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Modulation of Lipid Metabolism by Phytosterol Stearates and Black Raspberry Seed Oils

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Modulation of Lipid Metabolism by Phytosterol Stearates and Black Raspberry Seed Oils

by

Mark McKinley Ash

A THESIS

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Modulation of Lipid Metabolism by Phytosterol Stearates and Black Raspberry Seed Oils

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Naturally occurring compounds and lifestyle modifications as combination and monotherapy are increasingly used for dyslipidemia. Specifically, phytosterols and fatty acids have demonstrated an ability to modulate cholesterol and triglyceride metabolism in different fashions. In two separate studies, the lipid-lowering effects of black raspberry seed oil and three different phytosterol stearates were examined in order to elucidate the effects of these dietary components and the factors influencing their therapeutic actions.

The first study examined high cholesterol diets supplemented with crude and refined black raspberry seed oils, coconut oil or soybean oil. The crude and refined raspberry seed oil (RSO) treatments significantly altered lipid metabolism, lowering plasma and liver triglycerides while increasing cholesterol ester liver accumulation. Despite the typical reduction of phytosterol content in oils upon processing, both the composition and metabolic effects of the oils did not differ with the exception of increase bile acid excretion in the refined oil.

The second study investigated three phytosterol stearates varying in their phytosterol composition: sitosterol, stigmasterol, or sitostanol. All diets were high in cholesterol and fat to induce dyslipidemia. The phytosterol stearate treatments did not significantly lower plasma cholesterol levels; however, free cholesterol concentrations in the liver were beneficially reduced

by both the stanol stearate and sitosterol stearate dietary treatments. Fecal neutral sterol excretion was elevated in the stanol stearate and sitosterol stearate groups versus the cornstarch control, an effect absent in the stigmasterol stearate treatment, indicating that sitosterol stearate may be more effective than stigmasterol stearate at inducing neutral sterol loss via fecal excretion during this study at a 2.5% (g/g) dose.

In summary, both studies demonstrated beneficial but conservative impacts upon cholesterol metabolism by the specified treatments, indicating that RSOs may be a beneficial therapy and that phytosterol stearates do not dramatically differ in their effects upon cholesterol metabolism at a 2.5% dose.

Dedication

This work is dedicated to my love, my strength, and my joy: Kara, my wife, whom I would like to thank for her endless, loving support and her gracious, self-less attitude. She has definitely done more than her fair share of cooking and household chores during my time in graduate school. Her commitment to sharing in this journey of intellectual development and her willingness to ceaselessly and to joyfully entertain my scientific babblings make tangible the greatness of my debt. I could not be more blessed

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Introduction

Cardiovascular diseases (CVD) are collectively responsible for 29% of global deaths, according to data from the World Health Organization (W.H.O. September 2009). Because of the prevalence of these diseases and the ability to modify susceptibility to disease progression via lifestyle changes (diet and exercise) and drug therapies, considerable research has been devoted to one of the primary risk factors for the beginning of atherosclerosis and the plaque buildup that often progresses to cardiovascular diseases: plasma low-density lipoprotein concentrations (Lloyd-Jones, Adams et al.). While drug therapy involving statins, ezetimibe, or fenofibrates are effective in modulating and correcting dyslipidemia, other means are required for both low-risk individuals and patients experiencing adverse effects of drugs. Specifically, phytosterols have been historically utilized as an effective nutraceutical lipid-lowering therapy to aid in the prevention of CVD.

The purpose of this research was to examine the lipid-lowering effects of black raspberry seed oil and phytosterol stearates in two separate studies in order to further define the parameters for nutraceutical applications of these food products and gain a deeper understanding of how these therapies may exert their effects.

The first study consisted of examining the lipid-modulating effects of crude and refined black raspberry seed oil when added to high cholesterol and high fat diets in male Syrian hamsters. The raspberry seed oil (RSO) study included both a positive soybean oil control (known to beneficially decrease plasma cholesterol levels) and a negative coconut oil control (known to detrimentally elevate plasma cholesterol levels)(Jackson et al 1984). The two RSO diets differed in their extent of processing: the crude oil was not processed but the refined oil was refined and deodorized. Typically, the procedure reduces the phytosterol concentration of the oil by about one half.(Johansson and Hoffmann 1979, Mounts 1981) By design, the study sought to

determine whether the extent of oil processing affects the lipid-lowering capabilities of black raspberry seed oil, either due to different phytosterols concentrations or other factors.

The second study examined three different phytosterol stearates in comparison to a coconut oil negative control in order to determine whether there are distinct advantageous effects that distinguish the efficacy of different phytosterol stearates based upon the phytosterol component. Examining phytosterol esters in current common use, the study investigated their comparative ability to decrease plasma low-density lipoprotein cholesterol concentrations and other lipid metabolic parameters. Specifically, the study was designed to determine the effect of the sterol moiety attached to the fatty acid stearate in order to both understand the mechanism of action more comprehensively and to determine the most effective therapeutic option. The phytosterol stearates investigated were sitosterol stearate, stigmasterol stearate, and stigmastanol/sitostanol stearate (the collective dehydrogenation product of both sitosterol and stigmasterol, thus the compound will be referred to as stanol stearate in this text; Figure 1). By designing the study to examine the effects of sitosterol, stigmasterol, and the collective stanol moieties after controlling for the esterified stearate, insight into the effect of phytosterol structure was hoped to be achieved, specifically in regards to double-bond placement: (1) within the steroid ring (sitosterol); (2) the side-chain and the ring (stigmasterol); (3) or no double bonds (stanol).

In our investigations into the effect of black raspberry seed oil and phytosterol esters on LDL metabolism and plasma levels, we used the Charles River outbred Syrian hamster as our animal model because of the similarity to humans in response to a high fat, high cholesterol diet. (Dorfman et al 2005) When hamsters are challenged with a high fat, high cholesterol diet, they develop atherosclerosis and plaques that resemble those formed in humans. (Dorfman et al 2003, Mawatari et al 2004, Nistor et al 1987, Otto et al 1995) Furthermore, hamsters are a good model for investigating the progression of atherosclerosis not only because of similar plaque histology but because of similar cholesterol transport distribution. Cholesterol plasma transport mirrors the human condition more closely than other rodents, as hamsters carry a significant

portion of plasma cholesterol in low-density lipoproteins when placed on atherogenic diets (high fat, high cholesterol). (Lock et al 2005) When hamsters are placed on a high cholesterol diet (at least 0.1% g/g), low-density lipoprotein cholesterol levels become elevated, an effect that is sensitive to saturated fat intake in a similar manner as in humans, indicating appropriateness as a comparative atherosclerotic model. (Gajda et al 2007)

Although the majority of cholesterol synthesis occurs extrahepatically in all tissues, the liver contributes the most of any single tissue to the cholesterol synthetic pool; and thus takes a primary focus in cholesterol metabolism management. (Dietschy et al 1993, Spady and Dietschy 1983) Estimates of whole body cholesterol synthesis and metabolism place the same level of synthetic and regulatory importance on the liver in both humans and hamsters, indicating another shared similarity and further justifying the use of hamsters as a model of atherosclerosis.

Hamsters fed high saturated fat and high cholesterol diets begin developing aortic atherosclerotic lesions similar in structure and modeling to atherosclerosis in humans after only four weeks of dietary treatment. Because animals (and humans) tightly regulate cholesterol metabolism, it takes a significant amount of time to induce significant changes via dietary interventions; thus, we have designed the length of our studies to be four weeks in order to ensure lipid metabolism homeostasis. (Pien et al 2002)

Clarification of terms. The term “phytosterol” within this publication is used to refer to plant sterols and plant stanols collectively, whereas the terms “plant sterol” and “plant stanol” are used to refer to each specific species. Even though sitostanol and stigmastanol are equivalent structurally, the term “sitostanol” will be used to refer to the compound, which can be synthesized via hydrogenation of either sitosterol or stigmasterol.

Literature Review

I. Cardiovascular Disease and Atherogenesis

Because cardiovascular diseases (CVD) are globally the leading cause of death, research has investigated the etiology, pathology, and clinical treatment of the two major types of CVD: strokes and coronary heart disease (CHD)(NCEP 2002). Both major cardiovascular disease categories are associated with several risk factors including smoking, lack of physical activity, high blood pressure, and dyslipidemia (Lloyd-Jones et al 2010) Subsequently, research has investigated substances and therapeutic options that lower LDL cholesterol levels and thereby decrease CVD risk.(NCEP 2002) Atherosclerosis, a major pathological etiology of CVD, is the narrowing of the arteries of the vascular system due to cholesterol deposition, foam cell formation, and subsequently the initiation of fatty streaks. In response to the damage to the endothelial cells of the vascular tissue, clotting factors stimulate the formation of a thrombus to halt the degradation of the endothelial layer of the vessel. However, with increasing foam cell accumulation and inflammatory oxidation, the thrombus eventually ruptures, potentially blocking arteries and halting blood flow to organs and tissues.

The importance of Low-Density Lipoprotein (LDL) cholesterol in the disease process is paramount and has received much attention, as the oxidation or modification of LDL results in the migration of LDL into the intima of the artery and the genesis of an atherosclerotic plaque.(Matsuura et al 2008) Oxidized LDL is more rapidly taken up by macrophages in intima than native unmodified LDL, resulting in cholesterol accumulation in the cells, the formation of foam cells, and atherogenesis.(Matsuura et al 2008) Subsequently, high circulating LDL cholesterol concentrations in the bloodstream are a known risk factor for atherosclerosis and CHD.(Kiechl and Willeit 1999) Historically, this led to the recommendation of decreasing dietary

cholesterol intake in order to decrease circulating cholesterol levels. However, recent research has indicated that dietary cholesterol does not significantly contribute to atherosclerosis or the risk of CHD in the general population, as it increases both the LDL fraction of cholesterol and the HDL fraction, the anti-atherogenic fraction.(McNamara 2000) In fact, research has demonstrated that the fatty acid composition of the diet is a more important lifestyle modulator of LDL cholesterol levels, as high levels of saturated fatty acids and trans fatty acids intakes have been shown to result in elevated plasma LDL cholesterol concentrations (Oh et al 2005). Because of decreased risk of CHD and atherosclerosis associated with low LDL cholesterol concentrations, the National Cholesterol Education Program has recently designated the optimum plasma LDL target concentration to be <100 mg/dl (NCEP 2001).

Current therapeutic preventative action focuses on lowering the LDL cholesterol concentration in order to decrease atherosclerotic and CHD relative risk. Pharmacologically, statins or 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) inhibitors are used to decrease the synthesis of cholesterol within the body by inhibiting the activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. However, recent research has demonstrated that statins are not as safe as once believed, resulting in adverse events in 29.5% of patients in the case of simvastatin.(Kastelein et al 2008)

With high rates of side effects, statins are not the best option for everyone due to potential hepatotoxicity and other tissue damage. Even moderately dosed statin therapy is not the most appropriate choice for reducing CHD risk in the majority of the population because of the increased occurrence of adverse events, including liver function abnormalities and elevations in creatine kinase.(Silva et al 2007) Statins were shown to elevate serum alanine aminotransferase and aspartate aminotransferase levels, indicating a hepatotoxic response to treatment.(Kashani et al 2006) Furthermore, while statins are potent drugs for LDL concentration reduction, the consumption of phytosterol enriched margarines by statin users resulted in a 10% reduction of LDL cholesterol, whereas doubling the statin dose only produced an additional decrease in LDL

cholesterol of 3-7%.(De Jong et al 2008b) Thus, other treatment options must be considered in combination with statin treatments and as stand-alone preventative measures.

II. Effect of Phytosterols on Cholesterol Metabolism

While there are many compounds currently undergoing research for their plasma LDL cholesterol-lowering effects, plant sterols and stanols are among the most potent. Phytosterols decrease cholesterol absorption up to 40% and have been shown to effectively lower plasma total cholesterol, LDL cholesterol, and apo-B (the lipoprotein required for LDL formation) at a dosage of 1.8 g/day regardless of the initial plasma cholesterol condition of the patient.(Miettinen and Gylling 2004, Ntanios et al 2002) While naturally occurring plant stanols contribute only 5-10% to total phytosterol abundance observed in nature (Carr et al 2006), both free and esterified plant sterols and stanols lower LDL cholesterol levels in hypercholesterolemic, mildly hypercholesterolemic, and normal individuals.(Ntanios et al 2002)

In a typical modern diet, phytosterol intake is roughly 160-360 mg/day; thus, the dosage recommended simply recommends the increase of a nutritive substance already found in our food supply.(Morton et al 1995) The currently proposed mechanism responsible for the majority of phytosterol LDL-lowering relies upon the displacement of cholesterol from micelles, resulting in a reduction in the amount of cholesterol hydrolyzed and available for absorption.(Awika and Rooney 2004) The reduction in cholesterol absorption (both of dietary and biliary cholesterol) forces the body to increase cholesterol synthesis and LDL cholesterol uptake into the liver, clearing the blood of LDL cholesterol.(Carr et al 2006) However, in order to maximize practical applications of administration, plant sterols can be esterified with long chain fatty acids to increase their solubility in oil from 2% to 20% without impairing their ability to decrease cholesterol absorption.(Jandacek et al 1977, Mattson et al 1977) In a long term study over eighty-five weeks, Plant sterol and stanol ester consumption reduced LDL cholesterol 8.7% and 13.1%

respectively (De Jong et al 2008b). After one week, plant sterol consumption lowers serum cholesterol by 16%, cholesterol absorption by 40%, and fecal output of cholesterol by 36%; thus, plant sterol/stanols and phytosterol esters reduce plasma LDL cholesterol concentrations and CHD risk.(Gould et al 2007)

III. Clinical Aspects of Phytosterol Therapy

Because there is strong evidence of an association between elevated low-density lipoprotein cholesterol (LDL-C) levels and cardiovascular disease, LDL-C lowering therapies have received much attention, especially because they have demonstrated an ability to reduce relative disease risk even in individuals with average serum levels.(Downs et al 1998, Martin et al 1986, Stamler et al 1986) Furthermore, lowering serum LDL-C and increasing high-density lipoprotein cholesterol (HDL-C) has been shown to lead to a regression in atherosclerotic lesion progression.(Nissen et al 2006) Because common drug therapies, such as statins and fibrates, do induce adverse effects, much interest has been generated in using alternative LDL-C lowering strategies.(Florentin et al 2008, Kiortsis et al 2007) Adverse effects of statins have been shown to be dose-dependent, indicating any combination treatment that lowers the drug dose via substitution with phytosterol supplementation would be beneficial.(Golomb and Evans 2008)

Natural plant sterol intake

Plant sterols, stanols, and their collective esters are therapeutically used to lower LDL-C both as a monotherapy and in combination with drugs in both normocholesterolemic and hypercholesterolemic individuals (Ntanos et al 2002, Quilez et al 2003a). In a Northern Swedish population, investigators demonstrated an inverse correlation between plant sterol intake and serum LDL-C levels, suggesting the possibility that phytosterols may reduce serum cholesterol concentrations even at normal dietary intake levels (Klingberg et al 2008). Ostlund et al. (2003) demonstrated that human subjects fed phytosterol-free wheat germ oil incorporated into a breakfast muffin in place of wheat germ oil naturally high in phytosterols increased serum radiolabeled cholesterol enrichment by 42.8%, indicating that naturally occurring levels of phytosterols may attenuate cholesterol absorption even after a single dose (Ostlund et al 2003). This response was also demonstrated utilizing corn oils (Ostlund et al 2002b).

However, the reduction of cholesterol absorption does not necessitate that a significant decrease in LDL-C is obtained. While a phytosterol-deficient diet supplemented with 400 mg/d of phytosterols did not quite produce a statistical reduction of LDL-C ($p=0.077$), the natural phytosterol intake level decreased cholesterol absorption and increased both total fecal cholesterol and biliary cholesterol excretion (Racette et al 2009). Viewed altogether, a baseline dietary intake of phytosterols has a significant positive effect upon cholesterol metabolism (Racette et al 2009). Despite the possible importance of dietary phytosterols in cholesterol metabolism, supplemental doses of phytosterols are required to achieve maximal reductions of elevated LDL-C concentrations in many individuals.

Phytosterol Supplementation Efficacy and Dose

While natural phytosterol intakes may have hypocholesterolemic effects, a higher dose of phytosterols via supplementation dramatically and positively influences cholesterol metabolism, consistently lowering LDL-C about 10%. (Katan et al 2003) Wu et al. published a meta-analysis of phytosterol supplementation, concluding that administration lowers serum total cholesterol (TC), LDL-C, and triglycerides. (Wu 2009) Upon evaluation of the 20 publications analyzed (Wu 2009), only one demonstrated a significant triglyceride-lowering effect (Maki et al 2001). Most published meta-analyses are in agreement that phytosterols do not affect either serum triglycerides or high-density lipoprotein cholesterol (HDL-C). (Katan et al 2003, Law 2000, Moruise et al 2006) The meta-analysis of Katan et al. (2003) acknowledged the phytosterols lowered serum triglycerides in a few studies but asserted that the effect is sporadic and not supported by the general body of literature. (Katan et al 2003)

The main action of phytosterols is to lower LDL-C and subsequently total cholesterol. While 0.83, 1.6, and 3.24 grams per day (g/d) did not differ significantly in LDL-C lowering capacity, most meta-analyses conclude that phytosterols lower LDL-C dose-dependently up to 2 g/d with a maximal LDL-C decrease of 8-14%. (Hendriks and Weststrate 1999, Katan et al 2003, Law 2000) The meta-analysis of Moruise et al. was restricted to hypercholesterolemic trials only (four trials) but reached relatively the same conclusion: 2.3 g/d of phytosterols for 6.5 weeks lowered TC and LDL-C by 7 to 11% and 10-15%, respectively. (Moruise et al 2006) This is in agreement with the 2.15 g/d phytosterol dose calculated from the 8.8% maximal LDL-C decrease obtained from the meta-analysis LDL-C dose-response curve modeled by Demonty et al. (Demonty et al 2009) The placebo adjusted absolute LDL-C change increased with age but the percent decrease in LDL-C did not differ, achieving a maximum effect at 2 g/d of plant sterol or plant stanol equivalents with minimal benefit beyond 2.5 g/d (phytosterol ester dose is

sometimes expressed in terms of phytosterol equivalents to remove the fatty acid contribution to the dose weight).(Katan et al 2003) The eighty-four trial meta-analysis of Demonty et al. also demonstrated no difference between the LDL-C lowering of free and esterified phytosterols.(Demonty et al 2009)

Phytosterol Dose Frequency

Phytosterols have been administered within capsules, tablets, and food matrices via one or more doses a day and have demonstrated mixed results. When soybean phytosterol esters were incorporated into ground beef and administered once a day , TC and LDL-C were lowered by 9.3 and 14.6%,respectively(Matvienko et al 2002). However, other data demonstrate that a single morning dose does not lower TC or LDL.(AbuMweis et al 2006) In determining the dose-curve for LDL-C within the Demonty meta-analysis, the possibility of a dose-frequency effect upon LDL-C lowering was examined. When multiple doses of phytosterols were consumed and compared to single-dose treatments, LDL-C decreases with multiple doses were almost significantly greater than during a single dose ($p=0.054$). (Demonty et al 2009) However, this frequency of dose effect was confounded by dosage because multiple dose treatments tended to administer higher doses versus single dose trials.(Demonty et al 2009)

A meta-analysis by AbuMweis et al. (2008) determined that a single morning dose did not significantly affect LDL-C(AbuMweis et al 2008). In a trial, AbuMweis et al. (2009) specifically compared the effects of supplementing plant sterols three times a day versus a single dose and concluded that the a single morning dose was not as efficacious as multiple doses; however, based upon the data and the comparison of the LDL-C values after treatment, there was actually no difference, and the conclusion should have been equal effectiveness; however, it should be noted that the trial only lasted six days, and this may not have been sufficient time to

allow the frequency of dose effect to manifest.(AbuMweis et al 2009) Regardless, the frequency of dose may contribute to 5% of the variation within LDL-C changes, whereas the dose may account for 14%.(Demonty et al 2009) However, the results of the meta-analysis are merely suggestive of an effect and need to be verified with appropriately controlled trials to definitely decide the effect of consumption frequency upon LDL-C lowering capability.

Efficacy of Plant Sterols versus Plant Stanols

There are two forms of phytosterols administered to lower LDL cholesterol concentrations: sterols and stanols. The therapeutic differences between sterols and stanols are important to consider in order to maximize LDL-C lowering. However, there appear to be no significant differences between the two phytosterols in their ability to lower LDL-C.(Jones and Ntanios 1998) Hallikainen et al. demonstrated that 2 g/d of stanol ester or sterol ester as part of a low-fat diet lowered LDL-C with statistical equivalency by 12.7% and 10.4%,respectively.(Hallikainen et al 2000) Katan et al. also concluded no therapeutic difference between plant sterols and stanols in lowering LDL-C (Katan et al 2003).

While the equal short-term efficacy of phytosterols is commonly accepted, the equality of plant sterols and stanols over the long-term is debated. Few studies have examined the long-term efficacy of phytosterol supplementation. O'Neill et al. (2005) concluded in a meta-analysis of 14 trials that compared both phytosterols esters that plant stanols esters maintain their LDL-C lowering effect over time whereas plant sterols esters do not (O'Neill et al 2005). However, the meta-analysis used eleven trials that tested one of the phytosterols alone but only three of the trials featured both plant sterols and stanols; thus, the conclusions are far from conclusive. None of the three trials that compared stanol esters to sterol esters within the same study concluded that plant stanols were superior. Of the three head-to-head trials included, both Weststrate et al. and

Hallikainen et al. concluded no difference in LDL-C lowering between sterols and stanol esters, whereas Jones et al. demonstrated a greater absolute reduction in LDL-C concentrations upon plant sterol ester versus plant stanol ester treatment (Hallikainen et al 2000, Jones et al 2000, Weststrate and Meijer 1998).

The trials included in the meta-analysis by O'Neill et al. only lasted between three and four weeks making the "efficacy-diminishing effect" suggested unfounded based upon the trials included in the analysis. Furthermore, Hendriks et al. demonstrated that sterol esters maintained a LDL-C lowering effect over one year (Hendriks et al). Gylling et al. demonstrated that plant sterol and stanol esters administered over one year statistically equivalently lowered serum total cholesterol by 4.4% and 4.2%, respectively (LDL-C was not reported) (Gylling et al 2009). However, one long-term study in statin users resulted in a significant decrease in LDL-C of 13.1% for users of plant sterol esters but no decrease in the plant stanol treatment (De Jong et al 2008b). However, there was no control-adjusted statistical comparison between the treatments thus any conclusion comparing the two would be circumspet, as the authors even denoted that the study design was not to examine the difference between stanols and sterols. De Jong et al. further noted no difference between plant sterols/stanol ester-induced LDL-C lowering in statin users after sixteen weeks (De Jong et al 2008a). Some investigators suggested that these studies demonstrate that there is a decrease in efficacy of phytosterols overtime; however, it is possible a decrease in compliance is responsible for this loss of efficacy effect due to the free-living conditions (Miettinen and Gylling 2005). Altogether there seems to be no evidence to recommend plant stanol esters over plant sterol esters in hopes to maximize LDL-C decreases.

Food Matrix and Background Diet

Phytosterols have been incorporated into various food matrices and tested under many different background diets as cholesterol-lowering therapies. Specifically, phytosterols have variably but effectively lowered LDL-C when incorporated into margarine spreads, (low-fat) yogurt drinks, low-fat (fermented) milks, mayonnaise, salad dressings, chocolate, orange juice, vegetable juice, hard cheese, fresh cheese, milk tea, ground beef, muffins, croissants, bread, lemonade, vegetable oil, butter, tortilla chips, non-fat beverage, cereal, capsules, and tablets (Demonty et al 2009). The efficacy of phytosterols appears to function independently of dietary fat, thus there has been a movement to incorporate phytosterols into low-fat foods in order to promote a low-fat low cholesterol diet profile to further improve health lipid profiles (Chen et al 2009).

After a four week lead-in period on a National Cholesterol Education Program Step I diet, plant sterol esters at 1.1 g/d and 2.2 g/d lowered LDL-C by 7.6% and 8.4% (Maki et al 2001). Despite the variability of phytosterol efficacy in various food matrices, the meta-analysis by Demonty et al. concluded that neither the fat content of the food format nor the nature of the food as dairy or non-dairy significantly affected the LDL-C-lowering efficacy of the phytosterol product (Demonty et al 2009). However, the comparison of solid and liquid food demonstrated a significant effect upon the dosage curve, indicating that high phytosterol doses within solid matrices would yield a maximum LDL-C 5.2% greater than in liquid foods, but this difference was negligible at a dose of 2g/d (Demonty et al 2009).

The meta-analysis by AbuMweis et al. conflictingly concluded that LDL-C concentration reductions were greater when phytosterols were incorporated into fat spreads, mayonnaise, salad dressing, milk, and yogurt, versus croissants, muffins, orange juice, non-fat beverages, cereal bars, and chocolate (AbuMweis et al 2008). Clifton et al. demonstrated evidence of the

conclusion by AbuMweis that plant sterol esters have significantly different abilities to lower LDL-C when incorporated into different low-fat food matrices: (15.9%) milk (15.9%) > yogurt (8.6%) > bread (6.5%) = breakfast cereal (5.4%).(Clifton et al 2004) Plant stanol tablets have also been used in statin combination trials, demonstrating a decrease in LDL-C (Goldberg et al 2006). However, in a study comparing capsules and stanol lecithin tablets, the tablets led to a decrease in cholesterol, whereas the capsules did not.(McPherson et al 2005) Other trials have demonstrated that encapsulated phytosterol esters effectively lowered LDL-C.(Acuff et al 2007, Earnest et al 2007) For further reading on the incorporation of phytosterols into different food formats, see the review by St-Onge and Jones.(St-Onge and Jones 2003)

Therapeutic Combination Strategies

For individuals that cannot achieve LDL-C goals with diet and phytosterols alone, combination treatments have proven promising. Phytosterols have the potential to provide an adjunct therapy for the use of statins, fibrates, omega-3 fatty acids, niacin, and bile acid bind resins.

Statins. There is evidence that plant sterol and stanols produce an additive decrease in LDL-C for individuals on statin therapy.(Thompson 2005) Administration of plant sterol esters to individuals on statins has demonstrated an additional reduction in concentrations of TC by 5.7-7% and LDL-C by 9.1-10.3%.(Blair et al 2000, De Jong et al 2008a, Goldberg et al 2006) However, not all data demonstrate an additive effect of phytosterols upon statin treatment. A low dose of Simvastatin was compared to plant sterol esters alone and plant sterol esters in combination with statin treatment, non-HDL cholesterol was lowered by 12.8% (plant sterol ester (PSE)), 30.7% (simvastatin), and 35.4% (PSE+simvastatin) but the combination treatment did not

differ significantly from the simvastatin treatment alone, indicating no advantage to the combination versus simvastatin alone.

Fibrates. Fibrates are primarily used to significantly lower serum triglyceride levels up to 50%, often with the added secondary effects of increasing HDL cholesterol and reducing LDL-C. (Chapman 2003) However, fibrates can negatively effect serum lipids via elevating LDL-C levels in hypertriglyceride subjects. (Chapman 2003) Phytosterol combination with fibrates are a possible therapeutic option to simultaneously lower triglyceride levels and LDL-C, offsetting the variable effect of fibrates upon LDL-C. (Chapman 2003)

When plant sterols were added to the regimen of fibrate users and non-users following a strict low cholesterol diet (NCEP step 1), TC and LDL-C reduction were 8.5% and 11.1% in comparison to control spread reductions 0.0% and 1.3%, respectively, but there were no side-effects or changes in HDL-C concentrations (Fabienne Nigon 2001). While this study did not specifically examine phytosterols as an adjust therapy to fibrates, it demonstrated that phytosterols positively impact lipid blood profiles regardless of fibrate treatment.

The only other study examining this combination is a serial treatment regime of seven children without a control group that demonstrated progressive lowering of LDL-C by 50% as three treatments were added: dietary intervention, sitosterol, and finally bezafibrate (Becker et al 1992). While the LDL-C concentrations of these children decreased over the study time-period, a treatment effect cannot be concluded because the study was not controlled. However, the study does demonstrate that combination therapy of phytosterols and bezafibrate did not produce any side effects over the treatment period and the subsequent twenty-four months. Further research is required to determine the effect of this combination.

Ezetimibe. This therapeutic option is prescribed for its ability to block the intestinal absorption of cholesterol, which is believed to be mediated via the inhibition of Niemann-Pick

C1-like 1-mediated cholesterol absorption (Betteres and Yu 2009). Because both ezetimibe and plant sterols work to block absorption of cholesterol, it is not surprising that combination treatment with phytosterols and ezetimibe is no more effective than ezetimibe alone (Jakulj et al 2005).

Bile Acid-Binding Resin and Statins. Bile Acid Binding Resins function by promoting bile acid fecal loss and subsequent bile acid synthesis from cholesterol. In an uncontrolled before and after trial within hypercholesterolemic coronary patients on a low-fat, low-cholesterol diet, a low-dose simvastatin (20mg/d) was added for three months, before the addition of a plant stanol ester margarine for eight weeks, followed by the addition of cholestyramine (8 g/d) for another eight weeks. The addition of simvastatin lowered LDL-C by 39%, phytosterols by an additional 13%, and cholestyramine, to the cumulative 67% decrease (Gylling H 2002). While the study is not controlled and is only preliminary, more research should investigate this therapy both in the absence and presence of statins.

Omega-3 Fatty acids. Omega-3 fatty acid supplementation use is prevalent due to the anti-inflammatory and triglyceride-lowering capabilities of the lipids (Balk et al 2006). In monotherapy, omega-3 (3g/d) in the form of fish oil has demonstrated the ability to lower serum triglycerides up to 30% (NCEP 2002). When omega-3 fish oil and phytosterols were co-administered, total and LDL-cholesterol decreased statistically equivalent to sunola oil with plant sterol esters without attenuating the fish oil serum triglyceride lowering effect or the anti-inflammatory reduction of serum Tumor Necrosis Factor- α (Micallef and Garg 2008, Micallef and Garg 2009).

Omega-3 fatty acids have also shown promise upon incorporation into the phytosterol ester via esterification. Fish oil alone, fish oil sterol esters, and sunflower oil sterol esters were compared in a crossover study and fish oil esters both lowered LDL-C statistically equivalently to

sunflower oil sterol esters and reduced serum triglycerides by 40%) compared to the olive oil control diet (Demonty et al 2006). The reduction in serum triglycerides was greater than fish oil by itself. When sunflower oil, olive oil, and fish oil fatty acid phytosterol esters were compared to an olive oil control diet, there were no differences in total, LDL, or HDL cholesterol in comparison to the olive oil diet, but the fish oil phytosterol ester group did result in significantly lower fasting and postprandial triglycerides in comparison to the sunflower and olive oil phytosterol esters (Jones et al 2007).

Niacin. Niacin increases HDL cholesterol by 15-30% and reduces triglycerides by 20-35%. (NCEP 2002) Variably, Niacin has demonstrated some LDL-C lowering capabilities but only at high doses that are often unmanageable due to side-effects such as flushing, hepatotoxicity, and hyperglycemia in diabetic individuals. (NCEP 2002) Niacin not only favorably alters the serum lipid profile but has demonstrated a reduction in carotid artery plaque progression in individuals concomitantly on statins. (Lee et al 2009)

The combination of niacin and phytosterols has only been examined in the apoE mouse model of atherosclerosis and demonstrates no superiority of niacin and phytosterols versus phytosterols alone in either LDL-C lowering, serum triglycerides, HDL-C, or even lesion size. (Yeganeh et al 2005) However, this apoE knock-out model may be insufficient in addressing the combined action of niacin and phytosterols upon cholesterol metabolism because of the absence of apolipoprotein E may disturb the balance of lipoprotein clearance, as apolipoprotein E is transferable from VLDL to HDL, where the effects of its presence here are not completely understood. (Nguyen et al 2009) Regardless, only more research will elucidate whether there are possible clinical benefits to the combination of niacin and phytosterols.

Exercise. Because exercise has shown an ability to increase HDL-C and phytosterols do not typically affect HDL-C concentrations, a study examined the effect of plant sterol esters and

exercise in previously sedentary hypercholesterolemic individuals. While there is no no sterol-by-exercise interaction, and thus no synergistic effect, plant sterol supplementation appeared not to attenuate the positive HDL-C increase and TG lowering effect of the three day a week exercise program completed under supervision.(Varady et al 2004) Other data indicate that phytosterols may offset exercised-induced HDL increases.(Alhassan et al 2006) However, the results of phytosterols and exercise in combination are inconsistent and require more work to determine whether there are any consistent combination effects. For further reading on the effects of exercise and phytosterols, see the review by Marinangeli et al.(Marinangeli et al 2006)

Predicting Efficacy Based On Individuality

Baseline Serum Cholesterol Concentration. In an effort to maximize LDL-C-lowering efficacy, an understanding of the factors that influence the response of individuals to phytosterol therapy must be obtained. One of the most obvious factors is the baseline cholesterol levels of individuals and how that may influence the ability of phytosterols to have an effect upon the lipid profile. A meta-analysis displayed a significant correlation between the baseline LDL-C and the difference between the LDL-C change predicted (via dose) and the actual LDL-C change, indicating that some of the variance in individual responses is due to the baseline cholesterol values.(Demonty et al 2009) Another meta-analysis of five studies undertaken within the same laboratory to minimize sample analysis variation, plant stanol ester consumption resulted in larger absolute decreases in total and LDL cholesterol when patients had higher baseline serum levels but the percent decrease of cholesterol was not affected.(Naumann et al 2008) In other words, phytosterols reduce serum LDL-C more dramatically in individuals with higher baseline levels but consistently decrease serum LDL-C by a relatively constant percentage.

Baseline Serum Plant Sterol Concentrations. Cholesterol-standardized serum plant sterol concentrations are used as serum markers of baseline cholesterol absorption because of the correlation between the cholesterol-adjusted values and the fractional cholesterol absorption..(Miettinen et al 1990, Tilvis and Miettinen 1986) Because phytosterol therapy targets systemic absorption of cholesterol, there is some evidence that individuals who have higher baseline levels of cholesterol absorption may respond better to phytosterol treatment based upon the inverse premise that individuals with lower cholesterol absorption respond better to statin treatment.(Gylling and Miettinen 2002, Matthan et al 2009, Miettinen et al 1998) However, plant sterol concentrations appear to be hereditary and may not always be indicative of cholesterol absorption but variant function in the ATP-binding cassette sub-family G member 5/G member 8 (ABCG5/G8) that shuttles phytosterols out of the enterocyte and back into the lumen of the intestine, preventing systemic absorption (Berge et al 2002, Graf et al 2003). This concept is supported by the nature of the serum phytosterol response to phytosterol treatment.

Despite lowering cholesterol absorption, administration of plant sterols is known to increase serum concentrations of plant sterols. (Katan et al 2003) In individuals on phytosterol treatment, the nature of the relationship between serum plant sterols as biomarkers of cholesterol absorption has changed, because the serum concentrations increase despite the decrease in cholesterol absorption. Conversely, plant stanol therapy may not disrupt the association of cholesterol and serum plant sterol concentrations, as stanols are known to lead to minimal increases in serum phytosterol concentrations, whereas plant sterol supplementation substantially increases serum plant sterol concentrations.(De Jong et al 2008b, De Jong et al , Hallikainen et al 2000, Jones et al 2000) Even at 8.8 g/d of stanol esters, serum campestanol concentrations increased only 8.7 µg/dL after control adjustment whereas control-adjusted campesterol concentrations decreased by 153 ug/dL (Gylling et al). While plant sterols and stanols do consistently influence plasma phytosterol levels differently, the impact of elevated phytosterol serum concentrations during therapy is not fully understood and warrants more study to

determine the strength of the relationship between cholesterol absorption and serum phytosterols during phytosterol therapy in order to justify the usage of serum phytosterols as biomarkers of cholesterol absorption and to better manage the treatments of individuals who may or may not respond to absorption inhibition therapy.

Apolipoprotein E Genotype. Based upon the increased proportion of apoE4 genotype carriers among cardiovascular event cases in comparison to controls, a meta-analysis in the past concluded that there is an association between the apoE genotype locus and cardiovascular disease risk; apoE4 genotypes have an increased CHD risk versus apoE3 genotypes, whereas apoE2 genotypes have a decreased risk (Wilson et al 1996). While epidemiological data estimating the effect of the apoE genotype upon cardiovascular disease risk is commonly accepted, the evidence supporting the association between serum lipid variation and apoE genotype is not nearly as convincing. (Eichner et al 2002) Observational population studies have demonstrated that apoE4 phenotypic populations have statistically higher LDL-C concentrations than apoE3. (EJ Schaefer 1994, Howard et al 1998)

While some estimates indicate that the apoE genotype is responsible for 7% of serum lipid concentration variations across individuals, data from the Framingham offspring study demonstrated only a 1% variance and 2.1% variance in men and women, respectively, in LDL-C levels in response to the apoE locus (EJ Schaefer 1994, Ordovas 2009). Vanhanen et al. indicate that phytosterols decrease cholesterol more effectively in apoE4 genotypic individuals. (Vanhanen et al 1993) However, the data cited demonstrated no statistical comparison between the control-adjusted apoE4 and apoE3 genotypes but instead utilize a mere comparison to the control for each genotype individually, indicating that any conclusion of that nature is pre-emptive. (Vanhanen et al 1993) Sanchez-Muniz et al. concluded the opposite result, that phytosterol supplementation “may be of little value in apoE4 carriers;” however, upon closer examination, the same faulty comparison was made. (Sanchez-Muniz et al 2009)

Understanding why these conclusions were drawn aids in making sense of the rest of the literature regarding phytosterols and apoE genotype. Plat and Mensink (2002) did not observe a differential lowering of LDL-C based upon apoE genotype. Instead, the participants experienced a -0.42 mmol/L decrease of LDL-C across the treatment group regardless of apoE genotype (Plat and Mensink 2002). Geelen et al. (2002) demonstrated in a pre-trial apoE genotype screened, cross-over study administering plant sterol esters to normocholesterolemic individuals that despite an initial difference of elevated total cholesterol within the apoE4 group versus the apoE3 group, there were no significant differences according to apoE genotype in phytosterol-induced cholesterol-lowering (Geelen et al 2002). Ishiwata et al. provides further evidence that phytosterol ester cholesterol-lowering responses are not influenced by apoE genotype (Ishiwata et al). Hallikainen et al. (2000) demonstrated that apoE4 individuals had a greater LDL-C decrease than apoE3/3 individuals, providing some evidence of an apoE genotypic effect (Hallikainen et al 2000). Regardless, there is evidence that baseline total and LDL cholesterol serum concentrations are lower within individuals with apoE2 alleles versus apoE3 and apo4 alleles (Nissinen et al 2008, Plat and Mensink 2002).

IV. Safety Considerations

While the nutraceutical/function food safety literature has yet to reveal any deleterious side effects of phytosterols and phytosterol esters (PSE), the potent LDL cholesterol-lowering compounds do raise a few concerns. One potential negative impact of phytosterol fortification in the food supply stems from the induced elevation of serum levels upon plant sterol administration. Simulation studies have estimated that when multiple dietary items are replaced with phytosterol-fortified products, the median phytosterol intake may be 5.5 g/d in men and 4.6 g/d in women but climb up to 8.6 g/d or even 13 g/d if more liberal fortification is allowed (De Jong et al , Kuhlmann et al 2005). While the risk posed by an elevation in phytosterol supplementation is minimal in the majority of individuals, substantial phytosterol-fortification within food products may leave undiagnosed sitosterolemic individuals with a much higher risk of premature atherosclerosis due to the acceleration of serum phytosterol accumulation upon phytosterol-fortified food intake (Fernandez and Vega-Lopez 2005). The risk of phytosterol plasma elevation in sitosterolemic individuals will not be covered further in this review. However, it is worthy to note that even in unaffected individuals, serum phytosterols increase upon plant sterol intake but not upon plant stanol intake (Fernandez and Vega-Lopez 2005, Sudhop and von Bergmann 2004).

While the impact of elevated phytosterols is not completely understood, incorporation into aortic plaques is strongly associated with the proportion of serum phytosterols to cholesterol (Helske et al 2008). Furthermore, oxidized phytosterols are detected in the serum of sitosterolemic patients in a higher percentage than cholesterol, suggesting an increased atherogenicity (Plat et al 2001). However, the physiological relevance of the effects of oxidized phytosterols have yet to be fully elucidated. Regardless, the substantial decrease in the LDL-C concentration, which has demonstrated reliable atherogenicity, should potentially offset the

minimal (in magnitude comparison) increase in serum plant sterol levels despite the uncertainty of their atherogenicity.

After long-term consumption, plant sterol enriched products elevate serum plant sterol concentrations in humans (Fransen et al 2007). Elevations of sitosterol concentrations may be associated with an increased occurrence of major coronary events in men at high risk for CHD (Assmann et al 2006). However, in one animal study, the presence of foam cells in the aortic arch showed an inverse relationship with dietary plant sterol esters (PSE) ($P < 0.0001$), indicating that PSE may actually inhibit atherosclerotic development (Ntanios et al 2003). However, β -sitosterol was shown to cause apoptosis in human abdominal aorta endothelial cells (stronger toxic effect than cholesterol), indicating an increase in atherosclerotic risk but also a decrease in the viability of cancer cells (Rubis et al 2008).

Oxyphytosterols, the oxidization product of phytosterols, are another safety concern, as oxidized cholesterol is important in the genesis and progression of atherosclerosis. However, oxyphytosterols have been implicated to not be deleterious and may even have beneficial biological properties by LXR activation and subsequent expression regulation of the ABC gene family, resulting in decreased cholesterol absorption via ABCG5/G8 downregulation (Hovenkamp et al 2008). Lastly, plant sterols and stanols seem to evoke no negative drug interaction when administered and do not increase the risk of gallstone formation (Miettinen et al 2000, Vanstone et al 2002).

While there are possible detrimental impacts of phytosterol therapy, when viewed in lieu of the more substantiated and consistent reduction in cardiovascular disease risk (mediated by phytosterol-induced LDL cholesterol-lowering), the apparent risks of phytosterols are outweighed by the positive therapeutic benefit of reducing LDL cholesterol levels. However, more research needs to investigate the effect of plasma phytosterol concentration elevations following phytosterol treatment and the potential disease risk incurred. Potential problems of elevated plant

sterols are considered further in several reviews and will not be considered further here (Patel and Thompson 2006, Sudhop and von Bergmann 2004).

Serum Carotenoids and Lipid-soluble Vitamins

Phytosterol administration has often resulted in reductions in total lycopene and β -carotene plasma concentrations at the recommended dose, indicating a potentially detrimental side-effect in normocholesterol and hypercholesterolemic individuals (Katan et al 2003). Many investigators have expressed serum lipid-soluble vitamins and carotenoids in terms of lipid-standardized measures, often serum total cholesterol or combined total cholesterol and triglycerides (Christiansen et al 2001, Davidson et al 2001, Hallikainen et al 1999, Hallikainen and Uusitupa 1999, Hallikainen et al 2000, Hendriks and Weststrate 1999, Judd et al 2002, Maki et al 2001, Mensink et al 2002, Plat et al 2000, Raeini-Sarjaz et al 2002). While this adjustment is appropriate in examining the potential protective effects of carotenoids and fat soluble vitamins against the oxidation of LDL, it does not display the total serum concentrations available for delivery to tissues.

When considering the effect of phytosterols upon serum carotenoids and fat-soluble vitamins, both expressions should be displayed in order to gain insight both into oxidative potential of LDL and the possibility of nutrient deficiencies, as one measure may be significantly affected by phytosterol treatment whereas the other may not. However, this convention has not been followed. Noakes et al. briefly mentioned the statistically significant reduction in total plasma concentrations of α -tocopherol and carotenoids, but displayed the lack of difference between control for the lipid-standardized measures, and inappropriately concluded that plasma carotenoid concentrations were maintained with dietary advice to increase intake of vegetables (Noakes et al 2002). The proper conclusion was that plasma carotenoid concentrations were

reduced but their lipid-standardized measures were not. However, in three reviews this was reported as maintaining plasma carotenoid concentrations with no clarification that only lipid-standardized concentrations of carotenoids were maintained but not total serum levels (Berger et al 2004, Moreau et al 2002, Plat and Mensink 2005).

Phytosterol Effect on Total Lipid-Soluble Vitamins and Carotenoids. In considering total carotenoid and vitamin status, lipid-standardizing serum concentrations are inappropriate because they do not quantify the availability of the compound for delivery to tissues. According to the analysis of eighteen trials in meta-analysis by Katan et al., an intake of 1.5 g/d or more of phytosterols lowers α -carotene, β -carotene, and lycopene serum concentrations and thus total carotenoid serum status (Katan et al 2003). In many clinical trials, phytosterol total serum concentrations of carotenoids and/or tocopherol decreased but lipid-standardized concentrations did not (Davidson et al 2001, Hallikainen et al 1999, Hallikainen and Uusitupa 1999, Hallikainen et al 2000, Hendriks et al , Plat et al 2000). Decreases in carotenoids normally remain within the limits, but may be important for individuals with low intake (Judd et al 2002).

Despite the carotenoid-lowering effect of phytosterols, serum levels of retinol, indicating vitamin A status, have demonstrated no change (Christiansen et al 2001, Davidson et al 2001, Hallikainen and Uusitupa 1999, Hallikainen et al 2000, Judd et al 2002, Maki et al 2001, Mensink et al 2002, Ntanios et al 2002, Plat et al 2000, Raeini-Sarjaz et al 2002). While carotenoid systemic availability consistently decreases upon phytosterol supplementation, the presence of carotenoid-derived retinol is not modified, suggesting adequate nutrient status throughout the phytosterol treatment. For individuals with a greater need for vitamin A, such as pregnant and lactating women and children, the decrease in plasma carotene is an important concern (Richelle et al 2004).

β -carotene contributes to about 10-15% of the Recommended Daily Allowance of vitamin A via conversion, but the extent of this conversion depends to a substantial degree upon

the food matrix and the diet of the individual.(Strobel et al 2007, Wang et al 2008) However, in individuals with retinol concentrations of 1.7 $\mu\text{mol/L}$, β -carotene conversion to vitamin A was estimated to be 80% when consumed in the form of spirulina.(Wang et al 2008) Katan et al. concluded that even though low carotenoid levels have been associated with increased cancers, macular degeneration, and cardiovascular disease, the risks have yet to be backed by evidence beyond epidemiological trials.(Katan et al 2003)

Some investigators have demonstrated that carotenoids themselves are involved in immune function, thus decreased serum carotenoid concentrations may have an impact on health over the long-term, regardless of the impact upon serum concentrations retinol.(Palozza et al 2004, Ruhl 2007, Sharoni et al 2004) However, the physiological relevance of carotenoid-mediated functions independent of retinol need to be further examined to determine whether the impact of phytosterols on circulating carotenoid levels has any clinical importance. A six month trial investigating phytosterols demonstrated LDL-C lowering without a decrease in total serum carotenoid concentrations when administered as microcrystalline phytosterols.(Christiansen et al 2001) However, serum retinol concentrations were not evaluated. Because of the consistent carotenoid-lowering effect of phytosterols, long-term trials should evaluate both carotenoid and retinol status to ensure that there are no physiologically relevant negative long-term effects of phytosterols.

Potential Effect of Phytosterol on Serum Antioxidants and LDL Oxidation. Because carotenoids and tocopherols are primarily transported within lipoproteins, adjusting the serum concentrations in relation to the total serum cholesterol or serum lipid (total cholesterol + triglycerides) yields a lipid-standardized measure that relates the amount of the particular carotenoid to the lipid within the serum lipoproteins class responsible for its transport (Richelle et al 2004, Traber et al 1994, Wang et al 2007). While this does not provide a measure of serum carotenoid status, it is an appropriate approach when considering the antioxidant density of

lipoproteins and their subsequent potential to reduce the oxidative susceptibility of LDL. In some studies, the phytosterol treatment decreased both the lipid-standardized measure and the total serum concentrations, indicating both a reduction in carotenoid and vitamin status and a decrease in antioxidant density within lipoproteins.(Hendriks and Weststrate 1999, Judd et al 2002, Maki et al 2001, Mensink et al 2002)

However, while total serum carotenoids have been found to be related to oxidized LDL in older women in epidemiological studies, there is minimal evidence supporting this concept.(Beck et al 2008) LDL isolated from subjects on a high carotene diet failed to produce a protection against lipid peroxidation; in fact, only the tomato juice group (high in lycopene) demonstrated an anti-oxidative protective effect against LDL oxidation.(Bub et al 2000) A similar trial with a vitamin mix supplement demonstrated that antioxidants did not decrease LDL oxidation.(Abbey et al 1993, Brude et al 1997) Furthermore, a meta analysis found no link to support use of dietary antioxidants to prevent age-related macular degeneration, whereas another study found that only lutein plus zeaxanthin was associated to intima-media thickness—not β -carotene, retinol, or α -tocopherol (Chong et al 2007, Iribarren et al 1997). The data demonstrating any protective effect of anti-oxidants against LDL oxidation are unconvincing. Regardless, when examining the impact of phytosterol administration upon the anti-oxidant density of LDL, the lipid-standardized measure is appropriate.

Prevention of Carotenoid-Lowering Effect. In a bioavailability study within normocholesterolemic men, plant sterol equivalents as either free or esterified sterols both reduced both the area-under the curve (AUC) and the maximum concentration for [$^2\text{H}_8$]- β -carotene and retinyl palmitate over nine hours without a change in respective triglyceride concentrations, but the effect of plant sterol esters was significantly greater than those of the free sterols (AUC- β -carotene reduced 57% by esters and 48% by free sterols).(Richelle et al 2004)

In one study designed to determine if increasing dietary carotenoids via increased vegetable consumption was sufficient in offsetting phytosterol-induced carotenoid lowering, change was observed in the absolute plasma concentrations but reported in terms of the lack of change in lipid standardized values (TC+TG standardized). However, the plasma concentrations of lycopene, α/β carotene, and even the fat-soluble vitamin α -Tocopherol decreased significantly ($p < 0.001$) but the reported standardized measurements did not demonstrate this difference (Noakes et al 2002). Furthermore, the plasma lutein and α/β carotene levels increased in the control subjects from baseline, indicating that the subjects were increasing consumption of vegetables in a sufficient manner to induce significant serum carotenoid changes. However, upon standardization of these values, even these differences disappeared, which seems contrary with the absence of change in cholesterol levels in the control due to the lack of treatment. (Noakes et al 2002)

When hypercholesterolemic men consumed a low-fat 1.3 g/d plant sterol ester spread, dietary β -carotene serum concentrations decreased despite the dietary advice to increase carotenoid intake. (Colgan et al 2004) Under a controlled diet, 1.92 g/d of sterol esters and 1.76 g/d of stanol esters, neither the lipid-standardized or the total serum concentrations of retinol, α -tocopherol, γ -tocopherol, β -carotene, and other carotenoids were measured and shown to not decrease significantly according to either treatment versus the control. (Raeini-Sarjaz et al 2002) However, phytosterol therapy targets free-living individuals without dietary supervision, thus another approach to the prevention of phytosterol-induced serum carotenoid change must be implemented for feasible prevent in the population. However, in one study incorporating 0.33 and 0.59 mg/serving of β -carotene into the sterol ester treatment croissant and muffin but not the control (α -tocopherol done the same, half as much in the control), TC and LDL-C were lowered significantly by 0.38 mmol/L and 0.36 mmol/L, respectively, and the β -carotene, α -carotene, lycopene, α -tocopherol, and γ -tocopherol were not significantly reduced in comparison to the control, indicating that co-administration of carotenoids and phytosterols does not lower the net

carotenoid concentration in the serum regardless of its historical ability to decrease its absorption(Quilez et al 2003b). The data suggest that carotenoid supplementation should accompany phytosterol administration to minimize potential safety issues.

Pregnancy and Breastfeeding

A phytosterol-rich maternal diet increases the breast-milk phytosterols, and, subsequently, infant plasma phytosterols.(Mellies et al 1978) Because phytosterols are absorbed, albeit at a low level, and subsequently are present in breast-milk, the potential for phytosterol side-effects during pregnancy and post-partum care must be considered.(Ostlund et al 2002a) Phytosterol administration during pregnancy did not significantly affect serum LDL-C or total cholesterol during the first or third trimester of pregnancy or even during one month post-partum in a study with plant stanol esters (Benecol).(Laitinen et al 2009) Furthermore, the infants of the phytosterol group had significantly lower cholesterol-standardized serum β -carotene levels in comparison to the control infants at one month, but this difference disappeared after six months .(Laitinen et al 2009) The total serum β -carotene levels did not differ at one or six months of age, indicating adequate β -carotene status. However, lipid-standardized β -carotene levels were lower at one month than the control, an effect that disappeared by six months. Furthermore, plant stanol ester spread did not impact infant growth or gestation length.(Laitinen et al 2009) However, more research needs to demonstrate that the value of phytosterol supplementation during pregnancy outweighs the added risk of lipid-standardized β -carotene deficiency in neonates, but in the meantime phytosterols should not be recommended during pregnancy until therapeutic benefits are demonstrated.

Study I.

Crude and Refined Black Raspberry Seed Oils Significantly Lower Triglycerides and Moderately Affect Cholesterol Metabolism in Male Syrian Hamsters

Elevated plasma lipid concentrations are a major risk factor for atherosclerotic disease, including both low-density lipoprotein (LDL) cholesterol and triglyceride concentrations (Austin et al 1998, NCEP 2002). While potential severe side-effects accompany drug therapy, dietary components often elicit similar therapeutic benefits with minimal added risk of adverse events. In this study, we examined the ability of crude and refined black raspberry seed oil (*Rubus occidentalis*) to lower lipids in a hamster model of atherosclerosis fed a high-cholesterol (0.12% g/g), high fat (9% g/g) diet for four weeks. Black raspberry seeds are a species of caneberry seeds composed of high levels of polyunsaturated fatty acids, polyphenolic compounds (primarily ellagic acid), phytosterols, and α - and γ -tocopherols, yielding a promising potential lipid lowering dietary therapeutic (Bushman et al 2004).

Linoleic acid, an omega-6 fatty acid, and α -linolenic acid, an omega-3 fatty, are present in black raspberry seed oil (RSO) in high concentrations, thus the oil is a good terrestrial source of omega-3 fatty acids. Omega-6 polyunsaturated fatty acids have demonstrated the ability to lower LDL and HDL cholesterol concentrations (Kurushima et al 1995, Lecker et al 2010, Siri-Tarino et al), whereas omega-3 fatty acids are often used to reduce triglycerides in an effort to prevent and minimize risk of cardiovascular and diabetic disease accompanying hypertriglyceridemia (National Cholesterol Education Program NCEP 2002). While omega-6 fatty acids exert a regulatory role upon cholesterol metabolism, omega-3 fatty acids exert more of

an effect upon triglyceride concentrations, an effect believed to be mediated by alterations in triglyceride-rich very low-density lipoprotein (VLDL) secretion mediated by inhibition of sterol regulatory element binding protein-1 effector genes (Clarke 2001, Zuliani et al 2009) and hepatic β -oxidation via activation of peroxisome proliferator-activator receptor- α (PPAR- α) (Clarke 2001, Delarue et al 2004). Omega-6 polyunsaturated fatty acids have also demonstrated an ability to decrease VLDL lipid concentrations (Sessions and Salter 1994) and prevent hepatic LDL receptor gene suppression upon elevated hepatic cholesterol concentrations, thereby increasing hepatic uptake of LDL from the plasma (Kurushima et al 1995). Subsequently, when polyunsaturated fat sources replace saturated fatty acid-rich foods, both low-density lipoprotein cholesterol and high-density lipoprotein cholesterol are lowered in both hamsters (Dorfman et al 2005) and humans (Kralova Lesna et al 2008).

Raspberry seed oils also contain high levels of the polyphenol ellagic acid (Bushman et al 2004). While the antioxidant capacity of ellagic acid has been demonstrated (Hassoun et al 1997, Priyadarsini et al 2002), few investigations have examined the ability of polyphenol to modulate lipid concentrations. Within alcohol-induced liver steatotic rats, ellagic acid administration reduced the accumulation of lipids in the liver (Devipriya et al 2008). Within 0.5% cholesterol supplemented rabbits, 1% (w/w diet) ellagic acid significantly lowered plasma triglycerides and total, LDL, and HDL cholesterol in comparison to diets without ellagic acid (Yu et al 2005), indicating that ellagic acid may elicit lipid lowering effects; however, the full impact of ellagic acid requires more investigation.

While tocotrienols are not present to any great extent, tocopherols are highly concentrated in food products in black raspberry seed oils (Adhikari et al 2008). Tocopherol supplementation has demonstrated an inverse association with intima media thickness in epidemiology studies (Azen et al 1996, Rimm et al 1993). Tocopherols have been hypothesized to contribute to atherosclerotic disease prevention through the reduction of inflammation via their antioxidant characteristics (Devaraj et al 2007), but conflicting results have been obtained in

clinical trials (Albertini et al 2002, Bleys et al 2006). It is possible that the HDL lowering effect observed in the current study was exacerbated by tocopherols, which have shown some ability to downregulate cholesterol synthesis and HDL cholesterol secretion from Caco-2 cells (Landrier et al). However, α -tocopherols have shown no effect either plasma cholesterol and triglyceride concentrations or aortic foam cell accumulation in atherogenic diet fed hamsters (Parker et al 1995). Overall, there is little evidence supporting a therapeutic effect of vitamin E intake above the recommended dose on lipid metabolism (Schneider 2005). Regardless, the presence of tocopherols in the RSOs may contribute additively or synergistically to their overall lipid lowering efficacy.

The current study investigated black raspberry seed oil enriched in polyunsaturated fatty acids, ellagic acid, phytosterols, and α - and γ -tocopherols in both crude and refined oil form to determine the overall lipid lowering of this potential dietary therapeutic. High cholesterol diets supplemented with crude and refined black raspberry seed oils, or coconut oil (atherogenic control) were compared to a soybean oil diet without cholesterol in order to examine whether the crude and refined raspberry seed oil (RSO) treatments significantly altered lipid metabolism equivalently.

Methods and Materials

Thirty-seven male Syrian hamsters (Charles River Labs, Wilmington, MA) weighing 55-70 g were housed individually in polycarbonate cages with a bedding of sawdust. Upon arrival, the hamsters were randomly divided into groups of 9-10 hamsters, allowed free access to water, and fed dietary treatments ad libitum throughout the four week study. Hamsters were kept in a humidity controlled room at 25°C, using a 12-h light/dark cycle for the duration of the study. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska.

Hamsters were fed a modified AIN-93M diet supplemented with soybean oil, coconut oil, refined raspberry seed oil, or crude raspberry seed oil. Ingredients were mixed manually to achieve a fine blend in proportions outlined in Table 1. The soybean oil diet fatty acid intake was entirely from soybean oil (9% g/g), which provides all of the needed linoleic and linolenic essential fatty acids. However, because coconut oil does not have these essential fatty acids, 1% of the diet was soybean oil to ensure adequate linoleic and linolenic acid intake and 8% of the diet was provided by saturated fatty acid-rich coconut oil. Because black raspberry seed oil is rich in linoleic and α -linolenic acids (Table 2), the raspberry seed oil (RSO) diets consisted of a 9% RSO intake without soybean oil supplementation. The soybean oil diet was not supplemented with cholesterol to provide a basal lipid metabolism control for comparison, whereas the coconut oil and the RSO diets were supplemented with 0.12% (g/g) cholesterol. The coconut oil diet served as the atherogenic control. Each diet was portioned into two 1 kg bags and stored at -20°C. The AIN-93 mineral and vitamin mixes, casein, dextrinized cornstarch, and fibers were purchased from Dyets, Inc. (Bethlehem, PA). Choline bitartrate, L-cystine, and cholesterol were purchased from Sigma Chemicals (St. Louis, MO). Cornstarch, sucrose, and soybean oil were purchased from a local grocery store. Black raspberry seed oils were extracted via hot hexane, and the

refined oil was processed via acidification, degumming, bleaching, clay filtering, and deodorization as detailed in a prior publication (Adhikari et al 2008). The fatty acid composition of the raspberry seed oils was obtained via GC FAME analysis as described previously (Oh et al 2007) to determine whether there was a difference in the fatty acid or the phytosterol composition of the oil (Table 2). Notably, the crude and refined oils contained high levels of linoleic acid (omega-6), $54.3\% \pm 0.02\%$ (crude) and $55.3\% \pm 0.06\%$ (refined), and α -linolenic acid (omega-3), $32.2\% \pm 0.04\%$ (crude) and $31.4\% \pm 0.16\%$ (refined). The crude and refined raspberry seed oils contained 1268 ± 61 mg/kg and 1307 ± 89 mg/kg of sitosterol, the major phytosterol commonly present in vegetable oils. The obtained phytosterol concentrations were substantially lower than 0.73-1.10% phytosterol composition published by Winton and Winton in 1935 (Winton and Winton 1935). However, the statistically equivalent fecal phytosterol excretion support the compositional data obtained. While there were no differences between the oils in the parameters measured, both oils contained high levels of polyunsaturated fatty acids and sitosterol. Previous analysis of black raspberry seed oils extracted and/or processed in the same fashion demonstrated a significantly lower amount of total tocopherols in the refined oil (142 mg/100g oil) versus the crude oil (175 mg/100g oil) (Adhikari et al 2008).

Hamster body weights and food intakes were recorded weekly, and feces were collected during week four for pooled 7-day analysis of bile acid and sterol excretion. On the final day of the study (day twenty-eight), the animals were euthanized with CO₂ and blood was collected by cardiac puncture using 10 mL syringes containing 10 mg EDTA and immediately placed on ice after opening the thoracic cavity via incision. Within hours, plasma was isolated by centrifugation at $1000 \times g$ for 30 minutes at 4°C and either analyzed within four days or stored at -80°C until further analysis. Livers were excised, weighed, and stored at -80°C prior to analysis.

Table 1. Diet composition

	SO	CO	Refined RSO	Crude RSO
			g/kg	
Cornstarch	415.7	414.5	414.5	414.5
Dextrinized cornstarch	155.0	155.0	155.0	155.0
Casein	140.0	140.0	140.0	140.0
Sucrose	100.0	100.0	100.0	100.0
Coconut oil	---	80.0	---	---
Soybean oil	90.0	10.0	---	---
Raspberry seed oil, refined	---	---	90.0	---
Raspberry seed oil, crude	---	---	---	90.0
Insoluble fiber (cellulose)	40.0	40.0	40.0	40.0
Soluble fiber (guar gum)	10.0	10.0	10.0	10.0
Cholesterol	---	1.2	1.2	1.2
AIN-93 mineral mix	35.0	35.0	35.0	35.0
AIN-93 vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5

SO = soybean oil; CO = coconut oil; RSO = raspberry seed oil.

Table 2. Raspberry Seed Oil Lipid Composition

		SO ¹	CO ¹	Refined RSO	Crude RSO
Fatty Acids		% (w/w)			
Caproic	C6:0	N/D ²	0.5	N/D	N/D
Caprylic	C8:0	N/D	7.8	N/D	N/D
Capric	C10:0	N/D	5.8	N/D	N/D
Lauric	C12:0	N/D	43.2	N/D	N/D
Myristic	C14:0	0.1	17	N/D	N/D
Palmitic	C16:0	10.7	8.6	1.9 ± 0.01	2.1 ± 0.00
Palmitoleic	C16:1	0.1	N/D	N/D	N/D
Margaric	C17:0	0.1	N/D	N/D	N/D
Stearic	C18:0	4.4	2.9	0.8 ± 0.05	0.9 ± 0.00
Oleic	C18:1n-9	20.1	6.5	9.7 ± 0.05	9.7 ± 0.03
Vaccenic	C18:1 n-7	1.2	0.1	N/D	N/D
Linoleic	C18:2	53.3	1.6	55.3 ± 0.06	54.3 ± 0.02
Linolenic	C18:3	8.2	N/D	31.4 ± 0.16	32.2 ± 0.04
Arachidic	C20:0	0.3	N/D	0.4 ± 0.02	0.4 ± 0.00
11-Eicosenoic	C20:1	0.2	N/D	0.2 ± 0.03	0.2 ± 0.02
11,14-Eicosadienoic	C20:2	N/D	N/D	0.1 ± 0.05	0.1 ± 0.01
Docosanoic	C22:0	0.4	N/D	0.2 ± 0.01	0.2 ± 0.00
Phytosterols		mg/kg of RSO			
Sitosterol		1242	639	1307 ± 89	1268 ± 61
Total Phytosterols ³		2408	1177	1307 ± 89	1268 ± 61

SO = soybean oil; CO = coconut oil; RSO = raspberry seed oil; ²N/D = not detected. RSO values are means ± SEM, *n* = 3. SO and CO analyzed in singlet, *n* = 1. ANOVA Statistical comparison was performed for sitosterol analysis only and no treatment differences were detected (*P* > 0.05)

¹The SO and CO analyzed were standard stocks used in our laboratory in place of the exact oil used in the diet and are provided for subjective comparison. The percentages are not adjusted for unidentified peaks and thus represent total peak area percentage obtained from GC analysis.

³Sum of brassicasterol, campesterol, stigmasterol, sitosterol, and Δ⁵-avenasterol.

Plasma Lipid Analysis. Plasma concentrations of total cholesterol and triglycerides were analyzed in duplicate after 1:10 dilution using previously published enzymatic assays (Carr et al 1993). Regents were purchased from Roche Diagnostics, Indianapolis, IN and the plates were incubated at 37°C for ten minutes before the absorbances were measured at 505 nm. High-density lipoproteins (HDL) were isolated via precipitation of apo-B containing lipoproteins using a 1:1 dilution with 100 µL of plasma and 100 µL of precipitating reagent (Thermo Electron Corp, Melbourne, Australia) and HDL cholesterol was quantified in duplicate after 2:5 dilution in deionized water via measuring the total cholesterol of the supernatant (Carr et al 1993). Following incubation of ten minutes at 37°C, the samples were read at 505 nm. Plasma non-HDL cholesterol levels were calculated by subtracting plasma HDL cholesterol from plasma total cholesterol.

Liver Lipid Quantification. Approximately 0.3-0.4 g of frozen liver was minced and the exact weight recorded prior to an overnight chloroform/methanol (2:1, v/v) Folch extraction (Folch et al 1957) and subsequent heating for one hour at 50°C to ensure complete extraction. The samples were quantitatively filtered (rinsed three times with excess chloroform/methanol) through Whatman #41 filter paper into graduated conical screw cap tubes and the total volume was brought to 10 mL. After adding 2.0 mL of 0.88% KCl, the samples inverted five times to ensure mixing before complete phase separation via brief centrifugation. The lower phase volume was recorded before the upper phase was aspirated and discarded. Total cholesterol, free cholesterol, triglycerides and phospholipids were quantified enzymatically after solubilization of 0.5 mL lipid phase aliquots in Triton X-100 using the procedure of Carr et al. (Carr et al 1993). Total cholesterol and triglyceride regents were the same as used in the plasma analysis, whereas the free cholesterol and phospholipid regents were purchased from Wako Chemicals (Richmond, VA). Total cholesterol and free cholesterol aliquots were analyzed directly, whereas triglycerides and phospholipids were diluted 2:5 in deionized water prior to analysis. All samples

were analyzed in duplicate and incubated for ten minutes at 37°C after the addition of the appropriate reagent. Liver esterified cholesterol was quantified as difference between the obtained total cholesterol and free cholesterol.

Fecal Bile Acid and Neutral Sterol Extraction. Bile acids and neutral sterols were extracted individually on different occasions in the same manner from feces using chloroform/methanol (2:1, v/v) according to Folch et al (Folch et al 1957). Approximately 100 mg of ground fecal matter was placed in screw-top tubes, and the exact weight was recorded. 5 α -Cholestane was added to only the neutral sterol quantification samples as an internal standard to account for sterol loss during sample analysis. To ensure extraction efficiency, 0.7 mL of 0.5M HCl was added followed by 10 mL of chloroform/ methanol (2:1, v/v) to initiate the extraction. The samples were capped, vortexed, left overnight, and heated for fifteen minutes to ensure complete lipid extraction. After 2.0 mL of 0.88% KCl was added to each sample, the tubes were inverted several times and centrifuged for ten minutes at 1000 \times g to induce phase separation. For the bile acid extraction, the upper phase containing the bile acids was transferred to a clean tube without disturbing the lower phase. The lower phase was washed to recover any residual bile acids with an additional 3 mL of chloroform/methanol/water (3:48:47). Samples were inverted and centrifuged as before, and the upper phase was added to the bile acids from the first transfer. During the neutral sterol analysis, the upper bile acid phase was aspirated and discarded, and the lower phase was transferred to a new tube via decanting.

Bile Acid Analysis. Aliquots of the recovered upper phases were evaporated at 50°C under nitrogen in standardized glass cuvetts to remove the solvent prior to quantification using the 3 α -hydroxysteroid dehydrogenase method previously published (Sheltawy and Losowsky 1975). The samples were re-solubilized in 100 μ L of methanol and 3.5 mL of β -NAD in pH-adjusted CAPS buffer (0.2 mg/mL; pH 10.8) was added. After vortexing, the background absorbance at 340 nm was read and 0.4 mL of 3 α -hydroxysteroid dehydrogenase (0.75 units/mL of 0.01 M

phosphate buffer, pH 7.2) was added, followed by sample incubation at 37°C for thirty minutes. The absorbance was read again at 340 nm, and the difference between the post-enzymatic reaction and background was used to calculate the total bile acid concentration in the aliquots utilizing the 0.01 M cholic acid 0.1-0.5 mmol/assay standard curve dilution after adjusting for the difference in sample volume after the addition of the enzyme. Fecal bile acid excretion was calculated from the fecal concentration and the daily fecal output per 100g body weight. β -NAD, CAPs, Cholic acid, and 3 α -hydroxysteroid dehydrogenase were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Fecal Neutral Sterol Quantification. The collected lower phase was also evaporated at 50°C under nitrogen to remove the chloroform, utilizing excess solvent to concentrate the samples at the bottom of the tubes. Saponification was initiated with the addition of 1 mL of 1.0 M KOH in methanol. The tubes were flushed with nitrogen, capped, and heated at 50°C for one hour with frequent vortexing. After 1 mL of distilled water was added, samples were vortexed. Non-saponifiables were extracted via the addition of 3 mL of hexane, subsequent vigorous shaking for thirty seconds, brief centrifugation at 1000 x g, and the transferring of the upper hexane phase to new screw-top glass tubes. The lower phase was washed with an additional 3 mL of hexane, the samples were mixed vigorously, the phases separated via centrifugation, and the neutral sterol containing upper phase was added to the previously transferred hexane phase. Samples were dried under nitrogen at 50°C and were redissolved in equivalent volumes of hexane and quantified by gas chromatography using an AT-5 capillary column (Alltech, Deerfield, IL) with a 15.0°C/minute temperature ramp from an initial one minute hold at 270°C to a final hold at 300°C of fourteen minutes; an inlet temp of 270°C and a Flame Ionizing Detector temperature of 300°C.

Results

Dietary group hamster body weights were not significantly different during the study (Table 3). Food take did not differ except during the first week when the crude raspberry seed oil diet food intake was significantly lower than the soybean oil and coconut oil diets but not the refined raspberry seed oil diet. Because of the absence of any differences in intake during any other weeks, the effect was presumed to be due to initial adjustment to the diets (Table 4).

Plasma lipid analysis demonstrated significantly lower plasma total cholesterol concentrations in both raspberry seed oil (RSO) diets in comparison to the coconut oil atherogenic control but were not reduced to the extent of the soybean oil diet (Table 5). Non-HDL cholesterol concentrations were significantly higher and statistically equivalent in the coconut oil, crude RSO, and refined RSO diets than the soybean oil diet (Table 5). HDL cholesterol was statistically the highest in the coconut oil dietary group (4.07 ± 0.26 mmol/L) in comparison with the statistically equivalent crude and refined RSO diets (2.16 ± 0.10 mmol/L and 2.19 ± 0.15 mmol/L, respectively) (Table 5). The soybean oil group had the significantly lowest HDL cholesterol concentration of 2.09 ± 0.11 mmol/L. Plasma triglyceride concentrations were statistically and equivalently lower in the soybean oil (1.62 ± 0.16 mmol/L), crude RSO (1.75 ± 0.20 mmol/L), and refined RSO (2.32 ± 0.31 mmol/L) diets in comparison to the coconut oil diet (3.28 ± 0.51 mmol/L) (Table 5).

Liver masses were significantly lower in the soybean oil positive control group (4.50 ± 0.17 g/100 g of body weight) compared to the statistically equivalent cholesterol supplemented diets (Table 6). Liver free cholesterol concentrations were statistically higher and equivalent among the coconut oil (5.7 ± 0.2 μ mol/g), refined RSO (6.1 ± 0.1 μ mol/g), and crude RSO (6.0 ± 0.2 μ mol/g) diets in comparison to the soybean oil diet (4.7 ± 0.1 μ mol/g) (Table 6). The refined RSO diet (25.6 ± 1.4 μ mol/g) had the statistically highest liver esterified cholesterol

concentration in comparison all other diets, while the the statistically equivalent crude RSO ($21.2 \pm 2.1 \mu\text{mol/g}$) and coconut oil ($18.2 \pm 1.5 \mu\text{mol/g}$) diet means were significantly lower than the the soybean oil diet ($0.6 \pm 0.1 \mu\text{mol/g}$) (Table 6). Liver triglycerides were significantly elevated with the coconut oil diet ($7.8 \pm 0.7 \mu\text{mol/g}$) in comparison to all other diets. The soybean oil diet ($6.1 \pm 0.4 \mu\text{mol/g}$) liver triglyceride concentration was significantly lower than the coconut oil diet, statistically equivalent to the refined raspberry seed oil diet ($5.0 \pm 0.3 \mu\text{mol/g}$) but higher than the crude raspberry seed oil diet ($4.2 \pm 0.2 \mu\text{mol/g}$) despite statistical equivalency between both RSO diets (Table 6). Liver phospholipids were not significantly different between any of the diets (Table 6).

Fecal excretion of cholesterol and cholesterol derivatives produced from microorganism modification (neutral sterols) was equivalent among the cholesterol supplemented diets (coconut oil and both RSO groups) but significantly higher than the soybean oil control (Table 7). Phytosterol fecal excretion was significantly elevated with both RSO diets in comparison to the soybean and coconut oil diets (Table 7). Phytosterol fecal excretion tended to be higher in hamsters treated with the crude RSO diet compared to the refined RSO diet ($p = 0.0505$). Total sterol excretion was significantly higher and statistically equivalent among the refined RSO and the crude RSO diets in comparison to the coconut oil and the soybean oil groups, which did not differ significantly (Table 7). Fecal bile acid excretion was significantly elevated in the refined RSO diet in comparison to the other diets (Table 7). Fecal output did not differ significantly among the groups (Table 7).

Table 3. Hamster body weight

	SO	CO	Refined RSO	Crude RSO
	g			
Week 0	64.4 ± 1.5	65.4 ± 1.0	64.6 ± 1.1	64.8 ± 1.6
Week 1	77.6 ± 2.2	75.9 ± 1.5	74.3 ± 3.0	71.2 ± 3.6
Week 2	91.8 ± 4.1	89.7 ± 1.6	92.7 ± 3.3	86.9 ± 6.0
Week 3	102.0 ± 4.9	99.8 ± 2.4	106.2 ± 3.2	100.8 ± 5.6
Week 4	112.7 ± 5.5	106.9 ± 2.5	113.9 ± 3.1	109.9 ± 4.7

SO = soybean oil; CO = coconut oil; RSO = raspberry seed oil. Values are means ± SEM, *n* = 9-10. No treatment differences were detected (*P* > 0.05).

Table 4. Food intake

	SO	CO	Refined RSO	Crude RSO
	g			
Week 1	53.3 ± 2.9 ^b	53.2 ± 2.7 ^b	48.2 ± 2.8 ^{a,b}	38.9 ± 4.0 ^a
Week 2	59.2 ± 1.6	63.9 ± 3.5	61.6 ± 1.7	66.3 ± 4.1
Week 3	63.6 ± 4.0	63.0 ± 2.7	63.9 ± 2.0	65.4 ± 1.6
Week 4	54.3 ± 9.2	64.3 ± 3.8	61.9 ± 2.7	61.7 ± 1.3

SO = soybean oil; CO = coconut oil; RSO = raspberry seed oil. Values are means ± SEM, *n* = 9-10. ^{a,b} Means within a row having different superscripts are statistically different (*P* < 0.05).

Table 5 Plasma lipid concentrations

	SO	CO	Refined RSO	Crude RSO
	mmol/L			
Total cholesterol	2.74 ± 0.07 ^a	6.26 ± 0.27 ^c	5.23 ± 0.20 ^b	5.04 ± 0.27 ^b
non-HDL cholesterol	0.65 ± 0.09 ^a	2.19 ± 0.16 ^b	2.19 ± 0.15 ^b	2.16 ± 0.10 ^b
HDL cholesterol	2.09 ± 0.11 ^a	4.07 ± 0.26 ^c	3.05 ± 0.19 ^b	2.88 ± 0.25 ^b
Triglyceride	1.62 ± 0.16 ^a	3.28 ± 0.51 ^b	2.32 ± 0.31 ^a	1.75 ± 0.20 ^a

SO = soybean oil; CO = coconut oil; RSO = raspberry seed oil. Values are means ± SEM, *n* = 9-10.
^{a,b,c} Means within a row having different superscripts are statistically different (*P* < 0.05).

Table 6. Liver lipid concentrations

	SO	CO	Refined RSO	Crude RSO
	μmol/g			
Total cholesterol	5.3 ± 0.1 ^a	24.0 ± 1.6 ^b	31.6 ± 1.4 ^c	27.2 ± 2.2 ^b
Free cholesterol	4.7 ± 0.1 ^a	5.7 ± 0.2 ^b	6.1 ± 0.1 ^b	6.0 ± 0.2 ^b
Esterified cholesterol	0.6 ± 0.1 ^a	18.2 ± 1.5 ^b	25.6 ± 1.4 ^c	21.2 ± 2.1 ^b
Triglyceride	6.1 ± 0.4 ^b	7.8 ± 0.7 ^c	5.0 ± 0.3 ^{a,b}	4.2 ± 0.2 ^a
Phospholipid	22.6 ± 0.8	21.4 ± 0.3	22.7 ± 0.4	22.4 ± 0.6
	g/100 g of body weight			
Liver Weights	4.50 ± 0.17 ^a	5.50 ± 0.13 ^b	6.01 ± 0.20 ^b	5.73 ± 0.17 ^b

SO = soybean oil; CO = coconut oil; RSO = raspberry seed oil. Values are means ± SEM, *n* = 9-10.
^{a,b,c} Means within a row having different superscripts are statistically different (*P* < 0.05).

Table 7. Fecal output and fecal sterol and bile acid excretion

	SO	CO	Refined RSO	Crude RSO
	$\mu\text{mol} \times \text{day}^{-1} \times 100 \text{ g}^{-1} \text{ body weight}$			
Neutral sterols ¹	1.79 ± 0.18 ^a	4.09 ± 0.27 ^b	3.91 ± 0.22 ^b	4.02 ± 0.32 ^b
Phytosterols ²	1.75 ± 0.18 ^a	0.72 ± 0.06 ^a	3.13 ± 0.36 ^b	4.27 ± 0.43 ^b
Total sterols	3.54 ± 0.27 ^a	4.81 ± 0.29 ^a	7.04 ± 0.45 ^b	8.29 ± 0.68 ^b
Bile acids	0.84 ± 0.09 ^a	0.89 ± 0.07 ^a	1.20 ± 0.10 ^b	0.90 ± 0.07 ^a
	$\text{g} \times \text{day}^{-1} \times 100 \text{ g}^{-1} \text{ body weight}$			
Fecal output	0.725 ± 0.047	0.707 ± 0.016	0.665 ± 0.038	0.707 ± 0.046

SO = soybean oil; CO = coconut oil; RSO = raspberry seed oil. Values are means ± SEM, $n = 9-10$.

¹Sum of cholesterol, dihydrocholesterol, coprostanol, and coprostanone.

²Sum of brassicasterol, campesterol, stigmasterol, sitosterol, and sitostanol

^{a,b}Means within a row having different superscripts are statistically different ($P < 0.05$).

Discussion

The aim of this study was to examine the ability of black raspberry seed oil to lower lipids within hamsters as a model of atherosclerosis and to determine whether oil processing modifies any lipid lowering properties. Black raspberry seed oil is high in the polyunsaturated fatty acids linoleic acid and α -linolenic acid, the polyphenol ellagic acid, phytosterols, and α - and γ -tocopherols (Bushman et al 2004). Fatty acid analysis of the raspberry oils confirmed the high presence of linoleic and α -linolenic acid. While the oils consisted of an array of potential lipid modulating components, this study was not designed to test the individual contribution of each of these components to the lipid lowering efficacy of the oils but rather to examine the combined potential of the oil components to lower lipids depending on the extent of processing.

Despite the evidence that omega-6 polyunsaturated fatty acids lower LDL and HDL cholesterol concentrations (Kurushima et al 1995, Lecker et al 2010, Siri-Tarino et al), the data obtained from our study demonstrate a reduction in plasma HDL and total cholesterol concentrations upon replacing coconut oil with black raspberry seed oil rich in both linoleic and α -linolenic acids. While decreases in HDL cholesterol are associated with an increase in risk of atherosclerosis (NCEP 2002), polyunsaturated fatty acid intake typically reduces both LDL cholesterol and HDL cholesterol without modifying the ratio of LDL : HDL (Jackson et al 1984), a more useful measure for evaluating disease risk than LDL cholesterol or HDL cholesterol alone (Fernandez and Webb 2008). Even though both the crude and refined raspberry seed oil (RSO) treatments significantly lowered plasma total cholesterol and HDL cholesterol concentrations in comparison to the coconut oil atherogenic control without affecting the non-HDL fraction of cholesterol, an unexpected result in lieu of the ability of omega-6 fatty acids to attenuate the downregulation of low-density lipoprotein receptor in response to excess cholesterol in HepG2

cells (Yu-Poth et al 2005). While the lower HDL cholesterol observed with the RSO diets is potentially a negative effect, this was observed in comparison to saturated fatty acid-enriched coconut oil that typically increase HDL cholesterol (Kris-Etherton and Yu 1997); thus the net effect of black raspberry seed oil intake upon plasma cholesterol metabolism may simply not induce the HDL elevations observed with the coconut diet.

In humans, omega-3 fatty acids have demonstrated some ability to slightly increase LDL cholesterol (Fumeron et al 1991), proposing the possibility that the mixed omega-3 and omega-6 fatty acid profile of the raspberry seed oils may attenuated the lipid modulating effects of each other. The omega-3 fatty acid present in the raspberry seed oils, α -linolenic acid has been demonstrated to increase LDL cholesterol levels in hamsters fed α -linolenic acid in place of oleic acid when saturated fatty acid intake was held constant (Morise et al 2005). VLDL and LDL cholesterol concentrations are higher in hamster with omega-3 intake in place of omega-6 intake (Lu et al 1996). At 0.1% dietary cholesterol, plasma VLDL and LDL cholesterol concentrations were elevated in omega-3 fed hamsters compared to omega-6 fed hamsters (Mei-Huei et al 2005), indicating that the omega-3 fatty acid LDL cholesterol increasing effect may attenuate the omega-6 fatty acid LDL cholesterol lowering effect.

In the current study, the refined RSO elevated liver esterified cholesterol concentrations beyond the level of accumulation observed in the coconut oil diet, an effect that was absent in the crude RSO diet. The liver cholesterol ester accumulation in both RSOs enriched in the omega-6 fatty acid linoleic acid may have decreased acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) expression in the liver (Lee and Carr 2004), leading to the reduction of availability of cholesterol esters for incorporation into VLDL and subsequently reducing VLDL secretion.

While the plasma total and HDL cholesterol decreases and the accumulation of cholesterol in the liver observed may be attributed to the effects of the specific fatty acids, the other components of the oils (e.g., ellagic acid) that were not examined in our study may also contributed to these effects. While the high level of phytosterols may have contributed to the

lipid modulating effects observed, phytosterols typically lower low-density lipoprotein cholesterol, as has been demonstrated in both humans and hamsters (AbuMweis et al 2008, Guderian Jr et al 2007, Rasmussen et al 2006), but there was no decrease in non-HDL cholesterol. The current mechanism accepted to explain phytosterol-induced cholesterol lowering revolves around the displacement of micellar cholesterol with phytosterols (Jesch and Carr 2006), subsequently increasing fecal neutral sterol excretion and decreasing cholesterol absorption (Rasmussen et al 2006). However, there was no difference in fecal neutral sterol excretion between the coconut oil and the RSO diets. Phytosterols were present in the diet at 0.12-0.13% by weight of diet. While this is a low dose of phytosterols, doses as low as 0.24% of phytosterol esters (0.08% free phytosterol equivalents) have demonstrated efficiency in significantly lowering non-HDL cholesterol and total cholesterol (Lin et al 2004).

In our investigation, both black raspberry seed oil dietary treatments also beneficially lowered plasma triglyceride concentrations to the equivalent level observed in the soybean oil diet without added cholesterol, an effect likely the result of the high level of α -linolenic acid confirmed to be present. Omega-3 fatty acids are commonly used for their ability to consistently attenuate elevated plasma triglyceride concentrations (Sacks and Katan 2002). While eicosapentaenoic acid (found in fish oil) is typically used as a triglyceride-lowering therapy in hypertriglyceridemic individuals (Lewis 2009), our study with Syrian hamsters demonstrated a reduction in plasma and liver triglyceride concentrations with the supplementation of RSOs rich in α -linolenic acid in comparison to a saturated fatty acid-rich coconut oil control. The raspberry seed oils also significantly reduced liver triglycerides, indicating that RSOs may be effective therapeutic for treatment of hypertriglyceridemia. The crude oil even lowered liver triglycerides to the extent of the soybean diet, an effect that may be mediated by the high level of tocopherols in comparison to the refined oil demonstrated in another study utilizing the same processing procedure (Adhikari et al 2008).

The oil refinement process normally does not change the triglyceride fatty acid profile of oils to any great extent (even though it may reduce omega-3 fatty acid concentrations, overall polyunsaturated fatty acids remain at a consistent level)(Aro et al 2000), a finding confirmed within our study. In the case of the RSO treatment diets, the refining process did not change the fatty acid composition of the oils; thus, any treatment differences between the two oils cannot be attributed to the fatty acid profiles of the oils. While the oils equivalently lowered plasma and liver triglycerides, the crude RSO diet significantly decreased liver triglycerides to the low level of the soybean oil diet, indicating there may be some differences in lipid modulation by the oils. Despite no significant difference in triglyceride lowering efficacy between the crude and refined oils, cholesterol metabolism was significantly different between the two oils, reflecting the possible effects of components of the RSOs not quantified in this study, such as ellagic acid and tocopherols.

Our findings suggest that both refined and crude RSO effectively lowered plasma and liver triglyceride concentrations and plasma total cholesterol but increased liver cholesterol accumulation without decreasing plasma non-HDL, indicating both a null or potential detrimental impact on cholesterol metabolism and a dramatic beneficial reduction of hypertriglyceridemia. The major beneficial outcome of the RSO diets was the 46% and 29% reduction in plasma triglycerides by the crude RSO and refined RSO diets, respectively, compared to the atherogenic diet. Similarly, liver triglyceride concentrations in the crude and refined oil groups were significantly reduced 46% and 36%, respectively, compared to the coconut oil diet. Even though the refined RSO had the highest liver cholesterol accumulation, the diet also induced the greatest fecal bile acid excretion. While RSOs did not decrease the accumulation of cholesterol esters in the liver associated with high dietary cholesterol intake in hamsters, both crude and refined oils deserve further investigation to determine whether the absence of a non-HDL lowering effect is characteristic of the oil mixtures rich in linoleic and α -linolenic acid or simply an aberration of this study. Overall, RSOs demonstrated a null effect upon cholesterol metabolism despite the

presence of high levels of linoleic acid. However, RSOs rich in α -linolenic acid demonstrated a beneficial dramatic hypotriglyceridemic effect regardless of processing, displaying the beneficial decrease in triglycerides characteristic of omega-3 fatty acids (NCEP 2002).

In summary, both refined and crude black raspberry seed oils deserve a promising place as potential therapies for the management of dyslipidemia. The triglyceride lowering effect of black raspberry seed oil was not changed by the extent of oil processing, indicating that both oils confer dramatically positive benefits in hamsters. Furthermore, black raspberry seed oils may offer hypertriglyceridemic consumers an alternative to fish oil consumption to significantly lower plasma triglycerides, as demonstrated by the hypotriglyceridemic effect of both crude and refined oils in hamsters fed high levels of dietary cholesterol.

Study II.

Effect of Phytosterol Stearate Esters upon Cholesterol Metabolism in Charles River Golden Syrian Hamsters

Cardiovascular diseases (CVD) are collectively responsible for 29% of global deaths according to data from the World Health Organization (W.H.O. September 2009). Stroke and coronary heart disease are two prevalent cardiovascular diseases that share the same etiology: atherosclerotic plaque development. Individuals with elevated plasma low-density lipoprotein cholesterol concentrations are at a higher risk for atherosclerosis (NCEP 2002).

Esterified phytosterols have demonstrated the ability to decrease serum low-density lipoprotein cholesterol with equivalent efficacy as free phytosterols (Demonty et al 2009). Because fatty acids have lipid-modulating effects, the esterification of free fatty acids to phytosterols raises the possibility that the specific free fatty acid or phytosterol incorporated into the ester may positively or negatively affect therapeutic efficacy. Phytosterols esterified to fish oil fatty acids have demonstrated to be equally effective as sunflower oil phytosterol esters in lowering plasma cholesterol but have the added benefit of reducing plasma triglyceride concentrations (Demonty et al 2006), indicating that the fish oil phytosterol esters maintain the complimentary therapeutic effects of both the fatty acid and the phytosterol (Micallef and Garg 2008). While this study demonstrated no difference in plasma cholesterol lowering efficacy between the fish oil and sunflower oil esters, other investigations have demonstrated that the species of fatty acid incorporated into the phytosterol ester impacts cholesterol metabolism and the efficacy of the phytosterol ester. Phytosterols esterified to stearate-enriched fatty acid mixtures have demonstrated an ability to decrease plasma LDL cholesterol concentrations in

humans (Carr et al 2009) and a superior non-HDL cholesterol lowering efficacy than linoleate-enriched esters in hamsters (Rasmussen et al 2006). While previous research has focused on the impact of the fatty acid component of phytosterol esters, the effect of the specific phytosterol species incorporated has not been fully elucidated. In the current study, we compared the lipid lowering efficacy of three different phytosterols when incorporated into phytosterol stearate esters: stigmasterol, sitosterol, and sitostanol.

Stearic acid has demonstrated a neutral or cholesterol-lowering effect upon plasma low-density lipoprotein cholesterol concentrations whereas other saturated fatty acids have demonstrated hypercholesterolemic effects (Grundy 1994). Previous work in our laboratory has shown that stearic acid may even lower total cholesterol in comparison to palmitic, oleic, elaidic, and linoleic acids when co-administered in hamsters fed a 0.05% cholesterol diet (Schneider et al 2000). While this effect may disappear when dietary cholesterol intake increases (Imaizumi et al 1993), there does appear to be an increase in fecal neutral excretion upon increased dietary stearic acid intake (Schneider et al 2000). Stearate-enriched phytosterol esters have a dose-dependent response in non-HDL cholesterol lowering efficacy and a synergistic effect as evidenced by their increased efficacy in comparison to stearic acid and free phytosterols in combination (Guderian Jr et al 2007). Subsequently, we utilized phytosterol stearate esters in our investigation into the effect of the phytosterol species upon therapeutic effectiveness.

In order to determine the most efficacious phytosterol stearate combination with a feasible number of animals and treatment groups, we focused our attention upon sitosterol, stigmasterol, and sitostanol. Sitosterol, campesterol and stigmasterol are the most abundant plant sterols in nature, and the most readily available for incorporation into phytosterol esters (Lichtenstein and Deckelbaum 2001). However, campesterol is expensive and was deemed impractical both for our study and probably for the most cost-effective atherosclerotic risk reduction with phytosterol esters. Furthermore, some research has indicated that campesterol (9.6%) and campestanol (12.5%) is more highly absorbed than stigmasterol (4.8%) or sitosterol

(4.2%) (Jain et al 2008, Lutjohann et al 1995), presenting a potentially increased risk of negative effects of campesterol/campestanol phytosterol esters, as the accumulation of phytosterols may increase risk for atherosclerosis (Weingärtner et al 2009). Other more reliable methods have reported values as low as 0.04% for sitostanol and 1.9% for campesterol absorption (Ostlund et al 2002a). While there is little data supporting the hypothesis that serum phytosterols independently increase disease risk, campesterol was excluded from the current investigation and sitosterol, stigmasterol, and sitostanol were chosen for examination.

Our investigation included an atherogenic basal diet high in both cholesterol (0.12% g/g) and saturated fatty acid-enriched coconut oil with the addition of individual phytosterol stearates (2.5% g/g) within the treatment diets in place of cornstarch in the control. The three phytosterol stearate treatment diets consisted of sitosterol stearate, stigmasterol stearate, or sitostanol, allowing us to examine the effect of the specific phytosterol incorporated into the phytosterol stearate esters upon cholesterol lowering efficacy.

Materials and Methods

Experimental Animals and Diets. Thirty-nine male Syrian hamsters (Charles River Labs, Wilmington, MA) with body masses ranging from 56g to 70g were divided into experimental groups of 9-10 hamsters and separated into individual polycarbonate cages with a sawdust bedding. The hamsters were fed ad libitum for twenty-eight days and maintained in a humidity-controlled 25°C room with a twelve hour light/dark cycle. The hamsters were fed a semi-purified, atherogenic AIN-93M diet high in cholesterol (0.12% g/g) and coconut oil (8% g/g) (Table 1) (Reeves et al 1993). Hamsters fed a high cholesterol (0.1 g/100g) and saturated fat (10 g/100g) diet respond similarly to humans to dietary interventions and develop comparative pathologies, making the hamster an appropriate model for studies investigating atherosclerosis (Dorfman et al 2003). Treatment diets consisted of the replacement of cornstarch in the control diet with one of the following individual phytosterol stearates at 2.5% (g/g): stigmasterol stearate, sitosterol stearate, or sitostanol stearate (sitostanol is structurally equivalent to stigmasterol; Figure 1). The composition and purity of the administered phytosterol stearates was determined in triplicate using GC analysis after ester saponification, yielding purity values of 96.8% \pm 2.7% for sitostanol stearate, 98.5% \pm 0.2% for stigmasterol stearate, and 76.3% \pm 1.3% for sitosterol stearate (Table 3). The phytosterol stearates were synthesized from free phytosterols and stearoyl chloride purchased from TCI America (Portland, OR, USA) in the laboratory of Dr. Patrick Dussault (Department of Chemistry, University of Nebraska). The diets were prepared within our laboratory utilizing manual mixing and least-to-greatest addition order to maximize nutrient distribution. Diets were stored in 1 kg portions at -80°C. At the beginning of week four, a second batch of diets was made for the animals and the remaining amount of diet was divided equally among the hamsters to ensure adequate food availability until the diets were complete. The AIN-

93 mineral and vitamin mixes, casein, dextrinized cornstarch, and fibers were purchased from Dyets, Inc. (Bethlehem, PA). Choline bitartrate, L-cystine, and cholesterol were purchased from Sigma Chemicals (St. Louis, MO). Cornstarch, sucrose, and soybean oil were purchased from a local grocery store. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska.

Hamster feed intake and body weight records were maintained weekly. During week three, fecal matter was collected for four days after dual-isotope dose administration in soybean oil for the measurement of cholesterol absorption. Radio-labeled [^{14}C]cholesterol and [^3H]sitostanol were purchased from American Radiolabeled Chemicals (St. Louis, MO) with cholesterol ^{14}C labeled at the 4th carbon and sitostanol labeled with tritium atoms bound to the 5th and 6th carbon within the steroid ring of sitostanol to prevent metabolic catabolism. Bedding was collected for seven-day fecal pellet recovery and sterol excretion analysis at the end of week four.

On day twenty-eight, hamsters were euthanized with carbon dioxide, the thoracic cavity was exposed via incision, and blood was collected by cardiac puncture using 10 mL syringes prior to transfer to 10 mg EDTA containing tubes on ice. Plasma was isolated by centrifugation at 1000 x g for 30 minutes at 4°C, and the separated upper-phase was collected and stored on ice at 4°C for three days prior to storage at -80°C until analyzed. Whole livers were excised, weighed, and snap-frozen in liquid nitrogen prior to storage at -80°C.

Table 1. Diet composition

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	g/kg			
Cornstarch	404.5	379.5	379.5	379.5
Dextrinized cornstarch	155.0	155.0	155.0	155.0
Casein	140.0	140.0	140.0	140.0
Sucrose	100.0	100.0	100.0	100.0
Coconut oil	80.0	80.0	80.0	80.0
Soybean oil	20.0	20.0	20.0	20.0
Sitostanol stearate ¹	---	25.0	---	---
Stigmasterol stearate	---	---	25.0	---
Sitosterol Stearate	---	---	---	25.0
Insoluble fiber (cellulose)	40.0	40.0	40.0	40.0
Soluble fiber (guar gum)	10.0	10.0	10.0	10.0
Cholesterol	1.2	1.2	1.2	1.2
AIN-93 mineral mix	35.0	35.0	35.0	35.0
AIN-93 vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5

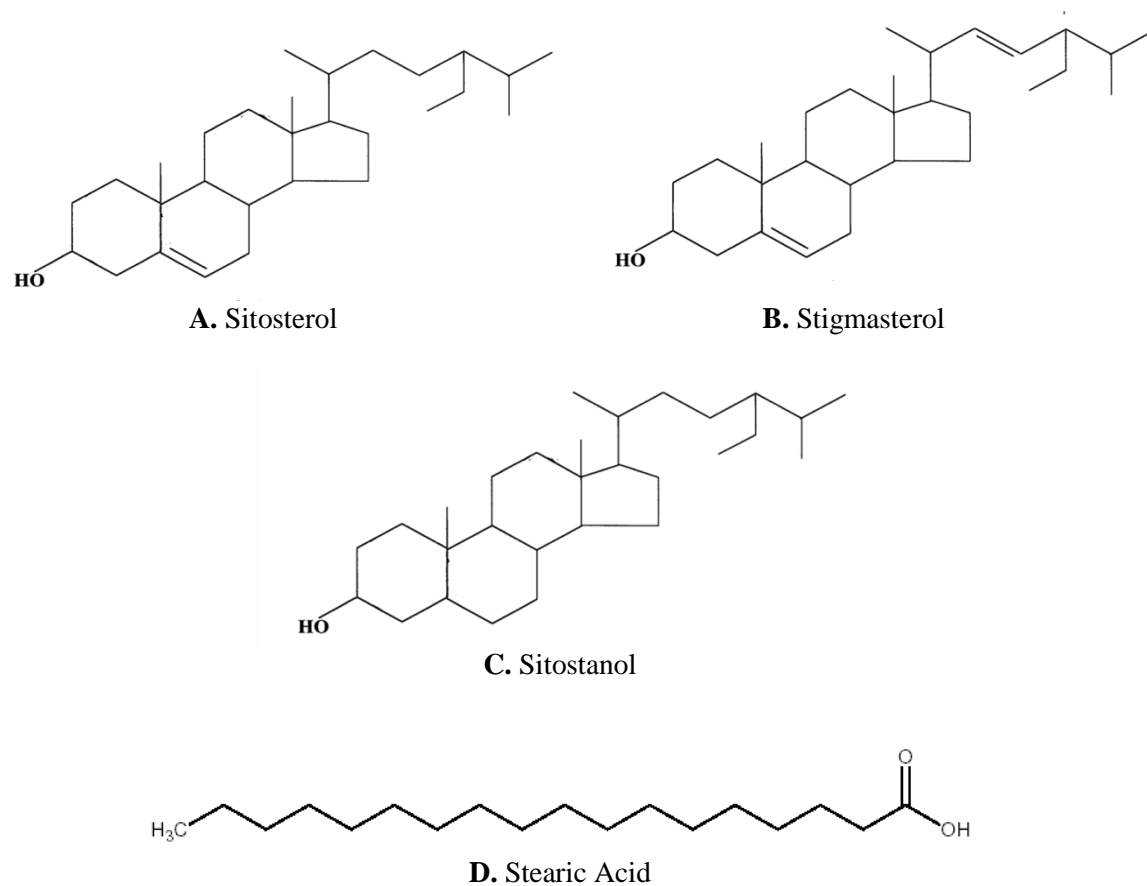


Figure 1. Chemical structures

Table 3. Phytosterol stearate treatment purity and composition

Phytosterol	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
		%	
Campesterol	1.6 ± 1.4 ^b	0.1 ± 0.2 ^b	9.5 ± 0.3 ^a
Stigmasterol	1.5 ± 1.3 ^b	98.5 ± 0.2 ^a	0.2 ± 0.3 ^b
Sitosterol	0.0 ± 0.0 ^b	1.0 ± 0.5 ^b	76.3 ± 1.3 ^a
Sitostanol	96.8 ± 2.7 ^a	0.4 ± 0.5 ^c	14.0 ± 1.2 ^b
Treatment Purity ¹	96.8 ± 2.7 ^a	98.5 ± 0.2 ^a	76.3 ± 1.3 ^b

Values are means ± S.D., n = 3.

¹Treatment purity denotes the percentage of the treatment phytosterol within the mixture.

^{a,b}Means within a row having different superscripts are statistically different ($P < 0.05$).

Dual-Isotope Cholesterol Absorption Assay. Cholesterol absorption was quantified via the dual-isotope [^{14}C]cholesterol and [^3H]sitostanol disintegrations per minute (dpm) ratio method previously published by Turley et al. (Turley et al 1994) and modified by our laboratory (Cai and Carr 1999, Schneider et al 2000). During week three on day fourteen, a 30 μL dose of $\sim 20 \mu\text{L}$ of 0.1 mCi/mL [^{14}C]cholesterol and 1 μL of 0.706 mCi/mL decay-adjusted [^3H]sitostanol in 2.4 mL of soybean oil was administered to each animal via gavage, and animals were switched to cages with new bedding to prevent contamination of fecal collection.

On day fifteen, the hamsters were again dosed with 25 μL . The bedding was collected on day eighteen, and the radioactive fecal pellets isolated with forceps. Three aliquots of the dual-isotope soybean dose were saved in 4 mL of Bio-Safe II cocktail (Research Products International) along with a Bio-Safe II blank to allow for quantification. Approximately 2 g of fecal pellets was placed into glass 20 x 150 tubes. After the addition of 95% ethanol (9 mL) and 50% KOH in water (1 mL), the samples were vortexed before overnight hot extraction at 80°C overnight. The extraction was halted with 3 mL of deionized water. After vortexing, hexane was added (7 mL), the samples were mixed by shaking and left overnight to allow for phase separation. The upper hexane layer was transferred to 7 mL scintillation vials directly. The scintillation vials were allowed to dry under exposure to Ultra Violet light over five days to breakdown chromophore compounds that could potential interfere with scintillation cocktail light propagation. BioSafe II scintillation cocktail (4 mL) was added to each sample and both the samples and the dose vials were read after vortexing using a dual-gated ^{14}C and ^3H procedure, yielding a isotope dose cholesterol-to-sitostanol ratio of 1.159 which was used to calculate animal cholesterol absorption from the following formula:

$$\text{Percent Cholesterol Absorption} = \frac{([\text{C}^{14}]_{\text{dose}}/[\text{H}^3]_{\text{dose}} - [\text{C}^{14}]_{\text{feces}}/[\text{H}^3]_{\text{feces}})}{([\text{C}^{14}]_{\text{dose}}/[\text{H}^3]_{\text{dose}})} \times 100 \%$$

Quantification of Plasma Lipids. Plasma total cholesterol was measured colorimetrically in 96-well plates using reagents as previously described (Carr et al 1993). The HDL fraction cholesterol quantification was performed using the total cholesterol assay after precipitation of apolipoprotein-B containing lipoproteins (VLDL and LDL) with a 1:1 dilution with *HDL Precipitating Reagent* (Thermo Electron Corp., Melbourne, Australia) and subsequent centrifugation at $>1000 \times g$ for 10 minutes to isolate the HDL lipoprotein fraction from whole plasma for analysis. Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol.

Quantification of Liver Lipids. Recording the exact weight, about 0.5g of each frozen liver sample was minced, and the lipids were extracted using the Folch extraction procedure (Folch et al 1957). After the addition of 5 mL chloroform/methanol (2:1, v/v) samples were flushed with nitrogen and extracted over the weekend and by heat for five hours at 50°C before filtration using Whatman #41 filter paper into 15 mL graduated conical glass tubes. The extraction tube and funnel were each washed three times with excess chloroform/methanol (2:1) to maintain quantitative technique, and the volume of each extract was brought to 10 mL prior to the additional of 2 mL of 0.88% KCL, vortexing, and phase separation by brief centrifugation. The lower phase volume was recorded, the upper phase was aspirated, and the lower phase was transferred to a clean tube, flushed with nitrogen, and stored at 4°C.

Triton X-100 (10% chloroform (v/v); 0.4 mL) was added to aliquots (0.5-0.1 mL) of the liver lipid extracts to maintain solubilization of the lipids upon the addition of 0.96 mL of deionized H₂O for aqueous enzymatic analysis as previously described (Carr et al 1993). Aliquot total cholesterol, free cholesterol, phospholipids, and triglycerides were quantified in duplicate. Total cholesterol and triglycerides were measured using the reagents used by Carr et al. obtained from Roche Diagnostics (Indianapolis, IN), whereas the free cholesterol and phospholipid reagents were purchased from Wako Chemicals (Richmond, VA). Liver esterified cholesterol was quantified as the difference between total and free cholesterol.

Quantification of Fecal Bile Acids. Bile acids were quantitatively extracted from approximately 100 mg of ground fecal matter, the exact mass was recorded, and the lipids extracted via the Folch procedure using 10 mL of chloroform/methanol (2:1, v/v) (Folch et al 1957) after the addition of 0.7 mL of 0.5 M HCl to maximize extraction efficiency. Samples were flushed with nitrogen, vortexed, and left at room temperature for extraction overnight prior to the addition of 2 mL of 0.88% KCl and phase separation via centrifugation at 1000 x g for 10 minutes. The upper phase was transferred to a graduated conical tube, and the lower phase was washed with 3 mL of chloroform/methanol/water (3:48:47, v/v), inverted six times, centrifuged for minutes at 1000 x g, and the upper phase added to the first upper phase. The total volume of the collected bile acid containing phases was recorded, and the upper phase was transferred to a new tube, flushed with nitrogen, and stored at 4°C until analysis.

Total bile acids were quantified using 3 α -hydroxysteroid dehydrogenase (Sheltawy and Losowsky 1975). Aliquots (2 mL) of the upper phase were evaporated at ~50°C under nitrogen in standardized glass cuvetts and re-solubilized in 100 μ L of methanol prior to the addition of 3.5 mL of β -NAD in pH-adjusted CAPS buffer (0.2 mg/mL; pH 10.8) to each sample and subsequent measurement of background absorbance at 340 nm. After the addition of 0.4 mL of 3 α -hydroxysteroid dehydrogenase (0.75 units/mL of 0.01 M phosphate buffer, pH 7.2), samples were incubated at 37°C for thirty minutes, and the absorbance was read at 340 nm. After adjusting for the difference in volume between the background and post-incubation absorbances measured, the difference in absorbance was used to quantify the total bile acids present in the fecal samples utilizing a 0.01 M cholic acid standard curve diluted to the range of 0.1-0.5 mmol/assay and allowing for accurate quantification. β -NAD, CAPS, cholic acid, and 3 α -hydroxysteroid dehydrogenase were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Free and Esterified Sterol Quantification. Fecal sterols were extracted from approximately 30 mg of ground fecal matter (exact weight recorded), using the Folch procedure (Folch et al 1957) and free sterols were measured via direct GC analysis whereas esterified sterols were quantified using a modification of the thin-layer chromatography (TLC) protocol published by Nissinen et al. (Nissinen et al 2007) after the addition of 0.1 mL of 300 µg of 5 α -cholestane (esterified sterol internal standard) and 300µg of lanosterol (free sterol internal standard). While Nissinen et al. (2007) utilized epicoprostanol as the free sterol fraction internal standard in analyzing human proximal jejunum samples, this was not deemed suitable for our fecal matter analysis because epicoprostanol (5 β -Cholestan-3 α -ol) is a stereoisomer of dihydrocholesterol (5 α -Cholestan-3 β -ol), a major metabolite of microfloral cholesterol modification commonly quantified in hamster fecal samples and incomplete separation of the two compounds was forecasted; thus lanosterol, which was not even detected in hamster fecal samples by Lee et al. (Lee et al 2005), was used. The lanosterol available was ~51% pure but demonstrated two prominent peaks upon GC analysis (n = 4) accounting for 35.83% \pm 0.20% (P1) and 33.30% \pm 0.21% of the observed peak area with an elution pattern that did not overlap with sterol peaks of interest (data not shown). Unfortunately, this purity test was only available after lanosterol addition to samples. Lanosterol proved to be an unreliable marker of free sterol migration during TLC analysis in our mobile phase (data not shown); subsequently, free sterol concentrations of the samples were analyzed via direct GC analysis.

After the addition of the internal standards to the fecal samples weighed into 15 mL conical graduated glass tubes, 0.2 mL of 0.5 M HCl and 5 mL of chloroform/methanol (2:1, v/v) were added to extract fecal lipids using the Folch protocol (Folch et al 1957). Samples were flushed with nitrogen, vortexed, and left at room temperature for extraction for one hour, vortexing every thirty minutes prior to the addition of 1 mL of 0.88% KCl (wt/v), inversion of the samples to ensuring mixing (six times), and centrifugation at 1000 x g for twenty minutes to induce phase separation. The lower phase volume was recorded, the upper phase was aspirated

and discarded, and the lower phase was carefully decanted into a new glass tubes to remove fecal debris before being flushed with nitrogen and stored at 4°C until TLC analysis (esterified sterols) or direct GC quantification (free sterols).

Because previous tests running the fecal samples on TLC plates demonstrated improper separation presumed to be caused by the high concentration of free and/or esterified phytosterols in the samples, only ~5 mg of each fecal sample was applied to the plate in a 1 inch band as determined to be the upper limit of reliable lipid separation of the current fecal samples (data not shown). In order to fall beneath this 5 mg of total lipid TLC lane mass application limit, previous testing data were used to estimate the appropriate aliquote volume to be 0.7 mL; thus 0.7 mL of each lower phase was transferred to a new tube, dried under nitrogen at 50°C, and transferred to a 1 inch-wide TLC plate lane twice with 50 µL of chloroform after gentle vortexed each time.. Each Whatman AL-SIL G TLC plate (Aluminum-Backed, 60Å Silica, 250 µm x 20 x 20 cm; Catalog # 4420-221) contained a standard lane containing phytosterol esters and free phytosterols.

The mobile phase consisted of 50 mL of heptane/ethyl ether (50:50, v/v) as described previously for separation of free and esterified sterols (Nissinen et al 2002), and it was changed after every two plates with fifteen minutes allowed prior to each run for mobile phase chamber equilibration. The plates were run for forty-five minutes, removed from the chamber, and allowed to dry for at least fifteen minutes. The standard lane was carefully liberated from the plate with a razor blade and developed in an iodine chamber to provide visual confirmation of separation of the the free and esterified sterol fractions before standardized cutting of all of the free and esterified fractions based upon the standard lane of each sample plate. The esterified fractions were cut from the plate, carefully folded to prevent silica-bound sample loss via plate flaking, and placed into clean individual glass tubes. The fractions were distributed in such a manner that left a mid-section between the esterified and free sterol fractions, leaving a possibility of sample loss upon incomplete separation. Analysis of six of the samples in this

region demonstrated that despite the possibility of the introduction of error, there was subjectively minimal presence of peaks in the TLC plate region. Chloroform was added to each tube in excess of that required to cover the aluminum-backed folded TLC plate fractions. Samples were flushed with nitrogen and stored at 4°C for less than one week.

The esterified sterol fractions were shaken for ten seconds and then centrifuged at 1000 x *g* for twenty minutes to pellet the silica at the bottom of the tube prior to decantation into new tubes. To maximize recovery, the TLC plate extraction was repeated with another excess of chloroform. The collected chloroform fractions were flushed with nitrogen, vortexed and shaken, and centrifuged for twenty minutes at 1000 x *g*. The esterified, mid-section, and free sterol TLC extracts in chloroform were all dried under nitrogen at 50°C, utilizing excess chloroform to wash down the sides and concentrate the samples at the bottom. The esterified fractions were saponified by 1 ml of 1 M KOH in methanol prior to leaving the nitrogen-flushed samples overnight at room temperature and several periods of 60- 80°C of heat and frequent vortexing to ensure complete saponification. Distilled water (1 mL) was added, the samples vortexed, and hexane (2 mL) was added to extract non-saponifiables. The samples were mixed using a multi-mixer set on high for five minutes, using haphazard grouping prior to phase separation via centrifugation for five minutes at 1000 x *g* and the transfer of upper hexane phases to new tubes. The extraction was repeated with an additional 2 mL of hexane to maximize recovery.

The esterified samples and a separate 0.7 mL aliquot of the same fecal lipid extracts utilized for the esterified analysis were then dried under nitrogen at 50°C. Following the addition of 100 µL of hexanes to the dried fecal samples and gentle vortexing, the dried samples were twice transferred to GC vials equipped with 300 µL inserts, yielding a final volume of 200 µL. All GC analyses were performed on an AT-5 capillary column (Alltech, Deerfield, IL) with helium as the carrier gas. The temperature program utilized a 15.0°C/minute temperature ramp from an initial one minute hold at 270°C to a final hold at 300°C of fourteen minutes, an inlet temp of 270°C, and a flame ionizing detector temperature of 300°C.

Statistical Analysis. Statistical One-way ANOVA analyses were performed using the GLM procedure (version 9.0; SAS Institute, Cary, NC, USA) and the simulated adjustment for multiple comparisons using the “/pdiff” option of “lsmeans” in SAS. The simulated adjustment was used instead of the Tukey to account for the different number of experimental units within each treatment group (control, n=9; phytosterol stearate treatments, n=10). The standard error of the mean was used to represent mean treatment variations displayed in both tables and figures with the exception of the phytosterol treatment purity, which used the standard deviation. The coefficient of variation was calculated for many of the assays via sample replicates (n=3-5) in order to quantifying the extent of intra-assay variation for the protocols (Table 4). Correlation analysis was calculated using the “PROC CORR” command in SAS (version 9.0; SAS Institute, Cary, NC, USA).

Table 4. Intra-assay variation: coefficient of variation for liver and fecal assays

Quantitative Assay	Intra-Assay Replicates	Coefficient of Variation ¹
	n	%
Liver		
Total Cholesterol	3	3.40%
Free Cholesterol	3	0.53%
Esterified Cholesterol	3	5.49%
Triglycerides	3	8.00%
Phospholipids	3	2.17%
Fecal		
Free Neutral Sterols	5	5.12%
Esterified Neutral Sterols	5	25.61%
Total Neutral Sterols	5	9.52%
Free Phytosterols	5	23.38%
Esterified Phytosterols	5	19.12%
Total Phytosterols	5	17.54%
Total Sterols	5	18.74%
Free Phytosterol Treatment	5	10.33%
Esterified Phytosterol Treatment	5	23.93%
Total Phytosterol Treatment	5	22.35%
Phytosterol Treatment FS/Total ²	5	34.30%
Phytosterol Treatment ES/Total ³	5	1.92%
Bile Acids	3	16.1%

¹Coefficient of variation (CV) calculated by dividing the standard deviation (σ) by the mean (μ).

²FS/Total = phytosterol treatment free sterols divided by phytosterol treatment total sterols.

³ES/Total = phytosterol treatment esterified sterols divided by phytosterol treatment total sterols.

Results

Body Weights, Food Intake, and Cholesterol absorption. Weekly hamster body weights (Table 5) and cumulative body weight gain (Figure 2) did not significantly differ ($P>0.05$) between diets during any of the study period weeks. Food intake measurements obtained weekly did not differ among the treatments for week one, week two, or week three ($P>0.05$). During week four, the dietary treatments had significantly different food intakes (Table 6). The control group dietary intake during week four was significantly greater than both the stanol stearate and stigmasterol stearate treatment groups but not the sitosterol stearate group. None of the phytosterol stearate treatments differed significantly in food intake during week four ($P>0.05$). Despite the significantly different feed intakes among the treatment groups, the average weekly intakes over the entire study did not statistically differ. Because of the difference in dietary feed intakes, the dietary intake data were fractionated to display the dietary intake of phytosterols and cholesterol based upon the dietary composition (Table 7). The dietary intake of phytosterols during week one, week two, and week three were statistically equivalent among the three phytosterol stearate treatments and differed from the control by design. During week four of the study, the dietary intake of sitosterol stearate was significantly higher than stigmasterol stearate but not sitostanol stearate. The stigmasterol stearate and stanol stearate treatment dietary phytosterol treatment intakes did not statistically differ ($P>0.05$). The average weekly dietary intake of the phytosterol stearate treatments did not differ during the study. Because the total treatment phytosterol fecal excretion did not differ statistically across the groups, the differences in treatment phytosterol intakes during week four were accepted as potential confounding variables within the current study without further consideration. The cholesterol dietary intakes only differed statistically during week four of the study, an effect that was not present when examining the average weekly dietary cholesterol intake. During week four, dietary intake of

cholesterol was highest in the control but did not differ statistically from the sitosterol stearate treatment group. The sitostanol stearate and stigmasterol stearate dietary cholesterol intakes were statistically lower than the control diet, but there was no statistical difference between any of the phytosterol stearate treatments. Despite no statistical difference in the average weekly cholesterol intake across the diets, cholesterol intake was also considered a potential confounding variable of the current study. Cholesterol absorption percentages ranged from $59.9\% \pm 3.0\%$ (sitostanol stearate) to $66.2\% \pm 3.0\%$ (sitosterol stearate) but did not differ significantly among the phytosterol stearate treatment groups or even in comparison to the control, indicating that the differences in intakes of phytosterol stearates and cholesterol did not result in any difference in the extent of intestinal absorption but may have confounded any treatment differences (Table 7).

Plasma Cholesterol. Plasma total cholesterol concentrations were unchanged by any of the treatments in comparison to the control ($P > 0.05$) after four weeks on the diets (Table 8). The plasma total cholesterol concentrations of the control (6.14 ± 0.23 mmol/L), sitostanol stearate (5.67 ± 0.34 mmol/L), stigmasterol stearate (5.87 ± 0.21 mmol/L), and sitosterol stearate (5.42 ± 0.26 mmol/L) were statistically equivalent. The non-HDL cholesterol concentrations of the control, sitostanol stearate, stigmasterol stearate, and sitosterol stearate groups were 3.83 ± 0.14 mmol/L, 3.45 ± 0.27 mmol/L, 3.50 ± 0.16 mmol/L, and 3.33 ± 0.17 mmol/L, respectively. However, the non-HDL cholesterol fractions did not significantly differ among any of the groups ($P > 0.05$). As expected, the HDL cholesterol concentrations of the treatment groups did not significantly differ, ranging from 2.09 ± 0.10 mmol/L (sitosterol stearate) to 2.36 ± 0.10 mmol/L (stigmasterol stearate).

Liver Cholesterol, Triglycerides, and Phospholipids. Liver total cholesterol concentrations were statistically similar across the four diets, ranging from 13.7 ± 1.8 $\mu\text{mol/g}$ in the sitostanol stearate treatment group to 18.9 ± 2.1 $\mu\text{mol/g}$ in the stigmasterol stearate treatment (Table 9). Free cholesterol liver concentrations significantly differed in response to the different

diets after weeks upon treatment (Table 9). The free cholesterol concentrations in the liver were highest and statistically equivalent in the control group ($4.7 \pm 0.1 \mu\text{mol/g}$) and the stigmasterol stearate groups ($4.5 \pm 0.0 \mu\text{mol/g}$). Both the control and the stigmasterol stearate treatment group had statistically elevated concentrations of free cholesterol in comparison to both the sitostanol stearate and sitosterol stearate treatments, which were statistically equivalent with respective means of $4.2 \pm 0.1 \mu\text{mol/g}$ and $4.2 \pm 0.1 \mu\text{mol/g}$. The calculated esterified cholesterol concentration means were unaffected by phytosterol treatment and no difference between any of the dietary groups was observed ($P > 0.05$). Neither the liver triglyceride nor the phospholipid concentrations statistically differed across the dietary treatment groups. The whole liver weights expressed as $\text{g} \times 100 \text{g}^{-1}$ of body weight did not significantly differ among the diets.

Table 5. Hamster body weight

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	g			
Week 0	61.4 ± 0.7	62.4 ± 1.0	64.5 ± 0.9	61.5 ± 1.1
Week 1	79.0 ± 2.4	79.7 ± 2.1	83.9 ± 2.2	78.5 ± 2.6
Week 2	95.2 ± 3.5	98.2 ± 2.1	103.3 ± 2.2	96.5 ± 3.6
Week 3	109.6 ± 2.9	109.6 ± 2.5	115.1 ± 2.6	109.3 ± 3.3
Week 4	117.4 ± 2.7	116.9 ± 2.8	120.7 ± 3.1	118.1 ± 2.7

Values are means ± SEM, $n = 9-10$. No treatment differences were detected using One-way Anova analysis with pdiff simulated comparisons ($P > 0.05$).

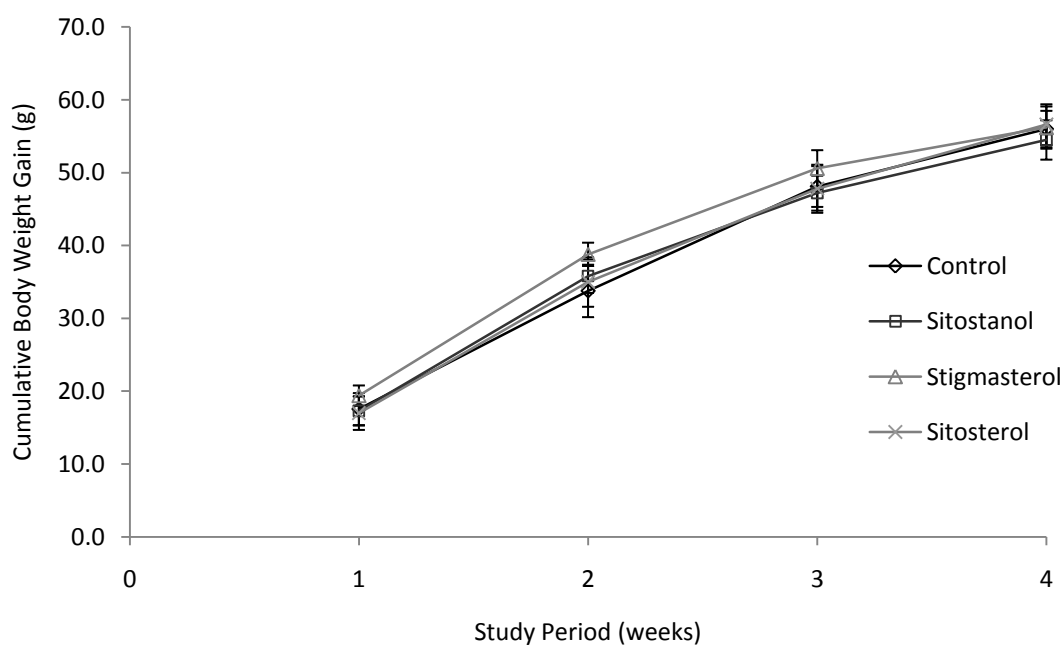


Figure 2. Cumulative body weight gain in hamsters during phytosterol stearate dietary treatment study. No treatment differences were detected using One-way Anova analysis with pdiff simulated comparisons ($P > 0.05$).

Table 6. Food intake

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	g/day			
Week 1	8.2 ± 0.5	8.7 ± 0.7	9.4 ± 1.0	8.0 ± 0.4
Week 2	12.0 ± 0.9	11.5 ± 1.0	10.9 ± 0.5	11.1 ± 0.7
Week 3	11.3 ± 0.6	12.2 ± 0.7	13.2 ± 0.4	12.2 ± 0.4
Week 4	7.5 ± 0.8 ^a	4.2 ± 0.5 ^b	3.2 ± 0.6 ^b	5.3 ± 0.4 ^{a,b}
Average	9.7 ± 0.6	9.2 ± 0.6	9.2 ± 0.5	9.2 ± 0.3

Values are means ± SEM, $n = 9-10$.

^{a,b}Means within a row having different superscripts are statistically different ($P < 0.05$).

Table 7. Dietary sterol intake¹ and cholesterol absorption

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	mg/day			
Phytosterols				
Week 1	0 ± 0 ^b	218 ± 17 ^a	234 ± 25 ^a	200 ± 11 ^a
Week 2	0 ± 0 ^b	289 ± 24 ^a	274 ± 12 ^a	277 ± 17 ^a
Week 3	0 ± 0 ^b	305 ± 16 ^a	331 ± 10 ^a	305 ± 10 ^a
Week 4	0 ± 0 ^c	106 ± 14 ^{a,b}	80 ± 16 ^b	133 ± 10 ^a
Average	0 ± 0 ^b	230 ± 16 ^a	230 ± 13 ^a	229 ± 8 ^a
Cholesterol				
Week 1	9.8 ± 0.6	10.5 ± 0.8	11.2 ± 1.2	9.6 ± 0.5
Week 2	14.4 ± 1.1	13.9 ± 1.2	13.1 ± 0.6	13.3 ± 0.8
Week 3	13.5 ± 0.7	14.7 ± 0.8	15.9 ± 0.5	14.6 ± 0.5
Week 4	9.0 ± 1.0 ^a	5.1 ± 0.7 ^b	3.8 ± 0.8 ^b	6.4 ± 0.5 ^{a,b}
Average	11.7 ± 0.7	11.0 ± 0.8	11.0 ± 0.6	11.0 ± 0.4
	%			
Cholesterol Absorption	61.6 ± 3.0	59.9 ± 3.0	63.0 ± 2.0	66.2 ± 3.0

¹Dietary phytosterols supplemented at 2.5% dosage (g/kg) in addition to basal phytosterol intakes for the phytosterol stearate treatments. Dietary cholesterol supplemented at 0.12% dosage (g/kg) in all diets. These percentages were used to calculate the sterol intake based upon total food intake during each week.

^{a,b,c} Means within a row having different superscripts are statistically different ($P < 0.05$).

Table 8. Plasma lipid concentrations

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	mmol/L			
Total cholesterol	6.14 ± 0.23	5.67 ± 0.34	5.87 ± 0.21	5.42 ± 0.26
non-HDL cholesterol	3.83 ± 0.14	3.45 ± 0.27	3.50 ± 0.16	3.33 ± 0.17
HDL cholesterol	2.31 ± 0.17	2.21 ± 0.10	2.36 ± 0.10	2.09 ± 0.10

Values are means ± SEM, $n = 9-10$. No treatment differences were detected using One-way Anova analysis with simulated comparisons ($P > 0.05$).

Table 9. Liver weight and lipid concentrations

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	μmol/g			
Total cholesterol	17.8 ± 2.1	13.7 ± 1.8	18.9 ± 2.1	15.8 ± 2.2
Free cholesterol	4.7 ± 0.1 ^a	4.2 ± 0.1 ^b	4.5 ± 0.0 ^a	4.2 ± 0.1 ^b
Esterified cholesterol	13.1 ± 2.1	9.5 ± 1.8	14.4 ± 2.0	11.5 ± 2.2
Triglyceride	5.0 ± 0.3	5.4 ± 0.4	4.6 ± 0.3	5.6 ± 0.4
Phospholipid	13.1 ± 0.3	12.6 ± 0.3	12.8 ± 0.3	12.5 ± 0.3
	g/100 g of body weight			
Liver	5.99 ± 0.13	5.97 ± 0.17	5.73 ± 0.14	5.92 ± 0.10

Values are means ± SEM, $n = 9-10$.

^{a,b} Means within a row having different superscripts are statistically different ($P < 0.05$).

Fecal Free and Esterified Neutral Sterols. While neutral free sterol excretion was statistically equivalent across the four dietary groups ($P>0.05$), esterified and total neutral sterol excretion were significantly different among the treatments (Table 10). The sitosterol stearate supplemented diet resulted in an increase in fecal esterified neutral sterols significantly greater than the other phytosterol esters or the control. Conversely, the control, sitostanol stearate, and stigmasterol stearate diets demonstrated statistically equivalent esterified neutral sterol excretion. Total neutral sterol excretion was significantly higher in the sitosterol stearate group than either the control or the stigmasterol stearate treatment but did not differ statistically from the sitostanol stearate treatment; however, the p-value of the comparison of the total neutral sterol excretion in the sitosterol stearate and sitostanol stearate dietary groups approached significance ($P=0.0556$). The control, sitostanol stearate, and stigmasterol stearate groups were statistically equivalent ($P>0.05$). The percent esterified neutral sterol excretion was significantly the highest in the sitosterol stearate dietary group ($37.78\% \pm 3.78\%$), whereas the stigmasterol stearate ($11.29\% \pm 0.58\%$) and sitostanol stearate ($4.07\% \pm 0.55\%$) treatments did not significantly differ despite the presence of a significantly higher percent esterified neutral sterol excretion in the stigmasterol stearate treatment versus the control ($1.81\% \pm 0.40\%$) (Table 10).

Fecal Free and Esterified Phytosterols and Total Sterols. Free phytosterol fecal excretion was equivalent and significantly higher in both the stigmasterol stearate and the sitosterol stearate treatments than the statistically equivalent control and sitostanol stearate groups (Table 10). Esterified phyosterol fecal excretion did not differ among the treatment groups but was significantly lower in the control group versus the phytosterol stearate diets. The total phytosterol excretion in the phytosterol stearates was statistically equivalent and greater than the control diet, reflecting the intended study design. The total sterol fecal excretion demonstrated an identical pattern with phytosterol treatment diets resulting in significantly higher amounts of total sterol excretion than the control ($P<0.05$).

While the absence of a significant effect on fecal neutral sterol excretion in response to sitostanol stearate and stigmasterol stearate treatments in comparison to the control may simply be due to a mirrored significantly different phytosterol stearate treatment intake during week four (fecal sterol measurements were quantified for week four), the presence of statistically equivalent fecal total phytosterol excretion levels indicate that the calculated difference in intake may not have manifested itself across the treatment groups to any statistically significant extent. Subsequently, data were analyzed disregarding the calculated difference in phytosterol stearate treatment intake during week four with the acknowledgement that the variable may confound our conclusions.

Free and Esterified Phytosterol Stearate Treatment Excretion. Free phytosterol treatment excretion was significantly higher in the sitosterol stearate treatment group than sitostanol stearate but not stigmasterol stearate (Table 10). Esterified phytosterol stearates differed significantly in that the sitostanol stearate treatment had a significantly higher excretion than either the stigmasterol stearate or the sitosterol stearate treatments, which were statistically equivalent. Total phytosterol treatment excretion among the phytosterol stearate diets was significantly higher in the sitostanol stearate group than both the stigmasterol stearate and sitosterol stearate treatments, which did not differ significantly. The percent of free treatment phytosterol excretion to total treatment phytosterol sterol excretion was significantly higher in the sitosterol stearate dietary treatment ($4.68\% \pm 0.90\%$) in comparison to the statistically equivalent sitostanol stearate ($0.88\% \pm 0.22\%$) and stigmasterol stearate diets ($2.45\% \pm 0.31\%$), indicating the level of net hydrolysis of the phytosterol treatments. The percent free phytosterol treatment fecal excretion mirrored the percent esterified neutral sterol excretion, demonstrating a significant correlation in a post-hoc statistical analysis ($r = 0.6304$, $p = <0.0001$; Figure 3).

Fecal Bile Acid Excretion and Fecal Output. The bile acid excretion did not differ significantly among any of the diet groups (Table 11). The fecal output was significantly higher in the sitostanol stearate and sitosterol stearate treatments than in the control but not in the

stigmasterol stearate treatment, which did not differ statistically from any of the diets (Table 11). This fecal output may simply reflect the significant difference in food intake observed during week 4 or it may be the result of treatment effects upon fecal excretion transit time. The effect will not be considered further in this study.

Table 10. Fecal sterol excretion

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	$\mu\text{mol} \times \text{day}^{-1} \times 100 \text{ g}^{-1} \text{ body weight}$			
Neutral sterols ¹				
Free	6.02 ± 0.32	7.39 ± 0.62	6.41 ± 0.37	6.07 ± 0.54
Esterified	0.11 ± 0.02 ^b	0.31 ± 0.05 ^b	0.84 ± 0.09 ^b	3.74 ± 0.45 ^a
Total	6.12 ± 0.31 ^b	7.70 ± 0.64 ^{a,b}	7.24 ± 0.46 ^b	9.80 ± 0.72 ^a
	%			
Esterified/Total	1.81 ± 0.40 ^c	4.07 ± 0.55 ^{b,c}	11.29 ± 0.58 ^b	37.76 ± 3.78 ^a
	$\mu\text{mol} \times \text{day}^{-1} \times 100 \text{ g}^{-1} \text{ body weight}$			
Phytosterols ²				
Free	1.90 ± 0.21 ^b	2.31 ± 0.39 ^b	4.47 ± 0.48 ^a	4.73 ± 0.72 ^a
Esterified	0.05 ± 0.01 ^b	129.74 ± 7.55 ^a	100.71 ± 6.38 ^a	125.59 ± 14.91 ^a
Total	1.95 ± 0.21 ^b	132.05 ± 7.55 ^a	105.18 ± 6.59 ^a	130.32 ± 14.97 ^a
Total Sterols ³	8.07 ± 0.44 ^b	139.75 ± 8.01 ^a	112.42 ± 6.86 ^a	140.13 ± 15.46 ^a
Phytosterol Treatment ⁴				
Free	N/A	1.13 ± 0.27 ^b	2.30 ± 0.32 ^{a,b}	3.51 ± 0.48 ^a
Esterified	N/A	129.74 ± 7.55 ^a	91.48 ± 6.03 ^b	85.97 ± 12.05 ^b
Total	N/A	130.87 ± 7.55 ^a	93.77 ± 6.15 ^b	89.47 ± 12.13 ^b
	%			
Free/Total ⁵	N/A	0.88 ± 0.22 ^b	2.45 ± 0.31 ^b	4.68 ± 0.90 ^a

N/A = Not applicable due to no phytosterol treatment in diet. Values are means ± SEM, $n = 9-10$.

¹Sum of cholesterol, dihydrocholesterol, coprostanol, and coprostanone.

²Sum of brassicasterol, campesterol, stigmasterol, sitosterol, and sitostanol.

³Total Sterols include both phytosterols and neutral sterols.

⁴Phytosterol treatment indicates quantification of the specific treatment phytosterol only.

⁵Free treatment phytosterol to total treatment phytosterol ratio is referred to as net hydrolysis.

^{a,b,c} Means within a row having different superscripts are statistically different ($P < 0.05$).

Table 11. Bile acid excretion and fecal output

	Control	Sitostanol Stearate	Stigmasterol Stearate	Sitosterol Stearate
	$\mu\text{mol} \times \text{day}^{-1} \times 100 \text{ g}^{-1} \text{ body weight}$			
Bile acids	1.38 ± 0.06	1.41 ± 0.08	1.53 ± 0.06	1.29 ± 0.09
	$\text{g} \times \text{day}^{-1} \times 100 \text{ g}^{-1} \text{ body weight}$			
Fecal Output	$0.830 \pm 0.03^{\text{b}}$	$0.979 \pm 0.02^{\text{a}}$	$0.931 \pm 0.03^{\text{a,b}}$	$0.971 \pm 0.05^{\text{a}}$

Values are means \pm SEM, $n = 9-10$.

^{a,b} Means within a row having different superscripts are statistically different ($P < 0.05$).

Post-hoc Correlation Analysis

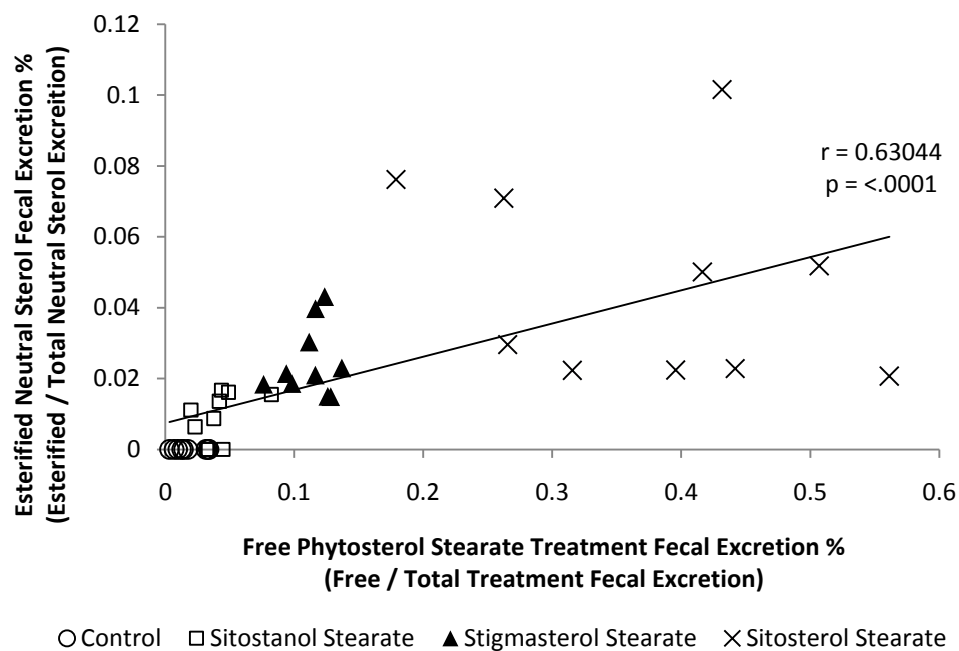


Figure 3. Post-hoc correlation analysis of esterified neutral sterol % and free phytosterol treatment sterol % excretion ($r = 0.63044$, $p = <0.0001$). Analysis of the correlation between esterified neutral sterol and free total phytosterol excretion demonstrated significance ($r = 0.43692$, $p = 0.0054$) as did analysis of the esterified neutral sterol and free phytosterol treatment excretion ($r = 0.65168$, $p = <0.0001$). Because the treatments consisted of purified phytosterol stearates, the analysis between percent esterified neutral sterol excretion and percent free phytosterol treatment excretion was chosen for display.

Discussion

This investigation sought to elucidate the effect of the phytosterol moiety incorporated into phytosterol stearate esters upon the lipid-lowering efficacy of the treatment at 2.5% (g/g) of diet. While purified phytosterol stearates and stearate-enriched phytosterol ester mixtures have demonstrated a serum cholesterol lowering effect in both humans (Carr et al 2009) and hamsters studies within our laboratory (Guderian Jr et al 2007, Rasmussen et al 2006), none of the phytosterol stearate treatment groups in our current study had significantly lower plasma total, non-HDL, or HDL cholesterol concentrations. We previously demonstrated an equivalent significantly greater decrease in non-HDL cholesterol in hamsters fed stearic acid-enriched phytosterol esters in comparison to soybean oil phytosterol esters (Rasmussen et al 2006). Utilizing a 5% (g/g) dietary dose, Rasmussen et al. (2006) demonstrated superior cholesterol lowering efficacy with increasing stearic acid enrichment in phytosterol esters indicating that the amount of stearic acid-enrichment synergistically results in a greater decrease of plasma cholesterol (Rasmussen et al 2006).

While our data may have been confounded by variable food intakes during week four, our phytosterol stearates resembled the effective purified phytosterol stearates but produced no lowering effect upon plasma cholesterol concentrations at half the 5.0% dose administered in the study by Rasmussen et al. (2006) indicating that possibly our treatment intake level was not sufficient to observe the same cholesterol lowering effect by phytosterol stearates despite the administration of ten times the 0.24% dose required to lower non-HDL cholesterol with phytosterol canola oil esters in hamsters (Lin et al 2004, Trautwein et al 2002). Dietary treatment with fish oil phytosterol esters at a 1.76% dose significantly lowered total cholesterol, an effect probably due to lowered HDL cholesterol (Demonty et al 2005). A decrease in HDL cholesterol, an effect reported by Rasmussen et al. (2006), was not even observed in the current study.

Guderian et al. (2007) administered the same beef tallow phytosterol esters (PSE) as Rasmussen et al. (2006) and observed a HDL cholesterol lowering effect at all doses (0.5% PSE, 1.0% PSE, and 5.0% PSE) but an effective non-HDL cholesterol lowering only with the 5.0% dose (Guderian Jr et al 2007). Furthermore, the 0.5% PSE diet elicited the decrease in HDL cholesterol and liver free cholesterol without the decrease in non-HDL cholesterol despite no decrease in cholesterol absorption (Guderian Jr et al 2007). With the exception of the HDL cholesterol lowering in the current study, we obtained remarkably similar mean cholesterol absorption percentages to the $62.0\% \pm 3.3\%$ measurement obtained for the 0.5% PSE treatment group within the study by Guderian et al. (2007) (Guderian Jr et al 2007).

While only the 5.0% PSE dosage demonstrated an ability to lower non-HDL despite the equivalent lowering of HDL cholesterol, a recent clinical study demonstrated that the beef tallow phytosterol esters significantly lowered LDL cholesterol in both normo- and hypercholesterolemic adults but did not decrease plasma HDL cholesterol concentrations (Carr et al 2009) at when administered within the recommended phytosterol ester intake range (Demonty et al 2009).

An explanation for this HDL cholesterol lowering in hamsters but not humans is required, as Bio-F₁B Syrian hamsters (BioBreeders) are a strain with increased sensitivity to dietary modifications and display high lipoprotein cholesterol distribution homology to humans (primarily transported within VLDL and LDL) (Trautwein et al 1993). It is possible that either the HDL cholesterol concentrations in the strain of hamster are more sensitive to dietary modification or that phytosterol stearates are affecting cholesterol metabolism differently in humans and hamsters, as the HDL cholesterol decrease was observed in hamsters even at the 0.5% PSE dose (Guderian Jr et al 2007).

While our investigation did not observe the HDL cholesterol lowering effect demonstrated by Guderian et al. (2007), several design differences may explain this difference. The current study utilized a cholesterol supplemented diet (0.12%) in Charles River outbred

hamsters, which are more resistant to dyslipidemia than the Bio F₁B hamsters fed a model Atkins diet with 90% lean ground beef diet and no added cholesterol by Guderian et al. (2007) (Dorfman et al 2003, Trautwein et al 1993). Because the level of dietary cholesterol has demonstrated an ability to modulate the effects of dietary fatty acids upon the distribution of and the concentration of lipoprotein cholesterol in hamsters (Lecker et al 2010, Mei-Huei et al 2004, Sessions and Salter 1994), the different atherogenic diets administered in our study and the study by Guderian et al. (2007) may be responsible for the differences observed. Regardless, there are currently no studies demonstrating a decrease in non-HDL cholesterol with phytosterol stearates at doses lower than 5.0% within hamsters despite the LDL cholesterol lowering effect observed in humans at the typical dietary recommended intake (Carr et al 2009), indicating that phytosterol stearate may act differently from other phytosterol esters depending upon the dose in hamsters (Guderian Jr et al 2007). In another study by (Guderian Jr et al 2007), beef tallow phytosterol esters administered at 5% PSE were more effective than a dietary treatment of 3% free phytosterols and 2% stearic acid, a treatment representing complete hydrolysis of a 5.0% dose of purified phytosterol stearate (Guderian Jr et al 2007), suggesting that incompletely hydrolyzed phytosterol stearates may function to lower cholesterol when given at the higher 5.0% dose.

Kobayashi et al. suggested a possible different mechanism of phytosterol stearate-induced cholesterol lowering based upon the absence of the effect at a dietary intake of 0.597% in rats (Kobayashi et al 2008). The 0.597% dose of purified phytosterol stearate did not induce cholesterol lowering but demonstrated a remarkable difference in the extent of net hydrolysis between the phytosterol oleates and the phytosterol stearates as examined by the extent of free phytosterols present in fecal matter, reporting a net hydrolysis of 99.477% for phytosterol oleate but a dramatically reduced net hydrolysis of 19.2% for phytosterol stearate (Kobayashi et al 2008). With 2.5% (g/g) phytosterol stearates, we observed a remarkably low level of net hydrolysis among the phytosterol stearate treatment groups in fecal matter. Furthermore, the

percent free phytosterol stearate treatment excretion (net hydrolysis) was significantly positively correlated to the percent excretion of esterified neutral sterol excretion ($r = 0.63044$, $p < 0.0001$; Figure 3), indicating that the increase in neutral sterol excretion observed with the sitosterol stearate diet may be caused by the higher level of net hydrolysis than the other phytosterol stearate treatments that were significantly lower both in fecal esterified neutral sterol excretion and in net hydrolysis. Specifically, the net hydrolysis was higher in the sitosterol stearate ($4.68\% \pm 0.90\%$) diet than the stigmasterol stearate ($2.45 \pm 0.31\%$) and the sitosterol stearate treatments ($0.88 \pm 0.22\%$).

Cholesterol esterase (PCE; EC 3.1.1.13) hydrolyzes esterified sterols, including both cholesterol and phytosterols, but the specificity of the enzyme for these different sterol and sitostanol esters differs, conferring a net difference in extent of hydrolysis, according to *in vitro* assay measurements (Brown et al 2009). Specifically, stearate esters were hydrolyzed with a $41.6 \pm 1.1\%$ activity in comparison to oleate esters, supporting the findings of Kobayashi et al. (2008) (Brown et al 2009). The *in vitro* assay measured the relative hydrolytic activity over 8 minutes at 37°C , a time period greatly condensed in comparison to the transit time and exposure of phytosterol esters to cholesterol esterase (Murthy and Ganiban 1988), indicating the feasibility of obtaining the great difference in net hydrolysis observed by Kobayashi et al. (2008) between phytosterol oleate and stearate esters. Our net hydrolysis data closely match the cholesterol esterase hydrolysis pattern demonstrated for specific phytosterol stearates within an *in vitro* model system by Brown et al. (2009) (Brown et al 2009). Specifically, our data are in agreement with the cholesterol esterase data in that there was no difference in the amount of net hydrolysis between the stigmasterol stearate and sitostanol stearate treatment groups but a greater net hydrolysis of sitosterol stearate (Brown et al 2009). Brown et al. demonstrated that cholesterol esterase hydrolyzes stigmasterol stearate at $12.6 \pm 1.6\%$ of the rate with which it hydrolyzes cholesterol oleate, supporting our high level of net unhydrolyzed stigmasterol stearate present in

the fecal matter. Furthermore, the equivalency of hydrolytic rate of both palmitate and stearate esters as demonstrated by Brown et al. (Brown et al 2009) supports the lack of difference between beef tallow phytosterol esters and purified phytosterol stearate esters demonstrated by Rasmussen et al. (Rasmussen et al 2006). Based upon our current data demonstrating an increased neutral sterol excretion that appears to mirror the extent of hydrolysis data, we have formulated the hypothesis that unhydrolyzed phytosterol stearate esters may lower cholesterol at high doses based upon an “oil phase” effect, solubilizing cholesterol within this phase and preventing micellar incorporation.

The current understanding of phytosterol-mediated cholesterol lowering primarily depends upon the mechanism of dietary phytosterol and cholesterol competition for micellarization, suggesting that displacement of cholesterol from micelles by phytosterol subsequently lowers cholesterol absorption (Jesch and Carr 2006). Work in our laboratory within a simulated *in vivo* mixed-micelle system has demonstrated that phytosterol esters neither incorporate into micelles themselves nor effect cholesterol micellar incorporation within our model micelle conditions (Carr TP and Brown AW, unpublished data). However, whether both of these effects hold true *in vivo* remains to be elucidated. The *in vitro* system used to model the effect of phytosterol esters upon cholesterol micellarization did not include cholesterol esterase, thus the possible dynamic hydrolysis and re-esterification effects of phytosterol stearates were not included in the model of phytosterol ester impact on cholesterol absorption. Furthermore, the absence of a phytosterol ester disruption of micellarized cholesterol supports our current data hypothesis, suggesting that the effect may be mediated only upon reaching high levels of phytosterol stearate intake that is inefficiently hydrolyzed by cholesterol esterase and may be responsible for the cholesterol lowering effect observed at a 5% dose despite assumed virtual lack of hydrolysis, an extrapolation from the data of Kobayashi et al. (2008) and the current study (Rasmussen et al 2006). Whether this theorized oil phase is an artifact of measurement generated upon the collection of monomeric phytosterol stearates present in the intestine or a physical

reality, the hypothesis provides a possible explanation for why phytosterol stearates did not lower plasma cholesterol within the current investigation at a dose of 2.5%.

In summary, sitosterol stearate elicited a slightly greater positive effect upon cholesterol metabolism than the other phytosterol stearate esters at a 2.5% (g/g) dose by elevating esterified neutral sterol excretion and lowering liver free cholesterol concentrations. However, this superior effect may or may not hold for more effective doses of phytosterol stearates, as we propose that the cholesterol lowering capacity of phytosterol stearates differs at different doses.

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