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Mechanisms of decreased cholesterol absorption mediated by phytosterols in the intestinal lumen

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MECHANISMS OF DECREASED CHOLESTEROL ABSORPTION MEDIATED BY PHYTOSTEROLS IN THE INTESTINAL LUMEN

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Phytosterols and their fatty acyl esters have been known for decades to lower LDL cholesterol, making them powerful nutraceuticals in lowering cardiovascular disease risk. The mechanisms by which phytosterols lower cholesterol, though, have been incompletely characterized. Three studies were executed to examine three aspects of cholesterol and phytosterol interactions in the intestinal lumen. In the first study, the ability of pancreatic cholesterol esterase to hydrolyze phytosterol esters was examined. Pancreatic cholesterol esterase hydrolyzed phytosterol esters, but the rate of hydrolysis proved sensitive to the structures of both the sterol and ester components. In the second study, cholesterol micellarization was challenged with phytosterols, phytosterol esters, and simulated hydrolysis products of phytosterol esters. Phytosterols inhibited cholesterol incorporation into micelles, but there was little difference in effects among the phytosterols. Investigation of the influence of fatty acids, simulating the effects of phytosterol ester hydrolysis, demonstrated a moderate increase in cholesterol micellarization in the presence of unsaturated, but not saturated, fatty acids. Intact esters, however, did not alter cholesterol micellarization, nor did the esters themselves incorporate into micelles. In the third study, phytosterol esters and ethers possessing

differential stability towards hydrolysis were incorporated into diets administered to hamsters. The lipids in the intestinal lumen of the hamsters did not show a hydrolysis-dependent partitioning of cholesterol between aqueous and oily phases, as was hypothesized. The extent of hydrolysis of phytosterol esters, however, was strongly and negatively correlated with cholesterol absorption efficiency. Treatment-associated changes to lipid profiles of the intestinal contents were also observed. These studies thus demonstrated that pancreatic cholesterol esterase is likely responsible for phytosterol ester hydrolysis in the intestine; competition for micellarization by free phytosterols explains some of their efficacy, while the exclusion of phytosterol esters from micelles implies hydrolysis must first take place for esters to affect this mechanism; and the extent of hydrolysis of phytosterol esters is a strong determinant of phytosterol ester efficacy. These conclusions may be useful in guiding phytosterol formulations for maximizing cholesterol-lowering efficacy.

Table of Contents

Figures and Tables

Chapter 1: Introduction[1]

1.1 General Introduction

Sterols serve a number of vital functions in biological systems, such as altering membrane fluidity, serving as precursors for bile acids to solubilize dietary lipids, and being precursors for steroid hormones such as testosterone and estrogen. Plants and animals both utilize sterols; however, the structures of sterols differ between these two taxonomic kingdoms: animals, and particularly mammals, primarily synthesize and accumulate cholesterol, while plants synthesize a number of different sterols. Herein, the plant-derived sterols will be collectively referred to as phytosterols. Unlike many other biomolecules where animals can utilize both plant- and animal-derived compounds, such as many fatty acids, carbohydrates, and amino acids, animals do not efficiently utilize phytosterols.

The investigation of the differences between cholesterol and phytosterol absorption, metabolism, and biological effects stretches back at least 80 years, beginning with observations that phytosterols are essentially excluded from absorption [1]. However, this exclusion from absorption does not preclude phytosterols from having biological effects in animals. While phytosterols have been ascribed a number of biological functions in animals, including antioxidant capabilities[2], anticarcinogenic properties (review [3]) and immune regulation[4], the most common and prominent use of phytosterols is to lower blood cholesterol, with the end goal of lowering the risk of heart disease. The

[1] *Portions of this introduction are excerpted from the author's contributions to "Cholesterol-lowering phytosterols: factors affecting their use and efficacy"*[1]

proposed mechanisms for the cholesterol-lowering properties of phytosterols have centered on pathways in which cholesterol is directly involved, including intestinal solubility, interaction with digestive enzymes, protein-mediated absorption, and gene regulation.

1.2 Phytosterols and cholesterol chemical structures

Phytosterols and cholesterol are both composed of a characteristic tetracyclic structure (Figure 1.1), which includes three cyclohexyl and one cyclopentyl ring. Additional common features include: a 3β-hydroxyl group, which lends itself to the 'ol' part of the 'sterol' name; a methyl group on the 10 and 13 carbons; and a branched alkyl chain on carbon 17. The sterols differ from each other, however, in the positions and numbers of double-bonds and additional alkyl groups. In particular, common phytosterols include additional alkyl groups on carbon 24, which is part of the carbon 17 branched alkyl sidechain. Campesterol includes a methyl group at this position, while stigmasterol, stigmastanol (aka: sitostanol), and sitosterol each contain an additional ethyl group.

Stigmasterol, stigmastanol, and sitosterol are distinguished from one another by the position and number of double bonds (Figure 1.1). Stigmasterol contains two double-bonds: one at carbon 22, and the other in the ring structure at carbon 5. Of the sterols discussed herein, the former double-bond is unique to stigmasterol, whereas the latter double-bond is common among all sterols, including cholesterol, with the exception of stigmastanol. Stigmastanol is a saturated phytosterol, thus making it technically a

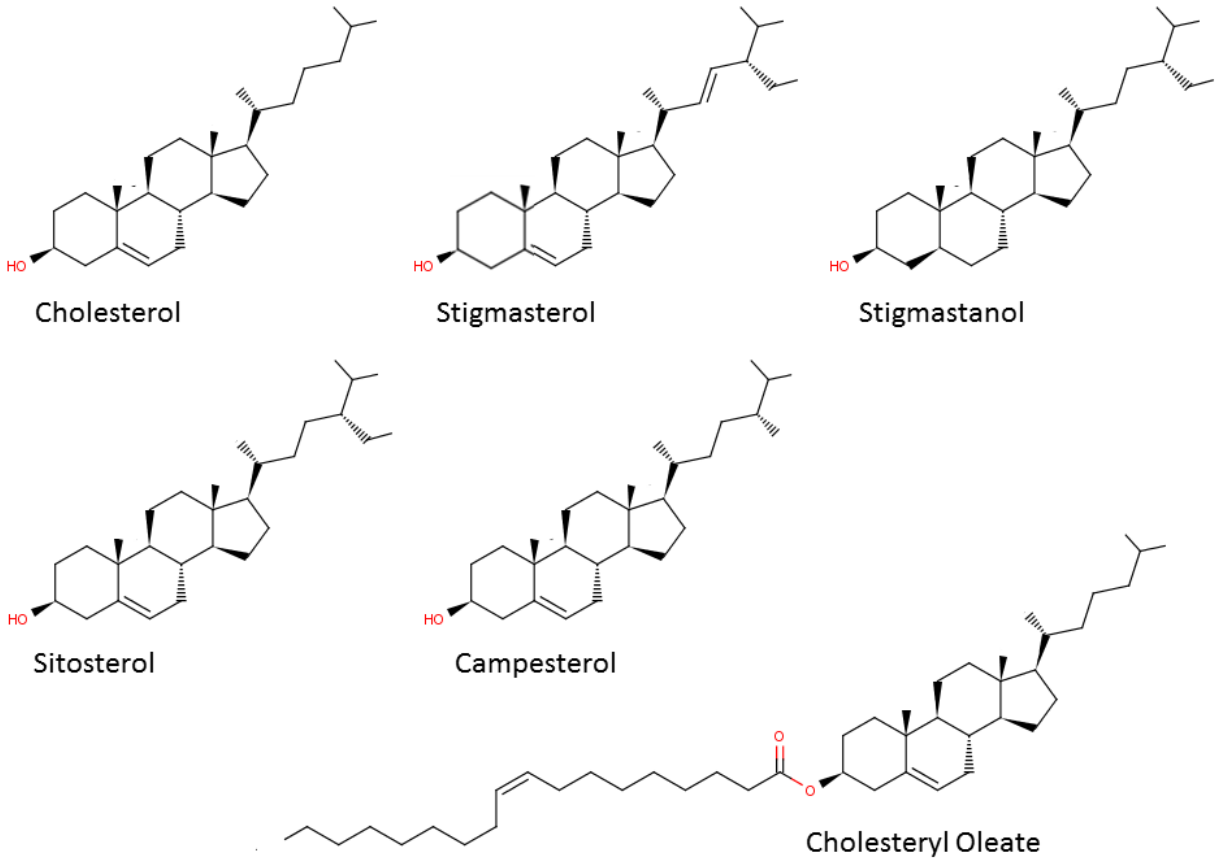

Figure 1.1. Structures of some common sterols. The differences in phytosterols that are common to Western diets include the double bond in the ring structure and alterations to the alkyl side chain. Cholesteryl oleate is shown as a representative sterol ester, defined by a fatty acyl chain connected through an ester bond to the 3β hydroxyl group of the sterol. Combinations of various fatty acids and sterols can create a wide variety of sterol ester species. Although chemically quite similar, these seemingly subtle differences among sterols or sterol esters can impart markedly different physiological effects.

phytostanol. For brevity, “phytosterols” will be used herein to indicate both plant sterols and plant stanols.

Considering the structural similarities among cholesterol and phytosterols, many of the differences among the sterols may seem to be minute. However, there are a number of factors to consider. First, the additional alkyl group on the carbon 17 sidechain creates a larger, more bulky hydrophobic group, which may affect enzyme-substrate interactions and solubility properties. Also, the double-bond contained in the carbon 17 sidechain of stigmasterol makes the alkyl chain rigid because the double-bond inhibits free rotation. Furthermore, the presence (sterols) or absence (stanols) of a double bond in the B ring results in different ring conformations. The seemingly subtle differences amongst the sterols result in a markedly different response of mammalian systems to cholesterol as opposed to the phytosterols.

1.3 Phytosterols lower cardiovascular disease risk

Cardiovascular diseases were collectively the number one cause of death in the United States in 2007 (the latest year in which data were available), accounting for one third of deaths[5]. Most of these deaths were attributable to ischemic heart diseases, in which a portion of the muscle in the heart does not receive adequate blood flow. A major cause of ischemia is atherosclerosis, which is characterized by inflammation and lipid-accumulation in the lumen of arteries, which decreases blood flow by narrowing the artery and can eventually lead to thrombosis, thereby completely blocking the artery with a blood clot.

The Framingham Heart Study is often credited with being the first to systematically investigate the links between blood cholesterol concentrations as a risk factor for cardiovascular diseases, and particularly the effects of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and total cholesterol[6][7]. The Framingham Heart Study, a long-term observational study, is now observing the third generation of participants from residents in Framingham, MA. The data from this study have allowed a number of prediction models of cardiovascular disease risk associated with a variety of risk factors, and has worked to separate environmental, genetic, and other effects through epidemiological modeling. In one such model, cholesterol concentrations were determined to be such a significant risk factor for coronary heart disease that all other risk factors, such as diabetes, smoking, and blood pressure, were converted into either cholesterol or LDL-C risk points, which were then compared to categories on a risk chart [8]. Clearly, blood cholesterol concentrations are an important risk factor for heart disease.

Cholesterol in the body comes from two sources: endogenous synthesis and dietary consumption. Dietary cholesterol contributes a relatively minor proportion of the cholesterol in the system, with an average of only 276 mg of cholesterol consumed per day [9]. In addition, dietary cholesterol minimally affects blood cholesterol, with estimates of only a 0.022-0.027 mg/dL difference in blood cholesterol for every 1 mg of dietary cholesterol consumed[10]. To put this into perspective, an individual with high cholesterol (>240 mg/dL) would need to consume an additional 100 mg of cholesterol per

day to increase their blood cholesterol by about 1%. Although dietary cholesterol alone seems to have little effect on blood cholesterol, dietary cholesterol is not the only source of cholesterol in the intestinal lumen. The gall-bladder secretes approximately 1000 mg of cholesterol per day into the small intestine[11]. Of the total cholesterol in the intestinal lumen, approximately 50% is absorbed, making inhibition of intestinal cholesterol absorption, not just decreasing dietary cholesterol, an important target for lowering blood cholesterol concentrations.

Phytosterols have been known for almost 60 years to lower blood cholesterol in humans, with initial hypotheses pointing to a disruption of cholesterol absorption [12]. Since then, many studies have been done to quantify the effects of phytosterols on blood cholesterol, with one meta-analysis indicating that phytosterols can lower LDL-C (which is considered atherogenic) by approximately 10% [13]. Phytosterols are therefore a powerful nutraceutical for lowering an atherosclerotic risk factor. In the decades since the initial discovery, a number of mechanisms have been proposed and investigated to explain the cholesterol-lowering properties of phytosterols.

1.4 Lowering cholesterol solubility in the intestinal lumen

Cholesterol in the intestinal lumen comes from both dietary and biliary sources, while the diet is the only substantial source of phytosterols [14]. Biliary cholesterol is secreted into the intestine in the form of mixed micelles primarily composed of a mixture of bile salts, phosphatidylcholine, and cholesterol [15]. Biliary secretions are important for efficient micellarization of dietary sterols, as well as other dietary components. Micellarization of

sterols, in turn, results in more efficient absorption, with small donor particles resulting in more efficient absorption [16], possibly by being able to better penetrate the unstirred water layer at the brush border membrane of the enterocyte.

Non-esterified dietary phytosterols compete with dietary cholesterol for micellarization, as well as displacing micellarized biliary cholesterol [17]. Because biliary cholesterol secretion contributes a greater amount of cholesterol to the intestinal lumen than the diet, the displacement of biliary cholesterol may be more important. The competition of phytosterols with cholesterol for micellarization occurs with free [17] phytosterols, but the effects of phytosterol esters on micellarization are unknown.

The solubility of cholesterol in micelles depends in part on the hydrophobicity of bile salts [18]. Besides simple competition for micellarization, phytosterols have also been shown to decrease the hydrophobicity index of bile salts in hamsters [19], thereby potentially decreasing cholesterol solubilization. The hydrophobicity index is employed to determine how hydrophobic a bile salt mixture is, such as that found in the intestine or bile [20]. How phytosterols may alter bile salt compositions has yet to be determined.

Another proposed mechanism by which phytosterols may decrease cholesterol solubility in the intestinal lumen was by simple co-crystallization, thereby making them insoluble. However, cholesterol and phytosterols are highly soluble in hydrolysis products of dietary lipids, making co-crystallization unlikely[21].

1.5 Inhibition of protein mediated sterol transport

One of the hallmarks of phytosterols' action is that they lower cholesterol absorption while being poorly absorbed themselves. In an average human, approximately 50% of cholesterol is systemically absorbed. Phytosterol absorption, however, is much lower, ranging from 0.04% for sitostanol and 1.9% for campesterol in one study [22], to 19% for campesterol in another study [23]. The differences in calculated absorption of phytosterols stem from the potential for a saturable absorption mechanism [22], as well as lower absorption values obtained through blood measurements when compared with higher absorption calculated through fecal sterol analysis. Though there is evidence that esterified sterols can be absorbed via protein mediated transport[24], it appears that free sterols are more efficiently absorbed *in vivo*.

Together, these observations implicate the necessity of protein-mediated cholesterol transporters. A number of proteins have been shown to be involved in intestinal uptake, including NPC1L1, SRBI, and CD36, and others implicated for enterocytic efflux, including ABCA1, ABCG5/G8 heterodimer, and ABCG1 (ABC transporter review[25]).

NPC1L1 has been implicated as being essential for high capacity cholesterol absorption, with only 15% cholesterol absorption occurring when NPC1L1 was knocked out [26]. Though at least one model indicated that NPC1L1 did not facilitate absorption of phytosterols [27], another model showed NPC1L1 facilitated sitosterol uptake about 40% as well compared to cholesterol [28], and still others have shown that phytosterol absorption is markedly decreased when NPC1L1 was knocked out in mice [29][30]. In

contrast, SR-BI and CD36 facilitate intestinal uptake of sterols to the membrane, but do not affect cholesterol absorption in mice [31]. These transporters are also present in the liver, and while it is known that they transport cholesterol across the canilicular membrane of the hepatocyte to bile, little work has been done to determine the transport of phytosterols involving these proteins, nor if there is direct competition between phytosterols and cholesterol for these transporters.

Another set of transporters important for sterol flux are the ATP Binding Casette (ABC) transporters, such as ABCA1, ABCG1, and ABCG5/G8. The hyperaccumulation of phytosterols commonly called sitosterolemia (so named because it was originally detected by hyper accretion of sitosterol[32]) has been attributed to a mutation in the sterol efflux heterodimer of ABCG5/G8 (sitosterolemia review[33]). The increase in phytosterols in individuals with malfunctioning ABCG5/G8 initially lead to the conclusion that this cotransporter was the phytosterol-specific efflux protein, but later research demonstrated that it, too, indiscriminately transported sterols[34]. ABCG5/G8 is also important in sterol secretion into bile [34].

ABCA1 is present in multiple tissues and is regarded as specifically donating sterols to ApoAI as part of the process of developing nascent HDL [35], while ABCG1 and ABCG4 deliver cholesterol to mature HDL [36]. However, these sterols do not appear to selectively transport phytosterols versus cholesterol, either [37][38]. Though there does not appear to be any direct evidence for phytosterols interacting with transport proteins, a

review of them is necessary for understanding the effects of enzymatic interactions and gene regulation of the transporters discussed next.

1.6 Interference with cholesterol-related enzymes

The interaction of phytosterols with cholesterol-metabolizing enzymes can dramatically affect their cholesterol lowering properties. One such enzyme, pancreatic cholesterol esterase (PCE), is involved in hydrolyzing sterol esters into unesterified sterols. Specificity of the enzyme depends on the sterol moiety, with cholesterol esters more rapidly hydrolyzed than phytosterol esters [39], and the fatty acyl moiety, with saturated esters being less well hydrolyzed than unsaturated [39] and di-hydroxystearate inhibiting the enzyme [40]. The importance of hydrolyzing esters is described above (micellarization). Because a relatively small amount of intestinal cholesterol is esterified [14], competition for pancreatic cholesterol esterase is likely not a large contributing mechanism for decreased cholesterol absorption.

Other enzymes related to sterol esterification are the acyl-CoA:cholesterol acyltransferases (ACAT). ACAT converts free sterols to sterol esters, which is the storage form of the sterols and the primary form in which cholesterol is incorporated into chylomicrons. ACAT isoforms have dramatically lower substrate specificity for phytosterols than for cholesterol [41][42][43], which results in more free phytosterols being available for return to the intestinal lumen from the enterocyte or removal from macrophages through such mechanisms as HDL-mediated reverse cholesterol transport. This difference in specificity does not, however, appear to be a driving factor for

lowering cholesterol absorption, as the presence of sitosterol along with cholesterol did not inhibit cholesterol esterification[41][44].

Enzymatic synthesis of cholesterol from steroid precursors includes a delta-24 reductase enzyme responsible for saturating the delta-24 bond on the cholesterol acyl-sidechain. The presence of a delta-22 desaturation, such as that found in stigmasterol, brassicasterol, and ergosterol inhibits the delta-24 enzyme, thereby inhibiting cholesterol synthesis[45]. The contribution of this inhibition to the cholesterol-lowering effects of phytosterols is not fully understood.

1.7 Regulating cholesterol-related genes and proteins

While most research supports the observation that phytosterol supplementation results in lowered cholesterol, not all sterols or animal models behave similarly. The expression and regulation of similar genes can differ dramatically depending on which sterols are studied, the model used (knock-out, organism, cell), and what tissue is investigated.

Once phytosterols are systemically absorbed, some differences in lipoprotein processing have been observed. When humans in a cross-over experiment were treated with a combination of psyllium and phytosterols, cholesterol ester transfer protein (CETP, an enzyme involved in transferring HDL-C to LDL) activity was lower than in a placebo period [46]. Similarly, upon supplementation of either 2 or 3 g/d of a phytostanol spread, CETP mass decreased [47], but not when participants were treated with phytostanols in a yogurt [48], potentially highlighting an oil versus aqueous dietary matrix effect.

In a transfected intestinal cell model, cholesterol and sitosterol suppressed NPC1L1 and HMGR expression to the same extent as 25-hydroxy cholesterol (an LXR ligand), but stigmasterol did not affect either of these. Conversely, stigmasterol upregulated SRBI and SREBP2 [49]. This would imply that the enterocyte would downregulate cholesterol absorption and synthesis when presented with sitosterol the same way it would when presented with cholesterol; however, stigmasterol did not demonstrate the same actions. Similar results of sitosterol were found in one Caco-2 cell model, with sitosterol upregulating ABCA1, APOAI, and LXR, but inhibiting FXR, as compared to cholesterol [50]. Another CaCO-2 cell model, though, indicated that although LXR agonists upregulated basolaterol sterol efflux, sitosterol itself had no effect on LXR regulated ABC transporter expression, [37].

In other models, stigmasterol has acted as an LXR agonist. In the adrenal glands of phytosterol-fed ABCG5/G8 knock-out mice (thus serving as a model for sitosterolemia), sterol regulation was severely disrupted, including significantly decreased total sterol content with little alterations to steroid hormone synthesis [51]. Adrenals may play an important role in circulating cholesterol homeostasis because they obtain cholesterol from HDL [52] by both SRBI (direct HDL-C absorption) dependent and independent mechanisms [53]. The effects of phytosterol treatment were demonstrated to be, in part, the result of altering LXR activation, which decreases expression of genes related to sterol synthesis, such as HMGR, and increases genes related to efflux, such as ABCA1. LXR was activated by stigmasterol, but not sitosterol. Similarly, HMGR protein

abundance was stunted by stigmasterol and campesterol, while only stigmasterol decreased LDLR and SRBI abundance.

The effects of stanol treatment are also not completely understood. Rabbits fed phytostanol esters had lower hepatic HMGR activity, as well as lower receptor-mediated LDL binding [54]. However, sitostanol supplementation in hamsters decreased circulating cholesterol without significantly altering gene expression [55].

The effects of phytosterols on the regulation of bile acid metabolism are also of interest. Farnesoid X Receptor (FXR) has become known as the "bile acid sensor"[56], and is important for bile acid and cholesterol homeostasis. Supplementing liver cells with a phytosterol derivative, stigmasterol acetate, proved to decrease FXR activity, even when bile acids were present [57], indicating that this derivative impairs bile acid homeostasis, which can result in cholestasis. Another analog, FH-VP4, was shown to downregulate bile acid synthesis and bile acid absorption in mouse livers[58]. The effects of non-derivatized phytosterols have yet to be determined.

Genetic factors may also affect response to phytosterol treatment. However, with the exception for those that carry the sitosterolemia gene, there is evidence that many of the common polymorphisms do not alter the beneficial cholesterol-lowering effects of phytosterol supplementation [59].

Discriminating between the effects of phytosterols themselves on gene expression and the secondary effects of altered cholesterol homeostasis has not been fully explored. For instance, the expression of NPC1L1 has also been shown to be upregulated by taurocholate and deoxycholate [16], leaving open the possibility for the alterations in bile composition as a result of phytosterol treatment [19] causing a secondary decrease in absorption through downregulating NPC1L1. In another example, monocytes from humans fed phytostanol mixtures had upregulated LDL receptors and HMGR mRNA, but both of these were found to be highly correlated with changes in cholesterol concentrations, confounding direct effects of increased phytosterols and decreased cholesterol concentrations on gene regulation[60].

Regulation itself does not necessarily directly correlate to alterations in cholesterol absorption. While mixed phytosterols and mixed phytostanols both increased gene expression of transporters such as ABCG5/G8 in one model, the cholesterol lowering effects were the same when ABCG5 was knocked-out[61]. Mixed phytosterols also had opposite effects on NPC1L1, ABCG5/G8, and ABCA1 in two different mouse models, even though phytosterol supplementation lowered cholesterol in both models[62]. Thus, directly linking changes in expression of cholesterol-related genes cannot yet explain the mechanisms of action.

1.8 Research direction

Several questions have yet to be answered regarding the mechanisms of action for phytosterols. First, although phytosterol esters are known to be hydrolyzed in the small

intestine, it is not known how they are hydrolyzed. One candidate is pancreatic cholesterol esterase, although both chemical reactions and promiscuous lipases could also explain hydrolysis. Second, although phytosterols are observed in the aqueous fraction of intestinal contents, which has historically been called a 'micellar phase,' their interactions with micelles are poorly characterized. The effects of individual phytosterols, phytosterol esters, and hydrolysis products of phytosterol esters may all differentially affect cholesterol incorporation into micelles, and thus also alter micelle-dependent cholesterol absorption. Third, and finally for the scope of this dissertation, the question of the efficacy of intact esters versus free phytosterols *in vivo* has not been adequately characterized. While studies show that administering phytosterol esters and free phytosterols both lower cholesterol and cholesterol absorption efficiency, the effects of the esters have always been confounded with their subsequent hydrolysis into free sterols. Further understanding these three mechanistic questions will help to better understand the physiological effects of phytosterols, as well as to potentially guide the development of more potent phytosterol formulations.

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Chapter 2: Plant sterol and stanol substrate specificity of pancreatic cholesterol esterase[2]

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2.1 Abstract

Consumption of plant sterols or stanols (collectively referred to as phytosterols) and their esters results in decreased LDL cholesterol, which is associated with decreased atherosclerotic risk. The mechanisms by which phytosterols impart their effects, however, are incompletely characterized. The objective of the present study is to determine if pancreatic cholesterol esterase (PCE; EC 3.1.1.13), the enzyme primarily responsible for cholesterol ester hydrolysis in the digestive tract, is capable of hydrolyzing various phytosterol esters, and to compare the rates of sterol ester hydrolysis *in vitro*. We found that PCE hydrolyzed palmitate, oleate, and stearate esters of cholesterol, stigmasterol, stigmastanol, and sitosterol. Furthermore, we found that the rate of hydrolysis was dependent on both the sterol and the fatty acid moieties in the following order of rates of hydrolysis: cholesterol > (sitosterol ≈ stigmastanol) > stigmasterol; oleate > (palmitate ≈ stearate) (differences were significant at $p<0.05$). The addition of free phytosterols to the system did not change hydrolytic activity of PCE,

[2]*This chapter has been adapted from the original published manuscript for consistency with the rest of this dissertation; no substantial changes have been made to content.*

while addition of palmitate, oleate, or stearate increased activity. Thus, PCE may play an important but discriminatory role *in vivo* in the liberation of free phytosterols to compete with cholesterol for micellar solubilization and absorption.

2.2 Introduction

Cardiovascular diseases are collectively the leading causes of death in the United States, with diseases of the heart accounting for over 25% of all deaths in the United States (1). Elevated circulating LDL cholesterol has long been considered a risk factor for the development of atherosclerotic lesions, which may ultimately lead to impaired blood circulation, heart attacks, and strokes. Several pharmaceutical and nutraceutical therapies are presently available to decrease LDL cholesterol, including the consumption of plant sterol and stanol esters (here, collectively referred to as phytosterol esters) (2).

Presently, one mechanism by which phytosterols are thought to exert their cholesterol-lowering effects is by decreasing the incorporation of cholesterol into micelles (3), thereby decreasing absorption and increasing excretion of cholesterol (4). In *in vitro* models, only free phytosterols are shown to be effective in displacing cholesterol (5), while the physiological effects are demonstrated *in vivo* by both free and esterified phytosterols. This leads to the hypothesis that phytosterol esters must be hydrolyzed to impart their cholesterol-lowering effects, which is supported by the observation that supplementation of phytosterol esters increases the amounts of free phytosterols and cholesterol in feces (4). Pancreatic cholesterol esterase (PCE; EC 3.1.1.13) has been suggested as the enzyme responsible for the hydrolysis of phytosterol esters. To date,

however, no research has been conducted to confirm whether PCE hydrolyzes these esters, much less whether PCE selectively hydrolyzes various sterol esters.

Efficient absorption of dietary cholesterol esters is dependent on hydrolysis by PCE, followed by the subsequent solubilization of free cholesterol by gall bladder secretions to form mixed micelles in the intestinal lumen; free phytosterols are also solubilized in this way (5). Furthermore, the putative intestinal cholesterol transporter Niemann-Pick C1-Like 1 (NPC1L1) transports free sterols but not sterol esters (6). Thus, it appears that the hydrolysis of cholesterol esters is necessary for cholesterol to be efficiently micellarized and absorbed, while hydrolysis of phytosterol esters may be necessary to impart their cholesterol-lowering properties.

PCE is a broad lipid-ester hydrolase, hydrolyzing other lipid carboxyl esters in addition to cholesterol esters (7, 8). The hydrolytic activity is not uniform across substrates, however, as the diacylglycerol lipase activity of PCE is greater than its triacylglycerol lipase activity (7), and the phospholipase A1 activity of PCE is greater than its phospholipase A2 activity (8). Because of the documented differences in substrate specificities, we hypothesized that PCE hydrolyzes phytosterol esters, and that the rate of hydrolysis depends on both the sterol and fatty acid moieties.

2.3 Materials and Methods

2.3.1 Reagents

Stigmasterol (95%) and stearoyl chloride (90%) were purchased from TCI America (Portland, OR). Sitosterol (75%), cholesteryl stearate (96%), cholesteryl palmitate (97%), and palmitoyl chloride (98%) were purchased from ACROS Organics (Geel, Belgium). Oleoyl chloride (85%), sodium cholate hydrate (99%), cholesteryl oleate (98%), oleic acid (99%), stearic acid (99%), Sylon BTZ, and palladium on carbon (Pd/C,10%) were purchased from Sigma-Aldrich (St. Louis, MO). Palmitic acid (99+%), Triton X-100, and porcine pancreatic cholesterol esterase (Cat. no. 0215067180) were obtained from MP Biomedicals (Irivine, CA). 5α-cholestane was obtained from Steraloids (Newport, RI). Cholesterol (95%) was obtained from Mallinckrodt OR (Paris, KY). W2 Raney Nickel (RaNi) was prepared by reaction of $NiAl_2$ alloy and NaOH as previously described (9).

2.3.2 Phytosterol ester preparation

Stigmasterol was used as supplied by the manufacturer in subsequent preparations.

Stigmastanol (aka: sitostanol) was prepared from stigmasterol as previously described (10, 11). Briefly, 1.06 g (1 mmol) of Pd/C was added to 400 mL of a 45 mM stirred solution of stigmasterol in 2-propanol. The reaction mixture was stirred under an atmosphere (balloon) of hydrogen gas at 60°C overnight, after which the Pd/C was removed by filtration through a pad of Celite. The resulting solution was concentrated at reduced pressure to provide a quantitative yield of pure stigmastanol (mp 139-140°C; literature 140°C (12)). The lack of a residual alkene was verified by ^{1}H NMR.

Sitosterol of relatively high purity (92%) was prepared through a three-step procedure (10). First, 4.4 g (10 mmol) of stigmasterol were hydrogenated using 3.4 g RaNi in 350 mL of ethyl acetate under an atmosphere (balloon) of hydrogen gas; the reduction step was monitored by GC/MS, using an AT-5 column (Alltech Associates, Inc., Deerfield, IL; 0.32 mm x 30 m). Once the hydrogenation had consumed most of the stigmasterol (typically 10 h), the RaNi was removed by filtration through a pad of Celite and the solvent was removed at reduced pressure. The crude residue was analyzed by ^{1}H NMR and determined to consist of a 7:80:13 mixture of stigmasterol, sitosterol, and stigmastanol, based upon the relative integration of the signals at 0.720, 0.702, and 0.671 ppm, respectively. Second, the mixture of sterols was dissolved in 100 mL of ether and treated drop-wise with 20mL of 0.6 M bromine in ether at room temperature. The reaction flask was then stoppered and stored at -20°C for 3 d. Crystals, which were residual stigmastanol (confirmed by TLC), were removed by filtration and the filtrate was concentrated in vacuum. The residue was purified by flash column chromatography with 10% ethyl acetate in hexane to furnish sitosterol 5,6-dibromide. Third, the dibromide was refluxed with 100 mL of 340 mM excess zinc in 1:1 ethanol:acetic acid for 3h. Solvent was removed and 50 mL of water was added; the suspension was extracted with three washes of 50 mL of dichloromethane. The organic layer was dried over sodium sulfate and solvent was removed under reduced pressure. The residue was recrystallized from hot acetone to furnish sitosterol as a white solid [mp 137-138°C; literature: 139°C (12)]. The sitosterol obtained (1.2 g, 29% yield) was determined to be 92% pure based upon ^{1}H NMR with impurities of stigmasterol (5%) and stigmastanol (3%).

Each phytosterol ester was generated as follows (illustrated for sitosteryl palmitate): 5.4 mmol of palmitoyl chloride were added drop-wise to a stirred mixture of 3.6 mmol of sitosterol and 0.95 mmol dimethylaminopyridine in 20 mL of dry pyridine at 50°C. The temperature was then increased to 70°C and stirred overnight. The reaction was cooled and diluted with 100 mL of water. The resulting suspension was acidified to a pH of 3-4 with 3 M HCl and subsequently extracted with three washes of 100 mL of dichloromethane. The combined organic layers were dried over sodium sulfate and solvent was removed under reduced pressure. The residue was recrystallized from hot ethyl acetate to furnish sitosteryl palmitate (2.2 g, 88% yield) as a white solid [mp 79-80°C; literature: 85.5°C (12)]. Analysis by ^{1}H NMR suggested the sitosteryl palmitate was 92% pure, and contained approximately 8% of a mixture of stigmasteryl and stigmastanyl palmitates.

2.3.3 PCE activity assay

PCE (100 U) was dissolved in 1 mL of 100 mM potassium phosphate buffer at pH 7.0, separated into 200 μL aliquots, and stored at -80°C until ready to use. Prior to use, an aliquot was thawed on ice and diluted to 2 U/mL of the same buffer. The stability of thawed PCE was determined by storing thawed PCE at 4°C for 0, 5, or 7 d. The hydrolytic activity of the enzyme was tested on aliquots of the same solubilized cholesteryl oleate solution and incubated as described later. No changes in activity were seen after 7 d (data not shown); regardless, freshly thawed enzyme was used when possible.

To test the substrate specificity of PCE, a routine enzyme assay was first developed and validated. Sterol esters (8 μmol) were dissolved in chloroform and added to 16 x 100 mm glass, screw top tubes and solvent was evaporated under N_2 at 50°C. Sodium cholate hydrate (100 mg), Triton X-100 (1 mL), and deionized water (8 mL) were added and tubes were capped with PTFE-lined lids. The solution was heated and stirred with a stir bar to 100°C until the solution turned white. Solutions were removed from heat and slowly cooled to 60°C with stirring, after which 1 mL of a 1M, pH 7.0 potassium phosphate buffer was added. The final composition of the mixture was 10 mL of 800 μM sterol ester, 1% (w/v) sodium cholate, 100 mM phosphate buffer, and 10% (v/v) Triton X-100. The optimal pH of the assay was determined to be 7.0 after testing a pH range of 6.0 to 8.0 (data not shown).

The effects of hydrolysis products on the hydrolysis of cholesteryl oleate by PCE were determined by adding 16 mM stearic, palmitic, or oleic acids, or 2.4 mM cholesterol, sitosterol, stigmasterol, or stigmastanol to solubilized cholesteryl oleate.

Aliquots of solubilized sterol ester (0.5 mL) were added to glass screw top tubes and preheated to 37°C. Dilute PCE (20 μL; 0.04 U) was added to each tube. Reactions were incubated at 37°C on a rocking platform. Hydrolysis proved to be linear through 16 min of incubation, and therefore subsequent incubation times were 8 min. Hydrolysis was stopped and lipids were extracted by Folch lipid extraction (13) by addition of 2 mL of ice cold 2:1 chloroform:methanol (v/v) containing 50 μM 5α-cholestane as an internal standard for GC analysis. Stopped reaction mixtures were vortexed for 10 s and

centrifuged at room temperature at 1000 x g for 10 min; the aqueous supernatant was aspirated and discarded. The chloroform in the infranatant was dried under nitrogen gas at 50°C; 1 mL of hexanes was then added to each sample, samples were vortexed, and the samples were again centrifuged at 1000 x g for 10 min. The hexanes-soluble supernatant was transferred to a GC vial, while any residual aqueous layer remained in the infranatant. Hexanes were evaporated at 50°C under nitrogen gas; TMS derivatives were prepared by addition of 100 μL of Sylon BTZ to each dried sample and subsequently transferred to a 300 μL GC vial insert. GC vials were capped using PTFE septa, and samples were allowed to derivatize for at least 30 min. Samples were analyzed by GC using an AT-5 column (Alltech Associates, Inc.).

2.3.4 Statistics

Variability among sets of reactions was accounted for by normalizing within-set rates of hydrolysis: relative activities were calculated by dividing the rate of hydrolysis of individual sterol ester reactions by the within-set rate of hydrolysis of cholesteryl oleate. Rates of hydrolysis were analyzed by a two-way ANOVA with sterol and fatty acid moieties as factors. Least squares means of relative rates of hydrolysis were calculated. The rates of hydrolysis of combinations of individual sterols and fatty acids were compared pairwise, with multiple comparisons being corrected for by using the Bonferroni adjustment. Statistics were computed using the mixed procedure of SAS software (version 9.1; SAS Institute Inc., Cary, NC).

2.4 Results

2.4.1 Method validity

Cholesteryl oleate was most effectively hydrolyzed at pH 7.0, with pH 6.0, 6.5, 7.5, and 8.0 retaining 89%, 80%, 84%, and 72% activity, respectively, relative to pH 7.0. Thus, all subsequent experiments were performed at pH 7.0. In addition, no appreciable ester synthesis was observed when PCE was added to a solution of cholesterol and oleic acid (data not shown). Storage of PCE for up to 7 d at 4°C did not alter its activity when measured at 8 and 16 min incubations (data not shown). These preliminary experiments ensure that the hydrolytic capabilities of PCE did not change over the course of a set of incubations because freshly thawed PCE was used for incubations whenever possible and was never used when stored at 4°C for more than 2 d.

Optimal incubation times were determined by incubating aliquots of a solution of cholesteryl oleate for 0, 0.25, 0.5, 1, 2, 4, 8, 15, 30, and 60 min (Figure 2.1). Shorter incubation times, specifically at 0.25 and 0.5 min (Figure 2.1 inset), had higher error among replicates and did not fit the overall curve well; longer incubation times, specifically at 30 and 60 min, were beyond the linear region necessary for determining enzyme kinetics. Therefore, subsequent serial reactions for each solubilized sterol ester were conducted with incubation times of 0, 1, 2, 4, 8, and 16 min (Figure 2.2); hydrolysis was linear for each ester through 8 min. Replicate hydrolysis measurements used only the 8 min time point to determine the initial velocity of the reaction.

2.4.2 Substrate Specificity

The rate of hydrolysis was affected by both the sterol and the fatty acid portions of the sterol esters. The average rate of hydrolysis of cholesterol esters was significantly greater than the average rate of hydrolysis of the esters of the three phytosterols (Figure 2.3; sterol effect: $p<0.0001$). Stigmastanol and sitosterol esters were not hydrolyzed statistically differently (56.4 ± 1.2% and 58.9 ± 1.2%, respectively, normalized to cholesterol esters), though at a greater rate than stigmasterol esters (29.3 ± 1.3%). Additionally, the rate of hydrolysis of sterol esters was significantly affected by the fatty acid moiety (Figure 2.3; ester effect: $p<0.0001$). Oleate esters, on average, were hydrolyzed most rapidly, while palmitate and stearate esters were not hydrolyzed statistically differently (45.8 ± 1.0% and 41.6 ± 1.1%, respectively, normalized to oleate esters).

Although there was a significant interaction between the sterol and ester effects (Figure 2.3; interaction effect: $p<0.0001$), in all cases the oleate esters were hydrolyzed more rapidly than the palmitate and stearate esters of the same sterol. Further, with the exception of stigmastanyl stearate, hydrolysis of sterol esters was in the order of cholesterol > (stigmastanol ≈ sitosterol) > stigmasterol esters within a particular acyl group; stigmastanyl stearate was hydrolyzed similarly compared to stigmasteryl stearate.

Across all sterol esters tested, cholesteryl oleate was hydrolyzed most rapidly, while stigmasteryl palmitate and stearate were hydrolyzed the least rapidly (12.9 ± 1.3% and 12.6 ± 1.6%, respectively, normalized to cholesteryl oleate). Among phytosterol esters,

stigmastanyl oleate and sitosteryl oleate were hydrolyzed at a similar rate that was greater than the other phytosterol esters tested (64.2 ± 1.3% and 59.8 ± 1.3%, respectively, normalized to cholesteryl oleate).

In an attempt to determine if the differences in rates of hydrolysis were the result of inhibition or activation of PCE by hydrolysis products, free sterols or free fatty acids were added to a solution of cholesteryl oleate. The addition of equimolar free sterols to cholesteryl oleate did not affect hydrolysis (Figure 2.4), nor did the addition of equimolar free fatty acids affect hydrolysis (data not shown). However, the addition of free fatty acids at a ratio more reflective of the human digestive tract appeared to stimulate hydrolysis (Figure 2.5).

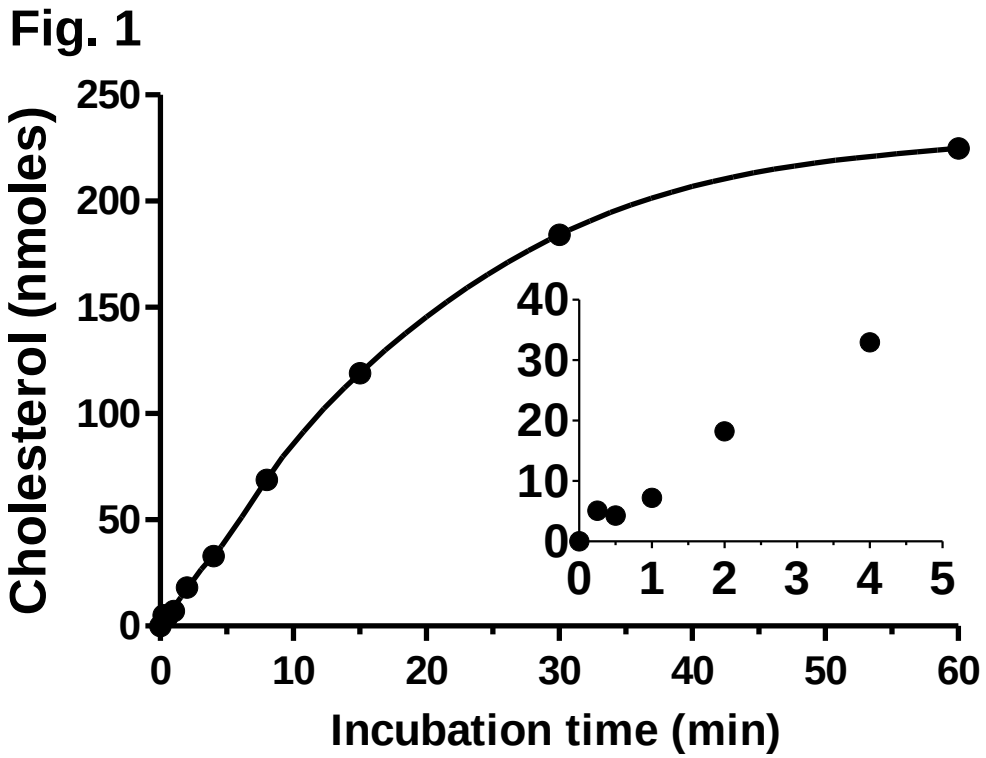

Figure 2.1. Confirming kinetic properties of PCE in the experimental assay conditions. Cholesteryl oleate was solubilized at a concentration of 800 μM in a 100mM pH 7.0 phosphate buffer containing 1% (w/v) sodium-cholate and 10% (v/v) Triton X-100 and hydrolyzed with PCE at 37ºC for the times indicated on the x-axis. Progression of hydrolysis was measured by the appearance of free cholesterol. The experimental conditions were sufficient to demonstrate the typical asymptotic curve expected of cholesterol ester hydrolysis by PCE. Incubation times greater than 15 min were well past the linear range needed for the approximation of the initial velocity of the reaction. Datapoints are connected with a spline. **Inset.** Close-up of incubation times less than 5 min. The first few incubation times were unpredictable and were omitted in subsequent experiments.

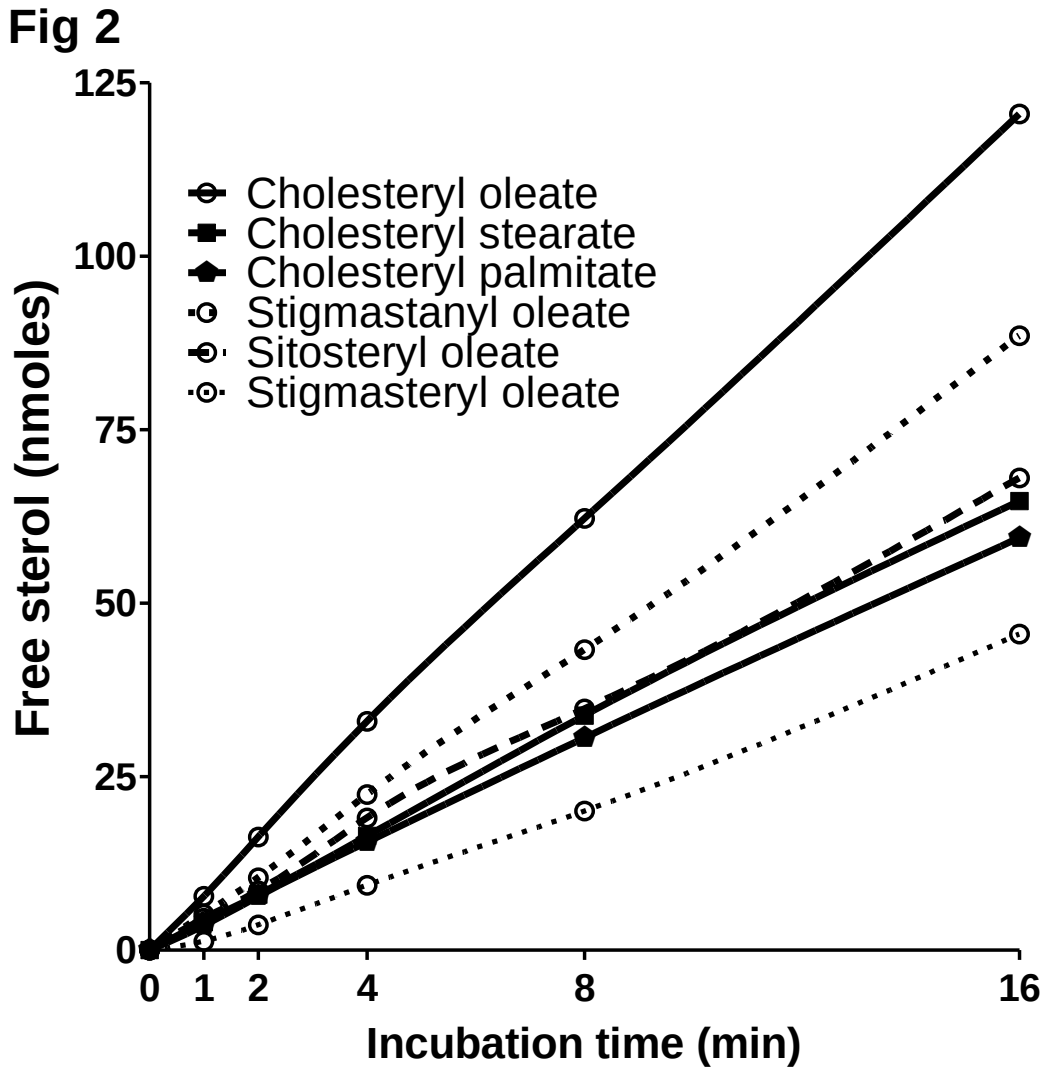

Figure 2.2. Determining the linear region of hydrolysis by PCE in the experimental assay conditions. Solubilized sterol esters at a concentration of 800 μM were hydrolyzed by PCE at 37ºC for the times denoted on the x-axis. Hydrolysis was measured by the appearance of the respective free sterols. All steryl esters tested were hydrolyzed linearly over time through at least the 8 min incubation time, and thus all subsequent experiments were conducted using 8 min incubations. For clarity, only cholesterol esters and the oleate esters of the phytosterols are shown, though palmitate and stearate esters of the three phytosterols tested were similarly linear.

Fig. 3

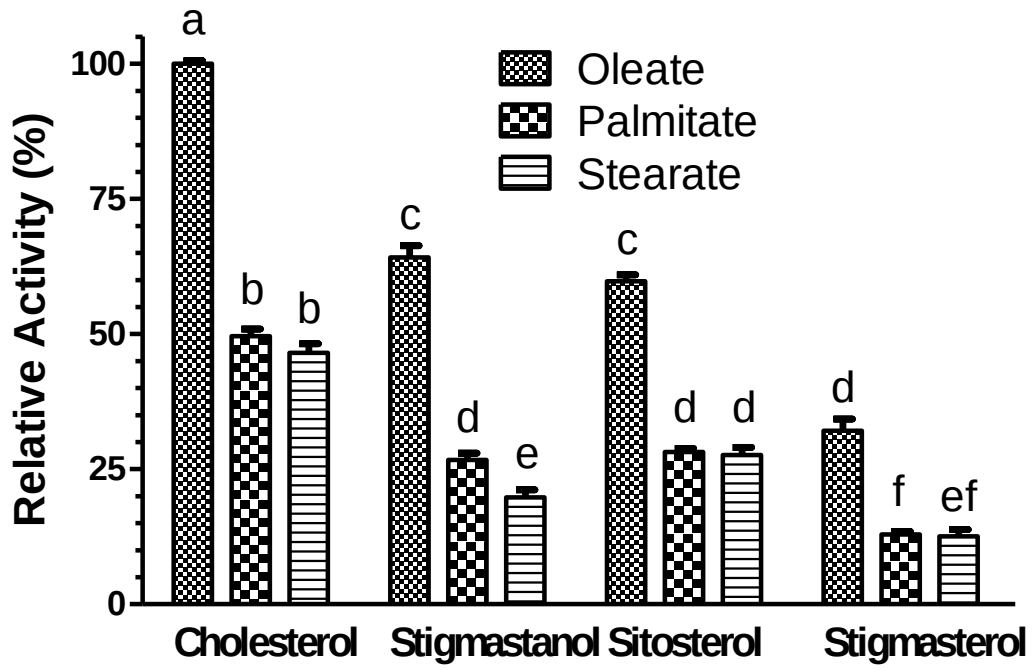

Figure 2.3. Determining relative rates of hydrolysis of 12 sterol esters. Solubilized sterol esters at a concentration of 800 μM were incubated in the presence of PCE at 37ºC for 8 min. Hydrolysis was measured by the appearance of the respective free sterols. Cholesteryl oleate was used as an external standard for each set of reactions and the average hydrolysis of cholesteryl oleate was set to 100% for each set of replicates. On average, cholesterol esters were hydrolyzed more rapidly than the phytosterol esters, with stigamstanol and sitosterol esters being hydrolyzed at an equal rate and stigmasterol esters being hydrolyzed the slowest. Oleate esters were hydrolyzed more rapidly than palmitate and stearate esters, which were generally hydrolyzed at an equal rate. The exception to the trend is stigmastanyl stearate, which did not differ when compared with stigmasteryl stearate, but was hydrolyzed more slowly than stigmastanyl palmitate. Bars represent means ± SEM; n=4-6 for all esters except cholesteryl oleate with n=18. Bars without common letters differ by a Bonferroni adjusted $p<0.05$.

Fig. 4

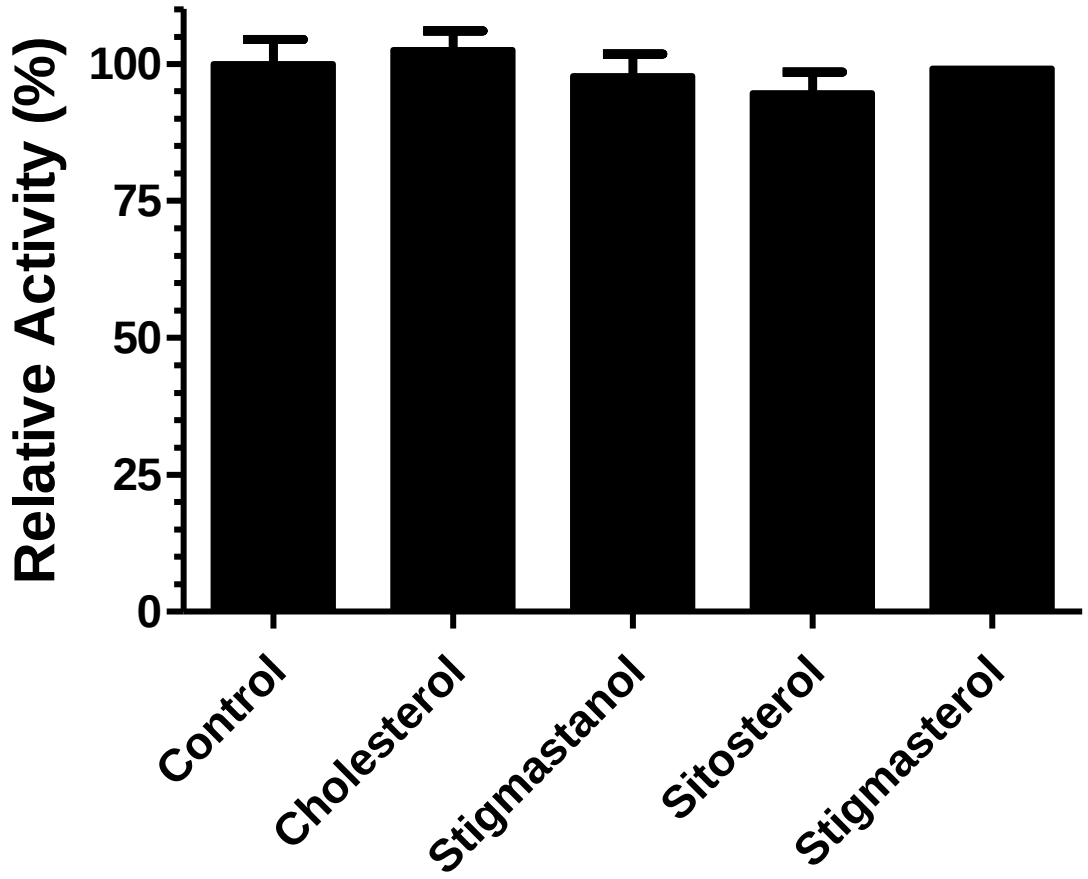

Figure 2.4. Testing for inhibition of PCE by free sterols. Each sterol was added at a concentration of 2.4 mM to an 800 μM solution of cholesteryl oleate and incubated in the presence of PCE for 8 min at 37ºC. No additional sterol was added to the control, and reactions were normalized to the mean of the control within replicates. Hydrolysis of cholesteryl oleate was measured by the appearance of free cholesterol. No significant differences were observed among the treatments. Bars represent means ± SEM.

Fig. 5

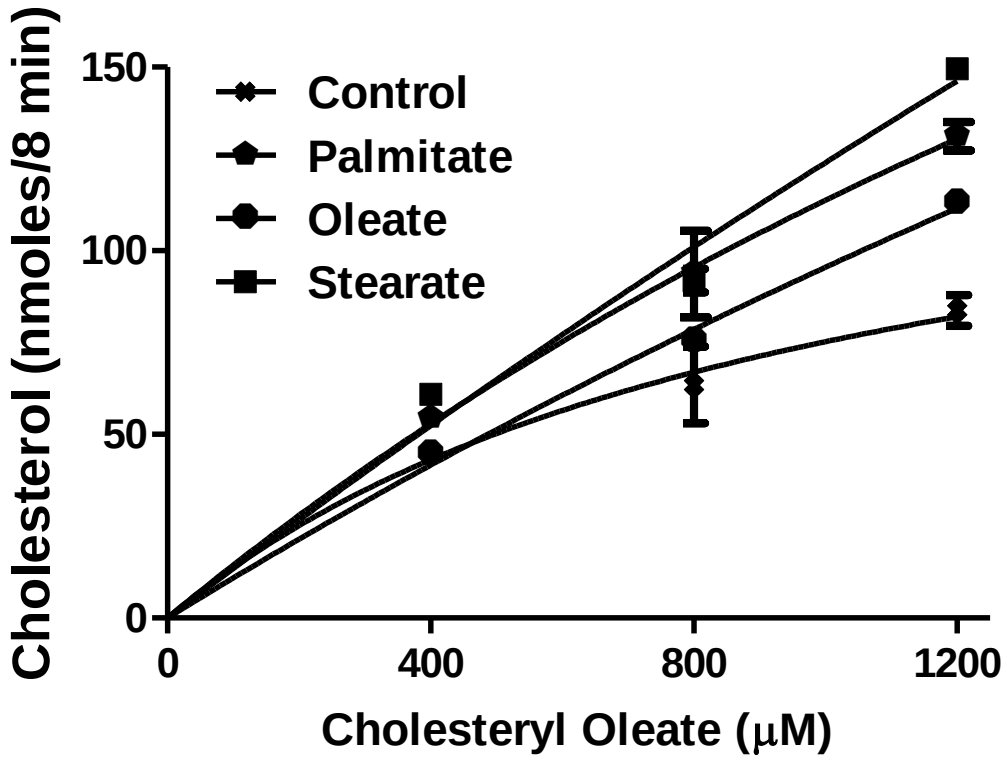

Figure 2.5. Testing for inhibition of PCE by free fatty acids. Solubilized cholesteryl oleate at the concentrations denoted on the x-axis were hydrolyzed in the presence or absence of 16 mM concentrations of each free fatty acid. Data points represent mean ± SEM of two replicates.

2.5 Discussion

Disrupting cholesterol micellarization and absorption in the intestine have been targets for decreasing cholesterol concentrations in the circulation. Ezetimibe, an inhibitor of NPC1L1 (14), and isocoumarin-derived compounds used to inhibit PCE (15) are just two examples of pharmaceutical manipulation of cholesterol absorption with the intent of decreasing atherosclerotic risk. At the forefront of nutraceutical therapies, phytosterols and their esters have been effective in altering intestinal sterol metabolism, though the complete characterization of their mechanisms of action has been elusive. In the present study, we created an *in vitro* model to investigate the hydrolysis of selected sterol esters by PCE and determined that PCE is capable of hydrolyzing a variety of sterol esters, though at varying rates.

The first consideration in creating our model system was to solubilize sterol esters in an aqueous system devoid of other potential substrates for PCE. PCE is a fairly indiscriminant carboxyl ester hydrolase, capable of hydrolyzing many of the lipids that are used in typical micelle preparations and that exist in micelles *in vivo,* including phospholipids (7) and acyl glycerols (8). Furthermore, PCE has been reported to catalyze the reverse reaction of ester synthesis (16), prompting us to create an aqueous model devoid of free fatty acids to avoid encouraging the reverse reaction of ester synthesis by altering the equilibrium. To avoid using free fatty acids, acyl glycerols, or phospholipids as amphipathic detergents, we chose to use Triton X-100 (17).

Other considerations for the model system included satisfying the bile salt dependency of PCE, and, in particular, the preference for a trihydroxy, rather than dihydroxy, bile salt (18), which was satisfied by the addition of sodium cholate; the potential for pH to affect the hydrolytic capability of pancreatic PCE (19); the potential for concurrent production and hydrolysis of sterol esters, which may have been prevented by the addition of greater than 20mM sodium cholate (20); and the ability to solubilize all esters at equal concentrations. We not only addressed these concerns, but validated that PCE can still perform enzymatic hydrolysis in our model system in a reproducible manner.

Once the model hydrolysis system was validated, we demonstrated that PCE, in addition to hydrolyzing various sterol esters, exhibited substrate specificity that was affected both by the sterol and fatty acid portions of the ester. Saturated esters were less well hydrolyzed than the unsaturated ester, with the palmitate and stearate esters being hydrolyzed approximately half as well as the oleate ester of any particular sterol ester. A similar disparity was demonstrated with a cholesterol esterase derived from rat testis, where cholesteryl stearate was hydrolyzed only 25% as well as cholesteryl oleate (21). Substrate specificity of PCE also appeared to be affected by certain structural elements of the individual sterols. The ethyl substitution on carbon 24 of the phytosterols is the consistent structural difference between the phytosterols and cholesterol, and the phytosterol esters were consistently hydrolyzed at a lower rate than cholesterol esters. The inclusion of the delta 22 double bond, which is the only structural difference between sitosterol and stigmasterol, resulted in a significant decrease in the relative activity of PCE. However, the presence or absence of the delta five double bond, the only structural

difference between sitosterol and stigmastanol, appeared to have little effect on the hydrolytic ability of PCE as evidenced by the similar hydrolysis when sitosterol and stigmastanol esters are compared. Thus, it could be that small changes to the side chain bonded to carbon 17 of these sterols may be more integral in conferring substrate specificity of PCE than the cyclic structure, though this would need to be confirmed by the hydrolysis of other sterol esters.

An unexpected result of this study was the increased hydrolysis of cholesteryl oleate observed upon addition of large concentrations of fatty acids. The amount of fatty acid used in these experiments roughly approximated the *in vivo* phytosterol ester to fatty acid ratio expected from the recommended daily intake of phytosterol esters (2 g/d; ~3 mmoles) and 50% hydrolysis of the average daily intake of triacylglycerols (100 g/d; ~226 mmoles of free fatty acids from the *sn*1 and *sn*3 positions), thus approximating the initial duodenal contents of these components. All three fatty acids increased the hydrolysis of cholesteryl oleate as compared with the solution initially devoid of fatty acids. Although not demonstrated statistically, it appeared that the more hydrophobic fatty acids promoted greater increases in PCE activity. An explanation of the activation of PCE by these fatty acids could be the formation of a more native lipid emulsion with which PCE could interact. This hypothesis is supported by the observation that only 40% of plant stanol esters were hydrolyzed *in vivo* on a low fat diet versus 70% on a normal fat diet (22).

Precedence exists for mammalian systems to distinguish between cholesterol and plant sterols, including the higher rate of plant sterol efflux via ABCG5/G8 co-transporters (23) and the higher rate of esterification of cholesterol as compared with sitosterol in the cytosol of proximal rat intestinal cells (24). Several human studies have also indicated a potential for discrimination among various sterols. In colectomized patients, ingested cholesterol esters were almost completely hydrolyzed (95%) by the time they reached the feces, while 90% of sitosterol esters and only 57% of stigmastanol esters were hydrolyzed (22). In another study, the effects of proximal digestion and absorption were distinguished by duodenal infusion of solubilized sterols and sterol esters and measuring the composition in the proximal jejunum. The percent of esterified sitosterol dropped from 64% to 27% and esterified stigmastanol from 92% to 39% from infusion to the proximal jejunum (25). While these studies support the present findings, confounding factors such as exposure to multiple digestive enzymes, variable emulsion structures, and diverse concentrations of substrates have made it impossible to conclusively determine the hydrolytic activity of PCE alone.

The interaction of phytosterol esters with PCE alone could decrease the hydrolysis of dietary cholesterol esters and thereby decrease their absorption. However, only a small portion of dietary cholesterol is in the esterified form (26); furthermore, the effects of phytosterol esters are demonstrated whether consumed only once daily or multiple times daily (27), which indicates that competition for PCE is likely not the only mechanism of action for phytosterols.

Here, we have used an *in vitro* model to demonstrate the potential for vastly different efficacies of phytosterol ester supplementation on cholesterol absorption that depends on phytosterol ester structure. However, it is unknown if the *in vitro* hydrolysis of phytosterol esters reflects hydrolysis *in vivo*, nor is it known by what mechanisms phytosterol ester supplementation most effectively decreases plasma cholesterol. Several proposed mechanisms include phytosterols interacting with cholesterol transporters, competing with cholesterol for micellar solubility, regulating cholesterol-related genes, and interacting with digestive enzymes (26). Except for the latter mechanism, the evidence supporting these mechanisms has not adequately explored whether phytosterol esters or free phytosterols are most effective at imparting their activity. In the present study phytosterol esters are shown to interact with at least one digestive enzyme, PCE, whereas the free phytosterols have no effect on cholesterol ester hydrolysis. Thus, it is the phytosterol ester that imparts its activity on PCE, at least *in vitro*.

2.6 Acknowledgments

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Chapter 3: Phytosterol ester constituents affect micellar cholesterol solubility in model bile[3]

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3.1 Abstract

Plant sterols and stanols (phytosterols) and their esters are nutraceuticals that lower LDL cholesterol, but the mechanisms of action are not fully understood. We hypothesized that intact esters and simulated hydrolysis products of esters (phytosterols and fatty acids in equal ratios) would differentially affect the solubility of cholesterol in model-bile mixed micelles in vitro. Sodium salts of glycine- and taurine-conjugated bile acids were sonicated with phosphatidylcholine and either sterol esters or combinations of sterols and fatty acids to determine the amount of cholesterol solubilized into micelles. Intact sterol esters did not solubilize into micelles, nor did they alter cholesterol solubility. However, free sterols and fatty acids altered cholesterol solubility independently (no interaction effect). Equal contents of cholesterol and either campesterol, stigmasterol, sitosterol, or stigmastanol (sitostanol) decreased cholesterol solubility in micelles by approximately 50% compared to no phytosterol present, with stigmasterol performing slightly better than sitosterol. Phytosterols competed with cholesterol in a dose-dependent manner,

[3]*This chapter has been adapted from the original published manuscript for consistency with the rest of this dissertation; no substantial changes have been made to content.*

demonstrating a 1:1 molar substitution of phytosterol for cholesterol in micelle preparations. Unsaturated fatty acids increased the micelle solubility of sterols as compared with saturated or no fatty acids. No differences were detected in the size of the model micelles. Together, these data indicate that stigmasterol combined with saturated fatty acids may be more effective at lowering cholesterol micelle solubility in vivo.

3.2 Introduction

Supplementation of human diets with plant sterols and stanols (collectively referred to here as phytosterols) and their esters can decrease serum LDL cholesterol concentrations [1], which is regarded as a modifiable risk factor for atherosclerosis. One way phytosterols lower serum LDL cholesterol is by disrupting intestinal cholesterol solubilization into micelles [2-5], which is a necessary step in the efficient absorption of cholesterol from the intestine [6]. However, the exact mechanisms for the actions of phytosterols have not been adequately defined.

Several studies have investigated the effects of individual sterols in simple systems of single bile acids with [2] or without [3-5] oleate or monoolein. However, the most quantitatively important source of cholesterol in the intestinal lumen is secreted from the gall bladder in a complex mixture of bile salts and phospholipids. In spite of the relevance to intestinal uptake of sterols, few studies have compared phytosterol solubilities and their effects on cholesterol solubility in a mixed micelle system similar to bile [7]. In addition, some phytosterol esters, namely sterol stearates [8], are superior to other phytosterols in their ability to lower serum cholesterol, yet the mechanisms by

which these esters impart their unique effects is unknown. Thus, the objectives of the present study were to examine the effects of 1) sterol esters, 2) simulated hydrolysis products of sterol esters (fatty acids and sterols in equal ratios), and 3) phytosterol concentration on micellar cholesterol solubility in a model-bile, mixed-micelle system.

3.3 Materials and Methods

3.3.1 Reagents

Cholesteryl oleate (98%), oleic acid (99%), stearic acid (99%), sodium taurodeoxycholate (>97%), and sodium glycodeoxycholate (>97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium glycocholate (98%) and granular phosphatidylcholine were purchased from ACROS Organics (Geel, Belgium). Sodium taurocholate (>97%) was purchased from Alfa Aesar (Ward Hill, MA, USA). 5α-Cholestane was obtained from Steraloids (Newport, RI, USA). Cholesterol (95%) was obtained from Mallinckrodt OR (Paris, KY, USA). Palmitic acid (99+%) was obtained from MP Biomedicals (Irvine, CA, USA). Sodium chloride, sodium azide, chloroform, and hexanes were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanol was purchased from VWR (West Chester, PA, USA). Linoleic and α-linolenic acids (99+%) were purchased from Nu-Chek Prep (Elysian, MN, USA). The total cholesterol kit, Chol, was purchased from Roche Diagnostics (Indianapolis, IN, USA). Phospholipids C and Free Cholesterol E colorimetric assay kits, and campesterol (98.7%) were obtained from Wako Chemicals USA, Inc. (Richmond, VA, USA). 24-Ethyl sterols were purchased or synthesized as previously described [9]. Briefly, stigmasterol (95%) was purchased from TCI America

(Portland, OR, USA) and partially or completely hydrogenated to make sitosterol or stigmastanol (a.k.a. sitostanol), respectively [10,11].

3.3.2 Model-bile mixed micelles

Simplified bile salt solutions modeling biliary bile salt compositions were limited to the glyco- and tauro- conjugates of cholate and deoxycholate in ratios similar to published values [7,12,13] for a final concentration of 52.5 mM total sodium salts of bile acids: glycocholate, 29.1 mM; taurocholate, 11.4 mM; glycodeoxycholate, 8.8 mM; and taurodeoxycholate, 3.4 mM. Concentrated stock solutions of phosphatidylcholine, free fatty acids, free sterols, and sterol esters were made in chloroform. Model-bile mixed micelles were created by combining stock solutions and evaporating chloroform under a stream of N2 at 50 °C, followed by addition of the 52.5 mM bile salt stock solution to create a 1 mL solution. All solutions had a final concentration of 52.5 mM bile salts and 15 mM phosphatidylcholine, with the concentrations of free sterols, free fatty acids, and sterol esters varying depending on the experiment. Lipids and bile acids were sonicated in an ice bath using a Branson 450 Sonifier (Branson, Danbury, CT) with probe tip at 30% of maximum output (400 W) for 15 min [7]. Immediately after sonication, micelles were separated by injecting 200 µL of the solution onto a 10/300 Superose 6 column using a Waters 600 Multisolvent Delivery System and eluted at 0.5 mL/min using a 6 mM bile salt eluent. Eluent was made by addition of 3:1 taurocholate/taurodeoxycholate (w/w) dissolved in 0.9% sodium chloride (w/v) with 0.01% sodium azide (w/v), followed by vacuum filtration using Millipore (Bedford, MA, USA) 47 mm, 0.2 µm pore-size, nylon filters. Fractions were collected every minute (0.5 mL) in 12x75 mm polystyrene tubes

(VWR) using an ISCO Retriever 500 (Lincoln, NE, USA). Individual fractions were analyzed for phosphatidylcholine using Wako's Phospholipids C Kit. Phosphatidylcholine-containing fractions indicated the presence of micelles [7,14], and were pooled for subsequent analysis of free sterols by GC.

3.3.3 Micelle sterol analysis

Free sterols were analyzed by extracting an aliquot of the pooled micelle-containing fractions with 5α-cholestane as an internal standard. The sample was dried under a stream of N2 at 60 °C and was extracted using a modified Folch procedure [15]. Briefly, 2 mL of chloroform/methanol (2:1, v/v) were added to each sample, samples were vigorously vortexed for 1 min followed by addition of deionized water for a final water content of 20% of the total volume. Samples were centrifuged at 1000 x g for 5 min to separate phases; the organic phase was transferred to a new tube, dried under a stream of N2, and resuspended in hexanes. Sterols were analyzed by GC using an HP (Wilmington, DE, USA) 5890 Series II Plus Gas Chromatograph equipped with an HP 6890 Autosampler, a 30 m DB-5 column (Agilent Technologies, Santa Clara, CA, USA), and a flame-ionizing detector. Peaks were identified by comparing retention times with that of known standards. For the experiments examining the effects of intact sterol esters on micellar cholesterol, total and free sterols were quantified enzymatically.

3.3.4 Statistics

Micellarization of individual sterols was analyzed as a randomized, incomplete block ANOVA, with the cholesterol stock solution used in a particular set serving as the

blocking factor. The effects of sterol and fatty acid combinations on the micellarization of cholesterol were analyzed as a two way ANOVA in a split-block design: sterols and fatty acids were the two factors; the cholesterol stock solution used for a particular preparation was a blocking effect; and the interaction of the sterol stock solution or the fatty acid stock solution with the blocking effect were random effects. Combinations were compared pairwise, and multiple comparison corrections were made using a simulation approach to account for unequal sample sizes. Significance was set at $p < 0.05$. The dose-dependent competition between stigmasterol and cholesterol for micellarization was modeled by fitting exponential and logarithmic functions to the asymptotic nature of the sterol contents of the micelles using ordinary least squares. Statistics were computed using the Mixed procedure of SAS software, with the exception of the functions in the sterol competition study, for which the Model procedure was used (version 9.2; SAS Institute, Cary, NC, USA).

3.4 Results

3.4.1 Micellar incorporation of free sterols

Initial tests demonstrated similar elution patterns and volumes to previous work [7]. The elution profile of a model-bile mixed micelle containing 3mM cholesterol, 52.5 mM bile salt, and 15 mM phosphatidylcholine is shown in Figure 3.1a.

3.4.2 Sterol esters and model-bile mixed micelles

Elution profiles of free cholesterol, total sterols (free plus esterified sterols), and phospholipids did not differ when cholesteryl oleate was included in a mixed micelle

containing free cholesterol, bile salt, and phosphatidylcholine as compared with a model micelle devoid of sterol ester (Figures 3.1a and 3.1b). When only cholesteryl oleate was added, with no free cholesterol, neither free nor esterified sterol was detected in the micelle fraction, and the elution pattern of phospholipids was altered (Figure 3.1c). The lack of incorporation of sterol esters in micelles was further shown by preparing model micelles with or without stearate esters of stigmasterol, sitosterol, stigmastanol, or cholesterol. Total sterol content (free plus esterified sterols) did not differ from free sterol content (2.22 ± 0.02 vs 2.19 ± 0.04 mM, respectively, pooled across preparations, n=4), indicating no measurable incorporation of sterol esters in the micelles. Additionally, the presence or absence of sterol esters in the preparation did not affect the free cholesterol content of the micelles.

3.4.3 Micelle solubility of individual sterols

The solubilities of free sterols within micelles prepared from solutions containing 3 mM cholesterol, campesterol, stigmasterol, sitosterol, or stigmastanol were quantified (Figure 3.2). Stigmasterol was incorporated into micelles quantitatively and significantly less as compared to cholesterol, sitosterol, and stigmastanol, while cholesterol, campesterol, sitosterol, and stigmastanol were equally incorporated.

3.4.4 Dose-dependent competition between sterols for micellar incorporation

Competition between stigmasterol and cholesterol for micelle incorporation was determined by adding 0, 1.5, 3, or 6 mM stigmasterol to model bile preparations containing 3 mM cholesterol (Figure 3.3). As stigmasterol content increased, cholesterol

content in the micelle decreased. Micellar cholesterol and stigmasterol contents were modeled as logarithmic and exponential functions, respectively. Cholesterol content was modeled as:

(1) $C = 1.83*EXP(-0.158*X)$

and stigmasterol content was modeled as:

(2) $S = 0.53*LN(1.073*(X+1))$

where

C = Final micellar cholesterol content in mM

S = Final micellar stigmasterol content in mM

X = Stigmasterol added to the preparation in mM

The amount of stigmasterol that would need to be added to decrease the cholesterol content by 50% according to equation 1 was 4.40 mM. This value corresponded closely to the solution of the two functions of X = 4.33 mM, where the amount of micellar stigmasterol and cholesterol are equal. Together, these values indicate approximately a 1:1 molar substitution of micellar cholesterol for stigmasterol. The observation that more stigmasterol needs to be added than cholesterol for a 1:1 competition further demonstrates a lower micellar solubility of stigmasterol. Because stigmasterol had a lower solubility than other sterols (Figure 3.2), other phytosterols may compete for micelle incorporation differently.

3.4.5 Free sterol and fatty acid effects on micellar sterol content

Mixed micelles containing 3 mM free cholesterol were made with or without 3 mM palmitic, stearic, oleic, linoleic, or α-linolenic acids, and with or without 3 mM free campesterol, stigmasterol, sitosterol, stigmastanol, or additional cholesterol. Micellar cholesterol and phytosterol concentrations were measured by GC. No statistical interactions were detected between fatty acids and sterols, so pairwise comparisons were conducted among preparations pooled across fatty acids ('sterol effects,' Table 3.1, pooled n=17 to 28) or among preparations pooled across sterols ('fatty acid effects,' Table 3.2, pooled n=15 to 19). The fatty acid effects were calculated only across micelle preparations that were relevant to the simulated hydrolysis of phytosterol esters (ie: preparations that contained both phytosterols and cholesterol).

Adding an additional 3 mM cholesterol to the micellar mixture (for a total of 6 mM cholesterol) resulted in a significant increase, although not a doubling, in the concentration of cholesterol in the micelles, indicating that the micelles are saturated with cholesterol at a concentration somewhere between 3 and 6 mM.

The presence of phytosterols significantly decreased micellar cholesterol, with the phytosterol-containing preparations solubilizing approximately 50% of the micellar cholesterol of the saturated micelles. Modest differences were observed among the phytosterols tested: a 9% difference in the amount of micellar cholesterol was observed for the most (stigmasterol) and least (sitosterol) effective phytosterols, although neither stigmasterol nor sitosterol differed from stigmastanol and campesterol. However, stigmasterol itself was less well incorporated compared with sitosterol, although again

neither stigmasterol nor sitosterol differed from stigmastanol or campesterol. Together, these differences in individual sterol solubilities resulted in stigmasterol-containing preparations having significantly less total sterol than the other preparations except for the preparations that contained only 3 mM instead of 6 mM total sterol. Stigmasterol also differed from the other phytosterol-containing preparations by containing a greater proportion of micellar total sterol that was cholesterol, despite having a lower magnitude of cholesterol incorporated.

The presence or absence of saturated fatty acids (palmitic and stearic) had no effect on the incorporation of cholesterol or phytosterols in micelles. However, the presence of unsaturated fatty acids (oleic, linoleic, and α-linolenic), as compared with the saturated and no fatty acids preparations, increased micellar cholesterol (1.23 mM vs 1.08 mM, respectively) and phytosterol (1.03 vs 1.15 mM, respectively) contents. Total sterol content was similarly affected, but the proportion of micellar total sterol that was cholesterol was unaffected, indicating that incorporation of unsaturated fatty acids increased the sterol capacity of micelle preparations. However, no differences were detected in phospholipid content, indicating an additive rather than substitutive change.

3.4.6 Micelle size

Elution volume was used as a surrogate marker of micelle size [16]. No differences were seen in elution volumes, indicating no detectable differences in micelle sizes across all sterol and fatty acid combinations (data not shown).

Table 3.1 Molar phospholipid and sterol compositions of model-bile mixed micelles formed with 3 mM cholesterol and with 0 or 3 mM additional sterol.[1]

Additional Sterol	None	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Stigmastanol
			mmol/L			
Cholesterol	2.02 ± 0.02 [a]	2.33 ± 0.02 [b]	1.15 ± 0.03 [cd]	1.09 ± 0.02 [c]	1.19 ± 0.02 [d]	1.17 ± 0.02 [cd]
Phytosterol [2]	0.00	0.00	1.11 ± 0.02 [a]	0.93 ± 0.02 [b]	1.16 ± 0.02 [a]	1.16 ± 0.02 [a]
Phospholipid	3.63 ± 0.03 [a]	3.20 ± 0.03 [b]	3.22 ± 0.04 [bc]	3.30 ± 0.03 [bc]	3.26 ± 0.03 [bc]	3.37 ± 0.03 [c]
Total Sterol	2.02 ± 0.03 [a]	2.33 ± 0.03 [b]	2.27 ± 0.04 [b]	2.02 ± 0.03 [a]	2.36 ± 0.03 [b]	2.33 ± 0.03 [b]
			mol%			
% Sterol as Cholesterol[2]	100.00	100.00	51.22 ± 0.63[a]	53.98 ± 0.43[b]	50.74 ± 0.43[a]	50.12 ± 0.43[a]

[1]Values represent means ± pooled SEM of 17 to 28 replicates, averaged across experiments that also included 0 or 3 mM fatty acids. Values with different superscripts within a row differ by $p < 0.05$.

[2]Phytosterol and mole percent data for the 'None' and 'Cholesterol' preparations were not compared to other preparations because neither contained phytosterols.

Table 3.2 Molar phospholipid and sterol compositions of model-bile mixed micelles formed with 3 mM phytosterol and with 0 or 3 mM free fatty acid.[1]

Fatty Acid	None	Palmitic	Stearic	Oleic	Linoleic	Linolenic
			mmol/L			
Cholesterol	1.09 ± 0.02[a]	1.08 ± 0.02[a]	1.07 ± 0.02[a]	1.25 ± 0.02[b]	1.22 ± 0.03[b]	1.22 ± 0.03[b]
Phytosterol	1.03 ± 0.02[a]	1.03 ± 0.02[a]	1.03 ± 0.02[a]	1.17 ± 0.02[b]	1.15 ± 0.03[b]	1.14 ± 0.03[b]
Total Sterol	2.12 ± 0.04[a]	2.11 ± 0.04[a]	2.10 ± 0.04[a]	2.42 ± 0.04[b]	2.37 ± 0.05[b]	2.35 ± 0.05[b]
Phospholipid	3.27 ± 0.03	3.25 ± 0.03	3.27 ± 0.03	3.32 ± 0.03	3.31 ± 0.04	3.30 ± 0.04
			mol%			
% Sterol as Cholesterol	51.44 ± 0.37	51.08 ± 0.39	51.13 ± 0.37	51.81 ± 0.37	51.73 ± 0.46	51.89 ± 0.46

[1]Values represent means ± pooled SEM of 15 to 19 replicates, averaged across experiments that also included 3 mM cholesterol and 3 mM additional phytosterol. Values with different superscripts within a row differ by $p < 0.05$.

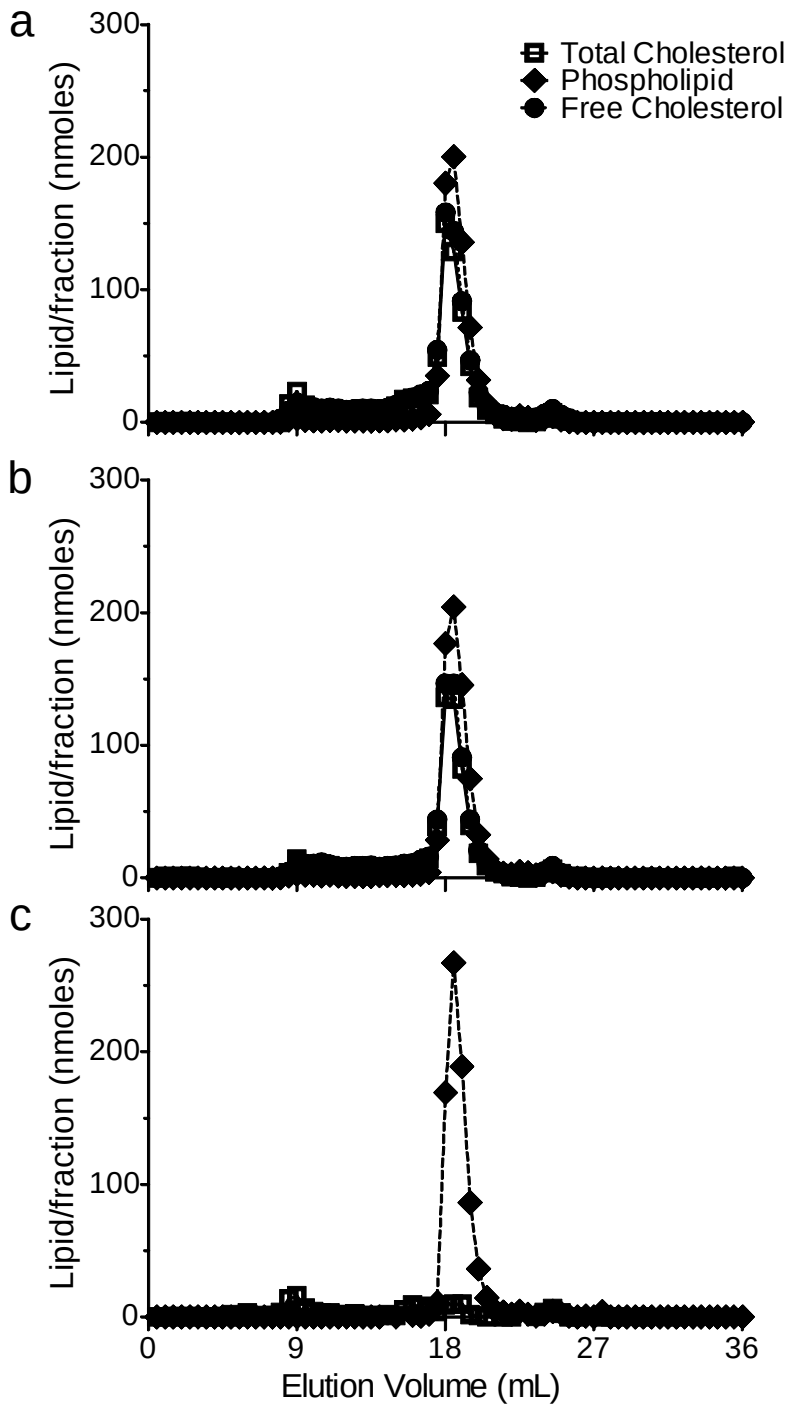

Figure 3.1. Elution profiles of micelles separated by size-exclusion chromatography. Model mixed micelles were made by sonicating 3 mM cholesterol (a), 3 mM cholesterol and cholesteryl oleate (b), or 3 mM cholesteryl oleate (c) in the presence of 52.5 mM mixed bile salts and 15 mM phosphatidylcholine, followed by separation on a Superose 6 column at a flow rate of 0.5 mL/min. Fractions (1 min, 0.5 mL) were collected and analyzed for cholesterol, phosphatidylcholine, and total sterol. Each panel represents 1 preparation.

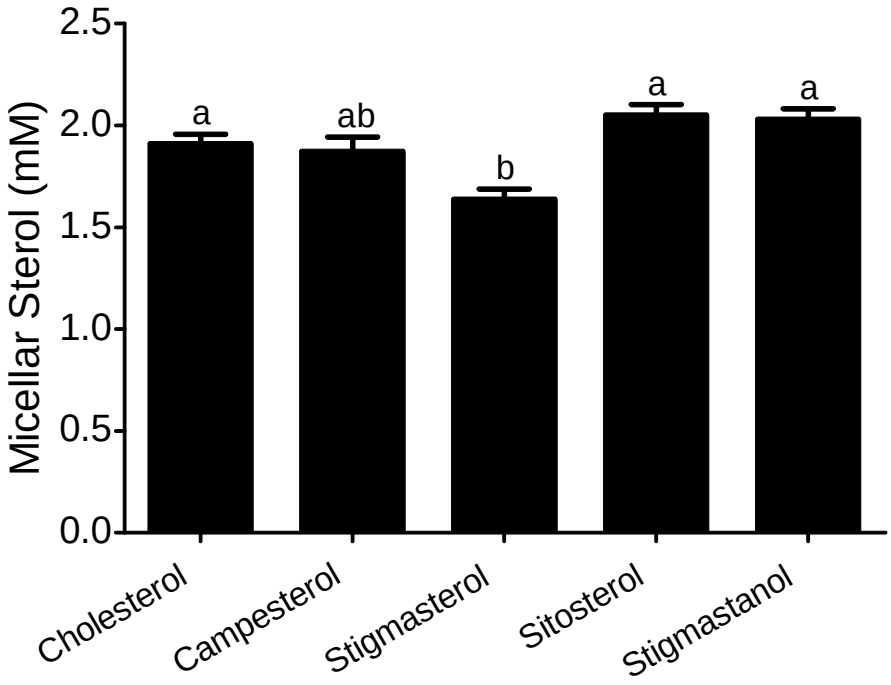

Figure 3.2. Sterol solubility in mixed micelles. Mixed micelles were prepared by sonicating 3 mM of each sterol in the presence of 52.5 mM mixed bile salts and 15 mM phosphatidylcholine. Micelles were isolated by size-exclusion chromatography, and sterols were quantified by GC. Data are represented as means ± pooled SEM, n=3; bars with differing letters indicate significantly different means, $p < 0.05$.

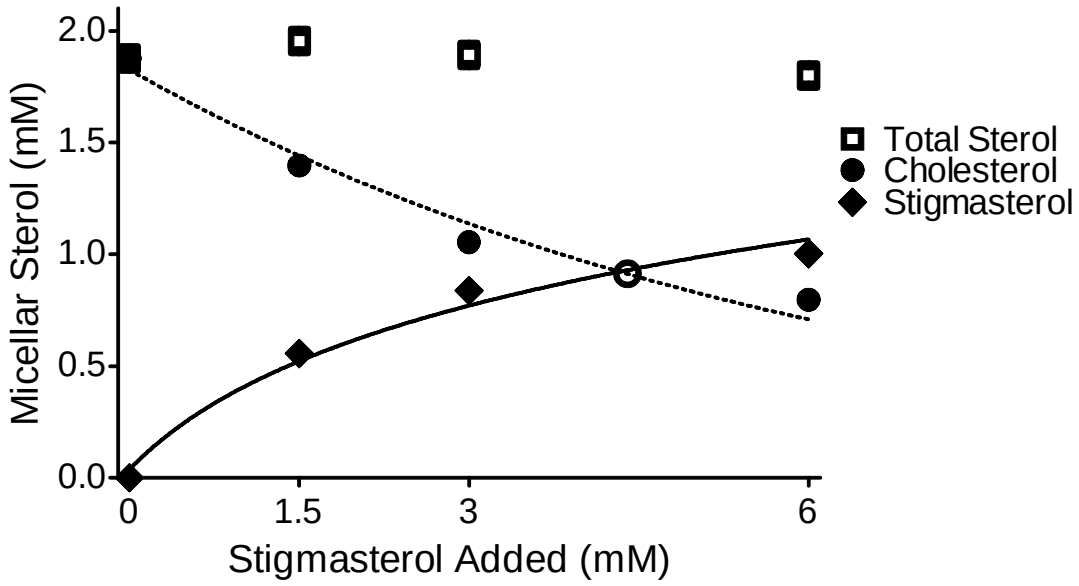

Figure 3.3. Competition of stigmasterol and cholesterol for micelle incorporation. Stigmasterol (0, 1.5, 3, or 6 mM) was sonicated in the presence of 3 mM cholesterol, 15 mM phosphatidylcholine, and 52.5 mM mixed bile salts. Micelles were isolated by size-exclusion chromatography, and cholesterol and stigmasterol were quantified by gas chromatography. Micellar cholesterol solubility, modeled as a function of added stigmasterol, is represented by the dashed line (Equation 1 in the text). Micellar stigmasterol solubility, modeled as a function of added stigmasterol, is represented by the solid line (Equation 2 in the text). The open circle indicates the concentration of added stigmasterol at which micellar cholesterol would be expected to be decreased by half, which closely coincides with the intersection of the curves. Data represent means ± pooled SEM, n=3; error bars may be eclipsed by the data markers.

3.5 Discussion

Phytosterols and their esters are potent nutraceuticals. Understanding the mechanisms by which phytosterols lower serum LDL cholesterol may be important in optimizing a more potent phytosterol treatment. Variations in the fatty acid [8] and sterol [17] components of phytosterol esters can result in different efficacies with regard to cholesterol lowering. One mechanism by which this difference could occur is through differences in rates of hydrolysis of the esters. However, we previously demonstrated no differences in the hydrolysis of sitosterol and stigmastanol esters or stearate and palmitate esters by pancreatic cholesterol esterase (EC 3.1.1.13) *in vitro*, and thus efficiency of hydrolysis likely does not directly determine the unique properties of some phytosterol esters [9]. Another mechanism for decreasing serum cholesterol concentrations could be diminishing cholesterol absorption by decreasing intestinal micellar cholesterol in the presence of mixtures of free fatty acids, free phytosterols, and/or intact phytosterol esters. We therefore examined the effects of sterol esters and their simulated hydrolysis products in a model-bile, mixed-micelle system to approximate the effects of dietary sterols on biliary cholesterol, which is quantitatively more important than dietary cholesterol with regards to cholesterol absorption [18].

The model-bile, mixed micelle system we used was created by using a simplified combination of bile acids [7,12,13], while the ratio of cholesterol to phytosterol approximated the relative amounts of the sterols in the intestinal lumen during phytosterol supplementation. On average, 1000 mg of cholesterol are secreted into the small intestine from the gall bladder daily [18], and the United States Cholesterol

Education Program recommends up to 2000 mg/d of phytosterols to lower serum cholesterol [19]. We therefore chose equal concentrations of sterols in the model micelle preparations to examine equal competition for micelle solubilization, as well as concentrations up to the 2:1 ratio of phytosterol to cholesterol that is expected in the intestinal lumen when consuming the recommended dose of phytosterols in order to examine dose-dependent effects. By examining equal ratios of phytosterol:cholesterol: free fatty acid, we also modeled the effects of complete hydrolysis of phytosterol esters on biliary cholesterol.

In the present study, we demonstrated that intact phytosterol esters do not alter cholesterol micelle solubility, nor do they incorporate at a detectable level into mixed bile-salt/phosphatidylcholine micelles. *In vivo* models, however, demonstrate that both free and esterified sterols impart cholesterol lowering effects [1]. This apparent discrepancy reinforces the concept that sterol esters must be first hydrolyzed to impart their cholesterol lowering properties. The direct influence of phytosterol esters on enterocytes or intestinal phase compositions, however, cannot be precluded. Indeed, Nissinen and colleagues detected esters and free sterols in both the oily and aqueous fractions of jejunal aspirates [20]. The presence of esters in what was labeled the 'micellar phase' in the jejunal contents, combined with the absence of esters in micelles in our model, may indicate that esters are suspended in small lipid droplets [21] or solubilized in an aqueous particle other than mixed bile salt micelles *in vivo*.

The effects of fatty acids on the micellar solubility of sterols have not been widely studied. Previous results implicating improved cholesterol-lowering effects of stearic acid-containing phytosterol esters [8] led us to investigate the simulated hydrolysis products of various phytosterol esters, with the hypothesis that the stearic acid-containing micelles would solubilize less cholesterol when compared to micelles prepared with other fatty acids. While our data did indicate a lower cholesterol content in stearic acid-enriched micelles, the effect was not unique to stearic acid, but was also seen in palmitic acid-enriched micelles as well as micelles prepared without fatty acids. In addition, the difference between cholesterol content in micelles that incorporated unsaturated and saturated fatty acids was small (1.23 mM vs 1.08 mM, respectively) compared to the 70% decrease in non-HDL cholesterol and the 85% decrease in cholesterol absorption observed in hamsters fed stearic acid esters of phytosterols [8]. Thus, the dietary effects of stearic acid-enriched phytosterol esters are likely not completely explained by decreasing the micellar solubility of cholesterol.

In a study of phytosterol esters derived from three oils used as supplements in humans (olive, sunflower, fish), no differences were observed among the treatment groups with regard to serum cholesterol concentrations [22], consistent with the similar cholesterol solubilization observed in the present study among micelles containing different unsaturated fatty acids. Conversely, another study showed that esterifying phytosterols with long chain omega-3 fatty acids synergistically lowered cholesterol, and the authors surmised that the effect was the result of decreasing micelle solubility [23]. Our data indicate the opposite: unsaturated fatty acids increased micellar cholesterol. Although the

only omega-3 fatty acid we investigated was α-linolenic acid whereas they used eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, our data suggest that if there is a unique effect of EPA and DHA on micellar cholesterol solubility that it is likely not the result of the degree of unsaturation or the omega-3 positioning of a double bond.

A number of studies have examined the thermodynamics of sterol solubilities in micelles and have demonstrated competition between cholesterol and other sterols for incorporation into micelles [4,5,7,24,25]. Although we also observed this competition, our results contrast markedly with those from several other studies. For instance, we found that four different phytosterols decreased cholesterol solubility to a very similar extent. Stigmasterol was, by a small margin, the most potent of the four, decreasing the concentration of micellar cholesterol and total solubilized sterols. This result is in contrast to a previous study on the solubility of cholesterol in sodium taurodeoxycholate micelles [4] that found stigmasterol to have the smallest effect among the sterols tested. The same study reported poor relative solubility of individual phytosterols in their system compared to cholesterol, whereas we observed almost equal solubility. The results of that study were consistent with ours in terms of total sterol solubility: in their binary (two sterol) systems, total sterol content was relatively equal. On the other hand, a third study that used micelles of sodium taurocholate and oleate demonstrated an approximately 50% decrease in total solubilized sterol as the amount of sitosterol was increased [2]. In contrast, our dose-dependence experiment demonstrated no decrease in total solubilized sterol, but instead showed a 1:1 substitution of stigmasterol for cholesterol. The

competition model we demonstrated does, however, reinforce the observations of Armstrong and Carey that micelles have a limited number of binding sites [5].

The disparities in results in sterol solubility studies probably reflect the differences in the micellar systems employed. The work reported here is based upon a model bile system, whereas other systems often use a single bile salt with or without another lipid component such as oleate. While these simpler systems do provide insight into the inherent solubility of various sterols in bile salt micelles, they may be less effective as models of intestinal micellar solubilization. Indeed, the lack of difference between individual phytosterols in our studies coincides with observations in clinical and animal trials. For example, similar decreases in serum cholesterol reduction were observed in humans upon administration of different combinations of phytosterols [26]. Analogous outcomes have been observed in rats [27] and hamsters (Ash and Carr, unpublished data) fed phytosterol esters varying only in the sterol component. It is likely that the differences in solubilities arise from the micelle composition, as the bile salt type and concentration both affect the solubility of sterols [5], as well as the presence of lyso- or phospholipids [28], oxidation of fatty acids [29], and amount of fatty acid [2]. The complexity of bile, then, is likely better able to accommodate different sterols than simpler systems. However, the decreased cholesterol solubilities when stigmasterol and saturated fatty acids are present may indicate that the combination of these components is more effective at decreasing cholesterol solubility in micelles than other combinations, though in vivo experiments must be conducted to validate these results on serum cholesterol and cholesterol absorption.

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Chapter 4: The extent of hydrolysis of phytosterol esters affects cholesterol absorption efficiency and lipids in the intestinal lumen

4.1 Abstract

Phytosterol esters lower cholesterol with different efficacies depending the fatty acid moiety of the ester. We hypothesized that differential hydrolysis of phytosterol esters affects the distribution of free cholesterol between the aqueous and oily phases of intestinal contents, altering its availability for absorption. Hamsters were fed an atherogenic diet (Control), or the Control with 5%: free phytosterols; phytosterols esterified to palmitic, oleic, or stearic acids; or phytosterols etherified to an octadecyl chain, thus representing different levels of expected hydrolysis. Contents were collected from the proximal intestine, aqueous and oily phases were separated, and lipids were analyzed by GC. Cholesterol absorption was measured by the dual radio-isotope method. As expected, the amount of free phytosterol in the phases differed among treatments: (free phytosterol = oleate esters) > (palmitate esters = stearate esters) > (stearate ethers = Control). However, no differences were observed in the partitioning of free cholesterol between the aqueous and oily phases. The extent of hydrolysis was significantly and negatively correlated with cholesterol absorption efficiency ($r=-0.6716$; $p<0.0001$); however, the Ether treatment lowered cholesterol to a greater extent than would be predicted by hydrolysis alone, and when removed from the model the extent of hydrolysis of phytosterol esters was more highly correlated ($r=-0.8460$; $p<0.0001$). Additionally, changes in the overall profile of the intestinal lipids appeared to be affected by the treatments, with the Ether treatment potentially increasing hydrolysis or altering phase

distributions of some non-cholesterol lipids. These results imply that it is the free, rather than esterified, phytosterol that decreases cholesterol absorption, but that another mechanism involving the hydrolysis or phase partitioning of non-cholesterol lipids may also be at play.

4.2 Introduction

Phytosterols and their esters consumed at recommended doses lower cholesterol by 10% on average[13], thereby acting as a powerful nutraceutical to lower cardiovascular disease risk. However, the mechanisms by which phytosterols lower cholesterol are incompletely characterized[63]. Previous studies have shown that phytosterols lower cholesterol incorporation in micelles[63][64], but intact phytosterol esters do not. The micellarization of cholesterol is essential for efficient absorption[65], and therefore the action of phytosterols competing for micellarization with cholesterol helps explain one mechanism of action.

Stearic acid esters of phytosterols have been shown in hamsters to lower cholesterol more effectively than phytosterols esterified to other fatty acids[66], and in humans the effect of phytosterol stearates are at least as effective as the average of 10%[67]. However, we have previously demonstrated that phytosterol stearates are poorly hydrolyzed by pancreatic cholesterol esterase [39], and intact esters do not affect cholesterol micellarization[63]. Therefore, it may be that another mechanism is responsible.

In the intestine, chyme and bile mix together and form three crude theoretical phases as characterized by ultracentrifugation: an oily phase, consisting primarily of hydrophobic compounds; an aqueous phase, composed of hydrophilic compounds as well as hydrophobic compounds that are solubilized in such amphipathic structures as micelles; and an insoluble pellet. Because phytosterols esterified to long chain saturated fatty acids are poorly hydrolyzed by cholesterol esterase, whereas the monounsaturated oleic acid esters are much more rapidly hydrolyzed [39], we hypothesized that the poor hydrolysis of the saturated phytosterol esters results in a persistent oily phase in which cholesterol is more soluble, and thus less readily available for absorption. This hypothesis implies that intact phytosterol esters would cause a significant difference in the partitioning of cholesterol between the oily and aqueous phases.

It is also possible that the presence of a large amount of poorly or non-hydrolyzable lipid may alter other lipid metabolism, as well. The ester bond is common in lipids, such as that found in cholesterol esters, phospholipids, and acylglycerols, and leaving the potential for phytosterol esters to be substrates for other lipolytic enzymes, and potentially competitive inhibitors.

Yet another mechanism could be unique effects of intact esters being absorbed. While absorbed slowly, the uptake of intact esters by membranes has been demonstrated [24]. Absorption of intact esters could have metabolic consequences without necessitating the typical physical-chemical interactions in the intestinal lumen, such as micellar competition for solubilization.

To examine the effects of the hydrolyzability of sterol esters on lipid metabolism and cholesterol absorption efficiency, dietary treatments were designed to provide a range of expected hydrolysis from 0% to 100% and the effects on cholesterol absorption efficiency and the lipid profiles of intestinal contents in hamsters.

4.3 Materials and methods

4.3.1 Reagents and Ingredients

Chloroform, hexanes, and sodium chloride were purchased from Fisher Scientific (Newport, RI). Methanol was purchased from VWR (West Chester, PA). Cholesterol and potassium chloride were obtained from Mallinckrodt OR (Paris, KY). 5α-cholestane was obtained from ACROS Organics (Geel, Belgium). Diisopropylfluorophosphate was purchased from EMD Biosciences (Gibbstown, NJ). Phenylboronic acid was purchased from TCI America (Portland, OR). AIN-93 mineral and vitamin mixes, casein, dextrinized cornstarch, guar gum, cellulose and coconut oil were purchased from Dyets, Inc. (Bethlehem, PA). Choline bitartrate, L-cystine, palmitic acid, stearic acid, and oleic acid were purchased from Sigma Chemicals (St. Louis, MO). ^{4}C-cholesterol and ^{3}H-sitostanol were purchased from American Radiolabeled Chemicals (St. Louis, MO). Cornstarch, sucrose, high oleic sunflower oil, and soy bean oil were purchased from a local grocery store. Soy bean sterols were generously donated by ADM (Decatur, IL). 18 megaohm water was produced in the lab (Millipore, Billerica, MA).

4.3.2 Experimental diets

Six experimental diets were prepared to create a range of non-esterified phytosterols in the intestine (in order from the most to least): non-esterified soy bean sterols (Free Sterol Diet), soy bean sterols esterified to oleic (Oleate Ester Diet), palmitic (Palmitate Ester Diet), and stearic (Stearate Ester Diet) acids, and an octadecyl soy bean sterol ether (Stearate Ether Diet), plus a Control Diet. Because the non-esterified phytosterols have a lower molecular weight than the esters and ether, the molar difference was compensated for by the addition of high-oleic sunflower oil. Therefore, the Free Sterol Diet approximated the complete hydrolysis of the phytosterol oleates. According to previous work[39], we expected: the oleate esters to be hydrolyzed to a much greater extent than the palmitate and stearate esters; the palmitate esters to be slightly more, if not equivalently, hydrolyzed compared to the stearate esters; and the ether was not expected to be hydrolyzed to any great extent because the ether bond is not the native target of carboxyl ester lipases such as pancreatic cholesterol esterase.

Each diet was an atherogenic modification[68] of the AIN-93M diet[69], with substitutions for the experimental compounds (Table 4.1). Each experimental compound replaced 5% w/w cornstarch compared to the control diet, with the exception of the Free Sterol Diet, in which 60% of the treatment compound weight (3% w/w of total diet) was soy bean phytosterols while the other 40% was high-oleic sunflower oil (as discussed above). Diets were pelleted and stored at -20°C.

4.3.3 Experimental compound synthesis

Esters were synthesized by first converting free fatty acids into the corresponding acid chlorides by reaction with oxalyl chloride in benzene in the presence of *N*,*N*-dimethylformamide as a catalyst. Following removal of solvent under vacuum, the fatty acid chloride was slowly added to a solution of non-esterified plant sterols and pyridine in ethanol-free chloroform. The reaction was stirred overnight after which the solution was washed with dilute aqueous HCl to remove pyridine. The organic layer was concentrated and the resulting solid was recrystallized from hot ethanol. The sterol esters were collected by vacuum filtration and analyzed for purity by TLC, and ^{1}H and ^{13}C NMR.

For the octadecyl phytosterol ether: an excess of sodium hydride (60% suspension in oil) was washed with a small volume of hexane under an atmosphere of nitrogen. The resulting pyrophoric powder was maintained at all times under an atmosphere of nitrogen. A solution of phytosterols in tetrahydrofuran was cautiously added to a suspension of the sodium hydride, followed by iodooctadecane. The octadecyl ether was purified by multiple recrystallizations from ethyl acetate, and analyzed for purity as described for the esters.

4.3.4 Animal Protocol

The use of animals was approved by the Institutional Animal Care and Use Committee of the University of Nebraska-Lincoln.

Thirty six 5 to 6 wk old, male Syrian hamsters (BioBreeders, Watertown, MA) were individually housed in polycarbonate cages with sawdust bedding in a temperature and humidity controlled animal facility at 25°C with a 12-hr light/dark cycle. Hamsters were acclimated for 4 d on the Control Diet, followed by randomization to the experimental diets, for a total of 29 d. Body weights were determined weekly, and food disappearance was determined twice weekly. Animals had access to fresh water at all times.

4.3.5 Cholesterol absorption efficiency

Cholesterol absorption efficiency was measured by a dual radioisotope method [70][71]. During the third week, hamsters were dosed with 1µCi [^{14}C]-cholesterol and 2µCi [^{3}H]-sitostanol dissolved in 50 µL of commercial vegetable oil and placed in cages with new bedding. Bedding was collected 3 d after dosing, and fecal matter collected and transferred to 20x150 mm glass screw-top test tubes with PTFE lined septa. 9 mL of 100% ethanol was added to each tube, followed by saponification with 1 mL of 50% KOH in a 50°C water bath over 2 d. Subsequently, 3 mL of DI H_20 were added, samples were mixed, and non-saponifiable lipids were extracted with 7 mL hexanes. Hexanes were transferred to 20 mL scintillation vials and samples were treated with UV light until pigmentation, which could potentially quench the signal, subsided (10 d). Hexanes were evaporated, 8 mL of Bio-Safe II scintillation cocktail was added, and DPM were measured on a dual channel scintillation counter (Packard Tricarb). The ratio of ^{14}C:^{3}H in each fecal sample was compared to the original ratio that was used to dose the animals to measure cholesterol absorption efficiency according to the following equation:

$$\left[\frac{\frac{^{14}C\ dose}{^{3}H\ dose} - \frac{^{14}C\ feces}{^{3}H\ feces}}{\frac{^{14}C\ dose}{^{3}H\ dose}}\right] \times 100$$

Thus, this method assumes that no radiolabelled sitosterol was absorbed.

4.3.6 Intestinal contents collection and phase separation

On d 29, the hamsters were euthanized in a 6x6 Latin Square design with the first factor accounting for differences in the amount of time samples waited for centrifugation (ie: order of euthanasia) and the second factor accounting for the rotor batch (6 samples per batch). Hamsters were anesthetized with carbon dioxide gas followed by exsanguination via cardiac puncture, after which the aorta was severed to assure death. Intestinal contents were collected via a modification of previous methods[72][73]: the gastrointestinal tract was removed, and the proximal end of the small intestine was clamped just distal to the pylorus with a haemostat. The proximal third of the small intestine was severed and the contents were collected by stripping the intestines with the handle of forceps into a 650 µL PCR tube. Samples were manipulated at or slightly above room temperature to discourage changes in the phase compositions of particle structures[74], as opposed to working on ice. Therefore, 6 µL of a lipase inhibitor cocktail was added to each PCR tube, the samples were mixed with a spatula, and the tube was centrifuged at room temperature for 5 min at 500 x g.

The contents of the tubes were transferred to 800 µL ultracentrifuge tubes (#344090; Beckman Coulter, Fullerton, CA, USA) and placed in adapters (#356860) for the buckets of the SW 55 Ti rotor. Samples were paired by weight to allow the maximum amount of

each sample to be transferred and still maintain a balanced rotor. Tubes were centrifuged under vacuum at less than 30°C for 60 min at 38,000 rpm (100,000 x g), followed by braking to 800 rpm and then free spinning until stopped. Samples were removed, layered with 50 µL of hexanes, and frozen at -80°C until analyzed.

The upper, oily phase was repeatedly washed with cold hexanes and the sample kept below -20°C until the phase was removed. The aqueous phased was removed by repeated washing with 0.9% NaCl in DI H_2O on ice. The resilient pellet in the bottom of the tube was left and stored at -80°C.

4.3.7 Lipid extraction

The hexanes in the oily phase were dried under nitrogen and reconstituted in a volume of hexanes containing 20 µg of 5α-cholestane as an internal standard.

The lipids in the aqueous phase were extracted by a method modeled after Folch, *et al*[75]. The aqueous sample was transferred in its entirety to a 50 mL tube and brought to a volume of 2 mL of 0.9% NaCl in DI H_2O. The contents were then brought to a final volume of 40 mL of 2:1 chloroform:methanol (v/v) with a total of 50 µg of 5α-cholestane as an internal standard. The samples were placed on a rocking platform for 1 hour, after which 8 mL of 0.88% KCl was added to each tube. Samples were inverted, phases separated by centrifugation at 1000 x g for 10 min, and the supernatant aspirated and discarded. The infranatant was transferred to a new tube and solvent evaporated at 50°C under a stream of nitrogen. The walls of the tube were washed with aliquots of

chloroform to collect the contents at the bottom of the tube, and the solvent was evaporated again. Extracts were reconstituted in 500 µL of chloroform and transferred to GC vials.

4.3.8 GC analysis

Lipd extracts were injected in their native state onto an AT-5 column (Alltech Associates, Inc., Deerfield, IL; 0.32mm x 30 m) installed in an Agilent 7890 GC equipped with an FID operated at 300°C. The inlet was operated in splitless mode at 270°C and constant pressure at 12 psi. The oven was set at 270°C for 1 min, followed by a 5°C/min ramp to 320°C and held for 100 min for a final run time of 111 min. Compounds were identified by retention times compared to external standards, and the treatment compounds (eg: soy sterols, esters, and ethers) were used as internal retention time standards. Several phytosterol peaks of interest partially or completely coeluted with other compounds in the complex lipid matrix; however, campesterol, campesterol esters, and campesterol ethers were completely resolved, and were used as markers of relative free and complexed sterols.

4.3.9 Statistics

Phytosterol ester and phytosterol ether hydrolysis data, cholesterol absorption measurements, and cholesterol phase distributions were analyzed as a mixed model ANOVA with the treatment as a fixed effect, and the order and batch of euthanasia as random effects for the Latin Square design. The observations from hamsters on the Control and Free diets were removed from the comparison of the extent of hydrolysis of

the esters and ethers because no esters or ethers were detected or expected in the intestinal contents of hamsters on these diets, therefore making the resulting variance of zero an artifact of the treatments themselves. Pearson correlation coefficients were calculated between the percent hydrolysis of the phytosterol esters or ethers and the percent cholesterol absorption, with the Control samples omitted because no treatment compounds were added and thus no percent hydrolysis could be calculated.

Lipid profiles of the aqueous and oily phases were examined independently by factor analysis. Groups of peaks with similar retention times were grouped into peak regions (Figure 4.1) and were expressed as a percent of total peak area, and these served as the variables for factor analysis. The internal standard peak (5α-cholestane) was removed, and the peaks representing esters and ethers were summed into one, non-contiguous peak region. Factors that explained more variability than average (ie: eigenvalues>1) were retained and optimized using orthogonal Varimax rotation. Factor scores were then tested for significant differences among treatments as a mixed model ANOVA with the factor scores for a particular factor as the response variable, treatments as the fixed effect, and the order and batch of euthanasia as random effects for the Latin Square design.

Results were considered significantly different if the p-value was less than 0.05. Multiple comparisons were corrected for by using the Tukey-Kramer method. Statistics were computed using SAS software (version 9.2; SAS Institute, Cary, NC); the Mixed procedure was used for the mixed model ANOVAs, and the Corr procedure was used to calculate Pearson correlation coefficients.

4.4 Results

4.4.1 Cholesterol phase distribution

The distribution of cholesterol between the aqueous and oily phases was measured by calculating the cholesterol in the aqueous phase as a percentage of the total cholesterol found in both the aqueous and oily phases (Figure 4.2A). No cholesterol esters were detected in our model, though none was fed in the diet. The majority of cholesterol was detected in the aqueous phase in all treatments (>90%). No differences were detected in the phase distribution of cholesterol ($p=0.3657$). The correlation coefficient between the moles of cholesterol in each phase was 0.21805 ($p=0.2083$), indicating an independent partitioning of cholesterol between the two phases (Figure 4.2B).

4.4.2 Hydrolysis of complexed phytosterols

The extents of hydrolysis of the phytosterol esters and ethers were estimated by comparing free campesterol in both the aqueous and oily phases to the total (free and esterified or etherified campesterol) in both phases. The estimates of hydrolysis varied significantly among treatments (Figure 4.3A). While the Free Sterol treatment was not compared to the others because it had a variance of 0 (all 100% hydrolysis; no esters were detected), it had the highest magnitude of hydrolysis, as expected. The Oleate group was next highest, and was greater than the other treatments that contained complexed phytosterols. Palmitate and Stearate esters were hydrolyzed to a similar extent, while the estimates of the hydrolysis of the Ethers were the lowest (11.2%).

4.4.3 Cholesterol absorption efficiency

Cholesterol absorption was significantly different amongst the various treatments (Figure 4.3B). The Control had the greatest magnitude of cholesterol absorption efficiency, though not significantly different than the Stearate or Palmitate treatments. However, the Ether group showed lower cholesterol absorption efficiency as compared to the Stearate treatment and the Control. The lowest cholesterol absorption efficiency was seen in the Oleate and Free groups, which were lower than the other four treatments.

To see how cholesterol absorption efficiency may be related to the rate of hydrolysis of the esters and ethers, Pearson correlation coefficients were calculated for cholesterol absorption versus the percent hydrolysis of phytosterol esters and ethers (Figure 4.3C). The correlation, including the Ether, Stearate, Palmitate, Oleate, and Free treatments, was negative ($r=-6716$; $p<0.0001$), indicating that as percent hydrolysis increases the cholesterol absorption efficiency decreases. However, the Ether appeared to be tightly clustered below the regression line, possibly leveraging the correlation toward 0. To investigate this, the line was refit omitting the Ether treatment, and the correlation resulted in a better fit ($r=0.8460$; $p<0.0001$).

4.4.4 Treatment effects on the overall lipid profile

The lipid profiles of the aqueous and oily phases were analyzed separately because a number of peak regions were not analogous between the two phases. Additionally, because the lipids were run natively and unpurified, the peaks were not all easily

identified. Thus, peaks with similar retention times were summed into peak regions for qualitative estimation of differences in lipid profiles.

In the oily phase, five factors were retained that accounted for a total of 89.6% of the variability in the data (Table 4.2). Of these five factors, factors 1 and 4 were significantly different among treatments ($p<0.05$, Figure 4.4A). Factor 1 was dominated by a single negative loading from the peak region immediately following the solvent peak, which likely represents non-esterified fatty acids. The other loadings were not as well distinguished for the first factor, but some of the highest loadings correspond with large peaks detected from the analysis of the lipids contained in the diet, which are likely di- and tri-acylglycerols. This indicates that when the purported di- and tri-acylglycerols are high, the amount of non-esterified fatty acids are low. The Control and Stearate Diets resulted in higher factor scores on the first factor, which were statistically greater than the Free Sterol Diet (Figure 4.4B). With regard to Factor 4, peak regions coinciding with the free phytosterol regions of the chromatogram stood out from the rest of the loadings on the Factor 4 axis, while the others tended to cluster around 0 (Figure 4.4A). The factor scores for the Free and Oleate Diets were significantly greater than the other four treatments, which were not significantly different from each other (Figure 4.4C). Because this factor seems to be related to the presence of phytosterols, the two diets representing the highest hydrolysis (Free and Oleate) would indeed be expected to have the highest positive loadings for this factor.

The aqueous phase analysis resulted in 6 retained factors explaining 89.2% of the variability in the data (Table 4.2). Of these, only factors 3, 4, and 6 were significantly different among treatments. For factor 3, the most extreme loadings were similar to those of factor 4 in the oily phase, which represented some of the regions consistent with free phytosterols; other more positive loadings were from regions soon after the solvent peak, likely representing free fatty acids and monoacylglycerols (Figure 4.5A). Thus, as expected, the Oleate and Free Sterol treatments resulted in factor scores that were significantly greater than the other four treatments. The loadings for Factor 4 of the aqueous phase were dominated by a negative loading on the purported free fatty acid peak, while the positive loadings were not distinct but tended to cluster around compounds with relatively short retention times such as phospholipids and some mono- and di-acylglycerols (data not shown). The Factor 4 scores for the Ether group were significantly lower than the Control and Stearate groups, while the other treatments were all not significantly different, indicating that the Ether is expected to have a relatively higher proportion of the post-solvent peak region with lower amounts of what may be mono- and di-acylglycerols and phospholipids (Figure 4.5C). Factor 6 was dominated by a positive loading on both the ester/ether peak regions and a peak around 9 min that was unique to the Ether diet (Figure 4.5A). The factor scores were also significantly greater for the Ether treatment than the other treatments (Figure 4.5D). Thus, Factor 6 likely represents the fact that the ethers were more easily detected as compared to the esters, and represents the presence of an unidentified peak unique to the Ether treatment.

Table 4.1. Experimental Diet Compositions.

Diet	Control	Palmitate Ester	Stearate Ester	Oleate Ester	Ether	Free Sterol
			g/kg			
Cornstarch	404.5	354.5	354.5	354.5	354.5	354.5
Dextrinized cornstarch	155.0	155.0	155.0	155.0	155.0	155.0
Casein	140.0	140.0	140.0	140.0	140.0	140.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Coconut oil	80.0	80.0	80.0	80.0	80.0	80.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0
Palmitate esters		50.0				
Stearate esters			50.0			
Oleate esters				50.0		
Ethers					50.0	
Free Plant Sterols						30.0
High Oleic Sunflower Oil						20.0
Insoluble fiber (Solka-Floc cellulose)	40.0	40.0	40.0	40.0	40.0	40.0
Soluble fiber (Guar gum)	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol	1.2	1.2	1.2	1.2	1.2	1.2
AIN-93 mineral mix (70% sucrose)	35.0	35.0	35.0	35.0	35.0	35.0
AIN-93 vitamin mix (98% sucrose)	10.0	10.0	10.0	10.0	10.0	10.0
L-Cystine	1.8	1.8	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
			%Energy[1]			
Carbohydrate	65.53	63.82	63.82	63.82	63.82	63.82
Protein	13.22	13.88	13.88	13.88	13.88	13.88
Lipid	21.25	22.30	22.30	22.30	22.30	22.30

[1]Estimates of %Energy from each macronutrient considers the contributions from the base diet and not changes in composition upon hydrolysis of phytosterol esters or the High-Oleic Sunflower Oil.

Table 4.2. Variance explained by each Factor

Oily Phase					
	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
	% of Variability[1]				
Pre-rotation	53.8	15.5	9.8	5.7	4.7
Post-rotation	43.6	15.2	13	9.7	8.0
Total variance explained (%)			89.56		

Aqueous Phase						
	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
	% of Variability[2]					
Pre-rotation	43.6	18.1	10.8	8.0	4.6	4.1
Post-rotation	33.8	20.1	12.1	12.0	6.0	5.3
Total variance explained (%)			89.23			

[1]The percent of variability expected for each factor by random chance is 4.0%
[2]The percent of variability expected for each factor by random chance is 3.3%

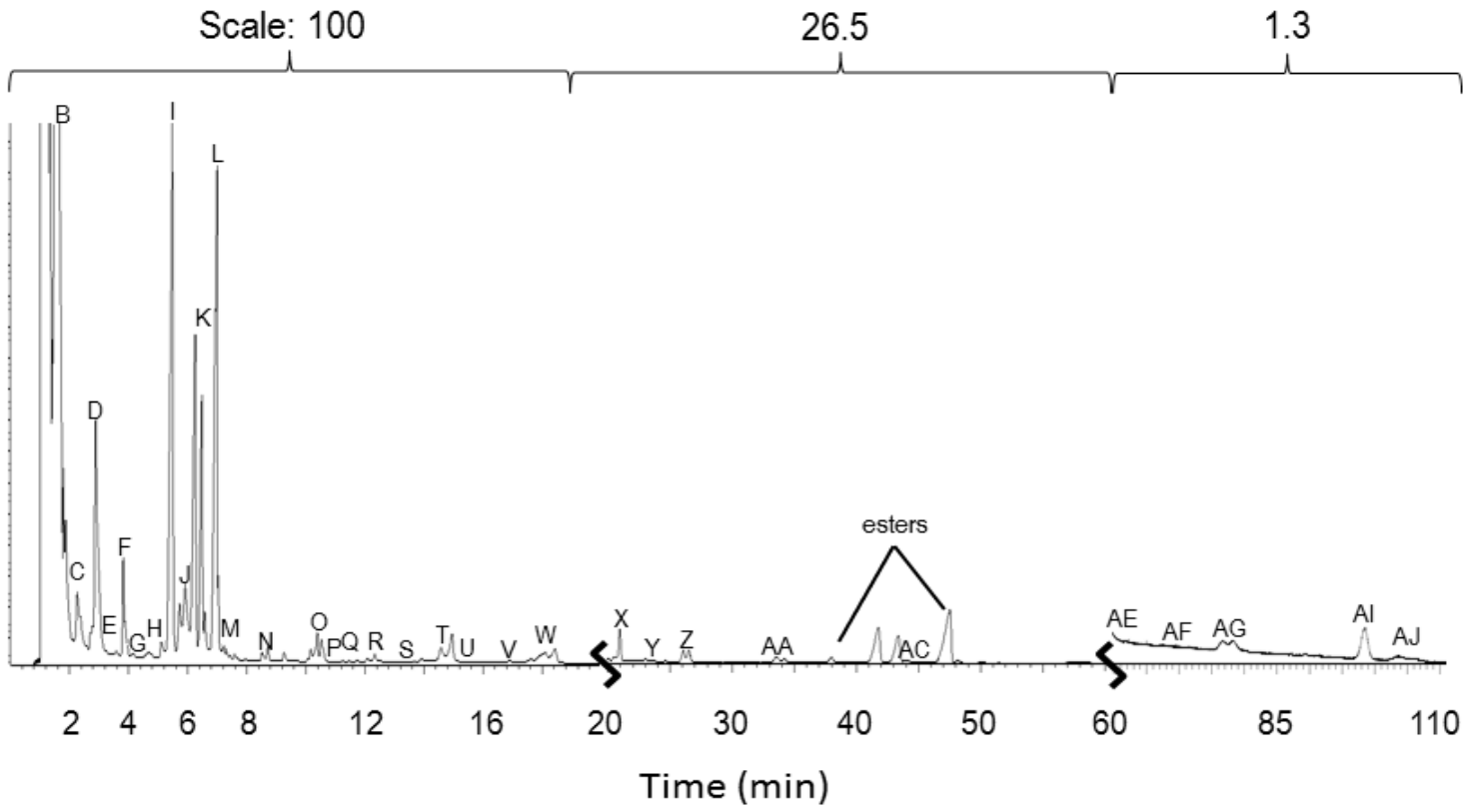

Figure 4.1. Peak region assignments for intestinal contents lipids. Aqueous and oily phase lipids separated from hamster intestinal contents were analyzed by gas chromatography and assigned to peak groups depending on retention time or previous identification. The 'esters' region is a non-contiguous peak region spanning an unrelated peak region identified as AC. Peak D represents 5 α cholestane, the internal standard; peak I is cholesterol; regions K and L represent phytosterols; region B and those close to it likely represent fatty acids and monoacylglycerols; phospholipids elute from 13-19 min; many other peaks regions represent unidentified di- and tri-acylglycerols. Note the differences in scales on both the time and response axes in the figure.

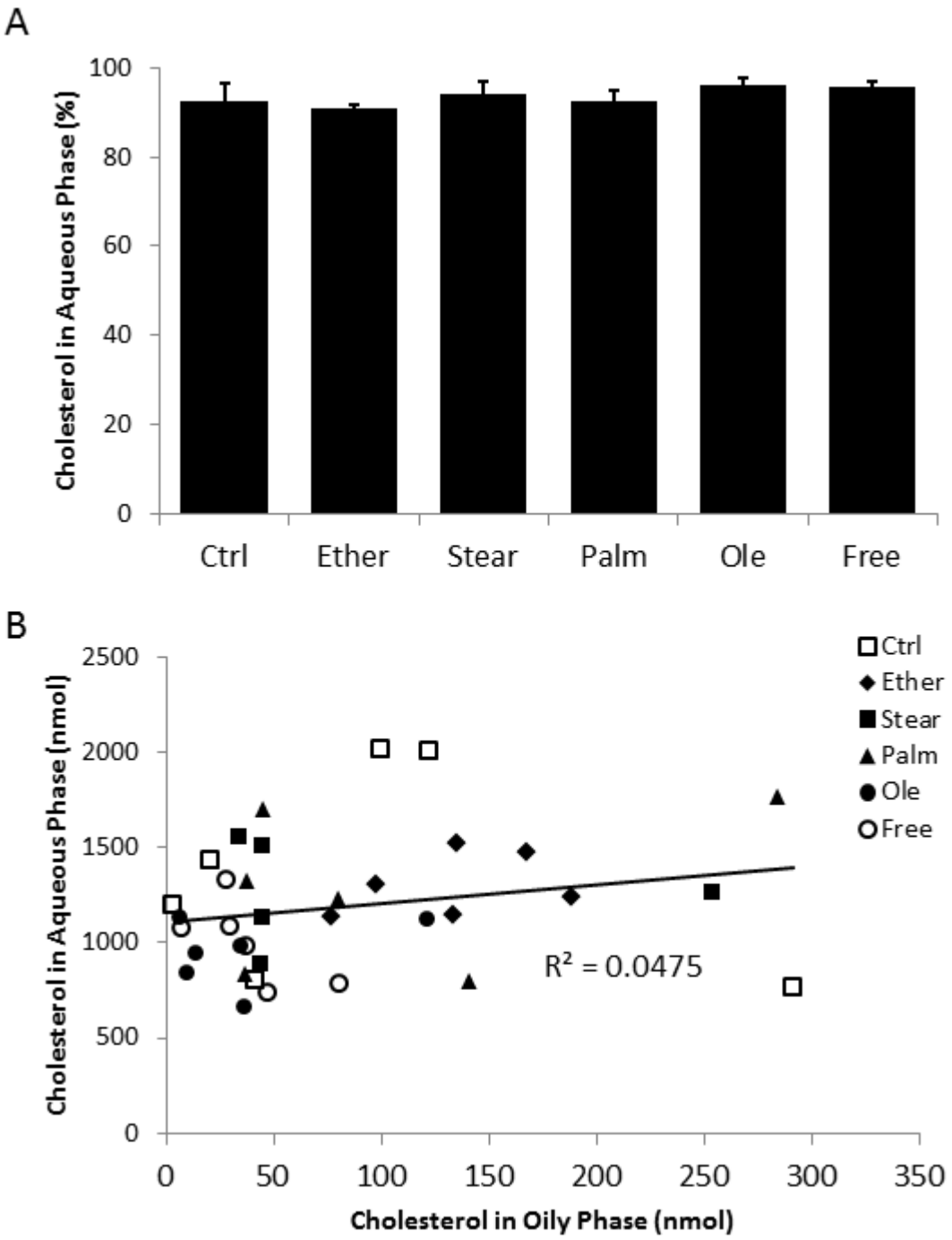

Figure 4.2. Phase partitioning of cholesterol. A) The proportion of cholesterol in the aqueous phase of the intestinal contents was calculated as a percentage of total cholesterol in the aqueous and oily phases. No significant differences were detected among treatments ($p>0.05$). **B)** No correlation existed between aqueous and oily phases, indicating they independently partitioned between phases.

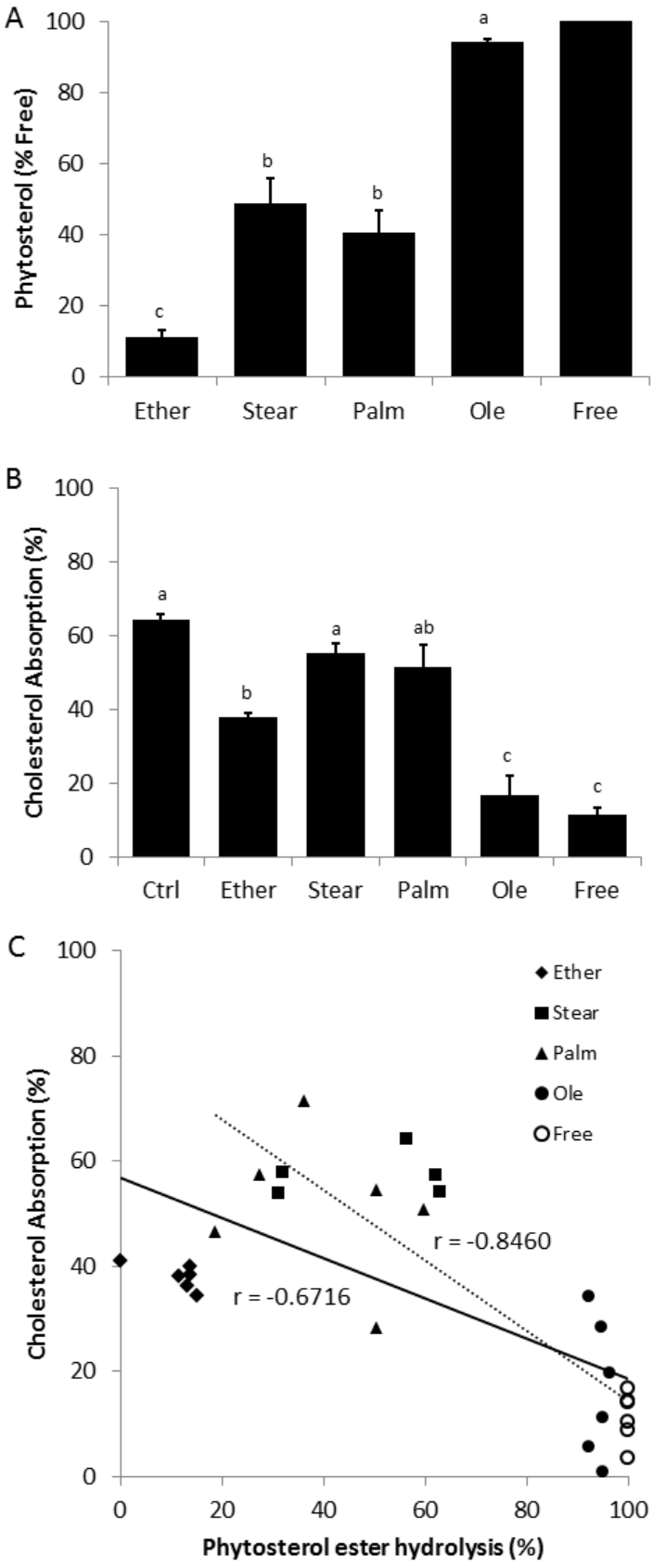

Figure 4.3. Phytosterol ester hydrolysis and cholesterol absorption efficiency. A) The proportion of phytosterol esters hydrolyzed in the hamster intestinal contents was estimated from the free and esterified or etherified campesterol in both the aqueous and oily phases. The Control diet was excluded from this analysis because no phytosterols or esters were included in the Contorl diet. **B)** Cholesterol absorption efficiency was determined by the dual fecal radioisotope method. **C)** The percent of hydrolyzed phytosterol ester as calculated in A was significantly associated with the cholesterol absorption efficiency calculated in B. The solid line represents the association of all treatments (except the Control, as described in A); because the Ether group appeared to be leveraging the line, the line was refit excluding the Ether group (dashed line). Bars represent means ± SEM; bars without common letters differ significantly ($p<0.05$).

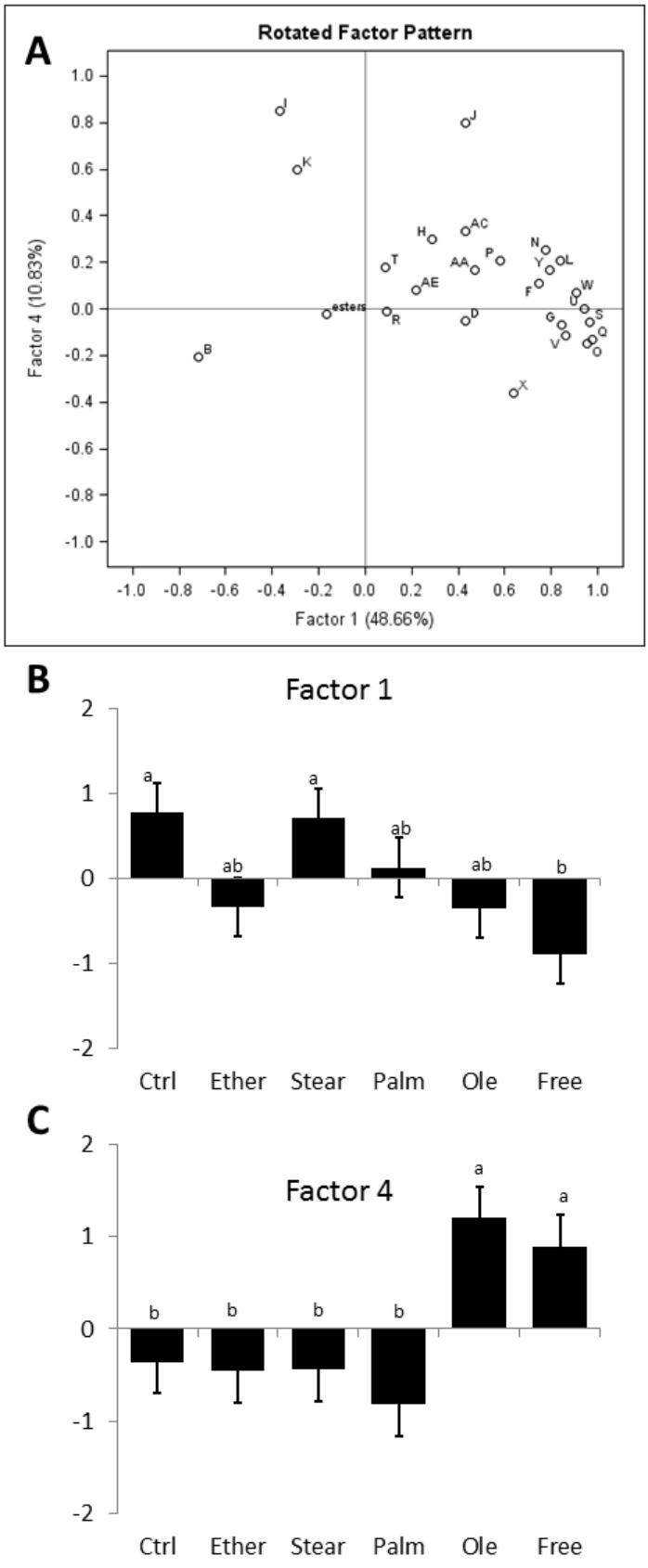

Figure 4.4. Factor analysis of lipid profiles from the oily phase of the intestinal contents. Peak regions identified in Figure 4.1 were analyzed using a factor analysis with an orthogonal Varimax rotation to identify patterns in the lipid profiles. **A)** Factor loadings for Factors 1 and 4 are plotted against each other. The percentages listed in the axis titles quantify the total percentage of variability in the data set explained by that factor. Factor scores across treatments for **B)** Factor 1 and **C)** Factor 4 were significantly different amongst treatments. Bars represent means ± SEM. Bars with no common letters differ significantly ($p<0.05$).

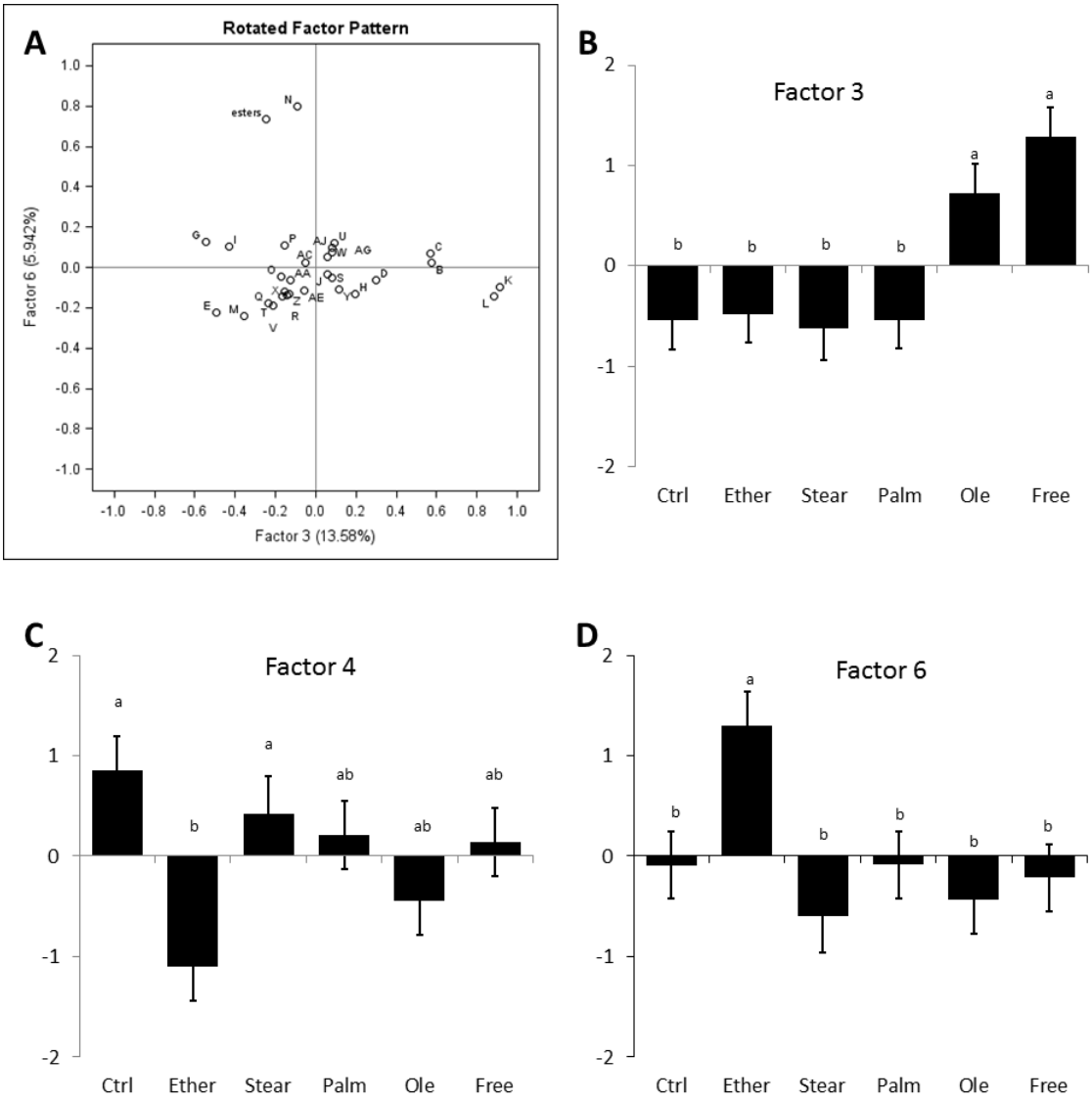

Figure 4.5. Factor analysis of lipid profiles from the aqueous phase of the intestinal contents. Peak regions identified in Figure 4.1 were analyzed using a factor analysis with an orthogonal Varimax rotation to identify patterns in the lipid profiles. **A)** Factor loadings for Factors 3 and 6 are plotted against each other. The percentages in the axis titles quantify the total percentage of variability in the data set explained by the factor. Factor scores for **B)** Factor 3, **C)** Factor 4, and **D)** Factor 6 were significantly different amongst treatments. Bars represent means ± SEM. Bars with no common letters differ significantly ($p<0.05$).

4.5 Discussion

The purpose of the present study was to determine if the extent of hydrolysis of phytosterol esters is a determining factor for the efficacy of phytosterols with regard to lowering cholesterol absorption efficiency. The treatments were designed to result in 0% hydrolysis in the Ether treatment, in which the ether bond was not expected to be appreciably hydrolyzed, to 100% hydrolysis in the Free Sterol treatment, in which all of the sterol that was added was not esterified. The treatments did, in fact, result in a wide range of hydrolysis, but the Ether group did not show 0% hydrolysis as expected. This can be partially explained by the presence of greater amounts of phytosterols in the Ether treatment than the other ester treatments (approximately 40 mg of free campesterol in the Ether group as compared to 20 mg or less in the other ester treatments, data not shown); however, this only explains a small part of the discrepancy. Two other explanations include: 1) the Ether was actually decomplexed through hydrolysis or elimination reactions, possibly through acid hydrolysis in the stomach; 2) the responses of the GC method to the ethers, esters, and free sterols are quite different, and although response factors were used to correct for these differences, there is the possibility the complete sample lipid matrix altered the response differently than the more simple dietary standards. Regardless, the order of hydrolysis was in the ranges and order expected.

Previous work has demonstrated that stearate esters have outperformed other esters with regard to lowering serum cholesterol[66][67], but they are hydrolyzed relatively poorly[39]. In the present study, we were unable to reproduce the higher efficacy of stearate esters on lower cholesterol absorption. In fact, we demonstrated that the

efficiency of cholesterol absorption was dependent on the extent of hydrolysis of phytosterol esters, with stearate esters being poorly hydrolyzed. We had hypothesized that the increased efficacy of stearate esters was through creating a persistent oily phase that solubilized cholesterol, making the cholesterol inaccessible for absorption. However, our data indicate that cholesterol was independently distributed between the two phases, and the treatments did not appear to have an effect on the proportion of cholesterol between the two phases. Other studies investigating the potential for cholesterol and free phytosterols to create insoluble crystals as a potential mechanism for the cholesterol-lowering properties of phytosterols showed that the mechanism is implausible, further noting that the sterols were increasingly soluble in increasingly polar lipids[21]. Thus, in the presence of various relatively polar hydrolysis products of lipids, particularly monoacylglycerols and non-esterified fatty acids, it seems the partitioning favors the aqueous phase.

Given the apparent hydrolysis-dependent lowering of cholesterol demonstrated here, the decrease in cholesterol absorption in response to the Ether treatment was unexpected. The cholesterol absorption efficiency for the Ether group clustered below correlation line for the ester and Free Sterol treatments, prompting the correlation to be recalcualted in the absence of the Ether, which fit the remaining data better. This leaves the possibility open that the Ether acts by another mechanism besides the most accepted micelle-competition model for phytosterols. In particular, there is the potential for intact esters and ethers to be absorbed intact, such as through the scavenger receptor type BI (SR-BI) protein-mediated transport[24]. Thus, there may be mechanisms at work that are independent of

the physical-chemical interactions typically seen through the micellar competition of phytosterols and cholesterol, such as alterations in expression of cholesterol-absorption related proteins such as NPC1L1. However, the present study can only indicate that a difference in cholesterol absorption efficiency did occur, with no evidence of a mechanism.

The effects of phytosterols and their esters on other lipid metabolism in the intestinal lumen has not been widely studied. However, the hydrolysis of lipids is integral to the efficient absorption of cholesterol, such as the activity of phospholipase A_2[76]. Thus, it is possible that another mechanism for phytosterol and phytosterol ester action in the intestinal lumen is through disrupting other lipid metabolism or altering biliary lipid secretions. We examined the differences in lipid metabolism through a factor analysis to measure how lipids varied across the entire lipid profile of the intestinal contents. Because the intestine is not a closed system and collecting intestinal contents occurs at different stages of digestion for each animal, measuring the magnitudes of individual lipids would have been insufficient to determine changes in the overall profile of the lipids. Significant differences in markers of di- and tri-acylglycerol hydrolysis, such as the appearance of free fatty acids and monoacylglycerols with the concomitant decrease in di- and tri-acylglycerols, showed that the phytosterol esters may, in fact, alter lipid metabolism in the small intestine. In particular, the Stearate and Control treatments had the lowest level of lipids in the chromatogram region associated with fatty acid generation, coupled with higher response in the region associated with acylglycerols, which would imply slower hydrolysis of dietary lipids. However, the method we used has

not adequately identified many of the peaks in the chromatograms. This means that although we have demonstrated a significant difference in several components of the lipid profiles, much of the conclusions are based on speculating what the peaks are in each peak region. Efforts are presently underway to better identify the full profile of lipids by adapting a recently published method[77] in addition to the peaks we have identified with a moderate degree of certainty.

Although the factor analysis first and foremost demonstrated that treatment compounds did affect the variability of the lipid profiles, the dichotomous changes expected with differences in lipid metabolism were not always seen. It was expected that the ester and ether peaks were going to be loaded oppositely from the free sterols; that is, when the esters and ethers were high the free sterols should be low and vice versa. However, only two factors had strong positive loadings for free sterols, with the Oleate and Free treatments having greater factor scores for these factors, but no negative loading on the ester/ether peak regions. Furthermore, to conclude that the differences in lipid profiles can be entirely attributed to differences in lipid metabolism within the intestinal lumen would be premature, as the effects of the treatments on bile lipids have not yet been characterized.

Overall, the present study reinforces that the free phytosterol appears to be essential for phytosterol ester-mediated decreases in cholesterol absorption efficiency. Furthermore, the partitioning of a majority of cholesterol into the aqueous phase supports previous research on the phase distribution of cholesterol[78]. However, the failure of the Ether

treatment to follow the patterns of the other treatments leaves open the possibility of other mechanisms by which intact esters may lower cholesterol, or perhaps there is a unique mechanism for sterol ethers. Finally, the alterations in lipid profiles warrant further investigation to determine if and how phytosterols and their esters alter lipid metabolism in the intestinal lumen.

4.6 Acknowledgments

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Chapter 5: General Discussion and Conclusions

The efficacy of phytosterols to effect the lowering of LDL cholesterol begs for phytosterols to be more closely studied to see if we can take cues from nature to derive more potent nutraceuticals or pharmaceuticals. Phytosterols occupy a unique niche among compounds known to lower cholesterol: they are structurally similar to cholesterol and yet exert most of their effects by being excluded from the body rather than absorbed. Other nutraceuticals that improve blood cholesterol profiles, such as omega 3 fatty acids and niacin, act through secondary means, including protein and gene regulation or altering the balance between inflammatory mediators. However, these properties are only realized upon absorption of the neutraceuticals. Phytosterols, on the other hand, exert much of their function directly, merely through physical-chemical interactions in the lumen of the intestine, and possibly through competition for enzymatic action.

In the work presented in this dissertation, a number of experiments were carried out to probe potential mechanisms for how phytosterols decrease cholesterol absorption, as well as to examine if various phytosterols or phytosterol esters possess unique qualities that provide them with improved efficacy. In the first study, the hydrolysis of phytosterol esters by cholesterol esterase was demonstrated. Previous work demonstrated that phytosterols are hydrolyzed *in vivo* in the small intestine[78], but the participation of cholesterol esterase had not been formally investigated. Because many of the lipolytic enzymes are considered to be promiscuous, it was worthy of investigating whether cholesterol esterase was capable of hydrolyzing phytosterol esters. Not only did we

demonstrate that cholesterol esterase-mediated hydrolysis of phytosterol esters was possible, but the rates of hydrolysis in our model system as compared to cholesterol esters were such that it is not unreasonable to assume they would explain a large portion of the phytosterol ester hydrolysis demonstrated *in vivo*. That is, the rates of hydrolysis of phytosterol esters were likely sufficient to explain hydrolysis in the intestine over the course of digestion, even though the hydrolysis of phytosterol esters was markedly lower than that of cholesterol esters. In addition, the cholesterol esterase study demonstrated that the enzyme appeared to have an active site that was affected more by the sterol side-chain than the saturation of the ring structure, as demonstrated by a marked decrease in hydrolysis with stigmasterol esters compared to sitosterol or stigmastanol esters. Furthermore, the saturated fatty acyl esters were hydrolyzed slower than the unsaturated ester we tested.

The unique effects of combinations of various phytosterols esterified to different fatty acids were tested in two hamster studies: one tested the cholesterol-lowering efficacy of different sterols esterified to the same fatty acid (all stearate esters of sitosterol, stigmasterol, and stigmastanol); and the other tested the effects of a mix of phytosterols esterified to different esters (soy bean sterols esterified to palmitate, oleate, or stearate). The former study was conducted by Mark Ash during my tenure at UNL. His results indicated that all of the esters behaved similarly, but also that the esters did not result in a lowering of cholesterol compared to an atherogenic control (unpublished data). The results also corroborated the results of the phytosterol esterase study in so far as the esters were poorly hydrolyzed.

The second study is represented by the intestinal contents work presented in this dissertation. The study initially was designed to compare the effects of phytosterols with different fatty acyl esters; however, because of the cholesterol esterase data highlighting differences in hydrolysis, the study evolved to also include a rate-of-hydrolysis component. This was accomplished by including a free phytosterol treatment to represent what would happen if 100% of a phytosterol ester was hydrolyzed; high-oleic sunflower oil was added to account for the difference in free fatty acid that would be produced from ester hydrolysis. In addition, a compound we expected to remain completely unhydrolyzed was added, which was a phytosterol etherified to an octadecyl group. Thus, the Free phytosterol treatment represented 100% hydrolyzed phytosterol oleates, while the Ether treatment represented completely unhydrolyzed phytosterol stearate esters. Although our results estimated that a portion of the ether was hydrolyzed, which may be explained to some extent by experimental error or unanticipated decomposition of the ether after consumption, the ether was significantly less well hydrolyzed compared to the ester treatments. Unlike the previous hamster study, in which different phytosterols were esterified to stearate, the use of treatments that represented different rates of hydrolysis dramatically altered cholesterol absorption efficiency dependent on the extent of hydrolysis. Furthermore, other data from this project generated by Trevor Carden demonstrated improvements to clinically relevant endpoints, as well, including altered liver esterified cholesterol (often considered the storage form of cholesterol) and blood cholesterol.

The effects of the extent of hydrolysis on cholesterol absorption efficiency reinforced the micellar competition experiments. In the micelle studies, intact esters neither incorporated into model micelles nor altered cholesterol incorporation into the micelles. However, free phytosterol did compete with cholesterol for incorporation into micelles, which is known to be necessary for the efficient absorption of cholesterol by the enterocyte[65]. While it was previously known that this competition could occur in some model systems, the prior studies were not as comprehensive. In particular, some studies used a spectrum of phytosterols but in models far removed from physiological conditions[79]. Conversely, Elliot Jesch's work, whose methods were the foundation for the micelle work presented herein, employed a more biologically relevant system but investigated only one phytosterol[64]. We expanded on his work to focus on more phytosterols, include model hydrolysis products of phytosterol esters, and investigate the effects of intact esters. We demonstrated that the free sterols not only competed with cholesterol for micellarization, but that the competition was relatively equal amongst the sterols, as well as being a concentration-dependent competition. Taken together, our results reinforce a primary mechanism of phytosterols lowering cholesterol absorption efficiency through a micellar competition model. However, these conclusions cannot preclude secondary mechanisms that are confounded with the presence of free phytosterols *in vivo*, such as alterations in cholesterol transporters, genes, and related proteins as a result of the free phytosterols internalized by the enterocyte[49] or transported to other tissues such as the liver[57].

A number of questions remain unanswered with regard to the mechanisms of phytosterol-dependent cholesterol lowering. First, the ether in the hydrolysis study resulted in a lower cholesterol absorption efficiency than the other intact esters. If hydrolysis-dependent micelle disruption was the predominant mechanism by which phytosterols lower cholesterol absorption, the ethers should have been the least effective treatment. The hypothesis that the persistence of the intact ether would dissolve more cholesterol into the oily phase was not observed, leaving the mechanism of the ether completely unanswered. Because intact ethers (as well as esters) can be absorbed, albeit to a small extent[80][24], there is the possibility that there are other metabolic changes occurring in the enterocyte, liver, or gall-bladder that are unique to the ether as a result of its absorption. Samples from the hamsters are presently being investigated to determine if, in fact, the ether was absorbed systemically.

Another question that remains unresolved is exactly how and to what extent the treatments from the hydrolysis study altered lipid profiles in the intestinal lumen. Because sample volumes are miniscule from hamster intestines, doing complex fractionation analyses like those of Hofmann and Borgström[15], in which the masses of lipid extracts were compared in longitudinal samples, was impractical. Although internal standards were added to each phase, and thus we should be able to estimate the mass of lipids in each fraction, this would rely on response factors to be calculated for each lipid, many of which have not been adequately identified as of yet. Employing factor analysis potentially avoids this problem, at least temporarily, by identifying patterns in the profiles. Thus, relative changes in peaks can be compared simultaneously without

concern for the calculated mass. The observation that the treatments appeared to alter the hydrolysis of triacylglycerols and diacylglycerols, as evidenced by the factors corresponding to increased fatty acids and decreased acylglycerols, raises the question of whether the treatments did, in fact, alter hydrolysis of other lipids. Mechanisms such as direct enzymatic inhibition, altering phase distributions of lipids, or alterations to gall-bladder lipids, could explain such phenomena. However, because the results are presently semi-quantitative, and possibly more qualitative at this point, it is premature to conclude that the treatments did alter lipid profiles in the two measured phases. The potential for phytosterols and phytosterol esters to influence other aspects of lipid metabolism has inspired further investigations, including refining the GC methods to better identify the lipids in the intestinal contents, and analyzing lipids from bile samples to investigate differences in biliary lipids. The possibility that phytosterols and phytosterols may be exerting their influence through a presently unidentified mechanism points to potentially exciting future studies.

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