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EFFECTS OF NATURAL AND SYNTHETIC PHYTOSTEROL ADMINISTRATION ON CHOLESTEROL METABOLISM IN NORMOLIPIDEMIC HUMANS

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ABSTRACT

Phytosterols play an important role in nature and as synthetic supplements for the modification of cholesterol metabolism in humans. The consensus is that the primary mechanism by which phytosterols modify lipid profiles is through altering the absorption of cholesterol. This thesis examined the effects of phytosterol mixtures on (i) *de novo* cholesterol synthesis rates and appearance of *de novo* cholesterol into the cholesterol ester pool through the use of deuterium incorporation, as well as, (ii) plasma lipid and sterol concentrations in normolipidemic humans. The initial investigation of this thesis compared the effects of diets based on either corn oil, olive oil, or olive oil plus phytosterol mixture on cholesterol metabolism. Each treatment was administered for 10 days in a cross-over design to 16 normolipidemic humans. In addition to confirming prior conclusions that corn oil was more effective than olive oil at decreasing plasma total and LDL-cholesterol concentration, this study was one of the first to determine that the differential effects on cholesterol metabolism observed in humans consuming corn versus olive oil is due, in part, to the higher concentrations of plant sterols naturally found in corn oil. The second study presented in this thesis investigated the differential effects on sterol metabolism of phytosterol mixtures either enriched with sitostanol or sitostanol-free administered for 10 days in 11 normolipidemic humans. The results of this study demonstrated that only the phytosterol mixture containing low doses of sitostanol decreased plasma total and LDL-cholesterol concentrations and increased the circulating HDL/LDL ratios; thus, the sitostanol-enriched phytosterol mixture was a more effective cholesterol-lowering agent.

RÉSUMÉ

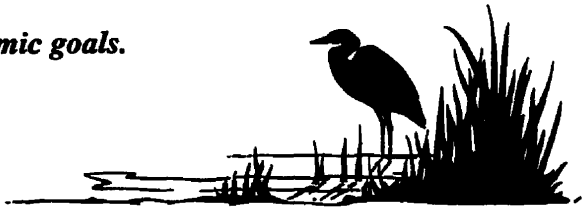
Les phytostérols jouent un rôle important dans la nature et comme suppléments synthétiques utilisés pour modifier le métabolisme du cholestérol chez l'homme. Il est généralement admis que la modification de l'absorption du cholestérol est le mécanisme premier par lequel les phytostérols changent les profils lipidiques. Cette thèse s'est intéressée aux effets qu'ont des préparations de phytostérols (i) sur le taux de synthèse du nouveau cholestérol et sur l'apparition de ce cholestérol nouvellement synthétisé dans le pool d'ester de cholestérol en utilisant l'incorporation du deutérium, et aussi, (ii) sur les concentrations plasmatiques de lipides et stérols chez des individus ayant des concentrations normales de lipides sanguins. Dans un premier temps, cette thèse a comparé les effets de diètes à base d'huile de maïs, d'huile d'olive ou d'huile d'olive enrichie de phytostérols sur le métabolisme du cholestérol. Pendant 10 jours, 16 individus ayant des concentrations normales de lipides sanguins ont reçu chaque traitement selon un design "cross-over". Cette recherche a permis de confirmer que l'huile de maïs est plus efficace que l'huile d'olive pour réduire le niveau plasmatique total du cholestérol LDL . De plus, elle a été une des premières à déterminer que cette différence sur le métabolisme du cholestérol était due, en partie, à la plus grande concentration de phytostérols contenue dans l'huile de maïs. Dans un deuxième temps, cette thèse s'est penchée sur les effets d'un mélange de phytostérols, enrichi ou non de sitostanol, sur le métabolisme des stérols chez 11 individus ayant des concentrations normales de lipides sanguins, individus auxquels cette préparation a été administré pendant 10 jours. Les résultats de cette étude ont démontré que seul le mélange de phytostérols enrichi d'une petite concentration de sitostanol avait abaissé le niveau plasmatique total du cholestérol LDL et augmenté le ratio HDL/LDL dans la circulation; le mélange de phytostérols enrichi de sitostanol était donc un agent réducteur de cholestérol plus efficace.

ACKNOWLEDGMENTS

I would like to acknowledge Diane MacDougall for her involvement in recruiting the first 8 subjects of this trial, creating the diets that were used in all the treatments, for having the whole study ready for me to just walk into so that I could recruit and study 8 more subjects, and a heart-filled thank you for her continual support during all phases of my thesis. Thank you to Kirsten Green and Jayne Rop for their excellent technical assistance in helping run the samples for the first 8 subjects and for their assistance during the sitostanol-containing phytosterol diet treatment. Thanks to Peter Oliveira for donating his time as a phlebotomist. I also gratefully acknowledge Dr. Laurie Chan's lab for their assistance in macronutrient analysis of the homogenized food mixtures. A special thank you to all the subjects for their time and dedication to the project. Finally a token of gratitude to my supervisor, Dr. Peter Jones, for the bountiful opportunities and challenges inherent in this project and for his support and confidence throughout my thesis experience.

This thesis is dedicated to my family.

With their continual love and support I was able to retain my sanity and attain my academic goals.



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1. *INTRODUCTION:*

High serum cholesterol levels are established risk markers for coronary heart disease (CHD). In normal individuals, every 1 mg/dl rise in plasma total cholesterol increases CHD risk by 1 - 2% (Kannel *et al* 1979; The Lipid Research Clinics Program 1984). It has been ascertained that plasma cholesterol levels are raised with consumption of fats containing saturated fatty acids (SFA), and reduced with fats high in monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids (Hegsted *et al* 1965, 1993; Mattson and Grundy 1985; Mensink and Katan 1989; Mazier and Jones 1997). Consequently, the current recommendations by the National Cholesterol Education Program Expert Panel (1988) for a healthy diet are that total fat should consist of less than 30% of total energy; where <10% are from SFA, <10% from PUFA, and 10%-15% from MUFA. Despite this knowledge, there is still dispute over the mechanisms by which unsaturated fats reduce circulating cholesterol levels in humans, and whether these mechanisms are constant for different unsaturated fats.

Dietary cholesterol is not the only contributor to serum cholesterol levels. Cholesterol can be synthesized by most, if not all, mammalian tissues from acetyl-CoA (Dietschy *et al* 1993). In fact, human endogenous cholesterogenesis represents at least 50% of total body influx (Dietschy *et al* 1993). The relatively recent use of deuterium oxide incorporation as a marker for cholesterol synthesis has provided a non-invasive, direct technique to monitor the effect of dietary fat consumption on cholesterol synthesis at the whole body level in a manner not previously possible. The use of this technique has demonstrated that, despite relatively similar actions on circulating cholesterol

concentrations, cholesterogenesis is greater in human subjects consuming 20% of total intake as corn oil versus olive oil or canola oil (Jones *et al* 1994). These findings suggest a fundamental difference in the mechanism by which these different plant oils elicit their cholesterol-lowering effect.

1.1 Components of plant oils effecting lipid metabolism

There is much interest in identifying nutritional factors which contribute to the development of hypercholesterolemia in otherwise healthy individuals. One such factor is dietary fat. The influence of dietary fat on serum cholesterol levels has endured considerable debate over the past forty years. Studies by Keys *et al* (1957) and Hegsted *et al* (1965) were two of the first investigations to suggest that the proportion of fatty acids in dietary fat consumed, rather than the percentage of energy supplied as fat, was a more effective predictor of serum cholesterol levels. Both studies derived predictive multiple regression equations associating serum cholesterol levels with dietary content of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA); they concluded that serum cholesterol levels were positively associated with SFA and negatively associated with PUFA. These investigators also concluded that monounsaturated fatty acids (MUFA) had no significant effect on serum cholesterol levels, thus, were deemed neutral.

The conclusions of these initial studies were generally accepted until Mattson and Grundy (1985) reported that an oil high in MUFA had the same cholesterol-lowering effect as an oil from the same plant source high in PUFA when fed in a formula diet. Following the release of these results, numerous reports have dealt with this issue but

without consistent results. In 1993, Hegsted *et al* performed a selective re-analysis of 77 studies and confirmed the results of the initial studies (Keys *et al* 1957; Hegsted *et al* 1965) where PUFA effectively lowered serum cholesterol levels and MUFA was neutral. A meta-analysis of 27 studies by Mensink and Katan (1992) concluded that saturated fatty acids had an elevating effect on plasma cholesterol, although the coefficient was about one-half of those computed by the previous studies (Keys *et al* 1957; Hegsted *et al* 1965). Both PUFA and MUFA appeared to lower cholesterol, but coefficients for both total and LDL-cholesterol lowering were significantly higher for PUFA. The meta-analysis also analysed data on HDL cholesterol, which suggested small but significant decreases in HDL with both PUFA and MUFA. Clearly, there is still dispute over the effects of fatty acids on plasma lipid levels.

There are a multitude of mechanisms that have been elucidated on how polyunsaturated fatty acids exert their cholesterol-lowering effects. The specific mechanisms whereby dietary unsaturated fatty acids exert their effects on cholesterol metabolism are not completely understood. Some metabolic consequences of polyunsaturated fats are listed in **Figure 1-1**.

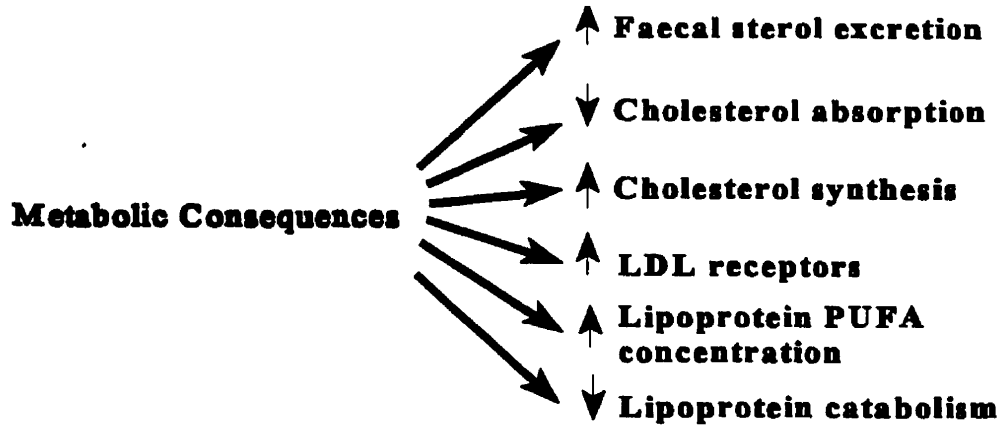


Figure 1-1: Metabolic consequences of polyunsaturated fats (Adapted from Mazier and Jones 1991)

There is still a lack of consensus and validation of these metabolic consequences as potential cholesterol-lowering mechanisms for PUFA. As well, due to the controversy over whether MUFA elicit any effects on lipid metabolism, little attention has been given to their cholesterol-lowering mechanisms. Investigations in animals have demonstrated the action of MUFA on liver metabolism appears to fundamentally differ from PUFA, with marked suppression of HMG-CoA (Fernandez *et al* 1990; Fernandez and McNamara 1991; Fernandez and McNamara 1994). This action has yet to be demonstrated in human subjects.

Throughout studies involving fatty acids, it has been assumed that the differential effect on serum cholesterol levels was caused entirely by the fatty acid content of the oils. However, where different plant sources were used to compare PUFA and MUFA, other confounding variables in dietary fats may have elicited an effect on cholesterol metabolism. It is well established that PUFA oils often contain more plant sterols than MUFA oils (Weihrauch and Gardner 1978) and investigations suggest that plant sterols have a

significant effect on plasma LDL concentrations even at relative low intakes (Grundy and Mok 1976; Lees *et al* 1977; Heinemann *et al* 1986, 1993; Pelletier *et al* 1995) . Consequently, it has been proposed that differences in serum cholesterol concentrations attributed previously to oleic and linoleic acids may have been due, in part, to the differential plant sterol content of various test oils. Investigations into this effect have yet to be conducted.

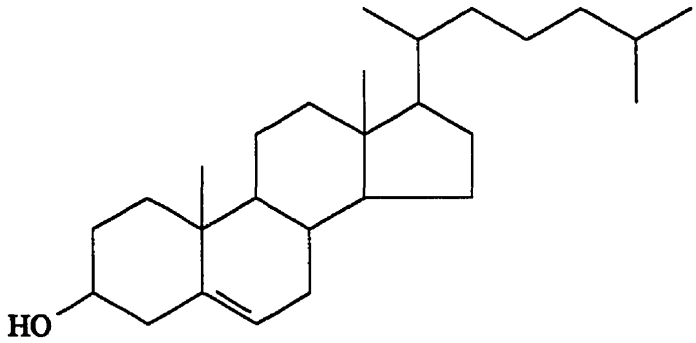
1.2 Physical and chemical properties of phytosterols

Phytosterols are structurally related to cholesterol, but differ in their side chain configuration (see **Figure 1-2**). Addition of a methyl or ethyl group at the 24 carbon atom of cholesterol leads to formation of campesterol or β-sitosterol, respectively. These two sterols, together with stigmasterol, are the most frequent plant sterols in nature (Heinemann *et al* 1991). β-sitosterol is the most predominant sterol in plants (Pollak and Kritchevski 1981). Saturated phytosterols such as campestanol and sitostanol are the products formed by 5α-hydrogenation of phytosterols. These 5α-saturated derivatives of phytosterols are found in only trace amounts in conventional diets (Cerqueira *et al* 1979).

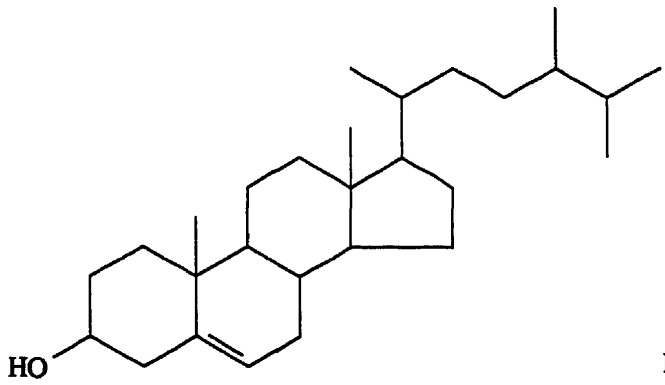
Since phytosterols are not endogenously synthesized in mammals, they are derived solely from intestinal absorption of dietary sources. In the United States, 160 - 360 mg/day of phytosterols are consumed in conventional diets (Weihrauch and Gardner 1978; Nair *et al* 1984); approximately twice this amount can be found in strictly vegetarian diets (Cerqueira *et al* 1979). However absorption of phytosterols is usually less than 5% of dietary levels, therefore only 0.3 - 1.7 mg/dl of phytosterols are found in human serum

under normal conditions (Ling and Jones 1995). These low plasma levels are also a result of preferential excretion of phytosterol over cholesterol via the biliary route (Lin *et al* 1984).

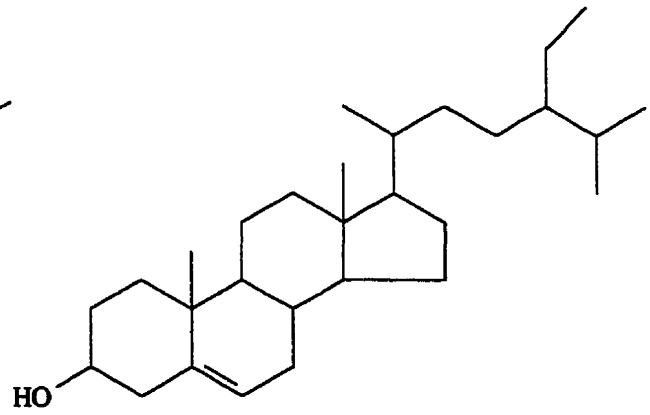
Even though it has been estimated that the average absorption of phytosterols is less than 5%, this value varies among phytosterols. Intestinal phytosterol absorption appears to be dependant on the side chain configuration of phytosterols, where absorbability of sterols decreases with an increase in the number of carbon atoms at C_{24} of the sterol side chain (Child and Kuksis 1983). With this in mind, **Figure 1-2** is helpful in illustrating that campesterol would be absorbed better than β-sitosterol, and absorption of stigmasterol would be minimal. The discovery that sitostanol (the 5α saturated derivative of β-sitosterol) is unabsorbable led to the belief that 5α-hydrogenation of a sterol resulted in an even further decrease in absorption of that sterol (Heinemann *et al* 1986). However, this assumption has been contrasted with campestanol (5α saturated derivative of campesterol) which has been shown to have a higher absorbability compared to campesterol (Heinemann *et al* 1993). Nevertheless, absorbability of phytosterols is still positively correlated with plasma cholesterol levels.



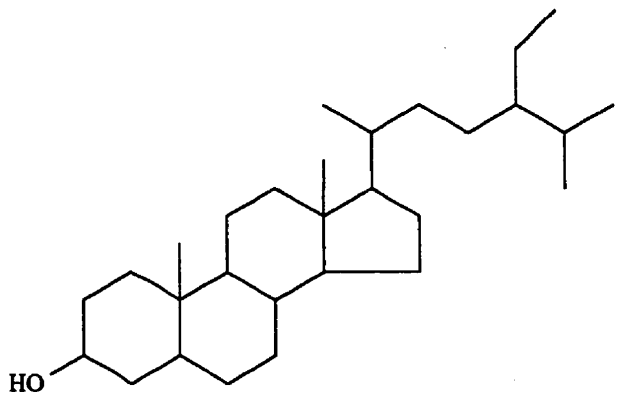
Cholesterol



Campesterol



Sitosterol



Sitostanol

Figure 1-2: Structures of Sterols

1.3 Potential mechanisms for phytosterol's effect on circulating cholesterol levels

In humans and experimental animals, phytosterols reduce absorption and re-absorption of cholesterol by acting at the level of the intestine as hypocholesterolemic agents. The mechanism by which phytosterols act is still unclear. Some proposed mechanisms are given in **Table 1-1**.

PHYTOSTEROLS ROLE IN LOWERING CHOLESTEROL ABSORPTION AND RE-ABSORPTION
I. Formation of a non-absorbable complex with cholesterol in the intestinal lumen
II. Reduction of the micellar solubility of cholesterol
III. Inhibition of cholesterol uptake at the absorptive site, possibly at the brush-border membrane
IV. Interference with cholesterol esterification or chylomicron formation in the intestinal mucosal cell

TABLE 1-1: Possible mechanisms by which phytosterol interfere with cholesterol absorption and re-absorption.
(Ikeda and Sugano 1983).

In light of these mechanisms, it has been suggested that the maximum effectiveness of the plant sterols can be realized only if they are present in the intestine simultaneously with cholesterol (Mattson *et al* 1982).

1.4 Review of literature that links the changes in plasma cholesterol concentration to phytosterol administration

1.4.1 Animal studies with phytosterols

Publication of experiments with phytosterols number close to two hundred. The first relevant report on the usefulness of plant sterols for regulating plasma cholesterol was made by Peterson (1951). In the study, chickens were fed a diet supplemented with 0.5 - 1% cholesterol, 0.5 - 1% mixed soy sterols, or a mixture of the two sterols. Following addition of dietary cholesterol to the basic diet, plasma cholesterol concentrations rose from 196 to 942 mg/dl. With the addition of soy sterols to the diet, plasma cholesterol concentrations did not rise above the normal level; nor did they rise after the diet was supplemented with both sterols. Additional analysis indicated that the effect of 0.05% cholesterol added to the diet was completely blocked by adding 0.25% or more of soy sterols, that is at a cholesterol to soy sterol ratio of 1:5.

The effects of the phytosterol termed sitosterol on serum cholesterol in rabbits were tested by Pollak (1953a). Rabbits were fed three diet cycles, the first containing 1 - 1.5 g cholesterol daily for 14 days; the second 1 g, 2 g, 3 g, 5 g, 6 g, 7 g, or 10 g sitosterol per day; or, the third where the two sterols were fed in proportions of 1:1, 3:1, 5:1, 6:1, 7:1, or 10:1 for 14 days. The results indicated that while the cholesterol supplement alone caused a 14- to 16-fold increase of serum cholesterol, sitosterol fed alone caused no increase in serum cholesterol. When cholesterol and sitosterol were fed in combination, dietary cholesterol's effect of increasing serum cholesterol concentrations was suppressed with increasing doses of sitosterol. At a sitosterol to cholesterol ratio of 7:1, dietary

cholesterol's effect of increasing plasma cholesterol concentrations was blocked completely.

Since the phytosterol preparations used in both the chicken and rabbit experiments contained only 75-80% of crude β-sitosterol, the effective sitosterol to cholesterol ratios were actually, 3.75:1 - 4.0:1 and 5.25:1 - 5.6:1, respectively. These ratios indicate that relative low levels of phytosterols are effective for cholesterol-lowering purposes.

Author(s)	Animal	Sterol	Phytosterol: Cholesterol dose	Prior state	Cholester-emia	Athero-sclerosis
Diller *et al* (1958;1960)	Chicken	Sitosterol	4%:2%	hyperchol-esterolemic	reduced	reduced
Herrmann *et al* (1959)	Rabbit	Sitosterol	3%:1%	hyperchol-esterolemic	reduced	reduced
Chandler *et al* (1979)	Cockerel chick	β-sitosterol	1%:1%	normo-lipidemic	reduced	ND

ND= no data

Table 1-2: Influence of phytosterols on cholesteremia and atherosclerosis in animals.

Most of the reports that ensued mirror the results in the above two papers. **Table 1-2** summarizes the outcomes of some of the more pertinent research. More recent studies have indicated that the phytosterol to cholesterol ratio for effective lowering of plasma lipid cholesterol levels is lower than previously believed necessary. Laraki *et al* (1991) demonstrated in Wistar rats that with the use of maize phytosterol (75% β-sitosterol) a phytosterol to cholesterol ratio of 1:1 was effective in reducing the lipid raising effects of a cholesterol overload, but a ratio of 4:1 was more effective. In 1993,

Laraki *et al* went on to show in Wistar rats that with the same maize phytosterol when the dietary phytosterol to cholesterol ratio is at least 1:1 and in the presence of dietary cholesterol excess, phytosterols do exert a regulatory role through decreasing both acetyl-CoA carboxylase and malic enzyme activities. A ratio of 2:1 enhanced this effect. Concurrently, liver fatty acids and cholesterol contents significantly decreased. These studies indicate that phytosterols may have two roles in reducing plasma and hepatic cholesterol: (i) reduction of cholesterol absorption, and (ii) the regulation of cholesterol metabolism key enzymes. The second role has yet to be demonstrated in human trials.

1.4.2 Human studies with phytosterols

Following the positive results of the initial rabbit experiment, Pollak (1953b) conducted the first human clinical study with β-sitosterol as a plasma cholesterol depressant in man. Basing his administration of phytosterols on those determined by past animal experiments, Pollak fed a daily dose of 5 - 7 g of crude sitosterol powder to 26 unselected subjects. The subjects' serum cholesterol levels ranged between 126 and 414 mg/dl, with a mean of 256 mg/dl; none of the subjects had clinical symptoms of atherosclerosis. Without dietary restriction, within seven days to two weeks of consuming phytosterols the range of circulating cholesterol levels was 131 - 264 mg/dl, with a mean of 173.5 mg/dl. The average decrease in serum cholesterol was 74 mg or 28%. It is important to note that for those whose cholesterol was <240 mg/dl, the decrease was 12.6%, but for those whose level was >240 mg/dl, the decrease was 22%, with up to 39% for those whose level exceeded 285 mg/dl prior to treatment. Upon cessation of the

sitosterol regimen, the serum cholesterol levels reversed to the original concentrations within two to three weeks. After a pause of 40 days, a second course of sitosterol had the same effect as the first course.

Pollak's (1953a) rabbit study initiated a cascade of clinical studies from 1952 to 1976, and became the model for these subsequent studies. As long as plasma cholesterol was not manipulated prior to sitosterol intake, those who followed the original recommendations with regard to selection of suitable subjects, dosage, and timing of sitosterol ingestion achieved results which were comparable to those of this prototype study (Pollak 1985). Often, the ensuing results were better then those of Pollack's landmark study (Pollak 1953a).

Petersen *et al* (1956) were the first to incorporate phytosterols into food by adding soy sterols to butter pats. Their 29 subjects, divided into two groups, were healthy and had normal plasma cholesterol concentrations, which apparently were not affected by ingesting three pats of butter daily. With the ingestion of 5.7 g/day of soy sterols in 42 g/day of butter, the average decrease in plasma cholesterol levels was 11%, thus further verifying the cholesterol lowering ability of phytosterols.

By comparing the cholesterol-lowering effects of corn oil, β-sitosterol, and α-tocopherol in 52 healthy humans for one week, Beveridge *et al* (1957) determined that administration of 7 g/day of β-sitosterol had equal cholesterol-lowering effects as corn oil (21 - 23%), whereas, α-tocopherol had no cholesterol-lowering effects. A follow-up study in 1958 by Beveridge *et al* fed healthy students corn oil plus β-sitosterol for 8 days and discovered that in combination, the decrease of plasma cholesterol was only 1.8%

better than on corn oil alone. However, the decrease did occur sooner than when corn oil alone had been administered.

In the same year, Farquhar and Sokolow (1958) published the results of their 46 week 7-phase study. Similar to the study by Beveridge *et al* (1957), this study demonstrated that the changes produced by β-sitosterol (18 g/day) and by safflower oil (PUFA-rich oil) were similar in magnitude. However, in contrast to the results found by Beveridge *et al* (1958), the combination of β-sitosterol and safflower oil had a 55% greater effect than either agent alone. This may indicate differences between the plasma cholesterol-lowering mechanisms of the two oils.

Thus, the question arises, what factors, other than fatty acid composition, could be responsible for lowering plasma lipid levels in these two oils. Weihrauch and Gardner (1978) determined that corn oil is a rich source of phytosterols, containing approximately three times more phytosterols than safflower oil (1390 mg/100 g versus 494 mg/100 g, respectively), the major component being β-sitosterol. This could be one possible reason for why the safflower oil study (Farquhar and Sokolow 1958) used 18 g/day compared to only 7 g/day used in the corn oil study (Beveridge *et al* 1958). Nevertheless, there is still a significant difference between the additive effects of phytosterols and the vegetable oils. Two possible conclusions could be that there is a difference in (i) the composition of the phytosterols administered or (ii) the plant source the phytosterols are derived from may explain the differential cholesterol-lowering mechanisms seen in each of the oils.

In 1976, Grundy and Mok set out to determine the optimal intake of β-sitosterol needed to markedly decrease the absorption of exogenous cholesterol and thus decrease

plasma lipid levels. Studies were carried out on 10 normo- and hyperlipidemic patients in a metabolic unit. The researchers concluded that administration of 3.0 g per day of β-sitosterol significantly reduced exogenous cholesterol absorption and plasma total cholesterol levels; surprisingly, however, no further reduction in either measurement was observed when the dose of β-sitosterol was increased to 9.0 g per day.

An amalgamation of clinical trials conducted over seven years by Lees *et al* (1977) evaluated the efficacy of plant sterol preparations from soy and tall oil sources administered as powder and oil-suspended forms on lowering the plasma cholesterol in a total of 46 patients with type II hyperlipoproteinemia. The maximal mean cholesterol lowering response to any preparation was 12%. The plasma lipid lowering capacity was comparable in both the sterol powders. The tall oil suspensions, however, were less effective in lowering plasma lipid levels. Nevertheless, when the tall oil suspensions were administered to patients in a metabolic ward in concentrations of 0.3 - 9 g/day, it was determined by sterol balance methods that 3 g/day was optimal for blocking exogenous cholesterol absorption. Consequently, from these past two studies it can be deduced that higher intakes of phytosterol mixtures do not necessarily lead to increased reductions in plasma lipid levels.

With the onslaught of long-term studies praising the cholesterol-lowering effects of bile acid sequestrants and other cholesterol-lowering pharmaceuticals, studies on phytosterols waned in the period of 1977 to 1985. However in the 1990's, there appears to be a slow but steady revival of studies examining plant sterols' efficacy as cholesterol-lowering agents. With the development of newer techniques, more variables can be

determined. For example, in the past, measuring rates of sterol synthesis *in vivo* in animals or in humans involved the difficult and unpleasant process of quantifying rates of cholesterol and bile acid output in the faeces each day (Grundy and Ahrens 1969). More recently, it has been demonstrated that these rates can be measured directly by administering animals water labelled with either tritium or deuterium (Dietschy *et al* 1993). The advantages of using deuterium are that it uses an intermediate time window (6-24 h), it is not overly invasive, and it measures synthesis directly (Jones *et al* 1993). As a result, rates of cholesterol synthesis can presently be measured with less difficulty and more directly in a number of different species. With such technological improvements and the increased sensitivity of equipment, studies are now able to concentrate on plasma lipoprotein cholesterol levels and ratios, cholesterol synthesis rates, and the differences between various phytosterols.

While the emphasis of studies so far has focused on the optimal amount of phytosterol needed to exert a plasma lipid lowering effect, research appears to be progressing toward determining what type of phytosterol is most effective in lowering plasma lipid levels. Heinemann *et al* (1993) investigated the differential intestinal absorption of various plant sterols. The results confirmed prior reports that there is a negative correlation between the ratio of sitosterol to cholesterol and the mass of cholesterol absorption (Pollak 1953a; Laraki *et al* 1991). Moreover, it was determined that by increasing the length of the side-chain of cholesterol the absorbability of the sterol also decreases (Child and Kuksis 1983). This study confirmed previous findings of an inverse relationship existing between the absorbability of plant sterols and their efficiency

in inhibiting cholesterol absorption (Heinemann *et al* 1986, 1991).

In 1994, Miettinen & Vanhanen compared the lipid-lowering efficiency of sitosterol, sitostanol, and sitostanol esters. They demonstrated that small amounts of dietary plant stanol esters (<1 g/day) resulted in small but significant decreases in serum total and LDL-cholesterol concentrations in hyperlipidemic subjects through a proportional decrease in cholesterol absorption. In turn, there was an increase in plasma cholesterol precursors, indicating a compensatory increase in cholesterol synthesis. The most effective results were seen with sitostanol esters dissolved in dietary fats. Plasma HDL-cholesterol concentrations were significantly, yet only slightly, increased in the sitostanol ester supplemented group, whereas the sitosterol and non-esterified sitostanol groups demonstrated no significant change.

To further test the effectiveness of sitostanol esters in reducing plasma lipid concentrations, Miettinen *et al* (1995) conducted a one-year, randomized double-blind study in 153 mildly hypercholesterolemic men and women. The mean one-year reduction in plasma total cholesterol was 10.2 percent in the sitostanol ester group, as compared with an increase of 0.1 percent in the control group. Reductions in LDL mirrored these effects, with a 14.1 percent decrease in the sitostanol group and only a 1.1 percent decrease in the control group. Similar cholesterol-lowering effects were seen with doses of 2.6 g and 1.8 g per day sitostanol ester. Serum HDL-cholesterol and triglyceride concentrations were not affected by the consumption of sitostanol ester.

There is, however, one report by Denke (1994) that demonstrated administration of 3 g/day of sitostanol in conjunction with a cholesterol-lowering diet did not significantly

lower LDL cholesterol levels in hypercholesterolemic men when compared with the diet alone. These results indicate that sitostanol's effectiveness as a cholesterol-lowering agent may be attenuated when the diet is low in cholesterol.

Although studies on β-sitosterol's efficacy as a cholesterol-lowering agent have been overshadowed by recent studies into sitostanol esters, research continues to demonstrate the effectiveness of β-sitosterol. Soybean phytosterols, which contain mostly β-sitosterol and no sitostanol, administered at a dose of only 740 mg per day were shown to decrease serum total cholesterol in normolipidemic humans by 10 percent, due mainly to a 15 percent decrease in LDL-cholesterol concentrations (Pelletier *et al* 1995). Thus, with sitostanol and β–sitosterol both shown to be effective cholesterol lowering substances, the next investigation to undertake would be to discern if there are any interactions between phytosterols that would result in an optimal plasma cholesterol-lowering mixture.

2. *STATEMENT OF PURPOSE*

Human studies investigating the effectiveness PUFA and MUFA in reducing plasma lipid concentrations have published varying results. The conclusion from Mensink and Katan (1992) meta-analysis of 27 trials published between 1970-1991 indicate that PUFA oils are more effective than MUFA oils at lowering plasma LDL-cholesterol concentrations, while at the same time decreasing HDL-cholesterol concentrations. Most of the studies within this analysis attribute the differential cholesterol-lowering effects observed between different plant oils exclusively to the fatty acid profile of the oils and do

not consider other confounding factors naturally present in plant oils from different sources. In a study where the same plant source was used, thus the only difference between the diets was the proportions of oleic acid and linoleic acid, there were similar decreases in plasma total and LDL-cholesterol concentrations in each diet (Mattson and Grundy 1985). Thus, it can be postulated that in studies where different plant sources are used to compare PUFA and MUFA, other confounding variables within the plant may be affecting cholesterol metabolism.

It is well established that PUFA oils often contain more plant sterols than MUFA oils (Weihrauch and Gardner 1978) and investigations suggest that phytosterols have a significant effect on plasma LDL-cholesterol concentrations, even at relative low intakes (Grundy and Mok 1976; Lees *et al* 1977; Heinemann *et al* 1986, 1993; Pelletier *et al* 1995) . Consequently, differences in serum cholesterol concentrations attributed previously to oleic and linoleic acids may have been partly due to differences in the plant sterol contents of various test oils. Investigation have yet to explore the possibility of plant sterols as a potential cholesterol-lowering factor in PUFA oils.

Recent investigations have determined that phytosterols are effective cholesterol-lowering agents. Despite the fact that the decrease in plasma lipid concentrations with phytosterol administration is not as effective as current pharmaceuticals, their low cost, low doses required, and lack of negative side-effects make them positive alternatives to pharmaceuticals, particularly in patients that display only mild familial hypercholesterolemia. This has lead to an onslaught of studies attempting to discover the type of phytosterol that will lead to superior cholesterol lowering. Sitostanol, the 5α-

derivative of sitosterol, has been shown to inhibit cholesterol absorption to a greater degree than sitosterol (Heinemann *et al* 1986, 1991). Consequently, sitostanol consumption has demonstrated superior decreases in plasma lipid concentrations when compared to sitosterol consumption, even at relatively low doses of 1.5 g per day. Nevertheless, supplementation with sitosterol has still exhibited potential as a cholesterol-lowering agent at optimal doses of 3 g per day (Pelletier *et al* 1995). With this knowledge, further investigation is warranted to determine if a synergistic effect would result from combining small amounts of sitostanol with sitosterol.

3. HYPOTHESES AND OBJECTIVES

3.1 Manuscript One

3.1.1 Null Hypotheses

1. *No difference will be observed in circulating plasma lipid levels when normolipidemic humans are fed diets based on:*
   - *(i) corn oil*
   - *(ii) olive oil*
   - *(iii) olive oil with plant sterols added at the level found in corn oil*

2. *No differences will be observed in free or esterified cholesterol de novo fractional synthetic rates when normolipidemic humans are fed diets based on:*

   (i) corn oil

   (ii) olive oil

   (iii) olive oil with plant sterols added at the level found in corn oil

3.1.2 Objectives:

I. To examine the effects of dietary and supplemental phytosterols on plasma lipid levels in normolipidemic humans fed corn oil- versus olive oil-based diets.

II. To determine if addition of supplemental phytosterols to an olive oil- based diet would alter the *de novo* cholesterol synthesis and esterification rates of normolipidemic humans when compared to olive oil- versus corn oil-based diets.

3.2 *Manuscript Two*

3.2.1 Null Hypothesis

When normolipidemic humans are fed diets based on either (i) olive oil, (ii) olive oil supplemented with a sitostanol-free phytosterol mixture, or (iii) olive oil supplemented with a sitostanol-enriched phytosterol mixture, there is no difference in the following physiological parameters:

1. *circulating plasma lipid levels*
2. *circulating plasma phytosterol levels*
3. *de novo cholesterol synthesis*

3.2.2 Objectives

I. To examine the effects of the addition of sitostanol-enriched and sitostanol-free phytosterol mixtures to an olive oil based diet on plasma total, LDL-, and HDL-cholesterol, and triglyceride concentrations in normolipidemic men and women.

II. To examine the effects of the addition of sitostanol to a phytosterol mixture on plasma phytosterol levels in normolipidemic men and women.

III. To compare *de novo* cholesterol synthesis rates in normolipidemic humans receiving olive oil and olive oil plus phytosterol mixtures containing sitostanol-enriched or sitostanol-free phytosterols.

Effects of the addition of corn oil phytosterols to an olive oil-based diet on cholesterol metabolism in normolipidemic humans

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ABSTRACT

To examine whether phytosterols in polyunsaturated oils account for their differential action on lipid metabolism compared with monounsaturated oils, sixteen normolipidemic individuals consumed three 10 day experimental diets containing corn oil (high in polyunsaturated fatty acids and plant sterols), olive oil (high in monounsaturated fatty acids and low in plant sterols), or olive oil supplemented with a plant sterol mixture resembling that naturally found in corn oil (high in monounsaturated fatty acids and plant sterols). During each phase, subjects consumed prepared solid food diets containing 33% of energy as fat, with two thirds of the dietary fat provided as the treatment. Plasma total cholesterol concentrations following both the olive oil and the olive oil supplemented with plant sterol treatments were significantly higher ($P<0.001$) than those following the corn oil treatment. Olive oil treatment resulted in significantly higher ($P<0.05$) plasma LDL-cholesterol and triglyceride concentrations compared to corn oil treatment. Addition of the plant sterol mixture to the olive oil diet resulted in suppression of the significant differences between LDL-cholesterol and triglyceride concentrations. Free cholesterol fractional synthetic rates determined by deuterium incorporation were lower ($P<0.05$) with olive oil treatment compared to corn oil treatment; the significance of this difference was abolished with the addition of phytosterols to the olive oil diet. These results suggest that plant sterols may be partly responsible for the differences in plasma cholesterol levels and synthesis observed between polyunsaturated and monounsaturated oils.

Supplementary key words phytosterols, corn oil, olive oil, polyunsaturated fatty acids, monounsaturated fatty acids, low-density lipoprotein cholesterol, triglycerides, cholesterol synthesis, deuterium incorporation

Although causes of coronary heart disease are multifactorial, high serum cholesterol concentrations are established risk markers. Dietary fat selection is known to exert a major influence on circulating cholesterol levels; they are raised with consumption of fats containing saturated fatty acids (SFA), and reduced with fats high in monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids (Hegsted *et al* 1965, 1993; Mattson and Grundy 1985; Mensink and Katan 1989; Mazier and Jones 1997). It has been postulated that consumption of MUFA results in a protective action on HDL concentrations whereas PUFA consumption results in a lowering of both LDL and HDL concentrations (Sirtori *et al* 1986; Baggio *et al* 1988; Mensink and Katan 1992; Mata *et al* 1992; Lichtenstein *et al* 1993); however, there is still need for consensus on this effect. In volunteers fed diets rich in PUFA versus other fatty acids, researchers have demonstrated enhanced remnant removal rates (Demacker *et al* 1991), enhanced faecal elimination (Connor *et al* 1969; Nestel *et al* 1975; Grundy 1975) and increased synthesis (Jones *et al* 1994; Mazier and Jones 1997) of cholesterol. Despite this knowledge, there is still dispute over mechanisms by which unsaturated fats reduce circulating cholesterol concentrations in humans, and whether these mechanisms are consistent across different unsaturated fats.

Oils high in PUFA are naturally abundant in plant sterols, whereas MUFA-rich oils such as olive oil have lower such concentrations. Research has demonstrated that cholesterol absorption efficiency varies inversely with the quantity of total plant sterols, in particular β-sitosterol, contained within an oil (Ikeda *et al* 1988; Heinemann *et al* 1993; Jones *et al* 1994). Thus, the enhanced turnover and synthesis of cholesterol observed following PUFA feeding could be the result of diminished absorption subsequent to micellular exclusion of cholesterol by plant sterols (Ikeda *et al* 1983). Consequently, the differential effect on circulation lipoprotein concentrations observed between oils high in PUFA and oils low in MUFA may be partly explained by the action of plant sterols contained within the PUFA-rich oil.

The present study was designed to investigate the influence of the addition of a plant sterol mixture similar to that found in corn oil to an olive oil diet on circulating plasma lipid concentrations and *de novo* cholesterol synthesis rates in normolipidemic humans. We hypothesized that the observed effects would not differ significantly from a corn oil diet which is naturally replete with plant sterols. The study also compared effects of corn oil and olive oil on the concentrations of serum lipoproteins and *de novo* cholesterol synthesis rates, and hypothesized there would be no difference among any of the diet treatments.

SUBJECTS AND METHODS

Subjects

Sixteen healthy volunteers with plasma total cholesterol <5.1 mmol/L, LDL-cholesterol <2.5 mmol/L, plasma triglycerides <2.5 mmol/L, and BMI<27 were recruited by poster and Internet advertisements. Subjects were assessed for personal and family history of high plasma lipid levels as well as willingness to participate in the dietary intervention program. Following verbal interviews, there was no indication of any chronic illness, they did not smoke, and were not taking any medication known to affect lipid metabolism such as lipid-lowering drugs, beta-blockers, diuretics, or hormones. Women selected were not pregnant and were not taking oral contraceptive agents. All subjects gave informed written consent. The study protocol was approved by the Human Investigation Ethical Review Committee of McGill University.

Experimental design

A randomized, cross-over design included three 10-day diet treatments. Each treatment period was separated by a minimum 2 week wash out period where the subjects consumed their habitual ad libitum diets. During each treatment subjects consumed a solid foods diet designed to contain 50% of energy as carbohydrate, 35% fat, and 15% protein. Non-oil constituents were identical across diets. Diets were designed to meet normal nutrient needs, as defined by Recommended Nutrient Intakes for Canadians (Health and Welfare Canada 1990). Diets contained 2/3 fat (approximately 21% of energy) as either (i) corn oil, (ii) olive oil, or (iii) olive oil plus a plant sterol mixture fed at a level of 0.4 g

per 1000 kcal. Phytosterols supplemented to the olive oil diet were chosen from capsules currently available in health food stores. The capsules chosen (Nu-Life Nutritional Products, Vancouver, Canada) were in a powder form, derived from vegetable oil, and had a similar composition to plant sterols found in corn oil. The powder phytosterol mixture was suspended in oil supplemented into each meal and administered in equal quantities at each meal. Three isocaloric meals per day were prepared, and wherever possible, treatment oils were used in all baking and cooking. All ingredients were weighed to the nearest 0.5 g. Meals were prepared and consumed on site under supervision at the Metabolic Research Unit, Macdonald Campus, McGill University. In rare cases when subjects could not be present for meals at the unit, pre-prepared meals were packaged for take-out. Subjects were continually monitored for compliance at meal times and requested not to eat or drink any additional food or beverages, except water, other than provided by the Metabolic Research Unit during each treatment period.

For each diet cycle, subjects received identical caloric loads individually determined by the Mifflin predictive equation (Mifflin *et al* 1990) and multiplied by an additional activity factor of 1.7 to yield total daily energy requirements for younger, active individuals (Bell *et al* 1985). Body weights were monitored daily during each diet cycle to ensure compliance and correct caloric intake. Any adjustments needed to caloric intake were made only within the first three days of the first cycle. This caloric load then remained constant over all three diet phases.

On days 9 and 10 of each dietary phase, duplicate fasting pre-breakfast blood samples were collected just before and 24 h after dosing with deuterium oxide (D_2O) for

the determination of plasma lipid concentrations and deuterium (D) incorporation into free and esterified cholesterol. An oral bolus dose of 0.7 g D_2O/kg estimated body water (99.8 atom percent excess, ICN Biomedicals, Montreal, Quebec) was administered at 8:00 am to each subject following the initial blood sample on day 9. Body water was estimated as 60% of body weight. Drinking water consumed during the subsequent 24 h included trace amounts of D_2O (1.4 g per kg water consumed).

Macronutrient analysis of diets

Complete 2 day meal cycle for each diet phase were chemically analysed for macronutrient content according to AOAC guidelines (1987). Moisture content was determined in duplicate samples of homogenized meal mixtures by lyophilizing (Flexi-Dry MP System, FTS Systems Inc, Stone Ridge, NY) at -80°C for 48 h. Triplicate freeze-dried samples were then ashed in an isothermic muffle furnace at 550°C for 24 h. For protein, nitrogen content was determined on freeze-dried samples using an automatic nitrogen analyser (Leco Corp., St. Joseph, MI). Crude fat was determined by pre-extracting samples with water; samples were then extracted with petroleum ether, followed by acid hydrolysis and a second petroleum ether extraction. Proximate compositions were reported as grams of macronutrient per 100 grams wet weight. Carbohydrate concentration of food samples was calculated by subtracting the sum of moisture, protein, crude fat, and ash values from 100 g. Final values are presented as percent of total energy.

Fatty acid and phytosterol analysis

Following lipid extraction (Folch *et al* 1957) and boron trifluoride methylation (Bannon *et al* 1982) of homogenized meal samples, fatty acid methyl esters were analysed on a Hewlett-Packard 5890 Series II gas-liquid chromatograph (GLC) equipped with flame ionization detectors and a 30 m x 0.25 mm ID fused-silica SP™-2330 capillary column (Supelco Inc., Bellefonte, PA). The carrier gas was helium at 1.0 ml/min with the inlet splitter set at 50:1. Temperature programmed runs were made as follows: initial temperature of 80°C for 1 min, ramp 10°C/min to 200°C, hold for 10 min, ramp 4°C/min to 250°C, hold 15 min, injector and detector set at 250°C. Fatty acid methyl esters were identified by matching retention times with 99% pure commercial standards (Nu-Chek Prep).

Phytosterol analysis was carried out following lipid extraction and saponification of the dietary oils and homogenized meals (Kuksis *et al* 1976). The nonsaponifiable lipid contents were analysed on a Hewlett-Packard 5890 Series II GLC equipped with a flame ionization detector and fitted with a 30 m x 0.25 mm ID SAC™-5 capillary column (Supelco Inc., Bellefonte, PA). The carrier gas was helium at 1.0 ml/min with the inlet splitter set at 50:1. Runs were isothermic at 275°C with the injector and detector heaters at 300°C. Sterol peaks were identified by comparison of retention times with those of authentic standards and quantitated with the use of a 5-α-cholestane internal standard (Sigma Chemical Co., St. Louis, MO).

Plasma lipid analysis

Duplicate fasting blood samples obtained on days 9 and 10 of each diet treatment were collected in vacutainer tubes containing EDTA (0.1%). Plasma and erythrocytes were separated within 2 h and stored at -80°C. With the use of an auto-analyser (Abbott VG Supersystem, Irving, TX), plasma total cholesterol (Allain *et al* 1974) and plasma triglyceride (TG) concentrations (Bucolo and David 1973) were assayed enzymatically (Sigma Co., St Louis, MO). HDL cholesterol concentration was measured similarly following dextran sulfate-magnesium precipitation of apo B-containing lipoproteins (Warnick *et al* 1985). LDL cholesterol concentrations were calculated by subtraction of TG/5 and HDL concentrations from total cholesterol using the Friedewald formula (Friedewald *et al* 1972). All samples from each subject were analysed in duplicate and within a single run.

Cholesterol synthesis determinations

Methods for determination of *de novo* cholesterol synthesis have been described previously (Jones *et al* 1988, 1993). Briefly, to determine the synthesis of free cholesterol in the rapidly turning over pool, erythrocyte total lipids were extracted in duplicate from 3 g erythrocyte samples and separated by thin-layer chromatography (TLC) with the use of hexane-diethyl ether-acetic acid 105:45:1.5 (v/v/v). For determination of deuterium enrichment of esterified cholesterol, plasma total lipids were similarly extracted from 2 g plasma and separated by TLC. Free cholesterol and cholesterol ester bands were identified with the use of standards and scraped from the TLC plates. In order to separate

deuterium enrichment of free fatty acids from that of cholesterol, cholesterol ester fractions were saponified from the silica gel with methanolic potassium hydroxide. Free cholesterol samples were eluted from the silica gel with hexane:choloroform:diethyl ether 5:2:1 (v/v/v). After drying the samples under nitrogen, the isolated cholesterol was transferred into pre-annealed Pyrex combustion tubes (18 cm x 6 mm) containing 0.5 g cupric oxide (BDH Chemicals, Toronto, ON) and 1 mm diameter silver wire (2 - 2.5 cm). Combustion tubes were then sealed under vacuum and heated at 520°C for 4 h. The water that ensued was then vacuum distilled into pre-annealed Pyrex reduction tubes (10cm x 6 mm) containing 0.06 g zinc reagent (Biogeochemical Laboratories, Bloomington, IN).

To measure plasma water deuterium enrichment, baseline plasma samples were diluted twofold while 24 h plasma samples were diluted sevenfold. These dilutions reduced the deuterium enrichment to within the range of working standards. These samples were sealed under vacuum into pre-annealed Pyrex reduction tubes containing 0.06 g of zinc. Tubes containing water from cholesterol and plasma samples were reduced at 520°C for 30 min before analysis of deuterium enrichment. Deuterium enrichment was determined by isotope ratio mass spectrometry (VG Isomass 903D, Cheshire, England). The mass spectrometer was calibrated daily against water standards of known isotopic composition. Samples for each subject were analysed in duplicate and concurrently by use of a single set of standards.

Calculations

Calculation of fractional synthetic rates (FSR) of free cholesterol in erythrocyte and esterified cholesterol in plasma is based on the methods using tritiated water by Dietschy and Spady (1984) as adapted by Jones *et al.* (1988;1993). In brief, synthesis is calculated using the difference between deuterium abundance of erythrocyte or plasma cholesterol at t=0 and t=24 h relative to the enrichment of the body water pool. As the enrichment plateau during constant ingestion of D_2O for erythrocyte or plasma cholesterol requires months to attain, the initial rate of uptake, independent of circadian rhythm, is highly linear. The initial rate provides a direct index of synthesis, independent of the total production rate. The equation for the FSR of cholesterol is expressed as the initial rate of change in cholesterol deuterium enrichment (Product) divided by the initial plasma water deuterium enrichment (Precursor) and is stated as follows:

$$\text{FSR (pools/day)} = \frac{\text{Product}}{\text{Precursor}} = \frac{\delta\ (^{o}/_{oo})_{\text{cholesterol}}}{\delta\ (^{o}/_{oo})_{\text{plasma water}} \times 0.478}$$

where $\delta_{\text{cholesterol}}$ and $\delta_{\text{plasma water}}$ refer to deuterium enrichments above baseline level over 24 h expressed as parts per thousand ($^{o}/_{oo}$) relative to the Standard Mean Ocean Water (SMOW) calibrated reference standard. The fraction 0.478 represents the proportion of protons in newly synthesized cholesterol which originate from water or water-sources (Jones 1990).

Statistical analysis

Descriptive data are expressed as mean ± SD, whereas inferential data pertaining to the objectives are expressed as mean ± SEM. The inferential data determined for each treatment group were analysed for within and between differences using a repeated measures analysis of variance (ANOVA). Due to the use of male and female subjects, gender effects were tested along with diet. Where the repeated measures ANOVA attained a significance of $P < 0.05$, specific group differences were evaluated using Tukey's pairwise comparisons post-hoc analysis. The significant between group differences were expressed as mean differences along with the 95% confidence interval (ie: mean difference (95% CI)).

RESULTS

Characteristics of study participants prior to the commencement of the study are shown in **Table 2-1**. The subjects were all young (23±2 years) with BMI (23.3±2.4 kg/m^2) and initial blood screening plasma lipid concentrations all within defined ranges. Average body weight did not significantly vary among the three diet periods (68.3±0.6 kg).

The macronutrient composition, fatty acid and sterol profiles, and cholesterol content of the diets are shown in **Table 2-2**. The diet was designed to contain 50% energy as carbohydrate, 35% fat, and 15% protein. Overall, there was good agreement between the analytical and calculated data. Carbohydrate, fat, protein, and cholesterol contents of the corn and olive oil diets were identical. Fatty acid composition and

phytosterol content variation between the diets were a direct reflection of the fatty acid and sterol composition of the oils (see **Table 2-3**). The supplemental phytosterol mixture administered was similar in composition to the phytosterols naturally found in corn oil (see **Table 2-4**). Due to the encasement of phytosterols found naturally in corn oil in an oil matrix, phytosterols found in nature may be incorporated into the micelle easier than synthetically administered phytosterol mixtures. Thus, in this study, the dose of the synthetic phytosterols was slightly higher than the dose naturally seen in the corn oil diet in order to ensure micellular incorporation of the synthetic phytosterols.

Plasma lipid level responses to diet treatment are shown in **Figure 2-1**. Plasma total cholesterol concentrations on the olive oil diet (3.71±0.15 mmol/L) and olive oil diet plus plant sterol (3.65±0.13 mmol/L) were significantly higher (P=0.0001) than on the corn oil diet (3.32±0.11 mmol/L). There was no significant difference between the olive oil and olive oil diet plus plant sterol diets (mean difference: 0.06 mmol/L (95% CI: -0.13 to 0.25 mmol/L)).

The olive oil treatment (2.17±0.12 mmol/L) resulted in significantly higher (P<0.05) plasma LDL-cholesterol concentrations than the corn oil treatment (1.99±0.12 mmol/L). With the addition of the plant sterol mixture to the olive oil diet (2.11±0.12 mmol/L), the plasma LDL-C concentrations were lower so there was no longer a significant difference from the corn oil diet (0.12 mmol/L (95% CI: -0.05 to 0.29)); however, this lower mean LDL-C concentration was not statistically different from the olive oil diet (-0.07 mmol/L (95% CI: -0.24 to 0.10 mmol/L)).

Plasma triglyceride concentrations responded in a similar manner with a significantly ($P<0.05$) higher plasma concentration of 0.15 mmol/L (95% CI: 0.01 to 0.28 mmol/L) following the olive oil treatment (0.85±0.07 mmol/L) when compared to the corn oil treatment (0.70±0.04 mmol/L). The addition of the phytosterol mixture to the olive oil diet resulted in plasma triglyceride concentrations following the same trend as was seen with LDL-C, with a non-significantly lower plasma concentration of -0.05 mmol/L (95% CI: -0.18 to 0.09 mmol/L) relative to the olive oil diet alone, yet the mean concentration of 0.80±0.06 mmol/L was no longer significantly different from the corn oil group (0.10 mmol/L (95% CI: -0.03 to 0.23 mmol/L)).

There were no significant differences among HDL-cholesterol concentrations following corn oil (1.05±0.05 mmol/L), olive oil (1.14±0.06 mmol/L), or olive oil plus plant sterols (1.17±0.05 mmol/L) treatments. There was a gender difference detected where female subjects had consistently higher ($P=0.03$) plasma concentrations of HDL-cholesterol than male subjects over each diet treatment. Nevertheless, both genders responded the same to each diet treatment with no within group differences or diet combined with gender group differences detected. For this reason, groups were not stratified.

Free cholesterol (FC) fractional synthetic rates (FSR) for the three diet treatments are shown in **Figure 2-2**. FC-FSR following the corn oil treatment (0.061±0.009 pools/day) was significantly higher (0.034 pools/day (95% CI: 0.008 to 0.059 pool/day); $P<0.05$) than the olive oil treatment (0.028±0.004 pools/day). With the addition of plant sterols to olive oil , the FC-FSR was higher by 0.019 pools/day, although this higher rate

was not statistically significant (95% CI: -0.007 to 0.044 pools/day). Nevertheless, this higher rate did render the FC-FSR of the plant sterol treatment group no longer significantly different from the corn oil treatment (-0.0152 pools/day (95% CI: -0.0410 to 0.0106 pools/day)).

Deuterium incorporation rates into esterified cholesterol did not differ among the corn oil (0.0197±0.0078 pools/day), olive oil (0.0225±0.0057 pools/day), and olive oil plus plant sterol (0.0198±0.0064 pools/day) treatments.

DISCUSSION

Although research has suggested that both MUFA and PUFA fats have the same influence on plasma lipid concentrations (Mattson and Grundy 1985; Mensink and Katan 1989; Nydahl *et al* 1994), a sizable body of data concludes that PUFA fats are more effective at lowering total and LDL-C concentrations (Sirtori *et al* 1986; Dreon *et al* 1990; Mensink and Katan 1992; Lichtenstein *et al* 1993; Jones *et al* 1994; Howard *et al* 1995; Mazier and Jones 1997). The results from the present study agree with this body of data demonstrating that olive oil consumption was associated with higher plasma total cholesterol, LDL-C, and triglyceride concentrations, as well as lower *de novo* cholesterol synthesis rates, when compared to corn oil consumption. HDL-C concentrations did not differ among any of the diet treatments.

Previous studies either attribute these differences between corn and olive oil entirely to the fatty acid content of the oils (Sirtori *et al* 1986; Lichtenstein *et al* 1993), or have only alluded to the differential phytosterol content of the oils as a potential factor

responsible for related variations in cholesterol metabolism (Grundy 1989; Jones *et al* 1994; Howard *et al* 1995). To our knowledge, the present study design is the first to systematically determine if phytosterols naturally present in corn oil could at least partly explain the differential cholesterol metabolism observed between these two oils. The addition of phytosterols to an olive oil diet did not significantly modulate plasma lipid concentrations or *de novo* cholesterol synthesis rates when compared to the olive oil diet; nevertheless, phytosterol administration did suppress the significant differences between the corn and olive oil treatments.

The second objective of this study was to determine if phytosterols play a role in modulating cholesterogenesis during corn oil feeding. Although somewhat counterintuitive, enhanced rates of cholesterogenesis with consumption of PUFA versus other fats have been observed previously (Moore *et al* 1968; Connor *et al* 1969; Nestel *et al* 1973; Grundy 1975; Jones *et al* 1994a, 1994b; Mazier and Jones 1997) and in the present study. These results suggest a fundamental difference in how various plant oils elicit their cholesterol-lowering effect. It was postulated that phytosterols in corn oil block absorption of dietary and biliary cholesterol, thereby reducing body pools and desuppressing cholesterol synthesis. In the present study, when phytosterols were added to the olive oil diet, cholesterol synthesis was enhanced so that there was no longer a significant difference from the corn oil diet; thus, suggesting that phytosterols are at least partly responsible for the differential effects on cholesterol synthesis observed between MUFA and PUFA oils. However, despite the changes observed in cholesterol metabolism with the addition of phytosterols to the olive oil diet, plasma total cholesterol

concentrations were not affected. Furthermore, although LDL-C and triglyceride concentrations and cholesterol synthesis rates were influenced by phytosterol supplementation, the results were not significantly different from the olive oil diet.

This study demonstrated higher plasma triglyceride concentrations following olive oil treatment than following corn oil treatment. In most clinical trials comparing effects of MUFA and PUFA oils, circulating triglyceride concentrations were not altered (Sirtori *et al* 1986; Dreon *et al* 1990; Lichtenstein *et al* 1993; Jones *et al* 1994a; Mazier and Jones 1997); however, epidemiological studies demonstrated that plasma triglycerides concentrations correlated positively with MUFA and negatively with PUFA (Takita *et al* 1996; Sonnenberg *et al* 1996). It has been well established that VLDL particles are the main transporters of triglyceride in plasma, therefore, plasma triglyceride concentrations have been categorized as surrogate indicators of plasma VLDL concentrations. In addition, dietary cholesterol has been shown to stimulate output of all VLDL lipids (Fungwe *et al* 1993). Therefore, it could be postulated that if corn oil phytosterols blocked dietary cholesterol from entering circulation, plasma VLDL concentrations would be expected to be suppressed during corn versus olive oil treatment. Furthermore, this study determined that the addition of phytosterols to the olive oil diet suppressed plasma triglyceride concentrations to levels that were no longer significantly different from the corn oil treatment; thus adding the theory that phytosterols in corn oil may be indirectly affecting triglyceride metabolism through decreasing absorption of dietary cholesterol.

Other dietary factors may also explain the differential cholesterol metabolism observed during corn versus olive oil consumption. Squalene is found in high

concentrations in olive oil (Grundy *et al* 1989; Miettinen and Vanhanen 1994; Howard *et al* 1995). Squalene may down-regulate hydroxy-methylglutaryl-CoA reductase activity through promotion of cholesterol synthesis further along its biosynthetic pathway. However, in general, the ingestion of squalene would be expected to enhance cholesterol synthesis, which was lower in the olive oil-treated subjects. Fatty acid compositional differences between the two oils may also account for dietary fat related effects on cholesterol metabolism. One mechanism by which dietary cis-unsaturated fatty acids lower LDL-cholesterol may possibly involve an alteration in membrane lipid composition or membrane fluidity that promotes enhance LDL receptor function, thereby leading to increased hepatic clearance of LDL (Kuo *et al* 1990a). Since PUFA oils would create a more fluid membrane than MUFA oils, it could be postulated that PUFA oils would lead to higher clearance of LDL than MUFA oils. It has been demonstrated that linoleate has a 50% greater molar potency in augmenting LDL metabolism than that of oleate (Kuo *et al* 1990b). Furthermore, in a study where polyunsaturated triacylglycerols were substituted for saturated triacylglycerols, LDL receptor activity increased from 25% to 80% of control and reduced LDL-cholesterol production rate from nearly 200% to 155% (Woollet *et al* 1992). In the same study, saturated triacylglycerols also caused a dose-dependent increase in the LDL-cholesterol production rate and markedly increased the plasma LDL-cholesterol level while the PUFA lipid did not affect either of these (Woollett *et al* 1992). This has yet to be demonstrated for MUFA lipids.

Although the results from this study did not demonstrate a strong relationship between phytosterol administration and modifications in cholesterol metabolism, even the

minor effects observed suggest that phytosterols naturally present in corn oil cannot be discounted as factors affecting cholesterol metabolism. One explanation for this lack of effect could be due to the form of phytosterols administered in this study. Phytosterols found naturally in oils are normally in the esterified and non-esterified forms and are encased in a matrix of oil (Weihrauch and Gardner 1978; Pollack and Kritchevski 1981). These characteristics increase phytosterol solubility, therefore, increase their susceptibility for uptake into intestinal micelles. It has been elucidated that phytosterols act primarily at the level of the micelle to displace cholesterol, therefore, absorption and reabsorption of cholesterol is decreased in the presence of phytosterols (Ikeda *et al* 1988). In the present study, phytosterols were administered as a non-esterified powder; thus, they were not in the same form as the natural phytosterols, and not as likely to displace cholesterol from the micelle. In order to bypass this effect, the non-esterified phytosterol powder was administered in a larger dose than that found naturally in corn oil, therefore allowing for competition with cholesterol for micellular inclusion; nonetheless, whether this actually occurred could not be verified.

In summary, our findings confirm that corn oil is superior to olive oil at lowering plasma total and LDL-cholesterol concentrations; however, no difference in HDL-C concentrations were detected between the two oils. The results also provide evidence that phytosterols are at least partly responsible for the differential effects between corn and olive oil on plasma lipid concentrations and cholesterol synthesis rates. However, due to the variability in phytosterol content among PUFA oils, the results from the current study are limited to corn oil and cannot be extrapolated to any other PUFA oils. Consequently,

it is recommended that further research be conducted investigating the contribution of phytosterols contained in other PUFA oils on lipid metabolism and exploring possible interactions between phytosterols and other dietary constituents of oils.

Table 2-1
Characteristics of Study Subjects

Age, y	22 ± 1	24 ± 2	23 ± 2
Body weight, kg	59 ± 5	77 ± 9	68 ± 11
Height, m	1.63 ± 0.09	1.78 ±0.05	1.71 ± 0.10
Body mass index, kg/m^2	22.3 ± 2.4	24.3 ± 2.1	23.3 ± 2.4
Total cholesterol, mmol/L	4.06 ±0.39	3.69 ±0.47	3.87 ±0.47
LDL-C, mmol/L	2.03 ±0.45	2.00 ±0.57	2.00 ±0.52
HDL-C, mmol/L	1.61 ±0.26	1.30 ±0.23	1.46 ±0.29
Triglycerides, mmol/L	0.92 ±0.18	0.86 ±0.32	0.89 ±0.25

Values expressed as mean ± SD.
Abbreviations: LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

Table 2-2
Composition of Experimental Diets

	[illegible]	[illegible]
	(% of total energy)	
Carbohydrate[1]	52.7 ± 3.7	52.4 ± 3.9
Protein	15.0 ± 1.5	15.0 ± 1.4
Fat	32.3 ± 2.8	32.7 ± 2.8
Fatty Acids	(% of total fat)	
Saturated:	**6.7**	**7.4**
C8:0	0.1	0.1
C10:0	0.4	0.2
C12:0	0.7	0.5
C14:0	0.6	0.6
C16:0	3.8	4.6
C18:0	1.2	1.5
MUFA:	**8.6**	**20.9**
C16:1n7	0.3	0.5
C18:1n9	8.3	20.4
PUFA:	**17.0**	**4.3**
C18:2n6	16.7	3.9
C18:3n3	0.3	0.4
	(mg/1000kcal)	
Cholesterol	128.9	128.9
Phytosterols:	(mg/1000kcal)	
Total	**218.9**	**66.6**
Campesterol	48.6	0.0
Stigmasterol	7.2	0.0
β-sitosterol	163.1	66.6

[1] Calculated by subtracting wet weights of moisture, protein, crude fat, and ash from 100g, therefore, overestimated by 1-5% due to crude fibre content.

Values expressed as means ± SD.

Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2-3
Composition of Dietary Oils

	[illegible]	[illegible]
	Phytosterol content	
PHYTOSTEROLS:	**(mg/100g)**	
Campesterol	210	0
Stigmasterol	65	0
β-Sitosterol	555	225
Sitostanol	ND	trace
Total	**830**	**225**
	Fatty acid composition	
FATTY ACIDS:	**(% of total fat)**	
Saturated:	**14**	**17.8**
C14:0	0.0	0.0
C16:0	12.3	16.5
C18:0	1.6	1.3
MUFA:	**28**	**72.9**
C16:1n7	0.0	0.9
C18:1n9	27.8	72.0
PUFA:	**58**	**9.3**
C18:2n6	55.5	8.2
C18:3n3	2.8	1.1

Abbreviations: ND, none detected; trace, trace amount detected <10 mg; MUFA, monounsaturated fatty acids; PUFA polyunsaturated fatty acids

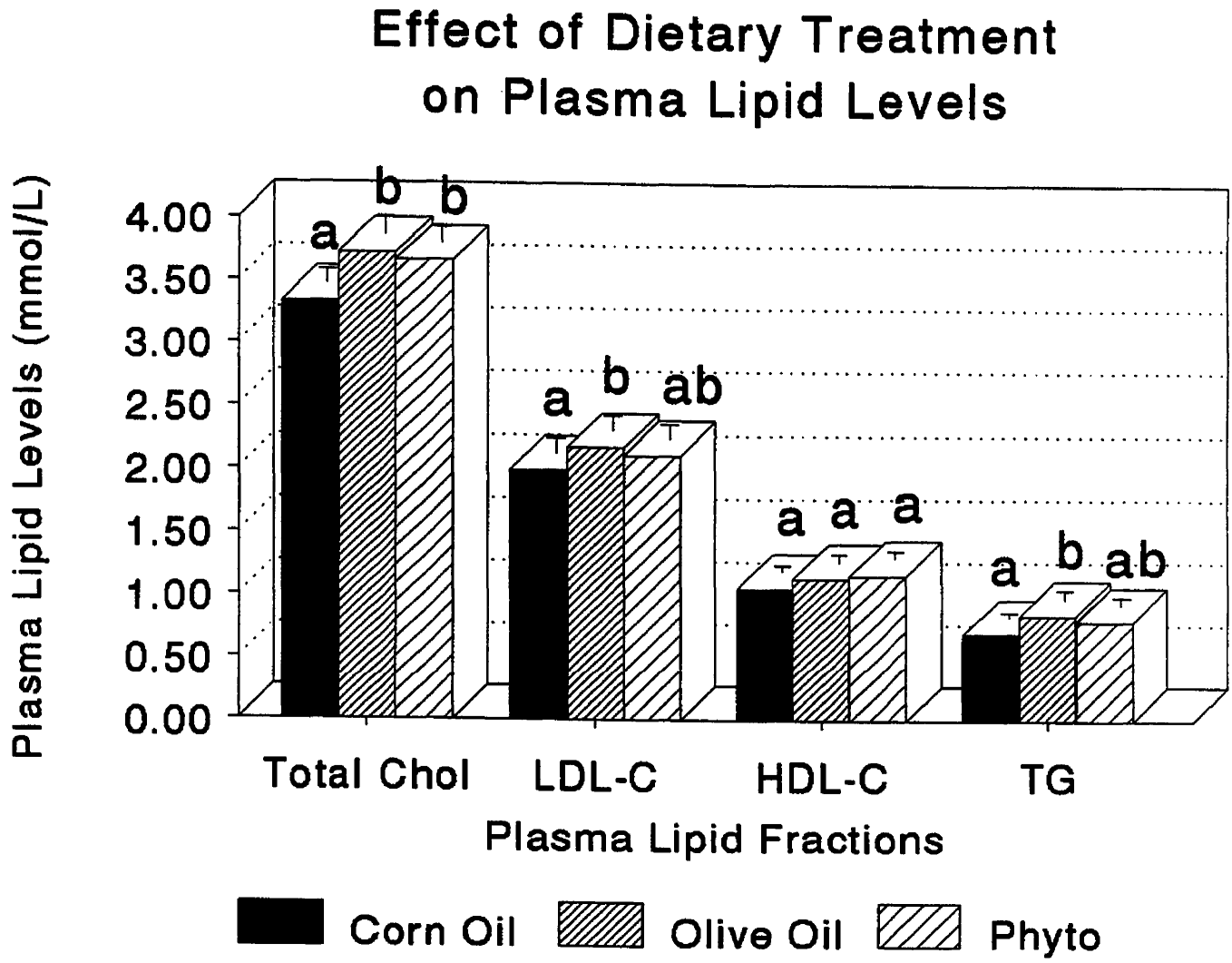
Table 2-4
Phytosterol Content of Each Diet Treatment

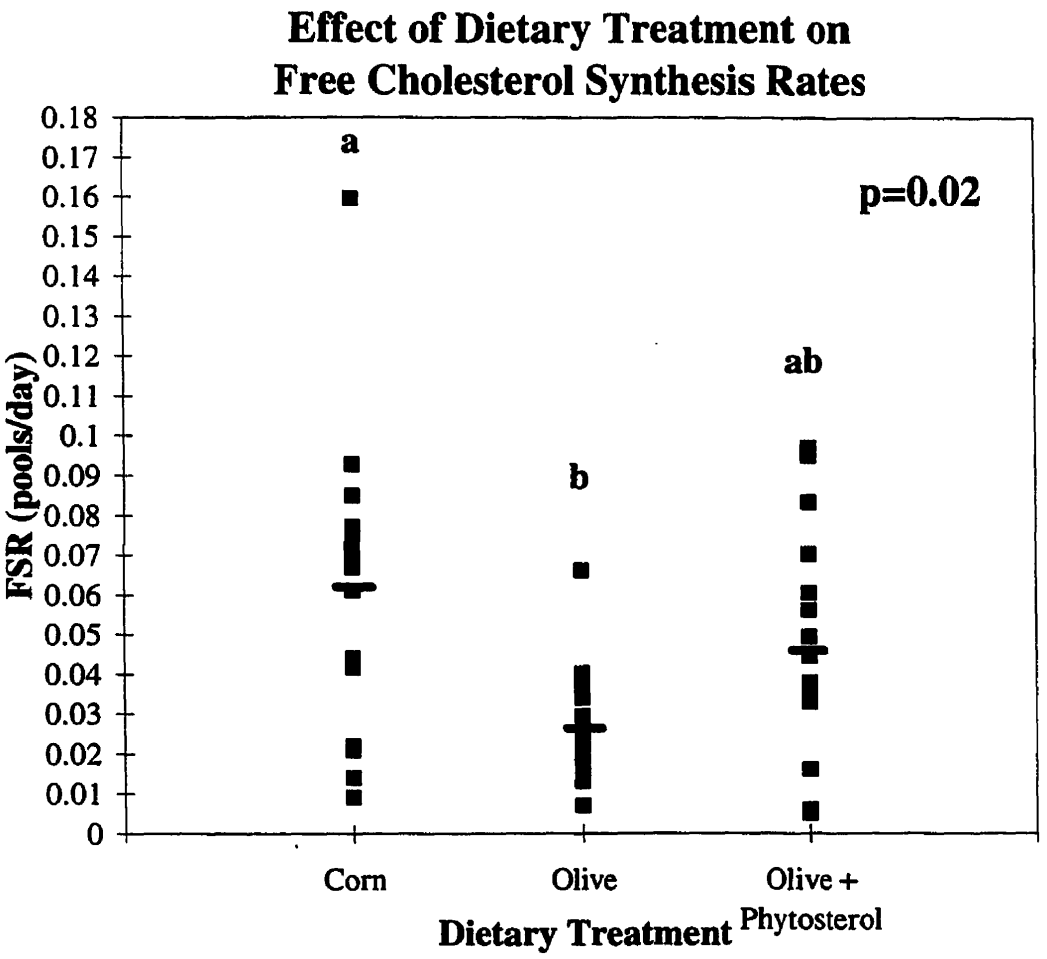
	Composition (% of total)		
Campesterol	**23.7**	**25.3**	**0.0**
Stigmasterol	**14.4**	**7.8**	**0.0**
β-Sitosterol	**61.9**	**66.9**	**100.0**
	Average daily dose (range, g)		
Total:	**1.45 (1.17, 1.69)**	**0.63 (0.49, 0.75)**	**0.22 (0.17, 0.26)**
β-Sitosterol	0.98 (0.81, 1.13)	0.47 (0.36, 0.56)	0.22 (0.17, 0.26)

FIGURE LEGENDS

Figure 2-1 Plasma lipid concentrations of total cholesterol (Total Chol), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglycerides (TG) in healthy male and female humans (n=16) consuming either a corn oil diet (Corn Oil), an olive oil diet (Olive Oil), or an olive oil plus phytosterol diet (Phyto) for 10 days. Results are expressed as mean $\pm$ SEM. Diet treatment group means within each parameter having different subscripts differ significantly ($P<0.05$ using Tukey's pairwise comparisons).

Figure 2-2 Free cholesterol fractional synthetic rates (FSR) in healthy male and female humans (n=16) consuming either a corn oil diet (Corn), an olive oil diet (Olive), or an olive oil plus phytosterol diet (Olive + Phytosterol) for 10 days. Dietary treatment group means having different subscripts differ significantly ($P<0.05$ using Tukey's pairwise comparisons).


Effect of Dietary Treatment
on Plasma Lipid Levels
Plasma Lipid Levels (mmol/L)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
b
b
a
b
ab
a
a
a
a
b
ab
Total Chol
LDL-C
HDL-C
TG
Plasma Lipid Fractions
Corn Oil
Olive Oil
Phyto


Effect of Dietary Treatment on
Free Cholesterol Synthesis Rates
FSR (pools/day)
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
b
ab
p=0.02
Corn
Olive
Olive + Phytosterol
Dietary Treatment

4. *TRANSITION*

Results from manuscript one demonstrated that the addition of phytosterols normally found in corn oil to an olive oil-based diet suppress the differences in cholesterol metabolism observed between corn and olive oil. Nevertheless, the sitostanol-free phytosterol mixture did not prove to be significantly different from the olive oil-based diet alone. With recent reports praising the plasma lipid lowering properties of sitostanol, the 5α-saturated derivative of β-sitosterol, a fourth diet cycle was added to the three diet cycles of manuscript one; the objective was to determine if the addition of sitostanol to a phytosterol mixture would result in a more effective cholesterol-lowering agent.

Manuscript two is based on data from eleven (6 men and 5 women) out of the original sixteen subjects from the previous three diet cycles. The subjects were administered a fourth treatment group composed of a phytosterol mixture enriched with 21% sitostanol added to the olive oil-based diet. This treatment group was compared to the olive oil alone and the olive oil plus sitostanol-free treatments described in manuscript one.

Effects of sitostanol-enriched versus sitostanol-free phytosterol mixtures on sterol metabolism in normolipidemic humans

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ABSTRACT

The following study examined the effects of adding low doses of sitostanol-free and sitostanol-enriched phytosterol mixtures to an olive oil based diet on sterol metabolism in normolipidemic humans. Eleven healthy normolipidemic subjects consumed three 10-day diets consisting of olive oil, olive oil supplemented with a sitostanol-free phytosterol mixture, and olive oil supplemented with a sitostanol-enriched phytosterol mixture. During each phase, subjects consumed prepared solid food diets containing 52% of energy as carbohydrate, 15% of energy as protein, and 33% of energy as fat with two-thirds of the dietary fat provided as olive oil. The addition of the sitostanol-enriched phytosterol mixture to the olive oil diet was the only dietary treatment that significantly lower plasma total and LDL-cholesterol concentrations (mean difference from olive oil alone: -0.238 mmol/L (95% CI: -0.436 to -0.040 mmol/L) and -0.279 mmol/L (95% CI: -0.421 to -0.137 mmol/L), respectively). Although there was no difference detected in plasma HDL-cholesterol concentrations among the treatment groups, the lower plasma LDL-cholesterol concentrations observed with the addition of the sitostanol-enriched phytosterol mixture to the olive oil diet resulted in a significantly higher plasma HDL/LDL cholesterol ratio (mean difference from olive oil alone: 0.145 (95% CI: 0.062 to 0.228)). Furthermore, the sitostanol-enriched phytosterol mixture resulted in a higher HDL/LDL ratio than the sitostanol-free phytosterol mixture (difference between the two phytosterol mixtures: 0.107 (95% CI: 0.026 to 0.188)). These results indicate that the cholesterol-lowering capacity of a phytosterol mixture containing primarily sitosterol is enhanced with the addition of low doses of sitostanol.

KEY WORDS Phytosterols, sitostanol, sitosterol, corn oil, olive oil, plasma lipid levels, cholesterol synthesis, deuterium

INTRODUCTION

The most common naturally-occurring plant sterols, campesterol and β-sitosterol, are consumed at a typical level of 160 to 360 mg/day (Weihrauch and Gardner 1978; Nair *et al* 1984). Despite their similarity to cholesterol, the presence of methyl and ethyl groups at the C24 position of the side chains of campesterol and β-sitosterol, respectively, results in their poor absorption (Heinemann *et al* 1986; 1991; 1993). Therefore, only small quantities, 0.3 - 1.7 mg/dl of phytosterols are found in human serum under normal conditions (Ling and Jones 1995). The possible therapeutic importance of plant sterols is based on the fact that they are capable of interfering with intestinal cholesterol absorption (Grundy and Mok 1976; Mattson *et al* 1982; Heinemann *et al* 1991, 1993), thus acting as cholesterol lowering agents (Lees *et al* 1977; Sugano *et al* 1977; Chandler *et al* 1979; Heinemann *et al* 1993; Vanhanen *et al* 1993; Miettinen *et al* 1995; Pelletier *et al* 1995).

Sitostanol, the 5α-saturated derivative of β-sitosterol, is virtually unabsorbed at the level of the intestine (Ikeda *et al* 1988; Vanhanen and Miettinen 1992) and appears to reduce the intestinal absorption of cholesterol more effectively than β-sitosterol (Sugano *et al* 1977; Heinemann *et al* 1991). Hence, recent studies (Heinemann *et al* 1993; Vanhanen *et al* 1993; Miettinen *et al* 1995) have demonstrated that 1.5 - 3.0 g per day of sitostanol efficiently reduces plasma total and LDL-cholesterol concentrations. Nonetheless, it has also been demonstrated that <1 g/day (Miettinen and Vanhanen 1994)

and 3 g/day (Denke 1994) of sitostanol have no effect on reducing plasma lipid levels. No studies have been performed in humans to determine the effect of combining low doses of sitostanol with other phytosterols on plasma lipid levels and sterol metabolism.

Consequently, the present study was undertaken to ascertain whether the presence of low doses of sitostanol (<0.5 g per day) in a phytosterol mixture containing primarily β-sitosterol could detectably interfere with cholesterol absorption, and subsequently result in enhanced synthesis and reduced circulating concentrations of cholesterol.

SUBJECTS AND METHODS

Subjects

Eleven healthy volunteers with plasma total cholesterol <5.1 mmol/L, LDL-cholesterol <2.5 mmol/L, plasma triglycerides (TG) <2.5 mmol/L, and BMI<27 were recruited by poster and Internet advertisements. After verbal screening which provided medical history and a physical examination by a physician, there was no indication of any chronic illness, they did not smoke and were not taking any medication known to affect lipid metabolism (lipid-lowering drugs, beta-blockers, diuretics, or hormones). All subjects gave informed consent on the understanding they could withdraw at any time from the study which was approved by the Ethical Committee of McGill University.

Experimental design

The diet treatments were administered for 10 days with a minimum 2 week wash out period between each phase where the subjects consumed their habitual ad libitum

diets. The olive oil and olive oil plus sitostanol-free phytosterol mixture treatments were randomized, however, the olive oil plus sitostanol-enriched phytosterol mixture (21% sitostanol mixture) treatment was administered a minimum of 4 weeks following the two diet cycles. For each 10 day diet cycle, subjects consumed a solid food diet contained 52% of energy as carbohydrate, 33% fat, and 15% protein with 2/3 of fat (approximately 21% of energy) administered as olive oil. The two phytosterol mixtures added to the olive oil diet contain either soy-based phytosterols (62% β-sitosterol) fed at a level of 0.4 g per 1000 kcal, or tall oil-based phytosterols (21% sitostanol enriched) administered at 1.5 g per 70 kg body weight. The diets were designed so as to meet normal nutrient needs, as indicated by Recommended Nutrient Intakes for Canadians (Health and Welfare Canada 1990).

The diets were administered as three isocaloric meals per day prepared using fresh, canned, and frozen ingredients purchased at a local supermarket; wherever possible, olive oil used in all baking and cooking. All ingredients were weighed to the nearest 0.5 g. Meals were prepared and consumed on site under supervision at the Metabolic Research Unit, Macdonald Campus, McGill University. In rare cases when subjects could not be present for meals at the unit, pre-prepared meals were packaged for take-out. Subjects were continually monitored for compliance during meal times and requested not to eat or drink any additional food or beverages, except water, other than that provided by the Metabolic Research Unit during each treatment. For each diet cycle, subjects received identical caloric loads individually determined by the Mifflin predictive equation (Mifflin *et al* 1990) and multiplied by an additional activity factor of 1.7 to yield total daily energy

requirements for younger, active individuals (Bell *et al* 1985). Body weights were monitored daily during each diet cycle to ensure compliance and correct caloric intake. Any adjustments needed to caloric intake were made only within the first three days of the first cycle. This caloric load then remained consistent over all three diet phases.

On days 9 and 10 of each dietary phase, fasting blood samples were collected just before and 24 h after dosing with deuterium oxide (D_2O) for the determination of plasma lipid levels, sterol levels, and deuterium (D) incorporation into cholesterol. An oral bolus dose of 0.7 g of D_2O per kilogram body water (estimated as 60% of body weight) was administered pre-breakfast on Day 9 at 8:00 am to each subject following the initial blood sample. Any water consumed during the following 24 h contained trace amounts of D_2O (1.4 g per kg water consumed).

Phytosterol analysis

Phytosterol analysis was carried out following lipid extraction and saponification of the plasma, phytosterol supplements, and homogenized meals (Kuksis *et al* 1976) . The nonsaponifiable lipid contents were analysed on a Hewlett-Packard 5890 Series II gas-liquid chromatograph equipped with a flame ionization detector and fitted with a 30 m x 0.25 mm ID SAC^{TM}-5 capillary column (Supelco Inc., Bellefonte, PA). The carrier gas was helium at 1.0 ml/min with the inlet splitter set at 50:1. Runs were isothermic at 275°C with the injector and detector heaters at 300°C. Sterol peaks were identified by comparison of retention times with those of authentic standards and quantitated with the use of a 5-α-cholestane internal standard (Sigma Chemical Co., St. Louis, MO). To

eliminate the effects of varying concentrations of lipoproteins transporting plant sterols, plasma phytosterol concentrations were expressed in terms of mmol/mol of total cholesterol (Miettinen and Vanhanen 1994).

Plasma lipid analysis

Fasting blood samples obtained on the morning of day 9 and 10 of each diet cycle were collected in vacutainer tubes containing EDTA (0.1%). Samples were centrifuged at 1500 rpm at 4°C for 15 min within 2 h of the blood draw, separated from erythrocytes, and stored at -80°C until further analysis. The only exception to this routine was the initial lipid screening values which were analysed immediately after centrifuging. With the use of an autoanalyser (Abbott VG Supersystem), total plasma cholesterol (Allain *et al* 1974) and triglyceride (TG) concentrations (Bucolo and David 1973) were assayed enzymatically using 75-100 µl plasma samples. HDL-cholesterol concentrations were measured similarly after precipitation of apo-B containing lipoproteins (Warnick *et al* 1985). LDL-cholesterol concentrations were calculated by subtraction of triglyceride and HDL concentrations from total cholesterol concentrations using the Friedewald formula (Friedewald *et al* 1972).

Cholesterol synthesis determinations

Methods have been described previously (Jones *et al* 1988, 1993). Briefly, erythrocyte total lipids were extracted from duplicate 3 g erythrocyte samples and separated by thin-layer chromatography (TLC). Separation by TLC was achieved with the

use of hexane-diethyl ether-acetic acid 105:45:1.5 (v/v/v). Cholesterol bands were identified, scraped from the TLC plates, and then eluted from the silica gel with the use of hexane:choloroform:diethyl ether 5:2:1 (v/v/v). After drying the samples under nitrogen gas, the resultant cholesterol was transferred into pre-annealed Pyrex combustion tubes containing 0.5 g cupric oxide and silver wire (2 - 2.5 cm). The combustion tubes were then sealed under vacuum and heated in an oven for 4 h at 520°C. The resultant water was then vacuum distilled into pre-annealed Pyrex reduction tubes containing 0.06 g zinc reagent. The resultant gas was analysed for deuterium enrichment.

In order to determine the deuterium enrichment of the precursor body water pool, plasma samples were diluted with tap water to bring enrichments within the range of the working standards. Diluted plasma samples were reduced over zinc in pre-annealed Pyrex tubes at 520°C before analysis of deuterium enrichment.

Deuterium enrichment was determined using isotope ratio mass spectrometry (VG Isomass 903D, Cheshire, England). The mass spectrometer was calibrated daily against water standards of known isotopic abundance. All samples for each subject were analysed in duplicate on the same day using a single standard set.

Calculations

Calculation of fractional synthetic rate (FSR) of cholesterol in RBC is based on the methods using tritiated water by Dietschy and Spady (1984) as adapted by Jones *et al* (1988;1993). In brief, synthesis is calculated using the difference between deuterium enrichment of erythrocyte cholesterol at t=0 and t=24 h relative to the enrichment of the

body water pool. The erythrocyte cholesterol represents the enriched product and the body water represents the precursor pool from which the cholesterol derives its deuterium. Therefore, the equation for the FSR of cholesterol is as follows:

$$\text{FSR (pools/day)} = \frac{\text{Product}}{\text{Precursor}} = \frac{\delta\ (^{o}/_{oo})_{\text{cholesterol}}}{\delta\ (^{o}/_{oo})_{\text{plasma water}} \times 22\ \text{D}/46\ \text{C}}$$

where $\delta_{\text{cholesterol}}$ and $\delta_{\text{plasma water}}$ refer to deuterium enrichments above baseline level over 24 h expressed as parts per thousand ($^{o}/_{oo}$) relative to the Standard Mean Ocean Water (SMOW) calibrated reference standard. The fraction 22 D (deuterium)/46 C(carbon) represents the proportion of protons in newly synthesized cholesterol which originate from deuterium enriched body water and NADPH (Jones 1990).

Statistical analysis

Descriptive data are expressed as mean $\pm$ SD, whereas inferential data pertaining to the objectives are expressed as mean $\pm$ SEM. The inferential data determined for each treatment group were analysed for within and between differences using repeated measures analysis of variance (ANOVA) with gender as a co-variate. Where the repeated measures ANOVA attained a significance of $P< 0.05$, specific group differences were evaluated using Tukey's pairwise post-hoc analysis. The significant between group differences were expressed as mean differences with the 95% confidence interval.

RESULTS

The characteristics of the study participants, including their baseline lipids, are shown in **Table 3-1**. The mean age of the six men and five women was 23±2 years and the average body mass index was 23.2±2.4 (mean ±SD). All subjects were normolipidemic with mean screening plasma total and LDL cholesterol levels of 3.8±0.5 and 2.0±0.6, respectively. The body weights of the volunteers were similar at the start of each study period and were stable throughout each study period (70.0±0.2 kg). All subjects completed every dietary treatment.

The two phytosterol mixtures administered differed in composition (see **Table 3-2**). Both mixtures contained approximately equal amounts of β-sitosterol, the difference between the mixtures was seen in the amount of sitostanol and stigmasterol. Small absolute daily dose of β-sitosterol were used during each of the phytosterol trials (0.99 g in sitostanol-free phytosterol mixture, 1.13 g in sitostanol-enriched phytosterol mixture). Both phytosterol mixtures were tolerated well by all subjects, with no reported side-effects.

Serum lipids

Gender effects were tested for all plasma lipid measurements and there was no significant difference between male and female subjects; therefore, results were analysed as one group. **Figure 3-1** summarizes the plasma lipid levels for all treatments. Total and LDL-cholesterol concentrations were lower with the addition of the sitostanol-enriched phytosterol mixture to the olive oil diet (mean difference from olive oil alone: -0.238

mmol/L (95% CI: -0.436 to -0.040 mmol/L) and -0.279 mmol/L (95% CI: -0.421 to -0.137 mmol/L), respectively). Furthermore, the sitostanol-enriched mixture appeared to be more effective than the sitostanol-free phytosterol mixture at lowering plasma total and LDL-cholesterol concentrations (mean difference between phytosterol groups: -0.231 mmol/L (95% CI: -0.425 to -0.038 mmol/L) and -0.220 mmol/L (95% CI: -0.360 to -0.082 mmol/L), respectively). The addition of the sitostanol-free phytosterol mixture did not change the circulating plasma concentrations of any lipid fraction.

The lower plasma LDL-cholesterol concentrations were reflected in significantly higher plasma HDL/LDL cholesterol ratios (0.145 (95% CI: 0.062 to 0.228)) when the sitostanol-enriched tall-oil based phytosterol mixture was added to the olive oil diet (see **Table 3-3**). Furthermore, the sitostanol-enriched phytosterol mixture consumption resulted in higher HDL/LDL ratios than consumption of the sitostanol-free phytosterol mixture (0.107 (94% CI: 0.026 to 0.188)).

Plasma phytosterol levels

Plasma phytosterol concentrations were extremely variable among individuals, therefore no significant differences were detected among any of the groups, however trends were detected between the means of the treatment groups (see **Figure 3-2**). Overall, plasma campesterol levels were higher than plasma sitosterol levels. This was exemplified in the ratio of campesterol to sitosterol (see **Table 3-4**). Sitostanol was not detected in the plasma of any of the groups, confirming the speculation that sitostanol is not absorbed into circulation. Plasma mean campesterol concentrations were highest in

the sitostanol-free phytosterol supplemented (28.91±5.72 mmol/mol (mean ± SEM)) group. Olive oil (19.57±3.67 mmol/mol) treatment resulted in slightly lower plasma mean campesterol concentrations, however, the sitostanol-enriched phytosterol mixture treatment demonstrated the lowest mean campesterol concentrations (13.43±4.03 mmol/mol).

Plasma sitosterol concentrations were considerably lower than plasma campesterol concentrations, with the olive oil (1.58±0.30 mmol/mol) and sitostanol-free phytosterol mixture (1.65±0.24 mmol/mol) treatments having higher concentrations. The sitostanol-enriched phytosterol mixture (3.04±1.12 mmol/mol) treatment appeared to have the highest concentration of sitosterol relative to cholesterol in the plasma, although, these differences were not statistically significant due to the inter-subject variability.

Campesterol to sitosterol (C:S) ratios varied from 1.05 to 46.50 (see **Table 3-4**). However, the C:S ratios did appear to follow the trend found with the plasma campesterol levels relative to cholesterol, with the sitostanol-free phytosterol mixture (18.65±3.97) treatment having the highest ratio, followed by the olive oil treatment (16.43±4.82), and the sitostanol-enriched phytosterol mixture (11.69±6.64) having the lowest overall C:S ratio.

Free cholesterol synthesis rates

Deuterium incorporation rates expressed as fractional synthetic rates (FSR, pools per day) are shown in **Figure 3-3**. Synthesis rates following the olive oil treatment (0.0295±0.005 pools/day) did not differ from either the sitostanol-free (0.0435±0.009

pools/day) or the sitostanol-enriched (0.0505±0.004 pools/day) phytosterol treatments. No significant differences were detected among the phytosterol treatments.

DISCUSSION

The present study demonstrated that the addition of a sitostanol-enriched phytosterol mixture to an olive oil based diet significantly decreased plasma total and LDL-cholesterol concentrations and increased HDL/LDL ratios in normolipidemic male and female volunteers. Furthermore, the sitostanol-enriched phytosterol mixture was more effective than the sitostanol-free phytosterol mixture at decreasing plasma total and LDL-cholesterol concentrations and increasing the HDL/LDL ratio. The addition of either phytosterol mixture to an olive oil based diet had no effect on *de novo* cholesterol synthesis rates or plasma phytosterol concentrations.

These data suggest a more effective plasma lipid lowering phytosterol mixture is created with the addition of sitostanol to a phytosterol mixture. This is best exemplified with the sitostanol-enriched phytosterol mixture being statistically more effective at increasing the HDL/LDL ratio than the sitostanol-free phytosterol mixture. The results from this study correlate well with recent studies investigating the effectiveness of sitostanol at lowering plasma lipid levels (Vanhanen *et al* 1993; Miettinen and Vanhanen 1994; Gylling *et al* 1995). The overall conclusions from these reports were that sitostanol supplementation effectively decreased both total and LDL plasma cholesterol concentrations with a corresponding increased in the HDL/LDL ratio. The key difference between this current project and previous research is the dose of phytosterol administered.

Previous studies have used 2 to 3 g of pure sitostanol, whereas, the present study only used an average of 0.32 g (range of 0.26 to 0.41 g), yet a similar response was induced in normolipidemic subjects. Clearly, further human research is required to investigate the optimal dose of this phytosterol mixture.

Cholesterol synthesis rates were monitored in this study through deuterium incorporation rates. The results indicate that there was no significant increase in *de novo* cholesterol synthesis rates with the addition of either phytosterol mixture to an olive oil based diet. Nevertheless, although not statistically significant, cholesterol synthesis rates appeared to be higher with the addition of the phytosterols mixtures to the olive oil diet. It has been proposed that absorption of cholesterol can be indirectly quantitated through monitoring cholesterol synthesis rates. If plasma cholesterol concentrations decrease and an increase in *de novo* cholesterol synthesis rates is induced, it can be inferred that absorption of cholesterol has decreased. In the present study, plasma cholesterol concentrations decreased, and although not statistically significant, *de novo* cholesterol synthesis rates were higher following phytosterol administration, therefore, cholesterol absorption may have been impaired during phytosterol administration.

Campesterol is a dietary plant sterol not synthesized in the body, and is absorbed to such a small extent that its serum concentration is less than one percent of the cholesterol value (Miettinen *et al* 1990). Nevertheless, it has been previously demonstrated that the serum concentration of campesterol is directly correlated with cholesterol absorption (Tilvis and Miettinen 1986; Miettinen *et al* 1990). In the present study, when plasma phytosterol concentrations were compared, no significant differences

were detected. Despite this lack of significance, there were distinct trends that are consistent with what is currently known about phytosterol absorption. Recent research has determined that a longer side chain at the C-24 position of the cholesterol molecule is negatively correlated with the absorption of that molecule (Heinemann *et al* 1993). Thus, campesterol, with an extra methanol at C-24 of the cholesterol molecule, is absorbed at a higher percentage than β-sitosterol, with an extra ethanol at the C-24 position. Furthermore, hepatic esterification of sitosterol is significantly lower than that of campesterol, therefore, its biliary elimination may exceed that of campesterol (Tavani *et al* 1982). Consequently, campesterol is found at higher levels in the plasma than sitosterol. The results of the present study followed this trend.

It has also been shown that the 5α-saturated derivative of β-sitosterol, better known as sitostanol, is almost completely unabsorbed (Heinemann *et al* 1991). Reports have indicated sitostanol may act not only to impede cholesterol absorption more effectively but also to impede the absorption of other phytosterols. This is demonstrated by comparing the level of plasma campesterol relative to cholesterol concentrations across the treatment groups. With the addition of sitostanol to the phytosterol mixture, there was a slight, but non-significant lower campesterol to cholesterol ratio. These effects are further exemplified in the plasma campesterol to sitosterol (C:S) ratio, with sitostanol-enriched phytosterol mixture supplemented diet appearing to display the lowest plasma C:S ratio, although again not statistically significant. However, this effect may have arisen due to the lower level of campesterol in the sitostanol-enriched phytosterol mixture. Consequently, further studies would be needed before it can be elucidated whether

sitostanol blocks campesterol absorption and thus induces lower plasma campesterol concentrations.

It has been proposed that the primary mechanism by which phytosterols achieve a change in lipid profile is through altering the absorption of cholesterol. With the lack of significant increases in both plasma campesterol concentrations and *de novo* cholesterol synthesis rates, it cannot be concluded from this study that cholesterol absorption was attenuated with the administration of either phytosterol mixture. Potential explanations could relate to the variability of responsiveness to phytosterol administration, the study populations being normolipidemic, the low-dose of sitostanol administered, or the short time span of each diet treatment.

In summary, the present experiment builds on existing knowledge to strongly suggest that at relatively low doses, the sitostanol-enriched phytosterol mixture effectively impedes cholesterol absorption, thus improving the plasma lipid profile through decreasing plasma total and LDL-cholesterol concentrations as well as increasing HDL/LDL ratios. Hence, with the strong negative correlation of the HDL/LDL ratio with relative risk for coronary heart disease and the lack of negative side effects reported for phytosterol administration, it can be postulated that supplementation with this sitostanol-enriched phytosterol mixture may be effective for individuals with suboptimal lipid levels. Clearly, more research is needed to determine the optimal effectiveness of this sitostanol-enriched phytosterol mixture for improving plasma lipid profiles in humans.

Table 3-1
Characteristics of the Study Subjects

Characteristic	Women (n=5)	Men (n=6)	Mean (n=11)
Age, y	21 ± 2	24 ± 2	23 ± 2
Body weight, kg	59 ± 3	80 ± 8	70 ± 12
Height, m	1.65 ± 0.11	1.78 ± 0.05	1.72 ± 0.11
Body mass index, kg/m^2	22.0 ± 2.5	24.9 ± 2.2	23.6 ± 2.6
Total cholesterol, mmol/L	4.11 ±0.39	3.62 ±0.47	3.84 ±0.49
LDL-C, mmol/L	2.03 ±0.62	1.93 ±0.62	1.98 ±0.57
HDL-C, mmol/L	1.65 ±0.31	1.36 ±0.23	1.49 ±0.28
Triglycerides, mmol/L	0.89 ±0.18	0.70 ±0.12	0.79 ±0.18

Values expressed as mean ± SD.

Abbreviations: LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

Table 3-2
Phytosterol Content of Treatment Groups

Phytosterol	[illegible]	[illegible]	Olive Oil
	Composition (% of total)		
Campesterol	**23.7**	**16.1**	**0.0**
Stigmasterol	**14.4**	**0.0**	**0.0**
β-Sitosterol	**61.9**	**62.0**	**100.0**
Sitostanol	**0.0**	**21.1**	**0.0**
	Average daily intake (range) (g)		
Total:	**1.46 (1.17 - 1.68)**	**1.71 (1.42 - 2.13)**	**0.22 (0.17 - 0.26)**
β-Sitosterol	0.99 (0.81 - 1.03)	1.13 (0.96 - 1.40)	0.22 (0.17 - 0.26)
Sitostanol	0.00	0.32 (0.26 - 0.41)	0.00
Campesterol	0.47 (0.36 - 0.55)	0.26 (0.20 - 0.32)	0.00

Abbreviations: SaF, sitostanol-free phytosterols; SaE, sitostanol-enriched phytosterols.

Table 3-3
The effect of phytosterol administration on circulating HDL/LDL cholesterol ratios

Subject	Gender	Diet	SaF	SaE
			HDL/LDL Ratio	
1	M	0.455	0.413	0.388
2	M	0.496	0.409	0.649
3	M	0.835	0.970	1.176
4	M	0.603	0.572	0.795
5	M	0.305	0.410	0.579
6	M	0.403	0.566	0.526
7	F	0.836	0.923	0.944
8	F	0.687	0.771	0.983
9	F	0.675	0.745	0.814
10	F	0.398	0.414	0.410
11	F	---	0.492	0.594
	MEAN:	**0.569** [a]	**0.608** [a]	**0.714** [b]
	±SEM:	**0.059**	**0.064**	**0.075**

Results are expressed as mean ± SEM. Treatment group means having different subscripts differ significantly ($P<0.05$ Tukey's pairwise comparisons). Abbreviations: M, male; F, female; SaF, sitostanol-free phytosterol mixture; SaE, sitostanol-enriched phytosterol mixture; HDL, high density lipoprotein;. LDL, low density lipoprotein.

Table 3-4
The effect of phytosterol administration on circulating campesterol to sitosterol ratios

Subject	Gender	Campesterol-to-Sitosterol Ratio		
		Olive	+SaF	+SaE
1	M	7.95	22.83	1.05
2	M	6.46	23.83	no sito
3	M	14.51	13.99	1.69
4	M	33.27	---	1.26
7	F	3.39	9.32	4.49
8	F	41.58	8.57	46.50
9	F	12.98	---	2.03
10	F	11.32	33.39	24.84
	MEAN:	**16.43**	**18.65**	**11.69**
	±SEM:	**4.82**	**3.97**	**6.64**

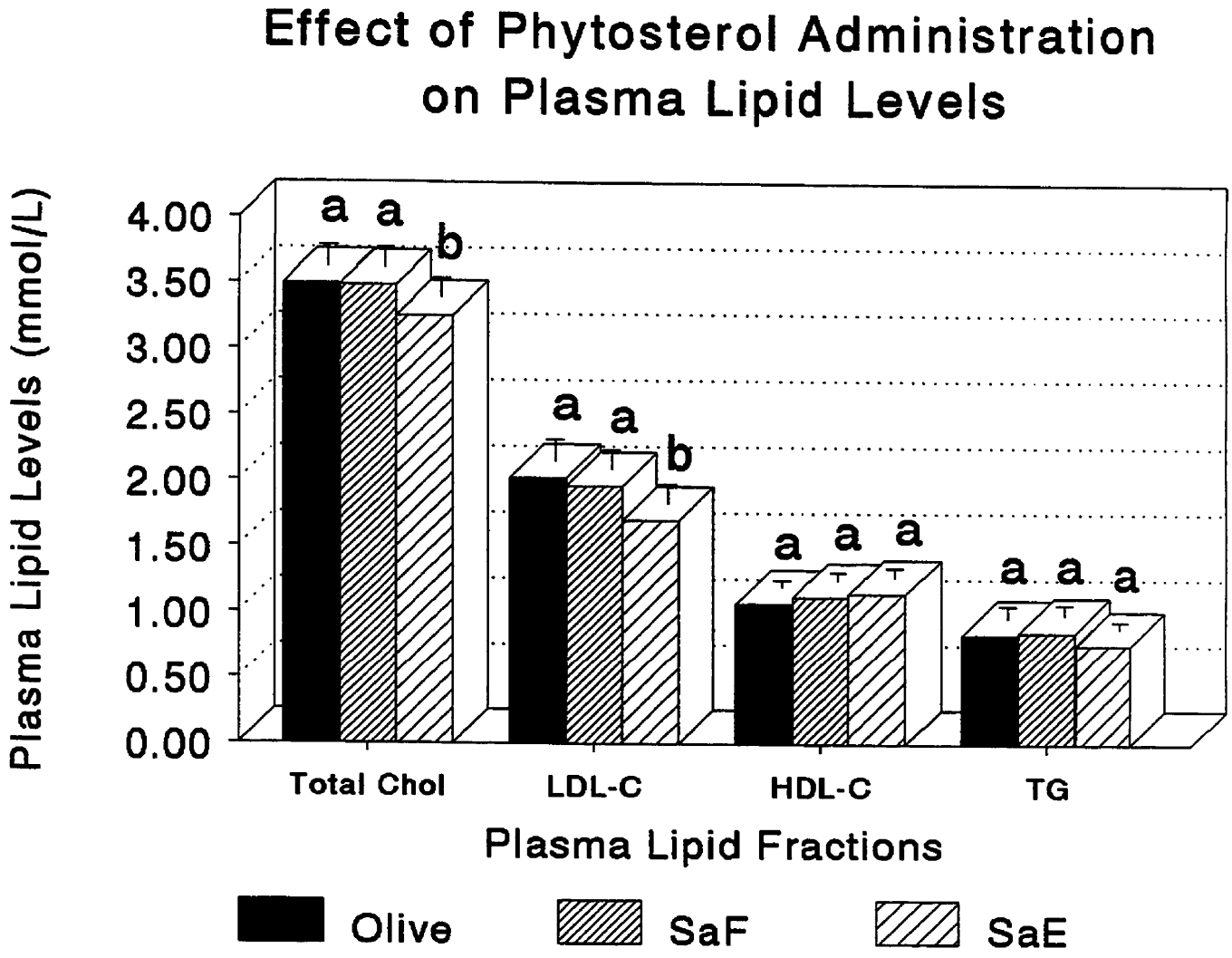
Results are expressed as mean ± SEM. Treatment group means having different subscripts differ significantly ($P < 0.05$ Tukey's pairwise comparisons). Abbreviations: M, male; F, female; SaF, sitostanol-free phytosterol mixture; SaE, sitostanol-enriched phytosterol mixture; no sito, no sitosterol detected in plasma; ---, no measurement available.

FIGURE LEGENDS

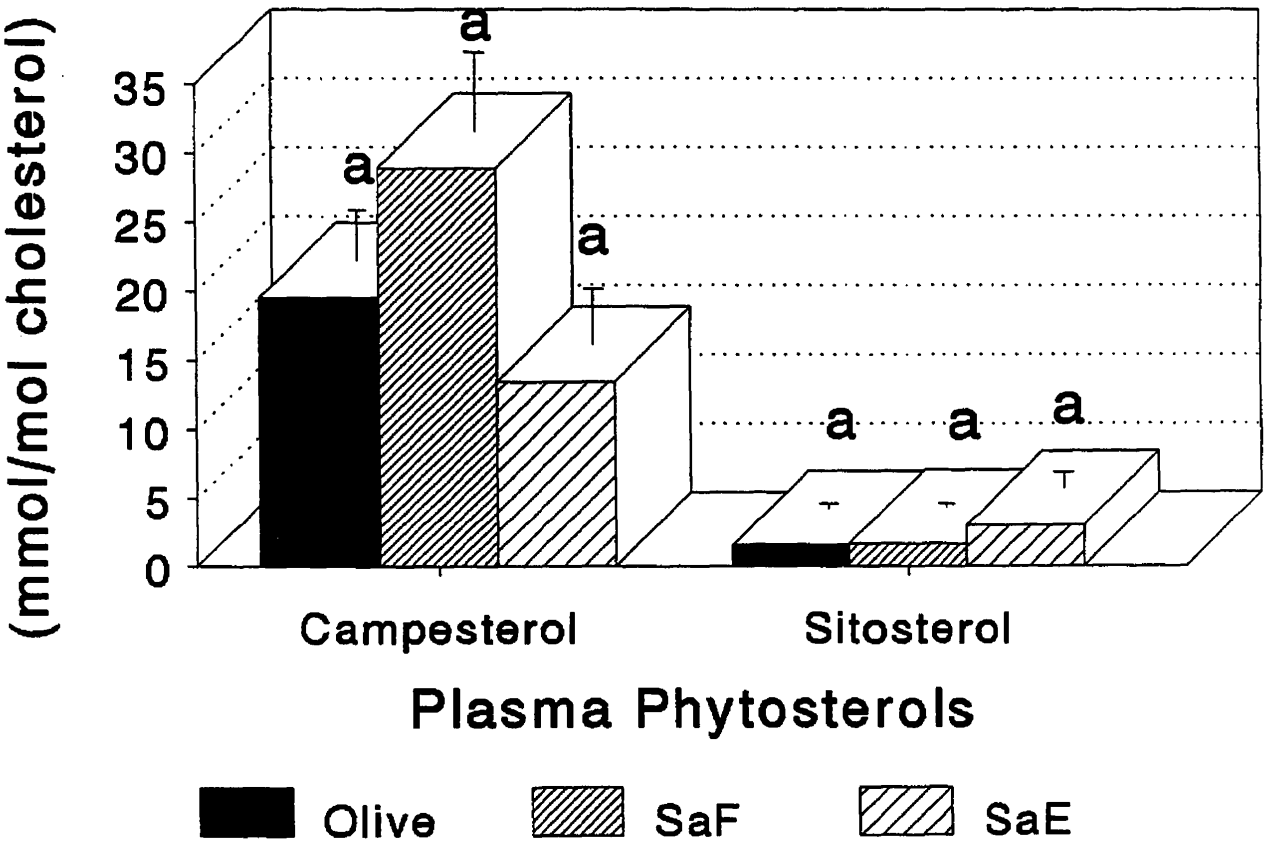
Figure 3-1 Plasma lipid concentrations of total cholesterol (Total Chol), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglycerides (TG) in healthy male and female humans (n=11) consuming either an olive oil diet alone (Olive Oil), an olive oil diet plus sitostanol-free phytosterol mixture (SaF), or an olive oil diet plus sitostanol-enriched phytosterol mixture (SaE) for 10 days. Results are expressed as mean ± SEM. Diet treatment group means within each parameter having different subscripts differ significantly ($P<0.05$ using Tukey's pairwise comparisons).

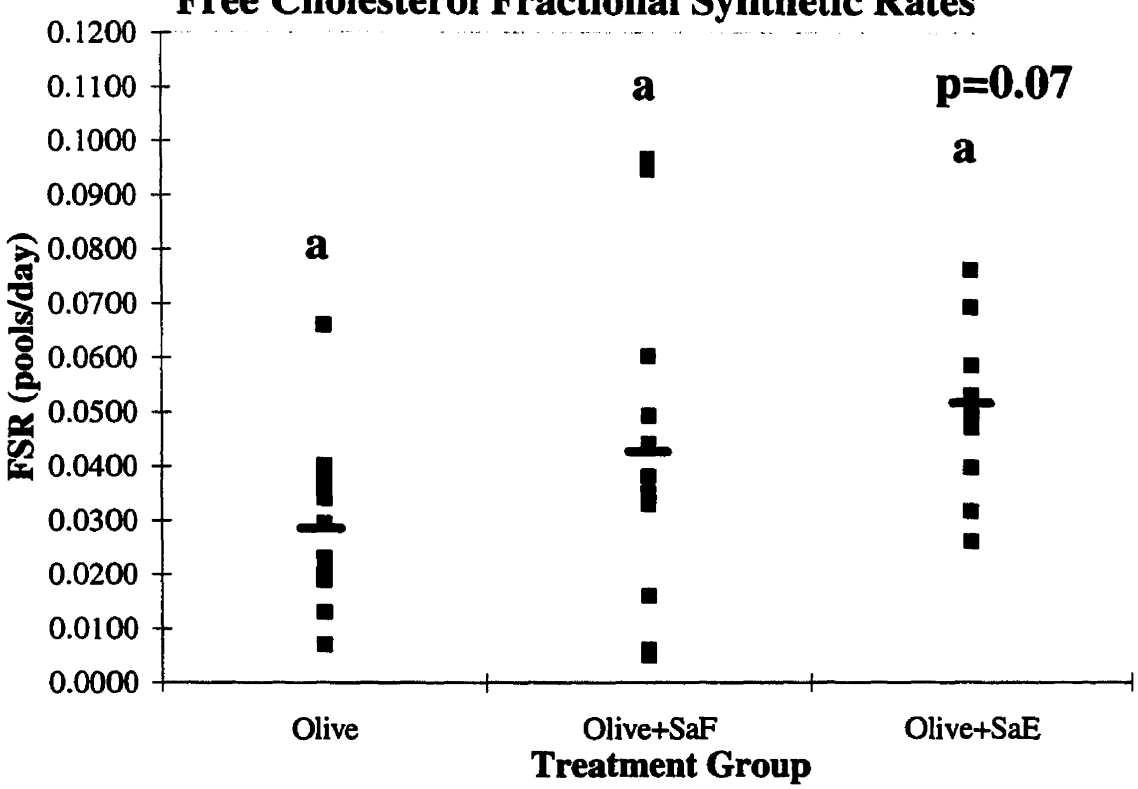
Figure 3-2 Plasma phytosterol concentrations expressed as mmol of plasma phytosterol per mol of plasma total cholesterol in healthy male and female humans (n=11) consuming either an olive oil diet alone (Olive Oil), an olive oil diet plus sitostanol-free phytosterol mixture (SaF), or an olive oil diet plus sitostanol-enriched phytosterol mixture (SaE) for 10 days. Results are expressed as mean ± SEM. Diet treatment group means within each parameter having different subscripts differ significantly ($P<0.05$ using Tukey's pairwise comparisons).

Figure 3-2 Free cholesterol fractional synthetic rates (FSR) in healthy male and female humans (n=11) consuming either an olive oil diet alone (Olive Oil), an olive oil diet plus sitostanol-free phytosterol mixture (SaF), or an olive oil diet plus sitostanol-enriched phytosterol mixture (SaE) for 10 days. Dietary treatment group means having different subscripts differ significantly ($P<0.05$ using Tukey's pairwise comparisons).


Effect of Phytosterol Administration on Plasma Lipid Levels
Plasma Lipid Levels (mmol/L)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a a b
a a b
a a a
a a a
Total Chol
LDL-C
HDL-C
TG
Plasma Lipid Fractions
Olive
SaF
SaE

Plasma Phytosterol Concentrations
Relative to Plasma Total Cholesterol




Effect of Phytosterol Administration on
Free Cholesterol Fractional Synthetic Rates
0.1200
0.1100
0.1000
0.0900
0.0800
0.0700
0.0600
0.0500
0.0400
0.0300
0.0200
0.0100
0.0000
FSR (pools/day)
a
a
p=0.07
a
Olive
Olive+SaF
Olive+SaE
Treatment Group

7. *SUMMARY AND FINAL CONCLUSIONS*

Manuscript one was one of the first studies to demonstrate that the differential effects observed with corn oil and olive oil on plasma lipid levels and cholesterol synthesis can be explained, in part, by the plant sterols content of corn oil. These results will help alleviate any doubts regarding the role of phytosterols in corn oil. Since it has been determined that polyunsaturated fatty acid based oils generally contain high levels of phytosterols, future projects could update the phytosterol levels of other polyunsaturated fatty acid oils and investigate the possible role they play in these plant oils. As well, these results may help future investigations design experiments that take into consideration phytosterols as a confounding variable when comparing fatty acids. Future projects could entail re-evaluation of prior reports comparing corn and olive oil, determining other factor within corn and olive oil that may affect cholesterol metabolism, and analysis of other factors within plant oils that may assist the cholesterol-lowering mechanisms of natural phytosterols.

Manuscript two concluded that plasma lipid profiles are improved in normolipidemic humans with a tall-oil based phytosterol mixture containing low doses (<0.5 g) and primarily β-sitosterol. These findings demonstrate that low doses of sitostanol may be effective as a cholesterol-lowering agent. However, due to the lack of affect reported in previous reports with this low level of sitostanol (<0.5g/day), a synergistic effect may also be occurring between the phytosterols when small amounts of sitostanol are combined with sitosterol. These results warrant further investigation of the efficiency of this mixture in humans with familial hypercholesterolemia. Other projects

that deserve further investigation are the interactions that may exist between phytosterols, optimal ratios of phytosterols that are required for improving plasma lipid levels, and the effect of these findings on hyperlipidemic humans.

The two investigations in this thesis were not without limitations. The primary problem with both studies was the dose and composition of the phytosterol mixtures administered. The vegetable oil-based phytosterol mixture administered in manuscript one was not exactly the same composition as the phytosterols found naturally in corn oil. The phytosterols were also administered as a non-esterified powder compared to the natural mixture in corn oil of esterified and non-esterified phytosterols in a matrix of oil. Therefore, the study increased the dosage of the phytosterol supplement to accommodate these differences. If more money and time was available to conduct the first study, phytosterol extracts could have been derived from corn oil and administered with the olive oil diet. However, the study had to rely on what was available in the market, so a phytosterol mixture with the approximate composition to corn oil was used. The second manuscript compared a vegetable oil-based sitostanol-free phytosterol mixture with a tall oil-based sitostanol-enriched phytosterol mixture. An ideal study design for the second investigation would have been to add sitostanol directly to the sitostanol-free phytosterol mixture, therefore, the only difference between the two mixtures would have been the sitostanol content. Nonetheless, again, we had to rely on what phytosterol mixtures were available to conduct the studies; thus, the phytosterol mixtures were slightly different with regard to phytosterol composition and dosage.

Other limitations of the two investigations in this thesis include the length of the treatments and the characteristics of the humans studied. The treatment period was only 10 days. Although this is a sufficient time to observe the initial change in lipid metabolism due to changes in diet, previous studies have indicated that it took 3 to 4 weeks to significantly reduce plasma total cholesterol concentrations with either 1.5 g/day sitostanol (Heinemann *et al* 1986) or 740 mg/day soybean phytosterols (Pelletier *et al* 1995). Studies over 12 months have indicated that plasma total cholesterol concentrations continue to decrease over 6 months with 1.8 g/day and over the entire 12 months with 2.6 g/day sitostanol ester (Miettinen *et al* 1995). Therefore, the use of 10 days in the present investigations may not have been long enough to observe the full effect of the phytosterol mixtures on sterol metabolism. Furthermore, prior studies recruited older hyperlipidemic subjects, whereas the present investigations attempted to manipulate the sterol metabolism of young healthy normolipidemic humans. These may have been factors leading to the lack of significant results obtained in the current studies.

Sample size was also a limitation in the second investigation. Due to financial constraints, phytosterol availability, and subject availability only 11 of the original 16 subjects were able to continue with the fourth diet treatment of the sitostanol-enriched phytosterol mixture. In order to attain a power of 80% to detect a 10% difference in plasma total cholesterol at the significance level of P=0.05, a minimum of 15 subjects would be required in a paired design; therefore, the use of 11 subjects in the second investigation decreased the power or sensitivity to detect differences among the treatments.

There are restrictions to the number of factors that can be measured during a human investigation. Firstly, baseline plasma lipid concentrations were not taken at the beginning of each treatment. It was postulated that subjects plasma lipid concentrations would return to baseline levels following a two week washout period, and the added stress of an additional baseline blood draw at the beginning of each diet treatment was not necessary. However, baseline plasma lipid concentrations would have been helpful to determine the change in plasma lipid concentrations over a treatment. Furthermore, this measurement would have served as a validation that the subjects plasma lipid concentrations returned to baseline following the two week washout periods. Secondly, measurement of the cholesterol content in the bile acid following each diet treatment would have assisted in determining if excess cholesterol was shuttled out of the body through bile acids or if it was returned to circulation when cholesterol synthesis was increased. However, this measurement would have involved collection of total daily faeces for a minimum of two days at the end of each treatment; an unpleasant measurement for both the subjects and the researchers.

Despite the limitations listed above, the results obtained in the investigations conducted in the present thesis contribute to current scientific knowledge. These limitations should be taken into consideration for future studies investigating the effects of natural and synthetic phytosterols on cholesterol metabolism.

REFERENCES:

Allain CC, Poon LS, Chan CS, Richmond W, and Fu PC. Enzymatic determination of total serum cholesterol. *Clinical Chem* 1974;20:470-475.

Association of Official Analytical Chemists. *Official methods of analysis of the Association of Official Chemists*, 15th ed. Arlington, VA: Association of Official Analytical Chemists, 1987.

Baggio G, Pagnan A, Muraca M, Martini S, Opportuno A, Bonanome A, Ambrosio GB, Ferrari S, Guarini P, Piccolo D, Manzato E, Corrocher R, and Crepaldi G. Olive oil-enriched diets: effects on serum lipoprotein levels and biliary cholesterol saturation. *Amer J Clin Nutr* 1988;47:960-964.

Bannon D, Craske JD, Har NT, Harper NL, and O'Rourke KL. Analysis of fatty acid methyl esters with high accuracy and reliability. II. Methylation of fats and oils with boron trifluoride-methanol. *J Chrom* 1982;247:63-69.

Bell L, Jones PJH, Telch J, Clandinin MT, and Pencharz PB. Prediction of energy needs for clinical studies. *Nutr Res* 1985;5:123-129.

Beveridge JRM. Role of fat in nutrition. II. Role of unsaturated fat in adult nutrition. *Am J Publ Hlth* 1957;47:1370-1380.

Beveridge JRM, Connell WF, Mayer GA, and White M. Hypocholesterolemic activity of corn oil fractions in human subjects. *Proc Can Fed Biol Soc* 1958;1:6-7.

Bucolo G and David H. Quantitative determination of serum triglycerides by the use of enzymes. *Clin Chem* 1973;19:476-482.

Cerqueira MT, Fry MM, and Connor WE. The food and nutrient intakes of the Tarahumara Indians of Mexico. *Am J Clin Nutr* 1979;32:905-915.

Chandler RF, Hooper SN, and Ismail HA. Antihypercholesterolemic studies with sterols: β-sitosterol and stigmasterol. *J Pharm Sci* 1979;68:245-247.

Child P and Kuksis A. Uptake of 7-dehydro derivatives of cholesterol, campesterol, and β-sitosterol by rat erythrocytes, jejunal villus cells, and brush border membranes. *J Lipid Res* 1983;24:552-565.

Connor WE, Witiak DT, Stone DB, and Armstrong ML. Cholesterol balance and faecal neutral steroid excretion and bile acid excretion in normal men fed dietary fats of different fatty acid composition. *J Clin Invest* 1969;48:1363-1375.

Demacker PNM, Reijnen IGM, Katan MB, Stuyt PMJ, and Stalenhoef AFH. Increased removal of remnants of triglyceride-rich lipoproteins on a diet rich in polyunsaturated fatty acids. *Eur J Clin Invest* 1991;21:1997-2003.

Denke MA. Lack of efficacy of low-dose sitostanol therapy as an adjunct to a cholesterol-lowering diet in men with moderate hypercholesterolemia. *Am J Clin Nutr* 1995;61:392-396.

Dietschy JM and Spady DK. Measurement of rates of cholesterol synthesis using tritiated water. *J Lipid Res* 1984;25:1469-1476.

Dietschy JM, Turley SD, and Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res* 1993;34:1637-1659.

Diller ER, Woods BL, and Harvey OA. Effect of beta-sitosterol on regression of hypercholesterosis and atherosclerosis in chickens. *Proc Soc Exp Biol Med* 1958;98:813-817.

Diller ER, Rose CL, and Harvey, OA. Effect of beta-sitosterol on regression of hyperlipidemia and increased plasma coagulability in the chicken. *Proc Soc Exp Biol Med* 1960;104:173-176.

Dreon DM, Vranizan KM, Drauss RM, Austin MA, and Wood PD. The effects of polyunsaturated fat vs. monounsaturated fat on plasma lipoproteins. *JAMA* 1990;263:2462-2466.

Farquhar JW and Sokolow M. Response of serum lipids and lipoproteins of man to beta-sitosterol and safflower oil: a long term study. *Circulation* 1958;17:890-899.

Fernandez ML, Yount NY, and McNamara DJ. Whole body and hepatic cholesterol synthesis rates in the guinea pig: effect of dietary fat quality. *Biochim Biophys Acta* 1990;1044:340-348.

Fernandez ML and McNamara DJ. Regulation of cholesterol and lipoprotein metabolism in guinea pigs mediated by dietary fat quality and quantity. *J Nutr* 1991;121:934-943.

Fernandez ML and McNamara DJ. Dietary fat saturation and chain length modulate guinea pig hepatic cholesterol metabolism. *J Nutr* 1994;124:331-339.

Folch J, Lees M, and Sloane SGH. A simple method for the extraction and purification of total lipids from animal tissues. *J Biol Chem* 1957;226:497-509.

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

Fungwe TV, Cagen LM, Cook GA, Wilcox HG, and Heimberg M. Dietary cholesterol stimulates hepatic biosynthesis of triglyceride and reduces oxidation of fatty acids in the rat. *J Lipid Res* 1993;34:933-941.

Grundy SM. Effects of polyunsaturated fats on lipid metabolism in patients with hypertriglyceridemia. *J Clin Invest* 1975;55:269-282.

Grundy SM. Monounsaturated fatty acids and cholesterol metabolism: implications for dietary recommendations. *J Nutr* 1989;119:529-533.

Grundy SM and Ahrens EH. Measurements of cholesterol turnover, synthesis, and absorption in man, carried out by isotope kinetic and sterol balance methods. *J Lipid Res* 1969;10:91-107.

Grundy SM and Mok HYI. Effects of low dose phytosterols on cholesterol absorption in man. In: *Lipoprotein Metabolism*, Greten, H (ed.). Springer, Berlin. 1976:119-124.

Gylling H, Siimes MA, and Miettinen TA. Sitostanol ester margarine in dietary treatment of children with familial hypercholesterolemia. *J Lipid Res* 1995;36:1807-1812.

Health and Welfare Canada. *Nutrient Recommendations: the report of the scientific review committee*. Ministry of Supply and Services, Ottawa, Canada, 1990

Hegsted DM, McGandy RB, Myers ML, and Stare FJ. Quantitative effects of dietary fat on serum cholesterol in man. *Am J Clin Nutr* 1965;17:281-295.

Hegsted DM, Ausman LM, Johnson JA, and Dallal GE. Dietary fat and serum lipids: an evaluation of the experimental data. *Am J Clin Nutr* 1993;57:875-883.

Heinemann T, Leiss O, and von Bergmann K. Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. *Atherosclerosis* 1986;61:219-223.

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

Heinemann T, Axtmann G, and von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur J Clin Invest* 1993;23:827-831.

Herrmann RG. Effect of taurine, glycine and beta-sitosterol on serum and tissue cholesterol in the rat and rabbit. *Circulation Res* 1959;7:224-227.

Howard BV, Hannah JS, Heiser CC, Jablonski KA, Paidi MC, Alarif L, Robbins DC, and Howard WJ. Polyunsaturated fatty acids result in greater cholesterol lowering and less triacylglycerol elevation than do monounsaturated fatty acids in a dose-response comparison in a multiracial study group. *Am J Clin Nutr* 1995;62:392-402.

Ikeda I and Sugano M. Some aspects of mechanism of inhibition of cholesterol absorption by β-sitosterol. *Biochim Biophys Acta* 1983;732:651-658.

Ikeda I, Tanaka K, Sugano M, Vahouny GV, and Gallo LL. Inhibition of cholesterol absorption in rats by plant sterols. *J Lipid Res* 1988;29:1573-1582.

Jones PJH, Scanu AM, and Schoeller DA. Plasma cholesterol synthesis using deuterated water in humans: effects of short term food restriction. *J Clin Invest* 1988;111:627-633.

Jones PJH. Use of deuterated water for measurement of short-term cholesterol synthesis in humans. *Can J Physiol Pharmacol* 1990;68:955-959.

Jones PJH, Leitch CA, Li Z-C, and Connor WE. Human cholesterol synthesis measurement using deuterated water: theoretical and procedural considerations. *Arterioscler Thromb* 1993;13:247-253.

Jones PJH, Lichtenstein AH, Schaefer EJ, and Namchuk GL. Effect of dietary fat selection on plasma cholesterol synthesis in older moderately hypercholesterolemic humans. *Arterioscler Thromb* 1994a;14:542-548.

Jones PJH, Lichtenstein AH, and Schaefer EJ. Interaction of dietary fat saturation and cholesterol level on cholesterol synthesis measured using deuterium incorporation. *J Lipid Res* 1994b;35:1093-1101.

Kannel WB, Catelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham study. *Ann Int Med* 1979;90:85-91.

Keys A, Anderson JT, and Grande F. Prediction of serum-cholesterol responses of man to changes in fats in the diet. *Lancet* 1957;2:959-966.

Kuksis A, Marai L, Myher JJ, and Geher K. Identification of plant sterols in plasma and red blood cells of man and experimental animals. *Lipids* 1976;11:581-586.

Kuo P, Winfeld M, and Loscalzo J. Effect of membrane fatty acyl composition on LDL metabolism in Hep G2 hepatocytes. *Biochemistry* 1990a;29:6626-6632.

Kuo P, Weinfeld M, Rudd MA, Amarante P, and Loscalzo J. Plasma membrane enrichment with cis-unsaturated fatty acids enhances LDL metabolism in U937 monocytes. *Arterioclerosis* 1990b;10:111-118.

Laraki L, Pelletier X, and Debry G. Effects of dietary cholesterol and phytosterol overload on wistar rat plasma lipids. *Ann Nutr Metab* 1991;35:221-225

Laraki L, Pelletier X, Mourot J, and Debry G. Effects of dietary phytosterols on liver lipids and lipid metabolism enzymes. *Ann Nutr Metab* 1993;37:129-133.

Lees AN, Mok HYI, Lees RS, McCluskey MA, and Grundy SM. Plant sterols as cholesterol-lowering agents: clinical trials in patients with hypercholesterolemia and studies of sterol balance. *Atherosclerosis* 1977;28:325-338.

Lichtenstein AH, Ausman LM, Carrasco W, Jenner JL, Gualtieri LJ, Goldin BR, Ordovas JM, and Schaefer EJ. Effects of canola, corn, and olive oils on fasting postprandial plasma lipoproteins in humans as part of a national cholesterol education program step 2 diet. *Arterioscler Thromb* 1993;13:1533-1542

Lin DS, Connor WE, and Phillipson BE. Sterol Composition of normal human bile. Effects of feeding shellfish (marine) sterols. *Gastroenterol* 1984;86:611-617.

Ling WH and Jones PJH. Mini-review of dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sciences* 1995;57:195-206.

The Lipid Research Clinics Program: The Lipid Research Clinics Coronary Primary Prevention Trial Results. I. Reduction in incidence of coronary heart disease. *JAMA* 1984;251:351-364.

Mata P, Alvarez-Sala LA, Rubio MJ, Nuno J, and De Ova M. Effects of long-term monounsaturate- vs polyunsaturated-enriched diets on lipoproteins in healthy men and women. *Am J Clin Nutr* 1992;55:846-850.

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

Mattson FH and Grundy SM. Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *J Lipid Res* 1985;26:194-202.

Mazier MJP and Jones PJH. Dietary fat quality and circulating cholesterol levels in humans: a review of actions and mechanisms. *Prog Food Nutr Sci* 1991;15:21-41.

Mazier MJP and Jones PJH. Diet fat saturation and feeding state modulate rates of cholesterol synthesis in normolipidemic men. *J Nutr* 1997 (in press).

Mensink RP and Katan MB. Effect of a diet enriched with monounsaturated or polyunsaturated fatty acids on levels of low-density and high-density lipoprotein cholesterol in healthy men and women. *N Engl J Med* 1989;321:436-441.

Mensink RP and Katan MB. Effect of dietary fatty acids on serum lipids and lipoproteins. A meta-analysis of 27 trials. *Arterioscler Thromb* 1992;12:911-919.

Miettinen TA, Tilvis RS, and Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am J Epidemiol* 1990;131:20-31.

Miettinen TA and Vanhanen H. Dietary sitostanol related to absorption, synthesis and serum level of cholesterol in different apolipoprotein E phenotypes. *Atherosclerosis* 1994;105:217-226.

Miettinen TA, Puska P, Gylling H, Vanhanen H, and Vartianinen E. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *N Engl J Med* 1995;333:1308-1312.

Mifflin MD, St Jeur ST, Hill LA, Scott BJ, Daugherty SA, Koh YD. A new predictive equation for resting energy expenditure in healthy individuals. *Am J Clin Nutr* 1990;51:241-247.

Moore RB, Anderson JT, Taylor HL, Keys A, and Frantz Jr ID. Effect of dietary fat on the fecal excreation of cholesterol and its degradation products in man. *J Clin Invest* 1968;47:1517-1525.

Nair PP, Turjman N, Kessie G, Calkins B, and Goodman G. Diet, nutrition and metabolism in populations at high and low risk for colon cancer. Metabolism of neutral sterols. *Am J Clin Nutr* 1984;40:931-936.

National Cholesterol Education Program Expert Panel. Detection, education, and treatment of high blood cholesterol in adults. *Arch Intern Med* 1988;148:36-69.

Nestel PJ, Havenstein N, Whyte HM, Scott TJ, and Cook LJ. Lowering of plasma cholesterol and enhanced sterol excreation with the consumption of polyunsaturated ruminants of fats. *N Engl J Med* 1973;288:379-382.

Nestel PJ, Havenstein N, Homma Y, Scott TW, and Cook LJ. Increased sterol excretion with polyunsaturated fat-high cholesterol diets. *Metabolism* 1975;24:189-198.

Nydahl MC, Gustafsson IB, and Vessby B. Lipid-lowering diets enriched with monounsaturated or polyunsaturated fatty acids but low in saturated fatty acids have similar effects on serum lipid concentrations in hyperlipidemic patients. *Am J Clin Nutr* 1994;59:115-122.

Pelletier X, Belbraouet S, Mirabel D, Mordret F, Perrin JL, Pages X, and Debry G. A diet moderately enriched in phytosterols lowers plasma cholesterol concentrations in normocholesterolemic humans. *Ann Nutr Met* 1995;39:291-295.

Petersen DW. Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. *Proc Soc Exp Biol Med* 1951;78:143-147.

Petersen DW, Nichols LW, Jr. Peck NF, and Chaikoff IL. Depression of plasma cholesterol in human subjects consuming butter containing soy sterols. *Fed Proc* 1956;65:569.

Pollak OJ. Successful prevention of experimental hypercholesteremia and cholesterol atherosclerosis in the rabbit. *Circulation* 1953a;7:696-701.

Pollak OJ. Reduction of blood cholesterol in man. *Circulation* 1953b;7:702-706.

Pollak OJ and Kritchevski D. Sitosterol. *Monographs on Atherosclerosis*, Vol. 10. Karger, Basel, 1981.

Pollak OJ. Effect of plant sterols on serum lipids and atherosclerosis. *Pharmac Ther* 1985;31:177-208.

Sirtori CR, Tremoli E, Gatti E, Montanari G, Sirtori M, Colli S, Gianfraceschi G, Maderna P, Dentone CA, Testolin G, and Galli C. Controlled evaluation of fat intake in the Mediterranean diet: comparative activities of olive oil and corn oil on plasma lipids and platelets in high-risk patients. *Am J Clin Nutr* 1986;44:635-642.

Sonnenberg LM, Quatromoni PA, Gagnon DR, Cupples LA, Franz MM, Ordovas JM, Wilson PWF, Schaefer EJ, and Millen BE. Diet and plasma lipids in women. 2. Macronutrients and plasma triglycerides, high-density lipoprotein, and the ratio of total to high-density lipoprotein cholesterol in women - the framingham nutrition studies. *J Clin Epidemiol* 1996;49:665-672.

Sugano M, Moriorka H, and Ikeda I. A comparison of hypocholesterolemic activity of β-sitosterol and β-sitostanol in rats. *J Nutr* 1977;107:2011-2019.

Takita T, Nakamura K, Kimira M, Yamada N, Kobayashi Y, and Innami S. Serum fatty acid compositions and lipid concentrations and their correlations. *J Clin Biochem Nutr* 1996;20:149-159.

Tavani DM, Nes WR, and Billheimer JT. The sterol substrate specificity of acyl CoA: cholesterol acyltransferase from rat liver. *J Lipid Res* 1982;23:774-781.

Tilvis RS and Miettinen TA. Serum plant sterols and their relation to cholesterol absorption. *Am J Clin Nutr* 1986;43:92-97.

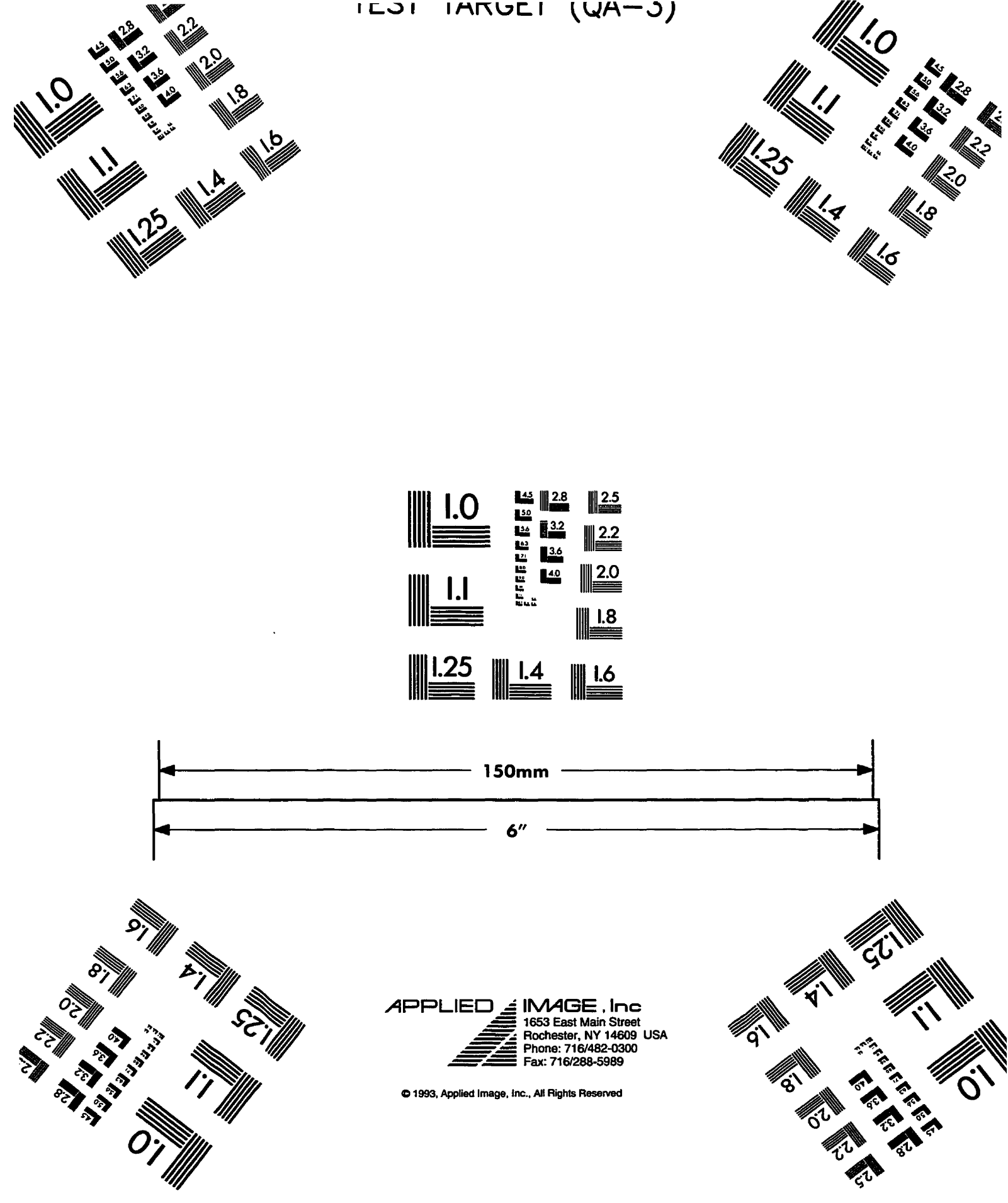
Vanhanen HT and Miettinen TA. Effect of unsaturated and saturated dietary plant sterols on their serum contents. *Clin Chem Acta* 1992;205:97-107.

Vanhanen HT, Blomqvist S, Ehnholm C, Hyvonen M, Juhiainen M, Torstila I, Miettinen TA. Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment. *J Lipid Res* 1993;34:1535-1544.

Warnick GR, Nouyen T, and Albers AA. Comparison of improving precipitation of methods for quantification of high-density lipoprotein cholesterol. *Clin Chem* 1985;31:217-222.

Weihrauch JL and Gardner JM. Sterol content of food of plant origin. *J Am Dietet A* 1978;73:39-47.

Woollett LA, Spady DK, and Dietschy JM. Saturated and unsaturated fatty acids independently regulate low density lipoprotein receptor activity and production rate. *J Lipid Res* 1992;33:77-88.



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