosterols (12)
44 and phytosterol esters (3). To date the relative effectiveness of these materials in a dietary
45 context where saturated fat and cholesterol intakes are at the higher end of the normal

46 physiological range has not been assessed nor have mechanisms of action been fully explored.
47 Therefore, the objective of this study was to examine the effect of unesterified sitosterol and
48 sitostanol supplementation on plasma lipid and phytosterol concentrations as well as cholesterol
49 absorption and synthesis in subjects consuming precisely defined diets.
50

51 **SUBJECTS AND METHODS**

52 **Subjects**

53 Ten male and six female otherwise healthy, free-living volunteers with primary familial
54 hyperlipidemia between the ages of 35 and 58 years were recruited. Female subjects were either
55 post-menopausal or had undergone a hysterectomy. Subjects were screened for total circulating
56 cholesterol and triacylglycerol (TAG) concentrations. Inclusion criteria included a plasma total
57 cholesterol concentration in the range of 5.2 to 9.0 mmol/L and TAG less than 3.5 mmol/L. Prior
58 to acceptance, subjects were required to provide a medical history as well as undergo a complete
59 physical examination. Fasting blood and urine samples were collected for serum biochemistry,
60 hematology, and urine analyses. Subjects were screened for chronic illness, including hepatic,
61 renal, thyroid, and cardiac dysfunction, prior to admission in the study. Subjects were required to
62 refrain from using drug therapy for hypercholesterolemia during and for at least 8 weeks prior to
63 the start of the study. Prior to study commencement, subjects received a thorough explanation of
64 the study protocol and were given the opportunity to discuss any queries with either the primary
65 investigator, physician, or study coordinator before signing the consent form. Baseline
66 characteristics of the study subjects are presented in **Table 1**. The experimental protocol was
67 approved by the Human Ethical Review Committee of the Faculty of Agriculture and
68 Environmental Sciences for the School of Dietetics and Human Nutrition at McGill University.

69

70 **Experimental design and diets**

71 The study was a randomized crossover double blind clinical trial. Subjects consumed
72 each of four dietary treatments. Each dietary treatment phase consisted of 21 feeding days. Each
73 feeding phase was separated by a four-week washout period during which time subjects returned
74 to consuming their habitual diets. In order to reduce the error term associated with diet
75 sequencing, subjects were randomly assigned to one of four predetermined Latin squares, where
76 each square possessed 4 sequenced phases and 4 subjects. In this manner, we ensured that the
77 crossover design was balanced.

78 The diets were comprised of solid foods, typical of those consumed in North America,
79 and provided as three meals per day in a 3-day rotating menu. The nutrient content of the basal
80 diet was calculated using Food Processor, a computerized dietary analysis system with a
81 Canadian database. Diets were designed based on Recommended Nutrient Intakes for Canadians
82 to provide 3000 kcal /70 kg individual /day. The Mifflin equation was used to estimate
83 individual subject basal energy requirements (13), which was then multiplied by an activity
84 factor of 1.7 to compensate for the additional energy needs of mildly to moderately active healthy
85 adults. If subjects gained or lost weight during the first week of each treatment phase, energy
86 adjustments were made to meet individual requirements and ensure that baseline body weights
87 were maintained. Dietary carbohydrate, fat, and protein made up 50, 35, and 15% of ingested
88 energy, respectively, with seventy percent of the fat provided as butter. Diets contained either
89 plant sterols (NS), plant stanols (SS), 50:50 mixture plant sterols and stanols (NSS), or a control
90 diet (control). The NS treatment consisted of purified phytosterols derived from soybeans and
91 contained sitosterol (43%), campesterol (26%), stigmasterol (17%), and other identified

92 phytosterols (10%). To achieve the SS treatment the same soybean phytosterols were
93 hydrogenated to produce a composition of sitostanol (66%) and campestanol (33%). Equal parts
94 of phytosterols and phytostanols were mixed together to create the NSS treatment. The control
95 product was cornstarch, as it strongly resembled the white powdery phytosterol containing
96 mixtures. The phytosterol and phytostanol mixtures, or cornstarch control, were blended into the
97 butter component of the diet at a dose of 1.8 g/day, warmed to 37°C, and administered equally
98 across the three daily meals. To achieve double blinding, containers containing the plant sterols,
99 stanols, and the cornstarch control were coded so that neither the researcher giving the test
100 mixture, nor the subject receiving it, knew its true identity. Diets were prepared in the metabolic
101 kitchen of the Mary Emily Clinical Nutrition Research Unit of McGill University. Subjects
102 consumed a minimum of 2 of the three daily meals each day at the unit under supervision. It was
103 required that all subjects consume breakfast at the unit with one of the other two meals was
104 available for take out.
105

106 **Protocol**

107 At the start (day 1) and end (day 22) of each dietary phase fasting blood samples were
108 taken for determination of circulating lipid levels. Ninety-six hours prior to the end of each
109 phase subjects provided a baseline blood sample prior to receiving an intravenous injection of 15
110 mg [25,26,26,26,27,27,27] $D_7$-cholesterol and a 75 mg oral dose of [3,4]$^{13}$C-cholesterol for
111 cholesterol absorption determination. The ratio of ingested [3,4]$^{13}$C-cholesterol to injected
112 [25,26,26,26,27,27,27] $D_7$-cholesterol enrichment in serum cholesterol after 24, 48, and 72 hrs
113 was taken as an indicator of the fractional cholesterol absorption rate. The $D_7$-cholesterol isotope
114 was prepared for injection by first, dissolving it in ethanol at a concentration of 5 mg/ml under

115 sterile conditions at the Royal Victoria Hospital pharmacy. The isotope/ethanol mixture was
116 then added drop-wise to a parenteral lipid emulsion **(Manufacturer)** for a total injectable
117 volume of 9 ml. Cholesterol synthesis was also determined at the end of each diet period using
118 the deuterium incorporation approach. Seventy-two hours following dosing with $^{13}C$-cholesterol
119 and $D_7$-cholesterol, subjects were dosed with 0.7 g/kg of estimated body water, deuterium oxide
120 (D) (99.8% atom percent excess, CDN Isotopes, Montreal, Quebec). Body water was estimated
121 to be 60% for calculation of the dose. Deuterium oxide was given immediately following a
122 fasting blood sample at approximately 08:00 hr on day 21 of each diet phase.
123

124 **Analyses**

125 *Plasma lipid concentrations*

126 Blood samples were centrifuged for 15 min at 1500 rpm to separate plasma from red
127 blood cells (RBC) and stored at –80°C until analysis. Plasma total, HDL cholesterol, and TAG
128 concentrations were analysed in quadruplicate with standardized reagents using a VP
129 Autoanalyser (Abbott Laboratories, North Chicago, IL, USA). Calibration of the analyser prior
130 to each run was performed as per the standardization protocol of the Canadian Reference
131 Laboratory. The Friedewald equation was used to calculate LDL cholesterol levels (14).
132

133 *Determination of cholesterol absorption*

134 Free cholesterol extracted from RBCs was used to determine $^{13}C$-cholesterol and
135 $D_7$-cholesterol enrichments. Lipid was extracted from the RBCs in duplicate using a modified
136 Folch extraction procedure (15). Thin layer chromatography (20x20 cm, 250 μ, Scientific
137 Adsorbents Inc., Atlanta, GA, USA) was used to separate free cholesterol from cholesteryl ester.

138 The free cholesterol band was then scraped from the silica gel plate and saponified with 0.5 M
139 methanolic KOH to eliminate any fatty acid contaminants. Free cholesterol extracts were dried
140 under nitrogen and transferred into 18 cm sealed combustion tubes (Vycor, Corning Glass
141 Works, Corning, NY, USA). Cupric oxide (0.6 g) and a 2 cm long piece of silver wire were
142 added and tubes sealed under vacuum for at least 5 min at less than 20 mtorr pressure. Dual
143 tracer labeled cholesterol samples were then combusted to D-enriched water and $^{13}C$-enriched
144 $CO_2$ over 4 hr at 520°C. The generated $CO_2$ was transferred under vacuum into Vycor tubes for
145 measurement of $^{13}C$ enrichment and thereafter, water was vacuum-distilled into sealed tubes
146 containing 0.06 g zinc (Biogeochemical Laboratories Indiana University, Bloomington, IN, USA)
147 for D enrichment analysis. Tubes containing the water and zinc were then reduced to D-labelled
148 hydrogen gas at 520°C for 30 min.

149 Nuclear magnetic resonance (NMR) was used to verify that the isotopic enrichments of
150 the tracers [3,4]$^{13}C$-cholesterol and $D_7$-cholesterol (CDN Isotopes, Pointe Claire, Quebec) were
151 greater than 99 atom percent excess. The $^{13}C$ enrichments of free cholesterol were measured by
152 differential isotope ratio mass spectrometry (IRMS) using an automated dual inlet system (SIRA
153 12, Isomass, Cheshire, UK). Enrichments were then expressed relative to PDB limestone
154 standard of the National Bureau of Standards (NBS). Linearity and gain of response of the SIRA
155 IRMS instrument were assessed using a reference tank $CO_2$ and NBS standards of known
156 isotopic enrichment. The D enrichments of free cholesterol were measured by differential IRMS
157 using a manually operated dual inlet system with electrical $H^{3+}$ compensation (VG Isomass
158 903D, Cheshire, UK). For D, enrichments were expressed relative to standard mean ocean water
159 (SMOW) and a series of standards of known enrichment from the NBS, analysed concurrently on

160 each day of measurement to correct for any variations in linearity of gain of response of the
161 IRMS.

162 The average $^{13}C$ and D enrichments of 48 and 72 hr RBC free cholesterol relative to
163 baseline (t=0) samples were used to calculate the cholesterol absorption coefficient (CAC) using
164 the ratio of orally ingested $^{13}C$-cholesterol to intravenously administered $D_7$-cholesterol as
165 described by Bosner et al. (16):

166

167 $$\text{CAC (pool/pool)} = \frac{\textit{del}\ ^{13}C}{\textit{del}\ D_7} \times \frac{\text{15 mg i.v. dose of } D_7\text{-cholesterol}}{\text{75 mg i.g. dose of } ^{13}C\text{-cholesterol}} \times \frac{7/46}{2/27} \times \frac{0.0112}{0.000155} \quad \text{(Eqn 1)}$$

168

169

170 where *del* (‰) for $^{13}C$ and $D_7$ is the difference between the average of the enriched samples at 48
171 or 72 hr and the baseline abundance (at t =0) in parts per thousand relative to PDB and SMOW
172 standards, respectively. The factors 7/46 and 2/27 reflect the ratios of labelled to unlabelled
173 hydrogen and carbon atoms in the cholesterol tracers, respectively. The constants 0.0112 and
174 0.000155 represent factors converting the part per thousand units to equivalent atom percent
175 excess for the PDB and SMOW scales, respectively.

176

177 *Determination of cholesterol biosynthesis*

178 Cholesterol biosynthesis was determined as the rate of incorporation of D from body
179 water into RBC membrane free cholesterol over the period between 72 and 96 hr at the end of
180 each feeding period. Deuterated water equilibrates quickly between intracellular and
181 extracellular water pools and permits direct determination of cholesterol formation rates (17).
182 Deuterium enrichment was measured in both RBC free cholesterol and plasma water. To

183 determine plasma cholesterol D enrichment, total RBC lipids were extracted and isolated using
184 the same procedure described above.

185 To measure deuterium enrichment of plasma water, additional plasma samples were
186 diluted 7 fold with water to reduce deuterium enrichment to within the normal analytical range.
187 Baseline samples were not diluted. Triplicate samples were then vacuum-distilled into zinc-
188 containing (0.06 g) Vycor tubes. Cholesterol and plasma water samples were then reduced to
189 hydrogen gas at 520°C for 30 min and analysed by differential IRMS, as previously described
190 above.

191 Cholesterol fractional synthesis rate (FSR) was taken to represent the RBC free
192 cholesterol D enrichment values relative to the corresponding plasma water sample enrichment
193 after correcting for the free cholesterol pool. The FSR represents that fraction of the cholesterol
194 pool that is synthesized in 24 hours and was calculated as per the formula (18):

195

196 $$\text{FSR (pools/day)} = (del_{\text{cholesterol}} / del_{\text{plasma}}) \times 0.478 \qquad \text{Eqn (2)}$$

197

198 where *del* (‰) for D cholesterol is the difference between enriched free cholesterol and plasma
199 water at 96 and 72 hr in parts per thousand relative to a SMOW standard. The factor 0.478
200 reflects the ratio of labelled H atoms replaced by D (22/46) during in vivo biosynthesis (18).

201

202 *Cholesterol turnover measured by $D_7$-cholesterol decay*

203 Plasma turnover of free unesterified cholesterol represents the rate of flux of incoming
204 sterol from synthesis and diet relative to that being esterified, eliminated, or transferred into other
205 pools. Turnover rates for RBC free cholesterol were determined from the decay rate of $D_7$-

206 cholesterol. Exponential curves were fitted to 24, 48, and 72 hr RBC $D_7$-cholesterol enrichments
207 after subtraction of baseline D abundance at 0 hr.

208 *Plasma phytosterol concentrations*

209 Plasma phytosterol concentrations were determined duplicate by gas-liquid
210 chromatography (GLC) from the nonsaponifiable material of plasma lipid as reported previously
211 (19). Briefly, 1.0 ml plasma samples were saponified with 0.5 M methanolic KOH for 1 h at
212 100°C and the nonsaponifiable materials were extracted with petroleum ether. Samples with 250
213 μg of 5-α cholestane added as an internal standard were injected into a GLC equipped with a
214 flame ionization detector (HP 5890 Series II; Hewlett Packard, Palo Alto, CA) and a 30-m
215 capillary column (SAC-5; Supelco, Bellefonte, PA). Detector and injector temperatures were
216 310°C and 300°C, respectively. Duplicate samples were run isothermically at 285°C. Phytosterol
217 peaks were identified by comparison with authenticated standards (Supelco, Bellefonte, PA).

218

219 *Statistics*

220 All data are expressed as the mean ± standard error mean (SEM). Lipoprotein
221 cholesterol, TAG, and phytosterol concentration, data at the beginning and end of each dietary
222 period were compared using an ANOVA design to identify treatment effects. When treatment
223 effects were identified as significant, a Tukey test was utilized for identification of significant
224 effects of diet at particular time-points. Student's paired t-tests were also used to compare
225 baseline values with final time-points within each diet. Cholesterol absorption, synthesis, and
226 turnover values were compared at the end of each treatment phase using an ANOVA. When
227 treatment effects were identified, a Tukey test was used for identification of significant effects of
228 each diet treatment. Pearson's correlation coefficient analysis was used to test for relationships

229 between variables. A level of statistical significance at $p<0.05$ was used in all analyses. The data
230 were analysed using proc-General Linear Model SAS (version 6.12) software.
231

232 **RESULTS**

233 Sixteen subjects were enrolled into the study. One male subject dropped out at the end of
234 the first feeding cycle due to difficulties with daily transportation to the unit each day. Therefore,
235 complete data for nine men and six women were collected and analysed as per the study protocol.
236 All individuals tolerated the diet without any reported adverse events. Subjects reported no
237 abnormal or atypical smell, taste, color, or mouth-feel effects when consuming any of the four
238 mixtures, thus were unable to distinguish between dietary treatments. There were no significant
239 mean group weight changes across any of the three treatment phases. Blood and urine samples at
240 the beginning and end of each phase for all 15 subjects were sent to LDS Diagnostic Laboratories
241 (Pointe Claire, Quebec), where complete blood counts (CBC), biochemistry (sequential multiple
242 analysis level C (SMAC)), and urinalyses were carried out. Results from all four phases of the
243 feeding trial remained within normal ranges throughout the study period and regular physical
244 exams revealed no suggestion of any clinical irregularities.
245

246 **Circulating lipids in response to treatment**

247 The concentrations of plasma lipids at the beginning and end of each treatment phase are
248 shown in **Table 2**. Total cholesterol concentrations measured across all phases of the feeding
249 trial showed significant variation between subjects. Plasma total cholesterol concentration was
250 decreased ($p<0.01$) by 7.8%, 11.9%, and 13.1%, in the NS, SS, and NSS groups, respectively,
251 versus control. LDL cholesterol levels were decreased ($p<0.03$) by 11.3%, 13.4%, and 16.0%, in

252 the NS, SS, and NSS groups, respectively, compared to control. Plasma TAG and HDL
253 cholesterol levels did not differ across diets. Over the study period, however, HDL cholesterol
254 was lower ($p<0.05$) in the NSS group.
255

256 **Cholesterol absorption in response to treatment**

257 Cholesterol absorption at the end of each feeding phase was taken as an average of the 48
258 hr and 72 hr measurements. Mean cholesterol absorption coefficient was decreased ($p<0.001$)
259 following ingestion of the NS, SS, and NSS diets (0.200 ± 0.03, 0.298 ± 0.04, 0.232 ± 0.04
260 pool/pool, respectively), compared to the control diet (0.454 ± 0.04 pool/pool) (**Table 4**).
261 Therefore, relative to control, absorption was reduced 56.0, 34.4, and 48.9 % for NS, SS, and
262 NSS groups, respectively. Absorption values for NS and SS groups were significantly ($p<0.05$)
263 different from each other, however, the NSS group was not significantly different from either the
264 NS or SS group.
265

266 **Cholesterol biosynthesis in response to treatment**

267 Cholesterol fractional biosynthesis rates were increased ($p<0.03$) following consumption
268 of all three treatment diets compared to the control diet. Fractional synthesis rate in the control
269 group was measured to be 0.040 ± 0.007 pool/d. Ingestion of both the SS and NSS diets
270 increased ($p<0.03$) fractional synthesis rates by 34.1% (0.059 ± 0.01 pool/day) and 45.5% (0.064
271 ± 0.007 pool/day), respectively, compared to control. Consumption of the NS diet also tended to
272 increase fractional synthesis rate by 25.0% (0.055 ± 0.008 pool/day), compared to control,
273 however, this increase was not statistically significant (**Table 4**). There was no difference
274 observed in synthesis between groups supplemented with phytosterols or phytostanols.

275

276 **Cholesterol turnover in response to treatment**

277 Turnover rates of cholesterol were calculated from $D_7$-cholesterol enrichment values
278 obtained during the 24 - 72 h period following injection of the isotope at the end of each
279 treatment phase. Turnover rates of free cholesterol, extracted from RBCs, were 0.381 ± 0.05,
280 0.346 ± 0.06, 0.324 ± 0.04, and 0.364 ± 0.04 pools/day, for NS, SS, NSS, and control diets,
281 respectively. There were no significant differences identified between any of the diets.

282

283 **Plasma plant sterol levels in response to treatment**

284 Plasma plant sterol concentrations and ratios relative to total cholesterol are presented in
285 **Table 3**. Plasma campesterol and sitosterol concentrations were not different between groups at
286 the beginning of each feeding phase. There were, however, changes between groups at the end of
287 phytosterol supplementation. Plasma campesterol and sitosterol levels were higher ($p<0.01$) in
288 the NS group compared to control, SS, and NSS groups. Mean plasma campesterol and sitosterol
289 concentrations were increased ($p<0.0001$) by 99.3% and 38.6%, respectively, compared to
290 control with consumption of the NS diet. Sitosterol concentrations were reduced ($p<0.01$) by
291 23.6 % with SS, compared to control. Campesterol levels were also lower with SS feeding,
292 however, this change did not reach significance. The NSS group produced little change in
293 circulating plant sterol levels.

294

295 **Associations between plasma lipid levels and kinetic measurements**

296 Across all subjects, both plasma total ($r = 0.42$, $P < 0.001$) and LDL cholesterol ($r = 0.35$,
297 $p<0.006$) levels varied directly with cholesterol absorption coefficient. Similarly, β-sitosterol ($r$

298 = 0.40, p<0.002) levels were found to vary directly with circulating LDL cholesterol
299 concentrations. Fractional synthesis rate varied inversely with LDL cholesterol ($r$ = -0.29,
300 p<0.03) levels, further supporting the compensatory relationship between cholesterol lowering
301 and increased synthesis rates. The sitosterol:campesterol ratio correlated directly with plasma
302 LDL cholesterol ($r$ = 0.49, p<0.0001) and inversely with FSR ($r$ = -0.42, p<0.0008). Notably,
303 neither campesterol nor the campesterol:cholesterol ratio correlated with cholesterol absorption
304 coefficient.
305

306 **DISCUSSION**

307 The major novel finding of the study is the demonstration that non-saturated, saturated,
308 and an equal part mixture of non-saturated/saturated phytosterols, in their unesterified form,
309 significantly and equally reduce both plasma total and LDL cholesterol concentrations. The
310 degree of cholesterol lowering observed is entirely due to the action of the plant sterols and
311 stanols, not the basal diet, since plasma total and LDL cholesterol concentrations marginally
312 increased on the control diet. This reduction in circulating cholesterol concentrations was
313 achieved through inhibition of intestinal cholesterol absorption as evidenced by lower absorption
314 coefficients, however, these reductions were accompanied by a partial compensatory de-
315 suppression of cholesterol synthesis, which may be an indication that other mechanisms are also
316 at work.

317 Despite the relatively high content of saturated fat and cholesterol in the basal diet, it was
318 shown that sterols and stanols are efficacious in lowering circulating total and LDL cholesterol
319 concentrations. It has been postulated that elevated intakes of dietary fat and cholesterol (400-
320 450 mg/day) may increase the effectiveness of the phytosterols in the intestinal lumen.

321 Unesterified plant sterols blended in butter and supplemented at a level of 0.74 g for 4 were
322 shown to decrease total and LDL cholesterol levels by 10 and 15 %, respectively, despite a
323 phytosterol dose of less than 1g/day (12). These authors attributed their results to the high
324 cholesterol intake obtained from butter. Several researchers, however, have achieved similar
325 degrees of cholesterol suppression when the total fat and cholesterol contents of the diet were
326 much lower (20, 21, 7). These and other studies have shown efficacy of plant sterols and stanols
327 when blended into a fat source such as margarine, butter, mayonnaise, or vegetable oils prior to
328 supplementation (22). Conversely, when provided as a powder-filled capsule as part of a low fat
329 diet, plant stanols failed to exert any lipid modulating effect (23), suggesting that the amount of
330 fat and cholesterol in the diet are not strong modulators in the effectiveness of plant sterols. It is
331 more likely that the effectiveness of plant sterols and stanols is more dependent on the vehicle in
332 which they are matrixed and added to the diet rather than the composition of the diet.

333 Although structurally very similar to cholesterol, plant stanols are believed to be
334 negligibly absorbed by the intestine (1), therefore, do not enter the cell and displace cholesterol at
335 the level of the micelle, interrupting absorption (24). Several methods exist to directly measure
336 cholesterol absorption, however, many require fecal collections and/or radio-labelled cholesterol
337 administration (25, 26). The current study is one of few to employ the dual-stable isotope
338 methodology which derives a coefficient of absorption for cholesterol through a time-step
339 comparison of the proportion of an oral labelled bolus of tracer cholesterol appearing in blood,
340 relative to the appearance of a bolus labelled with a second tracer administered intravenously (27,
341 16). The pattern of decay of the intravenous tracer permits correction of the oral tracer response
342 in plasma for loss into routes of excretion or deeper metabolic pools. Previously, selected ion
343 monitoring mass spectrometry has been used to measure isotope enrichments (16), whereas this

344 study used more sensitive isotope ratio mass spectrometry to improve precision with lower
345 isotopic dosages. The cholesterol absorption coefficient was determined by calculating the
346 average of the 48 and 72 hr time points using the approach described by Bosner et al. (16), who
347 showed that the plasma ratio of oral and intravenous tracers does in fact become constant
348 between 48 and 72 hrs after dosing which allows for an accurate measure of cholesterol
349 absorption.

350 The relative effect of unesterified sterols and stanols versus a control group on cholesterol
351 absorption has not been previously studied, particularly in the context of a rigidly controlled
352 dietary paradigm. A cholesterol absorption efficiency of 45.4 % in the control group is
353 comparable to values reported elsewhere (28, 16, 29). Similarly, a decrease in cholesterol
354 absorption rates of 34-56 %, following sterol and stanol supplementation, is in accordance with
355 data using sterol and stanol esters reported elsewhere in humans (29, 10, 6, 28). Present data
356 indicate that the decrease in circulating cholesterol concentrations in subjects supplemented with
357 unesterified phytosterols or phytostanols was due to this inhibition in the cholesterol absorption
358 efficiency.

359 Cholesterol absorption varied directly with both total and LDL cholesterol concentrations,
360 suggesting that circulating cholesterol levels are dependent on the uptake of cholesterol in the
361 intestine and that plant sterols and stanols effectively inhibited cholesterol absorption. The group
362 with the lowest absorption coefficient was not, however, the group with the greatest degree of
363 cholesterol lowering. The sterol group lowered cholesterol absorption 56 %, and raised synthesis
364 by 25 %, however, a smaller impact on cholesterol lowering was seen, compared to the other
365 groups. Although, the stanol group decreased the absorption coefficient by 34.4 % and raised
366 synthesis by the same amount (34.1 %), cholesterol levels fell more dramatically. Interestingly,

367 the 50:50 mix of sterols and stanols, decreased cholesterol absorption decreased 48.9 % and
368 synthesis increased almost 50 %, yet lowered cholesterol levels to the greatest degree. Free
369 sitosterol has been shown to more effectively lower cholesterol absorption compared to sitosterol
370 ester (28). However, most previous reports conclude that sitostanol more effectively inhibits
371 cholesterol absorption compared to sitosterol (1, 6, 7) or results in equal reductions in cholesterol
372 absorption efficiency (29, 10). Although results are in contrast to those previously reported, the
373 present protocol study enforced a strict dietary regimen ensuring that all subjects consumed
374 identical foods, in equal proportions, while maintaining a steady weight. This regimen
375 minimized several dietary confounders making the comparisons between groups more accurate.
376 Consumption of plant sterols and stanols significantly induced changes in circulating
377 plant sterols as well cholesterol concentrations, indicating mutually competitive inhibition
378 between all sterol forms (24). On this basis, plasma plant sterol concentrations have been used as
379 indicators of compliance. Absolute values and percent changes in campesterol and sitosterol
380 levels were similar to those previously reported following phytosterol feeding (10, 8, 22),
381 signaling that the subjects were in fact consuming the treatment. Plasma plant sterol
382 concentrations have also been used as an indirect measure of cholesterol absorption.
383 Specifically, serum campesterol concentration and the campesterol:cholesterol ratio have been
384 shown to correlate positively with intestinal cholesterol absorption. This association would be
385 expected to reflect cholesterol absorption under static dietary conditions. However, different
386 plant sterols are variably absorbed and metabolized, therefore, it is unclear whether the use of
387 campesterol is appropriate for measuring cholesterol absorption under conditions where plant
388 sterol/stanol intakes are changing. Supplementation with stanols inhibits cholesterol absorption
389 and has consistently produced decreases in sterol concentrations (10, 7, 22). During sitosterol

390 feeding, however, sitosterol and campesterol levels have been shown to increase (10) or remain
391 unchanged (11) despite a clear inhibition in the cholesterol absorption efficiency making this
392 correlation inapplicable as a method of estimating cholesterol absorption in any situation where
393 phytosterol intakes would be expected to change. In the present study plasma sterol levels
394 decreased with stanols and increased with sterols while levels remained similar to control with a
395 50:50 mix of sterols and stanols. Furthermore cholesterol absorption coefficient was not
396 associated with either campesterol concentration or the campesterol:cholesterol ratio, as would
397 be predicted from investigating stanols alone.

398 In summary, the present study demonstrates that in free form, sterol and stanol feeding
399 results in equal reductions in total and LDL cholesterol concentrations. Cholesterol absorption
400 was reduced in response to sterol and stanol feeding, and found to vary directly with reductions
401 in LDL cholesterol concentration. Cholesterol synthesis was increased, however, not to an extent
402 which prevented cholesterol lowering. In conclusion, both unesterified plant sterols and stanols
403 favorably lower LDL cholesterol, independent of the degree of hydrogenation in
404 hypercholesterolemic individuals.

405 **TABLE 1**

406 Baseline characteristics of the subjects[1]

| Variables | Men (n=9) | Women (n=6) | All (n=15) |
|---|---|---|---|
| Age (y) | 46.4 ± 2.7 | 49.8 ± 2.6 | 47.8 ± 1.9 |
| Weight (kg) | 92.2 ± 3.5 | 82.4 ± 7.3 | 88.3 ± 3.6 |
| Body mass index (kg/m$^2$) | 30.5 ± 1.5 | 31.3 ± 2.5 | 30.8 ± 1.3 |
| Lipids (mmol/L) | | | |
| Total cholesterol | 6.48 ± 0.43 | 6.57 ± 0.28 | 6.52 ± 0.27 |
| LDL cholesterol[2] | 4.31 ± 0.44 | 4.29 ± 0.28 | 4.30 ± 0.26 |
| HDL cholesterol[3] | 0.93 ± 0.04 | 1.42 ± 0.08 | 1.16 ± 0.08 |
| Triacylglycerols | 2.53 ± 0.36 | 1.86 ± 0.26 | 2.26 ± 0.25 |

407 [1]Values are expressed as mean ± SEM.
408 [2]Low-density lipoprotein cholesterol
409 [3]High-density lipoprotein cholesterol
410

410 **TABLE 2**

411

412 Plasma lipid levels at day 0 and day 21 of each dietary period[1]

| Lipid | NS | SS | NSS | C (control) |
|---|---|---|---|---|
| | | *mmol/L* | | |
| Total cholesterol | | | | |
| Day 0 | 5.97 ± 0.26 | 6.23 ± 0.26 | 6.40 ± 0.31 | 6.14 ± 0.33 |
| Day 21 | 5.54 ± 0.22 [b*] | 5.57 ± 0.22 [b***] | 5.59 ± 0.26 [b**] | 6.15 ± 0.25 |
| % Change | -6.3 ± 2.9 [b] | -10.4 ± 2.3 [b] | -11.6 ± 3.2 [b] | 1.5 ± 3.2 [a] |
| % Relative to control | -7.8 | -11.9 | -13.1 | |
| LDL-cholesterol[2] | | | | |
| Day 0 | 4.0 ± 0.20 | 4.11 ± 0.18 | 4.18 ± 0.23 | 4.06 ± 0.26 |
| Day 21 | 3.6 ± 0.17 [b*] | 3.59 ± 0.18 [b*] | 3.55 ± 0.17 [b**] | 4.01 ± 0.20 |
| % Change | -9.1 ± 2.9 [b] | -11.2 ± 3.0 [b] | -13.8 ± 3.2 [b] | 2.2 ± 5.4 [a] |
| % Relative to control | -11.3 | -13.4 | -16.0 | |
| Triacylglycerol | | | | |
| Day 0 | 1.91 ± 0.22 | 2.10 ± 0.24 | 2.21 ± 0.35 | 1.94 ± 0.20 |
| Day 21 | 1.85 ± 0.23 | 1.90 ± 0.20 | 1.99 ± 0.32 | 2.15 ± 0.33 |
| % Change | 2.2 ± 9.1 | -5.4 ± 6.7 | -3.1 ± 8.0 | 7.0 ± 6.3 |
| % Relative to control | -4.8 | -12.4 | -10.1 | |
| HDL-cholesterol[3] | | | | |
| Day 0 | 1.14 ± 0.08 | 1.17 ± 0.07 | 1.20 ± 0.09 | 1.19 ± 0.07 |
| Day 21 | 1.10 ± 0.07 | 1.14 ± 0.09 | 1.14 ± 0.09* | 1.16 ± 0.08 |
| % Change | -2.0 ± 2.9 | -2.6 ± 3.7 | -5.6 ± 2.5 | -2.3 ± 2.1 |
| % Relative to control | 0.3 | -0.3 | -3.3 | |
| LDL:HDL ratio[2,3] | | | | |
| Day 0 | 3.71 ± 0.29 | 3.69 ± 0.26 | 3.81 ± 0.39 | 3.62 ± 0.34 |
| Day 21 | 3.40 ± 0.25 | 3.48 ± 0.30 | 3.61 ± 0.42 | 3.73 ± 0.29 |
| % Change | -7.9 ± 3.2 | -6.4 ± 4.1 | -6.7 ± 6.4 | 4.0 ± 6.0 |
| % Relative to control | -11.9 | -10.4 | -10.7 | |

413 [1]Values are expressed as mmol/L ± SEM. Values carrying different superscript letters
414 indicate a significant difference between diets ($p<0.05$). Percent change is based on individual
415 data. Percent change relative to control is based on the mean of day 21.
416 *P<0.05; **P<0.01; ***P<0.001: significant differences within each diet (between day 0 and
417 21).
418 [2]Low-density lipoprotein cholesterol
419 [3]High-density lipoprotein cholesterol
420

420 **TABLE 3**

421

422 Plasma plant sterol levels at day 0 and day 21 of each dietary period[1]

| Phytosterol | NS | SS | NSS | C (control) |
|---|---|---|---|---|
| | | *µmol/L* | | |
| Campesterol | | | | |
| Day 0 | 14.1 ± 1.2 | 17.3 ± 3.0 | 13.8 ± 1.5 | 15.6 ± 2.1 |
| Day 21 | 24.3 ± 2.3 [a**] | 8.5 ± 0.8 [c**] | 17.7 ± 2.1 [b] | 12.7 ± 2.4 [bc] |
| % Change | 84.2 [a] | -43.1 [c] | 39.2 [b] | -10.9 [bc] |
| % Relative to control | 99.3 | -27.9 | 54.6 | |
| β-Sitosterol | | | | |
| Day 0 | 8.6 ± 1.2 | 9.2 ± 1.0 | 7.2 ± 0.7 | 8.4 ± 1.1 |
| Day 21 | 9.6 ± 1.0 [a] | 4.8 ± 0.5 [c**] | 7.4 ± 0.9 [b] | 7.0 ± 0.9 [b] |
| % Change | 26.3 [a] | -35.9 [c] | 10.0 [ab] | -12.3 [b] |
| % Relative to control | 38.6 | -23.6 | 22.3 | |
| β-Sitosterol:campesterol | | | | |
| Day 0 | 0.12 ± 0.01 | 0.12 ± 0.02 | 0.09 ± 0.01 | 0.10 ± 0.01 |
| Day 21 | 0.12 ± 0.01 | 0.06 ± 0.01 | 0.10 ± 0.01 | 0.11 ± 0.03 |
| % Change | 0.0 | -50.0 | 11.1 | 10.0 |
| % Relative to control | -10.0 | -60.0 | 1.1 | |
| Campesterol:total cholesterol | | | | |
| Day 0 | 1.87 ± 0.18 | 2.25 ± 0.31 | 1.95 ± 0.12 | 1.93 ± 0.14 |
| Day 21 | 2.65 ± 0.17 | 2.05 ± 0.32 | 2.53 ± 0.20 | 1.74 ± 0.18 |
| % Change | 41.7 | -8.9 | 29.7 | -9.8 |
| % Relative to control | 51.5 | 0.9 | 39.5 | |
| β-Sitosterol:total cholesterol | | | | |
| Day 0 | 0.20 ± 0.02 | 0.21 ± 0.03 | 0.17 ± 0.02 | 0.19 ± 0.02 |
| Day 21 | 0.30 ± 0.03 | 0.10 ± 0.01 | 0.25 ± 0.03 | 0.21 ± 0.06 |
| % Change | 50.0 | -52.4 | 47.1 | 10.5 |
| % Relative to control | 39.5 | -62.9 | 36.6 | |

423 [1]Values are expressed as mmol/L ± SEM. Values carrying different superscript letters
424 indicate significant differences between diets ($p<0.05$). Percent change is based on individual
425 data; percent change relative to control diet for day 21.
426 *P<0.05; **P<0.01; ***P<0.001: significant differences within each diet (between day 0 and
427 day 21).

References

1. Heinemann T, Leiss O, and von Bergmann K. Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. Atherosclerosis 1986;61:219-23.

2. Miettinen TA, Puska P, Gylling H, Vanhanen HT, and Vartiainen E. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. N Engl J Med 1995;333:1308-12.

3. Miettinen TA, and Vanhanen HT. Dietary sitostanol related to absorption, synthesis and serum level of cholesterol in different apolipoprotein E phenotypes. Atherosclerosis 1994;105:217-26.

4. Vanhanen HT, Blomqvist S, Ehnholm C, Hyvonen M, Jauhiainen M, Torstila I, and Miettinen TA. Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment. J Lipid Res 1993;34:1535-44.

5. Heinemann T, Pietruck B, Kullak-Ublick G, and von Bergmann K. Comparison of sitosterol and sitostanol on inhibition of intestinal cholesterol absorption. Agents Actions 1988;26:S117-S22.

6. Heinemann T, Kullak-Ublick GA, Pietruck B, and von Bergmann K. Mechanisms of action of plant sterols on inhibition of cholesterol absorption. Comparison of sitosterol and sitostanol. Eur J Clin Pharmacol 1991;40:S59-S63.

7. Gylling H, and Miettinen TA. Cholesterol reduction by different plant stanol mixtures and with variable fat intake. Metabolism 1999;48:575-80.

8. Jones PJH, Ntanios FY, Raeini-Sarjaz M, and Vanstone CA. Cholesterol lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men. Am J Clin Nutr 1999;69:1144-50.

9. Weststrate JA, and Meijer GW. Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolemic and mildly hypercholesterolemic subjects. Eur J Clin Nutr 1998;52:334-43.

10. Jones PJH, Raeini-Sarjaz M, Ntanios FY, Vanstone CA, Feng JY, and Parsons WE. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. J Lipid Res 2000;41:697-705.

11. Becker M, Staab D, and von Bergmann K. Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. J Pediatr 1993;122:292-6.

12. Pelletier X, Belbraouet S, Mirabel D, Mordret F, Perrin JL, Pages X, and Debry G. A diet moderately enriched in phytosterols lowers plasma cholesterol concentrations in normocholesterolemic humans. Ann Nutr Metab 1995;39:291-5.

13. Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, and Koh YD. A new predictive equation for resting energy expenditure in healthy individuals. Am J Clin Nutr 1990;51:241-7.

14. Friedewald WT, Levy RI, and Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without the use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.

15. Folch J, Lees M, and Stanley GH. A simple method for the isolation and purification of total lipids from animals tissues. J Biol Chem 1957;226:497-509.

16. Bosner MS, Ostlund RE, Osofisan O Jr, Grosklos J, Fritschle C. and Lange LG. Assessment of percent cholesterol absorption in humans with stable isotopes. J Lipid Res 1993;34:1047-53.

17. Jeske DJ and Dietschy JM. Regulation of rates of cholesterol synthesis in vivo in the liver and carcass of the rat measured using $^{3}H$ water. J Lipid Res 1980;21:364-76.

18. Jones PJH, Leitch CA, Li Z-C, and Connor WE. Human cholesterol synthesis measurement using deuterated water: theoretical and procedural consideration. Arterioscler Thromb 1993;13:247-53.

19. Ntanios F, and Jones PJH. Effects of variable dietary sitostanol concentrations on plasma lipid profile and phytosterol metabolism in hamsters. Biochim Biophys Acta 1998;1390:237-44.

20. Andersson A, Karlstrom B, Mohsen R, and Vessby B. Cholesterol-lowering effects of a stanol ester-containing low-fat margarine used in conjunction with a strict lipid-lowering diet. Eur Heart J Supplements 1999;1(Suppl S):S80-S90.

21. Hallikainen MA, and Uusitupa MIJ. Effects of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. Am J Clin Nutr 1999;69:403-10.

22. Jones PJH, MacDougall DE, Ntanios FY, and Vanstone CA. Dietary phytosterols as cholesterol-lowering agents in humans. Can J Physiol Pharm 1997;75:217-27.

23. Denke MA. 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 1995;61:392-6.

24. Piironen V, Linsay DG, Miettinen TA, Toivo J, and Lampi A-M. Review Plant sterols: biosynthesis, biological function and their importance to human nutrition. J Sci Food Agric 2000;80:939-66.

25. Grundy SM, Ahrens EH. Jr. and Davignon J. The interaction of cholesterol absorption and cholesterol synthesis in man. J Lipid Res. 1969;10: 304-15.

26. Turley SD, Herndon MW, and Dietschy JM. Reevaluation and application of the dual-isotope plasma ratio method for the measurement of intestinal cholesterol absorption in the hamster. J Lipid Res 1994; 35;328-39.

27. Zilversmit DB, and Hughes LB. Validation of a dual-isotope plasma ratio method for measurement of cholesterol absorption in rats. J Lipid Res 1974;15:465-73.

28. Mattson FH, Grundy SM, and Crouse JR. Optimizing the effect of plant sterols on cholesterol absorption in man. Am J Clin Nutr 1982;35:697-700.

29. Normén L, Dutta P, Lia Å, and Andersson H. Soy sterol esters and β-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. Am J Clin Nutr 2000;71:908-13.

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