Phytosterols that are naturally present in commercial corn oil significantly reduce cholesterol absorption in humans[1–3]

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ABSTRACT

Background: Although supplementing the diet with large quantities of phytosterols reduces cholesterol absorption and LDL-cholesterol concentrations, very little is known about the smaller amounts of phytosterols present naturally in food. Vegetable oils are the richest dietary source of phytosterols; corn oil contains 0.77% phytosterols by weight.

Objective: We tested the hypothesis that removing phytosterols from corn oil would increase cholesterol absorption when measured in single-meal tests containing corn oil as a source of fat.

Design: Free and esterified phytosterols were removed from corn oil on a kilogram scale by a new technique of competitive saturation adsorption to silica. Healthy subjects with a mean (±SEM) serum cholesterol concentration of 5.10 ± 0.18 mmol/L received an otherwise sterol-free test breakfast on 2 occasions 2 wk apart that contained 35 mg hexadeuterated cholesterol and 30–35 g of a corn oil preparation. The plasma enrichment of tracer was measured by negative ion mass spectrometry.

Results: Cholesterol absorption was 38.0 ± 10.2% higher after consumption of the sterol-free corn oil than after consumption of commercial corn oil with an identical fatty acid content ($P = 0.005$; $n = 10$). When corn oil phytosterols were added back to sterol-free corn oil at a concentration of 150 mg/test meal, cholesterol absorption was reduced by 12.1 ± 3.7% ($P = 0.03$; $n = 5$) and by 27.9 ± 9.1% ($P = 0.01$; $n = 10$) after inclusion of 300 mg phytosterols.

Conclusions: Phytosterols comprising < 1% of commercial corn oil substantially reduced cholesterol absorption and may account for part of the cholesterol-lowering activity of corn oil previously attributed solely to unsaturated fatty acids. *Am J Clin Nutr* 2002;75:1000–4.

KEY WORDS Phytosterols, oils, diet, cholesterol, mass spectrometry, deuterium

INTRODUCTION

Carefully controlled human trials have definitively established that different food fats produce clinically important changes in serum cholesterol concentration (1–4); however, the mechanism of action in the regulation of serum cholesterol by dietary fats is unknown. Because fatty foods contain >99% triacylglycerol (5), it has seemed safe to assume that the fatty acids themselves, derived from fat triacylglycerol by intestinal hydrolysis, are solely responsible for the cholesterol-lowering effects observed. However, formal evidence from human clinical trials supporting this position is quite limited and inconclusive (6, 7). Thus, the possibility exists that trace components of food fats may be involved in the regulation of serum cholesterol concentration.

Phytosterols constitute the largest nontriacylglycerol component of refined vegetable fats (5). They act within the intestine to reduce cholesterol absorption and lower LDL-cholesterol concentration without being absorbed themselves (8). Studies with properly formulated phytosterols showed that ≤300 mg phytosterols in a single dose, or 830 mg phytosterols/d, may have important effects on cholesterol metabolism (9, 10). These doses suggested to us that natural dietary phytosterols might also be physiologically active when compared with estimates of 100–500 mg phytosterols/d (or per 100 g fat) in the general diet (11, 12). We chose corn oil as a source of natural phytosterols because vegetable oils have much higher concentrations of phytosterols than do nonfatty vegetable foods and because corn oil is one of the richest sources of phytosterols among commonly used commercial oils (12). Our hypothesis was that cholesterol absorption during the consumption of test meals that contained corn oil would increase after corn oil phytosterols were removed.

To allow for the most direct comparison between purified corn oil triacylglycerol and commercial corn oil, we developed a method to remove both free and esterified phytosterols from bulk corn oil by using the principle of competition for adsorption to silica. We then compared cholesterol absorption in human subjects on 2 occasions after otherwise sterol-free test meals that

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TABLE 1
Efficiency of methods for removal of sterol esters and sterols from oils

	Solvent requirement	Triacylglycerol loading
	L/kg purified oil	*g/g silica*
Silica chromatography[1]	7500	0.017
Optimized silica chromatography[2]	2540	0.017
Saturation adsorption to silica (present method)	31	0.2

[1] From reference 14. Assuming 0.3 g oil applied to 18 g silica and eluted with 2250 mL solvent (scheme A).

[2] From reference 14. As above but assuming use of 3 washes with 4-column volumes of solvent.

contained 30–35 g of a corn oil preparation and deuterated cholesterol tracer. The concentration of the tracer cholesterol was quantified in plasma after each test by using a sensitive, negative-ion, mass spectroscopic procedure. We determined the effect on cholesterol absorption of commercial corn oil, purified corn oil, and purified corn oil with added corn oil phytosterols.

SUBJECTS AND METHODS

Subjects

Twenty-five healthy volunteers (20 women and 5 men) who had no medical or surgical illnesses and who were not taking prescription medications were studied. The characteristics of the subjects were as follows ($\bar{x}$ ± SEM): age, 39.2 ± 2.9 y; BMI (in kg/m^2), 26.4 ± 1.1; total cholesterol, 5.10 ± 0.18 mmol/L; triacylglycerol, 1.07 ± 0.14 mmol/L; LDL cholesterol, 3.28 ± 0.16 mmol/L; and HDL cholesterol, 1.32 ± 0.08 mmol/L. All subjects provided written, informed consent by completing forms that were approved by the Washington University Human Studies Committee.

Materials

Mixed hexanes (H 292) and isopropanol (A 516) were purchased from Fisher Scientific, St Louis. Silica gel of particle size 63–200 μm (S-2905) was obtained from Sigma, St Louis. Mazola corn oil (Bestfoods, Franklin Park, IL) was purchased from a local supermarket. [26,26,26,27,27,27-$^2H_6$]Cholesterol was purchased from Medical Isotopes, Pelham, NH. γ-Oryzanol was obtained from American Tokyo Kasei, Portland, OR.

Methods

Phytosterols were removed from the corn oil in 2 steps. First, free sterols and ferulic acid esters of sterols, which are more polar than triacylglycerol, were removed by diluting 4 kg corn oil with an equal amount of hexane and passing it over 1 kg dry silica gel in a 3-L, coarse sintered glass funnel with moderate vacuum (Corning Life Sciences, Acton, MA). Polar components were selectively bound to the silica while triacylglycerol and long-chain phytosterol esters were passed through it.

Long-chain fatty acid sterol esters were removed from the triacylglycerol in a second step. At least 1 kg of partly purified corn oil diluted in a larger amount of hexane was passed over 1 kg fresh, dry silica in a 3-L coarse sintered glass funnel (Corning Life Sciences). The residual unbound material was removed from the silica by washing it, without disturbing the gel, in aliquots with 3 L hexane, and most of the residual solvent was removed under vacuum. In this step, the excess triacylglycerol competes with long-chain phytosterol esters for binding to silica and saturates the silica so that the bound material does not contain phytosterol esters or other nonpolar materials. Triacylglycerols bound to the silica were 0.20–0.26 kg/kg dry silica gel. Purified corn oil triacylglycerols were eluted with 3 L 19.2% isopropanol:80.2% hexane (vol:vol), an azeotropic mixture, and suction was applied until the silica was dry after which it was heated at 80 °C overnight to prepare it for reuse. The solvents were recovered by distillation and the purified corn oil triacylglycerols were mechanically stirred while being heated at 90–100 °C for ≈2 h until the weight became constant.

The use of hexane and isopropanol is acceptable in the food oil industry (13). Phytosterols, for the readdition of sterols to sterol-free oil, were obtained by eluting the silica gel from the first purification step with the hexane:isopropanol mixture, removing the solvent and dissolving in 200 mL ethanol, saponifying with 13.7 mL 45% KOH and 86 mL H_2O at reflux for 30 min, diluting with water and ethyl ether, separating the organic phase, washing with 4.5% KOH and water, and evaporating the solvent.

Although purification of triacylglycerol from oils is trivial on a milligram or gram scale by use of liquid chromatography (14), the solvent cost is thousands of liters per kilogram, making it impractical for human studies requiring kilogram amounts, even when conditions are optimal (**Table 1**). The key points of our method are the use of saturating conditions to selectively bind a large amount of triacylglycerol to silica and the use of batch methods to minimize solvent consumption. The solvent cost of this procedure is reduced to 31 L/kg oil, and the solvents are recoverable by distillation at 65–70 °C. Although phytosterol esters can also be removed by reversed-phase adsorption, charcoal adsorption, or solvent extraction, it is our experience that each of these procedures requires larger amounts of solvent or produces a less than satisfactory purification.

The fatty acid composition of commercial corn oil and purified corn oil was determined by gas chromatography with flame ionization detection. Phytosterol composition of commercial corn oil was determined with mass spectrometry by using cholesterol as an internal standard. The sterol-free corn oil preparations had <5% of the original phytosterols, and no sterol bands were visible by thin-layer chromatography.

The concentration of deuterated cholesterol in total plasma cholesterol before and 4 and 5 d after the test meal was measured by using negative ion mass spectrometry of pentafluorobenzoyl cholesterol esters, as previously described (9, 15). Samples were chromatographed on a 15 m × 0.25 mm RTX-200 trifluoropropylmethyl polysiloxane column with film thickness of 0.5 μm (Restek, Bellefonte, PA) and analyzed on an Agilent Technologies 5973 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA) in negative-ion, chemical ionization mode with an ion source temperature of 160 °C and a methane reagent gas flow of 0.75 mL/min. Sterol detection was optimized by eliminating small air leaks at the column ends and by using a low methane flow rate.

Clinical protocol

Three independent clinical trials were each performed by using a randomized, double-blind crossover design. In each trial, cholesterol absorption was measured twice in each subject at an

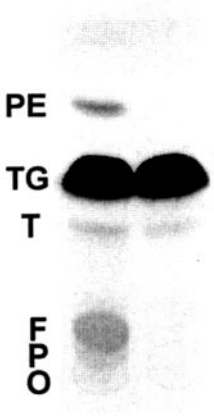

FIGURE 1. Thin-layer chromatogram of commercial corn oil (left) and purified corn oil (right). Silica gel sheets were eluted with 12% ethyl acetate in hexane and stained with iodine vapor. F, ferulic acid esters of phytosterols; O, origin; P, unesterified phytosterols; PE, long-chain fatty acid esters of phytosterols; T, γ-tocopherol; TG, triacylglycerol.

interval of 2 wk by using a meal with different types of corn oil. The test meal consisted of 120 g of a sterol-free pudding containing skim milk powder, cornstarch, sugar, water, and vanilla extract. Each portion contained 30.4 g carbohydrate and 5.4 g protein. Immediately before consumption, this meal was hand mixed with 30–35 g of a corn oil preparation containing 35 mg well-dissolved hexadeuterated cholesterol tracer to yield a meal with 1728–1912 kJ (413–457 kcal). Fasting plasma was collected before consumption of the meal and 4 and 5 d after consumption of the meal. Differences in the plasma hexadeuterated cholesterol concentration achieved in the 2 tests reflect changes in the efficiency of intestinal cholesterol absorption due to differences in the corn oil preparations used.

Study 1

Ten subjects received 35 g commercial corn oil (containing 270 mg intrinsic corn oil sterols) upon one occasion and 35 g purified corn oil triacylglycerol on the other.

Study 2

Five subjects received 30 g purified corn oil triacylglycerol on one occasion and 30 g purified corn oil triacylglycerol + 150 mg corn oil phytosterols upon the other.

Study 3

Ten subjects received 30 g purified corn oil triacylglycerol upon one occasion and 30 g purified corn oil triacylglycerol + 300 mg corn oil phytosterols on the other.

RESULTS

The 3 principal trace components of commercial corn oil, as judged by thin-layer chromatography, are long-chain fatty acid esters of phytosterols, ferulic acid esters of phytosterols (γ-oryzanol), and unesterified phytosterols (**Figure 1**). After processing, the purified triacylglycerol contained no free or esterified sterols. A small amount of γ-tocopherol (which is not a sterol) was present in both original and purified oils and is not resolvable by this technique. As shown in **Table 2**, the fatty acid composition of the commercial and purified oils was very similar. **Table 3** gives the phytosterol content and distribution of commercial corn oil, which contained 0.77% total phytosterols by weight.

TABLE 2
Fatty acid composition of oils used

Fatty acid	Corn oil	Purified corn oil
	% by wt	
14:0	0.0	0.2
16:0	10.4	10.8
16:1	0.1	0.1
18:0	2.1	2.3
18:1	28.7	30.9
18:2	57.5	54.8
18:3	1.2	0.9

Randomized, paired cholesterol absorption tests were performed in healthy subjects where various phytosterol-containing corn oil preparations were given during one test and sterol-free purified corn oil during the other. The concentration of hexadeuterated cholesterol tracer was measured in plasma 4–5 d after each test meal. In study 1, the effect of unpurified commercial corn oil was compared with sterol-free purified corn oil. Cholesterol absorption increased in all but one subject after the consumption of purified oil, and purification resulted in a 38.0 ± 10.2% mean increase in cholesterol absorption compared with the consumption of commercial corn oil ($P < 0.005$). To determine whether phytosterols were in fact the active agent, corn oil sterols were isolated and then added back to purified corn oil at 2 concentrations (studies 2 and 3). The composite data from these 3 independent clinical trials are shown in **Figure 2**,

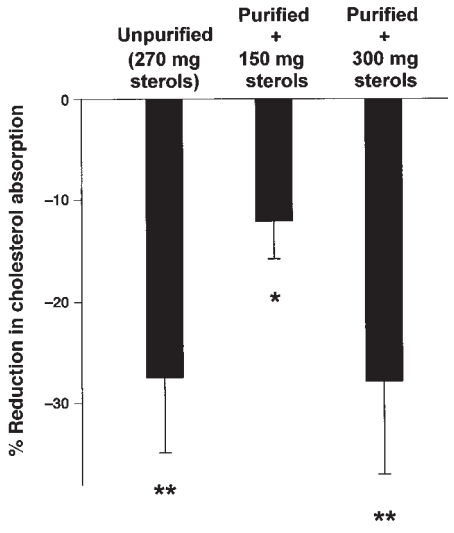

FIGURE 2. Mean (±SEM) reduction in plasma hexadeuterated cholesterol tracer concentration after consumption of corn oil sterols. Cholesterol absorption was determind in paired test meals comparing 1 of 3 phytosterol-containing corn oil preparations with purified sterol-free corn oil: unpurified corn oil compared with purified corn oil and purified corn oil containing 150 or 300 mg added corn oil sterols compared with purified corn oil. Plasma hexadeuterated cholesterol concentrations after purified corn oil ingestion were 0.557, 0.655, and 0.512 mmol/mol natural cholesterol in the 3 groups, respectively. $^{*}P < 0.05$. $^{**}P \leq 0.01$.

TABLE 3
Phytosterol composition of commercial corn oil determined by mass spectrometry

	Phytosterols	
	Weight	Amount
	% by weight	*%*
Sitosterol	0.593	76.7
Campesterol	0.147	19.0
Stigmasterol	0.014	1.8
Sitostanol	0.014	1.8
Campestanol	0.005	0.7
Total phytosterols	0.773	—

which expresses the results as a percentage reduction from the higher levels of absorption observed with purified corn oil. Supplementation of purified corn oil with 150 mg corn oil phytosterols resulted in a 12.1 ± 3.7% reduction in absorption ($P = 0.03$), whereas the addition of 300 mg sterols resulted in a reduction of 27.9 ± 9.1% ($P = 0.01$). By comparison, substituting unpurified commercial corn oil containing 270 mg phytosterols resulted in a reduction of 27.5 ± 7.4%. This suggests that phytosterols are the principal active agent in commercial corn oil. Each test breakfast contained 30–35 g corn oil, which provided ≈40% of energy as fat.

DISCUSSION

Although previous work has shown that extracted, concentrated phytosterols given as a dietary supplement reduce serum cholesterol concentration (8), there has been no evidence that the smaller amounts of phytosterols found in vegetable foods are bioactive. In the present study, we found that the natural concentrations of dietary phytosterols in corn oil, taken in amounts that might be consumed in some recommended diets at 40% of energy, have a substantial effect on the efficiency of intestinal cholesterol absorption. To show this, we took advantage of previous work showing that inhibitors of cholesterol absorption can have prominent effects during single-meal tests that can be quantitated by using nonradioactive, hexadeuterated cholesterol as an oral tracer followed by measurement of the plasma enrichment by using a sensitive mass spectrometric technique (9, 16). We further decreased the test variability by using a standard meal that was free of sterols, except for phytosterols, in the corn oil preparations and hexadeuterated cholesterol tracer. The magnitude of the observed effect on acute cholesterol absorption, a 38% increase in cholesterol absorption after the removal of corn oil phytosterols and a 28% reduction from the increased baseline after their readdition, was prominent and quite similar to reported reductions of 30–43% in cholesterol absorption efficiency with the administration of maximum effective doses of phytosterols and phytostanols in other studies (9, 17, 18). Thus, we believe it likely that phytosterols in natural foods, such as corn oil, meaningfully regulate circulating cholesterol concentration.

Corn oil phytosterols appear to have good bioavailability because a dose of only 150 mg in a test meal had a measurable effect on cholesterol absorption. This is similar to the low effective dose range of phytosterols emulsified with lecithin (9) and suggests that corn oil phytosterols become active during intraluminal digestion of the triacylglycerol. For reference, the mean phytosterol intake in a free-living group of middle-aged Finnish men was 279 mg/d (11). This same study found a significant, inverse correlation between fecal phytosterols and percentage cholesterol absorption. Our work suggests that this correlation may be due to a causal relation with dietary phytosterols contained in vegetable oils and perhaps other foods.

Sterol-free resynthesized oils were used in a previous study of 3 hyperlipidemic subjects, but unfortunately the experimental design did not allow any conclusions to be drawn about the effects of endogenous sterols on cholesterol metabolism (19). Early investigators also attempted to remove sterols and other nonsaponifiables from vegetable oils by vacuum distillation, but a complete separation was never achieved, rendering the studies difficult to interpret (6, 7). In our experience, the purification of oils by vacuum distillation resulted in poor removal of sterol esters (which have the same volatility as triacylglycerol) and unacceptable, heat-related degradation of unsaturated fatty acids. Our method of saturation adsorption to silica allows for the preparation of purified oils without extreme heat in sufficient quantities for clinical investigation so that the effects of natural oil phytosterols can be reinvestigated.

The limitations of the present study should be recognized. We have used only single meal tests, and chronic effects on LDL, although expected, have not yet been verified. It was shown previously that reducing serum cholesterol concentrations by use of phytosterols and neomycin appears to be closely related to a reduction in the percentage of cholesterol absorption (20). Our sterol-free test meals are not typical of recommended diets but do allow for the discovery of potential phytosterol effects hidden in natural baseline diets. Finally, we measured only the efficiency of cholesterol absorption rather than the absolute amount, which depends on endogenous biliary cholesterol secretion.

Nevertheless, our results have implications for the mechanism of action of dietary vegetable fats on lipoprotein concentrations. The substantial effect on the efficiency of intestinal cholesterol absorption shown here indicates that we cannot necessarily attribute all of the effects of vegetable oils to fatty acid structure. It is possible that both unsaturated fatty acids and phytosterols contribute to the beneficial effects of vegetable oils. For example, unsaturated oils increase hepatic LDL receptor activity, decrease LDL production, and increase LDL clearance (1). Although potential mechanisms for the involvement of fatty acids in this process are speculative, these actions are exactly what is anticipated from the known effect of phytosterols to reduce the delivery of dietary and biliary cholesterol to the liver (8). Thus, future work needs to focus on the independent contributions of phytosterol content and fatty acid structure to the regulation of LDL concentration. Dietary studies need to report explicitly the phytosterol content of the fats used. This will require the preparation of new and more accurate food tables because current knowledge of phytosterol intake on a population level is limited.

There are also implications for the industrial manufacturing process. The phytosterol content of oils is not fixed but can be considerably affected by the refining process (21). For example, there can be a 20-fold concentration of phytosterols in steam distillates removed from commercial oils during physical refining (22). If phytosterols are important to the natural diet, refining processes need to be optimized to remove the smallest feasible amount of phytosterols while producing acceptable products.

The present study and the work of many other investigators strongly suggest that phytosterols may be more important in human physiology than previously appreciated and may allow us

an additional means to reduce cholesterol concentrations and prevent atherosclerotic disease.

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