JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Supplementation of Test Meals with Fat-Free Phytosterol Products Can Reduce Cholesterol Micellarization during Simulated Digestion and Cholesterol Accumulation by Caco-2 Cells

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Phytosterols have been shown to reduce cholesterol absorption in humans. Supplementing phytosterols in fat-free formulations, however, has yielded controversial results. In the present study, we investigated the effect of supplementing test meals with different fat-free phytosterol products on cholesterol incorporation into mixed micelles during simulated digestion and accumulation of micellar cholesterol by Caco-2 cells: control orange juice (OJ), orange juice supplemented with either multivitamin/multimineral tablets (MVT), multivitamin/multimineral tablets containing phytosterols (MVT+P), and phytosterol powder (PP). These combinations were added to Ensure-based test meals and spiked with cholesterol of natural isotopic composition or $^{13}C_2$-cholesterol to differentiate external from endogenous cholesterol. After simulated gastric/small intestinal digestion, micelle fractions were analyzed for cholesterol enzymatically ($n = 6-20$/product) and by high-performance liquid chromatography−tandem mass spectrometry ($n = 12$/product) and added to Caco-2 cells to determine the accumulation of $^{13}C_2$-cholesterol ($n = 10-24$/product). As compared to OJ, PP and MVT+P significantly decreased cholesterol micellarization (determined enzymatically) by 70 ± 39 (mean ± SD) and 70 ± 39%, respectively ($P < 0.001$, Bonferroni). The stable isotope experiments revealed that both PP and MVT+P reduced cholesterol micellarization [by 25 ± 12 ($P = 0.055$) and 21 ± 8% ($P = 0.020$), respectively, Fisher's protected LSD test] and Caco-2 cell accumulation (by 28 ± 8 and 10 ± 8%, respectively; $P < 0.010$, Bonferroni). OJ+P did not inhibit micellarization or accumulation of cholesterol by Caco-2 cells. This study shows that fat-free phytosterol-containing products can significantly inhibit cholesterol micellarization and Caco-2 cell bioaccessibility, albeit to different extents depending on individual formulations. This is most likely explained by inhibition of cholesterol micellarization.

KEYWORDS: Phytosterols; food matrix; cholesterol absorption; micellarization; Caco-2 cells; isotopic label; mass spectrometry

INTRODUCTION

Phytosterols are components of plant cell membranes that are similar in structure to cholesterol. These compounds occur either in free form (e.g., sitosterol, stigmasterol, campesterol, and their Δ^5-hydrogenated forms referred to as stanols) or as esters. Human clinical trials have demonstrated that the consumption of 2−3 g/day phytosterols or phytostanols can reduce plasma total cholesterol and low-density lipoprotein cholesterol (LDL-C) concentrations (*1*, *2*) and, therefore, decrease the risk of cardiovascular diseases (*3*). The mechanism suggested for phytosterol-mediated inhibition of cholesterol uptake is controversial (*4*−*6*). Phytosterols have been reported to decrease the delivery of cholesterol to absorptive epithelial cells in the gut by competing with cholesterol for partitioning into mixed micelles. However, phytosterols also have been reported to increase the expression of transporter proteins such as the ATP binding cassette that facilitates the efflux of newly absorbed cholesterol back into the intestinal lumen (*6*−*8*).

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10.1021/jf061829l CCC: $37.00
Published on Web 12/20/2006

Consumer interest in reducing plasma cholesterol has resulted in the rapid development of new phytosterol-containing products by the food, nutraceutical, and pharmaceutical industries. Because of their limited water solubility, food-based strategies include incorporation of phytosterols/stanols predominantly into fat-containing products such as margarine and oils (*9*) but also into bread, milk, cereals (*10*), yogurt (*11*), lemonade (*12*), and organge juice (*13*). Incorporation of phytosterols into nonfat matrices has attracted attention because these foods are preferable delivery vehicles for hypercholesterolemic and overweight individuals. However, information on the effectiveness of delivering phytosterols in fat-free food matrices remains limited and ambiguous (*10*, *12*, *14*).

Proof of efficacy for newly developed food products or supplements for commercialization often requires lengthy and cost-prohibitive clinical trials. In vitro digestion procedures represent cost-effective tools for estimating the transfer of fat-soluble compounds from the food matrix to mixed micelles, a process that is essential for their delivery to absorptive epithelial cells (*15*). Differentiated cultures of Caco-2 human intestinal cells exhibit many of the biochemical and morphological characteristics of absorptive epithelial cells in the small intestine (*16*). Coupling in vitro digestion with Caco-2 cells has therefore been used to study the bioaccessibility [the maximum amount of a compound that is soluble in a synthetic gastrointestinal fluid and therefore potentially available for uptake across the intestinal lumen (*17*)] of various dietary factors, including iron (*18*), carotenoids (*15*, *19*), chlorophylls (*20*), and numerous drugs (*16*).

The objective of the present investigation was to assess the effect of supplementing test meals with several fat-free phytosterol formulations on the micellarization and intestinal cell accumulation of cholesterol. The amount of cholesterol micellarized during digestion was determined both spectrophotometrically and by liquid chromatography in combination with mass spectrometry [high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS)]. $^{13}C_2$-labeled cholesterol also was added to test meals to distinguish exogenous from endogenous cholesterol in Caco-2 cells to determine cellular accumulation of micellarized $^{13}C_2$-cholesterol and was quantified by HPLC-MS/MS.

MATERIALS AND METHODS

Study Design and Test Meals. Three phytosterol-containing products were tested for their ability to modify cholesterol bioaccessibility. Phytosterol-containing products included the following: orange juice (OJ+P, Minute Maid Premium Heartwise, Cargill Inc., Minneapolis, MN), multivitamin/multimineral tablets (MVT+P, Centrum Cardio prototype tablets, Wyeth Consumer Healthcare Inc., Madison, NJ), and phytosterol powder (PP, Corowise, Raw Material, Cargill Inc.). All phytosterol-containing products were originally based on Corowise PP containing 30−60% sitosterol, 20−30% campesterol, 15−30% stigmasterol, and <10% other phytosterols, containing 92.4% total sterols. Phytosterol-free control formulations were multivitamin/multimineral tablets (MVT; Centrum, Wyeth Consumer Healthcare Inc.) and orange juice (OJ; Minute Maid Premium Orange Juice, Cargill Inc.).

All products were blended into test meals as shown in **Table 1**. Solubility of the different products to be dissolved in OJ was tested in advance. At room temperature, MVT rapidly solubilized in OJ, MVT+P required about 1 h, and PP aggregated when added to OJ. Therefore, PP was added directly to the final test meal mixture in the reaction tubes. Resulting test meals were composed of 2 mL of Ensure Vanilla drink (Ross Abbott Laboratories, Columbus, OH), 2 mL of OJ, 20 μL of olive oil containing 540 μg of cholesterol of natural isotopic composition (enzymatic micellarization experiments) or (all other experiments) 540 μg of $^{13}C_2$-cholesterol (both Sigma, St. Louis, MO), and either no or 3800 μg of free phytosterols. Test meals were prepared in 50 mL screw cap polypropylene tubes and vortexed to mix constituents. Ensure was chosen as a test meal as it contains a diverse variety of nutrients and did not require further processing. OJ was chosen as a component of the test meal to simulate an additional drink consumed together with the test meal and has been used in a previous study (*13*).

Table 1. Composition of Test Meals Subjected to Simulated Digestion[a]

product	preparation/composition[d]	amounts used for test meal (mL)
Ensure Vanilla drink	6 g of fat, 40 g of CHO[e] (of which 18 g of sugars), 9 g of protein	2
	plus	
cholesterol in oil	540 μg of cholesterol dissolved in 20 μL of olive oil	0.02
	plus one of the following	
1. OJ	27 g of CHO (of which 24 g of sugars), 2 g of protein	2
2. OJ+P	as OJ, with 456 mg of phytosterols	2
3. MVT	1 tablet dissolved in OJ	2
4. MVT+P	1 tablet (456 mg of phytosterols) dissolved in OJ	2
5. PP	493 mg (456 mg of phytosterols) of PP dissolved in OJ	2

[a] All phytosterol products[b] were prepared in OJ. Final test meals were composed of aliquots of the OJ preparation (2 mL), Ensure Vanilla drink (2 mL), cholesterol in olive oil (20 μL) and contained either no or 3.8 mg of phytosterols.[c] [b] All based on Corowise phytosterol powder (30–60% sitosterol, 20–30% campesterol, 15–30% stigmasterol, and <10% other phytosterols), containing 92.4% total sterols. [c] The final concentration was 1.9 mg phytosterols/mL for all phytosterol containing test meals. [d] With the exception of cholesterol in oil, all amounts are given per a final volume of 240 mL. [e] Carbohydrates.

The prepared test meals were then used to study the effect of phytosterols on cholesterol micellarization during simulated gastric and small intestinal phases of digestion and accumulation of micellarized cholesterol by Caco-2 cells. Primary end points of the studies were the amount of cholesterol recovered in the micelles, measured either enzymatically (cholesterol of natural isotopic composition) or by HPLC-MS/MS ($^{13}C_2$-cholesterol) and the amount of $^{13}C_2$-cholesterol quantified in Caco-2 cells exposed to the control and phytosterol-containing test meals as measured by HPLC-MS/MS. Unless otherwise stated, all chemical supplies were obtained from Sigma or Fisher Scientific Co. (Pittsburgh, PA) and Gibco (Grand Island, NY) for all cell culture supplies. All chemicals used were of analytical or superior grade.

Cholesterol Solutions. The cholesterol solution of natural isotopic composition was prepared by weighing 13.5 mg of cholesterol into a sealable glass vial prior to the addition of 500 μL of olive oil (Filippo Berio, Salev, South Hackensack, NJ). The vial was capped, vortexed, and placed in a 37 °C water bath for 2 h to dissolve the cholesterol. The solution containing $^{13}C_2$-cholesterol was prepared similarly by mixing 13.5 mg of 3,4-$^{13}C_2$-cholesterol (Sigma) with olive oil. The final concentrations were 540 μg of cholesterol/20 μL of olive oil.

In Vitro Digestion. Pilot studies were performed to ensure complete digestion of oil droplets after simulated gastric and small intestinal digestion. Simulated gastric and small intestinal phases of digestion for each test meal were performed as described previously (*15*, *20*) with the exception that pure bile salts (0.8 mM glycodeoxycholate, 0.45 mM taurodeoxycholate, and 0.75 mM taurocholate) were added in place of crude bile extract for the small intestinal phase of digestion. This substitution was made since bile extract contains cholesterol, and we previously reported that micellarization of carotenoids was increased by addition of pure bile salts in place of the bile extract during simulated digestion (*19*). All test samples were digested at least in sextuples. After completion of simulated small intestinal digestion, aliquots of digesta were centrifuged for 35 min at 150000*g* at 4 °C (Beckman model L7-65, Palo Alto, CA), following a method described by Hernell et al. (*21*). The aqueous fraction was filtered (cellulose acetate, 0.22 μm

pores) to remove microcrystalline aggregates, and aliquots were stored at −70 °C or used directly to investigate cholesterol accumulation by Caco-2 cells.

Accumulation of Cholesterol from Micelles by Caco-2 Cells. Cultures of Caco-2 cells (HTB37, American Type Culture Collection, Rockville, MD; passage 26−34) were grown on plastic as described previously (*15*) and used to investigate accumulation of $^{13}C_2$-cholesterol when monolayers were 12−14 days postconfluent. Test medium was prepared by diluting filtered aqueous fractions with Dulbecco's modified Eagle medium (DMEM) supplemented with 1% nonessential amino acids and 2 mol/L glutamine (basal medium) 1:4 (v/v). Pilot studies confirmed that diluted aqueous (micellar) fractions from digested test meals did not compromise monolayer integrity (as analyzed by microscopic appearance), cell viability (as determined by trypan blue exclusion), and adherent cell protein per well during 4 h of exposure. In addition, monolayers used to determine $^{13}C_2$-cholesterol accumulation were observed hourly to ensure that treatments did not compromise morphological appearance, and the amount of cell protein adhered to the dish surface (1.9 ± 0.1 mg/well) was determined for an aliquot of sonicate from which cholesterol was extracted.

To determine accumulation of cholesterol from micelles generated during simulated digestion, monolayers were washed twice with basal medium at 37 °C before adding 2 mL test medium/well. After 4 h, the test medium was removed and monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) containing fatty acid-free bovine serum albumin (2 g/L) to remove micelles or their lipophilic contents adhering to the outer cell surface, followed by two washes with cold PBS (*19*). Cells were collected in 1.5 mL of ice-cold PBS and centrifuged at 800*g* (4 °C, 5 min), and cell pellets were stored at −70 °C under nitrogen for later analysis of cholesterol. Accumulation of $^{13}C_2$-labeled cholesterol was measured in 10−24 replicate wells/test sample. A larger number of control (OJ) measurements was done as compared to other test meals as the OJ control was always included as a control.

Enzymatic Analysis of Total Cholesterol. Total lipids were extracted following a method modified from Gamble et al. (*22*). In short, 1 mL aliquots of thawed aqueous fractions were extracted by adding 4 mL of chloroform:methanol (2:1, v/v). Mixtures were vortexed for 1 min and centrifuged (2000*g* for 5 min at 20 °C) to separate aqueous and organic phases. The extraction was repeated two times, and chloroform fractions were combined and dried at room temperature under nitrogen. The residue was resolubilized in 100 μL of 1% methanol in water. Total cholesterol was assayed enzymatically as described by Robyt and White (*23*), using a cholesterol kit from Stanbio (Boerne, TX). Absorbance was measured at 500 nm (Perkin-Elmer Lambda 25, Shelton, CT) and compared to an external cholesterol standard curve (range 0.1−4.0 mmol/L). Cholesterol values were corrected for interference using extracts prepared from a test meal blank (no added cholesterol) analyzed in parallel.

HPLC-MS/MS Analysis of Cholesterol. A detailed description of the HPLC-MS/MS method is described in (*24*). In short, to frozen cell pellets, 0.3 mL of NaCl (150 mol/L) was added and sonicated. After quantitatively adding ca. 0.4 μg of internal standard (cholesterol 2,2,3,4,4,6-d_6) in hexane, cell samples were extracted twice using 2 mL of chloroform:methanol (2:1, v/v). After centrifugation, the chloroform layers were combined and dried under nitrogen. The residue was dissolved in 1 mL of hexane and passed through 0.2 μm nylon filters. Filtered aqueous samples from centrifuged digesta (200 μL) were extracted three times following the same procedure.

Chromatographic separation and detection of cholesterols were achieved by HPLC-atmospheric pressure chemical ionization (APCI) tandem mass spectrometry, using a Waters 2695 HPLC (Waters, Milford, MA) and a tandem mass spectrometer (Quattro Ultima, Micromass Co. Ltd., Manchester, United Kingdom) following procedures previously described (*25, 26*). HPLC separation was achieved on a C-18 silica column (Luna 3 μm, 100 mm × 4.6 mm, Phenomenex, Torrence, CA). The mobile phase consisted of hexane:isopropanol (95:5, v/v) with a flow rate of 1 mL/min at 25 °C. LC-APCI/MS/MS quantification of cholesterol 3,4-$^{13}C_2$ and internal standard was achieved using characteristic fragmentations employing selected reaction monitoring (SRM) for simultaneous monitoring 3,4-$^{13}C_2$-cholesterol (371 > 149) and the internal standard (375 > 152). The limit of detection and quantification for cholesterol 3,4-$^{13}C_2$ was determined as 0.88 and 2.80 ng, respectively, based on the Environmental Protection Agency approach (*27*).

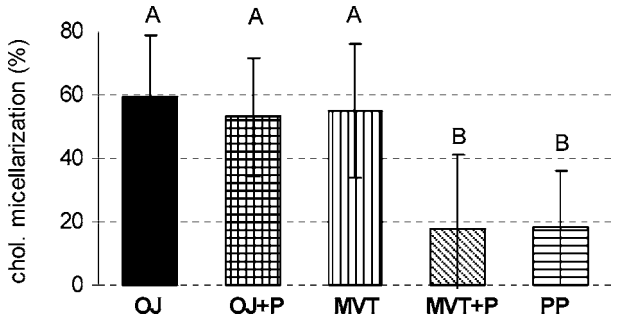

Figure 1. Recovery of cholesterol as determined enzymatically in micelles after simulated gastric/small intestinal digestion of a test meal (Ensure Vanilla drink) supplemented with phytosterols. Test samples included the following: OJ (n = 16), OJ+P (n = 20), MVT (n = 12), MVT+P (n = 12), and PP (n = 6). Error bars indicate ± 1 SD. Columns not sharing the same superscript are statistically significantly different (P < 0.05, Bonferroni).

Statistical Analysis. Data were analyzed using SPSS 14.0 (SPSS Inc. Chicago, IL). Normal distribution of data was verified by Q−Q plots and Kolmogorov−Smirnoff tests. The effect of different test meal supplements on cholesterol micellarization as determined enzymatically was analyzed using a linear mixed model followed by Bonferroni and LSD posthoc tests. Treatment (OJ, OJ+P, MVT, MVT+P, and PP) was set as a fixed factor, replica (if done) was set as a random factor nested within treatment, and percent inhibition of cholesterol accumulation was set as the dependent variable. Similarly, a linear mixed model was developed for studying the effect of different products on cholesterol micellarization and Caco-2 cell accumulation based on isotopic analyses. As all tested products were not studied altogether in any one experiment, conditions such as days postconfluency for the Caco-2 cells varied slightly. Thus, isotopic data were normalized to the mean of the control group (OJ), which was measured in each experiment, and expressed as percent inhibition of cholesterol micellarization and accumulation of micellar cholesterol by Caco-2 cells as compared to the control. The use of the linear mixed model procedure was preferred over the general linear model as it accounted for random effects, different sample sizes in an experiment, and estimated missing values and did not require constant variance. Unless otherwise stated, all values are given as arithmetic means ± standard deviations. A P value of <0.05 (two-sided) was considered statistically significant.

RESULTS

Cholesterol Recovery and Micellarization As Determined Enzymatically. Mean cholesterol recovery in the digested samples (prior to removal of the aqueous phase) was 92 ± 7% (range = 81−108%, n = 6−9 per group with a total of n = 36), with no significant differences between the different treatments, indicating only minor and reproducible losses during simulated gastric and small intestinal digestion. Mean cholesterol recovery in the filtered aqueous phase of the digesta (the micelle fraction) of digested OJ and MTV was 59 ± 19 (n = 16) and 55 ± 17% (n = 12), respectively, indicating that the majority of cholesterol was micellarized in control samples. The effect of the different supplemented products on cholesterol micellarization from the test meals is shown in **Figure 1**. Sample size varied due to practical limitations with additional testing for OJ+P, as this commercial product was expected to inhibit micellarization of cholesterol. Fewer PP samples were tested due to the robustness of its inhibitory effect on cholesterol micellarization. The relative high standard deviation, especially

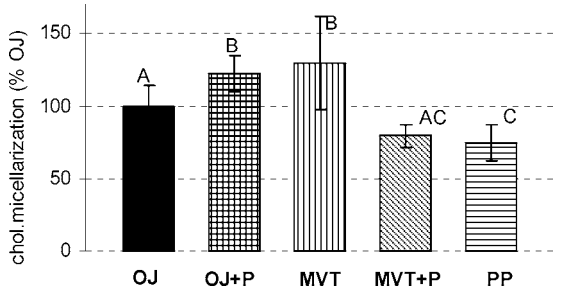

Figure 2. Micellarization of $^{13}C_2$-cholesterol during simulated digestion of test meals with and without phytosterol products ($n = 4$ except OJ $n = 8$, analyzed in triplicate by HPLC-MS/MS). Samples included OJ, OJ+P, MVT, MVT+P, and PP. Percent cholesterol micellarization is presented in comparison to OJ (control), which was set as 100%. Error bars indicate ± 1 SD. Columns not sharing the same superscript are statistically significantly different ($P < 0.05$, Fisher's protected LSD).

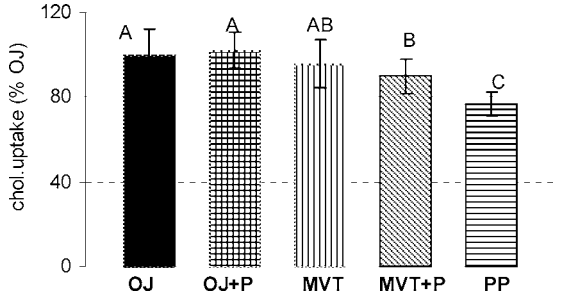

Figure 3. Cellular accumulation of micellarized $^{13}C_2$-cholesterol determined during simulated digestion of test meals containing the cholesterol isotope and different phytosterol containing products. Monolayers of Caco-2 cells were incubated for 4 h with medium containing micelles generated during digestion of meals containing the following: OJ ($n = 24$), OJ+P ($n = 10$), MVT ($n = 12$), MVT+P ($n = 12$), and PP ($n = 12$). Accumulation is presented in comparison to OJ (control), which was set as 100%. Error bars indicate ± 1 SD. Columns not sharing the same superscript are statistically significantly different ($P < 0.05$, Bonferroni).

for MVT+P and PP, can be explained by a relatively high absorbance of the blank sample for the enzymatic determination of cholesterol. This resulted in higher variations for samples with lower cholesterol concentrations. The overall *P* value (Fisher *F*-test) rejected the null hypothesis of all tested products not being significantly different ($P < 0.0001$). MVT+P showed the strongest inhibitory effect on cholesterol micellarization, with an average micellarization of 30 ± 39% of that of OJ ($P = 0.00001$, Bonferroni), followed by PP (30 ± 30%, $P = 0.0006$). OJ, OJ+P, and MVT were not significantly different from one another.

Micellarization of Cholesterol As Determined by Stable Isotope Techniques. Because the presence of the bile salts and phytosterols in the micellar fraction resulted in a high background for the enzymatic determination of micellarized cholesterol, it was decided to use the stable isotopically labeled cholesterol. The effect of the different products on cholesterol micellarization from the test meals is shown in **Figure 2**. The overall *P* value (Fisher *F*-test) rejected the null hypothesis of all tested products not being significantly different ($P = 0.0003$). PP exhibited the strongest inhibitory effect on cholesterol micellarization and was 75 ± 12% as compared to OJ (100 ± 14%, $P = 0.020$, Fisher's protected LSD test), followed by MVT+P (79 ± 8%, $P = 0.055$, Fisher's protected LSD test). Surprisingly, both the MVT and the OJ+P increased cholesterol micellarization as compared to the OJ to 130 ± 32 and 122 ± 13%, respectively ($P = 0.008$ and 0.038, Fisher's protected LSD test, respectively).

Caco-2 Cell Accumulation of Micellarized Cholesterol. The results for the accumulation of cholesterol by Caco-2 cells from micelles generated during simulated digestion of the meals supplemented with the different test products are displayed in **Figure 3**. One replicate sample (OJ) was lost during sample preparation. The overall *P* value (Fisher *F*-test) rejected the null hypothesis of all tested products being nonsignificantly different ($P < 0.0001$). PP had the strongest inhibitory effect on cholesterol accumulation by Caco-2 cells as compared to OJ and was 77 ± 8 vs 100 ± 9% ($P < 0.001$, Bonferroni), followed by MVT+P (90 ± 8%, $P = 0.01$, Bonferroni), with PP and MVT+P being significantly different ($P = 0.001$, Bonferroni). MVT and OJ+P were not significantly different from OJ, with a cholesterol accumulation of 96 ± 10 and 102 ± 10%, respectively.

DISCUSSION

The present study highlights the inhibitory effect of supplementing different fat-free phytosterol-containing products to test meals on both the cholesterol micellarization during simulated gastrointestinal digestion and the quantity of exogenous cholesterol accumulated by Caco-2 cells exposed to the micelle fraction. Both micellarization and accumulation of cholesterol by Caco-2 cell were studied to determine at which step(s) phytosterols decrease cholesterol transfer from the food matrix to absorptive epithelial cells. Cholesterol accumulation by Caco-2 cells exposed to digested test meals supplemented with phytosterols in powder form and from tablets was significantly reduced by about 23 and 10%, respectively, as compared to phytosterol-free orange juice. These results are in line with earlier Caco-2 cell studies demonstrating a 10−70% reduction of exogenous cholesterol accumulation when cultures were incubated with either micelles generated during digestion of milk (*4*) supplemented with nonesterified plant sterols or from oleate/bile acid micelles when supplemented with free β-sitosterol (*28*). Ingestion of 2−3 g of free or esterified phytosterols or stanols/day was reported to decrease cholesterol absorption by 25−56% in humans (*6*). This decrease in cholesterol uptake typically is associated with a 5−20% reduction of plasma LDL-C when consuming phytosterols for several weeks. Supplementation of less than 1.5 g phytosterols/day has been shown to be less effective or ineffective in decreasing plasma cholesterol levels (*6*), suggesting a certain ratio of phytosterols to cholesterol is needed to be efficient. In our study, the amount of cholesterol and phytosterols added to the test meals was 540 and 3800 μg, respectively. This is a comparable weight ratio of phytosterols to cholesterol (ca. 7) used in human studies, given a typical dietary cholesterol intake of ca. 160−400 mg/day (*6*).

Prior to cellular cholesterol uptake, incorporation into mixed lipid micelles is assumed to be important as cholesterol is poorly soluble in water and would not be available for uptake and possible transepithelial transport of cholesterol by the absorptive epithelium (*5*). In the present study, supplementation of phytosterols in powder and a tablet formulation reduced micellarization of cholesterol by 25 and 21%, respectively. This was comparable to the relative reduction in the quantity of exogenous cholesterol accumulated by Caco-2 cells as determined using stable isotope techniques. The enzymatic determination of cholesterol micellarization during simulated digestion suggested

even greater inhibitory effects of phytosterols on cholesterol micellarization from these two formulations. However, as phytosterols and bile salts may interfere with the enzymatic analysis of cholesterol, cholesterol micellarization was additionally determined by HPLC-MS/MS. On the basis of both enzymatic and HPLC-MS/MS results, the reduction in micellarization of cholesterol during simulated digestion likely accounts for the decreased accumulation of exogenous cholesterol in Caco-2 cells. Other reports have suggested that ATP binding cassette proteins located in brush border membranes of absorptive epithelial cells in small intestine could increase cholesterol efflux in the presence of phytosterols (*5*, *8*). If this was the case in our study, their effect on net cholesterol accumulation appears to be minimal. Moreover, the brief time of exposure of the cells to the micellar fraction containing phytosterols (4 h) may not be sufficient to markedly increase the quantities of such efflux proteins. Thus, the present study, similar to other in vitro studies, was not designed to provide detailed insights regarding the influence of individual or mixtures of phytosterols in the test material on apical efflux of cholesterol. In addition, we did not examine the possible impact of the phytosterols on esterification of accumulated cholesterol or its distribution among different classes of secreted lipoproteins (*28*, *29*). Further investigations examining the effects of long-term exposure of different mixtures of phytosterols on such processes affecting the apical efflux and basolateral secretion of cholesterol are merited.

We did not observe inhibitory effects of digested meals containing commercially available orange juice with phytosterols and the phytosterol-free tablets as compared to the orange juice alone on cholesterol accumulation by micelles and Caco-2 cells. On the contrary, HPLC-MS/MS results suggested that phytosterol-free tablets and orange juice containing phytosterols increased cholesterol micellarization, even though no effects were observed for cholesterol accumulation by Caco-2 cells. It is possible that minerals and vitamins present in the tablet formulation increased micelle stability and/or formation during digestion. For the orange juice containing phytosterols, we expected decreased partitioning of cholesterol into micelles and cholesterol accumulation by Caco-2 cells. A recent human feeding trial investigating the same phytosterol-containing orange juice showed that consuming 240 mL/day of this juice for 2 weeks significantly reduced total plasma cholesterol and LDL-C levels in humans by about 7 and 12%, respectively, as compared to orange juice not containing phytosterols (*13*). However, other phytosterol-containing products and the impact on micellarization during digestion were not investigated. One possible reason for the inhibitory effect of phytosterols on cholesterol absorption in the human study as compared to our study is that chronic intake of phytosterols may have increased expression of ABC binding transporter proteins enhancing cholesterol efflux into the intestinal lumen. It is also possible that the stability or solubility of the phytosterols in orange juice may have been compromised during in vitro digestion and subsequent determination of cholesterol accumulation by Caco-2 cells. Indeed, it has been shown that the ability of phytosterols to reduce cholesterol absorption can be affected by food matrix (*10*) and that poorly soluble crystalline phytosterols decrease cholesterol absorption to a lesser degree or are ineffective (*4*, *5*). To improve solubility in aqueous systems, phytosterols have to be coated by an emulsifier such as lecithin (*30*). Using this technique, fat-free beverages (a vanilla drink containing proteins and a lemonade drink, respectively) decreased cholesterol absorption (*31*) and total plasma cholesterol in humans (*12*). However, serving a fat-free beverage containing phytosterols for 21 days failed to affect plasma cholesterol in another study (*14*); no information was provided about how the phytosterols were solubilized. In our study, the phytosterol formulations were all based on proprietary techniques, and differences in production techniques, especially coating formulations, most likely explain the observed different effects on cholesterol micellarization and accumulation by Caco-2 cells.

Instead of using emulsifiers to improve phytosterol solubility, phytosterols also have been esterified to improve their solubility. Esterified phytosterols are better solubilized in fat than free phytosterols (*9*) and are expected to be more readily incorporated into mixed micelles (*30*). Because all phytosterol products used in this study were free, i.e., unesterified phytosterols, they should have exhibited comparable effects on cholesterol micellarization and Caco-2 cell accumulation. Other than the formulation of the phytosterols, test meals were identical.

In conclusion, the results from the present investigation demonstrate that micellarization and intestinal cell accumulation of exogenous cholesterol from test meals were inhibited by the presence of fat-free plant phytosterols in powder and tablet form but not from orange juice. It is suggested that phytosterol-mediated inhibition of cholesterol micellarization during the small intestinal phase of digestion certainly contributes to the observed decrease in cholesterol bioavailability from foods and formulations containing phytosterols.

ABBREVIATIONS USED

OJ, orange juice without phytosterols (control); OJ+P, orange juice with phytosterols; MVT, multivitamin/multimineral tablets without phytosterols (control); MVT+P, multivitamin/multimineral tablets with phytosterols; PP, phytosterol powder; LDL-C, low-density lipoprotein cholesterol; HPLC-MS/MS, high-pressure liquid chromatography coupled to mass spectrometry; SD, standard deviation.

ACKNOWLEDGMENT

We thank Judy Moreines from Wyeth Consumer Healthcare for her valuable input on the study and Rebecca Melick from the OSU Department of Human Nutrition for her assistance during the vitro digestion.

LITERATURE CITED

(1) Miettinen, T. A.; Gylling, H. Plant stanol and sterol esters in prevention of cardiovascular diseases. *Ann. Med.* **2004**, *36*, 126−34.

(2) Castro, I. A.; Barroso, L. P.; Sinnecker, P. Functional foods for coronary heart disease risk reduction: A meta-analysis using a multivariate approach. *Am. J. Clin. Nutr.* **2005**, *82*, 32−40.

(3) Huxley, R.; Lewington, S.; Clarke, R. Cholesterol, coronary heart disease and stroke: A review of published evidence from observational studies and randomized controlled trials. *Semin. Vasc. Med.* **2002**, *2*, 315−23.

(4) Pouteau, E. B.; Monnard, I. E.; Piguet-Welsch, C.; Groux, M. J.; Sagalowicz, L.; Berger, A. Non-esterified plant sterols solubilized in low fat milks inhibit cholesterol absorption-a stable isotope double-blind crossover study. *Eur. J. Nutr.* **2003**, *42*, 154−64.

(5) Ostlund, R. E. Phytosterols and cholesterol metabolism. *Curr. Opin. Lipidol.* **2004**, 15, *37*−41.

(6) Berger, A.; Jones, P. J. H.; Albumweiss, S. S. Plant sterols: Factors affecting their efficacy and safety as functional food ingredients. *Lipid Health Dis.* **2004**, *3*, 1−19.

(7) von Bergmann, K.; Sudhop, T.; Lutjohann, D. Cholesterol and plant sterol absorption: Recent insights. *Am. J. Cardiol.* **2005**, *96*, 10D−14D.
(8) Plat, J.; Mensink, R. P. Increased intestinal ABCA1 expression contributes to the decrease in cholesterol absorption after plant stanol consumption. *FASEB J.* **2002**, *16*, 1248−53.
(9) Thompson, G. R.; Grundy, S. M. History and development of plant sterol and stanol esters for cholesterol-lowering purposes. *Am. J. Cardiol.* **2005**, *96*, 3D−9D.
(10) Clifton, P. M.; Noakes, M.; Sullivan, D.; Erichsen, N.; Ross, D.; Annison, G.; Fassoulakis, A.; Cehun, M.; Nestel, P. Cholesterol-lowering effects of plant sterol esters differ in milk, yoghurt, bread and cereal. *Eur. J. Clin. Nutr.* **2004**, *58*, 503−509.
(11) Mensink, R. P.; Ebbing, S.; Lindhout, M.; Plat, J.; van Heugten, M. M. Effects of plant stanol esters supplied in low-fat yoghurt on serum lipids and lipoproteins, non-cholesterol sterols and fat soluble antioxidant concentrations. *Atherosclerosis* **2002**, *160*, 205−213.
(12) Spilburg, C. A.; Goldberg, A. C.; McGill, J. B.; Stenson, W. F.; Racette, S. B.; Bateman, J.; McPherson, T.; Ostlund, R. E. Fat-free foods supplemented with soy stanol-lecithin powder reduce cholesterol absorption and LDL cholesterol. *J. Am. Diet. Assoc.* **2003**, *103*, 577−581.
(13) Devaraj, S.; Jialal, I.; Vega-Lopez, S. Plant sterol-fortified orange juice effectively lowers cholesterol levels in mildly hypercholesterolemic healthy individuals. *Arterioscler., Thromb., Vasc. Biol.* **2004**, *24*, 25−28.
(14) Jones, P. J. H.; Vanstone, C. A.; Raeini-Sarjaz, M.; St.-Onge, M. P. Phytosterols in low- and nonfat beverages as part of a controlled diet fail to lower plasma lipid levels. *J. Lipid Res.* **2003**, *44*, 1713−1719.
(15) Garrett, D. A.; Failla, M. L.; Sarama, R. J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J. Agric. Food Chem.* **1999**, *47*, 4301−4309.
(16) Artursson, P.; Palm, K.; Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Delivery Rev.* **2001**, *46*, 27−43.
(17) Hamel, S. C.; Buckley, B.; Lioy, P. J. Bioaccessability of metals in snails for different liquid to solid ratios in synthetic gastric fluid. *Environ. Sci. Technol.* **1998**, *32*, 358−362.
(18) Glahn, R. P.; Wien, E. M.; Van Campen, D. R.; Miller, D. D. Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: Use of a novel in vitro method for rapid estimation of iron bioavailability. *J. Nutr.* **1996**, *126*, 332−339.
(19) Chitchumroonchokchai, C.; Schwartz, S. J.; Failla, M. L. Assessment of lutein bioavailability from meals and a supplement using simulated digestion and Caco-2 human intestinal cells. *J. Nutr.* **2004**, *134*, 2280−2286.
(20) Ferruzzi, M. G.; Failla, M. L.; Schwartz, S. J. Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in vitro digestion and caco-2 human cell model. *J. Agric. Food Chem.* **2001**, *49*, 2082−2089.
(21) Hernell, O.; Staggers, J. E.; Carey, M. C. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry* **1990**, *29*, 2041−2056.
(22) Gamble, W.; Vaughan, M.; Kruth, H. S.; Avigan, J. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* **1978**, *19*, 1068−1070.
(23) Robyt, J. F.; White, B. J. *Biochemical Techniques: Theory and Practice*; Waveland Press: Chicago, IL, 1987; p 246.
(24) Tian, Q.; Failla, M.; Bohn, T.; Schwartz, S. J. High-performance liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry determination of cholesterol uptake by Caco-2 cells. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 3056−3060.
(25) Briche, C.; Carter, D.; Webb, K. S. Comparison of gas chromatography and liquid chromatography mass spectrometric measurements for high accuracy analysis of cholesterol in human serum by isotope dilution mass spectrometry. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 848−853.
(26) Sandhoff, R.; Brugger, B.; Jeckel, D.; Lehmann, W. D.; Wieland, F. T. Determination of cholesterol at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *J. Lipid Res.* **1999**, *40*, 126−132.
(27) Berthouex, P. M.; Brown, L. C. Limit of detection. In *Statistics for Environmental Engineers*, 2nd ed.; Berthouex, P. M., Brown, L. C., Eds.; CRC Press: Boca Raton, 2002; pp 119−128.
(28) Field, F. J.; Born, E.; Mathur, S. N. Effect of micellar beta-sitosterol on cholesterol metabolism in CaCo-2 cells. *J. Lipid Res.* **1997**, *38*, 348−360.
(29) Iqbal, J.; Anwar, K.; Hussain, M. M. Multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells. *J. Biol. Chem.* **2003**, *278*, 31610−31620.
(30) Ostlund, R. E.; Spilburg, C. A.; Stenson, W. F. Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. *Am. J. Clin. Nutr.* **1999**, *70*, 826−831.
(31) Gremaud, G.; Dalan, E.; Piguet, C.; Baumgartner, M.; Ballabeni, P.; Decarli, B.; Leser, M. E.; Berger, A.; Fay, L. B. Effects of non-esterified stanols in a liquid emulsion on cholesterol absorption and synthesis in hypercholesterolemic men. *Eur. J. Nutr.* **2002**, *41*, 54−60.

Received for review June 29, 2006. Revised manuscript received November 2, 2006. Accepted November 16, 2006. This study was supported by Wyeth Consumer Healthcare Inc. and the Ohio Agricultural Research and Development Center.

JF061829L