Unilever

2854 '01 NOV 29 A11 :45

November 28, 2001

Dockets Management Branch (HFA-305)
Food and Drug Administration
5630 Fishers Lane, Rm. 1061
Rockville, Maryland 20852

Re: Docket Nos. 00P-1275 and 00P-1276
Food Labeling: Health Claims; Plant Sterol/Stanol Esters and Coronary Heart Disease (Interim Final Rule; reopening of comment period)
66 Fed. Reg. 50824 (October 5, 2001)

To Whom It May Concern:

On November 19, 2001, Unilever Bestfoods North America submitted comments to the above-referenced dockets. In those comments, on page 2, we cited a doctoral dissertation by M. Hallikainen entitled, *Role of plant stanol ester and sterol ester-enriched margarines in the treatment of hypercholesterolemia*. Enclosed please find a copy of this document for your reference.

Respectfully submitted,

N. Schnell

Nancy L. Schnell
Deputy General Counsel –
Marketing and Regulatory

enclosure

00P-1276

SUP 1

Unilever United States, Inc.
Lever House 390 Park Avenue New York, New York 10022-4698
Telephone (212) 888-1260

KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 251
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 251

MAARIT HALLIKAINEN

Role of plant stanol ester- and sterol ester-enriched margarines in the treatment of hypercholesterolemia

le of plant st ester- and sterol ester-enriched

Distributor:

Kuopio University Library
P.O.Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 163 430
Fax +358 17 163 410

Series editors:

Professor Esko Alhava, M.D., Ph.D.
Department of Surgery

Professor Martti Hakumäki, M.D., Ph.D.
Department of Physiology

Author's address:

Department of Clinical Nutrition
University of Kuopio
P.O.Box 1627
FIN-70211 KUOPIO
FINLAND

Supervisors:

Professor Matti Uusitupa, M.D.
Department of Clinical Nutrition
University of Kuopio

Docent Essi Sarkkinen, Ph.D.
Oy Foodfiles Ltd. and
Department of Clinical Nutrition
University of Kuopio

Reviewers:

Acting Professor, Docent Hannu Vanhanen, M.D.
Department of Medicine
University of Helsinki

Docent Terho Lehtimäki, M.D.
Department of Clinical Chemistry
University of Tampere

Opponent:

Emeritus Professor Tatu Miettinen, M.D.
Department of Medicine
University of Helsinki

ISBN 951-781-851-3
ISSN 1235-0303

University Printing Office
Kuopio 2001
Finland

Hallikainen, Maarit. Role of plant stanol ester- and sterol ester-enriched margarines in the treatment of hypercholesterolemia. Kuopio University Publications D. Medical Sciences 251. 2001. 90 p.
ISBN 951-781-851-3
ISSN 1235-0303

ABSTRACT

The effects of plant stanol esters or sterol esters on serum lipids and lipoprotein lipids, serum fat-soluble vitamins and carotenoids, serum cholesterol precursors as well as serum plant sterols and stanols were examined in mildly or moderately hypercholesterolemic men and women. Study I/II utilized a parallel study design, studies III/IV and V involved a repeated measures design. In study I/II 55 subjects were randomized after a 4-week baseline, high-fat, diet period into three experimental groups ingesting three low-fat margarines: wood stanol ester (WSEM), vegetable oil stanol ester (VOSEM) and control. The groups consumed the margarines for eight weeks as part of a low-fat, low-cholesterol diet. In study III/IV, each of 22 subjects consumed five different doses of plant stanol [target (actual) intake 0 (0), 0.8 (0.8), 1.6 (1.6), 2.4 (2.3), 3.2 (3.1) g/day] added as stanol esters to margarine for four weeks as part of a standardized habitual diet. The order of dose periods was randomly determined. In study V, 34 subjects consumed stanol ester (STAEST), sterol ester (STEEST) and control margarines as part of a cholesterol-lowering diet each for four weeks. The randomization was performed according to the Latin square model.

In study I, the low-fat WSEM and VOSEM margarines reduced serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) as part of a cholesterol-lowering diet significantly by 16-18% and 18-24%, respectively, from a high-fat baseline diet. An additional approximately 10% cholesterol-lowering effect of these margarines compared with the low-fat diet (control) was noted (I). There was no significant difference in the cholesterol-lowering efficacy between these test margarines (I). Study III showed that the effect of plant stanol esters on serum TC and LDL-C is dose-dependent. A significant reduction in serum TC and LDL-C was achieved with the stanol dose of 1.6 g/d, and increasing the dose from 2.4 g/d to 3.2 g/d did not offer additional cholesterol-lowering effect. In study V, no significant differences between the STAEST and STEEST margarines with respect to efficacy in reducing serum TC (9.2% vs. 7.3%, compared with control) and LDL-C (12.7% vs. 10.4%) in short-term were found.

Plant stanol esters or sterol esters did not affect serum fat-soluble vitamins (I, III, V). Their impact on serum carotenoids was minor (I/II, III, V) when the dietary intake of vegetables was ensured.

Plant stanol esters reduced serum plant sterol concentrations significantly already with the stanol dose of 0.8 g/d (III/IV) indicating that cholesterol absorption was effectively inhibited already with the small stanol ester doses. The findings of serum Δ7-lathosterol/TC ratio (an indirect indicator of cholesterol synthesis) indicated that cholesterol synthesis was stimulated by a stanol dose of 0.8 g/d, but no further increase was observed when the stanol dose was higher than 1.6 g/d (IV). The consumption of plant stanol esters increased serum sitostanol and campestanol concentrations by about twofold, but the concentrations remained extremely low, and they plateaued with a dose equal to or greater than the 0.8 g/d (III/IV).

In conclusion, plant stanol ester- and sterol ester-enriched margarines are an effective and safe way to achieve a reduction in serum cholesterol when they are consumed as part of a low-fat, low-cholesterol diet. The optimal dose of stanol ester is 1.6-2.4 g/d of stanols.

National Library of Medicine Classification WD 200.5.H8, QU 86, WA 722
Medical subject headings: anticholesterolemic agents; carotenoids/blood; cholesterol/blood; diet, fat-restricted; dietary fats; hypercholesterolemia; lipids/blood; margarine; phytosterols/therapeutic use; vitamins/blood

ACKNOWLEDGEMENTS

This work was carried out in the University of Kuopio, Department of Clinical Nutrition.

I wish to express my deepest gratitude to my principal supervisor, Professor Matti Uusitupa, M.D., now the rector of the University of Kuopio, for the possibility to do research work and for his support and professional guidance during these years.

I am also greatly indebted to my other supervisor, Docent Essi Sarkkinen, Ph.D. Her advice, unfailing support and encouragement have been indispensable at all stages of this work.

I am very grateful to the official reviewers of this work, Acting Professor, Docent Hannu Vanhanen, M.D., and Docent Terho Lehtimäki, M.D., for their constructive criticism and valuable suggestions.

I am very grateful for Professor Helena Gylling, M.D., for her expert advice and comments regarding the manuscripts of the articles and this thesis.

I want to express my gratitude to Arja Erkkilä, Ph.D., for analyzing the fatty acid composition of serum lipids, and Virpi Lindi, M.Sc., and Raisa Valve, Ph.D., for determining apo E genotypes. In addition, I want to thank Arja and Virpi for their invaluable help in so many different ways during these years.

I wish to thank Ms. Irja Kanniainen, Kaija Kettunen and Erja Kinnunen for skillfully performing the laboratory work. In addition, I wish to thank Professor Gerald Salen, M.D., and his coworkers for analyzing serum plant sterols in study I, and Ms. Leena Kaipiainen, Pia Hoffström and Ritva Nissilä for analyzing serum plant sterols and cholesterol precursors in studies III/IV and V.

I wish to thank nutritionists Soili Lehtomäki, M.Sc., for nutrition counseling (study V) and Niina Tapola, M.Sc., for her great help in managing the food records (studies I/II and III/IV).

I owe my special thanks to Helvi Vidgren, Ph.D., with whom I have shared successes and setbacks. Her constant support and encouragement during these years have meant a lot to me. I am also grateful to Professor Hannu Mykkänen, Ph.D., Leila Karhunen, Ph.D., Marjukka Kolehmainen, M.Sc., Anne Louheranta, M.Sc., and Ursula Schwab, Ph.D., for much good advice during these years. In addition, I want to thank the entire personnel of the Department of Clinical Nutrition, University of Kuopio for the pleasant working atmosphere.

I wish to thank statistician Pirjo Halonen, M.Sc., and Veikko Jokela, M.Sc., for their help concerning statistical analyses. I also wish to thank Ewen MacDonald, Ph.D., who revised the English language of this thesis.

I express my sincere thanks to all subjects who participated in these studies and made this work possible.

My warmest thanks belong to my parents, sisters and brothers and their families, other relatives and friends for their support during this work. In addition, I want especially thank my sister, Professor Vieno Piironen, Ph.D., for good "tips" and information concerning the plant sterol content of food items.

Last, but not least I want to thank my dear husband, Lauri, for his love and support so in the good and the bad days.

This study was financially supported by Raisio Benecol Ltd., Kuopio University, Kuopio University Hospital, the Finnish Cultural Foundation of Northern Savo and Juho Vainio Foundation, all of which I acknowledge with gratitude.

Kuopio, August 2001

Maarit Hallikainen

Maarit Hallikainen

ABBREVIATIONS

ACAT	Acyl-CoA:cholesterol acyltransferase
ANOVA	Analysis of variance
apo	Apolipoprotein
BMI	Body mass index
CAD	Coronary artery disease
DM	Diabetes mellitus
E%	Percent of energy
FCR	Fractional catabolic rate
FH	Familial hypercholesterolemia
FH-NK	Familial hypercholesterolemia - North Karelia mutation of low density lipoprotein receptor gene
FW	Fresh weight
GLC	Gas liquid chromatograph
GLM	General Linear Models
HDL-C	High density lipoprotein cholesterol
HDL-TG	High density lipoprotein triglyceride(s)
IDL-C	Intermediate density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
LDL-TG	Low density lipoprotein triglyceride(s)
MANOVA	Multivariate analysis of variance
MUFA	Monounsaturated fatty acid(s)
NCEP	National Cholesterol Education Program
P/S	Polyunsaturated to saturated fatty acids
PUFA	Polyunsaturated fatty acid(s)
RE	Retinol equivalents
SAFA	Saturated fatty acid(s)
Sitostanol	β-sitostanol
Sitosterol	β-sitosterol
STAEST	Stanol ester
STEEST	Sterol ester
TC	Total cholesterol
TG	Triglyceride(s)
TR	Transport rate
VLDL-C	Very low density lipoprotein cholesterol
VLDL-TG	Very low density lipoprotein triglyceride(s)
VOSEM	Vegetable oil stanol ester-enriched margarine
WSEM	Wood stanol ester-enriched margarine

Plant sterol and plant stanol products contain, in particular, β-sitosterol or β-sitostanol, respectively; and therefore many authors have used 'β-sitosterol' or 'β-sitostanol' when describing their products. In this thesis terminology 'plant sterols' and 'plant stanols', respectively, have been used, because preparations contain usually at least traces of other sterols. In addition, in this thesis the term 'plant sterols' is also used as a generic term to include free and esterified plant sterols as well as free and esterified plant stanols if no particular form of plant sterols was especially emphasized or specified.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals **(I-V)**:

I Hallikainen MA, Uusitupa MIJ. Effects of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. Am J Clin Nutr 1999;69:403-410.

II Hallikainen MA, Sarkkinen ES, Uusitupa MIJ. Effects of low-fat stanol ester margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentrations. Eur J Clin Nutr 1999;53:966-969.

III Hallikainen MA, Sarkkinen ES, Uusitupa MIJ. Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner. J Nutr 2000;130:767-776.

IV Hallikainen MA, Sarkkinen ES, Gylling H, Uusitupa MI. Plant stanol esters affect serum plant sterols, but not in serum cholesterol precursors in a dose-dependent manner in hypercholesterolemic subjects (submitted).

V Hallikainen MA, Sarkkinen ES, Gylling H, Erkkilä AT, Uusitupa MI. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. Eur J Clin Nutr 2000;54:715-725.

In addition, some unpublished results are presented.

CONTENTS

1 INTRODUCTION

Dietary changes alone result usually in a modest reduction (3-6%) in serum total (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations at the population level (1). Therefore, great interest has been focused on plant sterols which have a clear hypocholesterolemic effect and which can be added to normal food items.

Plant sterols, which resemble cholesterol structurally, are essential components of all plant cells. The most common plant sterols are β-sitosterol (sitosterol), campesterol and stigmasterol. The most common saturated forms of plant sterols are β-sitostanol (sitostanol) and campestanol. Since the 1950's, plant sterols have been known to have hypocholesterolemic properties (2). This is based on their ability to inhibit intestinal absorption of both dietary and biliary cholesterol. In the 1970s, plant sterols were marketed as cholesterol-lowering agents, however, owing to high doses, poor-solubility and their chalky taste, they were gradually displaced by new and more effective drugs, the statins. In the early 1990s, an innovation to transesterify plant sterols with fatty acids made it possible to add plant sterols to fat-containing food items (e.g. margarines) in a soluble-form without affecting their sensory properties.

Several clinical studies on plant stanol esters and sterol esters have shown the cholesterol-lowering efficacy of these agents (3). In most of the earlier studies, moderate rich or high-fat diets have been used. Two clinically relevant questions have remained; can plant stanol esters and sterol esters be effective also as part of a cholesterol-lowering diet and do they provide an additional cholesterol-lowering efficacy compared with a low-fat diet alone? Different amounts of plant stanols (0.7-4.0 g/d) have been used in evaluating the hypocholesterolemic effects of plant stanol esters. However, there are no studies in which the dose-response effect of stanol esters has been investigated with several different doses i.e. is there a dose of stanol ester beyond which no additional benefits can be obtained? The chemical structure of different plant sterols may affect cholesterol-lowering efficacy of these agents. However, comparative studies between stanol esters and sterol esters have yielded inconsistent results (4, 5).

The primary aim of the present studies was to investigate the role of stanol ester- and sterol ester-enriched margarines in lowering elevated serum cholesterol concentrations as part of a low-fat diet, and to determine the optimal dose of plant stanol esters in practice.

2 REVIEW OF LITERATURE

2.1 Plant sterols

2.1.1 Nomenclature and structure of plant sterols

Plant sterols, also called phytosterols, are steroid alcohols. They resemble cholesterol structurally in that they contain a tetracyclic cyclopenta[a]phenanthrene ring in the α-configuration, a 3β-hydroxyl group and an alkyl side chain at the C-17 carbon atom in the β-configuration (6, 7). The most common plant sterols are 4-desmethylsterols (8), which differ from cholesterol in their side-chain substitution (extra ethyl or methyl group) at the C-24 position, and/or an additional double bond in the side chain (Figure 1). The most common representatives of that structure are sitosterol (24α-ethylcholest-5-en-3β-ol), campesterol (24α-methylcholest-5-en-3β-ol) and stigmasterol (24α-ethylcholest-5,22-en-3β-ol). The double bond in the B ring can also be in a different position, accordingly these sterols can be categorized to Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$-sterols (9). The ring structure of plant sterols can also be saturated. The most common plant stanols are sitostanol (24α-ethylcholest-3β-ol), and campestanol (24α-methylcholest-3β-ol). Plant materials contain also minor amounts of 4α-monomethyl sterols and 4,4-dimethyl sterols, which are the precursors of plant sterols (8, 10).

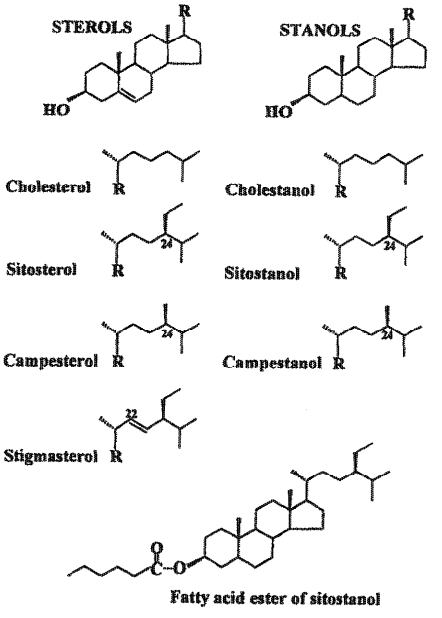


Figure 1. Structure of cholesterol and the most common plant sterols and their saturated forms, and as an example, the structure of the fatty acid ester of sitostanol is shown.

2.1.2 Occurrence of plant sterols in different plants

Plant sterols are not synthesized in the human body (11). Therefore, plant sterols are obtained only from the diet. Over 250 plant sterols and related compounds have been described in varying amounts in different plants and marine materials (8). Plant sterols can exist as free sterols, steryl esters (sterol esters) of fatty or phenolic acids, steryl glycosides or acylated steryl glycosides (12). The different fractions are thought to be located in different parts of the plant cell and to have several biological functions in plants, analogous to those of cholesterol in mammalian cells (12, 13). It has been hypothesized that free sterols, and to some extent steryl glycosides and acylated steryl glycosides, are incorporated into cell membranes and thus have structural and functional roles in cell membranes (12, 14). Plant steryl esters are believed to be located intracellularly and to represent mostly a storage and transport form of sterols (12, 13).

The plant sterol content in plants is not constant. Many factors, such as genetic factors, growth circumstances and time of plant harvest, as well as subsequent processing, may affect the concentration of sterols present in plants (15, 16). In addition, different analytical methods and sample preparation techniques may result in differences of sterol concentrations (17, 18).

Vegetable oils and vegetable oil-based products are regarded as the richest sources of plant sterols, followed by cereal and cereal-based products, nuts, seeds and legumes. The average plant sterol content of some foodstuffs is presented in Table 1. In plants, the predominant sterol is sitosterol followed by campesterol and stigmasterol. The other major plant sterols are avenasterol, stigmastenol and brassicasterol (19-22).

The total plant sterol content in the most frequently consumed vegetable oils has been reported to vary between 62 and 731 mg/100 g of oil (19, 21-25) (Table 1); rapeseed oil has the richest plant sterol content, whereas olive oil has the lowest content. Furthermore, small amounts of sitostanol have been found in hydrogenated coconut oil and soybean oil (19). The plant sterol content of vegetable oil-based margarines varies widely due to their different fat contents as well as source and proportion of vegetable oil in margarines (22, 26). Predominantly rapeseed oil based on soft margarines with a fat content of 40 to 80% have been reported to contain plant sterols 130-540 mg/100 g (26).

Although cereals and cereal products contain less plant sterols than vegetable oils (Table 1), they are nonetheless important sources of plant sterols due to their high daily dietary consumption. The plant sterol content in cereal grains has been reported to range from 23 to 178 mg/100 g of fresh weight (FW) (16, 19, 22, 27). Corn, rye and wheat are good sources of plant sterols, but oats are a poor source. Cereal grains, germ and bran fractions contain the most of the plant sterols, therefore whole grain flours are better sources of plant sterols than refined flours (16). The sterol content of rye breads has been reported to be 80-90 mg/100 g, whereas that of white bread has been reported to be only 40 mg/100 g (26). Moreover, bran fractions of rye, wheat and corn have been

found to contain appreciable amounts of plant stanols (16, 19, 28).

Vegetables, fruits and berries are generally not regarded as good sources of plant sterols (Table 1). In vegetables, the total sterol content has been reported to range from 3.8 to 50 mg/100 g FW (16, 22, 29). In general, cabbage is a good and potato is poor source of plant sterols. In fruits, the total sterol content has been reported to vary from 1.3 to 75 mg/100 g FW (22, 26, 29). Raspberry, lingonberry and blueberry have been found to contain moderate amounts of plant sterols (20-30 mg/100 g FW) (26).

Seeds, nuts and legumes, whose plant sterol content has been reported to vary between 22 mg and 714 mg/100 g (22, 30), are important sources of plant sterols in some diets. In addition, spices, coffee, cocoa and tea have also been reported to contain plant sterols, but they are not major dietary sources of plant sterols (22).

Table 1. Average plant sterol content in some foodstuffs.

Food item	Total plant sterol (mg/100 g)	References
Vegetable oils [a]		
Corn oil	472-952	22-24
Olive oil	62-232	22, 23, 25
Rapeseed oil	250-731	21-24
Soybean oil	221-328	19, 22-25
Sunflower oil	203-302	23, 25
Cereal grains [b]		
Barley	59-83	19[c], 26
Corn	178	22
Oats	23-52	19[c], 26, 27
Rye	91-110	19[c], 26
Wheat	60-76	19[c], 26
Seeds, nuts and legumes [b]		
Almonds	143	22
Peanuts	220	22
Sesame seeds	714	22
Soybeans	161	22
Vegetables and fruits [b]		
Brussels sprouts	24-43	22, 29
Carrot	12-16	22, 29
Cauliflower	18-40	22, 26, 29
Potato	3.8	29
Apple	12-13	22, 29
Avocado	75	26
Orange	24	29

[a] refined, except for olive oil which is virgin oil.

[b] per fresh weight

[c] calculated from reported sterol content of free and bound lipid of cereals.

Origin of plant sterols in plant sterol products

At present, the plant sterols used in clinical studies on plant sterol products are wood-based, derived predominantly from pine wood (tall oil) and/or vegetable oil-based, derived predominantly from soybeans, but also from rapeseed and sunflower oils (31). The plant sterol content differs depending on the source: plant sterols derived from wood contain approximately 90% sitosterol and 10% campesterol, while plant sterols derived from vegetable oils contain about 70% sitosterol and 30% campesterol (32-34). In addition, commercially available 'tall oil' sterols contain 15-20% by weight stanols (35, 36). Plant stanols can also be produced by hydrogenation of commercially available plant sterols (37).

2.1.3 Dietary intake of plant sterols

Food composition databases for plant sterols are still incomplete. Therefore, the calculations of the dietary intake of plant sterols are not accurate. This should be kept in mind when examining the published intake levels.

Vegetable oils, fats and cereal products are the most important sources of plant sterols in the average Western diet (38). The daily intake of plant sterols has been estimated to range from 150 to 400 mg/d (38-42) when the intake of plant stanols is estimated to be roughly 10% of the intake of plant sterols (43). However, the dietary intake of plant sterols seems to vary greatly among different populations depending primarily on the type and amount of plant food that is consumed. In some vegetarians, the intake of plant sterols has been reported to be almost 1 g/d (44), although also very low intake levels have been reported; in pure vegetarian Seventh Day Adventists, the intake of plant sterols has been reported to be only 89 mg/d, while in lacto-ovo-vegetarian and non-vegetarian Seventh Day Adventists it has been reported to be 344 mg/d and 231 mg/d, respectively (45).

2.1.4 Physical and technological properties of plant sterols

The physical properties of plant sterols may be critical in determining their ability to reduce cholesterol absorption and thus reduce serum cholesterol concentrations (described in more detail 2.5). Non-palatable and non-saponiable plant sterols have high melting points (8, 46), therefore at room temperature plant sterols are in a solid, crystalline form and their solubility in edible fats and oils is less than 1% (37, 47). The larger their side chains, the more hydrophobic the sterols become (46). Therefore, campesterol and sitosterol (C28 and C29) are more hydrophobic than cholesterol (C27). Furthermore, a double bond in side chain increases the hydrophilicity of sterols (46).

Transesterification of plant sterols with fatty acids of vegetable oils transforms crystalline plant sterol powder into a soluble form with fat-like properties (32). In this esterified form, plant sterols are readily incorporated into different foodstuffs such as margarines in sufficient amounts without changing their original texture and feel in the

mouth. Plant stanol esters and sterol esters can replace the hard fat used in the production of margarines and other spreads, and thus improve fatty acid composition as well as reduce the amount of fat of end products (37).

2.2 Plant sterols and serum lipids

2.2.1 Effects of plant sterols on serum total cholesterol and LDL cholesterol

In the following sections, first the cholesterol-lowering effects of free plant sterols and plant stanols and then corresponding effects of plant stanol esters and sterol esters are reviewed. Later, the results of comparison studies of plant sterols and plant stanols and dose-response studies are reviewed. Finally, factors affecting the cholesterol-lowering abilities of plant sterols are discussed.

2.2.1.1 Effects of free plant sterols

Since the early 1950's, plant sterols have been known to reduce serum cholesterol concentrations significantly in animals and humans (2). In 1951 Peterson (48) and in 1952 Pollak (49) reported in chickens and in rabbits, respectively, that simultaneous feeding with cholesterol and mixed soybean sterols reduced serum TC concentrations. Subsequently, this finding has been confirmed in many animal studies (2, 50).

Pollak was the first to show that plant sterols significantly reduce serum TC concentration in humans (51). In 1953, in his study with 26 healthy subjects, the consumption of 5-10 g/d of plant sterol mixture powder (75-80% of sitosterol) as part of a habitual diet resulted in a mean reduction of 28% in serum TC compared with the habitual diet alone. Since that study, and in particular, during the next fifteen years, numerous clinical studies on the hypocholesterolemic effects of plant sterols were carried out. Those studies have been reviewed by Pollak and Kritchevsky (2) and by Pollak (50). In summary, the studies have been controlled or non-controlled and have lasted from a single day to 45 months, with typical duration of 2-8 weeks. Subjects of both genders have mainly been normocholesterolemic, mildly to severely hypercholesterolemic or hypercholesterolemic with clinical evidence of atherosclerosis. The number of subjects has varied between 1 and 118, with an average of 20 subjects per trial. Subjects have mainly followed their habitual diet or a diet modified with regard to the intake of fat and cholesterol. Doses of plant sterol have been very large, up to 53 g/d, but a typical dose has been 5-18 g/d with a mean reduction of 10-20% in serum TC concentrations. However, the lipid responses to the intake of plant sterols seemed to be varied greatly within studies as well as among studies. Factors affecting the lipid responses are discussed in later. The assumption that large amounts of plant sterols are required to achieve a sufficient lipid response prevailed until the middle of the 1970s. Then Lees and Lees (52, 53) re-evaluated the effective dose of plant sterols and revealed that in most adult patients with type 2 hyperlipoproteinemia the maximal

cholesterol-lowering effect (9-12%) could be obtained with a dose of 3 g/d of tall oil sterols containing up to 95% sitosterol. The cholesterol-lowering efficacy of smaller doses of plant sterols has also been examined (54-57). The findings of these studies are described in the section of dose-response effect of plant sterols.

2.2.1.2 Effects of free plant stanols

In general, only a few studies have been made with free plant stanols. Interest in plant stanols arose when, in rat and rabbit studies, free plant stanols were found to be more effective in lowering serum TC than free plant sterols (58-61). In 1986, Heinemann et al. (62) reported that in 6 patients with hypercholesterolemia a daily dose as low as 1.5 g of plant stanol consumed as part of a diet containing <300 mg/d cholesterol and 35 percent of energy (E%) fat [polyunsaturated to saturated fatty acids (P/S)=1] reduced serum TC and LDL-C concentrations by up to 15% compared with the control period. Plant stanols were administered as capsules containing the stanols dispersed and partly dissolved in sunflower oil (62). In contrast to the findings of Heinemann et al. (62), in 33 men with mild to moderate hypercholesterolemia Denke (63) found that the consumption of 3 g/d of plant stanols did not reduce plasma TC and LDL-C significantly when these men had consumed plant stanols as gelatin capsules as part of a low-fat, low-cholesterol diet for 3 months. The most probable reason for this unexpected finding was that the capsules contained plant stanols suspended in sunflower oil i.e. not dissolved, and they were thus in a poorly soluble, less effective, form.

2.2.1.3 Effects of plant stanol esters and plant sterol esters

During the last decade, major interest has been focused on plant stanol esters and sterol esters and their efficacy in decreasing serum cholesterol concentrations. To date, the hypocholesterolemic effect of plant stanol esters or sterol esters has been shown in over 20 publications (4, 5, 31, 33, 34, 47, 54, 64-79). These intervention studies are described in Table 2 in which the percentage reductions in serum TC and LDL-C are presented mainly compared with a control group or period, but also in some studies they are related to baseline (33, 71, 74, 75, 78, 79). Most of these studies have been done in Finnish populations. When the results of various studies are compared, however, there do not seem to be differences in the cholesterol-lowering efficacy of stanol esters or sterol esters among populations in different countries.

The controlled studies have been carried out using a cross-over or parallel study design. The duration of trials has been short, typically 4-8 weeks, except in one study, which lasted for one year (68). The daily dose of plant stanols or sterols obtained from stanol ester or sterol ester products has ranged from 0.7-0.8 g to 8.6 g, a typical dose being 2-3 g/d. The number of subjects has varied between 7 and 318 per study. Most published studies in adults have been conducted in individuals with mild to moderate hypercholesterolemia, whereas normocholesterolemic individuals have participated in

Table 2. Intervention studies on the effects of plant stanol esters and sterol esters on serum TC and LDL-C concentrations.

First author (Ref)	Subjects	N(M/F)	Age, mean (range), y	Study design	Study diet		Dose (g/d)[a]	Duration (wk)	Results		
					SAFA (E%)	Chol. (mg/d)			Control TC/LDL-C (mmol/l)	Net reduction with test spreads (%)	
										TC	LDL-C
Adults											
Vanhanen (47)	HC	67(47/20)	46(25-60)	parallel	12	270	3.1 (STA)	6	5.9/3.7	-7	-9
Miettinen (54)	HC	31(22/9)[b]	45	parallel	12	326	0.7-0.8 (STA)	9	6.5/4.4	P=NS	-8(calc.)
Vanhanen (64)	HC	15(11/4)[c]	48(33-60)	parallel	..	295	0.7-0.8 (STA)	9	6.5/4.4	P=NS	P=NS
							1.8-1.9 (STA)	6	6.6/4.5	-9	-15
Gylling (65)	HC+DM type 2	11(11/-)	58	cross-over	..	341	3 (STA)	6	6.0/3.8	-6	-9
Gylling (67)	HC+DM type 2	7(7/-)	60	cross-over	..	233	3 (STA)	7	6.6/..	-11	-14
Miettinen (68)	HC	141	50(25-64)	parallel	14	321	1.8 (STA)	24	6.1/4.1	-9(calc.)	-9(calc.)
							2.6 (STA)	52		-10	-13
Niinikoski (69)	NC	24(8/16)	37(24-52)	parallel	..	..	2.2 (STA)	5	4.8/3.6[d]	-10	-13[d]
Gylling (70)	CAD	22(-/22)	51(48-56)	cross-over	<15	207	3 (STA)	7	6.0/3.7	-8	-15
Weststrate (4)	NC,HC	95	45(18-65)	cross-over	16	234	2.5 (STA)[e]	3.5	5.2/3.4	-7	-13
							3.3 (STE)			-8	-13
Gylling (33)	HC	23(-/23)	53(50-55)	cross-over	P/S 0.6	262	3.18 (STA) (camp:sito 1:11)	6	6.1/4.0[f]	-4	-8
							3.16 (STA) (camp:sito 1:2)			-6	-10
	HC	21(-/21)	53(50-55)	parallel	P/S 0.3	323	2.43 (STA) (camp:sito 1:13)[g]	5	6.3/4.2	-8	-12
Hendriks (31)	NC,HC	80	37(19-58)	cross-over	13	250	0.83 (STE)	3.5	5.2/3.1	-5	-7
							1.61 (STE)			-6	-9
							3.24 (STE)			-7	-10
Andersson (71)	HC	61(28/33)[h]	55(30-65)	parallel	8	240	2 (STA)[h]	8	6.6/4.5	-7	-7
					12	279	2 (STA)[h]		6.6/4.6[f]	-9	-12
Nguyen (72)	HC	318(157/161)	53	parallel	10	234	EU 2.2 (STA)[i]	8	6.0/4.0	-5	-5
							US 2.7 (STA)[i]			-7	-10
							US 1.6 (STA)[i]			-5	-4
Ayesh (73)	NC,HC	21	36(30-40)	parallel	..	..	8.6 (STE)	3 or 4[j]	5.2/3.3	-18	-23

Plat (34)	NC,HC	112(41/71)	33(18-65)	parallel	14	233	3.8 (STA)[k] 4.0 (STA)[k]	8	4.9/2.9	-8(calc.) -8(calc.)	-13(calc.) -11(calc.)
Vuorio (75)	FH-NK	4(2/2)	41(33-49)	parallel	<7	<200	2.24 (STA)	12	9.0/7.5[l]	-10(no stat.)	-11(no stat.)
	Healthy family members of FH-NK	16(3/13)	32(8-49)	parallel	<7 or 8-10[l]	<200 or <300[l]	2.24 (STA)	12	4.9/3.2[l]	-7	-12
Relas (79)	NC	11(11/-)	58	parallel	P/S 0.4	281	3 (STA)	2	4.6/..[f]	P=NS	..
Jones (5)	HC	15(15/-)	(37-61)	cross-over	10	..	1.96 (STE) 1.57 (STA)[m]	3	6.0/4.2	-9 P=NS	-13 -6
Miettinen (78)	Colect.	11	45(29-64)	parallel	..	..	2 (STA)[n]	1[n]	5.3/2.5[f]	-16	-14(calc.)
Plat (77)	NC,HC	39(11/28)	31(18-65)	cross-over	13	231	2.47 (STA)[o] 2.46 (STA)	4	5.0/3.0	-6 -7	-9 -10
Children											
Gylling (66)	FH	14(7/7) heteroz. 1(1/-) homoz.	9(2-15)	cross-over	14	114 (3.2 mg/ body WT)	2.8 (STA)	6	7.6/5.5 20.9/17.7	-11 -3(no stat.)	-15 -9(no stat.)
Williams (74)	NC,HC	19(8/11)	4(2-5)	cross-over[p]	11	172	2.9 (STA)	4	4.2/2.4[f]	-12	-16
Vuorio (75)	FH-NK	24(8/16)	9(3-13)	parallel	8-10[l]	<300[l]	2.24 (STA)	12	7.4/6.0[l]	-14	-18
Tammi (76)	NH,HC	72(40/32)	6	cross-over	11	152	1.5 (STA)	12	4.2/2.6	-5(calc.)	-7(calc.)

HC=hypercholesterolemia, DM=diabetes mellitus, NC=normocholesterolemia, CAD=coronary artery disease, FH-NK=Familial hypercholesterolemia - North Karelia mutation of low density lipoprotein receptor gene, Colect.=colectomized, FH=familial hypercholesterolemia

heteroz.=heterozygous, homoz.=homozygous

SAFA=saturated fatty acids, E%=percent of energy, P/S=polyunsaturated to saturated fatty acids, Chol.=dietary cholesterol, body WT=body weight

STA=plant stanols; Plant stanols have been added in esterified form to butter (33), vegetable oil-based mayonnaise (47, 54, 64), margarine and shortening (34, 77) or margarine (in all other studies). STE=plant sterols; Plant sterols have been added in esterified form to vegetable oil-based margarine. camp:sito=campestanol:sitostanol

calc.=calculated from mean values, no stat.= not statistically analyzed

.. =not reported

[a] Actual daily dose of added plant stanols or sterols as informed by investigators or as calculated from actual mean intake of test products (5, 47,

54, 64, 72).
[b] Plant stanol ester group (N=7) and control group (N=8)
[c] Control group consisted of 8 men.
[d] non-HDL-C
[e] Total amount of sterol was 2.7 g/d.
[f] baseline habitual diet
[g] Stanol ester mixture was added to butter. A control group consumed butter without added plant stanols.
[h] low-fat diet+low-fat stanol ester margarine group (N=19), low-fat diet+low-fat margarine (control) group (N=21), usual diet+low-fat stanol ester margarine group (N=21)
[i] EU 2.2=European-formula vegetable oil-based spread (3.0:6.2:2.6 g/actual daily dose, saturated:monounsaturated:polyunsaturated fatty acids); US 2.7=US-reformulated vegetable oil-based spread (2.0:7.9:4.6) and US 1.6= US-reformulated vegetable oil-based spread (2.1:7.7:4.3)
[j] For men the study period lasted 3 weeks and for women it was 4 weeks.
[k] 3.8 g/d of vegetable oil based plant stanols and 4.0 g/d of wood based plant stanols
[l] Adults followed the step 2 diet of the National Cholesterol Education Program (81), whereas children followed a step 1 diet. The comparisons have been made to baseline, step 2 or step 1 diet, respectively.
[m] Total amount of sterol was 1.76 g/d.
[n] Patients consumed light Benecol®. A significant reduction in TC was -3% (calc.) after 1 day and -11% (calc.) after 3 days.
[o] Dose of plant stanol ester has been consumed once per day at lunch.
[p] The other test period was a period of high-fiber diet. The comparison was made to the baseline value.

only a few studies. Furthermore, some studies have been conducted in adults with familial hypercholesterolemia - North Karelia mutation of low density lipoprotein receptor gene (FH-NK), women with coronary artery disease (CAD), men with type 2 diabetes mellitus (DM), or colectomized patients. In addition, there have been a few studies of normocholesterolemic, mildly to moderately hypercholesterolemic children, children with familial hypercholesterolemia (FH) or FH-NK. The age of men and women participating in these studies has varied between 18 and 65 years, a great part of them being over 40 years. The age of boys and girls has varied between 2 and 15 years.

In studies with normocholesterolemic (4, 31, 34, 69, 77) or mildly to moderately hypercholesterolemic subjects (4, 5, 31, 33, 34, 47, 64, 68, 71, 72, 77) a net reduction of 5-10% in TC and of 5-15% in LDL-C with the doses of 2-4 g/d of plant stanols or sterols has been observed compared with the control period or control group. In a landmark study (68) in subjects with mild to moderate hypercholesterolemia, the consumption of margarine enriched with plant stanol esters (2.6 g/d of stanols) for a year decreased serum TC and LDL-C concentrations by 10% and 14%, respectively, from the baseline values of the subjects and by 10% and 13%, respectively, from the values of the control group, which consumed margarine without added stanol esters.

In studies with normocholesterolemic or mildly to moderately hypercholesterolemic children aged 2-6 years, the consumption of plant stanol esters (1.5-2.9 g/d of stanols) for 4 or 12 weeks reduced serum TC by 5-12% and LDL-C by 7-16% (74, 76).

The role of plant sterol treatment in different types of lipid disorder is discussed later in the section on factors influencing on the cholesterol-lowering ability of plant sterols.

2.2.1.4 Comparison studies of plant sterols and plant stanols

Some studies have compared the cholesterol-lowering efficacy of plant sterols and plant stanols in free or in esterified form with inconsistent results.

In two comparison studies in hypercholesterolemic men and women (54, 55) with small doses of free plant sterols (0.7 g/d) and free plant stanols (0.6-0.7 g/d), no differences in cholesterol-lowering activity were found. However, in a comparison study in children with severe heterozygous FH, Becker et al. (80) found that 1.5 g/d of free plant stanols given as pastilles reduced serum LDL-C concentrations significantly more than 6 g/d of free plant sterols. The reductions were 33% after 3 months and 29% after 7 months vs. 20% after 3 months, respectively.

In the comparison study of Weststrate and Meijer (4), a soybean sterol ester margarine and a stanol ester margarine (Benecol®) were found to reduce plasma TC and LDL-C concentrations equally effectively, despite the fact that these two margarines differed in the amount of total sterols (3.25 g/d vs. 2.74 g/d, sterol ester margarine vs. Benecol®), in the degree of esterification of sterols and stanols (65% vs. >98.5%) and in fatty acid composition [less saturated (SAFA) and monounsaturated (MUFA) fatty acids and more linoleic acid in sterol ester margarine than in Benecol®]. However,

recently, Jones et al. (5) demonstrated that plant sterol esters reduced plasma LDL-C concentrations more efficiently than stanol esters. In that cross-over study, however, the amount of total sterols was different (1.96 g/d vs. 1.76 g/d, sterol ester vs. stanol ester margarine). Furthermore, the number of subjects was small; only 15 subjects completed the study. In addition, the subjects were randomly assigned to one of six predetermined Latin squares, where each square included three sequenced periods and three subjects. Therefore, the findings in that study might be due to different amounts of total sterols, the methods of randomization and/or the small number of subjects.

2.2.1.5 Dose-response effect of plant sterols

In general, information on the dose-response effect of plant sterols for lowering serum TC and LDL-C is scarce, since in most studies only one dose has been used to evaluate the cholesterol-lowering efficacy of plant sterols. Some studies, however, have evaluated directly the dose-response relationship.

Minimum daily dose

In earlier studies it has been suggested that at least 1 g/d of plant sterols or stanols should be consumed before a clinical response in serum cholesterol is reached (54, 55, 64). This suggestion was based on findings from studies (54, 55, 64) that mainly included a small number of subjects. In those studies, the consumption of 0.6-0.8 g/d of plant sterols, stanols or stanol esters (calculated as free stanols) added to 50 g/d of rapeseed oil-based mayonnaises or spreads resulted in a significant or non-significant reduction of 2-8% (calculated from mean values) in TC and LDL-C. The typical feature of those studies was that serum LDL-C decreased significantly (up to 15%) during the rapeseed oil run-in period. In later studies, more consistent findings have been found with small doses of plant sterols. In two studies (31, 56) a daily intake of 0.80-0.83 g of soybean- or other edible oil-derived plant sterols added as free or in esterified form to margarine reduced TC and LDL-C by 4-7% compared with the control. In addition, in one study 0.74 g/d of free soybean sterols added to butter reduced TC and LDL-C by 10-15% (57).

In conclusion, according to the above-mentioned studies, it seems that a daily dose as little as 0.7-0.8 g of plant sterols or stanols can reduce serum cholesterol concentrations significantly, though of that dose the cholesterol-lowering effect remains below 10%.

Maximum daily dose

In the 1960s Beveridge et al. (82) reported that supplementing 0.3 g/950 kcal (0.87 g/d) of free plant sterols to a diet significantly decreased (-12.72 mg/dl, -0.30 mmol/l) plasma TC. With the greatest supplementation tested (6.4 g/950 kcal, 20.4 g/d), the decrease was 35.43 mg/dl (-0.90 mmol/l, -20%) suggesting that the sterol increments progressively reduced the concentrations of plasma TC (82). In later studies, however, it

has been suggested that the relationship between the intake of plant sterols and serum TC and LDL-C concentration is curvilinear rather than linear. In a series of four clinical trials with type 2 hyperlipoproteinemic adults, Lees and Lees (53) found that an intake of 3 g/d of tall oil sterol suspension or powder reduced plasma TC by 9-12%, but increasing the dose of tall oil sterol suspension from 3 to 6 g/d did not further lower plasma TC. Lees and Lees (53) also confirmed those findings in a cholesterol-balance study. In that study, 3 g/d of tall oil sterols reduced cholesterol absorption markedly, and increasing the dose from 3 to 9 g/d did not achieve any further gains (53). In children, however, the same researchers observed that an intake of 3 g/d of tall oil sterol resulted in only a 3% reduction in TC ($P=0.03$), whereas an intake of 6 g/d ($P=0.06$) achieved a 10% reduction; therefore they suggested that in children, in contrast to adults, 6 g/d of free plant sterols may be more effective than 3 g/d (53).

The optimal dose of plant stanol esters and sterol esters has also been evaluated. Miettinen et al. (68) found that a dose of 2.6 g/d of plant stanols as stanol esters reduced serum TC and LDL-C concentrations slightly, but significantly more (about 0.2 mmol/l) than a dose of 1.8 g/d of plant stanols. They concluded, however, that for practical purposes both doses possessed similar cholesterol-lowering effects. Similarly, Nguyen et al. (72) found that a US-reformulated vegetable oil-based spread containing 2.7 g/d of plant stanols as stanol esters reduced serum LDL-C significantly more than a similar spread containing 1.6 g/d of plant stanols (difference 0.24 mmol/l). On the contrary, Hendriks et al. (31) found no significant differences in cholesterol-lowering effects between the doses of 0.83, 1.61 and 3.24 g/d of plant sterols as sterol esters. However, 95% confidence intervals (compared with the control) suggested that the higher the sterol ester dose, the greater the reduction in plasma cholesterol (4.9% to 6.8% for TC and 6.7% to 9.9% for LDL-C, respectively) (31).

The optimal dose has also been assessed by Wester in his review (83) in which he compared TC and LDL-C responses for different stanol ester doses in different studies. He concluded that the curvilinear dose-response curve plateaus at an intake equivalent to about 2.2 g/d of stanols and that optimal cholesterol-lowering effect is obtained with daily intake of plant stanol esters corresponding to 2-3 g stanols. Furthermore, it has been suggested that increasing the dose above 3 g/d may not lead to any further reductions in serum TC and LDL-C (83, 84). The narrow range of dose responsiveness has been proposed to be a consequence of the compensatory increase in cholesterol synthesis that can be observed after consumption of high doses of plant sterols or stanols (84). This suggestion is based on the findings of Vanhanen et al. (64), in which the intake of about 2 g/d of plant stanols, but not a dose of about 0.8 g/d, was considered to increase cholesterol synthesis by 2 mg/body weight/d. However, the rate of synthesis does not replenish the lost cholesterol, leading to a net reduction of serum cholesterol.

Time needed to response

Plant sterols have been found to reduce serum cholesterol concentrations within 2-3 weeks of the initiation of treatment (62, 72, 74, 85). However, those studies have not actually assessed the minimum time needed to observe an effect of plant sterols on serum cholesterol concentrations. In a one year-long study Miettinen et al. (68), reported that although the reduction in serum TC and LDL-C concentrations had occurred mainly during the first three months, the concentrations tended to continue to fall throughout the study. Lees and Lees (53) reported that a reduction in plasma TC reached during the first 10 months due to the consumption of soy sterols did not diminish during 3 years with their continued consumption. With cessation of ingestion of plant sterols, the serum cholesterol concentrations have been found to return to the initial value within 2-3 weeks (62, 68, 72, 74, 85).

In one specific group, colectomized patients, a significant reduction in serum TC was found already after one day of the consumption of plant stanol esters, and the steady state was reached within just one week (78).

2.2.1.6 Factors influencing on the cholesterol-lowering ability of plant sterols

Large between-subjects variation in cholesterol responses to intake of plant sterols has been reported in many studies (2, 5, 53, 63, 86). Several factors such as gender, age, body weight, initial value of serum cholesterol, type of lipid disorder and genetics as well as experimental diets and plant sterol products can influence the cholesterol-lowering ability of plant sterols (2, 15, 50). However, only a few studies have focused systematically on these issues. Therefore, conclusions have mainly been drawn by comparing findings of different studies, which might have been performed very different study designs.

Gender, age, body weight and initial value of serum cholesterol

No differences in cholesterol-lowering response to plant sterol administration between **genders** have been reported (4, 47, 51, 54, 56, 64, 77).

Serum cholesterol concentration varies with **age** (87). Findings of the effects of age on lipid responses induced by plant sterols have been conflicting in those few studies in which children, adolescents and adults or at least two of these age groups have participated (2, 53, 75, 88). According to the meta-analysis of 14 intervention trials with different groups of adult subjects by Law (89), the reduction in the concentration of LDL-C at each stanol or sterol dose is significantly greater in older people than in younger people. Plant stanol or sterol doses ≥2 g/d have been found to reduce serum LDL-C significantly by an average of 0.54 mmol/l (14%) in people aged 50-59 years, by 0.43 mmol/l (9%) in those aged 40-49 years and by 0.33 mmol/l (11%) in those aged 30-39 years (89).

In the few studies in which effects of **body weight** on lipid responses to plant sterols

have been examined, no differences in cholesterol-lowering efficacy between subjects with normal weight and those who are overweight have been observed (53, 72).

The higher the **initial concentration of serum TC,** the greater the reduction in TC which has been observed in several (51, 68, 70, 76, 78), but not in all plant sterol studies (4).

Type of lipid disorder

The type of lipid disorder seems to affect outcomes of plant sterol treatment. In several studies the consumption of plant sterols has been shown to reduce serum TC and LDL-C significantly in subjects with primary moderate hypercholesterolemia (2, 5, 33, 47, 64, 68, 71, 72). Subjects with clinical evidence of atherosclerosis or documented coronary heart disease have been found to respond fairly well to plant sterol treatment (2, 62, 70). Recently, in women with **CAD** (70) the consumption of plant stanol esters (3 g/d of stanols) added to rapeseed oil-based margarine reduced serum TC and LDL-C by 8-15% compared with the control period and by up to 13-20% compared with the baseline diet values. In addition, the use of stanol ester margarine was found to normalize serum LDL-C (<2.6 mmol/l) in about every three women with CAD, especially those with high baseline absorption and low synthesis of cholesterol.

Type 2 DM is associated with accelerated atherosclerosis. In a small number of mildly hypercholesterolemic men with type 2 DM (65, 67) stanol esters (3 g/d of stanols) reduced serum TC and LDL-C concentrations by 6-11% and 9-14%, respectively, compared with the control period. In addition, serum LDL-C was found to be reduced, in particular, in the dense fraction, which is considered to be the most atherogenic LDL particle (90, 91).

Genetics

The finding of the effects of **apolipoprotein (apo) E** genotype or phenotype on lipid responses for an intake of plant sterols have been controversial: in earlier studies it has been suggested that reduction of LDL-C would be more consistent in subjects with the ε4 allele than in those with homozygous ε3 alleles (47, 54), but in later studies no differences have been found among genotype or phenotype groups (34, 66).

Heterozygous FH subjects, especially children, seem to benefit from plant sterol treatment. In FH children, the consumption of free plant sterols (6 g/d) (80, 92) or free plant stanols (1.5 g/d) (80) as pastilles or the consumption of plant stanol esters (2.8 g/d of stanols) added to rapeseed oil-based margarine (66) has been reported to cause a 11-26% reduction in serum TC and a 15-33% reduction in serum LDL-C compared with the control. However, in one homozygous FH boy (66), the reductions were only 3% and 9%, respectively, being in line with the earlier suggestion that monogenic hypercholesterolemia of the homozygous type will not respond or respond only poorly to these compounds (2). In a genetically homogenous FH population containing both

children aged 3-13 years and adults all carrying the FH-NK deletion (75) the consumption of stanol ester margarine (2.24 g/d of stanols) as part of a National Cholesterol Education Program (NCEP) (81) step 1 (children) or step 2 (adults) diet, reduced serum TC and LDL-C by 14-18% in children and by 10-11% in adults compared with the cholesterol-lowering diet the subjects had followed for at least a year before the study. However, in one heterozygous FH-NK child, the serum LDL-C concentration was reported to slightly increase during the trial (75).

Experimental diets

The composition of the diet may have an effect on cholesterol-lowering efficacy of plant sterol treatment. In most studies, the comparisons have been made with a **control or run-in diet** being similar to the study diet except added plant sterols. In some studies (55, 64, 70) replacing the usual dietary fats with rapeseed oil-based products containing substantial amounts of unsaturated fats and thus natural plant sterols, has reduced serum LDL-C significantly, up to 15%, already during the run-in or control diet period alone. Therefore, when a small dose of plant sterol has been added to that diet, no additional cholesterol-lowering effect has been achieved (55, 64). In some studies the comparisons have been made with the **habitual (baseline) diet** of the subjects that might have been varied greatly between subjects (33, 62, 68, 70, 71, 74, 78). Naturally, the reduction in LDL-C is numerically slightly greater when the comparison has been made against the habitual diet than to the control diet (68, 70, 71).

In most studies a **study diet** has contained a moderate or substantial amount of dietary fat and SAFA and in some cases also large amounts of cholesterol. In those studies, serum TC and LDL-C have been reduced by an average of 5-15% with plant sterols compared with the control (4, 31, 33, 34, 47, 64, 68, 77). Despite the opposite finding of Denke (63) with free plant stanols (discussed more in section 2.2.1.2), plant stanol esters have been found to be effective also as part of a low-fat, low-cholesterol diet (71, 72, 75). Recently, Andersson et al. (71) showed that the cholesterol-lowering effect of low-fat stanol ester margarine was additive, when consumed as part of a cholesterol-lowering diet. The reductions in serum TC and LDL-C were -15% and -19%, respectively, with combination of the low-fat stanol ester margarine (2 g/d of stanols) and the low-fat diet. The respective reductions were -8% and -12% with the low-fat control margarine and low-fat diet, and -9% and -12% with the low-fat stanol ester margarine and usual diet.

Plant sterol products

Serum cholesterol-lowering efficacy of plant sterols may also vary as a result of the composition, form and dose of plant sterols as well as the physical state and consumption frequency of the plant sterol products. The influence of plant sterol dose on outcomes has been discussed in earlier in this thesis.

It has generally been suggested that the greater the sitosterol or sitostanol content in a preparation, the greater will be its hypocholesterolemic efficacy. However, findings on the importance of the **composition** of plant sterols have been inconsistent. Lees and Lees (52, 53) observed that a smaller amount of tall oil sterols (93% sitosterol, with the remainder mostly consisting of campesterol) than soybean sterols (60-65% sitosterol and 35-40% mostly campesterol) was needed to achieve a similar cholesterol-lowering effect. However, recently, wood-based (about 90% sitostanol and 10% campestanol) and vegetable oil-based (about 70% and 30%, respectively) stanol esters dissolved into margarines have been found to reduce serum TC and LDL-C equally effectively (33, 34). In addition, a mixture of sitostanol containing tall oil plant sterols (about 62% sitosterol, 21% sitostanol, 16% campesterol and 1% campestanol) blended into dietary fats has been reported to reduce TC and LDL-C significantly (36, 86). In some earlier studies, stigmasterol, but not γ-sitosterol, has been reported to reduce serum TC concentration (2). Furthermore, rice bran or sheanut oils containing 4,4'-dimethyl sterols (1.7-3.2 g/d) esterified mainly with ferulic (rice bran) and cinnamic and acetic acids (sheanut oil) have not been found to lower serum TC and LDL-C significantly (4, 56).

There are no comparison studies between free and esterified **forms** of plant sterol in contrast to comparisons between the chemical forms of plant sterols. Free plant stanols have been proposed to reduce serum LDL-C more than free plant sterols with large doses (80), but not with small doses (54, 55). Findings of the comparisons between the esterified forms of plant sterols and stanols have been inconsistent (4, 5).

The **physical state of preparation** (vehicle of plant sterols) seems to have a crucial role in determining the cholesterol-lowering efficacy of plant sterols. Powdered plant sterols as such or used in capsules, tablets or granules have been found to be more effective than when they are in suspension (2, 52, 53). Furthermore, dissolving of plant sterols free or in esterified form into dietary fats seems to increase their cholesterol-lowering efficacy when a significant reduction in LDL-C can be reached already with small doses of plant sterols. Findings of two studies in which plant stanol ester-enriched spreads with different fatty acid compositions have been compared, have been contradictory. In one study (33), no differences between rapeseed oil- (monoene-rich) based margarine (3.16-3.18 g/d of stanols) and butter (2.43 g/d of stanols) have been found. However, in another study (72) US-reformulated vegetable oil-based spread (2.7 g/d of stanols) reduced serum LDL-C more than European-formula vegetable oil-based spread (2.2 g/d of stanols). The difference has been suggested to partly be due to the fact that the former spread contained less SAFA and more MUFA and polyunsaturated fatty acids (PUFA) than the latter spread (see Table 2). However, the difference might also partly be due to the different daily intakes of plant stanol esters.

In earlier studies, the consumption of plant sterols with meals, **consumption frequency** (2-3 times/d) and amount of consumption in relation to dietary cholesterol have been suggested to be critical in their cholesterol-lowering abilities (2, 82, 93, 94),

however, these proposals have recently been questioned, at least with respect to the plant stanol esters (77).

2.2.2 Effects of plant sterols on other serum lipids and lipoproteins, and apolipoproteins

Effects on high-density lipoprotein cholesterol

In general, in most studies the consumption of plant sterols has not been found to affect the concentration of serum high-density lipoprotein cholesterol (HDL-C) compared with the control (4, 5, 31, 34, 36, 47, 54, 56, 57, 62-64, 66-71, 74-78, 80, 84, 86, 92). However, in some studies, serum HDL-C concentration has increased (33, 65), while two studies reported decreases in HDL-C (80, 88). The effects of plant stanol esters on subclasses HDL_2-C and HDL_3-C have not been different from that of the control group or the control period (66, 95).

Effects on intermediate and very low-density lipoprotein cholesterol

Effects on serum intermediate density lipoprotein cholesterol (IDL-C) or very low-density lipoprotein cholesterol (VLDL-C) have been examined in a few studies. In some studies, plant stanols or plant stanol esters have not been found to affect IDL-C (54, 65, 67, 71) or VLDL-C concentrations significantly (54, 63, 66, 70, 71, 95), whereas in others, the reduction in serum IDL-C (66, 70) or VLDL-C (65, 67) has been significant compared with the control.

Effects on total and lipoprotein triglycerides

In general, persons who have had serum triglyceride (TG) concentration greater than 3 mmol/l were excluded from intervention studies. In most studies, plant sterols have not been reported to have any significant effect on serum TG concentration (5, 31, 33, 34, 36, 47, 54, 56, 62-68, 70-72, 74-78, 80, 86, 88, 92). However, in some studies or subtrials, serum TG concentration has increased moderately (52, 53, 80, 96) while in others it has decreased (52, 53).

Compared with the control, plant stanol esters have not been found to change concentrations of serum very low-density lipoprotein triglycerides (VLDL-TG) or intermediate density lipoprotein triglycerides (IDL-TG) significantly (66, 70, 71, 95). In one study, serum low-density lipoprotein triglycerides (LDL-TG) decreased slightly, but significantly, compared with the control (70); but in other studies these effects have not been significant (66, 71, 95). Furthermore, no significant changes have been observed in serum high-density lipoprotein triglycerides (HDL-TG) (66, 70, 71) or in subclasses HDL_2-TG and HDL_3-TG (66, 95).

Effects on apolipoproteins

Apo AI and apo B are the major structural components in HDL-C and LDL-C

particles, respectively (97). There are only a few studies in which the effects of plant sterols on apolipoproteins have been investigated. In most of these studies, no significant changes in apo AI concentrations have been found (67, 77, 80, 92, 95, 98). On the other hand, in one study, the consumption of plant stanol esters resulted in a slight, but significant, increase in serum apo AI concentration in parallel to the increase in HDL-C concentration (65). However, in that study, the fractional catabolic rate (FCR) and transport rate (TR) for apo AI were unchanged by plant stanol esters (65). No significant changes in apo AII have been found (65, 67).

Similarly to LDL-C, the consumption of plant sterols has significantly reduced apo B concentrations (65, 67, 80, 92, 95). However, this is not without exception (98). The significant reduction in apo B has been found to result from a significantly diminished TR for LDL apo B (65).

2.3 Absorption and metabolism of plant sterols

2.3.1 Absorption

Absorption under normal conditions

In humans, cholesterol is absorbed in the duodenum and proximal jejunum (99). However, in rat studies plant sterols appear to be absorbed in a somewhat wider region than cholesterol (100-102). Under normal conditions, the concentrations of plant sterols in serum are very low, on average 0.3-1.0 mg/dl (11, 103), and the concentrations of plant stanols are even lower, on average 0-0.03 mg/dl (36, 104). Plant sterol concentrations have been reported to be greater in women than in men (105), and in hypercholesterolemic than in normocholesterolemic subjects (2, 106). Low serum and tissue concentrations have been suggested to be a consequence of poor absorption rate of plant sterols (107-110) and their rapid biliary elimination (11). The poor absorption of plant sterols has been thought to be due to their poor micellar solubility (46, 111, 112), their slow transport rate through the outer surface of mucosal cell to an intracellular site (101, 113) and/or their inadequate esterification rate (112-116). The extent and rates of absorption vary among the different plant sterols; intestinal absorption of plant sterols has been observed to decrease as the number of carbon atoms at the C-24 side chain increases (46, 100, 109, 117) and with saturation of the nucleus double bond of the sterol (118). However, the latter finding is not without exception (109). In humans, sitosterol has been found to be absorbed less ($\leq$5%) than campesterol (9.6-16%) and both are absorbed less than cholesterol (30-50%) (11, 109, 119). Sitostanol is virtually non-absorbable, whereas campestanol has been found to be absorbed 5.5-12.5% (109, 119, 120). Consistent with these findings, the fecal recovery of ingested sitostanol in humans has been found to be over 95% (65, 70, 78, 80).

Effects of enhanced intake of plant sterols on their serum concentrations

The effects of the intake of natural plant sterol containing foodstuffs, spreads enriched with plant sterol esters or with stanol esters on serum plant sterols are presented in Table 3.

Serum plant sterol concentrations have been found to reflect intestinal absorption of cholesterol (103, 105), but also to reflect the intake of plant sterols in diet (121). In hypercholesterolemic subjects, the consumption of naturally plant sterols containing rapeseed oil or foodstuffs based on that (mean daily intake about 30-120 mg of campesterol and 40-220 mg of sitosterol) has been found to increase serum campesterol concentrations or the ratio to TC significantly by on average 9-65% from baseline value (47, 54, 55, 64, 70, 71, 104, 121, 122). In contrast, the consumption of spreads enriched with sterol esters (497-810 mg/d of campesterol and 883-1509 mg/d of sitosterol) has been found to increase serum campesterol significantly on average by up to 93% compared with control (4, 5). The effects of the intake of plant sterols on serum sitosterol have generally been smaller than their effects on campesterol. Sitosterol can inhibit absorption of campesterol and vice versa (54, 55). In contrast to the above-mentioned findings, the consumption of commercially prepared infant formulas enriched with vegetable oils (300-400 mg/d of plant sterols), and low-cholesterol, plant sterol rich diets (924-943 mg/d of plant sterols) have been reported to increase serum plant sterol concentrations by three- to fivefold in infants and hypercholesterolemic children or adolescents, respectively, compared with infants receiving breast or cow's milk and children or adolescents consuming self-chosen diets (123). The researchers speculated that the result might be due to that before adulthood, the ability to reject the absorption of plant sterols is not sufficiently matured (123).

The effects of free plant sterol supplementation on serum plant sterol concentrations have been investigated in some studies. Lees and Lees (52, 53) reported in subjects with type 2 hyperlipoproteinemia that the consumption of soy sterol suspension containing 18 g/d of plant sterols (about 6.3 g/d of campesterol and 10.8-11.7 g/d of sitosterol) caused high plasma campesterol concentrations (range 4-21 mg/dl), while sitosterol concentrations remained quite low (range 0.73-0.75 mg/dl). Furthermore, in the same study, the consumption of tall oil sterols of 3 g/d (about 2.85 g/d sitosterol) did not increase plasma sitosterol concentration over 2.5 mg/dl in any of subjects. These trials did not report any baseline values. On the other hand, in children or adolescents with type 2 hyperlipoproteinemia, the supplementation of plant sterols of 12 g/d (about 11.2 g/d sitosterol) increased plasma sitosterol concentrations by about 68% (no statistical significance reported) compared with placebo (88).

Table 3. Effects of the intake of natural plant sterol containing foodstuffs, spreads enriched with plant sterol esters or with plant stanol esters on serum plant sterols in some studies.

Source/Reference	Campesterol	Sitosterol	Campestanol	Sitostanol
Natural plant sterol containing foodstuffs [a]				
Vanhanen and Miettinen (55)	↑	↑	..	(↑)
Vanhanen et al. (47)	↑	↑	..	(↑)
Miettinen and Vanhanen (122)	↑	↑	..	..
Vanhanen et al. (64)	↑	↑	..	(↑)
Gylling et al. (70)	↑	(↑)	..	..
Sarkkinen et al. (121)	↑	(↑)	..	..
Gylling et al. (104)	↑	(↑)	(↑)	(↑)
Spreads enriched with plant sterol esters				
Weststrate and Meijer (4)	↑	↑	ND	ND
Jones et al. (5)	↑	↑	..	..
Spreads enriched with plant stanol esters				
Vanhanen et al. (47)	↓	↓	..	(↑)
Weststrate and Meijer (4)	↓	↓	ND	ND
Gylling and Miettinen (33)	↓	↓	↑	↑
Gylling et al. (104)	↓	↓	↑	↑
Nguyen et al. (72)	↓	↓	↑	↑
Tammi et al. (76)	↓	↓	..	↑
Jones et al. (5)	↓	↓	..	..

[a] Rapeseed oil, rapeseed oil-based mayonnaises, rapeseed oil-based margarines
↑ increased serum plant sterols; ↓ decreased serum plant sterols; The thickness of arrow reflects the magnitude of change. () nonsignificant change, .. not reported, ND not detectable

The consumption of plant stanols or stanol esters has been found to decrease serum campesterol and sitosterol concentrations or ratios to TC significantly, by on average 10-50% compared with control or baseline (4, 5, 33, 47, 54, 55, 64-68, 70-72, 75, 76, 78-80, 104), but also non-significant changes in sitosterol have been found (71, 79). Furthermore, stanol esters have not been found to affect serum avenasterol significantly (71, 78). The greater the ratio of campesterol or sitosterol to TC in baseline or during the run-in period, the greater the decreases induced by the plant stanol esters (47, 66, 68, 70, 75, 78). Although the consumption of a sitostanol-containing (about 0.3 g/d) plant sterol mixture has not been found to affect serum campesterol and sitosterol concentrations (36, 86), daily doses as low as 0.6-0.8 g of stanols in free or in esterified form have been found to effectively reduce serum campesterol and sitosterol (54, 55, 64). Furthermore, so far only in colectomized patients has the rapidity with which plant stanol esters can lower serum plant sterols been examined (78). A significant reduction in serum campesterol and sitosterol has been observed already on the first day of stanol ester margarine consumption and the reduction has been observed to plateau during the first week (78).

Although in previous studies (4, 54, 62, 71) plant stanols, and especially sitostanol, have been suggested to be virtually nonabsorbable, in recent studies (33, 72, 76, 78, 104), it has been shown that the consumption of mixture of stanol esters (about 0.26-0.95 g/d of campestanol and 2.21-2.91 g/d of sitostanol) can increase serum plant stanol concentrations or the ratio to TC by two- to fivefold (about 5-12 μg/dl and 12-26 μg/dl, respectively). The relative increase in campestanol appears to be greater than the increase in sitostanol. However, the values of campestanol and sitostanol have still only been 3-15% and 10-19% of the values of campesterol and sitosterol, respectively (78, 104). The different findings between the previous and the recent studies might be due to the preparation used or improved analytical methods. Large amounts of sitostanol in diet have been found to effectively inhibit absorption of campestanol (33, 124). In colectomized patients, the increase of plant stanols in serum has been observed to plateau within 7-18 days after the initiation of stanol ester margarine consumption which has been proposed to be due to their increased biliary secretion (78).

Absorption in subjects with phytosterolemia

In phytosterolemia (sitosterolemia), which is a very rare autosomal recessively inherited lipid storage disease described first in 1974 (125), absorption of plant sterols is high, varying between 16 and 63% (119, 125-127). The characteristics for this disease are xanthomas, early developed of coronary atherosclerosis and hemolysis (128, 129). Increased amounts of plant sterols (sitosterol, campesterol, stigmasterol and avenasterol) and 5α-stanols (cholestanol, 5α-sitostanol and 5α-campestanol) have been found in blood (119, 125, 127, 130, 131) and virtually all tissues except brain (125, 132). The vast majority of plant sterols must be of dietary origin, since they cannot be synthesized endogenously (11, 131). However, 5α-stanols are probably produced endogenously from the corresponding unsaturated sterols, because diet naturally contains some cholestanol, but virtually no plant stanols (130, 133). In phytosterolemic individuals, serum cholesterol concentrations may be normal or elevated (119, 130, 131, 134). In addition to enhanced absorption of plant sterols, their reduced biliary removal by the liver and decreased cholesterol synthesis has been suggested to contribute to this disease (119, 126, 127, 131, 135, 136). In phytosterolemic heterozygotes, serum plant stanol and sterol concentrations are generally not elevated, since these individuals have an almost normal absorption rate and rapid biliary sterol elimination (120, 137). However, moderately increased serum plant sterol concentrations have also been reported (138). Recently, enhanced intake of plant stanols as stanol esters (120) and plant sterols as sterol esters (139) has been reported to increase serum plant stanol and plant sterol concentrations, respectively, to similar levels as found in healthy subjects.

2.3.2 Metabolism

In general, the turnover and biliary excretion of plant sterols are more rapid than that

of cholesterol (11, 140), but slower than that of plant stanols (59). In humans, plant sterols have been found to circulate mainly in LDL, but also in HDL and to lesser extent in VLDL particles (105), either in esterified or unesterified forms (11, 127, 141). In rats, when sterols were intravenously injected, more plant stanols than plant sterols have been found in the esterified form in serum (59). In humans, the stanol metabolism is still incompletely known.

Very little is known about the distribution of plant sterols in tissues of the body. The findings seem to differ somewhat among studies depending on the tissues examined and the administration routes of plant sterols (oral or intravenous). Campesterol has been reported to be the dominant plant sterol in the tissues of rabbits (142). In animal studies, plant sterols have been observed to be deposited mainly in the liver, small intestine, kidney, adipose tissue, adrenal gland and ovaries (59, 140, 142-144). Incorporation of plant stanols into plasma, liver and other tissues has been found to be negligible (61). In humans, Gould et al. (145) reported that small amounts of ingested plant sterols are absorbed and distributed throughout the body. The greatest amounts of plant sterols have been observed in liver, spleen, kidney, lungs, plasma and red blood cells with the lowest amounts in aorta and blood vessels (145).

Under normal conditions, Δ5-plant sterols have not been found to be converted enzymatically to stanols in the liver (128). In addition, plant sterols are not converted to bile acids in humans (146), although in previous studies some conversion was claimed to occur (11). Unabsorbed plant sterols have been found to be converted to 24-methyl- and 24-ethyl-coprostanol and coprostanone by intestinal bacteria (147), but no similar conversion has been found with plant stanols (148). Small amounts of plant sterols may also be excreted into skin surface lipids (149).

2.4 Effects of plant sterols on serum cholesterol precursors and cholestanol

Squalene and cholesterol precursor sterols

Cholesterol synthesis has been shown to be stimulated compensatorily in response to cholesterol malabsorption and to depletion of the hepatic cholesterol pool by plant sterols (64, 65, 67, 70, 78). However, in some studies no consistent changes in cholesterol synthesis, as measured by using deutered water, have been found (5, 36, 86).

Figure 2 shows a scheme of cholesterol synthesis via certain main cholesterol precursor sterols in humans. Δ8-cholestenol, desmosterol and Δ7-lathosterol in serum have been found to reflect cholesterol synthesis, while squalene has been found to reflect that less consistently (103, 150-152). A high baseline precursor sterol ratio to TC has been reported to predict a small reduction in serum cholesterol in some stanol ester studies (70, 78, 104), because according to cholesterol homeostasis, the efficacy of cholesterol absorption is low in these subjects. However, in a specific population, FH children, opposite findings have also been presented (66).

In most studies the consumption of plant stanol esters has been found to increase serum Δ8-cholestenol (33, 47, 64-66, 70, 75, 76, 78, 79, 104), desmosterol (33, 47, 65-67, 70, 75, 76) and Δ7-lathosterol (33, 47, 64-66, 70, 72, 75, 76, 78, 79, 104) concentrations or the ratio to TC significantly, on average by 8-68%, compared with the baseline or control. However, also non-significant changes in serum Δ8-cholestenol (64, 67, 71), desmosterol (64, 71, 78, 79) or Δ7-lathosterol (67, 71) have been reported. No significant changes in serum squalene have been found (47, 54, 64-67, 78, 79). Furthermore, no congruent effects on serum cholesterol precursors with low daily dose of plant sterols (about 0.7-0.8 g/d of stanols or sterols) have been observed (54, 64). One reason for that might be that the consumption of rapeseed oil has enhanced serum cholesterol precursor concentrations already during the run-in period. In most studies (64, 65, 67, 70), but not in all (54, 78) the results of serum cholesterol precursor sterols have been in accordance with the findings of sterol balance studies.

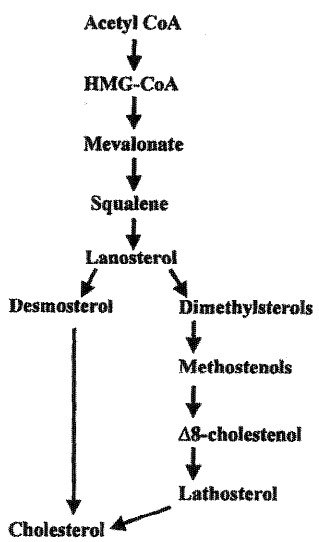


Figure 2. Scheme of cholesterol synthesis via certain main precursor sterols in human serum. HMG-CoA=3-hydroxy-3-methylglutaryl-coenzyme A

Cholestanol

Serum cholestanol, a metabolite of cholesterol, has been reported to reflect the cholesterol absorption efficacy and inversely that of cholesterol synthesis (153). Thus the changes in cholestanol are parallel those seen with plant sterols (background diet non-supplemented with plant sterols) and are opposite to those of cholesterol precursor sterols (153). In most studies, the serum cholestanol concentration or the ratio to TC was significantly lowered on average 6-15% by plant sterols or stanols (33, 54, 65-67, 70, 75, 78, 104), but also non-significant changes have been reported (47, 55, 64, 71, 79).

2.5 Hypocholesterolemic mechanisms of plant sterols

Many theories have been presented about the possible hypocholesterolemic mechanisms of plant sterols. The generally accepted view is that plant sterols inhibit the absorption of dietary and biliary cholesterol from intestine when sufficient amount of plant sterols are present in the intestine. This is supported by many animal and human studies (5, 53, 54, 64, 65, 67, 70, 78, 93, 112, 154), in which even greater than 50% reductions in cholesterol absorption have been reported. Hypocholesterolemic mechanisms of plant sterols have mainly been investigated with rats or other small animals whose lipid metabolism differs from that of humans. In addition to inhibition of cholesterol absorption, other possible hypocholesterolemic mechanisms of plant sterols are reviewed in this section.

Inhibition of absorption of cholesterol

The interference of intestinal absorption of cholesterol by plant sterols is likely related to their close chemical structure to cholesterol. However, the precise mechanisms of action through which plant sterols inhibit the cholesterol absorption and increase its excretion are not totally understood. Among the possible mechanisms are: an inhibition of mixed micelles formation, changes in micellar solubilization, competition with cholesterol for uptake to the brush border membrane, intracellular esterification or/and incorporation into chylomicrons (155). Figure 3 presents a schematic model of the inhibition of cholesterol absorption and lowering of serum LDL-C.

At present, the reduced micellar solubility of cholesterol is thought to be the major mechanism through which plant sterols inhibit cholesterol absorption (111, 157, 158). Solubilization of cholesterol to mixed micelles is essential for intestinal absorption of cholesterol (99). Slota et al. (111) found that increasing the amount of free plant sterols in mixed micellar solution reduced the solubility of cholesterol below that predicted by an equimolar replacement of cholesterol. *In vitro*, free plant sterols, which are more hydrophobic than cholesterol, have been reported to have a lower capacity but higher affinity for binding to cholic acid micelles, and thus to displace cholesterol from micelles with a favorable free energy change (46).

In *in vitro* studies, high amounts of plant sterols in the donor have been reported to be required before they can inhibit uptake of cholesterol to brush border membrane (115, 117, 157, 158). Previously, it was believed that cholesterol crosses the mucosal cell membrane by simple diffusion (99), however, recently, it has been found that uptake of cholesterol to brush border membrane is also energy-independent, protein-mediated process (159-162). Some researchers have suggested that plant sterols might compete with cholesterol for binding to the protein(s) which facilitate sterol uptake in the small-intestinal brush border membrane (16) such as scavenger receptor of class B

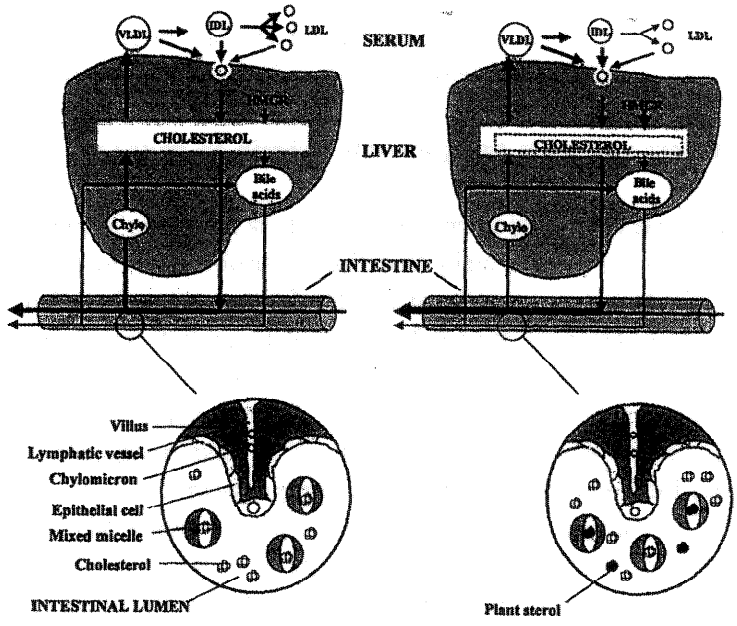


Figure 3. Schematic presentation of inhibition of cholesterol absorption and that of LDL-C lowering by plant sterols adapted by Miettinen and Gylling (156). Left panel: normal situation without plant sterol addition; Right panel: situation with plant sterol addition.
Plant sterols displace cholesterol from mixed micelles when less cholesterol is taken up to epithelial cells (enterocytes). Less cholesterol in packed into nascent chylomicrons, excreted in lymph and transported in chylomicron remnants (Chylo) to liver. As a consequence, the hepatic cholesterol pool is reduced (broken line). This, in turn, stimulates cholesterol synthesis and probably LDL receptor activity. The receptors pick up especially VLDL and IDL particles, precursors of LDL, resulting in reduced production and serum concentration of LDL. Bile acid synthesis is unaffected. HMGR= 3-hydroxy-3-methylglutaryl-coenzyme A reductase

type I (163) or for protein(s) which facilitate intracellular transfer (115).

With respect to intracellular steps, the quantity of plant sterols taken up by intestinal cells may be insufficient to inhibit cholesterol processing, i.e. esterification or incorporation into chylomicrons (157). It seems that plant sterols do not interfere with nor compete with cholesterol for acyl-CoA:cholesterol acyltransferase (ACAT)- or cholesterol esterase-catalyzed esterification in intestinal mucosa (2, 114, 157, 164) and therefore, these enzymes cannot account for plant sterol inhibition of cholesterol absorption. However, there are also some exceptions as presented by Pollak and Kritchevsky (2).

In general, only a minimal amount of the administered plant sterols has been found to be recovered in the lymph compared with cholesterol (112). However, there is little information available about the competition of plant sterols with cholesterol for incorporation into chylomicrons. It has been suggested that transport of cholesterol is

preferential relative to plant sterols during intracellular transport of sterols from plasma membrane to microsomal membranes and to the chylomicrons (165).

Effects of composition or physical state of plant sterols on inhibition of cholesterol absorption

A great part of published studies has been made with plant sterol mixtures containing mainly sitosterol. The effects of different plant sterols on inhibition of cholesterol absorption have been compared only in *in vitro* or animal studies. In most of these studies, the effects of stigmasterol (110, 164) have appeared to be similar to sitosterol, the effects of campesterol (164) and fucosterol (110, 157) have been weaker.

In animal and human studies, free plant stanols have been found to inhibit the cholesterol absorption more efficiently than free plant sterols (58, 61, 166, 167). They appear to be better as reducing micellar solubility of cholesterol (167) and increasing excretion of cholesterol (60, 61, 80, 166). When Heinemann et al. (166) compared effects of free plant stanols and sterols on cholesterol absorption in normo- or hypercholesterolemic volunteers directly by using an intestinal perfusion technique, they found that the cholesterol absorption declined during plant sterol and stanol infusion (3.6 μmol/min for both) by almost 50% and 85%, respectively.

Esterification, however, seems to make unsaturated plant sterols comparable to the saturated counterparts in inhibition of cholesterol absorption. Normén et al. (168) used a continuous isotope feeding method and demonstrated that unsaturated soy sterol esters could inhibit cholesterol absorption as efficiently as stanol esters (cholesterol absorption 38% vs. 39%) when those had been consumed in the same way in small buns spread with butter. Jones et al. (5) utilized a dual stable isotope ratio technique and demonstrated that plant sterol esters and stanol esters dissolved into margarines reduced cholesterol absorption on average by 36% and by 26%, respectively.

In addition to esterification, solubility of plant sterols and thus their efficacy to reduce cholesterol absorption can be increased by using phospholipids (lecithin) or dietary fats as a vehicle to deliver plant sterols into the small intestine. When plant sterols are offered in a soluble form into the intestine, they might replace and precipitate cholesterol from the absorbable micelles more effectively, and with smaller doses, than might be possible with a crystalline form (94, 169, 170). Recently, Ostlund et al. (170) found that 0.3 g and 0.7 g of plant stanols in lecithin micelles reduced cholesterol absorption significantly by 34% and 37%, respectively. Instead, 1 g of plant stanol powder reduced that only by 11%. The former finding is consistent with the study of Vanhanen (64), in which 0.7-0.8 g/d of stanols as stanol esters dissolved in mayonnaise was reported to reduce efficiently cholesterol absorption. On the other hand, Mattson et al. (94) observed that free plant sterols reduced cholesterol absorption more effectively than oleate esters of plant sterols (42% vs. 33% reduction in cholesterol absorption). However, in that study, free and esterified plant sterols were added to food in different

ways: the former was mixed with omelet and the latter was dissolved in frying fat. It has generally been suggested that before plant sterol esters or stanol esters can inhibit cholesterol absorption, they have to be hydrolyzed to free plant sterols or stanols, respectively, in the intestine (83, 94). Hydrolysis is normally rapid (148), with about 50% of administered plant stanol esters being hydrolyzed in a 50-cm segment of duodenum (148). Since only the sterol monohydrate can affect micellar binding, the reason for different results in the study of Mattson et al. (94) might be incomplete hydrolyzation of sterol esters in the intestine lumen.

In rats, the ability of free plant sterols to alter cholesterol absorption has been compared with that of plant sterols esterified with fatty acids with various chain-lengths or with various degrees of saturation. In those studies both free and esterified plant sterols with acetate (93, 171), decanoate (93) or oleate (93, 171), but not with propionate or palmitate (171) have been observed to cause a similar decrease in cholesterol absorption.

Although in earlier studies it has been proposed that both plant sterols and cholesterol have to be present in diet simultaneously to achieve optimal efficacy in inhibition of cholesterol absorption (2, 94), in a recent study (77) that suggestion has been challenged. Since the researchers (77) did not detect any difference in cholesterol-lowering efficacy between the consumption frequency of plant stanol esters, they hypothesized that plant stanols or stanol esters remain in the intestinal lumen or possibly in or associated with the enterocytes and thus affect micellar solubility of intestinal cholesterol and ultimately cholesterol absorption.

Other hypocholesterolemic mechanisms

In experimental animals, administration of free plant sterols intraperitoneally or subcutaneously has also been reported to cause reduction in serum TC concentrations (155, 172). Thus, it has been proposed that plant sterols may have intrinsic hypocholesterolemic effects via mechanisms other than those involving cholesterol absorption (155). On the other hand, some researchers have suggested that part of the infused plant sterols may be secreted by the liver into the bile and may then impair cholesterol absorption (173). This has been challenged by others claiming that the amount of plant sterols secreted into bile is too low to inhibit cholesterol absorption (174). Only infusion of a large amount (100 mg) of free plant sterols has been observed to achieve even a partial inhibition in cholesterogenesis (175). An increase in the tissue plant sterol pool has not been observed to reduce 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity, the rate-limiting enzyme of cholesterol synthesis when free plant sterols have been fed (176, 177) or infused intravenously to rats (173, 174). In fact, the enzyme activity has been found to increase twofold in plant sterol-fed rats (176).

In animals, the effects of plant sterols on cholesterol 7α-hydroxylase activity, the rate-limiting enzyme of conversion of cholesterol to bile acids, have been conflicting

(140, 155, 173, 174, 176). In humans, no changes in bile acid synthesis (65, 67, 70) or bile acid composition (78) have been found. In addition, the consumption of plant sterols has not been found to increase bile acid excretion in feces in most studies (54, 64, 65, 67, 70, 78, 168), though one study with plant stanols did report opposite findings (80).

Plant sterols have been observed to be able to inhibit esterification of exogenous cholesterol by ACAT in rat liver (116), and thus to increase cholesterol excretion in bile.

Depletion of intracellular cholesterol in the liver induced by plant stanol esters could be hypothesized to upregulate LDL receptor activity (Figure 3). The receptor may effectively pick up VLDL-C and IDL-C particles resulting in their reduced conversion to LDL and in decreased LDL-C concentrations (65, 67). This hypothesis is based on findings that FCR of LDL apo B remained unchanged and that serum VLDL-C and IDL-C concentrations decreased significantly with the consumption of stanol esters (65, 67).

2.6 Side effects of plant sterols

2.6.1 Effects of plant sterols on serum fat-soluble vitamins and carotenoids

As mentioned earlier, plant sterols inhibit cholesterol absorption from intestine. Therefore, it has been thought that plant sterols might also interfere with the absorption of carotenoids and fat-soluble vitamins. To date, the effects of plant sterols on the absorption of carotenoids and fat-soluble vitamins have been evaluated only by measuring their serum concentrations. In the circulation, tocopherols and carotenoids are transported in lipid particles, therefore changes in these carrier particles may also alter the concentrations of tocopherols and carotenoids. Thus, in the published studies, changes in serum tocopherols or carotenoids have been standardized against simultaneous changes in TC (33, 72, 75, 178), LDL-C (76, 77), total glycerol+TC (4) or TG+TC (31, 71).

Fat-soluble vitamins

Plant sterols have not been found to have significant effects on serum concentrations of retinol (33, 71, 72, 75-77, 178), 25-hydroxyvitamin D_3 (31, 33, 71, 72, 76, 178) or vitamin K (31, 179). Serum α-tocopherol concentrations have reduced significantly, but after lipid standardization, the α-tocopherol concentrations have remained almost unchanged (31, 33, 71, 76, 77, 178). Only the effects of plant stanol esters on serum δ-, γ- or β-+γ-tocopherol have been studied. No significant changes in δ-tocopherol concentration (77) or in γ- (71) or β-+ γ-tocopherols (77) after lipid standardization have been detected.

Carotenoids

There have been significant reductions in serum concentrations of α-carotene, β-carotene or α-+β-carotene, even after lipid standardization, after ingestion of plant sterols reported in several (4, 31, 33, 72, 75-77, 80, 178), but not in all (56, 71) studies. The effects on serum lycopene concentrations have been inconsistent. In one study, plant stanol esters have been found to affect serum lycopene also after lipid standardization (4), but not in the others (71, 77). Moreover, free plant sterols (56), but not sterol esters (4, 31), have been found to affect plasma lycopene after lipid standardization. In one study, stanol esters have not been reported to cause any significant reduction in serum phytofluene, lutein/xeaxanthin and β-cryptoxanthin after lipid standardization (77). However, in that study, serum carotenoid as well as tocopherol concentrations have been reported to be slightly lower when stanol esters had been consumed three times per day than consumed once per day (77).

2.6.2 Hormonal effects of plant sterols

Earlier findings in rodents and fish have suggested that plant sterols have effects on the reproductive system, and in particular that they possess estrogenic activity (180-182). However, in recent studies no evidences of estrogenic activity or effects on the reproductive system have been found. Turnbull et al. (183) stated that vegetable oil-based stanols did not increase the proliferation of estrogen-responsive MCF-7 human breast cancer cells at the doses tested (up to 10^{-4}M). Baker et al. (184) observed that plant sterols did not bind to the immature rat uterine estrogen receptor at doses up to 10^{-4} M or to stimulate the transcriptional activity of the human estrogen receptor in a recombinant yeast strain at doses up to $2x10^{-4}$ M. In addition, vegetable oil- or wood-based stanol esters (183) or vegetable oil-based plant sterols or sterol esters (184) had no estrogenic potential in an *in vivo* rat uterotrophic assay. In two-generation reproductive toxicity studies, no adverse effects on the reproduction or development of male and female rats over two generations were found when the rats were fed a diet containing plant stanol esters in concentration of 8.76% (equivalent to 5% total stanols) (185) or sterol esters in concentration of 8.1% (equivalent to 5% total sterols) (186). In addition, no embryotoxic, fetotoxic, or teratogenic effects were found when rats were fed diet containing stanol esters in a concentration of 8.76% (equivalent to 5% of total stanols) (187).

In humans, effects of plant stanol esters or sterol esters on serum female sex hormone concentrations have been investigated in few short-term studies. Gylling et al. (70, 188) did not find changes in serum estradiol concentrations in postmenopausal women with CAD when the women had consumed stanol esters (3 g/d of stanols) for seven weeks. Furthermore, Ayesh et al. (73) did not observe any biologically relevant effects on serum female sex hormone concentrations in normocholesterolemic or hypercholesterolemic women when they had consumed sterol esters (8.6 g/d of sterols)

for four weeks.

Plant sterols have been used in the treatment of benign prostatic hyperplasia, because they have been found to ease urologic symptoms and improve measures of flow (189).

2.6.3 Other adverse effects of plant sterols in humans

In general, in the vast majority of clinical trials, oral administration of plant sterols has been well tolerated and without any side effects. However, some adverse effects have been reported. In one study, some subjects described mild constipation when they had consumed 3-6 g/d of tall oil sterols (53). Diarrhea has also been reported to occur occasionally in some studies (50). One subject reported a skin reaction when he had used sterol ester margarine (8.6 g/d of sterol) (73). In addition, in two children, appetite was depressed during the first two weeks of plant sterol treatment (92).

Minor changes in routine hematology and clinical chemistry parameters have been reported. However, the values have remained within the normal ranges (4, 31, 71-73). No significant effects on coagulation or fibrinolytic parameters have been observed (34) when subjects have consumed stanol ester margarine (3.8-4 g/d of stanols) for eight weeks. Furthermore, no significant changes in the formation of bile acids or neutral sterol metabolites (190) or the bacterial profile of the gut microflora (73) or urine parameters (5, 73) have been observed when subjects had consumed sterol ester margarine containing up to 8.6 g/d of total sterols.

3 AIMS OF THE STUDY

The general aim of these studies was to examine the role of stanol ester- and sterol ester-enriched margarines in lowering elevated serum cholesterol concentrations with different study designs. In addition, the safety of plant stanol esters and sterol esters was evaluated by measuring the serum concentrations of carotenoids and fat-soluble vitamins as well as concentrations of plant stanols and plant sterols during the intervention studies.

Specific questions in the separate studies were as follows:

1. Do the low-fat margarines enriched with plant stanol esters offer an additional cholesterol-lowering effect to a cholesterol-lowering diet alone **(I)**.

2. Do the margarines enriched with plant stanol esters or sterol esters reduce serum TC and LDL-C concentrations as part of a low-fat, low-cholesterol diet **(I, V)**.

3. Do the low-fat margarines enriched with wood- or vegetable oil-derived plant stanol esters differ in their abilities to lower serum cholesterol concentrations **(I)**.

4. Do the margarines enriched with plant stanol esters or sterol esters differ in their abilities to lower serum TC and LDL-C concentrations **(V)**.

5. Do plant stanol esters reduce serum TC and LDL-C concentrations in a dose-dependent manner and what is the optimal dose of plant stanol esters **(III)**.

6. Do plant stanol ester- or sterol ester-enriched margarines affect serum fat-soluble vitamin or carotenoid concentrations as part of a cholesterol-lowering diet or a standardized habitual diet **(I, II, III, V)**.

7. How do different doses of plant stanol esters affect cholesterol metabolism using serum cholesterol precursors as biomarkers and how do the stanol esters affect serum plant sterol and stanol concentrations **(IV)**.

4 SUBJECTS AND METHODS

Detailed descriptions of subjects and methods are presented in the original publications (I-V).

4.1 Subjects

A total of 128 mildly to moderately hypercholesterolemic men and women with normal or slightly elevated body weight [body mass index (BMI) 19-30 kg/m^2] were recruited to the studies from the occupational health care system (I/II, V), from former studies carried out in the Department of Clinical Nutrition, University of Kuopio (I-V) and from the local society of the Finnish Heart Association (III/IV). In addition, employees of the city of Kuopio were recruited to study V. To be included in the studies the subject had to have normal liver, kidney and thyroid function. They were not allowed to have DM, gastrointestinal diseases, lipid-lowering drug treatment, alcohol abuse (>45 g ethanol/day) or irregular eating habits. The subjects were requested to maintain any medication, and to keep their weight, alcohol consumption, smoking habits and physical activity constant during the studies. All study protocols were approved by the Ethics Committee of the University of Kuopio, and all subjects gave their informed consent.

Study I/II

A total of 60 hypercholesterolemic subjects met the inclusion criteria, which were for serum TC 5.4-7.5 mmol/l, for serum TG <3.0 mmol/l, and for age 20-60 years. Five subjects dropped out at the beginning of the run-in period for personal reasons. Ten of the subjects were smokers.

Study III/IV

A total of 26 subjects were recruited to the dose-response study. To be included to the study, the subjects had to have serum TC of 5.0-8.5 mmol/l and TG <3.5 mmol/l after the pre-trial period, and to be aged 25-65 years. Four subjects dropped out during the study due to personal reasons, prolonged infection (bronchitis, stomatitis), prostatitis or use of a plantago ovata product (Visiblin®) for constipation. One of the subjects was a smoker.

Study V

A total of 42 subjects were recruited to the study, in which the main inclusion criteria were as follows: serum TC 4.8-7.0 mmol/l and TG below 2.5 mmol/l at screening, and age 30-65 years. Eight subjects dropped out during the study due to personal reasons or poor commitment. Two subjects were smokers.

Baseline characteristics of the subjects completed studies I-V are shown in Table 4.

Table 4. Baseline characteristics of the subjects in studies I-V.

	Study I/II			Study III/IV	Study V
	Control group	WSEM group	VOSEM group		
N(men/women)	17(6/11)	18(8/10)	20(6/14)	22(8/14)	34(15/19)
Age (years)	46.0 ± 8.2	43.2 ± 8.2	40.8 ± 9.3	50.5 ± 11.7	48.8 ± 8.1
BMI (kg/m^2)	25.7 ± 3.5	25.6 ± 4.0	24.2 ± 3.0	26.3 ± 3.4	24.9 ± 2.4
Serum TC (mmol/l)	5.93 ± 0.64	6.36 ± 0.76	6.15 ± 0.79	6.87 ± 1.28	6.24 ± 0.83
Serum TG (mmol/l)	1.24 ± 0.66	1.42 ± 0.67	1.25 ± 0.39	1.24 ± 0.43	1.11 ± 0.52

Mean ± SD. WSEM=wood stanol ester-enriched margarine, VOSEM=vegetable oil stanol ester-enriched margarine

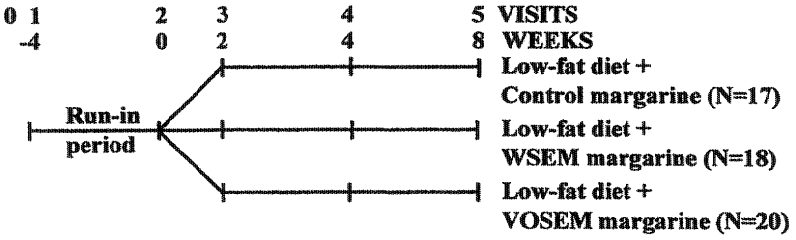
4.2 Study designs

In study I/II, a double blind, parallel study design was used (Figure 4). The subjects were randomly assigned into three low-fat [control, wood stanol ester (WSEM) and vegetable oil stanol ester (VOSEM)] margarine groups taking smoking habits and phase of menstrual cycle into account. All groups followed the experimental, low-fat and low-cholesterol diet for eight weeks preceded by a 4-week run-in, high-fat diet period.

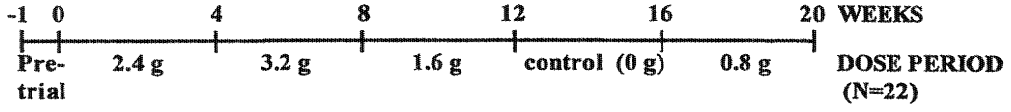
In study III/IV, a randomized single blind repeated measures design was used (Figure 4). After a 1-week pre-trial period, all subjects consumed five different plant stanol doses in the same randomly determined order [2.4 g, 3.2 g, 1.6 g, control (0 g), 0.8 g].

In study V, a double-blind randomized repeated measures design with three test margarines [control, stanol ester (STAEST) and sterol ester (STEEST)] and three periods of 4-week duration was used (Figure 4). The randomization was made according to a Latin square model. The experimental periods were preceded by a 2-week pre-trial period. During the entire study, the subjects followed a low-fat diet.

Study I/II



Study III/IV



Study V

Period	Subjects (groups) 1	2	3
I	STEEST margarine, N=11 (C)	STAEST margarine, N=12 (A)	Control margarine, N=11 (B)
II	STAEST margarine, N=11 (A)	Control margarine, N=12 (B)	STEEST margarine, N=11 (C)
III	Control margarine, N=11 (B)	STEEST margarine, N=12 (C)	STAEST margarine, N=11 (A)

Figure 4. Study designs.

4.3 Methods

4.3.1 Diets

Margarines

Test margarines were low-erucic acid rapeseed oil-based margarines (Raisio Group Plc, Raisio, Finland) except in study I/II where the spread used in the run-in period was a milk fat-based spread. Plant stanol esters (I-V) were prepared using commercially available plant sterols by recrystallization, hydrogenation to form plant stanols, and esterification with low-erucic acid rapeseed oil-based fatty acids to produce fatty acid esters of plant stanols. The respective plant sterol esters (V) were prepared by recrystallization, and esterification with low-erucic acid rapeseed oil-based fatty acids to produce fatty acid esters of plant sterols. In study I/II, the WSEM margarine contained plant stanol esters were derived from wood sterols (Ultra sitosterol, Kaukas Oy, Finland), and the VOSEM margarine contained plant stanol esters from vegetable oils (Archer Daniels Midland Co, Decatur, IL). In studies III/IV and V, the stanol ester margarines were prepared from wood and vegetable sterols (DRT, Les Derives Resiniques & Terpeniques Granel S.A. Dax Cedex, France and Archer Daniels Midland Co, Decatur, IL, respectively). In study V, the sterol ester margarine was prepared from vegetable oil-based sterols (Archer Daniels Midland Co, Decatur, IL).

The daily dose of the test margarines was 25 g (I/II, III/IV) or 20 g (V) taken in 2 to 3 portions with meals. The fatty acid composition and the amount of plant stanols and sterols in daily dose of the test margarines are presented in Table 5. The low-fat control and test margarines contained absorbable fat 35% and 32%, respectively (I/II). In the other studies, the test margarines contained absorbable fat 68-70% (III/IV) or 70-71% (V). The theoretical (planned intake x actual composition of spread) daily intake of plant stanols was 2.34 g in the WSEM group and 2.20 g in the VOSEM group (I/II). In study III/IV, the amount of plant stanols in the test margarines differed slightly from that planned being 0.81 g (planned 0.8 g), 1.56 g (planned 1.6 g), 2.29 g (planned 2.4 g) and 3.03 g (planned 3.2 g) per daily dose. In addition, the theoretical daily amount of total plant sterols and stanols was 2.02 g in the STAEST margarine and 2.06 g in the STEEST margarine (V). The control margarines did not contain added stanols or sterols, neither did the spreads used during the run-in or pre-trial periods. All test spreads were fortified with vitamin A [550 μg as retinol equivalents (RE), I/II; 445 μg RE, III/IV; 870 μg RE, V] and vitamin D (7 μg, I/II; 6.4 μg, III/IV; 7 μg, V) per 100 g spread. This kind of fortification of margarines is a normal procedure, in fact it is stipulated legally in Finland.

The subjects received the coded tubs of test margarines when visiting the study unit. They were given detailed instructions on how to use of test spreads. Furthermore, the subjects were asked to record the use of test fats daily (I-V) and to return the empty and partly empty tubs and the extra tub of test margarine to the study unit at the end of each

Table 5. The target daily intake of absorbable fat, fatty acids and plant sterols and plant stanols (g) from daily margarine dose (25 g in I/II and III/IV and 20 g in V) /their actual mean intake.

	Study I/II			Study III/IV					Study V		
	Control	**WSEM**	**VOSEM**	**Dose 0 g**	**Dose 0.8 g**	**Dose 1.6 g**	**Dose 2.4 g**	**Dose 3.2 g**	**Control**	**STAEST**	**STEEST**
Absorbable fat	8.8/8.6	8.0/7.9	8.0/7.8	17.5/17.8	17.4/17.7	17.2/17.6	17.0/17.3	17.2/17.3	14.2/14.1	14.0/13.9	14.0/13.9
Fatty acids											
PUFA	2.1/2.1	2.1/2.0	2.1/2.1	3.8/3.9	3.9/4.0	3.8/3.9	3.8/3.9	3.9/3.9	3.1/3.1	3.1/3.1	3.2/3.2
MUFA	4.1/4.1	4.2/4.2	4.1/4.0	8.5/8.7	8.9/9.0	8.7/8.9	8.3/8.4	8.7/8.8	7.1/7.1	7.0/7.0	6.9/6.8
SAFA	2.0/2.0	1.1/1.1	1.1/1.1	4.2/4.3	3.8/3.9	3.8/3.9	3.9/4.0	3.7/3.7	3.2/3.2	3.3/3.3	3.3/3.3
Total plant stanols	0/0	2.34/2.31	2.20/2.16	0/0	0.81/0.82	1.56/1.59	2.29/2.33	3.03/3.05	0/0	1.92/1.91	0.09/0.09
Sitostanol	0/0	2.15/2.13	1.50/1.47	0/0	0.62/0.63	1.19/1.22	1.74/1.77	2.30/2.32	0/0	1.43/1.42	0.06/0.06
Campestanol	0/0	0.19/0.19	0.70/0.69	0/0	0.19/0.19	0.37/0.38	0.55/0.56	0.73/0.74	0/0	0.49/0.49	0.02/0.02
Total plant sterols	0.05/0.05	0.10/0.10	0.15/0.15	0.10/0.10	0.13/0.13	0.15/0.15	0.17/0.17	0.21/0.21	0.09/0.09	0.10/0.10	1.98 /1.96
Brassicasterol	0.01/0.01	-	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	0.01/0.01	-	0.06/0.06
Campesterol	0.02/0.02	0.04/0.04	0.06/0.06	0.04/0.04	0.05/0.05	0.05/0.05	0.06/0.06	0.06/0.06	0.03/0.03	0.04/0.04	0.57/0.56
Sitosterol	0.03/0.03	0.07/0.07	0.08/0.08	0.05/0.05	0.07/0.07	0.09/0.09	0.10/0.10	0.12/0.12	0.04/0.04	0.06/0.06	1.00/0.99
Stigmasterol	-	-	-	-	-	-	-	-	-	-	0.34/0.34
Total plant sterols and stanols	0.03/0.03	2.44/2.41	2.35/2.30	0.10/0.10	0.93/0.94	1.70/1.74	2.46/2.50	3.24/3.27	0.09/0.09	2.02/2.01	2.06/2.04

period (III/IV, V). The packages and the test spread left over were weighed and the result recorded (III/IV, V).

Diets

The goals of fat composition of diets are presented in Table 6. In study I/II, during the run-in period, a diet rich in fat and SAFA was used. During the experimental diet period of study I/II as well as during the entire study V, the subjects followed a diet which resembled the NCEP step 1 diet (81). In study III/IV, subjects followed a standardized background diet, which resembled their habitual diet, throughout the study.

Table 6. Goals for the composition of diets during the studies.

	Study I/II		**Study III/IV**	**Study V**
	Run-in	Experimental	Pre-trial and experimental	Pre-trial and experimental
Fat (E%)	36-38	28-30	34	<30
SAFA (E%)	16-18	8-10	<12	8-10
MUFA (E%)	14	12	14	12-14
PUFA (E%)	6	8	8	5-7
Cholesterol (mg/MJ)	35.7	23.8	23.8	23.8

The diets were composed of normal Finnish food items. In studies I/II and V, all subjects received individual oral and written instructions on the isocaloric diet including the precise amounts and quality of foods to be eaten by main food groups: fats, dairy products, meat and meat products, cereals, fruits and berries, and leaf vegetables and roots. In study III/IV, the diet plan included only precise amounts and quality of fat and cheese, and the precise quality of liquid milk and meat products. Depending on the goals for the amount of fat and the fatty acid composition of the different studies, the diets were adjusted with moderate rich fat (I/II, run-in) or low-fat and fat free dairy and meat products (I-V), sunflower (I/II, run-in) or rapeseed (I-V) oil, salad dressing (III/IV, V), milk fat-based spread (I/II, run-in) or vegetable oil-based margarines (I-V). The diet plans were made for several energy levels. The energy requirement of each subject was estimated by a 4-day food record kept before the study (I/II) and by the Harris-Benedict formula (I-V) added with the energy need due to physical activity (191). The body weight was not allowed to change more than ±1 kg during the studies. Therefore, if necessary the energy intake level was changed in order to ensure stable body weight. The feasibility of diet was improved by providing the test spreads, vegetable oils and liquid milk products (I/II) or test margarines, vegetable oil, salad dressing and low-fat cheese (III/IV, V) to the participants free of charge.

4.3.2 Evaluation of the feasibility of the diets

Food records

Adherence to the background diet was monitored by 3-day (III/IV) or 4-day (I/II, V) food records kept before the end of the run-in period (I/II) or the pre-trial period (III/IV, V) and by 4-day food records kept before the end of each experimental period (III/IV, V) or 3 times during the experimental period (I/II). One of the recording days was a weekend day or the person's day off from work. The subjects recorded their food consumption after consulting a portion size booklet containing photographs of food (192). At study visits, the amounts and qualities of foods in the records were checked by the nutritionist for completion, filling in data that were lacking.

The diets were planned and the nutrient intake in food records were calculated using the Micro-Nutrica® dietary analysis program (version 2.0, Finnish Social Insurance Institute, Turku, Finland). The food composition database is based mainly on analyses of the Finnish food and international food composition tables (193). In addition, the database was updated for the purposes of each study.

Fatty acid composition of serum cholesteryl esters

Fatty acid composition of serum cholesteryl esters was determined as an objective marker of dietary adherence in studies III/IV and V.

4.3.3 Height, body weight and blood pressure

Height was measured on the first visit of the studies to the closest 0.5 cm. Body weight with light clothing was measured at every visit with a digital scale. Blood pressure (I/II, V) was measured in the sitting position from right arm using a digital blood pressure monitor (Hem-705c, Omron Corporation, Japan) after the subjects had rested for 5-10 min. Two measurements were taken, with the mean being used in the analyses.

4.3.4 Laboratory measurements

Fasting blood samples were taken in study I/II at the beginning of the run-in (-4 wk) and the experimental diet (0 wk) periods and at weeks 2, 4 and 8. In the other studies, fasting blood samples were taken at the beginning of the pre-trial (-1 wk, III/IV or -2 wk, V) period, at the beginning of the first experimental period (0 wk) and at the middle and the end of each experimental period. Serum lipids were determined from blood samples at every visit, but the main comparisons were made between the mean values of the weeks 0 and 8 (I) or among the mean values at the end of each experimental period (III, V). Samples for other variables were taken only at the beginning and the end of the studies (I-V), at the beginning and the end of the experimental diet period (I/II) or at the end of each experimental period (III/IV, V). Since the phase of menstrual cycle may affect cholesterol concentration (194), in study I/II in those women with a menstrual

cycle, the main blood samples were taken at the same time of the cycle, and in studies III/IV and V, the end measurement of each period was performed at days 5-10 of the cycle.

Serum samples for analysis of apo AI and B, carotenoids and fat-soluble vitamins, cholesterol precursors, plant sterols and cholestanol, as well as fatty acid composition were stored at –70 °C until analysis.

Routine laboratory measurements

The blood samples for the routine laboratory examinations (B-Hemoglobin, B-Thrombocytes, S-Thyroid stimulating hormone, S-Alanine aminotransferase, S-Gamma glutamyltransferase and S-Creatinine) were drawn at the beginning and the end of each study. The samples were analyzed with standardized methods.

Fatty acid composition of serum cholesteryl esters

In the analysis of fatty acid composition of serum cholesteryl esters, serum samples were extracted with chloroform-methanol (2:1, vol:vol), and lipid fractions (cholesteryl esters, triglycerides and phospholipids) were separated with an aminopropyl column (195). Fatty acids were analyzed in a gas liquid chromatograph (GLC) (Hewlett-Packard 5890 series II, Hewlett-Packard Company, Waldbronn, Germany) equipped with a 25-m long FFAP-column. Fatty acids are presented as molar percentage of total fatty acids.

Serum total and lipoprotein lipids and apolipoproteins

Lipoproteins were separated by ultracentrifugation for 18 h at density 1.006 to remove the VLDL fraction. HDL in the infranatant was separated from LDL by precipitation of LDL with dextran sulfate and magnesium chloride (196). LDL-C was calculated as the difference between the mass of cholesterol in the infranatant and HDL, and VLDL-C was calculated as the difference between the whole serum and the infranatant. Enzymatic photometric methods were used for the determination of cholesterol and TG from whole serum and separated lipoproteins using commercial kits (Monotest® Cholesterol and Triglyceride GPO-PAP, Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) with a Kone Specific Clinical Analyser (Kone Ltd., Espoo, Finland). The coefficients of variation between measurements for serum TC were 1.3-2.1% (I), 0.9-1.6% (III) and 1.3-1.4% (V), for TG were 3.0-4.7% (I), 1.6-2.4% (III) and 1.7-1.9% (V), for HDL-C were 1.1-1.4% (I), 1.2-1.9% (III) and 1.1-1.2% (V), and for HDL-TG were 1.6% (I), 1.9% (III) and 1.1% (V).

Analyses of apolipoproteins were based on the measurement of immunoprecipitation enhanced by polyethylene glycol at 340 nm (197). A Kone Specific Clinical Analyzer and apo AI and apo B reagents from Kone Corporation (Espoo, Finland) were used. The coefficients of variation within measurements for serum apo AI were 1.5-2.3% (I), 2.8-5.5% (III) and 3.7-4.9% (V), and for apo B were 1.2-1.8% (I), 1.8-2.2% (III) and 1.7-

2.8% (V).

Serum cholesterol precursors, plant sterols and cholestanol

In study I, serum plant sterols were quantified from nonsaponifiable serum materials by GLC (Hewlett-Packard 5890A, Palo Alto, CA) (126) equipped with a 25-m long silica CP-Sil 5-CB capillary column (Chrompack, Raritan, NJ). 5α-cholestane and 5β-coprostanol were used as internal standards.

In studies III/IV and V, serum cholesterol precursors, plant sterols, cholesterol and cholestanol were measured by GLC (HP 5890 Series II, Hewlett Packard, Delaware, Little Falls, USA) (198, 199) equipped with a 50-m long Ultra 1 capillary column (methyl-polysiloxane) (Hewlett Packard, Delaware, Little Falls, USA) for cholestanol, squalene, Δ8-cholestenol, Δ7-lathosterol, desmosterol, campesterol and sitosterol, and equipped with a 50-m long Ultra 2 capillary column (5% Phenyl-methyl siloxane) (Hewlett Packard, Delaware, Little Falls, USA) for sitostanol and campestanol. Serum cholesterol precursors, plant sterols and cholestanol were determined in duplicate from the same samples and the mean value of two measurements was used in the statistical analyses. 5α-cholestanol for cholesterol and epi-coprostanol for cholesterol precursors, plant sterols and cholestanol were used as internal standards.

Serum carotenoids and fat-soluble vitamins

Serum carotenoids and fat-soluble vitamins were analyzed with a high performance liquid chromatographic system (Perkin-Elmer, Norwalk, CT) on a C_{18} column (Waters, Milford, MA) (200-202) except in study V where serum 25-OHD_3 was analyzed with a radioimmunoassay method (25-OHD_3 I125 RIA KIT, DiaSorin, Stillwater, MN).

Apo E genotypes

Apo E genotypes were analyzed with the polymerase chain reaction-restriction fragment length polymorphism method described by Tsukamoto et al. (203) with a slight modification.

4.3.5 Questionnaires

At the beginning of each study, previous and present diseases, current medication, alcohol and tobacco consumption, physical activity, use of vitamins or other nutrient supplements were interviewed using a structured questionnaire. Alcohol and tobacco consumption and physical activity were reviewed also at the end of each study. The possible adverse effects and symptoms were enquired repeatedly based on a structured questionnaire (III/IV,V) except in study I/II where a non-structured interview was used at every study visit.

4.3.6 Statistical methods

Statistical methods are reported in detail in the original publications I-V. The data were analyzed with SPSS for Windows 6.0 (I/II, III/IV) or Windows 7.5 (V) statistics program (SPSS, Chicago, IL, USA) (204-206).

Normal distribution and homogeneity of variance were checked before further analyses. Overall changes in continuous variables were analyzed with analysis of variance for repeated measurements [SPSS procedures MANOVA (I/II, III/IV) or GLM (V)]. In study V, GLM was used to assess the effect of the order of margarine consumption periods, carry-over effect and gender on the main end-point variables among the different experimental margarine periods. In further analyses, Student's test in between-group comparisons (I/II) and paired t-test (I/II, III/IV) or GLM (V) within-group comparisons were used. Statistical significances for the response variables (I/II) were tested with a single measurement simple factorial analysis of variance (ANOVA) followed by Student's t-test. In study I/II if the initial concentration differed significantly among the study groups, the concentrations were adjusted in the between-groups comparisons by dividing the response variable with the initial concentration. Variables which were not normally distributed even after logarithmic transformation or non-continuous variables were tested with the Friedman two-tailed ANOVA, Mann-Whitney test, Kruskal-Wallis test, Chi-square test or Wilcoxon matched-paired signed rank test. The analysis of covariance was used for checking whether some variables had effects on lipid responses. In addition, for some variables of interest, Pearson correlation coefficient were calculated. To control the overall α level, Bonferroni adjustment was used. The results are expressed as means ± SDs, means ± SEMs or means.

5 RESULTS

5.1 Baseline characteristics

Body weight and blood pressure

During study I/II body weight decreased slightly and similarly (mean decrease 1.1-1.2 kg, P<0.001-0.01) within the control, WSEM and VOSEM groups. During studies III/IV and V body weight remained unchanged. There were no significant changes in systolic or diastolic blood pressure in studies I/II and V.

Routine laboratory measurements

Blood hemoglobin and thrombocytes, and serum γ-glytamyl and alanine amino transferase and creatinine were all within the normal ranges in all studies, even if there were slight, but significant changes in these variables.

Side effects

No adverse effects related to the use of test margarines were recorded. The symptoms (gastrointestinal or skin) reported by the participants were slight and they occurred occasionally and were not related to any particular experimental period or test product.

5.2 Feasibility of the diets

The compliance with the use of test margarines was good. The average daily consumption of the test margarines was between 96-102% of the target amount in all studies. The actual daily intake of plant stanols and plant sterols from test margarines is shown in Table 5.

The actual intakes of the energy nutrients during the different studies are shown in Figure 5. The goals for the composition of experimental diets were well achieved in all studies. Actually, in study I/II during the low-fat diet the mean intakes of fat, SAFA and cholesterol were lower than the goal (step 1) (81) and the intake of dietary cholesterol (mean 137-161 mg/d, 18-21 mg/MJ/d) achieved the goal of the step 2 diet (<200 mg/d) and the intake of SAFA (mean 6.8-7.3 E%/d) was near to these goals (7 E%/d) in all study groups. The intake of nutrients did not differ among the study groups.

During study III/IV, the mean intake of SAFA was slightly, but significantly, greater during the control period than during the 2.4 g dose period. The mean intake of alcohol was slightly higher in the control period than during the 1.6 g, 2.4 g and 3.2 g dose periods owing to the fact that the eve of May Day and May Day occurred at the end of the control period. In addition, the mean intake of fiber was slightly lower during the control and 0.8 g dose periods than during the 2.4 g and 1.6 g dose periods. However, according to analyses of covariance, the differences in the intake of these nutrients

during the different dose periods did not interfere with the results.

During study V, the mean intake of SAFA was within the goal, the mean intake of fat (30.0-31.1 E%) met almost the goal and the mean intake of cholesterol (166-179 mg/d, 20-22 mg/MJ/d) was even lower than the goal. There were no significant differences in the intake of nutrients among the different test margarine periods.

There were no significant changes in the intake of β-carotene or fat-soluble vitamins during any of the studies. The mean intakes of β-carotene, vitamin A and E were 3056-4726 μg/d, 812-1337 μg RE/d and 10.4-16.8 mg/d, respectively, in all studies.

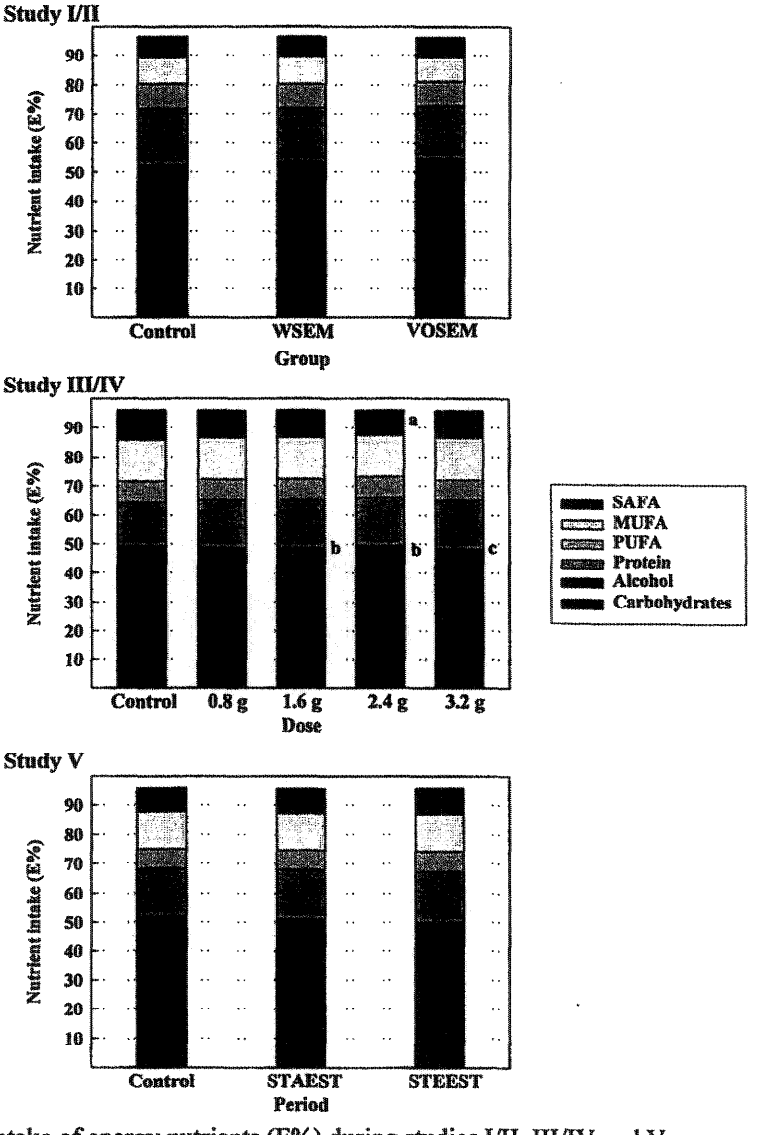


Figure 5. Actual intake of energy nutrients (E%) during studies I/II, III/IV and V.
a $P<0.001$, b $P<0.05$, c $P<0.01$ vs. control.

Fatty acid composition of serum cholesteryl esters

There were no major differences in the fatty acid composition of serum cholesteryl esters during the experimental periods (III/IV, V), and the results of this biomarker paralleled the food records (Table 7). Proportions of fatty acids which reflect the intake of SAFA (myristic acid, palmitic acid and stearic acid), the intake of MUFA (oleic acid), and the intake of PUFA (γ-linolenic acid, α-linolenic acid, dihomo-γ-linolenic acid and their metabolites) did not differ among the experimental periods (III/IV, V).

5.3 Serum total lipids, lipoprotein lipids and apolipoproteins

Serum TC and LDL-C

Study I

Serum TC and LDL-C decreased significantly in all study groups during the low-fat, low-cholesterol diet (Figure 6). The reduction in serum TC was significantly greater in both the WSEM and VOSEM groups compared with the control group (mean reduction 18.3% and 15.7% vs. 7.7% from baseline value, respectively). Furthermore, the reduction in LDL-C was significantly greater in the WSEM group compared with the control group (mean 23.6% vs. 9.9%, $P<0.01$). There were no significant differences in the reduction of TC or LDL-C compared with the baseline between the WSEM and VOSEM groups.

Study III

Serum TC and LDL-C decreased in a dose-dependent manner and significant decreases in serum TC and LDL-C concentrations were reached with the daily stanol dose equal to or greater than 1.6 g (Figure 6). The percentage mean reductions in TC compared with the control were 2.8%, 6.8%, 10.3% and 11.3% with the daily doses of 0.8 g, 1.6 g, 2.4 g and 3.2 g, respectively. The respective reductions for LDL-C were 1.7%, 5.6%, 9.7% and 10.4%. The reduction in serum TC was significantly greater with the doses of the 1.6 g/d, 2.4 g/d and 3.2 g/d than with the dose of the 0.8 g/d, but the reduction in serum LDL-C was significantly greater only with the doses of the 2.4 g/d and 3.2 g/d compared with the 0.8 g/d dose.

Study V

There were no significant differences in cholesterol-lowering efficacy between the STAEST and STEEST margarines (Figure 6). The STAEST and STEEST margarines resulted in significantly lower serum TC (mean 9.2% and 7.3%, respectively) and LDL-C (12.7% and 10.4%, respectively) concentrations compared with the control.

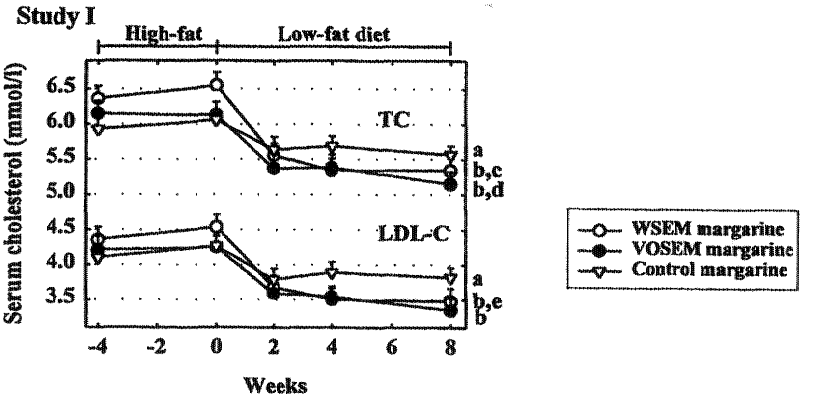
Table 7. Fatty acid composition of serum cholesteryl esters during studies III/IV and V.

	Study III/IV					Study V		
Fatty acid, mol%	**Control (0 g)**	**Dose 0.8 g**	**Dose 1.6 g**	**Dose 2.4 g**	**Dose 3.2 g**	**Control**	**STAEST**	**STEEST**
Myristic acid	0.96 ± 0.20	0.94 ± 0.18	0.96 ± 0.20	1.02 ± 0.20	1.02 ± 0.19	1.17 ± 0.33	1.14 ± 0.36	1.22 ± 0.42
Palmitic acid	12.05 ± 0.72	12.06 ± 0.69	12.07 ± 0.59	12.23 ± 0.79	12.03 ± 0.68	12.46 ± 0.87	12.37 ± 0.86	12.45 ± 0.89
Palmitoleic acid	3.11 ± 1.26	3.05 ± 1.05	3.24 ± 1.28	3.38 ± 1.35	3.17 ± 1.30	3.80 ± 0.88	3.68 ± 0.91	3.58 ± 0.80
Stearic acid	0.55 ± 0.12	0.51 ± 0.15	0.49 ± 0.18[a]	0.50 ± 0.19	0.45 ± 0.13[a,b]	0.78 ± 0.15	0.67 ± 0.13[c]	0.67 ± 0.13[c]
Oleic acid	20.29 ± 1.06	20.04 ± 1.64	19.91 ± 1.34	20.51 ± 1.87	19.95 ± 1.45	21.22 ± 1.50	21.07 ± 1.49	20.66 ± 1.52[d]
Linoleic acid	53.78 ± 3.08	54.04 ± 3.62	53.79 ± 3.17	52.50 ± 4.02[b]	53.72 ± 3.29	51.45 ± 3.21	52.27 ± 3.27	52.20 ± 3.37
γ-linolenic acid	0.78 ± 0.38	0.69 ± 0.28	0.71 ± 0.28	0.80 ± 0.32	0.74 ± 0.34	0.65 ± 0.29	0.63 ± 0.32	0.59 ± 0.26
α-linolenic acid	1.12 ± 0.17	1.03 ± 0.15	1.06 ± 0.13	1.10 ± 0.18	1.03 ± 0.13[e]	1.07 ± 0.20	1.00 ± 0.16	0.99 ± 0.23
Dihomo-γ-linolenic acid	0.48 ± 0.10	0.49 ± 0.10	0.50 ± 0.08	0.51 ± 0.10	0.49 ± 0.08	0.52 ± 0.15	0.49 ± 0.10	0.53 ± 0.17
Arachidonic acid	4.78 ± 0.83	5.00 ± 0.97	5.02 ± 1.00	4.99 ± 1.02	4.93 ± 0.88	4.58 ± 0.95	4.50 ± 0.89	4.63 ± 0.82
EPA	1.59 ± 0.73	1.59 ± 0.67	1.64 ± 0.61	1.85 ± 0.73	1.87 ± 0.76	1.68 ± 1.00	1.57 ± 0.66	1.86 ± 1.04
DHA	0.54 ± 0.13	0.56 ± 0.16	0.60 ± 0.17[e]	0.62 ± 0.17[a]	0.61 ± 0.16[e]	0.63 ± 0.18	0.60 ± 0.19	0.63 ± 0.20

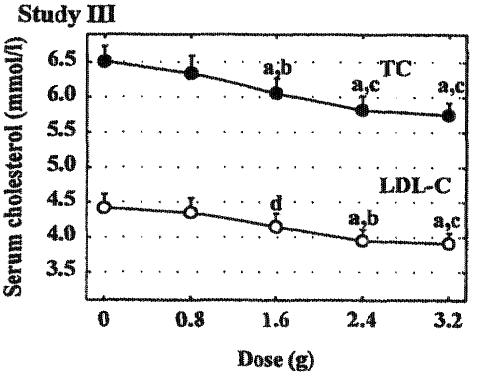
Mean ± SD. EPA=Eicosapentanoic acid, DHA=Docosahexanoic acid

Study III/IV:
[a] P<0.01, [e] P<0.05, vs. control
[b] P<0.05 vs. 0.8 g dose

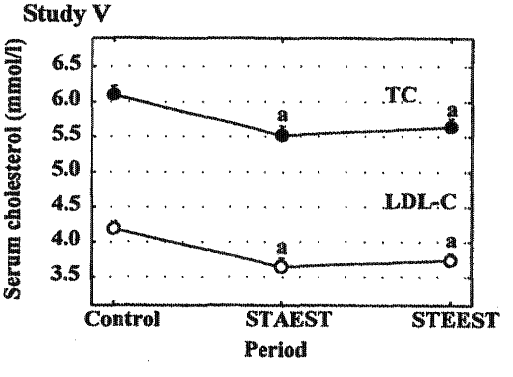
Study V:
[c] P<0.001, [d] P<0.01 vs. control



a P<0.01, b P<0.001 change within-group from 0 to 8 weeks
c P<0.001, d P<0.05, e P<0.01 vs. control group



a P<0.001, d P<0.05 vs. control dose
b P<0.05, c P<0.001 vs. 0.8 g dose



a P<0.001 vs. control margarine

Figure 6. Serum TC and LDL-C concentrations (mmol/l) during studies I, III and V. Mean ± SEM.

Variation in LDL-C responses to plant stanol esters or sterols esters

Serum LDL-C increased in one subject in the VOSEM group and in three subjects in the control group (I). The greater the stanol ester dose, the smaller the number of non-responders (III). However, there was no subject who did not respond at least one of the four stanol ester doses (III). In addition, in both STAEST and STEEST margarine periods (V) serum LDL-C increased slightly in five subjects and in two of them with both test margarine periods. When the reduction in LDL-C was examined in thirteen subjects who participated in at least two of three studies, no real non-responders were found.

Serum HDL-C, VLDL-C and TG

Serum HDL-C concentrations remained almost unchanged in all studies.

In study I, serum VLDL-C decreased within all study groups, but only significantly within the VOSEM group (0.47 ± 0.24 mmol/l to 0.34 ± 0.18 mmol/l, 0 to 8 wk). There were no significant differences in the reduction in VLDL-C among the three study groups (I). In study III, the serum VLDL-C was significantly lower with the doses of 1.6 g/d (0.39 ± 0.22 mmol/l), 2.4 g/d (0.38 ± 0.29 mmol/l) and 3.2 g/d (0.34 ± 0.23 mmol/l) compared with control value (0.62 ± 0.25 mmol/l), but there were no significant differences in serum VLDL-C concentrations between any two stanol doses. No significant changes in serum VLDL-C were found during study V.

There were no significant changes in serum total TG (I,III,V), VLDL-TG (I), LDL-TG (I) or HDL-TG (I) concentrations among the study groups (I) or in serum TG among the experimental periods (III, V). However, there was a significant decrease in the serum VLDL-TG concentration (0.96 ± 0.62 mmol/l to 0.77 ± 0.64 mmol/l, 0 to 8 wk) within the WSEM group, and an increase in the serum LDL-TG concentration (0.27 ± 0.06 mmol/l to 0.30 ± 0.08 mmol/l, 0 to 8 wk) within the control group (I).

Serum apo AI and B

Serum apo AI was reduced on average by 9.0%, 8.6%, and 6.1% from the baseline within the WSEM, VOSEM and control groups, respectively, $P<0.01$-0.05 (I). However, no significant differences in the changes of apo AI were found among the study groups (I) or among the experimental periods (III, V).

Serum apo B was reduced significantly in all studies and the reduction paralleled the reduction in LDL-C. In study I, the mean reduction was 19.2%, 13.7% and 5.2% from the baseline, within the WSEM, VOSEM and control groups, respectively, $P<0.001$-0.05. In study III, a significant reduction (8.7%) in apo B was reached already with the lowest stanol dose (0.8 g/d). With the other doses (1.6 g/d, 2.4 g/d and 3.2 g/d) the mean reduction of apo B was 9.3%, 10.2% and 13.7%, respectively. In addition, the STAEST margarine resulted in a 10.7% reduction and the STEEST margarine in a 10.4% reduction in serum apo B concentrations compared with the control margarine, $P<0.001$

for both.

5.3.1 Non-dietary factors affecting serum lipid responses

No differences between **genders** or different **age** groups (Figure 7) were found in serum lipid responses to plant stanol esters or sterol esters in any of studies I, III and V. No differences in the lipid results between normal weight and slightly over**weight** subjects were found (I, III, V). In addition, there were no significant correlations between the **initial value of LDL-C** and the percentage reduction in LDL-C in any of the studies when subjects were divided to tertiles of the initial LDL-C concentrations.

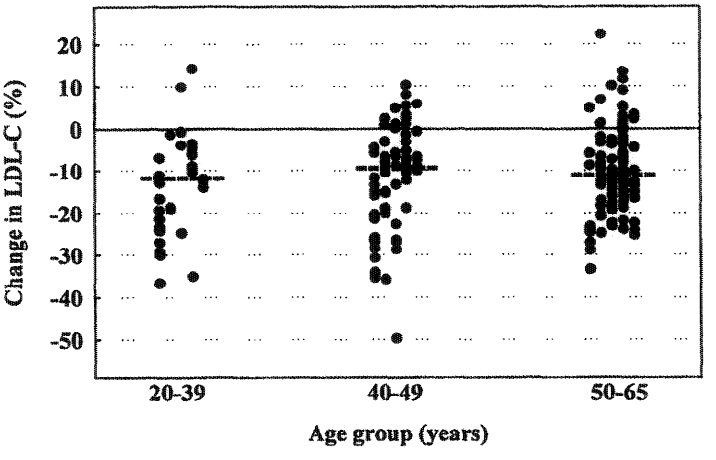


Figure 7. Serum LDL-C response (%) in different age groups during studies I, III and V.

The changes in LDL-C were similar for those subjects with **apo E genotype** 3/3 and those with apo E allele 4 (3/4 or 4/4) in both experimental groups during study I (Figure 8). However, in the control group, the reduction in LDL-C seemed to be somewhat greater in those with apo E3/4 than those with apo E3/3. Also in study III, serum LDL-C reduced similarly in two apo E groups with different stanol ester doses. In study V, the subjects with apo E3/4 had a significantly greater reduction in LDL-C during the STAEST margarine period than during the STEEST margarine period. However, there were no significant differences in the reduction in LDL-C in those with apo E3/3 between the two test margarine periods or between the two apo E groups during either experimental period.

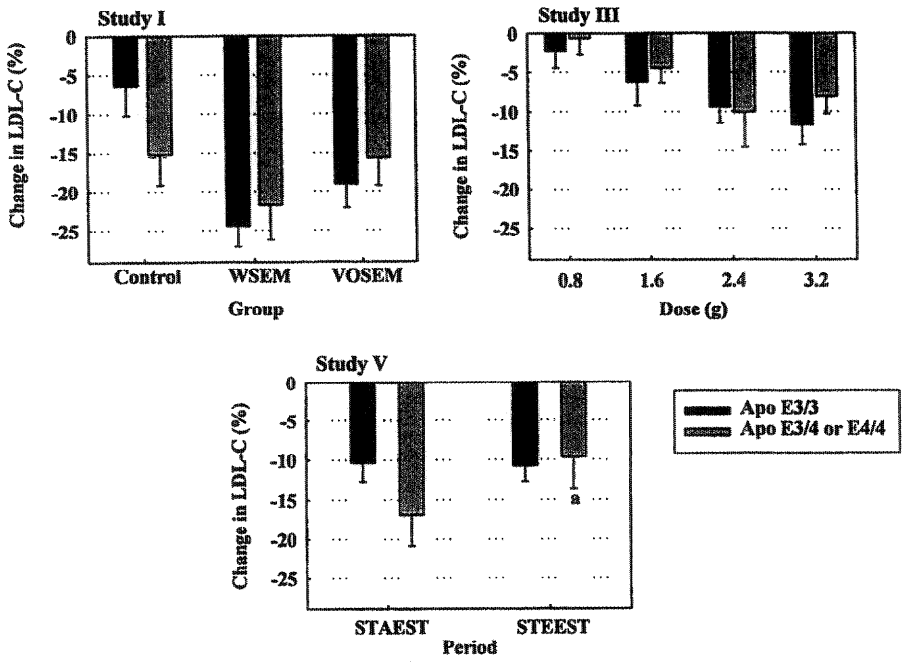


Figure 8. Changes in serum LDL-C (%) in different apo E groups during studies I, III and V. a $P<0.05$ STAEST vs. STEEST in the subjects with apo E3/4. Mean ± SEM.

5.4 Serum cholesterol precursors and cholestanol

In general, the changes in the concentrations of serum cholesterol precursors were small. The most pronounced changes were found in serum Δ8-cholestenol and Δ7-lathosterol concentrations, especially in their ratios to serum TC (Figure 9). In study V, also the increase in the ratios of the serum desmosterol and squalene to TC reached statistical significance (Figure 9).

The serum Δ7-lathosterol/TC ratio (IV) did not increase in a dose-dependent manner but plateaued with a dose equal to or greater than 1.6 g/d (Figure 9).

Serum cholestanol concentration reduced significantly with daily doses of the 1.6 g, 2.4 g and 3.2 g in comparison with the control (IV; Table 2). However, the serum cholestanol/TC ratio remained almost unchanged during that study. In study V, the serum cholestanol concentration was lower during the STAEST and STEEST margarine periods than during the control (V; Table 5). The ratio was also significantly lower during the STEEST margarine period than during the control or STAEST margarine period.

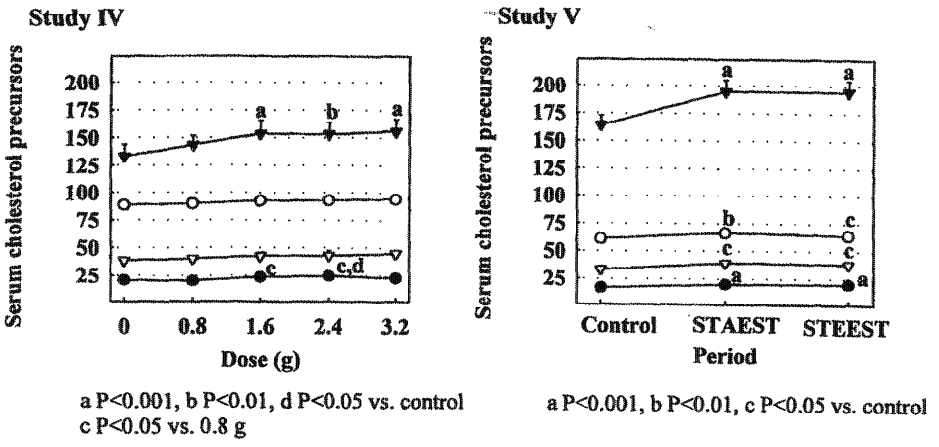


Figure 9. Serum cholesterol precursors Δ7-lathosterol (▼), desmosterol (○), squalene (▽) and Δ8-cholestenol (●) (10^{-2} μg/mg of TC) during studies III/IV and V. Mean ± SEM.

5.5 Serum plant sterols

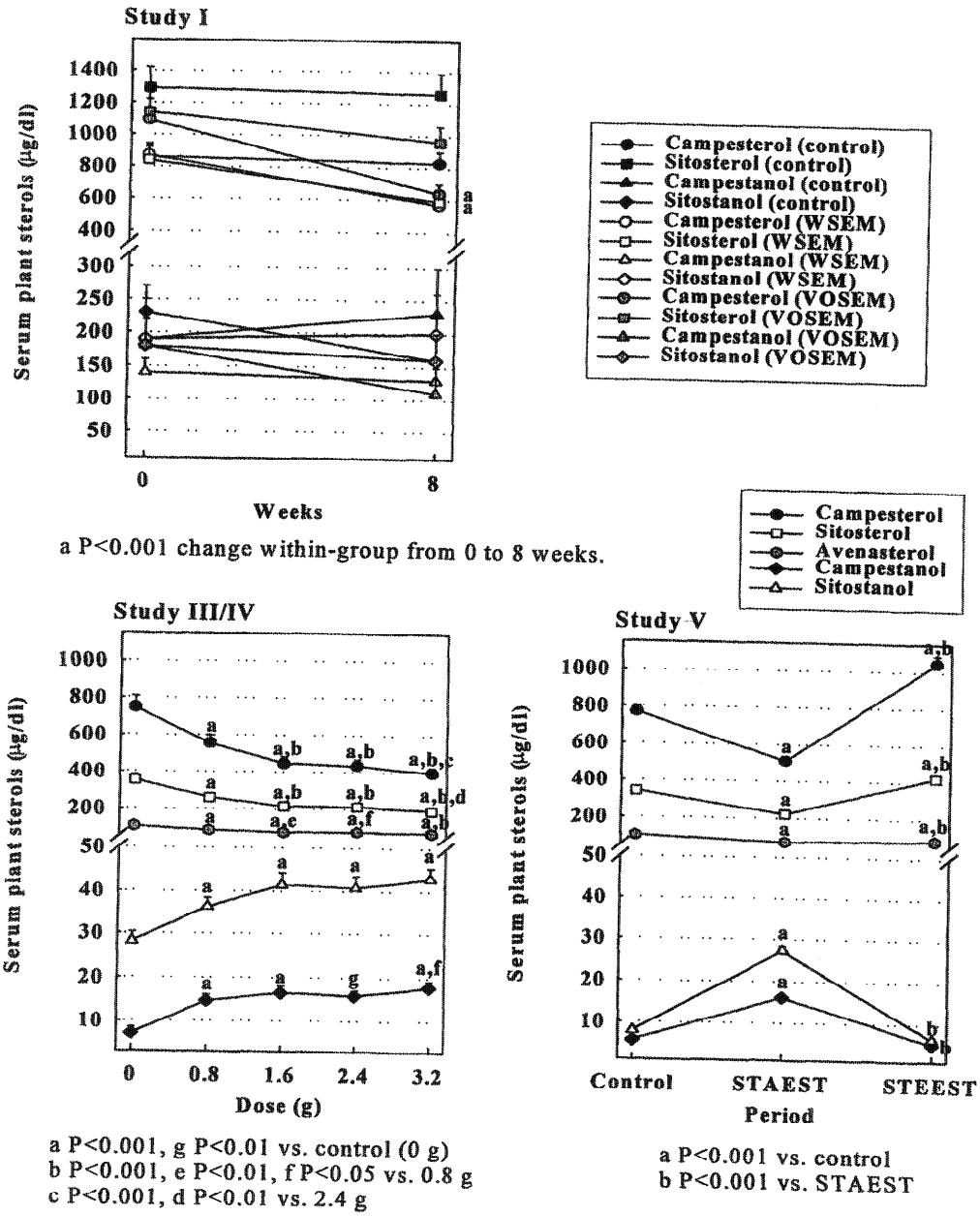
The consumption of plant stanol esters containing margarines (I, III/IV, V) resulted in a significant reduction in serum campesterol concentration (Figure 10) and the greater the stanol ester dose, the greater the reduction in serum campesterol (III/IV). The consumption of STEEST margarine increased serum campesterol concentration significantly (V).

In study I, serum sitosterol concentrations did not decrease significantly within the WSEM and VOSEM groups. However, in study III/IV, a significant decrease in sitosterol concentration occurred already with a dose of 0.8 g/d (Figure 10). In study V, serum sitosterol concentration decreased significantly with the STAEST margarine and increased significantly with the STEEST margarine compared with the control (Figure 10).

The changes in serum avenasterol (III/IV, V) were parallel, but somewhat smaller than the changes in serum campesterol and sitosterol (Figure 10). Furthermore, the changes in the ratios of serum campesterol, sitosterol or avenasterol to serum TC paralleled the changes in their serum concentrations.

Serum campestanol and sitostanol concentrations did not change significantly within any of the three study groups during study I (Figure 10). Similarly, during the STEEST margarine period (V) those concentrations remained close to the control values. On the other hand, in studies III/IV and V, significant increases in serum campestanol and sitostanol concentrations were found during the stanol ester margarine periods (Figure 10). Serum campestanol concentration became doubled already with the stanol dose of 0.8 g/d compared with the control, and the concentration increased only slightly further with greater doses (III/IV). The greatest increment in serum sitostanol concentration was

achieved also with the dose of 0.8 g/d, and only a minor further increase was found with the greater stanol ester doses (III/IV). Similarly to serum plant sterol concentrations, the changes in the ratios of serum sitostanol and campestanol to serum TC paralleled the changes in their serum concentrations in studies I, III/IV and V.



a P<0.001 change within-group from 0 to 8 weeks.

a P<0.001, g P<0.01 vs. control (0 g)
b P<0.001, e P<0.01, f P<0.05 vs. 0.8 g
c P<0.001, d P<0.01 vs. 2.4 g

a P<0.001 vs. control
b P<0.001 vs. STAEST

Figure 10. Serum plant sterol and stanol concentrations (µg/dl) during studies I, III/IV and V. Mean ± SEM.

5.6 Serum carotenoids and fat-soluble vitamins

Plant sterols and stanols had the greatest effect on serum β-carotene concentration (Figure 11). There was a significant reduction in absolute serum β-carotene concentration within the WSEM and VOSEM groups (I/II) and during the STAEST and STEEST margarine periods (V), but no significant reduction in the β-carotene concentration was found during the different stanol dose periods (III). When the serum β-carotene concentration was related to the serum TC concentration, no significant differences in the changes of the ratio were found in any of the studies. Within the control group, the changes in serum α-carotene were small and only the reduction in the α-carotene/TC ratio was statistically significant (II). The changes in the α-+β-carotene paralleled the changes in β-carotene. However, in study V, serum α-+β-carotene concentration did not differ significantly between the STAEST margarine and the control periods as it did between the STEEST margarine and the control periods. Serum lycopene and lycopene/TC did not change significantly within the WSEM and VOSEM groups (II) or during the STAEST and STEEST margarine periods (V). In women, serum lycopene concentration was significantly greater with the control and the dose of 0.8 g/d than with the dose of 2.4 g/d, and the lycopene/TC ratio was significantly greater with the dose of 0.8 g/d than with the dose of 2.4 g/d and control (III). However, no significant differences in the serum lycopene concentrations were found in men during the same study (III).

Serum retinol concentration remained almost unchanged in all studies. There were no major changes in the serum 25-OHD_2 concentration during study I or in serum 25-OHD_3 concentration during studies III and V. The concentration of 25-OHD_3 increased significantly within the WSEM, VOSEM and control groups, and the increase was significantly smaller in the WSEM than in the VOSEM group (I; Table 5). However, there was no significant difference in the percentage increase of 25-OHD_3 among the study groups.

Serum α-tocopherol concentrations were reduced significantly within the WSEM, VOSEM and control groups (I; Table 5). The reduction differed significantly only between the WSEM and control groups. Serum α-tocopherol concentration was significantly lower with all test doses compared with the control, and in addition, the concentration was significantly lower with the dose of 3.2 g/d than with the dose of 0.8 g/d (III; Table 6). Furthermore, the serum α-tocopherol concentration was significantly lower during the STAEST and STEEST margarine periods than during the control period (V; Table 6). However, after relating α-tocopherol to TC there were no significant changes among the study groups (I) or experimental periods (III, V). The changes in serum γ-tocopherol concentrations were smaller than the changes in serum α-tocopherol concentrations (III and V; Table 6). Only in study III was the concentration significantly lower with the stanol doses of 2.4 g/d and 3.2 g/d than with

the control. The γ-tocopherol/TC ratio remained almost unchanged during these two studies (III, V). In addition, the changes in serum α-+γ-tocopherol paralleled the changes in serum α-tocopherol (III, V).

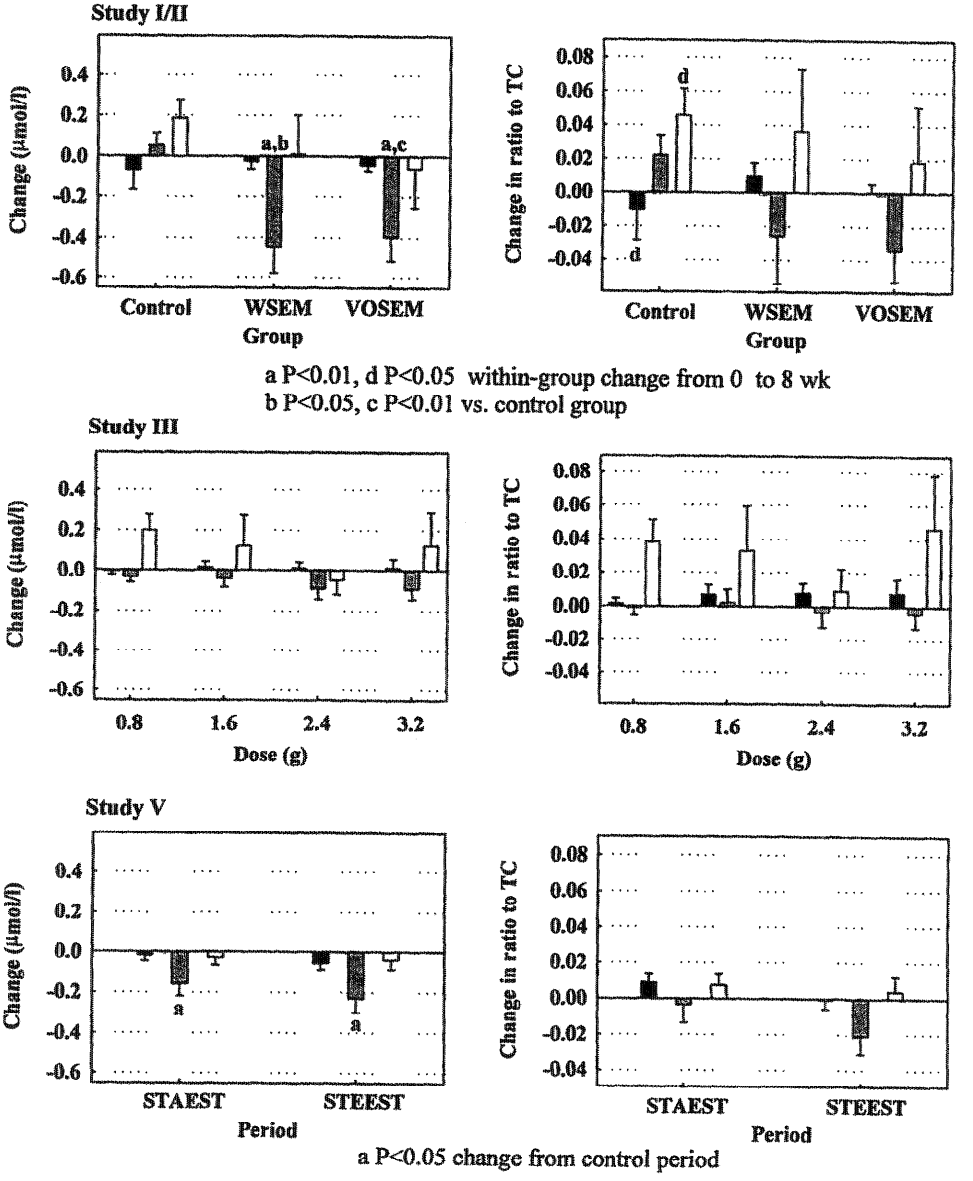


Figure 11. Changes in serum α-carotene (black), β-carotene (gray) and lycopene (white) concentrations (µmol/l) and their ratios to TC (mmol/mol of TC) during studies I/II, III and V. Mean ± SEM.

6 DISCUSSION

6.1 Subjects and study designs

Subjects representing the potential users of plant sterol-enriched products i.e. middle-aged, normal weight or slightly overweight and mildly or moderately hypercholesterolemic individuals were recruited into the studies. The subjects completing the studies did not differ from those who dropped out with respect to background variables. The subjects can be considered to be well representative of the target study population.

In study I/II a parallel **study design** with a 4-week high-fat diet period and a 8-week low-fat diet period was used, because our aim was to examine how much stanol ester-enriched low-fat margarines could potentiate the lipid reduction induced by a low-fat diet alone compared with the typical Western diet. On the other hand, because in studies III/IV and V the main interest was to compare the different stanol ester doses or the different test margarines, the repeated measures design with 4-week experimental periods was chosen as the study design. The length of the experimental period in study I/II was eight weeks instead of four weeks, because the implementation of a low-fat diet takes a longer time than four weeks, and thus study I/II could not be arranged with the cross-over design.

In studies III/IV and V, the subject consumed each test margarine in a randomized order, which was determined as a whole study group (III/IV) or according to the Latin square model (V). The randomization as a group in study III/IV was chosen for practical reasons; there were five test doses and if each subject had been randomized separately or according to the Latin square model, there would have to have been many more subjects in the study. The benefit of the repeated measures design is that it increases the power of the study by eliminating the between-individual variation. In addition, the randomization according to the Latin square model in study V enabled us to control the effect of time. On the contrary, the randomization as a group in study III/IV did not allow to control for the effect of time but did allow to control for systematic bias due to the order of periods. However, should time have affected the results of that study, the greatest reduction in serum cholesterol would have been expected to occur during the first dose period (2.4 g/d), but this was not true the case in that study. Moreover, in study V no effect of time was observed.

The disadvantage in the repeated measures design is a possible carry-over effect. To eliminate the carry-over effect, it is possible to use wash-out periods between test periods or to use a sufficiently long study period. In studies III/V and V, the duration of the experimental period was four weeks and the main comparisons were made among the mean values at the end of each experimental period. It is well-known that effects of plant stanols and plant sterols become apparent within a short time. In earlier studies, it

has been shown that plant sterols reduce cholesterol concentrations within 2-3 weeks of the initiation of treatment (4, 15, 72, 74). Furthermore, in one specific patient group, colectomized patients, a significant reduction in serum cholesterol was found already after one day of the consumption of plant stanol esters and a new steady state was reached within seven days (78). Moreover, the serum cholesterol concentration has been shown to return to its initial value within 2-3 weeks, upon cessation of the ingestion of plant sterols (4, 15, 68, 74). In addition, in dietary intervention studies, a new steady state in the cholesterol concentration has been attained within 2 weeks (207). Altogether, the four-week study period in studies III/IV and V can be considered as being sufficiently long enough to eliminate any carry-over effects of the previous period, and long enough to illustrate the effects of a given test margarine on serum cholesterol concentration.

6.2 Compliance of subjects and feasibility of the diets

All subjects participating in the present studies were free-living. In order to gain the dietary goals and eliminate changes in body weight, the subjects received precise oral and written instructions about isocaloric diets, which were individually tailored to each subject within the set dietary goals. The subjects themselves took care of the practical implementation with normal Finnish foodstuffs and only the test spreads and the other study products (vegetable oils, salad dressings, low-fat cheeses or liquid milk products) were given to them free of charge. In some other plant sterol studies, all meals have been prepared entirely by the research unit (36, 71, 86) to improve the compliance of the subjects and to diminish the variability of the background diets. However, the lipid results of our studies were comparable to the result of those studies. In the present studies, the subjects were well motivated to participate in the studies and only three out of seventeen dropped out due to lack of commitment while other reasons for dropping outs were not connected to motivation. In addition, there were only minor changes in medication and life-style of the subjects during the studies and they did not affect the results. Therefore, our free-living setting was sufficiently strict to allow us to evaluate the test diet at the level demanded by the hypothesis. The advantage of our chosen procedure is that it gives a more realistic view on the effects of the study products and experimental diets on examining variables in free living individuals under normal conditions than providing of prepared meals to subjects or studies in a metabolic unit setting.

In study I/II, test margarines were enriched with stanol esters derived from wood (WSEM) or vegetable oils (VOSEM) and in the other studies (III/IV, V) they were a blend of wood and vegetable oils. The sterol esters were only vegetable oil-based as has been the case in the other published sterol ester studies (4, 5, 31). In most of previous studies, plant stanol esters have been derived from wood (47, 64-68). However, recently

published studies, blends of wood and vegetable oils have been used (4, 71, 72, 77, 78). The amount of absorbable fat, fatty acid composition of the test margarines and the esterification degree of plant stanols or sterols in each study was identical (I-V). However, there were slight differences in the total amount of plant stanols between the WSEM and VOSEM margarines (difference 0.18 g/d; I/II) and between the amount of planned and actual plant stanols in the different stanol ester doses (≤0.15 g/d of stanols; III/IV). These differences, however, were minor and therefore, it can be assumed that they have any impact on the results.

The dietary compliance of the subjects was monitored by three to four consecutive days of food records (I-V) and by measuring the fatty acid composition of serum cholesteryl esters (III/IV, V) regularly. The use of these two methods ensured the reliability of the dietary follow-up. The three to four consecutive days food recording has been found to be sufficient to estimate the intake of energy nutrients at a group level (208, 209). However, for the monitoring cholesterol intake more recording days may be needed (208). In study I, the mean of data from the three four days' food records was used as the estimate of nutrient intake. In addition, in all studies the subjects followed a certain dietary regimen. This diminished within-person variation and thus decreased the number of recording days required to obtain reliable information on nutrient intake (210).

The subjects were given oral and written instructions on how to keep the food records. In addition, the nutritionist reviewed the records during the study visits to complement data that were lacking. It is known that subjects attempt to please the nutritionist by manipulating their records to match the dietary goals of the study (211, 212). Therefore, it was emphasized to the subjects that they have to report their true food intake to ensure the reliability of the study. In addition, the results of fatty acid composition of serum cholesteryl esters (III/IV, V), which is an objective marker of dietary adherence in terms of quality of fat, reflecting the fatty acid composition of a diet during the past 3-4 weeks (213, 214), suggests that the subjects were honest in their reporting. The subjects were aware of the use of this objective measurement.

According to the food records (I-V), the experimental diets met well the goals for fatty acid composition and dietary cholesterol. In study I/II, the consumption of the low-fat test margarines combined with the low-fat diet enabled the achievement of the goals of the step 2 diet (81) in the intake of fat and dietary cholesterol. Also the intake of SAFA was close to the goal of the step 2 diet (81) in all three study groups. No significant differences were found in the nutrient intake among the groups of study I/II or among the experimental margarine periods of study V. In addition, the minor differences in the intake of SAFA, alcohol and fiber during the different dose periods (III/IV) had a non-significant effect on the serum lipid responses caused by the stanol esters. The fatty acid composition of serum cholesteryl esters was similar during the different experimental periods indicating that the background diets of these studies did

not change. In addition, the similar serum fatty acid composition as the biomarker confirms good compliance to the use of test margarines and intended fat modification.

6.3 Serum total lipids, lipoprotein lipids and apolipoproteins

Effects of plant stanol esters as part of a cholesterol-lowering diet

The low-fat WSEM and VOSEM margarines (I) combined to the cholesterol-lowering diet reduced serum TC and LDL-C concentrations on average by 16-18% and 18-24%, respectively, from the high-fat baseline diet. The reductions seen here were greater than those achieved (range of mean reduction 4-10% and 5-15%, respectively) in several studies with mildly to moderately hypercholesterolemic subjects in which full-fat stanol ester margarine combined to a habitual moderate rich or rich fat diet has been used (33, 34, 68). In fact, our results (I) indicate that combining plant stanol esters to a low-fat margarine and as part of a strict low-fat, low-cholesterol diet, can reduce serum cholesterol concentration nearly as much as some cholesterol-lowering drugs (215, 216). In addition, our findings show that low-fat stanol ester margarines do provide an additional, about 10% reduction, in serum cholesterol concentrations to that which can be obtained with a strict cholesterol-lowering diet alone as was recently also shown with a similar study design by Anderson et al. (71). This additional benefit is remarkable, because the dietary changes have been reported to obtain only a 3-6% reduction in serum cholesterol at the population level (1).

Our findings that the stanol ester (I, V) and sterol ester (V) margarine reduced serum TC and LDL-C significantly as part of a low-fat, low-cholesterol diet are contrary to the earlier suggestion that plant stanols and sterols are ineffective when the diet is low in cholesterol. This suggestion is based on the findings of Denke (63), in which 3 g/d of plant stanols taken in capsules and as part of a low-fat and low-cholesterol diet reduced serum TC and LDL-C only slightly. However, the most probable reason for Denke's finding was that plant stanols in capsules were suspended, not dissolved, in sunflower oil and were thus in a poorly soluble, less effective, form. The findings of Denke have now also been rejected in several other studies (70-72, 75-77). The finding that plant stanols can reduce serum cholesterol concentrations even when combined with a low-cholesterol intake, indicates that they must inhibit both the absorption of dietary as well as biliary cholesterol.

In the present studies, as in many others (4, 34, 65-72) the daily dose of plant stanol esters was taken in 2-3 portions with meals. However, most recently Plat et al. (77) demonstrated that it is not necessary to eat plant stanol esters simultaneously with dietary cholesterol or with each meal to obtain the optimal cholesterol-lowering effect.

Serum HDL-C and TG concentrations remained almost unchanged, in agreement with previous studies (5, 34, 47, 64, 66-68, 71, 72, 74-78).

Dose-response effect of plant stanol esters

In study III, plant stanol esters reduced the serum cholesterol concentrations in a dose-dependent manner. The significant reduction in serum TC and LDL-C concentrations was reached with a daily stanol dose equal to or greater than 1.6 g compared with the control. Furthermore, increasing the daily dose of plant stanol from 2.4 g to 3.2 g did not provide additional cholesterol-lowering effect. Therefore, based on the present findings and the findings of the other stanol ester or sterol ester studies (31, 68, 72) the optimal daily stanol or sterol dose seems to be about 1.6-2.4 g. These findings indicate that the dose-response of plant stanols or sterols on serum cholesterol is curvilinear and that the response plateaus with a dose of equal to or greater than 2.4 g/d. Above that level of plant stanol or sterol, the cholesterol-lowering efficacy increases only marginally. These findings are interpreted to mean that the ability of plant stanols or sterols to disturb the cholesterol absorption from intestine is dependent on their relative amounts in the intestine. Therefore, if there is an excessive amount of plant stanol or sterol in the intestine in relation to cholesterol, no additional benefit can be obtained by increasing the dose of stanol ester or sterol ester. In adults, each day between 1000-1500 mg of cholesterol, either of biliary or dietary origin, enters the lumen of the small intestine (97). This could be one reason why the reduction plateaus with the dose of 2.4 g/d.

In general, the changes in serum apo B paralleled the changes in serum LDL-C. However, a dose as low as of 0.8 g/d resulted in a significant reduction in apo B concentrations compared with the control, although with that dose the reduction in serum LDL-C was small and non-significant. In recent studies, it has been suggested that as little as 0.7-0.8 g/d of plant sterols or stanols are needed to achieve a significant reduction in serum cholesterol (31, 54, 56, 57). In this study, only one blood sample was taken at the end of each dose period. Therefore, the slight, but non-significant, reduction during the 0.8 g dose period in serum cholesterol concentrations might be concealed by within-subject variation in serum cholesterol concentrations. Within-subject variation in serum cholesterol concentration is 5-10% (217, 218). In addition, the power of the study (0.8) was calculated to detect a 0.4-0.5 mmol/l difference in TC response between different doses. Therefore, the number of subjects (N=22) was too small to observe such small reductions in serum cholesterol as being statistically significant.

Variation in serum LDL-C responses to plant stanol esters or sterol esters

In one study about 8% of subjects were reported to be non-responders to the stanol ester treatment (104). However, we did not find any real non-responders when we compared the reduction in LDL-C in thirteen subjects who participated in at least in two of our three studies. Although there were subjects who did not respond to stanol ester or sterol ester treatment in one study, in another study their LDL-C concentrations did decrease in response to the treatment. In addition, it seems that the more strict the

background diet, the fewer non-responders found in the present studies.

Origin and form of plant stanols or plant sterols

In study I, the wood and vegetable oil-based stanol esters enriched margarines reduced serum TC and LDL-C concentrations equally effectively, as was recently also stated by Gylling and Miettinen (33) and Plat and Mensink (34). In addition, in study V the cholesterol-lowering effect of plant sterol esters and stanol esters (saturated form of plant sterols) did not differ significantly. The findings in two recent comparison studies have been inconsistent (4, 5): in one study soy oil-based sterol ester margarine and stanol ester margarine (Benecol®) reduced serum cholesterol concentrations similarly (4), but in the second study sterol esters reduced serum LDL-C concentrations somewhat more than stanol esters (5). In some earlier studies it has been suggested that free plant stanols reduce serum cholesterol concentrations more effectively than free plant sterols (58-61, 80). Therefore, there might be differences in the cholesterol-lowering efficacy between the large and small doses of plant sterols and stanols. However, with the doses used currently, there does not seem to be differences in the efficacy of plant sterols and stanols. Furthermore, based on recent findings (56, 170) it seems that the vehicle by which plant sterols or stanols are delivered to the small intestine is a more critical factor determining their ability to disturb the cholesterol absorption and thus reduce serum cholesterol concentrations than the degree of saturation of plant sterols.

6.4 Non-dietary factors affecting serum lipid responses

In none of the studies (I, III, V) were there any differences detected between **genders** in serum lipid responses on plant stanol esters or sterol esters in agreement with other studies (4, 47, 54, 64, 77).

Although it has been suggested that the subjects **aged** 30-39 years would have a lower LDL response to plant stanol or plant sterol treatment compared with those aged 40-49 or 50-59 years (89), the findings of the present studies I, III and V do not confirm this suggestion.

No differences in lipid responses between the subjects with normal **weight** and those who were slightly overweight were found in any of the studies (I, III, V) in accordance with the findings of other studies (53, 72). Furthermore, the changes in body weight can not be considered to have any confounding effect on serum lipid results, since in studies III and V the body weight remained unchanged and in study I the significant decrease in body weight was only marginal and similar within the three study groups. In study I, the decrease in body weight was primarily ascribed to the lower intake of energy during the experimental period than during the run-in period (mean 7.1-7.8 MJ/d vs. 8.0-8.7 MJ/d). In turn, this was attributed to the low-fat diet, all of the subjects could not eat the

planned amount of food, which would have covered their energy requirements.

The **initial value** of serum LDL-C did not affect the magnitude of response to plant stanol esters or sterol esters in any of the present studies (I, III, V). This is in contrast to the findings of several other studies (68, 70, 76, 78), although also similar findings to ours have been presented (4).

In previous studies, the results of the effects of **apo E genotype** or phenotype on LDL-C responses to plant stanols or plant sterols have been controversial (34, 47, 54, 66). According to secondary analyses performed in studies I, III and V, there were no significant differences in LDL-C response between the apo E3/3 and apo E3/4 genotype groups. Surprisingly, the subjects with apo E3/4 had a greater percentage reduction in serum LDL-C during the STAEST period than during the STEEST period (V). It is difficult to assess the validity of this finding, since there are no previous reports in which the effects of plant stanols on serum cholesterol concentrations in different apo E groups have been compared with that of plant sterols. Therefore, this finding should be verified in prospective studies.

6.5 Serum non-cholesterol sterols as a marker of cholesterol metabolism

The concentrations of serum plant sterols (campesterol and sitosterol; I, III/IV, V) and cholestanol (IV, V) were determined to evaluate cholesterol absorption (103, 105, 153), whereas the concentrations of serum cholesterol precursors (Δ8-cholestenol and Δ7-lathosterol and desmosterol; IV, V) were measured to estimate cholesterol synthesis (103, 150-152).

Serum plant sterol concentrations, and in particular the serum campesterol concentration, have been found to reflect cholesterol absorption efficacy from intestine in individuals on a normal background diet without plant sterol or stanol supplementation (103, 105). As in previous plant stanol or stanol ester studies (4, 5, 33, 47, 54, 55, 64-68, 70-72, 75, 76, 78, 79, 104) in the present studies, stanol esters reduced serum campesterol concentrations significantly. Compared with the control the significant reduction in serum plant sterol concentration was already attained with a stanol dose of 0.8 g/d (III/IV) indicating that plant stanols can effectively inhibit intestinal absorption of cholesterol even at low doses. This confirms the findings of the previous studies in which 0.6-0.8 g/d of plant stanols in free or esterified form caused marked reductions in serum plant sterol concentrations (54, 55, 64). Our findings are also in agreement with the studies where 0.7-0.8 g/d of plant stanol as stanol esters effectively reduced intestinal absorption of cholesterol as measured by the continuous isotope feeding method (54, 64). The greater the stanol ester dose, the greater the reduction in serum campesterol, indicating an even greater reduction in cholesterol absorption with the higher daily stanol doses of 1.6 g, 2.4 g and 3.2 g (III/IV). On the other hand, the increased serum plant sterol concentrations with the STEEST margarine

reflected the absorption of plant sterols from that margarine rather than the increased cholesterol absorption.

In studies IV and V, serum cholestanol, a metabolite of cholesterol, seemed to be a weak marker of cholesterol absorption. However, this is not surprising, in view of the fact that most published reports of the effects of plant stanol esters on serum cholestanol have been inconsistent (33, 47, 54, 55, 64-67, 70, 71, 75, 78, 79, 104).

Cholesterol malabsorption and depletion of hepatic cholesterol pool induced by plant sterols have been suggested to stimulate a compensatory increase in endogenous cholesterol synthesis (64, 65, 67, 70, 78). The marker of that is the enhanced concentrations of serum cholesterol precursors, and particularly the elevated serum Δ7-lathosterol/TC ratio (103, 151, 152). In studies IV and V, the enhanced serum Δ7-lathosterol/TC ratio indicated that cholesterol synthesis had increased in compensation for the cholesterol malabsorption and the decrease in hepatic cholesterol. The plant stanol dose of 0.8 g/d increased serum Δ7-lathosterol/TC only slightly and this agreed with earlier findings (54, 64). Furthermore, serum Δ7-lathosterol/TC plateaued with the stanol dose of 1.6 g/d which might indicate that cholesterol synthesis does not increase in a dose-dependent manner but the endogenous cholesterol synthesis reaches its maximum level with a stanol dose of 1.6-2.4 g/d (III/IV). Despite the increase in cholesterol synthesis, plant stanols and sterols induced a marked decrease in serum TC and LDL-C concentrations. This can be ascribed to that the decreased hepatic cholesterol pool in addition to increased cholesterol synthesis enhanced LDL receptor activity resulting in a reduction of serum levels of cholesterol rich particles (32).

6.6 Serum concentrations as a marker of absorption of plant sterols and plant stanols

Absorption of plant sterols and stanols is a key issue for the evaluation of systemic effects possibly caused by the consumption of plant sterol ester or stanol ester. Therefore, concentrations of plant sterols and plant stanols in serum were determined in studies I, III/IV and V.

Under normal conditions, serum plant sterol concentrations are very low, only about 1/1000 of the serum cholesterol concentration, and the concentrations of plant stanols in serum are even lower. During the present studies, both plant sterol and plant stanol concentrations in serum remained very low. Similarly to the other plant sterol studies (4, 5, 53), in study V the consumption of the sterol ester-enriched margarine increased serum campesterol and sitosterol concentrations. Furthermore, in studies III/IV and V, the consumption of the stanol ester-enriched margarines increased serum campestanol and sitostanol concentrations confirming the findings of other recently published studies (33, 72, 76, 78, 104) that also plant stanols are absorbed, but that the absorbed amounts were very small compared with the daily intake of plant stanols from the test

margarines. However, the findings of study I differed from the findings of studies III/IV and V and the recent reports (33, 72, 76, 78, 104). In study I, serum campestanol and sitostanol concentrations did not change significantly within any of the three study groups, and the serum concentrations in that study were higher than in studies III/IV and V. The differences in the higher values of serum stanols might partly be due to the analytical method, especially different column used. In study I, the column probably did not differentiate sitostanol from avenasterol, and therefore, the sitostanol values might represent a mixture of sitostanol and avenasterol rather than sitostanol alone. However, that cannot explain the higher sitostanol values entirely. Therefore, the reason for the higher serum sitostanol and campestanol concentrations remains unresolved. Presumably, the used analytical method is also one reason for the findings of the several earlier intervention studies, in which sitostanol has been suggested to be virtually nonabsorbable (4, 54, 62, 64). In study I, serum plant sterol concentrations were also higher than in studies III/IV and V. Furthermore, in that study serum sitosterol concentration was exceptionally higher than serum campesterol concentration.

The increases in serum campesterol and campestanol concentrations were greater than that in serum sitosterol and sitostanol, respectively, when the consumed amounts were taken into account, reflecting the better absorption rates of campesterol and campestanol compared with sitosterol and sitostanol, respectively. That is in accordance with the findings of absorption studies (109, 119) as well as clinical trials (4, 5, 33, 76, 78, 104). The differences in the absorption rates between the individual plant sterols and plant stanols were attributable to the small differences in their chemical structures (i.e. the extra carbon atom in the side chain of sitosterol and sitostanol) which make campesterol and campestanol more readily absorbable.

According to the findings of study III, the absorption of plant stanols seems to plateau already at a stanol dose of 0.8 g/d (III), since with higher doses the increases of sitostanol and campestanol were only minor. However, in addition to negligible absorption of plant stanols, the low serum concentrations could also be a consequence of fast and effective clearance of absorbed stanols (11). To date, there are no published data on whether the unsaturated plant sterol concentrations increase in a dose-dependent manner or whether they level off with higher plant sterol doses.

6.7 Serum carotenoids and fat-soluble vitamins

Since plant stanol esters and sterols esters inhibit intestinal absorption of cholesterol, they may also affect the absorption of carotenoids and fat-soluble vitamins. Therefore, those concentrations were determined in studies I/II, III and V. Furthermore, the concentrations of serum carotenoids and tocopherols were related to the concentrations of serum TC, since carotenoids and tocopherols are transported in lipoprotein particles, such as LDLs (219-221). Therefore, changes in serum cholesterol concentrations are

reflected in serum carotenoid and tocopherol concentrations.

Serum carotenoids (α- and β-carotene and lycopene) and fat-soluble vitamins (retinol, vitamin D and tocopherols) were all within the normal range in the present studies. Similarly to the results of several other studies (31, 33, 71, 72, 75-77, 178) plant stanol esters and sterol esters had no effect on the concentrations of serum retinol, vitamin D and the concentrations of tocopherols related to serum TC (I, III, V). Furthermore, plant stanol esters and sterol esters had no effect on the concentrations of serum α-carotene or lycopene (II, III, V). Although in women there were differences in serum lycopene between the different dose periods (III), the differences were not related to the stanol ester dose. Women had also lower serum lycopene concentrations than men, which could partly be due to their older age (222). The greatest effects of plant stanol esters and sterol esters focused on serum β-carotene concentrations. However, after relating changes in β-carotene to the simultaneous changes in TC, the changes were minor and non-significant. As in the recent dose-response study of Hendriks et al. (31) with plant sterol esters, in study III the effects of plant stanol esters on carotenoids and fat-soluble vitamins were not dose-dependent but rather dependent on the achieved reduction in serum cholesterol. Therefore, it can be assumed that the reduction in serum carotenoid concentration plateaus with the same dose (2.4 g/d of stanols) as the reduction in serum cholesterol does. In addition, because the effects were not dose-dependent, it can be proposed that there are some other factors e.g. nutrient density of the background diet, which could affect the fluctuations on serum carotenoid concentrations rather than the dose of plant stanol or sterol. In the present studies the background diet was standardized including instructions about the intake of vegetables, but this was probably not the case in most of the other studies (4, 31, 33, 72, 178). Therefore, the variability in composition of background diets might explain why in most of the other plant sterol studies (4, 31, 33, 72, 75, 178) the effects of plant stanols or sterols on serum β- or α-+β-carotene concentrations have been greater than those seen here even after relating the changes in β- or α-+β-carotene concentrations to the changes in lipid concentrations.

Finally, according to the present studies, the changes in serum carotenoids were minor and possibly clinically unimportant. In addition, the findings of present studies showed that by ensuring the intake of vegetables in the diet, a reduction in carotenoid concentrations induced by plant stanol esters or sterol esters can easily be prevented. It should be noted that although in context of plant sterols much attention has been paid to their effect on serum carotenoids, the clinical importance of β-carotene has diminished due to reports of the harmful effects following β-carotene supplementation (223, 224).

6.8 Adverse effects

According to the laboratory tests no changes in the health status of the subjects in any of the present studies were found. In addition, the recorded gastrointestinal and skin symptoms were slight and occurred occasionally and were not related to any particular experimental or control period or test product. These findings are in accord with the findings of the previous stanol ester or sterol ester studies (4, 5, 31, 34, 71, 72, 76, 225).

Are systemic effects possible? According to the present findings in studies I, III/IV and V and the findings of the other studies in healthy subjects (4, 5, 33, 72, 104) it can be postulated that the absorbed amounts of plant stanols from stanol ester margarines and plant sterols from sterol ester margarines are so small that the systemic effects are most unlikely. In addition, even with the long-term use of stanol ester-enriched margarine, the increase in plant stanol concentrations has been minor (104). There is, however, a specific group, phytosterolemic patients, to whom the consumption of plant sterols and stanols is harmful. In phytosterolemia (119, 125-127) absorption of plant sterols is elevated and thus the serum plant sterol concentrations are very high, about 100 times greater than those detected in study V with sterol ester margarine. In that disease, serum plant stanol concentrations are also enhanced. High concentrations of plant sterol in serum are suggested to have atherogenic potential (134). However, in phytosterolemic heterozygotes, the consumption of plant sterols or stanols in esterified form has recently been reported to increase only slightly serum plant sterol or stanol concentrations, respectively (120, 139). Based on some earlier animal studies (180-182) it has been suggested that plant sterols and stanols may have hormonal effects. However, no relevant effects on serum female sex hormone concentrations in normocholesterolemic or hypercholesterolemic women (73) or women with CAD (70, 188) have been found in clinical studies.

6.9 Clinical implications

Based on the present studies and the other plant stanol ester (4, 5, 34, 47, 64, 68, 71, 72, 77) or sterol ester studies (4, 5, 31, 73) it can be concluded that subjects with mild or moderate hypercholesterolemia and high intestinal absorption rate of cholesterol most likely would benefit from plant stanol ester or sterol ester treatment. However, also subjects with type 2 DM (65, 67) or CAD (70) and children with heterozygous FH (66) have been found to respond favorably to stanol ester treatment. In mildly or moderately hypercholesterolemic subjects serum LDL-C is typically reduced by 10-15%, and a reduction of that magnitude has been proposed to decrease the risk of heart disease by about 25% (89).

Treatment of hypercholesterolemia begins usually with diet therapy. The diet generally recommended for hypercholesterolemic individuals is low in total fat

(≤30E%), and in SAFA (<10E%) and dietary cholesterol <300 mg/d (81, 226). If serum cholesterol concentrations are still elevated after dietary changes, individuals could be advised to replace their usual spreads with stanol ester- or sterol ester-containing spreads. Based on the available data, the daily plant stanol or sterol dose should be about 2 g to achieve the optimal cholesterol-lowering benefit. The daily dose can be taken two to three times per day, but also once per day may be equally effective (77). If the above-mentioned dietary modifications do not normalize the elevated serum cholesterol concentrations, the finally step is cholesterol-lowering drug therapy possibly combined with the dietary stanol esters. The combining of these two has been found to potentiate the cholesterol-lowering effects of drugs such as statins, neomycin and cholestyramine in mildly dyslipidemic men with type 2 DM (67), in women with CAD (70) or adults with FH-NK (75). Another advantage of combining a cholesterol-lowering drug and stanol esters is that the dose of drug can possibly be reduced and thus the risk of side effects associated with higher drug doses can be diminished (227).

The number of stanol esters or sterol esters containing products on the market continues to expand. This increases the need for knowledge about foodstuffs and nutrition in health care, in the food retail trades and in the food industry. The amount of stanol esters or sterol esters in products should be planned so that the dosage would be easy to assess, even when different stanol ester- or sterol ester-containing food products being consumed during the day. The consumers and patients must be counseled on the effective and safe dose of plant stanols or sterols as well as on how to incorporate these food products into their daily diet. In addition, written instructions should be available for all consumers.

7 SUMMARY AND CONCLUSIONS

The aim of the present studies was to investigate the effects of stanol ester and sterol ester margarines on serum lipids and lipoprotein lipids. Furthermore, the dose-response effect of plant stanol esters was examined. Cholesterol-lowering efficacy of plant stanol esters and sterol esters was also compared. In addition, the safety of plant stanol esters or sterol esters was evaluated by measuring the serum concentrations of carotenoids and fat-soluble vitamins, as well as concentrations of plant sterols and plant stanols. Altogether 111 mildly to moderately hypercholesterolemic subjects participated in the three different studies.

The results of the present studies can be summarized as follows:

1. The low-fat margarines enriched with plant stanol esters offered an **additional**, clinically significant reduction (8-14%) in serum TC and LDL-C to that obtained with a cholesterol-lowering diet alone.
2. The margarines enriched with plant stanol esters or sterol esters reduced serum TC and LDL-C concentrations effectively when used as part of **a low-fat, low-cholesterol** background diet.
3. There was no significant difference in the cholesterol-lowering efficacy of two low-fat margarines enriched with **wood- or vegetable oil**-derived plant stanol esters.
4. The margarines enriched with **stanol esters or sterol esters** did not differ in their ability to reduce serum TC and LDL-C concentrations.
5. Plant stanol esters reduced serum TC and LDL-C concentrations in a **dose-dependent** manner. The significant reduction was achieved with the daily stanol dose of 1.6 g. Increasing the daily dose from 2.4 g to 3.2 g did not provide additional cholesterol-lowering effect.
6. Plant stanol esters or sterol esters did not affect **serum fat-soluble vitamins**. The effects of plant stanol esters or sterol esters on **serum carotenoids** were minor and possibly clinically unimportant when plant stanol esters or sterol esters were consumed as part of a cholesterol-lowering diet or a standardized habitual diet and the intake of vegetables was ensured.
7. Plant stanol esters reduced **serum** plant **sterol concentrations** significantly already with a dose 0.8 g/d of stanols, indicating that cholesterol absorption was effectively reduced already with the small stanol ester doses. As judged from the serum Δ7-lathosterol/TC ratio, it is considered that plant stanol esters stimulated cholesterol synthesis. However, the synthesis did not seem to increase further when the daily stanol doses increased from 1.6 g to 2.4 g or 3.2 g. The consumption of plant stanol esters increased serum sitostanol and campestanol concentrations by about twofold, but the concentrations remained extremely low, and they plateaued with doses equal to or greater than the 0.8 g/d.

In conclusion, plant stanol ester- and sterol ester-enriched margarines reduce serum cholesterol concentrations effectively as part of the diet recommended for subjects with elevated serum cholesterol concentrations. Furthermore, the effects on serum carotenoids are minor and possibly clinically unimportant when stanol ester and sterol ester margarines are consumed as part of a recommended and healthy diet.

8 REFERENCES

1. Tang JL, Armitage JM, Lancaster T, Silagy CA, Fowler GH, Neil HA. Systematic review of dietary intervention trials to lower blood total cholesterol in free-living subjects. BMJ 1998;316:1213-1220.
2. Pollak OJ, Kritchevsky D. Sitosterol. Monographs on Atherosclerosis. New York: Basel; 1981.
3. Moghadasian MH, Frohlich JJ. Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. Am J Med 1999;107:588-594.
4. Weststrate JA, Meijer GW. Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. Eur J Clin Nutr 1998;52:334-343.
5. Jones PJ, Raeini-Sarjaz M, Ntanios FY, Vanstone CA, Feng JY, Parsons WE. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. J Lipid Res 2000;41:697-705.
6. IUPAC, The nomenclature of steroids (recommendations 1989), International Union of Pure and Applied Chemistry and International Union of Biochemistry and Molecular Biology [cited 10 August 2000]. Available: http://www.chem.qmw.ac.uk/iupac/steroid.
7. Mead JF, Alfin-Slater RB, Howton DR, Popják G. Biosynthesis of cholesterol and related substances. In: Lipids: chemistry, biochemistry and nutrition. New York: Plenum Press; 1986. p. 295-367.
8. Akihisa T, Kokke W, Tamura T. Naturally occurring sterols and related compounds from plants. In: Patterson GW, Nes WD, editors. Physiology and Biochemistry of Sterols. Champaign, IL: American Oil Chemists' Society; 1991. p. 172-228.
9. Nes WR. Multiple roles for plant sterols. In: Stumpf PK, Mudd JB, Nes WD, editors. The metabolism, structure, and function of plant lipids. New York: Plenum Press; 1987. p. 3-9.
10. Hartmann M-A, Benveniste P. Plant membrane sterols: isolation, identification, and biosynthesis. Methods Enzymol 1987;148:632-650.
11. Salen G, Ahrens EH Jr, Grundy SM. Metabolism of β-sitosterol in man. J Clin Invest 1970;49:952-967.
12. Wojciechowski ZA. Biochemistry of phytosterol conjugates. In: Patterson GW, Nes WD, editors. Physiology and biochemistry of sterols. Champaign, IL: American Oil Chemists' Society; 1991. p. 365-395.
13. Dyas L, Goad LJ. Steryl fatty acyl esters in plants. Phytochemistry 1993;34:17-29.
14. Demel RA, De Kruyff B. The function of sterols in membranes. Biochim Biophys Acta 1976;457:109-132.
15. Jones PJH, MacDougall DE, Ntanios F, Vanstone CA. Dietary phytosterols as cholesterol-lowering agents in humans. Can J Physiol Pharmacol 1997;75:217-227.
16. Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi A-M. Review. Plant sterols: biosynthesis, biological function and their importance to human nutrition. J Sci Food Agric 2000;80:939-966.
17. Jonker D, van der Hoek GD, Glatz JFC, Homan C, Posthumus MA, Katan MB. Combined determination of free, esterified and glycosilated plant sterols in foods. Nutr Rep Int 1985;32:943-951.
18. Toivo J, Lampi A-M, Aalto S, Piironen V. Factors affecting sample preparation in the gas chromatographic determination of plant sterols in whole wheat flour. Food Chem 2000;68:239-245.
19. Dutta PC, Appelqvist L-Å. Saturated sterols (stanols) in unhydrogenated and hydrogenated edible vegetable oils and in cereal lipids. J Sci Food Agric 1996;71:383-391.
20. Kochhar SP. Influence of processing on sterols of edible vegetable oils. Prog Lipid Res 1983;22:161-188.
21. Toivo J, Piironen V, Kalo P, Varo P. Gas chromatographic determination of major sterols in edible oils and fats using solid-phase extraction in sample preparation. Chromatographia 1998;48:745-750.
22. Weihrauch JL, Gardner JM. Sterol content of foods of plant origin. JADA 1978;73:39-47.
23. Trautwein EA, van Leeuwen A, Erbersdobler HF. Plant sterol profiles and squalene concentrations in common unrefined and refined vegetable oils. In: Sandberg A-S, Andersson H, Amadò R, Schlemmer U, Serra F, editors. COST 916. Bioactive inositol phosphates and phytosterols in foods.

Göteborg, Sweden, 23 to 25 October 1997. Luxembourg: Office for Official Publications of the European Communities; 1999. p. 79-82.
24. Ferrari RA, Schulte E, Esteves W, Brühl L, Mukherjee KD. Minor constituents of vegetable oils during industrial processing. J Am Oil Chem Soc 1996;73:587-592.
25. Salminen R. Kasvisterolit sekä kolesteroli ravintorasvoissa. Master's thesis. Helsinki: University of Helsinki; 1997.
26. Piironen V, Toivo J, Lampi A-M. Natural sources of dietary plant sterols. J Food Comp Anal 2000;13:619-624.
27. Määttä K, Lampi A-M, Petterson J, Fogelfors BM, Piironen V, Kamal-Eldin A. Phytosterol content in seven oat cultivars grown at three locations in Sweden. J Sci Food Agric 1999;79:1021-1027.
28. Seitz LM. Stanol and sterol esters of ferulic and p-coumaric acids in wheat, corn, rye, and Triticale. J Agric Food chem 1989;37:662-667.
29. Normén L, Johnsson M, Andersson H, van Gameren Y, Dutta P. Plant sterols in vegetables and fruits commonly consumed in Sweden. Eur J Nutr 1999;38:84-89.
30. USDA Nutrient Database for Standard Reference, Release 13. SR13 Page reports [cited 15 August 2000]. Available: http://www.nal.usda.gov/fnic/foodcomp/Data/SR13/reports/sr13page.htm.
31. Hendriks HFJ, Weststrate JA, van Vlient T, Meijer GW. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. Eur J Clin Nutr 1999;53:319-327.
32. Cater NB. Historical and scientific basis for the development of plant stanol ester foods as cholesterol-lowering agents. Eur Heart J Supplements 1999;1 (Suppl S):S36-S44.
33. Gylling H, Miettinen T. Cholesterol reduction by different plant stanol mixtures and with variable fat intake. Metabolism 1999;48:575-580.
34. Plat J, Mensink RP. Vegetable oil based versus wood based stanol ester mixtures: effects on serum lipids and hemostatic factors in non-hypercholesterolemic subjects. Atherosclerosis 2000;148:101-112.
35. Ling WH, Jones PJH. Enhanced efficacy of sitostanol-containing versus sitostanol-free phytosterol mixtures in altering lipoprotein cholesterol levels and synthesis in rats. Atherosclerosis 1995;118:319-31.
36. Jones PJH, Howell T, MacDougall DE, Feng JY, Parsons W. Short-term administration of tall oil phytosterols improves plasma lipid profiles in subjects with different cholesterol levels. Metabolism 1998;47:751-756.
37. Wester I. Cholesterol-lowering effect of plant sterols. Eur J Lipid Sci Technol 2000:37-44.
38. Morton GM, Lee SM, Buss DH, Lawrence P. Intakes and major dietary sources of cholesterol and phytosterols in the British diet. J Human Nutr Dietet 1995;8:429-440.
39. de Vries JHM, Jansen A, Kromhout D, et al. The fatty acid and sterol content of food composites of middle-aged men in seven countries. J Food Comp Anal 1997;10:115-141.
40. Phillips KM, Tarragó-Trani MT, Stewart KK. Phytosterol content of experimental diets differing in fatty acid composition. Food Chem 1999;64:415-422.
41. Schothorst RC, Jekel AA. Oral sterol intake in the Netherlands: evaluation of the results obtained by GC analysis of duplicate 24-h diet samples collected in 1994. Food Chem 1999;64:561-566.
42. Hirai k, Shimazu C, Takezoe R, Ozeki Y. Cholesterol, phytosterol and polyunsaturated fatty acid levels in 1982 and 1957 Japanese diets. J Nutr Sci Vitaminol 1986;32:363-372.
43. Czubayko F, Beumers B, Lammsfuss S, Lütjohann D, von Bergmann K. A simplified micro-method for quantification of fecal excretion of neutral and asidic sterols for outpatient studies in humans. J Lipid Res 1991;32:1861-1867.
44. Vuoristo M, Miettinen TA. Absorption, metabolism, and serum concentrations of cholesterol in vegetarians: effects of cholesterol feeding. Am J Clin Nutr 1994;59:1325-1331.
45. Nair PP, Turjman N, Kessie G, et al. Diet, nutrition intake, and metabolism in populations at high and low risk for colon cancer. Dietary cholesterol, β-sitosterol, and stigmasterol. Am J Clin Nutr 1984;40:927-930.
46. Armstrong MJ, Carey MC. Thermodynamic and molecular determinants of sterol solubilities in bile salt micelles. J Lipid Res 1987;28:1144-1155.
47. Vanhanen HT, Blomqvist S, Ehnholm C, et al. 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.
48. Peterson DW. Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. Proc Nutr Soc 1951;78:143-147.
49. Pollak OJ. Prevention of hypercholesterolemia in the rabbit: successful prevention of cholesterol atherosclerosis. Reduction of blood cholesterol in man. Circulation 1952;VII:459.
50. Pollak OJ. Effect of plant sterols on serum lipids and atherosclerosis. Pharmac Ther 1985;31:177-208.
51. Pollak OJ. Reduction of blood cholesterol in man. Circulation 1953;VII:702-706.
52. Lees RS, Lees AM. Effects of sitosterol therapy on plasma lipid and lipoprotein concentrations. In: Greten H, editor. Lipoprotein metabolism. Berlin: Springer; 1976. p. 119-124.
53. Lees AM, Mok HYI, Lees RS, McCluskey MA, Grundy SM. Plant sterols as cholesterol-lowering agents: clinical trials in patients with hypercholesterolemia and studies of sterol balance. Atherosclerosis 1977;28:325-338.
54. Miettinen TA, Vanhanen HT. Dietary sitostanol related to absorption, synthesis and serum level of cholesterol in different apolipoprotein E phenotypes. Atherosclerosis 1994;105:217-226.
55. Vanhanen HT, Miettinen TA. Effects of unsaturated and saturated dietary plants sterols on their serum contents. Clin Chim Acta 1992;205:97-107.
56. Sierksma A, Weststrate JA, Meijer GW. Spreads enriched with plant sterols, either esterified 4,4-dimethylsterols or free 4-desmethylsterols, and plasma total- and LDL-cholesterol concentrations. Brit J Nutr 1999;82:273-282.
57. Pelletier X, Belbraouet S, Mirabel D, et al. A diet moderately enriched in phytosterols lowers plasma cholesterol concentrations in normocholesterolemic humans. Ann Nutr Metab 1995;39:291-295.
58. Ikeda I, Kawasaki A, Samezima K, Sugano M. Antihypercholesterolemic activity of β-sitostanol in rabbits. J Nutr Sci Vitaminol 1981;27:243-251.
59. Ikeda I, Sugano M. Comparison of absorption and metabolism of β-sitosterol and β-sitostanol in rats. Atherosclerosis 1978;30:227-237.
60. Sugano M, Kamo F, Ikeda I, Morioka H. Lipid-lowering activity of phytostanols in rats. Atherosclerosis 1976;24:301-309.
61. Sugano M, Morioka H, Ikeda I. A comparison of hypocholesterolemic activity of β-sitosterol and β-sitostanol in rats. J Nutr 1977;107:2011-2019.
62. Heinemann T, Leiss O, von Bergmann K. Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. Atherosclerosis 1986;61:219-223.
63. 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.
64. Vanhanen HT, Kajander J, Lehtovirta H, Miettinen TA. Serum levels, absorption efficiency, faecal elimination and synthesis of cholesterol during increasing doses of dietary sitostanol esters in hypercholesterolaemic subjects. Clin Sci 1994;87:61-67.
65. Gylling H, Miettinen TA. Serum cholesterol and cholesterol and lipoprotein metabolism in hypercholesterolaemic NIDDM patients before and during sitostanol ester-margarine treatment. Diabetologia 1994;37:773-780.
66. Gylling H, Siimes M, Miettinen TA. Sitostanol ester margarine in dietary treatment of children with familial hypercholesterolemia. J Lipid Res 1995;36:1807-1812.
67. Gylling H, Miettinen TA. Effects of inhibiting cholesterol absorption and synthesis on cholesterol and lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic men. J Lipid Res 1996;37:1776-1785.
68. Miettinen TA, Puska P, Gylling H, Vanhanen H, Vartiainen E. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. N Eng J Med 1995;333:1308-1312.
69. Niinikoski H, Viikari J, Palmu T. Cholesterol-lowering effect and sensory properties of sitostanol ester margarine in normocholesterolemic adults. Scand J Nutr 1997;41:9-12.
70. Gylling H, Radhakrishnan R, Miettinen TA. Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary

sitostanol ester margarine. Women and dietary sitostanöl. Circulation 1997;96:4226-4231.
71. Andersson A, Karlström B, Mohsen R, Vessby B. Cholesterol-lowering effects of a stanol ester-containing low-fat margarine used in conjunction with a strict lipid-lowering diet. Eur Heart J Supplements 1999;1 (Suppl S):S80-S90.
72. Nguyen TT, Dale LC, von Bergmann K, Croghan IT. Cholesterol-lowering effect of stanol ester in a US population of mildly hypercholesterolemic men and women: a randomized controlled trial. Mayo Clin Proc 1999;74:1198-1206.
73. Ayesh R, Weststrate JA, Drewitt PN, Hepburn PA. Safety evaluation of phytosterol esters. Part 5. Faecal short-chain fatty acid and microflora content, faecal bacterial enzyme activity and serum female sex hormones in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester- enriched margarine. Food Chem Toxicol 1999;37:1127-1138.
74. Williams CL, Bollella MC, Strobino BA, Boccia L, Campanaro L. Plant stanol ester and bran fiber in childhood: effects on lipids, stool weight and stool frequency in preschool children. J Am Coll Nutr 1999;18:572-581.
75. Vuorio AF, Gylling H, Turtola H, Kontula K, Ketonen P, Miettinen TA. Stanol ester margarine alone and with simvastatin lowers serum cholesterol in families with familial hypercholesterolemia caused by the FH-North Karelia mutation. Arterioscler Thromb Vasc Biol 2000;20:500-506.
76. Tammi A, Rönnemaa T, Gylling H, et al. Plant stanol ester margarine lowers serum total and low-density lipoprotein cholesterol concentrations of healthy children: the STRIP project. J Pediatr 2000;136:503-510.
77. Plat J, van Onselen ENM, van Heugten MMA, Mensink RP. Effects on serum lipids, lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines and shortenings enriched with plant stanol esters. Eur J Clin Nutr 2000;54:671-677.
78. Miettinen TA, Vuoristo M, Nissinen M, Järvinen HJ, Gylling H. Serum, biliary, and fecal cholesterol and plant sterols in colectomized patients before and during consumption of stanol ester margarine. Am J Clin Nutr 2000;71:1095-1102.
79. Relas H, Gylling H, Miettinen TA. Effect of stanol ester on postabsorptive squalene and retinyl palmitate. Metabolism 2000;49:473-478.
80. Becker M, Staab D, von Bergmann K. Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. J Pediatr 1993;122:292-296.
81. National Cholesterol Education Program. Second report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel II). Circulation 1994;89:1329-1445.
82. Beveridge JMR, Haust HL, Connell WF. Magnitude of the hypercholesterolemic effect of dietary sitosterol in man. J Nutr 1964;83:119-122.
83. Wester I. Dose responsiveness to plant stanol esters. Eur Heart J Supplements 1999;1 (Suppl S):S104-S108.
84. Nguyen TT. The cholesterol-lowering action of plant stanol esters. J Nutr 1999;129:2109-2112.
85. Farquhar JW, Smith RE, Dempsey ME. The effect of beta sitosterol on the serum lipids of young men with arteriosclerotic heart disease. Circulation 1956;XIV:77-82.
86. Jones PJH, Ntanios FY, Raeini-Sarjaz M, Vanstone CA. Cholesterol-lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men. Am J Clin Nutr 1999;69:1144-1150.
87. Kreisberg RA, Kasim S. Cholesterol metabolism and aging. Am J Med 1987;82:54-60.
88. Schlierf G, Oster P, Heuck CC, Raetzer H, Schellenberg B. Sitosterol in juvenile type II hyperlipoproteinemia. Atherosclerosis 1978;30:245-248.
89. Law M. Plant sterol and stanol margarines and health. BMJ 2000;320:861-864.
90. Austin MA, Breslow JL, Hennekens CH, Buring JE, Willett WC, Krauss RM. Low-density lipoprotein subclass patterns and risk of myocardial infarction. JAMA 1988;260:1917-1921.
91. Crouse JR, Parks JS, Schey HM, Kahl FR. Studies of low density lipoprotein molecular weight in human beings with coronary artery disease. J Lipid Res 1985;26:566-574.
92. Becker M, Staab D, von Bergmann K. Long-term treatment of severe familial hypercholesterolemia in children: effect of sitosterol and bezafibrate. Pediatrics 1992;89:138-142.
93. Mattson GH, Volpenhein RA, Erickson BA. Effect of plant sterol esters on the absorption of dietary

cholesterol. J Nutr 1977;107:1139-1146.
94. Mattson FH, Grundy SM, Crouse JR. Optimizing the effect of plant sterols on cholesterol absorption in man. Am J Clin Nutr 1982;35:697-700.
95. Blomqvist SM, Jauhiainen M, van Tol A, et al. Effect of sitostanol ester on composition and size distribution of low- and high-density lipoprotein. Nutr Metab Cardiovasc Dis 1993;3:158-164.
96. Kudchodkar BJ, Horlick L, Sodhi HS. Effects of plant sterols on cholesterol metabolism in man. Atherosclerosis 1976;23:239-248.
97. Grundy SM. Cholesterol and atherosclerosis: diagnosis and treatment. New York: Gower Medical Publishing; 1990.
98. Weisweiler P, Heinemann V, Schwandt P. Serum lipoproteins and lecithin:cholesterol acyltransferase (LCAT) activity in hypercholesterolemic subjects given β-sitosterol. Int J Clin Pharmacol Ther Toxicol 1984;22:204-206.
99. Turley SD. Dietary cholesterol and the mechanisms of cholesterol absorption. Eur Heart J Supplements 1999;1 (Suppl S):S29-S35.
100. Bhattacharyya AK. Uptake and esterification of plant sterols by rat small intestine. Am J Physiol 1981;240:G50-G55.
101. Sylvén C. Influence of blood supply on lipid uptake from micellar solutions by the rat small intestine. Biochim Biophys Acta 1970;203:365-375.
102. Sylvén C, Nordström C. The site of absorption of cholesterol and sitosterol in the rat small intestine. Scand J Gastroenterol 1970;5:57-63.
103. Miettinen TA, Tilvis RS, Kesäniemi 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.
104. Gylling H, Puska P, Vartiainen E, Miettinen TA. Serum sterols during stanol ester feeding in a mildly hypercholesterolemic population. J Lipid Res 1999;40:593-600.
105. Tilvis RS, Miettinen TA. Serum plant sterols and their relation to cholesterol absorption. Am J Clin Nutr 1986;43:92-97.
106. von Bergmann K, Prange W, Lütjohann D. Metabolism and mechanism of action of plant sterols. Eur Heart J Supplements 1999;1 (Suppl S):S45-S49.
107. Hassan AS, Rampone AJ. Intestinal absorption and lymphatic transport of cholesterol and β-sitostanol in the rat. J Lipid Res 1979;20:646-653.
108. Lütjohann D, Meese CO, Crouse JR, von Bergmann K. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. J Lipid Res 1993;34:1039-1046.
109. Heinemann T, Axtmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. Eur J Clin Invest 1993;23:827-831.
110. Vahouny GV, Connor WE, Subramaniam S, Lin DS, Gallo LL. Comparative lymphatic absorption of sitosterol, stigmasterol, and fucosterol and differential inhibition of cholesterol absorption. Am J Clin Nutr 1983;37:805-809.
111. Slota T, Kozlov NA, Ammon HV. Comparison of cholesterol and β-sitosterol: effects on jejunal fluid secretion induced by oleate, and absorption from mixed micellar solutions. Gut 1983;24:653-658.
112. Ikeda I, Tanaka K, Sugano M, Vahouny GV, Gallo LL. Discrimination between cholesterol and sitosterol for absorption in rats. J Lipid Res 1988;29:1583-1591.
113. Sato Y, Nishikawa K, Aikawa K, et al. Side-chain structure is critical for the transport of sterols from lysosomes to cytoplasm. Biochim Biophys Acta 1995;1257:38-46.
114. Field FJ, Mathur SN. β-sitosterol: esterification by intestinal acylcoenzyme A:cholesterol acyltransferase (ACAT) and its effect on cholesterol esterification. J Lipid Res 1983;24:409-417.
115. Compassi S, Werder M, Weber FE, Boffelli D, Hauser H, Schulthess G. Comparison of cholesterol and sitosterol uptake in different brush border membrane models. Biochem 1997;36:6643-6652.
116. Tavani DM, Nes WR, Billheimer JT. The sterol substrate specificity of acyl CoA:cholesterol acyltransferase from rat liver. J Lipid Res 1982;23:774-781.
117. Child P, 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.

118. Bhattacharyya AK. Differences in uptake and esterification of saturated analogues of cholesterol by rat small intestine. Am J Physiol 1986;251:G495-500.
119. Lütjohann D, Björkhem I, Beil UF, von Bergmann K. Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment. J Lipid Res 1995;36:1763-1773.
120. Salen G, Xu G, Tint GS, Batta AK, Shefer S. Hyperabsorption and retention of campestanol in a sitosterolemic homozygote: comparison with her mother and three control subjects. J Lipid Res 2000;41:1883-1889.
121. Sarkkinen ES, Uusitupa MIJ, Gylling H, Miettinen TA. Fat-modified diets influence serum concentrations of cholesterol precursors and plant sterols in hypercholesterolemic subjects. Metabolism 1998;47:744-750.
122. Miettinen TA, Vanhanen H. Serum concentration and metabolism of cholesterol during rapeseed oil and squalene feeding. Am J Clin Nutr 1994;59:356-363.
123. Mellies M, Glueck CJ, Sweeney C, Fallat RW, Tsang RC, Ishikawa TT. Plasma and dietary phytosterols in children. Pediatrics 1976;57:60-67.
124. Xu G, Salen G, Tint GS, Batta AK, Shefer S. Campestanol (24-methyl-5α-cholestan-3β-ol) absorption and distribution in New Zealand white rabbits: effect of dietary sitostanol. Metabolism 1999;48:363-368.
125. Bhattacharyya AK, Connor WE. β–sitosterolemia and xanthomatosis. A newly described lipid storage disease in two sisters. J Clin Invest 1974;53:1033-1043.
126. Salen G, Shore V, Tint GS, et al. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. J Lipid Res 1989;30:1319-1330.
127. Miettinen TA. Phytosterolaemia, xanthomatosis and premature atherosclerotic arterial disease: a case with high plant sterol absorption, impaired sterol elimination and low cholesterol synthesis. Eur J Clin Invest 1980;10:27-35.
128. Lütjohann D, von Bergmann K. Phytosterolaemia: diagnosis, characterization and therapeutical approaches. Ann Med 1997;29:181-184.
129. Salen G, Shefer S, Nguyen L, Ness GC, Tint GS, Shore V. Sitosterolemia. J Lipid Res 1992;33:945-955.
130. Salen G, Kwiterovich POJ, Shefer S, et al. Increased plasma cholestanol and 5α-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis. J Lipid Res 1985;26:203-209.
131. Lin HJ, Wang C, Salen G, Lam KC, Chan TK. Sitosterol and cholesterol metabolism in a patient with coexisting phytosterolemia and cholestanolemia. Metabolism 1983;32:126-133.
132. Salen G, Horak I, Rothkopf M, et al. Lethal atherosclerosis associated with abnormal plasma and tissue sterol composition in sitosterolemia with xanthomatosis. J Lipid Res 1985;26:1126-1133.
133. Skrede B, Björkhem I, Bergesen Ø, Kayden HJ, Skrede S. The presence of 5α-sitostanol in the serum of a patient with phytosterolemia, and its biosynthesis from plant steroids in rats with bile fistula. Biochim Biophys Acta 1985;836:368-375.
134. Glueck CJ, Speirs J, Tracy T, Streicher P, Illig E, Vandegrift J. Relationships of serum plant sterols (phytosterols) and cholesterol in 595 hypercholesterolemic subjects, and familial aggregation of phytosterols, cholesterol, and premature coronary heart disease in hyperphytosterolemic probands and their first-degree relatives. Metabolism 1991;40:842-848.
135. Nguyen LB, Salen G, Shefer S, Tint GS, Shore V, Ness GC. Decreased cholesterol biosynthesis in sitosterolemia with xanthomatosis: diminished mononuclear leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and enzyme protein associated with increased low-density lipoprotein receptor function. Metabolism 1990;39:436-443.
136. Bhattacharyya AK, Connor WE, Lin DS, McMurry MM, Shulman RS. Sluggish sitosterol turnover and hepatic failure to excrete sitosterol into bile cause expansion of body pool of sitosterol in patients with sitosterolemia and xanthomatosis. Arterioscler Thromb 1991;11:1287-1294.
137. Salen G, Tint GS, Shefer S, Shore V, Nguyen L. Increased sitosterol absorption is offset by rapid elimination to prevent accumulation in heterozygotes with sitosterolemia. Arterioscler Thromb 1992;12:563-568.
138. Hidaka H, Nakamura T, Aoki T, et al. Increased plasma plant sterol levels in heterozygotes with

sitosterolemia and xanthomatosis. J Lipid Res 1990;31:881-888.
139. Stalenhoef AF, Hectors M, Demacker PN. Effect of plant sterol-enriched margarine on plasma lipids and sterols in subjects heterozygous for phytosterolaemia. J Intern Med 2001;249:163-166.
140. Subbiah MTR, Kuksis A. Differences in metabolism of cholesterol and sitosterol following intravenous injection in rats. Biochim Biophys Acta 1973;306:95-105.
141. Dobiášová M. Lecithin:cholesterol acyltransferase and the regulation of endogenous cholesterol transport. Adv Lipid Res 1983;20:107-194.
142. Bhattacharyya AK, Lopez LA. Absorbability of plant sterols and their distribution in rabbit tissues. Biochim Biophys Acta 1979;574:146-153.
143. Boberg KM, Skrede B, Skrede S. Metabolism of 24-ethyl-4-cholesten-3-one and 24-ethyl-5-cholesten-3β-ol (sitosterol) after intraperitoneal injection in the rat. Scand J Clin Lab Invest Suppl 1986;184:47-54.
144. Sanders DJ, Minter HJ, Howes D, Hepburn PA. The safety evaluation of phytosterol esters. Part 6. The comparative absorption and tissue distribution of phytosterols in the rat. Food Chem Toxicol 2000;38:485-491.
145. Gould RG, Jones RJ, LeRoy GV, Wissler RW, Taylor CB. Absorbability of β-sitosterol in humans. Metabolism 1969;18:652-662.
146. Boberg KM, Einarsson K, Björkhem I. Apparent lack of conversion of sitosterol into C_{24}-bile acids in humans. J Lipid Res 1990;31:1083-1088.
147. McNamara DJ, Proia A, Miettinen TA. Thin-layer and gas-liquid chromatographic identification of neutral steroids in human and rat feces. J Lipid Res 1981;22:474-484.
148. Miettinen TA, Gylling H. Serum cholesterol lowering properties of plant sterols. In: Sandberg A-S, Andersson H, Amadò R, Schlemmer U, Serra F, editors. COST 916. Bioactive inositol phosphates and phytosterols in foods. Göteborg, Sweden, 23 to 25 October 1997. Luxembourg: Office for Official Publications of the European Communities; 1999. p. 85-91.
149. Bhattacharyya AK, Connor WE, Lin DS. The origin of plant sterols in the skin surface lipids in humans: from diet to plasma to skin. J Invest Dermatol 1983;80:294-296.
150. Miettinen TA. Detection of changes in human cholesterol metabolism. Ann Clin Res 1970;2:300-320.
151. Björkhem I, Miettinen T, Reihnér E, Ewerth S, Angelin B, Einarsson K. Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver. J Lipid Res 1987;28:1137-1143.
152. Kempen HJ, Glatz JF, Gevers Leuven JA, van der Voort HA, Katan MB. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. J Lipid Res 1988;29:1149-1155.
153. Miettinen TA, Tilvis RS, Kesäniemi YA. Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. Metabolism 1989;38:136-140.
154. Ntanios FY, Jones PJH. Dietary sitostanol reciprocally influences cholesterol absorption and biosynthesis in hamsters and rabbits. Atherosclerosis 1999;143:341-351.
155. Ling WH, Jones PJH. Dietary phytosterols: a review of metabolism, benefits and side effects. Life Sci 1995;57:195-206.
156. Miettinen TA, Gylling H. Sitostanol-ester margarine. In: Yalpani M, editor. New technologies for healthy foods & nutraceuticals. Westborough, MA: ATL Press, Inc. Science Publishers; 1997. p. 71-83.
157. Ikeda I, Tanaka K, Sugano M, Vahouny GV, Gallo LL. Inhibition of cholesterol absorption in rats by plant sterols. J Lipid Res 1988;29:1573-1582.
158. Ikeda I, Sugano M. Some aspects of mechanism of inhibition of cholesterol absorption by β-sitosterol. Biochim Biophys Acta 1983;732:651-658.
159. Compassi S, Werder M, Boffelli D, Weber FE, Hauser H, Schulthess G. Cholesteryl ester absorption by small intestinal brush border membrane is protein-mediated. Biochem 1995;34:16473-16482.
160. Thurnhofer H, Hauser H. Uptake of cholesterol by small intestinal brush border membrane is protein-mediated. Biochem 1990;29:2142-2148.

161. Schulthess G, Compassi S, Boffelli D, Werder M, Weber FE, Hauser H: A comparative study of sterol absorption in different small-intestinal brush border membrane models. J Lipid Res 1996;37:2405-2419.
162. Boffelli D, Weber FE, Compassi S, Werder M, Schulthess G, Hauser H. Reconstitution and further characterization of the cholesterol transport activity of the small-intestinal brush border membrane. Biochem 1997;36:10784-10792.
163. Hauser H, Dyer JH, Nandy A, et al. Identification of a receptor mediating absorption of dietary cholesterol in the intestine. Biochem 1998;37:17843-17850.
164. Field FJ, Born E, Mathur SN. Effect of micellar β-sitosterol on cholesterol metabolism in CaCo-2 cells. J Lipid Res 1997;38:348-360.
165. Child P, Kuksis A. Uptake and transport of sterols by isolated villus cells of rat jejunum. Can J Biochem 1980;58:1215-1222.
166. Heinemann T, Kullak-Ublick G-A, Pietruck B, von Bergmann K. Mechanisms of action of plant sterols on inhibition of cholesterol absorption. Eur J Clin Pharmacol 1991;40 (Suppl. 1):S59-S63.
167. Ikeda I, Tanabe Y, Sugano M. Effects of sitosterol and sitostanol on micellar solubility of cholesterol. J Nutr Sci Vitaminol 1989;35:361-369.
168. Normén L, Dutta P, Lia Å, Andersson H. Soy sterol esters and β-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. Am J Clin Nutr 2000;71:908-913.
169. Grundy SM, Mok HY. Determination of cholesterol absorption in man by intestinal perfusion. J Lipid Res 1977;18:263-271.
170. Ostlund RE Jr., Spilburg CA, Stenson WF. Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. Am J Clin Nutr 1999;70:826-831.
171. Best MM, Duncan CH. Effects of the esterification of supplemental cholesterol and sitosterol in the diet. J Nutr 1958;65:169-181.
172. Malini T, Vanithakumari G. Rat toxicity studies with β-sitosterol. J Ethnopharmacol 1990;28:221-234.
173. Boberg KM, Åkerlund JE, Björkhem I. Effect of sitosterol on the rate-limiting enzymes in cholesterol synthesis and degradation. Lipids 1989;24:9-12.
174. Shefer S, Salen G, Bullock J, et al. The effect of increased hepatic sitosterol on the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and cholesterol 7α-hydroxylase in the rat and sitosterolemic homozygotes. Hepatol 1994;20:213-219.
175. Kakis G, Kuksis A. Effect of intravenous infusion of Intralipid, cholesterol, and plant sterols on hepatic cholesterogenesis. Can J Biochem Cell Biol 1984;62:1-10.
176. Raicht RF, Cohen BI, Shefer S, Mosbach EH. Sterol balance studies in the rat. Effects of dietary cholesterol and β-sitosterol on sterol balance and rate-limiting enzymes of sterol metabolism. Biochim Biophys Acta 1975;388:374-384.
177. Ide T, Gotoh Y, Sugano M. Dietary regulation of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase and cholesterol synthetic activities in fasted-refed rats. J Nutr 1980;110:158-168.
178. Gylling H, Puska P, Vartiainen E, Miettinen TA. Retinol, vitamin D, carotenes, and α-tocopherol in serum of a moderately hypercholesterolemic population consuming sitostanol ester margarine. Atherosclerosis 1999;145:279-285.
179. Nguyen TT, Dale LC. Plant stanol esters and vitamin K. Mayo Clin Proc 1999;74:642-643.
180. Malini T, Vanithakumari G. Antifertility effects of β-sitosterol in male albino rats. J Ethnopharmacol 1991;35:149-153.
181. MacLatchy DL, Van Der Kraak GJ. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol 1995;134:305-312.
182. Mellanen P, Petänen T, Lehtimäki J, et al. Wood-derived estrogens: Studies in vitro with breast cancer cell lines and in vivo in trout. Toxicol Appl Pharmacol 1996;136:381-388.
183. Turnbull D, Frankos VH, Leeman WR, Jonker D. Short-term tests of estrogenic potential of plant stanols and plant stanol esters. Regul Toxicol Pharmacol 1999;29:211-215.
184. Baker VA, Hepburn PA, Kennedy SJ, et al. Safety evaluation of phytosterol esters. Part 1. Assessment of oestrogenicity using a combination of in vivo and in vitro assays. Food Chem Toxicol 1999;37:13-22.
185. Whittaker MH, Frankos VH, Wolterbeek APM, Waalkens-Berendsen DH. Two-generation

reproductive toxicity study of plant stanol esters in rats. Regul Toxicol Pharmacol 1999;29:196-204.
186. Waalkens-Berendsen DH, Wolterbeek AP, Wijnands MVW, Richold M, Hepburn PA. Safety evaluation of phytosterol esters. Part 3. Two-generation reproduction study in rats with phytosterol esters-a novel functional food. Food Chem Toxicol 1999;37:683-696.
187. Slesinski RS, Turnbull D, Frankos VH, Wolterbeek APM, Waalkens-Berendsen DH. Developmental toxicity study of vegetable oil-derived stanol fatty acid esters. Regul Toxicol Pharmacol 1999;29:227-233.
188. Gylling H, Miettinen TA. Efficacy of plant stanol ester in lowering cholesterol in postmenopausal women and patients with diabetes. In: Nguyen TT, editor. New developments in the dietary management of high cholesterol. Postgraduate Medicine Special Report. Minneapolis, MN: McGraw-Hill Healthcare Information group; 1998. p. 39-43.
189. Wilt T, Ishani A, MacDonald R, Stark G, Mulrow C, Lau J. Beta-sitosterols for benign prostatic hyperplasia (Cochrane review). In The Cochrane Library, Issue 2, 2001. Oxford: Update Software.
190. Weststrate JA, Ayesh R, Bauer-Plank C, Drewitt PN. Safety evaluation of phytosterol esters. Part 4. Faecal concentrations of bile acids and neutral sterols in healthy normolipidaemic volunteers consuming a controlled diet either with or without a phytosterol ester-enriched margarine. Food Chem Toxicol 1999;37:1063-1071.
191. Alpers DH, Clouse RE, Stenson WF. Protein and Calorie Requirements. In: Manual of nutritional therapeutics. Toronto: Little, Brown and Company; 1986. p. 131-159.
192. Haapa E, Toponen T, Pietinen P, Räsänen L. Annoskuvakirja (in Finnish). Helsinki: National Public Health Institute; 1985.
193. Rastas M, Seppänen R, Knuts L-R, Karvetti R-L, Varo P. Nutrient composition of foods. Helsinki: Publications of the Social Insurance Institution; 1993.
194. Cullinane EM, Yurgalevitch SM, Saritelli AL, Herbert PN, Thompson PD. Variations in plasma volume affect total and low-density lipoprotein cholesterol concentrations during the menstrual cycle. Metabolism 1995;44:965-971.
195. Ågren JJ, Julkunen A, Penttilä I. Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column. J Lipid Res 1992;33:1871-1876.
196. Penttilä IM, Voutilainen E, Laitinen O, Juutilainen P. Comparison of different analytical and precipitation methods for the direct estimation of high-density lipoprotein cholesterol. Scand J Clin Lab Invest 1981;41:353-360.
197. Fruchart JC, Kora I, Cachera C, Clavey V, Duthilleul P, Moschetto Y. Simultaneous measurement of plasma apolipoproteins A-I and B by electroimmunoassay. Clin Chem 1982;28:59-62.
198. Miettinen TA. Cholesterol metabolism during ketoconazole treatment in man. J Lipid Res 1988;29:43-51.
199. Miettinen TA, Koivisto P. Non-cholesterol sterols and bile acid production in hypercholesterolaemic patients with ileal bypass. In: Paumgartner G, Stiehl A, Gerok W, editors. Bile acid and cholesterol in health and disease. Lancaster: MTP Press; 1983. p. 183-187.
200. Driskell WJ, Bashor MM, Neese JW. Beta-carotene determined in serum by liquid chromatography with an internal standard. Clin Chem 1983;29:1042-1044.
201. Kaplan LA, Miller JA, Stein EA. Simultaneous measurement of serum retinol, tocopherols, carotenes, and carotenoids by high performance liquid chromatography. J Clin Lab Anal 1987;1:147-152.
202. Parviainen MT. The fat-soluble vitamins A, D and E - their metabolism, binding-proteins and determination from human serum. Doctoral thesis. Tampere: University of Tampere; 1983.
203. Tsukamoto K, Watanabe T, Matsushima T, et al. Determination by PCR-RFLP of apo E genotype in a Japanese population. J Lab Clin Med 1993;121:598-602.
204. Norusis MJ. SPSS for Windows Base System User's Guide. Release 6.0. Chicago, IL: SPSS; 1993.
205. SPSS® Base® 7.5 for Windows®. User's Guide. Chigaco: SPSS Inc.; 1997.
206. SPSS Advanced Statistics™ 7.5. User's Guide. Chicago: SPSS Inc.; 1997.
207. Sandström B. Review: Quality criteria in human experimental nutrition research. Eur J Clin Nutr 1995;49:315-322.

208. Jackson B, Dujovne CA, DeCoursey S, Beyer P, Brown EF, Hassanein K. Methods to assess relative reliability of diet records: minimum records for monitoring lipid and caloric intake. JADA 1986;86:1531-1535.
209. Buzzard M. 24-hour dietary recall and food record methods. In: Willett W, editor. Nutritional Epidemiology. 2 ed. New York: Oxford University Press; 1998. p. 50-73.
210. Bingham SH, Nelson M, Paul AA, Haraldsdottir J, Løken EB, van Staveren WA. Methods for data collection at an individual level. In: Cameron ME, van Staveren WA, editors. Manual on methodology for food comsumption studies. New York: Oxford University Press; 1988. p. 53-106.
211. Worsley A, Baghurst KI, Leitch DR. Social desirability response bias and dietary inventory responses. Hum Nutr Appl Nutr 1984;38:29-35.
212. Kristal AR, Andrilla CHA, Koepsell TD, Diehr PH, Cheadle A. Dietary assessment instruments are susceptible to intervention-associated response set bias. JADA 1998;98:40-43.
213. Hunter D. Biochemical indicators of dietary intake. In: Willet W, editor. Nutritional epidemiology. 2 ed. New York: Oxford University Press; 1998. p. 174-243.
214. Glatz JF, Soffers AE, Katan MB. Fatty acid composition of serum cholesteryl esters and erythrocyte membranes as indicators of linoleic acid intake in man. Am J Clin Nutr 1989;49:269-276.
215. Pyörälä K, De Backer G, Graham I, Poole-Wilson P, Wood D on behalf of the Task Force. Prevention of coronary heart disease in clinical practice. Recommendations of the Task Force of the European Society of Cardiology, European Atherosclerosis Society and European Society of Hypertension. Eur Heart J 1994;15:1300-1331.
216. Watts GF, Burke V. Lipid-lowering trials in the primary and secondary prevention of coronary heart disease: new evidence, implications and outstanding issues. Curr Opin Lipidol 1996;7:341-355.
217. Durrington PN. Biological variation in serum lipid concentrations. Scand J Clin Lab Invest Suppl 1990;198:86-91.
218. Garde AH, Hansen ÅM, Skovgaard LT, Christensen JM. Seasonal and biological variation of blood concentrations of total cholesterol, dehydroepiandrosterone sulfate, hemoglobin A_{1c}, IgA, prolactin, and free testosterone in healthy women. Clin Chem 2000;46:551-559.
219. Clevidence BA, Bieri JG. Association of carotenoids with human plasma lipoproteins. Methods Enzymol 1993;214:33-46.
220. Ogihara T, Miki M, Kitagawa M, Mino M. Distribution of tocopherol among human plasma lipoproteins. Clin Chim Acta 1988;174:299-305.
221. Behrens WA, Thompson JN, Madere R. Distribution of α-tocopherol in human plasma lipoproteins. Am J Clin Nutr 1982;35:691-696.
222. Vogel S, Contois JH, Tucker KL, Wilson PW, Schaefer EJ, Lammi-Keefe CJ. Plasma retinol and plasma and lipoprotein tocopherol and carotenoid concentrations in healthy elderly participants of the Framingham Heart Study. Am J Clin Nutr 1997;66:950-958.
223. Omenn GS, Goodman GE, Thornquist MD, et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Eng J Med 1996;334:1150-1155.
224. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. N Eng J Med 1994;330:1029-1035.
225. Gylling H. Studies of plant stanol esters in different patient populations. Eur Heart J Supplements 1999;1 (Suppl S):S109-S113.
226. Suomen sisätautilääkärien yhdistyksen, Suomen kardiologisen seuran, Suomen verenpaineyhdistyksen, Kunnallislääkärit-yhdistyksen, Suomen teollisuuslääketieteen yhdistyksen, ja Suomen sydäntautiliiton työryhmä. Sepelvaltimotaudin ehkäisy käytännön lääkärityössä. Recommendations of the Task Force of the European Society of Cardiology, European Atherosclerosis Society, and European Society of Hypertension. Suomen lääkäril 1996;51:783-802.
227. Grundy SM. Stanol esters as a dietary adjunct to cholesterol lowering therapies. Eur Heart J Supplements 1999;1 (Suppl S):S132-S138.

I

Hallikainen MA, Uusitupa MIJ.
Effects of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects.
Am J Clin Nutr 1999;69:403-410.

Effects of 2 low-fat stanol ester–containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects[1–3]

Maarit A Hallikainen and Matti IJ Uusitupa

ABSTRACT

Background: Full-fat sitostanol ester–containing margarine reduces serum total and LDL cholesterol, but the effect of plant stanol ester–containing margarine as part of a low-fat, low-cholesterol diet has not been studied.

Objective: We investigated the cholesterol-lowering effects of 2 novel, low-fat stanol ester–containing margarines as part of a low-fat diet recommended for hypercholesterolemic subjects.

Design: In a parallel, double-blind study, 55 hypercholesterolemic subjects were randomly assigned after a 4-wk high-fat diet (baseline) to 3 low-fat margarine groups: wood stanol ester–containing margarine (WSEM), vegetable oil stanol ester–containing margarine (VOSEM), and control margarine (no stanol esters). The groups consumed the margarines for 8 wk as part of a diet resembling that of the National Cholesterol Education Program's Step II diet. The daily mean total stanol intake was 2.31 and 2.16 g in the WSEM and VOSEM groups, respectively.

Results: During the experimental period, the reduction in serum total cholesterol was 10.6% ($P < 0.001$) and 8.1% ($P < 0.05$) greater and in LDL cholesterol was 13.7% ($P < 0.01$) and 8.6% ($P = 0.072$) greater in the WSEM and VOSEM groups, respectively, than in the control group. Serum campesterol concentrations decreased 34.5% and 41.3% ($P < 0.001$) in the WSEM and VOSEM groups, respectively. Serum HDL cholesterol, sitostanol, campestanol, β-carotene, and fat-soluble vitamin concentrations did not change significantly from baseline.

Conclusions: We conclude that the low-fat, plant stanol ester–containing margarines are effective cholesterol-lowering products in hypercholesterolemic subjects when used as part of a low-fat, low-cholesterol diet. They offer an additional, clinically significant reduction in serum cholesterol concentrations to that obtained with a low-fat diet alone. *Am J Clin Nutr* 1999;69:403–10.

KEY WORDS Cholesterol, low-fat diet, plant stanol esters, sitostanol, campestanol, campesterol, apolipoproteins, hypercholesterolemia, margarine, humans

INTRODUCTION

An increased concentration of LDL cholesterol is the main risk factor for atherosclerotic vascular disease. Considerable efforts have focused on different measures to lower elevated concentrations of LDL cholesterol, such as dietary and pharmacologic measures.

Plant sterols, structurally resembling cholesterol, reduce serum cholesterol concentrations by inhibiting the absorption of both dietary and biliary cholesterol from the small intestine (1, 2). Sitostanol, the saturated form of sitosterol, has been shown to be most effective in this respect (2, 3). Because sitostanol is virtually unabsorbable, it has been considered a safe way to reduce elevated serum cholesterol concentrations. Several studies have shown that 2.0–3.0 g sitostanol from full-fat sitostanol ester–containing margarines or mayonnaises significantly reduces serum total and LDL-cholesterol concentrations without affecting HDL-cholesterol or serum triacylglycerol concentrations (4–9). However, the effect of plant stanols delivered in low-fat margarines on elevated cholesterol concentrations as part of a recommended low-fat, low-cholesterol diet (10) has not been studied.

Therefore, we investigated to what extent the 2 low-fat margarines enriched with wood or vegetable oil–based plant stanols would reduce serum total and LDL-cholesterol concentrations as part of a low-fat, low-cholesterol diet and whether or not these 2 low-fat plant stanol ester–containing margarines would lower serum cholesterol concentrations equally.

SUBJECTS AND METHODS

Subjects

Altogether, 91 subjects were screened for the study from the occupational health care system and former studies carried out at the Department of Clinical Nutrition, University of Kuopio, Kuopio, Finland. To be included in the study, subjects had to have a serum total cholesterol concentration of 5.4–7.5 mmol/L; to have a serum triacylglycerol concentration <3.0 mmol/L; to

[1] From the Department of Clinical Nutrition, University of Kuopio, Kuopio, Finland.

[2] Supported by Raisio Benecol Ltd, Raisio, Finland.

[3] Address reprint requests to MA Hallikainen, Department of Clinical Nutrition, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland. E-mail: Maarit.Hallikainen@uku.fi.

Received January 29, 1998.

Accepted for publication August 10, 1998.

TABLE 1
Baseline characteristics of the subjects in the 3 study groups[1]

Variables	WSEM group (n = 8 M, 10 F)	VOSEM group (n = 6 M, 14 F)	Control group (n = 6 M, 11 F)
Age (y)	43.2 ± 8.2	40.8 ± 9.3	46.0 ± 8.2
Weight (kg)			
Men	83.0 ± 9.5	80.5 ± 12.8	80.4 ± 7.5
Women	64.6 ± 11.0	61.9 ± 7.5	68.1 ± 12.2
Body mass index (kg/m^2)	25.6 ± 4.0	24.2 ± 3.0	25.7 ± 3.5
Waist circumference (cm)			
Men	93.4 ± 8.1	94.4 ± 9.3	94.8 ± 8.4
Women	81.2 ± 12.1	78.1 ± 9.4	83.8 ± 12.8
Lipids (mmol/L)			
Total cholesterol	6.36 ± 0.76	6.15 ± 0.79	5.93 ± 0.64
LDL cholesterol	4.36 ± 0.76	4.21 ± 0.89	4.10 ± 0.60
HDL cholesterol	1.36 ± 0.38	1.37 ± 0.32	1.27 ± 0.27
Triacylglycerols	1.42 ± 0.67	1.25 ± 0.39	1.24 ± 0.66
Blood pressure (mm Hg)			
Systolic	123 ± 8	121 ± 11	127 ± 17
Diastolic	79 ± 6	79 ± 9	81 ± 9

[1] $\bar{x}$ ± SD. There were no significant differences among groups. WSEM, wood stanol ester–containing margarine; VOSEM, vegetable oil stanol ester–containing margarine.

be aged 20–60 y; to have normal liver, kidney, and thyroid function; to not be taking any lipid-lowering drugs or other drugs that might affect lipid concentrations; to be willing to participate; and to not be an abuser of alcohol. On the basis of these criteria, 60 subjects were selected for the study. Five subjects dropped out at the beginning of the run-in period for personal reasons. These subjects did not differ in initial serum lipid concentrations, weight, or lifestyle habits from the 55 subjects who completed the study. Five subjects used low-estrogen oral contraceptives, 6 used postmenopausal estrogen medication, and 3 used calcium channel blockers, diuretics, or both for the treatment of hypertension or ischemic heart disease. Ten of the subjects were smokers. The subjects were requested to maintain their weight, alcohol consumption, smoking habits, and physical activity during the study. Baseline characteristics of the subjects are shown in **Table 1**. The study protocol was approved by the Ethics Committee of the University of Kuopio and all subjects gave their informed consent.

Study design

This double-blind, parallel, randomized study consisted of a 4-wk run-in period (high-fat diet) and an 8-wk experimental period (low-fat, low-cholesterol diet). In 2 subjects, the experimental diet period lasted only 6 wk because of a trip abroad.

Routine laboratory measurements were taken at the screening visit and at the last visit of the study to ensure normal health status. In addition, medical history, drug use, smoking habits, alcohol consumption, and physical activity were reviewed with a questionnaire at the same time points. The subjects started the study by following a high-fat diet for 4 wk. At the end of the run-in period, the subjects were randomly assigned into 3 experimental groups: wood stanol ester–containing margarine (WSEM), vegetable oil stanol ester–containing margarine (VOSEM), and control margarine. Smoking and the phase of menstrual cycle were taken into account in the randomization. After randomization, the subjects followed a low-fat diet for the next 8 wk. Fasting blood samples were taken at the beginning of the run-in (−4 wk) and the experimental diet (0 wk) periods and at weeks 2, 4, and 8. Body weight and side effects were recorded at each visit.

Diets

The composition of the low–erucic acid rapeseed oil–based low-fat margarines (Raisio Group, Raisio, Finland) is presented in **Table 2**. The control margarine contained 35% of energy as fat and no added plant stanols. The 2 test margarines contained 40% of energy as fat and were prepared with use of commercially available plant sterols (wood sterols: Ultra sitosterol, Kaukas Oy, Finland; vegetable sterols: derived principally from soy oil, Archer Daniels Midland Co, Decatur, IL) by recrystallization, hydrogenation to form plant stanols, and esterification to produce fatty acid esters of the obtained plant stanols. The subjects consumed 25 g low-fat margarine/d as part of their low-fat, low-cholesterol diet. The theoretical daily intake of stanols was 2.34 g (2.15 g sitostanol and 0.19 g campestanol) in the WSEM group and 2.20 g (1.50 g sitostanol and 0.70 g campestanol) in the VOSEM group. Vitamin A (5.5 μg/g) and vitamin D (0.07 μg/g) were added to all 3 spreads. The subjects received coded tubs of the test margarines when visiting the laboratory and they were asked to record daily the consumption of the test margarines.

During the run-in period, dietary goals were to consume 36–38% of energy as fat (16–18% as saturated, 14% as monounsaturated, and 6% as polyunsaturated fat), 20% as protein, and 40–44% as carbohydrate. During the experimental period, the diet resembled the Step I diet of the National Cholesterol Education Program (10) and provided 28–30% of energy as fat (8–10% as saturated, 12% as monounsaturated, and 8% as polyunsaturated fatty acids), 20% as protein, and 50–52% as carbohydrate. The goal for cholesterol intake was 35.7 and 23.8 mg/MJ during the run-in and experimental periods, respectively. Except for the 3 test margarines, the diets were composed of normal Finnish food items. The fatty acid compositions were adjusted by changing the quality of spreads, vegetable oils, and liquid milk products during the different study periods. During the run-in

TABLE 2
Composition of low-fat wood stanol ester–containing (WSEM), vegetable oil stanol ester–containing (VOSEM), and control margarines[1]

Nutrients	WSEM margarine	VOSEM margarine	Control margarine
		g	
Fat	10.0	10.0	8.8
Total stanols	2.34	2.20	—
Total unsaturated sterols	0.10	0.15	0.05
Fatty acids			
Polyunsaturated	2.05	2.13	2.10
trans Polyunsaturated	0.03	0.05	0.05
Monounsaturated	4.18	4.08	4.13
trans Monounsaturated	0	0	0.03
Saturated	1.10	1.13	2.03

[1] Values are per 25 g spread.

period, a milk-fat based spread (a blend of 0.6 g milk fat and 0.2 g vegetable oil/g spread), a small amount of rapeseed oil, and 1.5%-fat milk were consumed. During the experimental period, a low-fat test margarine, sunflower oil, and skim or 1.0%-fat milk were used. The compliance of the subjects was improved by providing the spreads, vegetable oils, and liquid milk products free of charge.

The subjects received detailed written and oral instructions about the diets, including the precise amounts of food to be eaten and the quality of food, by main food groups. The diets were calculated for 9 energy intakes: 6.7, 7.6, 8.4, 9.2, 10.1, 10.9, 11.8, 12.6, and 13.4 MJ/d. The energy requirement of each subject was estimated from a 4-d food record that subjects completed before the study and by using the Harris-Benedict formula (11), to which energy needs as a result of physical activity were added.

Adherence to the diets was monitored by examining a 4-d (completed on 3 weekdays and 1 weekend day) food record once during the run-in period and 3 times during the experimental period. The subjects recorded their food consumption after consulting a booklet containing photographs of food portions (12) aimed to help them estimate portion sizes. At every study visit, the subjects met a dietitian who advised them on the practical management of the diets and checked their food records. The diets were planned and the nutrients in the food records were calculated by using the MICRO-NUTRICA dietary analysis program (Finnish Social Insurance Institute, Turku, Finland). The values for the food-composition database were taken from Finnish food analyses and international food-composition tables (13).

Laboratory measurements

Venous blood samples were obtained after a 12-h overnight fast. After ultracentrifugation and precipitation (14), enzymatic colorimetric methods were used to determine cholesterol and triacylglycerols from whole serum and separated lipoproteins by using commercial kits (Monotest Cholesterol and Triacylglycerol GPO-PAP; Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) with a Kone Specific Clinical Analyzer (Kone Ltd, Espoo, Finland).

Serum samples for β-carotene, fat-soluble vitamins, apolipoprotein (apo) A-I, apo B, and plant sterols were stored at −70 °C until analyzed at the end of the study. A Kone Specific Clinical Analyzer and apo A-I and apo B reagents from Kone Corporation were used to analyze apolipoproteins based on immunoprecipitation enhanced by polyethylene glycol at 340 nm. β-Carotene and fat-soluble vitamins were analyzed by HPLC (Perkin-Elmer, Norwalk, CT) on a C_{18} column (Waters, Milford, MA) (15, 16). Serum plant sterols were measured by gas-liquid chromatography (model 5890A; Hewlett-Packard, Palo Alto, CA) equipped with a (0.25 mm internal diameter) 25-m fused silica CP-Sil 5-CB capillary column (Chrompack, Raritan, NJ) (17).

Statistical analyses

Statistical analyses were performed with SPSS for WINDOWS 6.0 statistics program (SPSS Inc, Chicago). Normal distribution of variables was checked with the Shapiro-Wilks test (18). Differences in serum lipid variables were analyzed with repeated-measures multivariate analysis of variance (MANOVA) followed by Student's t test in between-group analyses and paired t test in within-group analyses. Statistical significance for the continuous response variables (serum lipids, fat-soluble vitamins, apolipoproteins, and mean plant sterols) were tested with a single-measurement, simple-factorial ANOVA followed by Student's t test. Logarithmic transformations were used when appropriate. If the initial concentration differed significantly among groups, the concentration was adjusted in the between-groups comparisons by dividing the response variable by the initial concentration. In addition, variables that were not normally distributed, even after logarithmic transformation, and noncontinuous variables were tested with the Kruskal-Wallis test, the chi-square test, or Wilcoxon's matched-pairs signed-rank test. Bonferroni adjustment was used to control the overall α level. The results are expressed as means ±SDs.

RESULTS

Baseline characteristics

There were no significant differences in baseline characteristics among the study groups (Table 1). Body weight decreased marginally during the study in all groups (1.2 ± 1.1, 1.2 ± 1.0, and 1.1 ± 1.3 kg in the WSEM, VOSEM, and control groups, respectively; NS among groups). Physical activity and smoking habits remained stable and no side effects were reported.

Feasibility of the diets

The mean consumption of the test margarines was 98.9%, 98.0%, and 98.0% of the scheduled amount in the WSEM, VOSEM, and control groups, respectively. Thus, the actual daily mean stanol intakes were 2.31 ± 0.03 g (2.13 ± 0.03 g sitostanol and 0.19 ± 0.00 g campestanol) in the WSEM group and 2.16 ± 0.12 g (1.47 ± 0.08 g sitostanol and 0.69 ± 0.04 g campestanol) in the VOSEM group. The small differences in the sitostanol and campestanol intakes were significant between the experimental groups ($P < 0.001$).

There were no significant differences in habitual nutrient intakes before the study among the groups. Nutrient intakes during the experimental diet periods remained stable and were not significantly different among the 3 groups (**Table 3**). Furthermore, the dietary goals were well achieved by all groups. In fact, the mean intake of fat, saturated fatty acids, and dietary cholesterol during the experimental period was even lower than the dietary goals. Energy intake was 44–54 kJ/d lower on average during the experimental than during the run-in period.

TABLE 3
Actual composition of the diets during the study in the 3 study groups[1]

Nutrients	Run-in period			Experimental period[2]		
	WSEM group ($n = 18$)	VOSEM group ($n = 20$)	Control group ($n = 17$)	WSEM group ($n = 18$)	VOSEM group ($n = 20$)	Control group ($n = 17$)
Energy (MJ/d)	8.7 ± 2.0	8.4 ± 1.4	8.0 ± 1.9	7.8 ± 1.4	7.7 ± 1.5	7.1 ± 1.3
Fat (% of energy)	40.6 ± 3.6	39.9 ± 4.6	41.1 ± 3.0	26.4 ± 3.3	25.6 ± 3.9	26.5 ± 3.1
Saturated fatty acids (% of energy)	16.8 ± 1.8	16.9 ± 2.1	16.9 ± 1.5	7.0 ± 1.4	6.8 ± 1.7	7.3 ± 1.6
Monounsaturated fatty acids (% of energy)	14.6 ± 1.6	14.3 ± 2.1	15.0 ± 1.4	8.9 ± 1.5	8.1 ± 1.5	8.6 ± 1.4
Polyunsaturated fatty acid (% of energy)	6.4 ± 1.0	5.9 ± 0.8	6.3 ± 0.7	8.3 ± 0.7	8.3 ± 1.2	8.5 ± 1.2
Protein (% of energy)	16.7 ± 1.9	16.0 ± 1.5	17.0 ± 1.5	18.4 ± 1.6	18.1 ± 2.1	19.2 ± 2.1
Carbohydrate (% of energy)	40.1 ± 3.7	40.7 ± 5.0	39.2 ± 2.6	51.2 ± 4.1	51.8 ± 4.9	50.8 ± 4.8
Alcohol (% of energy)	1.4 ± 1.9	2.2 ± 3.2	1.4 ± 1.8	2.6 ± 3.4	3.1 ± 3.9	2.1 ± 2.3
Cholesterol (mg/MJ)	26 ± 7	34 ± 5	38 ± 7	21 ± 7	18 ± 5	19 ± 5
Fiber (g/MJ)	2.9 ± 0.7	2.9 ± 0.8	2.7 ± 0.5	3.7 ± 0.6	4.0 ± 1.1	4.0 ± 1.0
Vitamin A (μg RE/d)	1258 ± 983	1187 ± 635	1140 ± 548	880 ± 394	928 ± 260	972 ± 323
β-Carotene (μg/d)	3725 ± 3437	4142 ± 3320	3804 ± 1826	3259 ± 2201	3388 ± 1384	3056 ± 920
Vitamin E (mg/d)	11.7 ± 2.2	11.0 ± 2.1	11.2 ± 2.5	16.8 ± 3.0	16.7 ± 3.4	16.8 ± 4.1
Vitamin D (μg/d)	3.1 ± 2.5	3.0 ± 2.4	2.4 ± 1.5	4.9 ± 2.3	4.2 ± 2.0	4.1 ± 1.2

[1] $\bar{x}$ ± SD. There were no significant differences among groups. WSEM, wood stanol ester–containing margarine; VOSEM, vegetable oil stanol ester–containing margarine; RE, retinol equivalents.
[2] Nutrient intakes are presented as the mean from the 3 food records.

Serum lipids and lipoproteins

There were no significant differences between baseline (−4 wk) and 0-wk (at randomization) serum lipids and lipoproteins among the 3 groups. During the run-in period, serum total or lipoprotein lipid concentrations did not change significantly in any of the 3 groups. No significant differences were found between men and women and therefore the results are presented for both sexes combined.

Serum total and LDL-cholesterol concentrations decreased significantly within all study groups during the experimental period. Most of the reduction in serum total and LDL-cholesterol concentrations was achieved after 2 wk. The serum total cholesterol concentration decreased by 18.3%, 15.7%, and 7.7% in the WSEM, VOSEM, and control groups, respectively. The reduction was significantly greater in the WSEM (10.6%, $P < 0.001$) and VOSEM (8.1%, $P < 0.05$) groups than in the control group, but no significant differences were found between the 2 experimental groups (**Table 4**). The serum LDL-cholesterol concentration decreased by 23.6%, 18.4%, and 9.9% in the WSEM, VOSEM, and control groups, respectively. There were significant differences only in the absolute (0.73 mmol/L, $P < 0.01$) and percentage (13.7%, $P < 0.01$) reductions in LDL-cholesterol concentrations between the WSEM and the control groups. The difference in percentage reduction in LDL-cholesterol concentration (8.6%) between the VOSEM and control groups was almost significant after Bonferroni correction ($P = 0.072$). Furthermore, there were no significant differences in absolute or percentage changes between the WSEM and VOSEM groups.

Serum HDL-cholesterol concentrations did not change significantly from baseline in any of the study groups, whereas VLDL cholesterol decreased significantly at 8 wk only in the VOSEM group (Table 4). However, there were no significant differences in VLDL-cholesterol concentrations among the groups at the end of the study. Serum VLDL triacylglycerols decreased significantly from baseline only in the WSEM group (Table 4) and serum HDL-triacylglycerol concentrations did not change significantly in any of the groups (data not shown). LDL triacylglycerols at 8 wk (0.30 ± 0.08 mmol/L) were significantly greater than those at baseline (0.27 ± 0.06 mmol/L) in the control group. There were no significant differences in total, VLDL, or LDL triacylglycerols among the groups at the end of the study.

The decrease from baseline in apo B concentrations at 8 wk in the WSEM (by 0.23 ± 0.16 g/L, 19.2%; $P < 0.001$), VOSEM (by 0.15 ± 0.14 g/L, 13.7%; $P < 0.001$), and control (by 0.06 ± 0.01 g/L, 5.2%; $P < 0.05$) groups was significant and paralleled the decrease in LDL-cholesterol concentrations in all groups. Although HDL cholesterol remained unchanged, apo A-I decreased significantly from baseline in the WSEM (by 0.17 ± 0.17 g/L, 9.0%; $P < 0.01$), VOSEM (by 0.15 ± 0.16 g/L, 8.6%; $P < 0.01$), and control (by 0.10 ± 0.16 g/L, 6.1%; $P < 0.05$) groups at 8 wk. Furthermore, the ratio of apo A-I to apo B increased by 14.3% and 8.3% in the WSEM and VOSEM groups, respectively, but the increase was significant ($P < 0.001$) only in the WSEM group.

Serum β-carotene and fat-soluble vitamins

Serum retinol concentrations did not change significantly in the 3 groups. The absolute concentration of serum β-carotene and α-tocopherol concentrations decreased significantly in the WSEM and VOSEM groups, but in the control group the change in serum β-carotene and α-tocopherol concentrations was not significant (**Table 5**). There was a significant difference in the absolute change in serum β-carotene between the experimental groups and the control group; however, the difference in the absolute change in serum α-tocopherol was significant only between the WSEM and control groups. However, there were no significant changes in serum β-carotene or α-tocopherol concentrations among the groups when the values were related to the serum total cholesterol concentration, ie, when vitamin concentrations were divided by serum total cholesterol concentrations. In fact, the ratio of serum α-tocopherol to total cholesterol increased significantly in all groups.

Serum 25-hydroxyergocalciferol (ercalcidiol) concentrations did not change significantly, whereas the absolute concentration of 25-hydroxycholecalciferol (calcidiol) increased significantly in all groups, but the increase was significantly smaller in the WSEM than in the VOSEM group (Table 5). However, there was

TABLE 4
Serum lipids in the 3 study groups during the experimental period[1]

	WSEM group (n = 18)	VOSEM group (n = 20)	Control (n = 17)
Total cholesterol (mmol/L)[2]			
0 wk	6.55 ± 0.78[3]	6.13 ± 0.81	6.06 ± 0.54
4 wk	5.34 ± 0.74	5.38 ± 0.85	5.69 ± 0.56
8 wk	5.34 ± 0.76[4]	5.15 ± 0.78[4]	5.57 ± 0.49[5]
P (MANOVA)[6]	<0.001	<0.001	<0.01
Change (from 0 to 8 wk)[7]	−1.21 ± 0.61[8]	−0.98 ± 0.59[9]	−0.48 ± 0.49
LDL cholesterol (mmol/L)[10]			
0 wk	4.54 ± 0.72	4.25 ± 0.85	4.27 ± 0.59
4 wk	3.50 ± 0.69	3.54 ± 0.69	3.89 ± 0.62
8 wk	3.48 ± 0.77[4]	3.45 ± 0.76[4]	3.82 ± 0.56[5]
P (MANOVA)[6]	<0.001	<0.001	<0.01
Change (from 0 to 8 wk)[7]	−1.06 ± 0.45[11]	−0.80 ± 0.50	−0.45 ± 0.59
HDL cholesterol (mmol/L)			
0 wk	1.44 ± 0.38	1.41 ± 0.38	1.36 ± 0.26
4 wk	1.38 ± 0.30	1.32 ± 0.37	1.35 ± 0.27
8 wk	1.41 ± 0.33	1.36 ± 0.31	1.37 ± 0.26
P (MANOVA)[6]	NS	NS	NS
Change (from 0 to 8 wk)	−0.03 ± 0.17	−0.05 ± 0.18	0.01 ± 0.15
VLDL cholesterol (mmol/L)			
0 wk	0.57 ± 0.35	0.47 ± 0.24	0.42 ± 0.27
4 wk	0.46 ± 0.22	0.51 ± 0.30	0.46 ± 0.26
8 wk	0.45 ± 0.34	0.34 ± 0.18[12]	0.38 ± 0.28
P (MANOVA)[6]	NS	<0.01	NS
Change (from 0 to 8 wk)	−0.13 ± 0.40	−0.13 ± 0.21	−0.04 ± 0.28
Total triacylglycerols (mmol/L)[10]			
0 wk	1.45 ± 0.70	1.24 ± 0.50	1.25 ± 0.68
4 wk	1.16 ± 0.54	1.36 ± 0.61	1.19 ± 0.59
8 wk	1.26 ± 0.67	1.13 ± 0.45	1.33 ± 0.80
P (MANOVA)[6]	<0.05	NS	NS
Change (from 0 to 8 wk)	−0.20 ± 0.55	−0.11 ± 0.41	0.08 ± 0.31
VLDL triacylglycerols (mmol/L)			
0 wk	0.96 ± 0.62	0.76 ± 0.49	0.82 ± 0.63
4 wk	0.73 ± 0.52	0.88 ± 0.56	0.76 ± 0.55
8 wk	0.77 ± 0.64[12]	0.63 ± 0.37	0.85 ± 0.75
P (MANOVA)[6]	<0.05	NS	NS
Change (from 0 to 8 wk)	−0.19 ± 0.50	−0.13 ± 0.38	0.03 ± 0.28

[1] WSEM, wood stanol ester–containing margarine; VOSEM, vegetable oil stanol ester–containing margarine; MANOVA, repeated-measures multivariate analysis of variance.
[2,10] Significant group-by-time interaction (MANOVA): [2] $P < 0.001$, [10] $P < 0.01$.
[3] $\bar{x} \pm$ SD.
[4,5,12] Significantly different from 0 wk (paired t test): [4] $P < 0.001$, [5] $P < 0.01$, [12] $P < 0.05$.
[6] Significant difference in overall within-group changes.
[7] Significant difference among groups, $P < 0.01$ (ANOVA).
[8,9,11] Significantly different from control group (Student's t test and Bonferroni correction): [8] $P < 0.001$, [9] $P < 0.05$, [11] $P < 0.01$.

no significant difference in the percentage increase in calcidiol concentrations among the study groups.

Plant sterols

Baseline concentrations of serum sitostanol and campestanol did not change significantly over the 8-wk study period in the WSEM, VOSEM, and control groups: sitostanol (from 4.6 ± 4.3 to 4.8 ± 7.4 μmol/L, 4.3 ± 5.5 to 3.8 ± 5.3 μmol/L, and 5.5 ± 5.3 to 3.8 ± 4.8 μmol/L, respectively); campestanol (from 3.5 ± 3.0 to 3.2 ± 3.2 μmol/L, 4.5 ± 7.4 to 2.7 ± 5.2 μmol/L, and 4.7 ± 7.9 to 5.7 ± 9.2 μmol/L, respectively). Serum campesterol concentrations did not change significantly in the control group but decreased significantly from baseline ($P < 0.001$) in both experimental groups: from 21.7 ± 6.5 to 14.2 ± 6.0 μmol/L (34.5% change) in the WSEM group and from 27.2 ± 18.7 to 16.0 ± 9.5 μmol/L (41.3% change) in the VOSEM group. Furthermore, serum campesterol concentrations were still significantly decreased in both experimental groups after correction for the reduction in serum cholesterol. In addition, serum sitosterol concentrations tended to decrease in both the experimental groups, but not significantly so.

DISCUSSION

In the present study, the wood- and vegetable oil–based plant stanol ester–containing margarines (WSEM and VOSEM groups, respectively), as part of a low-fat diet, reduced more markedly both serum total and LDL-cholesterol concentrations than did

TABLE 5
Serum β-carotene, retinol, α-tocopherol, calcidiol, and ercalcidiol concentrations and ratios of β-carotene to total cholesterol and of α-tocopherol to total cholesterol in the 3 study groups during the experimental period[1]

	WSEM group (n = 18)	VOSEM group (n = 20)	Control group (n = 17)
β-Carotene (μmol/L)[2]			
0 wk	1.66 ± 1.10	1.47 ± 0.79	1.00 ± 0.37
8 wk	1.22 ± 0.97[3]	1.07 ± 0.54[3]	1.06 ± 0.42
Change (from 0 to 8 wk)[4,5]	−0.44 ± 0.57[6]	−0.40 ± 0.54[7]	0.05 ± 0.26
Retinol (μmol/L)			
0 wk	2.50 ± 0.72	2.21 ± 0.81	2.30 ± 0.66
8 wk	2.36 ± 0.68	2.12 ± 0.82	2.21 ± 0.70
Change (from 0 to 8 wk)	−0.14 ± 0.45	−0.09 ± 0.40	−0.09 ± 0.29
α-Tocopherol (μmol/L)[2]			
0 wk	51.49 ± 8.17	45.10 ± 9.68	44.58 ± 9.86
8 wk	45.27 ± 6.97[8]	41.51 ± 9.30[8]	43.45 ± 9.46
Change (from 0 to 8 wk)[4,9]	−6.22 ± 5.04[7]	−3.59 ± 4.29	−1.13 ± 3.34
Calcidiol (nmol/L)[10]			
0 wk	67.47 ± 23.73	62.48 ± 21.57	73.66 ± 44.26
8 wk	80.62 ± 22.96[11]	96.23 ± 33.68[8]	103.19 ± 43.30[8]
Change (from 0 to 8 wk)[9]	13.15 ± 22.75[12]	33.75 ± 25.72	29.53 ± 24.12
Ercalcidiol (nmol/L)			
0 wk	38.14 ± 42.39	41.19 ± 28.99	63.44 ± 32.76
8 wk	49.06 ± 33.58	42.70 ± 34.65	72.00 ± 41.47
Change (from 0 to 8 wk)	10.92 ± 27.87	1.51 ± 22.42	8.56 ± 32.30
β-Carotene:total cholesterol			
0 wk	0.27 ± 0.21	0.24 ± 0.13	0.17 ± 0.07
8 wk	0.24 ± 0.23	0.21 ± 0.10	0.19 ± 0.08
Change (from 0 to 8 wk)	−0.03 ± 0.12	−0.03 ± 0.08	0.02 ± 0.05
α-Tocopherol:total cholesterol			
0 wk	7.86 ± 0.81	7.40 ± 1.41	7.38 ± 1.55
8 wk	8.50 ± 0.90[8]	8.06 ± 1.35[8]	7.80 ± 1.58[11]
Change (from 0 to 8 wk)	0.65 ± 0.50	0.66 ± 0.66	0.42 ± 0.75

[1] $\bar{x}$ ± SD. WSEM, wood stanol ester–containing margarine; VOSEM, vegetable oil stanol ester–containing margarine; MANOVA, multivariate analysis of variance.

[2,10] Significant group-by-time interaction (MANOVA): [2] $P < 0.01$, [10] $P \leq 0.05$.

[3,8,11] Significantly different from 0 wk (paired t test): [3] $P < 0.01$, [8] $P < 0.001$, [11] $P < 0.05$.

[4] Initial concentrations were nearly significantly different by ANOVA (β-carotene, $P = 0.063$; α-tocopherol, $P = 0.053$) among the study groups; therefore, initial concentrations were taken into account in the between-groups comparisons by dividing the response variable by the initial value.

[5,9] Significant difference among groups (ANOVA): [5] $P < 0.01$, [9] $P < 0.05$.

[6,7] Change significantly different from change in control group (Student's t test with Bonferroni correction): [6] $P < 0.05$, [7] $P < 0.01$.

[12] Significantly different from VOSEM group, $P < 0.05$ (Student's t test with Bonferroni correction).

the low-fat diet alone in subjects with elevated serum total cholesterol concentrations. The cholesterol-lowering effects of the 2 plant stanol ester–containing margarine diets did not differ significantly. These findings indicate that low-fat plant stanol ester–containing margarines, when part of a low-fat diet (10), can reduce serum cholesterol concentrations almost as much as cholesterol-lowering drugs (19, 20).

There have been no studies of the effects on serum cholesterol concentrations of plant stanols as part of a strictly and frequently monitored low-fat, low-cholesterol diet. Moreover, earlier studies used full-fat margarines and mayonnaises (4–9), whereas the present study used low-fat stanol ester–containing margarines (40% of energy as fat, including 9% nonabsorbable stanols). In contrast with Denke's study (21), we found that stanol esters can significantly lower serum cholesterol concentrations even in those with a low cholesterol intake. Note that nonesterified sitostanol suspended in safflower oil and packed into gelatin capsules was used in Denke's study.

The novel finding that plant stanols can reduce serum cholesterol concentrations, even in conjunction with a markedly low dietary cholesterol intake, indicates that plant stanols must inhibit not only the absorption of dietary cholesterol but also that of biliary cholesterol. This is supported by the findings of earlier studies of plant stanol (2, 4, 7, 9), in which the fecal excretion of neutral sterols increased despite a constant dietary cholesterol intake. In addition, in the present study the serum campesterol concentration, which is known to reflect intestinal cholesterol absorption (22, 23), decreased significantly in both stanol ester groups, which agrees with the findings of earlier studies (2, 4, 7, 22, 23). In some studies of plant stanol in diabetic subjects (4, 24), the biliary secretion of cholesterol, which normally ranges from 600 to 1000 mg/d (25), was found to increase significantly (11–16%) (4, 24). An average of 50% of the cholesterol that enters the small intestine is reabsorbed (25). Cholesterol absorption was shown to decrease by 60% in diabetic patients with a daily intake of 3 g sitostanol delivered as fatty acid esters (4, 24).

Sitostanol has been shown to be virtually unabsorbable (26–28), but 12.5% of campestanol was found to be absorbed in a study of intestinal perfusion in humans (29). However, the results from the present study indicate that the absorption of campestanol was also negligible when campestanol was fed as part of a stanol blend containing substantial amounts of sitostanol

(65%). In the present study, the serum campesterol concentration decreased significantly and campestanol decreased nonsignificantly in both stanol ester groups. Therefore, the vegetable oil–based sterol blend can be used after saturation to stanol without an increase in serum campestanol concentration. The absorption of campestanol might be possible when it is not ingested as part of a blend containing competitive components like sitostanol. When campestanol is used as part of the stanol blend that contains substantial amounts of sitostanol, as was used in the present study, campestanol seems not to be absorbed at all (30).

The 2 low-fat test margarines were intended to differ from each other only with respect to the origin of the plant stanols, with the VOSEM margarine containing more campestanol and less sitostanol than the WSEM margarine. However, the actual daily intake of total plant stanol was 6.5% higher in the WSEM than in the VOSEM group. The cholesterol-lowering effect of sitostanol is well documented in the literature, but the effects of campestanol have not been studied, probably because of practical problems in obtaining pure campestanol in reasonable amounts. However, it has been shown in rats (31) that the oleate ester of campesterol can decrease the absorption of dietary cholesterol with the same efficacy as free β-sitosterol, stigmasterol, or the oleate ester of β-sitosterol. Furthermore, recent data from free-living humans indicate that rapeseed oil–derived campesterol could reduce cholesterol absorption and thus reduce serum cholesterol concentration (32). On the basis of these data, campestanol can also be expected to reduce cholesterol absorption. Thus, the difference in stanol compositions is not likely to have an effect on the present results.

On the basis of the food records during both study periods the adherence to the diets was good. Actually, during the low-fat diet the intakes of fat, saturated fatty acids, and dietary cholesterol were even lower than the dietary goals. Note that the intake of dietary cholesterol achieved the goal of the Step II diet of the National Cholesterol Education Program (<200 mg/d) (10), and the intake of saturated fatty acids was close to these goals (<7% of energy) in all study groups. Despite the frequent monitoring, there was a slight decrease in body weight in all study groups during the experimental period. The decrease in weight was primarily due to the lower intake of energy during the experimental than during the run-in period. However, because the weight change was marginal in all groups and because there were no significant differences in weight change among the groups, the decrease in weight cannot explain the findings of the present study.

Low-fat stanol ester–containing margarines appeared to have little effect on serum concentrations of retinol and ercalcidiol. The serum absolute concentration of β-carotene and α-tocopherol decreased significantly in both the stanol ester–containing margarine groups, but this would be expected because β-carotene and α-tocopherol are transported in serum in lipoproteins, whose concentrations decreased during the experimental diet periods. When the serum β-carotene concentrations were related to the serum total cholesterol concentrations, the decrease was not significant in either of the low-fat stanol ester–containing margarine groups. In addition, the decrease in serum α-tocopherol concentration was ascribed to the changes in serum cholesterol concentrations because the ratio of serum α-tocopherol to total cholesterol actually increased significantly in all of the test margarine groups. These findings agree with the findings of Gylling et al (33). The increase in calcidiol concentrations was significantly smaller in the WSEM than in the VOSEM group. However, there were no significant differences among the groups in percentage changes in calcidiol or absolute calcidiol concentrations at the end of the study.

In conclusion, both the low-fat WSEM and VOSEM margarines when used as part of a low-fat, low-cholesterol diet are effective in reducing serum cholesterol concentrations with apparently equal efficacy in subjects with elevated serum cholesterol concentrations. In addition, these margarines offer an additional, clinically significant reduction in serum cholesterol concentrations to that obtained with a low-fat diet alone.

We thank ES Sarkkinen for reviewing the manuscript and laboratory nurses Kaija Kettunen, Erja Kinnunen, and Irja Lyytikäinen for technical assistance.

REFERENCES

1. Heinemann T, Kullak-Ublick G-A, Pietruck B, von Bergmann K. Mechanisms of action of plant sterols on inhibition of cholesterol absorption. Comparison of sitosterol and sitostanol. Eur J Clin Pharmacol 1991;40(suppl):S59–63.
2. Becker M, Staab D, von Bergmann K. Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. J Pediatr 1993;122:292–6.
3. Heinemann T, Leiss O, von Bergmann K. Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. Atherosclerosis 1986;61:219–23.
4. Gylling H, Miettinen TA. Serum cholesterol and cholesterol and lipoprotein metabolism in hypercholesterolaemic NIDDM patients before and during sitostanol ester-margarine treatment. Diabetologia 1994;37:773–80.
5. Vanhanen HT, Kajander J, Lehtovirta H, Miettinen TA. Serum levels, absorption efficiency, faecal elimination and synthesis of cholesterol during increasing doses of dietary sitostanol esters in hypercholesterolaemic subjects. Clin Sci 1994;87:61–7.
6. Gylling H, Siimes MA, Miettinen TA. Sitostanol ester margarine in dietary treatment of children with familial hypercholesterolemia. J Lipid Res 1995;36:1807–12.
7. Miettinen TA, Puska P, Gylling H, Vanhanen H, Vartiainen E. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. N Engl J Med 1995; 333:1308–12.
8. Niinikoski H, Viikari J, Palmu T. Cholesterol-lowering effect and sensory properties of sitostanol ester margarine in normocholesterolemic adults. Scand J Nutr 1997;41:9–12.
9. Gylling H, Radhakrishnan R, Miettinen TA. Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary sitostanol ester margarine. Women and dietary sitostanol. Circulation 1997;96:4226–31.
10. National Cholesterol Education Program. Second report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (adult treatment panel II). Circulation 1994;89:1329–445.
11. Alpers DH, Clouse RE, Stenson WF. Protein and calorie requirements. In: Manual of nutritional therapeutics. Toronto: Little, Brown and Company, 1986:131–59.
12. Haapa E, Toponen T, Pietinen P, Räsänen L. Annoskuvakirja. (Portion size booklet.) Helsinki: Social Insurance Institution, 1985 (in Finnish).
13. Rastas M, Seppänen R, Knuts L-R, Karvetti R-L, Varo P, eds. Nutrient composition of foods. Helsinki: Publication of Social Insurance Institution, 1993.
14. Penttilä IM, Voutilainen E, Laitinen O, Juutilainen P. Comparison of different analytical and precipitation methods for the direct estimation of high-density lipoprotein cholesterol. Scand J Clin Lab Invest 1981;41:353–60.

15. Driskell WJ, Bashor MM, Neese JW. Beta-carotene determined in serum by liquid chromatography with an internal standard. Clin Chem 1983;29:1042–4.
16. Parviainen MT. The fat-soluble vitamins A, D and E—their metabolism, binding-proteins and determination from human serum. Acta Univ Tamperensis 1983;159:49–54.
17. Salen G, Shore V, Tint GS, et al. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. J Lipid Res 1989;30:1319–30.
18. Norusis MJ. SPSS for Windows base system user's guide, release 6.0. Chicago: SPSS Inc, 1993.
19. Pyörälä K, De Backer G, Graham I, Poole-Wilson P, Wood D. Prevention of coronary heart disease in clinical practice. Recommendations of the Task Force of the European Society of Cardiology, European Atherosclerosis Society and European Society of Hypertension. Eur Heart J 1994;15:1300–31.
20. Watts GF, Burke V. Lipid-lowering trials in the primary and secondary prevention of coronary heart disease: new evidence, implications and outstanding issues. Curr Opin Lipidol 1996;7:341–55.
21. 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;61:392–6.
22. Tilvis RS, Miettinen TA. Serum plant sterols and their relation to cholesterol absorption. Am J Clin Nutr 1986;43:92–7.
23. Miettinen TA, Tilvis RS, Kesäniemi YA. Serum plant sterol and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J Epidemiol 1990;131:20–31.
24. Gylling H, Miettinen TA. Effect of inhibiting cholesterol absorption and synthesis on cholesterol and lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic men. J Lipid Res 1996;37:1776–85.
25. Grundy SM. Cholesterol and atherosclerosis: diagnosis and treatment. New York: Gower Medical Publishing, 1990.
26. Hassan AS, Rampone AJ. Intestinal absorption and lymphatic transport of cholesterol and β-sitostanol in the rat. J Lipid Res 1979;20:646–53.
27. Czubayko F, Beumers B, Lammsfuss S, Lütjohann D, von Bergmann K. A simplified micro-method for quantification of fecal excretion of neutral and acidic sterols for outpatient studies in humans. J Lipid Res 1991;32:1861–7.
28. Lütjohann D, Meese CO, Crouse JR, von Bergmann K. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in human. J Lipid Res 1993;34:1039–46.
29. Heinemann T, Axtmann G, von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. Eur J Clin Invest 1993;23:827–31.
30. Vanhanen HT, Miettinen TA. Effects of unsaturated and saturated dietary plant sterols on their serum contents. Clin Chim Acta 1992;205:97–107.
31. Mattson FH, Volpenhein RA, Erickson BA. Effect of plant sterol esters on the absorption of dietary cholesterol. J Nutr 1977; 107:1139–46.
32. Sarkkinen ES, Uusitupa MIJ, Gylling H, Miettinen TA. Fat-modified diets influence serum concentrations of cholesterol precursors and plant sterols in hypercholesterolemic subjects. Metabolism 1998;47:744–50.
33. Gylling HK, Puska P, Vartiainen E, Miettinen TA. Serum retinol, α-tocopherol, carotenes and lipid peroxide production during serum cholesterol lowering by sitostanol ester margarine in a mildly hypercholesterolemic population. Circulation 1996;94(suppl 1):578 (abstr).

II

Hallikainen MA, Sarkkinen ES, Uusitupa MIJ.
Effects of low-fat stanol ester margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentrations.
Eur J Clin Nutr 1999;53:966-969.

European Journal of Clinical Nutrition (1999) 53, 966-969

http://www.stockton-press.co.uk/ejcn

Effects of low-fat stanol ester enriched margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentrations

MA Hallikainen[1]*, ES Sarkkinen[1] and MIJ Uusitupa[1]

[1]*Department of Clinical Nutrition, University of Kuopio, Kuopio, Finland*

Objective: To investigate the effects of low-fat stanol ester margarines on concentrations of serum carotenoids.
Design: A randomized parallel double-blind study design consisting of a 4-week run-in (high-fat diet) and an 8-week experimental (low-fat, low-cholesterol diet) period. During the experimental diet period subjects consumed low-fat wood stanol ester (WSEM), vegetable oil stanol ester (VOSEM) or control (no stanol esters) margarine daily. The daily mean total stanol intake was 2.31 and 2.16 g in the WSEM and VOSEM groups, respectively.
Setting: Outpatient clinical trial with free-living subjects.
Subjects: Altogether, 60 hypercholesterolaemic subjects were selected for the study out of 91 originally screened. The study was completed by 55 subjects.
Main outcomes measures: Serum α- and β-carotene and lycopene determined by the HPLC.
Results: Serum α-carotene concentration did not change significantly in either of the experimental groups, whereas β-carotene concentration decreased significantly in the WSEM and VOSEM groups ($P < 0.01$), and the change differed significantly ($P < 0.05$ and $P < 0.01$, respectively) from that of the control group. Decrease in $\alpha+\beta$-carotene concentration was significantly greater ($P < 0.05$) in both experimental groups than in the control group. However, the change in α-, β- or $\alpha+\beta$-carotene/total cholesterol ratio did not differ significantly among the groups. No significant changes were found in serum lycopene or lycopene/total cholesterol ratio in both experimental groups.
Conclusions: Low-fat stanol ester margarines appeared to have little effect on serum concentrations of α-, β- or $\alpha + \beta$-carotene, or lycopene.
Sponsorship: Grant to the University of Kuopio by Raisio Benecol Ltd, Raisio, Finland.
Descriptors: plant sterols; carotenoid; lycopene; α-carotene; β-carotene; cholesterol

Introduction

Plant sterols have been found to reduce serum cholesterol concentrations by inhibiting the absorption of both dietary and biliary cholesterol from the small intestine (Heinemann *et al*, 1991; Becker *et al*, 1993; Gylling *et al*, 1997). They may also reduce the concentrations of serum carotenoids, particularly serum β-carotene concentration (Gylling et al, 1996). In a recently published study it was observed that plant sterol-enriched margarines reduced plasma lycopene as well as $\alpha+\beta$-carotene concentrations, even if the changes in plasma lipid concentrations were taken into account (Weststrate & Meijer, 1998). Reduction in serum carotenoid concentrations can be undesirable, because there are indications that carotenoids could have beneficial effects on human health (Gerster 1993; Mayne, 1996). The most studied carotenoid in this respect is β-carotene (Gerster 1993; Mayne, 1996).

Lycopene is one of the most abundant carotenoids in human blood and tissues; it has not been found to have a provitamin A activity, but it has been found to have antioxidant properties (Clinton, 1998). Therefore, we investigated the effects of two low-fat margarines enriched with wood- or vegetable oil-based plant stanol esters on serum lycopene and α- and $\alpha + \beta$-carotene concentrations as part of a low-fat diet. Results on serum lipids have been published elsewhere (Hallikainen & Uusitupa,1999).

Methods

Subjects, study design, diets and laboratory measurements have been described in more detail elsewhere (Hallikainen & Uusitupa, 1999).

Subjects

Altogether 60 hypercholesterolaemic subjects were selected for the study out of 91 originally screened subjects. The study was completed by 55 subjects, whose baseline characteristics are shown in Table 1. The study protocol was approved by the Ethics Committee of the University of Kuopio and all subjects gave their written consent.

*Correspondence: M Hallikainen, Department of Clinical Nutrition, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland.
E-mail Maarit.Hallikainen@uku.fi.
Contributors: MIJU was responsible for study design. MAH recruited the subjects, gave nutrition counsel, analysed and interpreted the data and wrote the manuscript. MIJU and ESS contributed to planning the diets, interpreting the data and writing the manuscript.
Guarantor: M Uusitupa
Received 18 March, 1999; revised 14 June 1999; accepted 30 June 1999

Table 1 Baseline characteristics of the subjects, intake of plant stanols and serum carotenoid concentrations during the study[a]

	WSEM (N=18)	*VOSEM (N=20)*[b]	*CONTROL (N=17)*	*P-values*[c]
Men/women	8/10	6/14	6/11	
Age (y)	43.2±8.2	40.8±9.3	46.0±8.2	NS
Body mass index (kg/m^2)	25.6±4.0	24.2±3.0	25.7±3.5	NS
Intake of plant stanols from test margarines (g/d)	2.31±0.03	2.16±0.12	0	
Sitostanol	2.13±0.03	1.47±0.08	0	
Campestanol	0.19±0.0	0.69±0.04	0	
Serum concentrations of carotenoids				
α-carotene (μmol/l):				
At 0 weeks	0.33±0.23	0.33±0.20	0.35±0.39	NS
At 8 weeks	0.31±0.19	0.29±0.11	0.28±0.10	NS
Changes (from 0 to 8 weeks)	−0.02±0.16	−0.04±0.13	−0.07±0.40	NS
P-values[d]	NS	NS	NS	
β-carotene (μmol/l):				
At 0 weeks	1.66±1.10	1.47±0.79	1.00±0.37	0.063
At 8 weeks	1.22±0.97	1.07±0.54	1.06±0.42	NS
Changes (from 0 to 8 weeks) [e]	−0.44±0.57*	−0.40±0.54†	0.05±0.26	0.002
P-values[d]	0.002	0.001	NS	
α+β-carotene (μmol/l):				
At 0 weeks	2.00±1.24	1.80±0.91	1.35±0.49	NS
At 8 weeks	1.53±1.11	1.36±0.61	1.34±0.46	NS
Changes (from 0 to 8 weeks)	−0.47±0.65*	−0.44±0.59*	−0.02±0.53	0.004
P-values[d]	0.010	0.010	NS	
Lycopene (μmol/l):				
At 0 weeks	0.96±0.67	1.04±0.92	0.58±0.42	0.072
At 8 weeks	0.97±0.54	0.98±0.57	0.77±0.37	NS
Changes (from 0 to 8 weeks)[e]	0.01±0.82	−0.06±0.84	0.19±0.38	NS
P-values[d]	NS	NS	NS	
α-carotene/total cholesterol ratio:				
At 0 weeks	0.05±0.04	0.06±0.04	0.06±0.07	NS
At 8 weeks	0.06±0.04	0.06±0.03	0.05±0.02	NS
Changes (from 0 to 8 weeks)	0.01±0.03	0.00±0.02	−0.01±0.07	NS
P-values[d]	NS	NS	0.018	
β-carotene/total cholesterol ratio:				
At 0 weeks	0.27±0.21	0.24±0.13	0.17±0.07	NS
At 8 weeks	0.24±0.23	0.21±0.10	0.19±0.08	NS
Changes (from 0 to 8 weeks)	−0.03±0.12	−0.03±0.08	0.02±0.05	NS
P-values[d]	NS	NS	NS	
α+β-carotene/total cholesterol ratio:				
At 0 weeks	0.32±0.24	0.30±0.16	0.23±0.09	NS
At 8 weeks	0.30±0.26	0.27±0.12	0.24±0.09	NS
Changes (from 0 to 8 weeks)	−0.02±0.14	−0.03±0.09	0.01±0.10	NS
P-values[d]	NS	NS	NS	
Lycopene/total cholesterol ratio:				
At 0 weeks	0.15±0.11	0.18±0.18	0.10±0.06	NS
At 8 weeks	0.18±0.11	0.20±0.12	0.14±0.08	NS
Changes (from 0 to 8 weeks)	0.04±0.16	0.02±0.14	0.05±0.07	NS
P-values[d]	NS	NS	0.010	

[a]Values are means±s.d. [b]$N = 19$ for results concerning lycopene and lycopene/total cholesterol ratio. [c]Indicates the significance of the difference among the groups analysed with a single measurement simple factorial analysis of variance (ANOVA) test (age, body mass index, β-carotene, lycopene and ratios of β-carotene/total cholesterol and lycopene/total cholesterol) or the Kruskal–Wallis one way ANOVA test (α-carotene, α + β-carotene and ratios of α-carotene/total cholesterol and α+β-carotene/total cholesterol). [d]Indicates the significance of the difference within the group during the experimental period analysed with a paired *t*-test or the Wilcoxon matched–paired signed rank test. [e]The initial concentrations were almost significantly different among the study groups analysed with ANOVA, and therefore the initial concentrations have been taken into account in the between–groups comparisons by dividing the response variable by the initial value *$P < 0.05$, †$P < 0.01$ indicate the significances of the differences between the experimental study groups and the control group analysed with Student's *t*-test or the Mann–Whitney U-test with Bonferroni correction.

Study design

The study has carried out with a parallel double-blind study design. All subjects started the study with a 4-week run-in (high-fat diet) period. At the end of the run-in period the subjects were randomized into one of the three experimental groups: wood stanol ester margarine (WSEM), vegetable oil stanol ester margarine (VOSEM) or control margarine. After randomization the subjects followed a closely instructed, and strictly and frequently monitored low-fat, low-cholesterol diet which resembled Step 2 of the National Cholesterol Education Program (1994) for the next 8 weeks. As part of that diet the subjects consumed 25 g rapeseed oil-based low-fat margarine (Raisio Group Plc, Raisio, Finland) daily. The daily dose of the test margarines was taken in two to three portions in connection with the meals. The two test margarines contained about 31% and the control margarine about 35% absorbable fat. The theoretical daily intake of total stanol was 2.34 g (of which 2.15 g was sitostanol and 0.19 g campestanol) and 2.20 g (of which 1.50 g was sitostanol and 1.70 g campestanol) in the WSEM and VOSEM groups, respectively. The control margarine did not contain added plant stanols.

The subjects recorded the consumption of the test margarines daily in the follow-up diaries and the adherence to the diets was monitored by a 4-day (3 weekdays and 1 weekend day) food record kept three times during the experimental period.

Laboratory measurements

Venous blood samples were obtained after a 12 h overnight fast. Enzymatic colorimetric methods were used for the determination of serum total and lipoprotein lipids using commercial kits (Monotest® Cholesterol and Triglyceride GPO-PAP, Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) and a Kone Specific Clinical Analyzer (Kone Ltd, Espoo, Finland). Serum carotenoids were obtained at the beginning and the end of the experimental diet period and were analysed at the end of the study from samples stored at −70°C, using a high-performance liquid chromatography system (Perkin Elmer, USA) equipped with a C18 column (Nova-pak Waters, USA), and detected at 450 nm, using a Uvikon 735 LC detector (Germany) (Driskell *et al*, 1983; Kaplan *et al* 1987).

Statistical analyses

Statistical analyses were performed with SPSS for Windows 6.0 statistics program (SPSS, Chicago, IL, USA). Values of serum carotenoids were analysed by the analyses of variance for repeated measurements (MANOVA), followed by a single-measurement simple factorial analysis of variance (ANOVA) test and Student's *t*-test or paired *t*-test, or the Kruskal–Wallis one-way ANOVA test followed by Mann–Whitney U-test. Logarithmic transformations were used when appropriate. To control the overall α level, Bonferroni adjustment was made. The results are expressed as means $\pm$ s.d.

Results

Results on baseline characteristics, intake of nutrients, changes in serum lipids and also β-carotene have been presented elsewhere (Hallikainen & Uusitupa, 1999).

The intake of nutrients during the experimental diet periods was stable and did not differ among the three groups. The mean intakes of fat and saturated fatty acids were 25.6–26.5% of energy (E%) and 6.8–7.3 E%, respectively, in the three study groups. The mean daily intake of dietary cholesterol was 137–161 mg, achieving the goal of the Step 2 diet of the National Cholesterol Education Program (< 200 mg/d; National Cholesterol Education Program, 1994). Actual daily intakes of plant stanols as mean values are presented in Table 1.

During the experimental period serum total and LDL cholesterol decreased significantly in all three groups. The net decrease in serum total cholesterol concentration was 10.6% ($P < 0.01$) and 8.1% ($P < 0.05$), and in LDL cholesterol 13.7% ($P < 0.01$) and 8.6% ($P = 0.072$) in the WSEM and VOSEM groups, respectively, compared with the control group.

Serum β-carotene decreased significantly within both experimental groups ($P < 0.01$), but increased slightly, but non-significantly, within the control group. The changes differed significantly between the experimental groups and the control group. However, no significant differences among the groups were found in serum β-carotene after standardization for serum total cholesterol concentration.

There were no significant changes in serum α-carotene concentration or α-carotene/total cholesterol ratio within the WSEM and VOSEM groups (Table 1). Within the control group that ratio decreased slightly, but statistically significantly. Serum $\alpha + \beta$-carotene concentration decreased within all groups, but only within the WSEM and VOSEM groups did the decrease reach statistical significance (Table 1), and it differed significantly from that of the control group. However, there were no significant changes in serum $\alpha+\beta$-carotene concentrations after standardization for serum total cholesterol concentration within any of the three study groups or differences in the changes among the groups. Serum lycopene and lycopene/total cholesterol ratio did not change significantly in the experimental groups, but the ratio increased significantly in the control group (Table 1).

Discussion

In the present study the wood stanol ester margarine and the vegetable oil stanol ester margarine had only a small effect on serum concentrations of α- and β-carotene, the sum of $\alpha+\beta$-carotene or lycopene, in particular when changes were related to the changes in serum total cholesterol concentrations. Before lipid standardization serum β-carotene concentration decreased significantly; however this was not unexpected, because serum LDL-cholesterol concentrations decreased significantly in the present study and in circulation carotenoids are transported in lipoproteins, mainly in the LDL fraction, but also in HDL and to a minor extent in VLDL fraction (Clevidence & Bieri, 1993).

There are only a few studies (Gylling *et al*, 1996; Weststrate & Meijer, 1998; Hendriks, *et al.* 1999) in which the effects of plant stanols or plant sterols on serum or plasma carotenoid concentrations have been investigated. In the study of Gylling *et al* (1996), after lipid standardization serum β-carotene concentration tended to decrease more in two sitostanol ester margarine (sitostanol intake 3 g/d and 2 g/d) groups than in the control group, whereas unstandardized and lipid standardized serum α-carotene concentration decreased similarly in all three groups. In the study of Weststrate and Meijer (1998) the plant sterol enriched margarines (sterol intake 1.5–3.3 g/d) also significantly decreased lipid standardized plasma $\alpha + \beta$-carotene concentrations. Moreover, Hendriks *et al* (1999) found that the daily dose of 0.83 and 3.24 g plant sterols decreased lipid standardized plasma $\alpha + \beta$-carotene more than the 1.61 g daily dose. The effects of plant sterols and plant stanols on plasma lycopene concentrations were smaller than the effects on plasma carotenes (Weststrate & Meijer, 1998; Hendriks *et al*, 1999). In particular, the results of Hendriks *et al* (1999) indicate that other factors, i.e. nutrient density of background diet, could affect the fluctuations of serum carotenoid concentrations rather than the dose of plant stanol or plant sterol.

The differences between our findings and the findings of Gylling *et al* (1996), Weststrate & Meijer (1998) and Hendriks *et al* (1999) might be due to the differences in composition of background diets during the studies. In our study subjects received detailed written and oral instructions about the low-fat (high-nutrient density) diets, specifying amounts and quality of food by main food groups, including vegetables. The background diets in other studies were not so closely standardized since only the consumption

of the test margarines was instructed and subjects followed their habitual, moderate-fat or high-fat diet.

One reason for divergent results could be a seasonal variation in the intakes of carotenoids. However, according to earlier studies there is only a weak correlation between lycopene intake and serum lycopene concentration (Campbell *et al*, 1996; Clinton 1998).

According to our findings the effects of plant sterols on serum carotenoid concentrations were minor and clinically non-important, but additional studies will be needed to determine the long-term effects of plant sterols on serum carotenoid profile. At the moment, it seems to be reasonable to advise healthy food rich in vegetables for people using stanol ester margarine to reduce elevated serum cholesterol concentrations.

References

Becker M, Staab D & von Bergmann K (1993): Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. *J. Pediatr.* **122**, 292–296.

Campbell DR, Gross MD, Martini MC, Grandits GA, Slavin JL & Potter JD (1994): Plasma carotenoids as biomarkers of vegetable and fruit intake. *Cancer Epidemiol. Biomarkers Prev.* **3**, 493–500.

Clevidence BA & Bieri JG (1993): Association of carotenoids with human plasma lipoproteins. *Meth. Enzymol.* **214**, 33–46.

Clinton SK (1998): Lycopene: chemistry, biology, and implications for human health and disease. *Nutr. Rev.* **56**, 35–51 (review).

Driskell WJ, Bashor MM & Neese JW (1983): Beta-carotene determined in serum by liquid chromatography with an internal standard. *Clin. Chem.* **29**, 1042–1044.

Gerster H (1993): Anticarcinogenic effects of common carotenoids. *Int. J. Vitam. Nut. Res.* **63**, 93–121 (review).

Gylling HK, Puska P, Vartiainen E & Miettinen TA (1996): Serum retinol, α-tocopherol, carotenes and lipid peroxide production during serum cholesterol lowering by sitostanol ester margarine in a mildly hypercholesterolemic population. *Circulation.* **94** (Suppl. I), 578 (abstract).

Gylling H, Radhakrishnan R & Miettinen TA (1997): Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary sitostanol ester margarine. Women and dietary sitostanol. *Circulation.* **96**, 4226–4231.

Hallikainen MA & Uusitupa MIJ (1999): Effects of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. *Am. J. Clin. Nutr.* **69**, 403–410.

Heinemann T, Kullak-Ublick GA, Pietruck B & von Bergmann K (1991):Mechanisms of action of plant sterols on inhibition of cholesterol absorption. *Eur. J. Clin. Pharmac.* **40**, (Suppl. 1), S59–S63.

Hendriks HFJ, Weststrate JA, van Vlient T & Meijer GW (1999): Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J Clin. Nutr.* **53**, 319–327

Kaplan LA, Miller JA & Stein EA (1987): Simultaneous measurement of serum retinol, tocopherols, carotenes, and carotenoids by high performance liquid chromatography. *J. Clin. Lab. Anal.* **1**, 147–152.

Mayne ST (1996): Beta-carotene, carotenoids, and disease prevention in humans. *FASEB.* **10**, 690–701 (review)

National Cholesterol Education Program (1994): Second report of the Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel II). *Circulation.* **89**, 1329–1445.

Weststrate JA & Meijer GW (1998): Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* **52**, 334–343.

III

Hallikainen MA, Sarkkinen ES, Uusitupa MIJ.
Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner.
J Nutr 2000;130:767-776.

liver, kidney and thyroid function, willingness to participate, no lipid-lowering medication, no unstable coronary heart disease, no alcohol abuse (>45 g of ethanol/d) and no irregular eating habits. Four subjects dropped out during the study: one during the pretrial period due to personal reasons, one during the first dose period due to prolonged constipation for which medication possibly affecting serum lipids (a plantago ovata product, Visiblin®) was prescribed, one during the second-dose period due to prolonged infection (bronchitis, stomatitis) and one during the fourth-dose period due to prostatitis. One subject had a hormone-releasing intrauterine device, one subject used hormone substitution medication, four subjects used postmenopausal hormone replacement therapy, and one subject used a calcium channel blocker and one subject renin-angiotensin system affecting medication for the treatment of hypertension, but he stopped the medication in the middle of the third-dose period. One of the subjects was a smoker. The subjects were requested to maintain their medication, weight, alcohol consumption, smoking habits and physical activity constant during the entire study. Baseline characteristics of 22 subjects are shown in **Table 1**.

The subjects gave their informed consent for the study, and study protocol was approved by the Ethics Committee of the University of Kuopio.

Study design. The study was carried out from January to June 1998 at the Department of Clinical Nutrition, University of Kuopio with a randomized single-blind, repeated measures design. After a 1-wk pretrial period, all subjects consumed five different doses of plant stanol added as plant stanol ester into the rapeseed oil-based margarine. Each dose was taken for 4 wk in the same order. The order of dose periods was randomly determined and was as follows: 2.4, 3.2, 1.6, 0 (control) and 0.8 g.

Routine laboratory measurements were taken to ensure normal health status at the first and at the last visit of the study. In addition, previous and present diseases, current medication, alcohol and tobacco consumption, physical activity, use of vitamins or other nutrient supplements were interviewed by a structured questionnaire at the first visit of the study. Alcohol and tobacco consumption and physical activity were reviewed also at the last visit to the study unit. Furthermore, possible changes in diseases, medication, use of vitamin or nutrient supplements were recorded during the study. Blood samples were taken from fasting subjects at the beginning of the pretrial period (−1 wk), at the beginning of the first dose period (0 wk) and at the middle and the end of each period (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 wk). A study period of 4-wk long was used to achieve the steady state with the present dose. Body weight was recorded at each visit. The possible adverse effects and symptoms were interviewed using a structured questionnaire at the end of each dose period.

TABLE 1

Baseline characteristics of the hypercholesterolemic human subjects during the study[1]

Variables	
Men, *n*	8
Women, *n*	14
Age, *y*	50.5 ± 11.7
Weight, *kg*	
Men	84.0 ± 9.7
Women	67.5 ± 10.2
Body mass index, *kg/m²*	
−1 wk	26.3 ± 3.4
0 g (16 wk)	26.2 ± 3.5
0.8 g (20 wk)	26.2 ± 3.5
1.6 g (12 wk)	26.3 ± 3.5
2.4 g (4 wk)	26.2 ± 3.5
3.2 g (8 wk)	26.4 ± 3.5
Lipids, *mmol/L*	
Total cholesterol	6.87 ± 1.28
LDL cholesterol	4.81 ± 1.07
HDL cholesterol	1.48 ± 0.35
Triglycerides	1.24 ± 0.43
Glucose, *mmol/L*	5.4 ± 0.4
Apolipoprotein E genotypes, *n*	
3:3	14
3:4	8

1 Values are means ± SD.

Diet. The composition of low erucic acid rapeseed oil-based margarines (Raisio Group Plc., Raisio, Finland) is presented in **Table 2**. The total amount of fat in the test margarines ranged between 70 and 81% and the amount of absorbable fat 68 and 70%. Added plant stanol ester-containing spreads were prepared using commercially available plant sterols by recrystallization, hydrogenation to form plant stanols and esterification to produce fatty acid esters of the obtained plant stanols. The daily dose of the test margarine was 25 g taken in two to three portions with meals. The daily amounts of total stanols based on the actual amount of stanols in the test spreads were 0 g containing no added stanols, 0.81 g (planned 0.8 g) consisting of 0.62 g sitostanol and 0.19 g campestanol, 1.56 g (planned 1.6 g) consisting of 1.19 g sitostanol and 0.37 g campestanol, 2.29 g (planned 2.4 g) consisting of 1.74 g sitostanol and 0.55 g campestanol and 3.03 g (planned 3.2 g) consisting of 2.30 g sitostanol and 0.73 g campestanol. During the pretrial period, the spread did not contain added stanols. Vitamin A (4.45 μg retinol equivalents/g) and vitamin D (0.064 μg/g) were added to each spread.

The subjects received the coded tubs of test margarines when visiting the study unit. To verify the precise daily dose of test fat 180 g, i.e., weekly dose of the plant stanol ester margarine was packed into the one tub and subjects were advised to mark the fat spread slightly with a knife into the seven equal parts in advance. One tub of test fat per week of the test period was delivered and one extra tub for the occasions of unexpected loss or damage of test products. The subjects were asked to record the use of test fats daily, and furthermore, to return the empty and partly empty tubs and the extra tub of test margarine to the study unit at the end of each period. The packages and the test spread left over were weighed and results recorded.

Subjects followed a standardized background diet throughout the study. The composition of the background diet resembled the habitual diet of the subjects and was the following: 34% of energy (E%)[3] from fat including <12 E% saturated, 14 E% monounsaturated and 8 E% polyunsaturated fat, and 23.8 mg/MJ dietary cholesterol. The subjects received oral and written instructions on the diet by food groups at their own energy level. The diet plan included precise amounts and quality of fat and cheese (low-fat), and only the precise quality of liquid milk (fat-free or low-fat) and meat products (low-fat). The diet plan was made for eight energy levels (6.7–12.6 MJ/d). Energy requirement of the subjects was estimated according to Harris-Benedict formula to which the energy needs as a result of physical activity were added (Alpers et al. 1986). The feasibility of the background and test diets was improved by providing test margarines, rapeseed oil, salad dressing and low-fat cheese for the subjects free of charge.

If the subject's habitual diet did not meet the goal for the composition, the diet was modified during the pretrial period. Adherence to the background diet was monitored by 3-d food records kept before the end of the pretrial period and by 4-d food records kept before the end of each dose period. One of the recording days was a weekend day or the person's day off from work. The subjects recorded their food consumption after consulting a booklet containing photographs of food portions (Haapa et al. 1985). At study visits, the amounts and qualities of foods in the records were checked by the nutritionist for completion, filling in data that were lacking.

The nutrient intake was calculated using the Micro-Nutrica® dietary analysis program (version 2.0; Finnish Social Insurance Institute, Turku, Finland). The food composition database is based on analyses of the Finnish food and international food composition

[3]Abbreviations used: apo , apolipoprotein; E%, energy percentage.

TABLE 2

Composition of daily dosage (25 g) of the control and plant stanol ester margarines

	Dose				
	0 g (Control)	0.8 g	1.6 g	2.4 g	3.2 g
	g/25 g spread				
Nutrients					
Total fat	17.5	18.2	18.7	19.3	20.2
Absorbable fat[1]	17.5	17.4	17.2	17.0	17.2
Total stanols	0	0.81	1.56	2.29	3.03
Total unsaturated sterols	0.10	0.13	0.15	0.17	0.21
Fatty acids:					
Polyunsaturated	3.8	3.9	3.8	3.8	3.9
Trans polyunsaturated	0.12	0.03	0.06	0.08	0.03
Monounsaturated	8.5	8.9	8.7	8.3	8.7
Trans monounsaturated	0.06	0	0	0	0
Saturated	4.2	3.8	3.8	3.9	3.7

[1] Absorbable fat = total fat − total stanol. The test margarines were rapeseed oil-based margarines. Rapeseed oil contains naturally unsaturated sterols. In the test margarines, a degree of sterol saturation of plant stanol ester was >97%, and therefore the unsaturated plant sterols of the plant stanol ester margarines were partly derived from the sterols contained in the vegetable oils and fats used and partly from the plant stanol ester.

tables (Rastas et al. 1993). In addition, the database was updated for the purposes of the present study.

Laboratory measurements. All measurements were done and venous blood samples were obtained after a 12-h overnight fast by using standardized methods. Body weight was measured with a digital scale. Since the phase of menstrual cycle may have an effect on serum cholesterol concentration (Cullinane et al. 1995) in premenopausal women, the study measurements at each dose period were performed at d 5–10 of the cycle.

Lipoproteins were separated by ultracentrifugation for 18 h at a density of 1.006 kg/L to remove VLDL fraction. HDL in the infranatant was separated from LDL by precipitation of LDL with dextran sulfate and magnesium chloride (Penttilä et al. 1981). LDL cholesterol was calculated as the difference between the mass of cholesterol in the infranatant and HDL, and VLDL cholesterol was calculated as the difference between the whole serum and the infranatant. Enzymatic photometric methods were used for the determination of cholesterol and triglycerides from whole serum and separated lipoproteins using commercial kits (Monotest® Cholesterol and Triglyceride GPO-PAP; Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) and a Kone Specific Clinical Analyser (Kone, Espoo, Finland).

Serum samples for α- and β-carotene, lycopene and fat-soluble vitamins, and apolipoprotein A-I (apo A-I) and B and plant sterols were stored at −70°C until analyzed at the end of the study. Analyses of apo were based on the measurement of immunoprecipitation enhanced by polyethylene glycol at 340 nm. A Kone Specific Clinical Analyzer and apo A-I and apo B reagents from Kone Corporation were used.

Serum α- and β-carotene, lycopene and fat-soluble vitamins were analyzed by the HPLC system (Perkin-Elmer, Norwalk, CT) equipped with a C18 column (Waters, Milford, MA) (Driskell et al. 1983, Kaplan et al. 1987, Parviainen 1983).

Serum plant sterols were measured by gas-liquid chromatograph (HP 5890 Series II, Hewlett Packard, Delaware, Little Falls, Wilmington, DE) from nonsaponifiable serum material equipped with 50-m long Ultra 1 capillary column (methyl-polysiloxane) [Hewlett-Packard, Little Falls, DE] for plant sterols and equipped with a 50-m long Ultra 2 capillary column (phenyl-methyl-siloxane) (Hewlett-Packard) for sitostanol and campestanol (Miettinen 1988, Miettinen and Koivisto 1983). Serum plant sterols were determined twice from same samples, and the mean values of two determinations were used in the statistical analysis.

Plasma glucose was analyzed by enzymatic photometric method using reagent Granutest 100 (Merck, Darmstadt, Germany) with a Kone Specific Clinical Analyser (Kone).

Apo E genotypes were analyzed with the restriction fragment length polymorphism-polymerase chain reaction method described by Tsukamoto et al. (1993) with a slight modification.

Statistical analyses. All statistical analyses were performed with SPSS for windows 6.0.1 statistics program (SPSS, Chicago, IL). The results are given as means ± SD in text and tables, and as means ± SEM in figure.

The main comparison was made among the mean values at the end of each dose period. In the Results and Discussion sections only these end measurements and their percentage changes are presented. The percentage changes were calculated comparing the end measurements of each dose period to the end measurement of the control period. To eliminate the effects of changes in lipoprotein concentrations, serum carotenoid, tocopherol and plant sterol values are given besides crude concentrations also in terms of mmol/mol of cholesterol, which express ratios to total cholesterol.

Normal distribution of variables was checked with Shapiro Wilks test before the further analyses (Norusis 1993). If a variable was not normally distributed, statistical analysis was made after logarithmic transformation. Repeated measures ANOVA was used to compare the overall changes in continuous variables among different dose periods. Two-tailed comparisons with paired t test were used in the further analyses. For variables (intake of alcohol, fiber and vitamin A, and serum α-carotene, β-carotene, lycopene and campestanol) which were not normally distributed not even after logarithmic transformation Friedman Two-tailed ANOVA test and Wilcoxon's matched-pairs signed rank test or Mann-Whitney test was used. To control the overall α level, Bonferroni adjustment was used. Wilcoxon's matched-pairs signed rank test was used to compare alcohol consumption, smoking habits and physical activity, which were reviewed by the questionnaires at the beginning and at the end of the study.

Power of the study was 0.80 based on assumption to be able to detect a 0.4–0.5 mmol/L difference in serum total cholesterol response between the different doses with the present number of subjects and probability for type I error $\alpha = 0.05$.

RESULTS

Baseline characteristics. Baseline characteristics of the subjects are presented in Table 1. Blood hemoglobin and thrombocytes, and serum thyroid stimulating hormone, γ-glutamyl and alanine amino transferase and creatinine were all within the normal ranges at the beginning and the end of study. Body mass index did not change significantly during the study (Table 1). Physical activity, alcohol consumption and

TABLE 3

Actual composition of the diet during consumption of the different doses (0, 0.8, 1.6, 2.4 and 3.2 g) of plant stanols[1]

Nutrients	Dose period					
	0 g (Control)	0.8 g	1.6 g	2.4 g	3.2 g	*P*-values[2]
Energy, *MJ/d*	7.9 ± 2.3	7.8 ± 2.0	7.4 ± 2.5	7.3 ± 2.0	7.7 ± 2.2	0.020[3]
Fat, *% of energy*	34.3 ± 4.9	33.4 ± 4.9	33.4 ± 4.3	32.5 ± 5.4	33.5 ± 4.2	0.319
Saturated fatty acids, *% of energy*	10.3 ± 2.2	9.4 ± 1.9	9.3 ± 1.3	8.5 ± 2.1[4]	9.3 ± 2.2	0.001
Monosaturated fatty acids, *% of energy*	13.9 ± 2.2	14.1 ± 2.5	14.2 ± 2.3	13.9 ± 2.7	14.2 ± 2.0	0.869
Polyunsaturated fatty acids, *% of energy*	7.5 ± 1.2	7.2 ± 1.1	7.2 ± 1.3	7.3 ± 1.2	7.2 ± 0.8	0.576
Proteins, *% of energy*	15.0 ± 1.8	16.3 ± 1.8	16.5 ± 1.8	16.4 ± 2.1	16.6 ± 2.2	0.003[3]
Carbohydrates, *% of energy*	46.5 ± 5.0	46.5 ± 5.7	47.5 ± 4.5	48.5 ± 6.0	47.2 ± 4.7	0.160
Alcohol, *% of energy*	2.9 ± 3.0	2.5 ± 4.7	1.2 ± 2.0[5]	1.3 ± 3.2[5]	1.3 ± 1.9[6]	0.002
Cholesterol, *mg/MJ*	20 ± 6	23 ± 8	21 ± 7	21 ± 8	23 ± 9	0.303
Fiber, *g/MJ*	2.9 ± 0.7	3.0 ± 0.7	3.4 ± 0.6[5,7]	3.6 ± 1.1[6,7]	3.3 ± 0.8	<0.001
Vitamin A, *μg RE/d*[8]	812 ± 351	885 ± 621	1106 ± 785	908 ± 465	1000 ± 654	0.682
β-carotene, *μg/d*	3288 ± 1931	3113 ± 2589	4098 ± 2902	4054 ± 2700	3518 ± 2118	0.193
Vitamin D, *μg/d*	3.8 ± 1.4	5.4 ± 3.7	3.9 ± 1.5	4.7 ± 2.4	4.5 ± 2.5	0.267
Vitamin E, *mg/d*	11.1 ± 2.7	10.7 ± 2.8	10.4 ± 2.8	11.1 ± 3.3	10.7 ± 3.2	0.523

[1] Values are means ± SD, $n = 22$. Subjects kept 4-d food record before the end of each dose period.
[2] Indicates the significance of the differences for overall changes during the dose-response study analyzed with repeated measures ANOVA or Friedman two-way ANOVA test (alcohol, fiber and vitamin A).
[3] There were no significant differences in intake of energy or protein in pairwise comparisons after Bonferroni correction among the dose periods.
[4,5,6] Significantly different from the control period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [4] $P < 0.001$, [5] $P < 0.05$, [6] $P < 0.01$.
[7] Significantly different from the 0.8-g dose period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): $P < 0.01$.
[8] RE = Retinol equivalents.

smoking habits remained stable according to questionnaires. During the study, five subjects had gastrointestinal symptoms (stomach pain/discomfort, flatulence or constipation), and two subjects had skin symptoms (eczema, itching or dry skin). The symptoms occurred occasionally and they were not related to the dose of stanol ester.

Feasibility of the diet. The mean daily consumption of margarine was between 25.2 and 25.5 g during the different dose periods. Thus the actual mean daily intake of stanol was 0.82 ± 0.0 g (0.63 ± 0.0 g sitostanol and 0.19 ± 0.0 g campestanol), 1.59 ± 0.02 g (1.22 ± 0.02 g sitostanol and 0.37 ± 0.0 g campestanol), 2.33 ± 0.05 g (1.77 ± 0.04 g sitostanol and 0.56 ± 0.01 g campestanol) and 3.05 ± 0.09 g (2.32 ± 0.07 g sitostanol and 0.74 ± 0.02 g campestanol) in the 0.8, 1.6, 2.4 and 3.2 g dose periods, respectively.

The actual composition of the diet during the different dose periods is presented in **Table 3**. There were no significant differences in the intake of fat, monounsaturated and polyunsaturated fatty acids, cholesterol, carbohydrates nor in the intake of fat-soluble vitamins and β-carotene among the different dose periods. However, the intake of saturated fatty acids was significantly lower (1.8 E%) during the 2.4-g dose period than during the control period, but there were no significant differences in the intake of saturated fatty acids between any other two dose periods. Furthermore, the intake of alcohol was significantly lower (difference 1.6–1.7 E%) during the 3.2, 2.4 and 1.6 g dose periods than during the control period. The intake of fiber was significantly lower (difference 0.5–0.6 g/MJ) during the control and 0.8-g dose periods than during the 2.4- and 1.6-g dose periods.

According to analyses of covariance performed, these differences in the intake of saturated fatty acids, alcohol and fiber among the different dose periods did not interfere with the results.

Serum lipids and lipoproteins. The concentrations of serum lipids, lipoproteins and apo at the end of each dose period are shown in **Table 4**. **Figure 1** (A,B,C) presents the percentage differences in serum total cholesterol, LDL cholesterol and apo B compared to the control dose, respectively.

The mean concentration of serum total cholesterol was significantly lower at the end of the 3.2-, 2.4- and 1.6-g dose periods than at the end of the 0.8-g dose and control periods (Table 4). There were no significant differences in the mean concentration of serum total cholesterol between the 3.2- and the 2.4-g dose periods, between the 2.4- and the 1.6-g dose periods, or between the 0.8-g dose and the control periods. The mean concentration of the serum total cholesterol tended to be lower ($P = 0.054$) at the end of the 3.2- than at the end of the 1.6-g dose period.

There were no significant differences in the mean concentration of the serum LDL cholesterol among the 3.2-, 2.4- and 1.6-g dose periods, or between the dose periods of the 1.6 g and the 0.8 g, or the 0.8 g and the control (Table 4). The mean concentration of the serum LDL cholesterol was significantly lower at the end of 3.2- and 2.4-g dose periods than at the end of the 0.8-g dose and control periods. Furthermore, serum LDL cholesterol concentration was significantly lower at the end of the 1.6-g dose than at the end of the control period. The percentage changes in serum total and LDL cholesterol concentration calculated in reference to control were parallel to the changes in mean concentration (Fig. 1).

The mean concentration of serum VLDL cholesterol did not differ significantly among the 3.2-, 2.4-, 1.6- and 0.8-g dose periods (Table 4). Furthermore, there was no significant difference in the mean concentration of serum VLDL cholesterol between the 0.8-g dose and the control periods. The mean concentration of the serum VLDL cholesterol was significantly

TABLE 4

Serum lipids, lipoproteins and apolipoproteins during the different doses (0, 0.8, 1.6, 2.4 and 3.2 g) of plant stanols in hypercholesterolemic human subjects[1]

	Dose period						
	0 g (Control)	0.8 g	1.6 g	2.4 g	3.2 g	*P*-values[2]	*P*-values[3]
				mmol/L			
Total cholesterol	6.51 ± 1.03	6.34 ± 1.17	6.06 ± 0.98[4,5]	5.82 ± 0.92[4,6]	5.75 ± 0.78[4,6]	<0.001	<0.001
LDL cholesterol	4.42 ± 0.95	4.35 ± 1.00	4.15 ± 0.87[7]	3.95 ± 0.75[4,5]	3.91 ± 0.69[4,6]	<0.001	<0.001
HDL cholesterol	1.51 ± 0.30	1.51 ± 0.30	1.52 ± 0.31	1.50 ± 0.37	1.50 ± 0.32	0.954	0.899
VLDL cholesterol	0.62 ± 0.25	0.49 ± 0.27	0.39 ± 0.22[4]	0.38 ± 0.29[4]	0.34 ± 0.23[4]	<0.001	0.060
Triglycerides	1.40 ± 0.65	1.20 ± 0.46	1.26 ± 0.63	1.29 ± 0.64	1.16 ± 0.56	0.136	0.614
				g/L			
Apolipoprotein A-I	1.43 ± 0.19	1.40 ± 0.21	1.42 ± 0.19	1.39 ± 0.23	1.40 ± 0.22	0.493	0.690
Apolipoprotein B	1.08 ± 0.23	0.98 ± 0.21[4]	0.97 ± 0.20[4]	0.97 ± 0.21[4]	0.92 ± 0.15[4]	<0.001	0.035

[1] Values are means ± SD.
[2] Indicate the significance of the differences for overall changes during the dose-response study analyzed with repeated measures ANOVA.
[3] Indicate the significance of the differences for overall changes among the 0.8, 1.6, 2.4 and 3.2 dose periods repeated measures ANOVA.
[4,7] Significantly different from the control period (paired *t* test and Bonferroni correction): [4] $P < 0.001$, [7] $P < 0.05$.
[5,6] Significantly different from the 0.8-g dose period (paired *t* test and Bonferroni correction): [5] $P < 0.05$, [6] $P < 0.001$.

lower at the end of the 3.2-, 2.4- and 1.6-g dose periods than at the end of the control period.

There were no significant changes in serum HDL cholesterol and total triglyceride concentrations during the entire study (Table 4).

Serum apo B decreased significantly at the dose of 0.8 g (8.7%, $P < 0.001$) compared to the control (Fig. 1). There were no significant differences in the mean concentration or percentage decrease of serum apo B in pairwise comparisons after Bonferroni correction among the 3.2-, 2.4-, 1.6- and 0.8-g dose periods (Table 4, Fig. 1).

Serum apo A-I concentration did not change significantly during the study (Table 4). Furthermore, there were no significant differences in serum apo A-I/apo B ratio among the 3.2-, 2.4-, 1.6- and 0.8-g dose periods. However, apo A-I/apo B ratio was significantly higher at the 3.2-, 1.6- and 0.8-g dose periods than at the control period and in addition, the apo A-I/apo B ratio tended to be higher ($P = 0.068$) at the 2.4-g dose than at the control period.

In a secondary analysis, there were no significant differences in the percentage changes of LDL cholesterol concentration between apo E 3:3 ($n = 14$) and 3:4 ($n = 8$) groups (−2.3 vs. −0.6%, −6.2 vs. −4.5%, −9.4 vs. −10.1% and −11.8 vs. −8.1%, apo E 3:3 vs. 3:4, at the 0.8-, 1.6-, 2.4- and 3.2-g dose periods vs. reference to control, respectively).

Plant sterols. The higher the dose, the lower the serum plant sterol concentration was (**Table 5**). The mean values of serum campesterol were 4.75 ± 3.01 μmol/L (24.5 ± 11.6%) to 8.67 ± 4.76 μmol/L (44.7 ± 10.4%) lower at the end of the test dose periods compared to the control period. Serum campesterol concentration was significantly lower at the end of the 3.2-, 2.4- and 1.6-g dose periods than at the end of the 0.8-g dose period. Furthermore, serum campesterol concentration was lower at the end of the 3.2-g dose period than at the end of the 2.4-g dose period. The changes in campesterol/total cholesterol ratio were parallel with the changes in absolute serum campesterol concentration (Table 5).

Changes in serum sitosterol and avenasterol concentrations were parallel with the changes in serum campesterol concentration (Table 5). Serum sitosterol concentrations were 2.21 ± 1.41 μmol/L (25.2 ± 10.7%) to 3.83 ± 2.02 μmol/L (43.9 ± 8.7%) lower at the end of the test dose periods compared to the end of the control period.

Serum plant stanol concentrations rose with increasing the dose. However, their concentrations remained very low in serum throughout the entire study. The serum concentrations of campestanol were 0.19 ± 0.11 μmol/L to 0.27 ± 0.16 μmol/L greater at the end of the test dose periods than at the end of the control period. In addition, serum campestanol concentration was significantly greater at the end of the 3.2-g dose period than at the of 0.8-g dose period. The campestanol/total cholesterol ratio was significantly greater (difference 0.04 ± 0.03–0.06 ± 0.03 mmol/mol of cholesterol) at the end of all dose periods than at the end of the control period (Table 5). In addition, the ratio was significantly greater at the end the 3.2-g dose period than at the end of the other test dose periods.

The serum sitostanol concentration was significantly greater (difference 0.19 ± 0.21–0.36 ± 0.25 μmol/L) at the end of all dose periods than at the end of the control period, but among the test dose periods there were no significant differences in serum sitostanol concentration (Table 5). Changes in the sitostanol/total cholesterol ratio (increase from 0.05 ± 0.04 to 0.08 ± 0.05 mmol/mol of cholesterol) were parallel to the absolute changes in sitostanol concentration, except that the ratio was significantly greater also at the end of the 3.2-g than at the end of the 0.8-g dose period.

Carotenoids and fat-soluble vitamins. There were no significant changes in serum retinol, α-carotene, β-carotene or α + β-carotene concentrations nor their ratios to the serum total cholesterol concentrations during the different dose periods (**Table 6**).

Serum lycopene concentrations differed significantly between men and women, and therefore, the results of serum lycopene are presented separately for men and women (Table 6). There were no significant differences in the serum lycopene concentrations in the men throughout the study. However, in

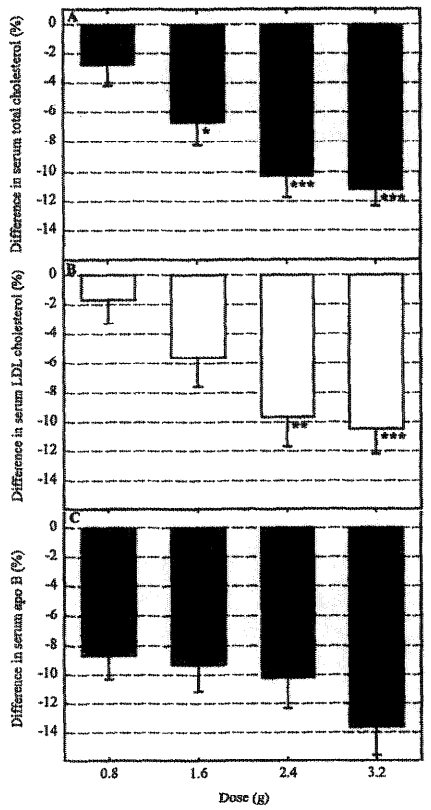


FIGURE 1 Serum total cholesterol (A), LDL cholesterol (B) and apolipoprotein (apo) B (C) concentration (%) during the different doses (0.8, 1.6, 2.4 and 3.2 g) of plant stanols in reference to the control (0 g/d) dose. Values are means ± SEM, $n = 22$. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ significantly different from the 0.8-g dose period (paired t test and Bonferroni correction). In pairwise comparisons after Bonferroni correction, there were no significant differences in percentage reductions of total and LDL cholesterol among the 1.6-, 2.4- and 3.2-g dose periods. In addition, there were no significant differences in percentage reduction of apo B concentrations among the doses of 0.8–3.2 g.

women, serum lycopene concentrations were significantly greater at the end of the control and 0.8-g dose periods than at the end of the 2.4-g dose period (Table 6). In women, the lycopene/total cholesterol ratio was significantly greater at the end of the 0.8-g dose period than at the end of 2.4-g dose and control periods (Table 6).

Serum α-tocopherol concentration was significantly lower at the end of all experimental dose periods than at the end of the control period. In addition, serum α-tocopherol concentration was significantly lower at the end of the 3.2-g dose period than at the end of the 0.8-g dose period (Table 6). Serum γ-tocopherol concentration was significantly lower only at the end of the 3.2-g and the 2.4-g dose periods than at the end of the control period (Table 6). Furthermore, the changes in serum $\alpha+\gamma$-tocopherol concentration were parallel to the changes in the serum α-tocopherol concentration during the trial (Table 6). However, after relating the serum α-, γ- and $\alpha+\gamma$-tocopherol to the serum total cholesterol concentration, there were no significant differences among the different periods.

Serum 25-hydroxycholecalciferol concentration was significantly lower at the end of the control than at the end of the 0.8-g dose period (Table 6). There were no significant differences in serum 25-hydroxycholecalsiferol concentration between any other dose periods after the Bonferroni correction.

DISCUSSION

The main purpose of the present study was to determine what the dose is of plant stanol ester beyond which no apparent additional benefit can be obtained and the lowest dose which has clinically significant cholesterol-lowering effect. Therefore, we determined the dose-response curve for serum total and LDL cholesterol with different doses of plant stanol ester. Significant reduction of serum total and LDL cholesterol concentration was reached with the dose of 1.6 g stanol and increasing the dose of stanol to the 2.4 or 3.2 g did not provide clinically significant additional effect. It should be noticed that the dose of 2.4 g decreased slighly more (0.2 mmol/L) serum cholesterol concentrations than the dose of 1.6 g, but this was not statistically significant.

Why does the cholesterol-lowering effect of stanol ester seem then to level off with higher doses? After hydrolysis, the cholesterol absorption is dependent on micelle formation, and the amount and type of bile acids influence this micelle formation and consequently cholesterol absorption. It is believed that the cholesterol-lowering effect of plant sterols and stanols is based on their competition with cholesterol incorporation into mixed micelles (Ikeda and Sugano 1998). It can be assumed that if there occurs an excessive amount of plant stanols in the small intestine to that of cholesterol, no additional benefit can be obtained with increasing doses of plant stanol esters. In adults, 1000–1500 mg of cholesterol, biliary and dietary origin, enters the lumen of small intestine daily. Therefore, the full saturation effect would be reached with doses of around 2.0–3.0 g of plant stanols, as also suggested by the present results.

The dose of 0.8 g stanol did not significantly affect serum total and LDL cholesterol concentrations, but resulted in 8.7% decrease in apo B concentration in comparison to the control dose, suggesting a reduction of apo B containing particles even with a low dose of stanol ester. The results of the present study are parallel to the results of previous studies, in which sitostanol has reduced total and LDL cholesterol concentrations, when the ingestion of sitostanol has been from 1.5 g (Becker et al. 1993, Heinemann et al. 1986) up to 3.4 g/d (Gylling and Miettinen 1994, Gylling et al. 1995 and 1997, Hallikainen and Uusitupa, 1999, Miettinen et al. 1995, Niinikoski et al. 1997, Vanhanen et al. 1993 and 1994, Weststrate and Meijer 1998). Our results are also in agreement with earlier studies (Hendriks et al. 1999, Miettinen and Vanhanen 1994, Vanhanen et al. 1994) in which it has been shown that at least 0.8–1 g/d of plant sterol should be consumed before clinically remarkable cholesterol-lowering effects can be observed. Stanol ester inhibits the cholesterol absorption so that less dietary and endogenous cholesterol enters via portal circulation the liver. Depletion of intracellular cholesterol in the liver could result in the upregulation of LDL receptor activity and con-

TABLE 5

Serum plant sterol and plant stanol concentrations during the different doses (0, 0.8, 1.6, 2.4 and 3.2 g) of plant stanols in hypercholesterolemic human subjects[1]

	Dose period						
	Control (0 g)	0.8 g	1.6 g	2.4 g	3.2 g	*P*-values[2]	*P*-values[3]
				μmol/L			
Campesterol	18.60 ± 7.31	13.85 ± 5.16[4]	11.11 ± 4.40[4,5]	10.84 ± 3.88[4,5]	9.94 ± 3.26[4,5,6]	<0.001	<0.001
Sitosterol	8.48 ± 3.43	6.26 ± 2.37[4]	5.17 ± 2.17[4,5]	5.15 ± 1.83[4,5]	4.65 ± 1.70[4,5,7]	<0.001	<0.001
Avenasterol	2.51 ± 0.67	2.00 ± 0.42[4]	1.76 ± 0.33[4,8]	1.84 ± 0.29[4,9]	1.72 ± 0.31[4,5]	<0.001	<0.001
Campestanol	0.18 ± 0.16	0.36 ± 0.17[4]	0.41 ± 0.19[4]	0.39 ± 0.14[10]	0.44 ± 0.17[4,9]	<0.001	<0.001
Sitostanol	0.67 ± 0.25	0.87 ± 0.25[4]	1.00 ± 0.32[4]	0.98 ± 0.31[4]	1.03 ± 0.29[4]	<0.001	0.086
				mmol/mol of cholesterol			
Campesterol	3.42 ± 1.25	2.72 ± 1.00[4]	2.19 ± 0.81[4,5]	2.18 ± 0.78[4,5]	2.04 ± 0.68[4,5]	<0.001	<0.001
Sitosterol	1.55 ± 0.56	1.23 ± 0.45[4]	1.02 ± 0.40[4,5]	1.04 ± 0.35[4,5]	0.95 ± 0.33[4,5,7]	<0.001	<0.001
Avenasterol	0.46 ± 0.09	0.39 ± 0.07[4]	0.35 ± 0.06[4,5]	0.37 ± 0.05[4,9]	0.35 ± 0.05[4,9]	<0.001	<0.001
Campestanol	0.03 ± 0.04	0.07 ± 0.04[4]	0.08 ± 0.04[4]	0.08 ± 0.03[10]	0.09 ± 0.04[4,9,11,12]	<0.001	<0.001
Sitostanol	0.12 ± 0.05	0.17 ± 0.06[4]	0.20 ± 0.06[4]	0.19 ± 0.05[4]	0.21 ± 0.05[4,9]	<0.001	0.019

[1] Values are means ± SD, $n = 22$.

[2] Indicate the significance of the differences for overall changes during the dose-response study analyzed with repeated measures ANOVA or Friedman two-way ANOVA test (campestanol).

[3] Indicate the significance of the differences for overall changes among the 0.8, 1.6, 2.4 and 3.2 dose periods repeated measures ANOVA or Friedman two-way ANOVA test (campestanol).

[4,10] Significantly different from the control period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [4] $P < 0.001$, [10] $P < 0.01$.

[5,8,9] Significantly different from the 0.8-g dose period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [5] $P < 0.001$, [8] $P < 0.01$ [9] $P < 0.05$.

[11] Significantly different from the 1.6-g dose period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [11] $P < 0.05$.

[6,7,12] Significantly different from the 2.4-g dose period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [6] $P < 0.001$, [7] $P < 0.01$, [12] $P < 0.05$.

sequently cause an enhanced clearance of apo B containing particles. It has been hypothesized by Gylling and Miettinen (Gylling and Miettinen 1994, Miettinen and Gylling 1999) that decreased VLDL and IDL cholesterol concentrations caused by removal of these cholesterol-rich particles also results in decrease of their conversion to LDL. This phenomenon might explain the small discrepancy in reduction of LDL cholesterol and apo B concentrations with the dose of 0.8 g, because one might expect a greater reduction in LDL cholesterol based on the change in apo B concentration which is the major apo in LDL particle. The other possibility is that LDL particles in circulation after ingestion of 0.8 g dose of stanol are more cholesterol-rich, but the results on VLDL levels in the present study support the first-described explanation, the enhanced clearance of apo B containing particles.

Serum campesterol concentration is shown to correlate positively with intestinal cholesterol absorption (Miettinen et al. 1990, Tilvis and Miettinen 1986). In the present study, serum campesterol, sitosterol and avenasterol concentrations were significantly lower already at the end of the 0.8-g dose period compared with the control period, reflecting that plant stanols inhibit effectively intestinal cholesterol absorption even with the small dose of stanol ester. Both in former human and animal absorption studies, sitostanol has been found to be minimally absorbed and campestanol to some extent (Hassan and Rampone 1979, Heinemann et al. 1993, Lütjohann et al. 1993, Xu et al. 1999). In this study, higher serum concentrations of sitostanol and campestanol at the end of the test dose periods as compared to the control period indicate that small amounts of sitostanol and campestanol are absorbed from the intestine. However, it should be noticed that the absorbed amounts are really neglible compared to the given dose; at the stanol dose of 0.8 and 3.2 g the increase of serum sitostanol in reference to control was only about 0.19 μmol/L (80.7 μg/L) and 0.36 μmol/L (149.2 μg/L), respectively. The respective increase in serum campestanol was 0.19 μmol/L (75.2 μg/L) and 0.27 μmol/L (108.3 μg/L). These results are in-line with the results of Gylling et al. (1999). Besides the negligible absorption of plant stanols, the low-serum concentrations could also result from the fast and effective clearance of absorbed stanols.

In the present study, all subjects consumed each test margarine in the same randomly determined order, and each subject served as his/her own control. The benefit of the present study design is that it eliminates the between-individual variation. The order of the dose periods was randomized to control for systematic bias due to the order of periods. According to the chosen design, the main comparisons were made between the mean values at the end of each period. Dose period of a 4-wk duration can be considered sufficient to eliminate the carry-over effect of the previous dose period to the next one, and in addition, to bring out the effects of a given dose on serum cholesterol concentrations. In earlier studies it has been shown that plant sterols reduce cholesterol concentrations within 2–3 wk of initiation of treatment (Jones et al. 1997). That is also in agreement with our previous study (Hallikainen and Uusitupa 1999). On the other hand, the serum cholesterol concentration returned to an initial value

TABLE 6

Serum carotenoids and fat-soluble vitamins during the different doses (0, 0.8, 1.6, 2.4 and 3.2 g) of plant stanols in hypercholesterolemic human subjects[1]

	Dose period						
	Control (0 g)	0.8 g	1.6 g	2.4 g	3.2 g	*P*-values[2]	*P*-values[3]
				μmol/L			
Retinol	2.92 ± 0.64	2.95 ± 0.66	2.84 ± 0.62	2.91 ± 0.57	2.89 ± 0.62	0.672	0.579
α-Carotene	0.28 ± 0.21	0.28 ± 0.23	0.30 ± 0.22	0.29 ± 0.19	0.29 ± 0.26	0.734	0.639
β-Carotene	0.64 ± 0.56	0.61 ± 0.54	0.60 ± 0.50	0.55 ± 0.45	0.55 ± 0.46	0.151	0.714
α- +β-Carotene	0.93 ± 0.76	0.88 ± 0.76	0.90 ± 0.72	0.85 ± 0.62	0.84 ± 0.69	0.373	0.504
Lycopene[4]							
Men	1.55 ± 1.27	1.76 ± 1.10	1.77 ± 1.37	1.67 ± 1.22	1.69 ± 1.31	0.645	0.896
Women	0.76 ± 0.41	0.95 ± 0.40	0.79 ± 0.51	0.56 ± 0.23[5,6]	0.89 ± 0.92	0.007	0.009
α-Tocopherol	46.56 ± 7.65	43.35 ± 6.43[7]	42.13 ± 6.79[8]	41.89 ± 7.27[8]	40.58 ± 6.17[6,8]	<0.001	0.017
γ-Tocopherol	2.12 ± 0.93	2.04 ± 0.79	1.93 ± 0.79	1.79 ± 0.79[5]	1.85 ± 0.79[5]	0.024	0.083
α + γ-Tocopherol	48.32 ± 8.37	45.25 ± 7.16[5]	44.17 ± 7.45[7]	43.86 ± 8.02[8]	41.97 ± 6.68[6,8]	<0.001	0.005
				nmol/L			
25-Hydroxycholecalciferol	64.65 ± 24.08	80.66 ± 32.80[5]	63.65 ± 25.69	66.96 ± 30.32	71.96 ± 30.23	0.025	0.030
				mmol/mol of cholesterol			
α-Carotene	0.05 ± 0.04	0.05 ± 0.05	0.05 ± 0.05	0.05 ± 0.04	0.05 ± 0.05	0.382	0.436
β-Carotene	0.10 ± 0.11	0.10 ± 0.11	0.11 ± 0.10	0.10 ± 0.09	0.10 ± 0.10	0.474	0.871
α + β-Carotene	0.15 ± 0.15	0.15 ± 0.15	0.16 ± 0.15	0.15 ± 0.13	0.15 ± 0.14	0.709	0.639
Lycopene[4]							
Men	0.27 ± 0.22	0.31 ± 0.18	0.32 ± 0.25	0.31 ± 0.22	0.33 ± 0.25	0.319	0.930
Women	0.12 ± 0.07	0.16 ± 0.08[5]	0.14 ± 0.11	0.10 ± 0.05[6]	0.16 ± 0.18	0.037	0.030
α-Tocopherol	7.18 ± 0.74	6.91 ± 0.74	6.99 ± 0.72	7.21 ± 0.74	7.06 ± 0.66	0.034	0.219
γ-Tocopherol	0.32 ± 0.14	0.32 ± 0.11	0.32 ± 0.12	0.31 ± 0.13	0.32 ± 0.12	0.963	0.953
α + γ-Tocopherol	7.40 ± 0.74	7.15 ± 0.75	7.22 ± 0.73	7.50 ± 0.84	7.28 ± 0.64	0.198	0.172

[1] Values are means ± SD, $n = 22$ except $n = 20$ for results concerning γ-tocopherol and α + γ-tocopherol, and in addition, for lycopene $n = 8$ in men and $n = 10$ in women.

[2] Indicates the significance of the differences for overall changes during the dose-response study analyzed repeated measures ANOVA or Friedman two-way ANOVA test (α-carotene, β-carotene, α + β-carotene and lycopene).

[3] Indicates the significance of the differences for overall changes among the 0.8, 1.6, 2.4 and 3.2 dose periods analyzed repeated measures ANOVA or Friedman two-way ANOVA test (α-carotene, β-carotene, α + β-carotene and lycopene).

[4] The changes in serum lycopene concentrations differed between men and women during the different dose periods, and therefore the results were expressed and the analyses were made separately to men and women.

[5,7,8] Significantly different from the control period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [5] $P < 0.05$, [7] $P < 0.01$, [8] $P < 0.001$.

[6] Significantly different from the 0.8-g dose period (paired *t* test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [6] $P < 0.05$.

within 2–3 wk, upon cessation of the ingestion of plant sterols (Farquhar et al. 1956, Heinemann et al. 1986).

The differences in the nutrient intake among the different dose periods were occasional and minor. Thus the differences in lipid responses among the dose periods can be ascribed to the differences in the amount of active compound rather than differences in background diet. Furthermore, body mass index did not change significantly during the study.

Serum VLDL cholesterol concentration was significantly lower at the 3.2-, 2.4- and 1.6-g dose periods compared with the control period. The significant decrease could be due to the effect of plant stanols, but it could more likely be ascribed to slightly, but significantly, higher alcohol consumption, which might have increased VLDL cholesterol concentration at the end of the control period (Steinberg et al. 1991). Temporary increased alcohol consumption was probably due to the fact that the eve of May Day and May Day were at the end of the control period. In Finland, alcohol consumption belongs to the celebration of May Day.

It has been assumed that sitostanol ester could reduce serum total and LDL cholesterol concentration more effectively in subjects with the apo E allele 4 than those with allele 2 or 3 (Miettinen and Vanhanen 1994, Vanhanen et al. 1993), but our results do not support this assumption. In the present study there were no significant differences in percentage reduction in LDL cholesterol between subjects with apo genotype 3:3 and 3:4 during the different dose periods. However, when interpreting this result, it should be kept in mind that the sizes of two apo E genotype groups were rather small.

During the study there were no significant changes in the concentrations of serum retinol, α- and β-carotene, and in the concentration of serum tocopherols related to the serum total cholesterol concentrations. Serum lycopene concentration did not change significantly in men during the study, whereas in

women there were significant differences among the different dose periods even after standardization for serum total cholesterol concentration. However, the differences were not related to the dose of stanol ester. Women had lower concentrations of serum lycopene than men, which could be due partly to their older age (Vogel et al. 1997) (mean age 52 vs. 42 y, women vs. men). Also the changes in serum 25-hydroxycholecalsiferol were not related to the dose of stanol ester. Based on previous plant sterol studies (Gylling et al. 1996, Hallikainen and Uusitupa 1999, Hendriks et al. 1999, Weststrate and Meijer 1998), it seems that plant sterol would have some effect on serum carotenoid concentrations. However, the results are variable: Before lipid standardization serum carotenoid concentrations might have decreased significantly, but after lipid standardization these changes have not usually been great or significant (Gylling et al. 1996, Hallikainen and Uusitupa 1993, Hendriks et al. 1999, Weststrate and Meijer 1998). These differences in the results can not be due to different carotenoid contents in test margarines, because both test and control margarines have been similarly vitaminized in those studies (Gylling et al. 1996, Hallikainen and Uusitupa 1999, Hendriks et al. 1999, Weststrate and Meijer, 1998). Therefore, the differences in the results might be a consequence of variability in composition of background diets. The diets in the studies of Gylling et al. (1996), Hendriks et al. (1999) and Weststrate and Meijer (1998) were not standardized like in our earlier (Hallikainen and Uusitupa 1999) or present study. In our studies the instructions for intake of vegetables were given to the subjects. According to our findings, the effects of plant sterol on serum carotenoid concentrations were minor and clinically unimportant. However, additional studies will be needed to discover long-term effects of plant sterol on carotenoid concentrations.

In conclusion, significant reduction of serum total and LDL cholesterol concentrations is reached with the dose of 1.6 g stanol, and increasing the dose of stanol from 2.4 g to 3.2 g does not provide clinically significant additional benefits. Interestingly, the 0.8-g dose of stanol resulted in 8.7% reduction in apo B concentration. Serum plant stanol concentrations rose slightly with the dose; however, their concentrations remained extremely low in serum.

ACKNOWLEDGMENTS

The authors thank Raisa Valve for the analysis of apolipoprotein E genotypes and Helena Gylling, Leena Kaipiainen, Ritva Nissilä and Pia Hoffström for the analyses of serum plant sterols and plant stanols.

LITERATURE CITED

Alpers, D. H., Clouse, R. E. & Stenson, W. F. (1988) Protein and Calorie Requirements. In: Manual of Nutritional Therapeutics, pp. 131–159. Little, Brown and Company, Toronto.

Becker, M., Staab, D. & von Bergmann, K. (1993) Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. J. Pediatr. 122: 292–296.

Cullinane, E. M., Yurgalevitch, S. M., Saritelli, A. L., Herbert, P. N. & Thompson, P. D. (1995) Variations in plasma volume affect total and low-density lipoprotein cholesterol concentrations during the menstrual cycle. Metabolism 44: 965–971.

Driskell, W. J., Bashor, M. M. & Neese, J. W. (1983) Beta-carotene determined in serum by liquid chromatography with an internal standard. Clin. Chem. 29: 1042–1044.

Farquhar, J. W., Smith, R. E. & Dempsey, M. E. (1956) The effect of beta sitosterol on the serum lipids of young men with arteriosclerotic heart disease. Circulation XIV: 77–82.

Gylling, H. & Miettinen, T. A. (1994) Serum cholesterol and cholesterol and lipoprotein metabolism in hypercholesterolaemic NIDDM patients before and during sitostanol ester-margarine treatment. Diabetologia 37: 773–780.

Gylling, H., Puska, P., Vartiainen, E. & Miettinen, T. A. (1996) Serum retinol, α-tocopherol, carotenes and lipid peroxide production during serum cholesterol lowering by sitostanol ester margarine in a mildly hypercholesterolemic population. Circulation 94(Suppl. 1): 678. (abs.).

Gylling, H., Puska, P., Vartiainen, E. & Miettinen, T. A. (1999) Serum sterols during stanol ester feeding in a mildly hypercholesterolemic in a mildly hypercholesterolemic population. J. Lipid Res. 40: 593–600.

Gylling, H., Radhakrishnan, R. & Miettinen, T. A. (1997) Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary sitostanol ester margarine. Women and dietary sitostanol. Circulation 96: 4226–4231.

Gylling, H., Siimes, M. & Miettinen, T. A. (1995) Sitostanol ester margarine in dietary treatment of children with familial hypercholesterolemia. J. Lipid Res. 36: 1807–1812.

Haapa, E., Toponen, T., Pietinen, P. & Räsänen, L. (1985) Annoskuvakirja. (Portion size booklet.) Sosial Insurance Institution, Helsinki, Finland (in Finnish).

Hallikainen, M. A. & Uusitupa, M. I. J. (1999) Effects of two low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. Am. J. Clin. Nutr. 69: 403–410.

Hassan, A. S. & Rampone, A. J. (1979) Intestinal absorption and lymphatic transport of cholesterol and β-sitostanol in rats. J. Lipid Res. 20: 646–653.

Heinemann, T., Axtmann, G. & von Bergmann, K. (1993) Comparison of intestinal absorption of cholesterol with different plant sterols in man. Eur. J. Clin. Invest. 23: 827–831.

Heinemann, T., Leiss, O. & von Bergmann, K. (1988) Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. Atherosclerosis 61: 219–223.

Hendriks H. F. J., Weststrate J. A., van Vlient T. & Meijer G. W. (1999) Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. Eur. J. Clin. Nutr. 53: 319–327.

Ikeda, I. & Sugano, M. (1998) Inhibition of cholesterol absorption by plant sterols for mass intervention. Curr. Opin. Lipidol. 9: 527–531.

Jones, P. J. H., MacDougall, D. E., Ntanios, F. & Vanstone, C. A. (1997) Dietary phytosterols as cholesterol-lowering agents in humans. Can. J. Physiol. Pharmacol. 75: 217–227.

Kaplan, L. A., Miller, J. A. & Stein, E. A. (1987) Simultaneous measurement of serum retinol, tocopherols, carotenes, and carotenoids by high performance liquid chromatography. J. Clin. Lab. Anal. 1: 147–152.

Lütjohann, D., Meese, C. O., Crouse, J. R. & von Bergmann, K. (1993) Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in human. J. Lipid Res. 34: 1039–1046.

Miettinen, T. A. (1988) Cholesterol metabolism during ketoconazole treatment in man. J. Lipid Res. 29: 43–51.

Miettinen T. A. & Gylling H. (1999) Regulation of cholesterol metabolism by dietary plant sterols. Curr. Opin. Lipidol. 10: 9–14.

Miettinen, T. A. & Kolvisto, P. (1983) Non-cholesterol sterols and bile acid production in hypercholesterolaemic patients with ileal bypass. In: Bile Acid and Cholesterol in Health and Disease (Paumgartner, G., Stiehl, A. & Gerok, W., ed.) pp. 183–187. MTP Press, Lancaster.

Miettinen, T. A., Puska, P., Gylling, H., Vanhanen, H. & Vartiainen, E. (1995) Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. N. Engl. J. Med. 333: 1308–1312.

Miettinen T. A., Tilvis R. S. & Kesäniemi Y. A. (1990) Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am. J. Epidemiol. 131: 20–31.

Miettinen, T. A. & Vanhanen, H. T. (1994) Dietary sitostanol related to absorption, synthesis and serum level of cholesterol in different apolipoprotein E phenotypes. Atherosclerosis 105: 217–226.

Niinikoski, H., Viikari, J. & Palmu, T. (1997) Cholesterol-lowering effect and sensory properties of sitostanol ester margarine in normocholesterolemic adults. Scand. J. Nutr. 41: 9–12.

Norusis, M. J. (1993) SPSS for Windows Base System User's Guide. Release 6 0: SPSS, Chicago, IL.

Parviainen, M. T. (1983) The fat-soluble vitamins A, D and E - their metabolism, binding-proteins and determination from human serum. Acta Universitatis Tamperensis 159: 49–54.

Penttilä, I. M., Voutilainen, E., Laitinen, O. & Juutilainen, P. (1981) Comparison of different analytical and precipitation methods for the direct estimation of high-density lipoprotein cholesterol. Scand. J. Clin. Lab Invest. 41: 353–360.

Pollak, O. J. & Kritchevsky, D. (1981) Phytosterols-sitosterol as cholesterol depressants. In: Sitosterol. Monographs on Atherosclerosis. Vol. 10, pp. 60–118. Karger, Basel, Switzerland.

Rastas, M., Seppänen, R., Knuts, L.-R., Karvetti, R.-L. & Varo, P. (1993) Nutrient Composition of Foods. Social Insurance Institution, Helsinki, Finland.

Steinberg, D., Pearson, T. A. & Kuller, L. H. (1991) Alcohol and atherosclerosis Ann. Intern. Med. 114: 967–976.

Tilvis R. S. & Miettinen T. A (1986) Serum plant sterols and their relation t cholesterol absorption. Am. J. Clin. Nutr. 43: 92–97.

Tsukamoto, K., Watanabe, T., Matsushima, T., Kinoshita, M., Kato, H., Hashmoto, Y., Kurokawa, K. & Teramoto, T. (1993) Determination by PCR

IV

Hallikainen MA, Sarkkinen ES, Gylling H, Uusitupa MI. Plant stanol esters affect serum plant sterols, but not in serum cholesterol precursors in a dose-dependent manner in hypercholesterolemic subjects.

Plant stanol esters affect serum plant sterols, but not serum cholesterol precursors in a dose-dependent manner in hypercholesterolemic subjects

Maarit A. Hallikainen, Essi S. Sarkkinen, Helena Gylling and Matti I. J. Uusitupa
Department of Clinical Nutrition, University of Kuopio

Background and Aims: *Plant stanol/sterol esters lower serum cholesterol concentrations due to decreased cholesterol absorption from intestine. Measurements of serum cholesterol precursors and plant sterols can be used as an estimate of cholesterol synthesis and absorption, respectively. We studied the effects of different doses of plant stanol esters on serum cholesterol precursor, and plant sterol and stanol concentrations in hypercholesterolemic subjects in order to get more comprehensive view on the hypocholesterolemic actions of plant stanols.*

Methods and Results: *In a single-blind study each of 22 subjects consumed five different doses of plant stanol [target (actual) intake 0 (0), 0.8 (0.8), 1.6 (1.6), 2.4 (2.3), 3.2 (3.1) g/day] added as stanol esters to margarine for four weeks. The order of dose periods was randomly determined. As compared with control (0 g) a significant increase in the serum Δ7-lathosterol/total cholesterol ratio, a main indicator of cholesterol synthesis, was found with the dose of 1.6 g ($P<0.001$). This ratio leveled off with higher doses. Serum campesterol and sitosterol concentrations, main indicators of cholesterol absorption efficiency, decreased by 25 - 45% by the doses from the 0.8 g to 3.2 g ($P<0.001$ for all). The increases in serum campestanol and sitostanol concentrations were significant with all stanol ester doses, but the increase was only slight after the dose of the 0.8 g.*

Conclusions: *Judged from the serum Δ7-lathosterol/total cholesterol ratio cholesterol synthesis may not further increase when the dose of stanol is higher than 1.6 g. Absorption of cholesterol is inhibited already by the 0.8 g stanol dose.*

Serum plant sterol concentrations have been found to reflect intestinal cholesterol absorption in subjects on normal diets (unsupplemented with plant sterols or stanols) (1, 2). In turn, serum cholesterol precursors have been found to reflect endogenous cholesterol synthesis (2-4).

Plant stanol/sterol esters containing products are commonly used for cholesterol-lowering, because of their ability to inhibit the absorption of cholesterol. In addition to the inhibition of cholesterol, plant stanols inhibit the absorption of plant sterols, and thus reduce their serum concentrations, but at the same time plant stanol concentrations have been found to increase to a small extent (5-7). Although plant stanol esters containing products are widely studied, the effects of the different doses of plant stanol esters on the serum concentrations of campesterol and sitosterol as well as campestanol and sitostanol have not been investigated systematically as have been investigated the effects of them on serum lipid responses.

The effects of serum plant stanol esters on cholesterol synthesis have been investigated in several studies by determining serum cholesterol precursors (5, 6, 8-14), using sterol balance technique (9-13) or deuterated water (15). However, published data on the dose responsiveness of plant stanol esters on cholesterol synthesis is scarce and contradictory. Therefore, it is still unclear, whether the cholesterol synthesis is stimulated in a dose dependent manner as a response of cholesterol malabsorption and hepatic cholesterol depletion or whether it levels off with the greater doses.

The aim of this study was to investigate the effects of different doses of plant stanol esters on cholesterol synthesis by determining serum cholesterol precursors. In addition, the effects of different doses of plant stanol esters on serum plant sterol and plant stanol concentrations were investigated to obtain information on the absorption of cholesterol, plant sterols, and plant stanols.

METHODS

Subjects

Altogether 26 (10 men and 16 women) subjects were recruited to the study (16). The mean age of 22 subjects who completed the study was 50.5 ± 11.7 y and the mean body mass index (BMI) was 26.3 ± 3.4 kg/m^2. The mean baseline total and LDL cholesterol concentrations were 6.87 ± 1.28 and 4.81 ± 1.07

Table 1. The daily intake of plant stanols and sterols during different dose periods from 25 g of the control and stanol ester margarines/ the actual mean daily intake of them (16).

	Dose				
	Control (0 g)	0.8 g	1.6 g	2.4 g	3.2 g
Total plant stanols (g)	0/ 0	0.81/ 0.82	1.56/ 1.59	2.29/ 2.33	3.03/ 3.05
Sitostanol (g)	0/ 0	0.62/ 0.63	1.19/ 1.12	1.74/ 1.77	2.30/ 2.32
Campestanol (g)	0/ 0	0.19/ 0.19	0.37/ 0.37	0.55/ 0.56	0.73/ 0.74
Total plant sterols (g)	0.10/ 0.10	0.13/ 0.13	0.15/ 0.15	0.17/ 0.17	0.21/ 0.21

mmol/L, respectively. The study protocol was approved by the Ethics Committee of the University of Kuopio, and all subjects gave their written consent.

Study design

The study was carried out with a randomized single blind repeated measures design. After a 1-wk pre-trial period all subjects consumed five different doses of plant stanols added as plant stanol ester into the rapeseed oil-based margarine. Each dose was taken for 4 wk in the same randomized order. The order was randomly determined and was as follows: 2.4 g, 3.2 g, 1.6 g, 0 g (control) and 0.8 g.

The routine laboratory measurements were taken to ensure normal health status. Alcohol and tobacco consumption, physical activity, possible medication and use of vitamins or other nutrient supplements were reviewed by structured questionnaires. Blood samples were taken from fasting samples at the beginning of the pre-trial period (-1 wk), at the beginning of the first dose period (0 wk), and at the middle and the end of each period (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 wk). Serum lipids were determined from blood samples at every visit, but the main comparisons were made among the mean values at the end of each dose periods. Samples for serum cholesterol precursors, plant sterols and cholestanol were taken only at the end of each dose periods (16). A study period of 4-wk long was used to achieve the steady state with each dose. Body weight was recorded at each visit.

Diet

Low-erucic acid rapeseed oil-based test margarines were prepared by Raisio Group Plc. (Raisio, Finland). The composition of the test margarines has been described previously in detail (16). The amounts of total stanols based on the actual amount of stanols in the test margarines are presented per daily dose (25 g) in Table 1. The amount of absorbable fat in the test margarines was 68 - 70 %. Added plant stanol ester containing margarines were prepared using wood and vegetable sterols (DRT, Les Derives Resiniques & Terpeniques Granel S.A. Dax Cedex, France and Archer Daniels Midland Co, Decatur, IL, respectively). The daily dose of the test margarines was taken in 2 to 3 portions with the meals. The subjects recorded the use of the test margarines daily, and in addition, the nutritionist weighed the returned packages and test spread left over and recorded the result at the end of each period.

Subjects followed a standardized background diet, which resembled their habitual diet, throughout the study. The composition of diet was the following: 34 % of energy (E%) from fat, including <12 E% saturated, 14 E% monounsaturated and 8 E% polyunsaturated fat, and 23.8 mg/ MJ dietary cholesterol. The subjects received oral and written instructions on the isocaloric diet and the use of the test margarines (16). Adherence to the background diet was monitored by 3-d food record kept before the end of the pre-trial period and by 4-d food record (three weekdays and one weekend day or day off from work) kept before the end of each dose periods. The nutrient intake was calculated using the Micro-Nutrica® dietary analysis program (version 2.0, Finnish Social Institute, Turku, Finland). The food composition database is based on the combination of analyses of the Finnish food and values derived from international food composition tables (17).

Laboratory measurements

Venous blood samples were obtained after a 12-h overnight fast. An enzymatic photometric method was used for the determination of serum total cholesterol using commercial kits (Monotest ® Cholesterol and Triglyceride GPO-PAP, Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) and a Kone Specific Clinical Analyser (Kone Ltd., Espoo, Finland). The coefficient of variation between measurements for serum total cholesterol was 0.9 - 1.6%.

Serum cholesterol, cholesterol precursors (Δ8-cholestenol, Δ7-lathosterol, desmosterol and squalene), plant sterols (sitosterol, campesterol, avenasterol, sitostanol and campestanol) and cholestanol, a metabolite of cholesterol, were analyzed at the end of the study from the samples stored at -70°C. Cholesterol, cholesterol precursors, plant sterols and cholestanol were measured by gas-liquid chromatograph (GLC) (HP 5890 Series II, Hewlett Packard, Delaware, Little Falls, Wilmington, DE) (18, 19) from nonsaponifiable serum material equipped with 50-m long Ultra 1 capillary column (methyl-polysiloxane, Hewlett Packard, Delaware,

Little Falls, DE), and sitostanol and campestanol with a 50-m long Ultra 2 capillary column (Phenyl-methyl-siloxane, Hewlett Packard). 5α-cholestane was used as an internal standard in cholesterol analysis and epi-coprostanol in other analyses. Serum cholesterol, cholesterol precursors, plant sterols and cholestanol were determined in duplicate from the same samples and the mean value of two determinations were used in the statistical analysis. To eliminate the effect of changes in serum cholesterol, serum cholesterol precursors, plant sterol and cholestanol values are presented besides absolute concentrations also in terms of mmol/mol of cholesterol (GLC), which express ratios to total cholesterol.

Statistical analyses

All statistical analyses were performed with SPSS for windows 6.0.1 statistics program (SPSS, Chicago, IL, USA) (20). Variables, which were normally distributed were tested with repeated measures ANOVA. Two-tailed comparisons with paired t test were used in the further analyses. For variables which were not normally distributed not even after logarithmic transformation Friedman Two-tailed ANOVA test and Wilcoxon's matched-pairs signed rank test was used. To control the overall α level, Bonferroni adjustment was made. A P-value of <0.05 was considered significant. The results are expressed as the means ± SDs or means in text and tables and as means ± SEMs in figure.

RESULTS

Results on the intake of nutrients and the changes in serum lipids have been presented elsewhere in detail (16). Serum total cholesterol concentration reduced (calculated in reference to control) by 2.8% (P=0.384), 6.8% (P<0.001), 10.3% (P<0.001) and 11.3% (P<0.001) when plant stanol doses from 0.8 g to 3.2 g were consumed (Figure 1). There were no significant changes in BMI (varied between 26.2 ± 3.5 and 26.4 ± 3.5 kg/m^2) during the study. In addition, physical activity, smoking habits and alcohol consumption remained stable according to questionnaires.

Diet

The mean daily consumption of test margarines was between 25.2 and 25.5 g. Actual daily intakes of plant stanols as mean values are presented in Table 1. During the dose periods the mean intake of fat was 32.5 - 34.3 E%, saturated fatty acids 8.5 - 10.3 E%, alcohol 1.2 - 2.9 E%, dietary cholesterol 20 - 23 mg/MJ and dietary fiber 2.9 - 3.6 g/MJ (16).

Serum cholesterol precursors and cholestanol

There were only small and non-consistent changes in the absolute values of serum cholesterol precursor concentrations during the different dose periods (Table 2). Serum Δ7-lathosterol/total cholesterol ratio, the main indicator of cholesterol synthesis (2-4), increased compared with the control by 13.3 - 19.4% by the doses from the 0.8 to 3.2 g, and the increase was significant with the doses of 1.6 g, 2.4 g and 3.2 g (Figure 1). Serum Δ8-cholestenol/total cholesterol ratio did not increase so consistently than Δ7-lathosterol/total cholesterol ratio did, however, the ratio was significantly greater with the dose of the 2.4 g than with the control, and also greater with the doses of the 1.6 g and 2.4 g than with the dose of the 0.8 g (data not shown). In addition, the ratios of serum desmosterol and squalene to total cholesterol rose along with the dose, but not significantly (data not shown).

Serum cholestanol concentration, which reflects cholesterol absorption (21), reduced significantly with the doses of the 1.6 g, 2.4 g and 3.2 g compared with the control (Table 2). However, the serum cholestanol/total cholesterol ratio remained almost unchanged during the entire study (data not shown).

Serum plant sterols

Serum campesterol, sitosterol and avenasterol concentrations decreased significantly during all dose periods compared with the control period. The average serum campesterol concentration was 24.5% to 44.7%, sitosterol concentration was 25.2% to 43.9% and avenasterol concentration was 19.2% to 29.4% lower with the doses from 0.8 g to 3.2 g. Furthermore, the serum concentrations of these three plant sterols were significantly lower with the doses of the 1.6 g, 2.4 g and 3.2 g compared with the dose of the 0.8 g indicating a greater reduction of cholesterol absorption with higher daily intake of plant stanols. In addition, serum campesterol and sitosterol concentrations were significantly lower with the dose of the 3.2 g than with the dose of the 2.4 g. The changes in the ratios of plant sterols to total cholesterol paralleled the changes in absolute serum plant sterol values. The results on the campesterol/total cholesterol ratio are shown in Figure 1.

Serum plant stanol concentrations remained very low throughout the study (Table 2). Serum campestanol concentration was doubled with the dose of the 0.8 g compared with the control.

Table 2. Serum cholesterol precursors, plant sterols and cholestanol (μmol/L) during the different dose periods.

	Period						
	Control (0 g)	Dose 0.8 g	Dose 1.6 g	Dose 2.4 g	Dose 3.2 g	P^a	P^b
Squalene	1.97 ± 0.68	1.89 ± 0.41	1.99 ± 0.60	1.96 ± 0.74	2.05 ± 0.58	0.916	0.824
Δ8-cholestenol	1.11 ± 0.43	1.03 ± 0.36	1.19 ± 0.49	1.25 ± 0.55	1.12 ± 0.48	0.022	0.016
Δ7-lathosterol	7.31 ± 2.83	7.40 ± 2.23	7.80 ± 2.83	7.72 ± 2.61	7.74 ± 2.76	0.295	0.519
Desmosterol	4.84 ± 1.10	4.63 ± 0.95	4.71 ± 1.10	4.71 ± 1.17	4.66 ± 1.03	0.706	0.921
Campesterol	18.60 ± 7.31	13.85 ± 5.16^{c}	11.11 ± 4.40^{c,d}	10.84 ± 3.88^{c,d}	9.94 ± 3.26^{c,d,e}	<0.001	<0.001
Sitosterol	8.48 ± 3.43	6.26 ± 2.37^{c}	5.17 ± 2.17^{c,d}	5.15 ± 1.83^{c,d}	4.65 ± 1.70^{c,d,f}	<0.001	<0.001
Avenasterol	2.51 ± 0.67	2.00 ± 0.42^{c}	1.76 ± 0.33^{c,g}	1.84 ± 0.29^{c,h}	1.72 ± 0.31^{c,d}	<0.001	<0.001
Campestanol	0.18 ± 0.16	0.36 ± 0.17^{c}	0.41 ± 0.19^{c}	0.39 ± 0.14^{i}	0.44 ± 0.17^{c,h}	<0.001	<0.001
Sitostanol	0.67 ± 0.25	0.87 ± 0.25^{c}	1.00 ± 0.32^{c}	0.98 ± 0.31^{c}	1.03 ± 0.29^{c}	<0.001	0.086
Cholestanol	7.63 ± 1.62	7.25 ± 1.40	6.95 ± 1.25^{c}	7.03 ±1.11^{i}	6.93 ±1.28^{c}	<0.001	0.253

Values are mean ± SD, n=22.

[a] indicates the significance of the differences for overall changes during the different dose periods

[b] indicates the significance of the differences for overall changes among the 0.8, 1.6, 2.4 g and 3.2 g dose periods analyzed with analysis of repeated measures ANOVA or Friedman Two-way ANOVA test (campestanol)

[c, i] Significantly different from the control period (paired t test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [c] P<0.001, [i] P<0.01.

[d, g, h] Significantly different from the 0.8 g dose period (paired t test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [d] P<0.001, [g] P<0.01, [h] P<0.05.

[e, f] Significanly different from the 2.4 g dose period (paired t test or Wilcoxon's matched-pairs signed rank test and Bonferroni correction): [e] P<0.001, [f] P<0.01.

Furthermore, these values increased slightly with the greater doses. Similarly, the greatest increase in serum sitostanol concentration was already reached with the 0.8 g dose and the increase was only slightly higher with the greater doses. The changes in the ratios of campestanol and sitostanol to total cholesterol paralleled the respective changes in absolute serum campestanol and sitostanol values.

DISCUSSION

The main result of the present study is that serum Δ7-lathosterol/total cholesterol ratio, the main indicator of endogenous cholesterol synthesis (2-4), increased compensatorily due to cholesterol malabsorption induced by plant stanols. Interestingly, the increase did not occur in a dose-dependent manner. Because the ratio did not actually increase with the dose greater than the 1.6 g, this probably mean that the endogenous cholesterol synthesis may have reached its maximum level with the doses of 1.6 - 2.4 g of plant stanols. Despite this increased cholesterol biosynthesis plant stanol esters induced (7 - 11 %) decreases in serum total cholesterol concentrations. This can be attributed to depletion of cholesterol in hepatocytes, which, on the other hand, results in an enhanced LDL receptor activity and consequently reduced serum concentrations of cholesterol rich LDL, IDL and VLDL particles (22).

Serum campesterol concentration has been shown to reflect cholesterol absorption efficiency from intestine (1, 2). In the present study, the greater the stanol ester dose was the greater the reduction in serum campesterol concentration or its ratio to serum total cholesterol was. Furthermore, significant reductions in them were reached already with the 0.8 g stanol dose indicating that plant stanols can effectively inhibit intestinal cholesterol absorption even with a rather small dose. This finding is in agreement with the findings of some previous studies where 0.6 - 0.8 g/d of plant stanols (unesterified or esterified form) were shown to reduce serum plant sterol concentrations up to 30 % (9, 10, 23). Our finding agrees also with the reports in which 0.7-0.8 g/d of plant stanol as stanol esters effectively reduced intestinal absorption of cholesterol as measured by the continuous isotope feeding method (9, 10).

Cholesterol absorption has been found to decrease by up to 60% with a daily intake of 3 g

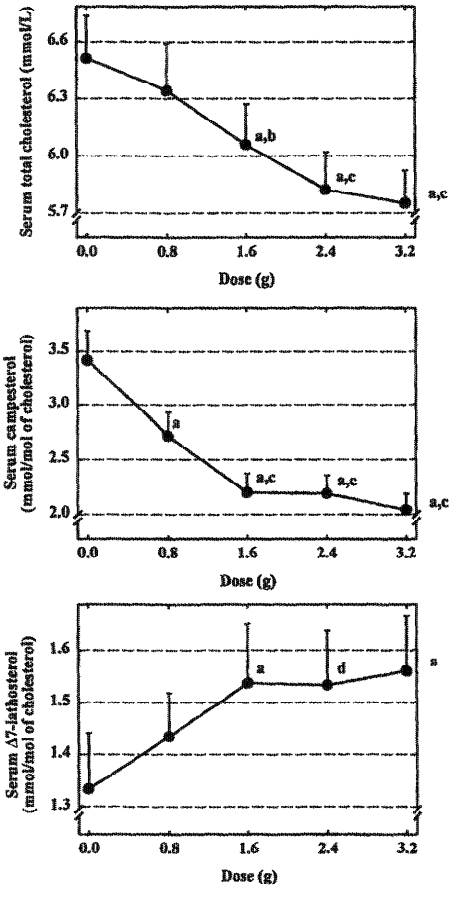


Figure 1. Serum total cholesterol (mmol/L), serum campesterol (mmol/mol of cholesterol) and Δ7-lathosterol (mmol/mol of cholesterol) during the different doses (0 g , 0.8 g, 1.6 g, 2.4 g and 3.2 g) of plant stanols. Values are mean ± SEM, n=22.
[a] P<0.001, [d] P<0.01 significantly different from the 0 g dose period (paired t-test with Bonferroni correction).
[b] P<0.05, [c] P<0.001 significantly different from 0.8 g dose period (paired t-test with Bonferroni correction). In pairwise comparisons after Bonferroni correction there were no significant differences in serum total cholesterol, campesterol and Δ7-lathosterol between the 1.6 g, 2.4 g and 3.2 g dose periods.

plant stanol delivered as fatty acid esters when the absorption was investigated with peroral double-isotope and continuous feeding method (11, 13). In addition, the serum campesterol/total cholesterol ratio was reduced by about 46% (11, 13). In the present study serum campesterol concentration and its ratio to total cholesterol were reduced by 45% and 39%, respectively, with the greatest stanol ester dose, indicating somewhat smaller decrease in cholesterol absorption than in the former studies. Furthermore, there was a discrepancy between the reduction in serum campesterol and serum cholesterol concentrations. This is likely to be due to the fact that plant sterols are not synthesized in the human body while cholesterol synthesis is stimulated compensatorily to a reduced absorption of dietary cholesterol as also shown in the present study.

Serum plant stanol concentrations are normally very low, on an average 0-0.74 μmol/L (0-30 μg/dl) (5, 24) compared with serum plant sterol concentrations (7-20 μmol/L; 300-800 μg/dl) (2, 25) or serum total cholesterol concentration (4.5-6.5 mmol/L). Although in the present study campestanol and sitostanol concentrations in serum remained extremely low (0.36-0.44 μmol/L and 0.87-1.03 μmol/L, respectively) throughout the study, their concentrations rose with all stanol ester doses. Relatively, the greatest increase in serum campestanol and sitostanol concentrations was reached already with the 0.8 g stanol dose, and the increases in them with the greater doses were only slight suggesting that the absorption of plant stanols might be leveled off already with small doses of stanol ester. Besides negligible absorption of plant stanols the low serum concentrations of plant stanols could also result from the fast and effective clearance of absorbed stanols (25). Our finding is parallel the previous plant stanol studies where the increase of serum campestanol and sitostanol concentration has been found to be minor (5, 6) even if stanol ester was consumed in long-term (5).

In conclusion, the compensatory increase in serum Δ7-lathosterol/total cholesterol ratio as a response to cholesterol malabsorption did not occur in a dose-dependent manner. This suggests that cholesterol synthesis may be leveled off with the stanol dose equal to or greater than 1.6 g. Serum campesterol concentration and its ratio to total cholesterol was reduced significantly already with the 0.8 g dose which indicates an effective cholesterol absorption inhibition even with the small doses of stanol ester. The observed further reduction of serum cholesterol with the dose of 1.6 - 3.2 g may be due to other mechanism than diminished cholesterol absorption. Finally, based on serum sitostanol and campestanol concentration

measurements their absorption is extremely low.

ACKOWLEDGEMENTS

We thank Leena Kaipiainen, Pia Hoffström, Ritva Nissilä, Irja Kanniainen, Erja Kinnunen and Kaija Kettunen for excellent laboratory work. This study was financially supported by Raisio Benecol Ltd., Raisio, Finland.

REFERENCES

1. Tilvis RS, Miettinen TA (1986) Serum plant sterols and their relation to cholesterol absorption. Am J Clin Nutr 43:92-97
2. Miettinen TA, Tilvis RS, Kesäniemi YA (1990) Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J Epidemiol 131:20-31
3. Björkhem I, Miettinen T, Reihnér E, Ewerth S, Angelin B, Einarsson K (1987) Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver. J Lipid Res 28:1137-1143
4. Kempen HJ, Glatz JF, Gevers Leuven JA, van der Voort HA, Katan MB (1988) Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. J Lipid Res 29:1149-1155
5. Gylling H, Puska P, Vartiainen E, Miettinen TA (1999) Serum sterols during stanol ester feeding in a mildly hypercholesterolemic population. J Lipid Res 40:593-600
6. Gylling H, Miettinen T (1999) Cholesterol reduction by different plant stanol mixtures and with variable fat intake. Metabolism 48:575-580
7. Hallikainen MA, Sarkkinen ES, Gylling H, Erkkilä AT, Uusitupa MI (2000) Comparison of the effects of plant sterol ester and plant stanol ester- enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. Eur J Clin Nutr 54:715-725
8. Vanhanen HT, Blomqvist S, Ehnholm C, Hyvönen M, Jauhiainen M, Torstila I, Miettinen TA (1993) Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment. J Lipid Res 34:1535-1544
9. Vanhanen HT, Kajander J, Lehtovirta H, Miettinen TA (1994) Serum levels, absorption efficiency, faecal elimination and synthesis of cholesterol during increasing doses of dietary sitostanol esters in hypercholesterolaemic subjects. Clin Sci 87:61-67
10. Miettinen TA, Vanhanen HT (1994) Dietary sitostanol related to absorption, synthesis and serum level of cholesterol in different apolipoprotein E phenotypes. Atherosclerosis 105:217-226
11. Gylling H, Miettinen TA (1994) Serum cholesterol and cholesterol and lipoprotein metabolism in hypercholesterolaemic NIDDM patients before and during sitostanol ester-margarine treatment. Diabetologia 37:773-780
12. Gylling H, Radhakrishnan R, Miettinen TA (1997) Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary sitostanol ester margarine. Women and dietary sitostanol. Circulation 96:4226-4231
13. Gylling H, Miettinen TA (1996) Effects of inhibiting cholesterol absorption and synthesis on cholesterol and lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic men. J Lipid Res 37:1776-1785
14. Andersson A, Karlström B, Mohsen R, Vessby B (1999) Cholesterol-lowering effects of a stanol ester-containing low-fat margarine used in conjunction with a strict lipid-lowering diet. Eur Heart J Supplements 1 (Suppl S):S80-S90
15. Jones PJ, Raeini-Sarjaz M, Ntanios FY, Vanstone CA, Feng JY, Parsons WE (2000) Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. J Lipid Res 41:697-705
16. Hallikainen MA, Sarkkinen ES, Uusitupa MIJ (2000) Plant stanol esters affect serum cholesterol concentrations of hypercholesterolemic men and women in a dose-dependent manner. J Nutr 130:767-776
17. Rastas M, Seppänen R, Knuts L-R, Karvetti R-L, Varo P (1993) Nutrient composition of foods. Social Insurance Institution, Helsinki
18. Miettinen TA (1988) Cholesterol metabolism during ketoconazole treatment in man. J Lipid Res 29:43-51
19. Miettinen TA, Koivisto P (1983) Non-cholesterol sterols and bile acid production in hypercholesterolaemic patients with ileal bypass. In: Paumgartner G, Stiehl A, Gerok W (ed) Bile acid and cholesterol in health and disease. MTP Press, Lancaster, pp 183-187
20. Norusis MJ (1993) SPSS for Windows Base System User's Guide. Release 6.0. SPSS, Chicago, IL
21. Miettinen TA, Tilvis RS, Kesäniemi YA (1989) Serum cholestanol and plant sterol levels in relation to cholesterol metabolism in middle-aged men. Metabolism 38:136-140
22. Cater NB (1999) Historical and scientific basis for the development of plant stanol ester foods as cholesterol-lowering agents. Eur Heart J Supplements 1 (Suppl S):S36-S44
23. Vanhanen HT, Miettinen TA (1992) Effects of unsaturated and saturated dietary plants sterols on their serum contents. Clin Chim Acta 205:97-107
24. Jones PJH, Howell T, MacDougall DE, Feng JY, Parsons W (1998) Short-term administration of tall oil phytosterols improves plasma lipid profiles in subjects with different cholesterol levels. Metabolism 47:751-756
25. Salen G, Ahrens EH, Jr, Grundy SM (1970) Metabolism of β-sitosterol in man. J Clin Invest 49:952-967

V

Hallikainen MA, Sarkkinen ES, Gylling H, Erkkilä AT, Uusitupa MI. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet. Eur J Clin Nutr 2000;54:715-725.

European Journal of Clinical Nutrition (2000) 54, 715–725

www.nature.com/ejcn

Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low-fat diet

MA Hallikainen[1]*, ES Sarkkinen[1], H Gylling[2], AT Erkkilä[1] and MIJ Uusitupa[1,3]

[1]*Department of Clinical Nutrition, University of Kuopio, Kuopio, Finland;* [2]*Department of Medicine, University of Helsinki, Helsinki, Finland; and* [3]*Kuopio University Hospital, Kuopio, Finland*

Objective: To investigate cholesterol-lowering effects of stanol ester (STAEST) and sterol ester (STEEST)-enriched margarines as part of a low-fat diet.
Design: According to a Latin square model randomized double-blind repeated measures design with three test margarines and three periods.
Setting: Outpatient clinical trial with free-living subjects.
Subjects: Thirty-four hypercholesterolaemic subjects completed the study.
Interventions: Subjects consumed three rapeseed oil-based test margarines (STAEST, STEEST and control (no added stanols or sterols)) as part of a low-fat diet each for 4 weeks.
Results: Mean daily intake of total plant sterols plus stanols was 2.01–2.04 g during the two test margarine periods. In reference to control, serum total cholesterol was reduced by 9.2 and 7.3% with the STAEST and STEEST margarine, respectively ($P<0.001$ for both). The respective reductions for low-density lipoprotein (LDL) cholesterol were 12.7 and 10.4% ($P<0.001$). The cholesterol-lowering effects of the test margarines did not differ significantly. The presence of apolipoprotein E4 allele had a significant effect on LDL cholesterol response during the STAEST margarine only. Serum sitosterol and campesterol increased by 0.83 and 2.77 mg/l with the STEEST ($P<0.001$), respectively and decreased by 1.18 and 2.60 mg/l with the STAEST margarine ($P<0.001$). Increases of serum sitostanol and campestanol were 0.11 and 0.19 mg/l with the STAEST margarine ($P<0.001$), repsectively. No significant changes were found in serum fat-soluble vitamin and carotenoid concentrations when related to serum total cholesterol.
Conclusions: STAEST and STEEST margarines reduced significantly and equally serum total and LDL cholesterol concentrations as part of a low-fat diet.
Sponsorship: Grant to the University of Kuopio by Raisio Benecol Ltd, Raisio, Finland.
Descriptors: cholesterol-lowering diet; hypercholesterolaemic; plant sterols; plant stanols; carotenoids; vitamins; apolipoprotein E
European Journal of Clinical Nutrition (2000) **54,** 715–725

Introduction

Great interest has been focused on the cholesterol-lowering properties of plant sterols and stanols (Jones *et al*, 1997; Pollak & Kritchevsky, 1981), and there are now commercial products available aimed at helping to reduce elevated serum total cholesterol concentrations. Based on previous studies 2.0–3.0 g/day plant stanols from full-fat sitostanol ester margarine or mayonnaises as part of a moderately rich or high-fat diet significantly reduces serum total and low-density lipoprotein (LDL) cholesterol concentrations without affecting high-density lipoprotein (HDL) cholesterol or triglyceride concentrations (Gylling *et al*, 1995,1997; Gylling & Miettinen, 1994; Miettinen *et al*, 1995; Niinikoski *et al*, 1997; Vanhanen *et al*, 1994). In our own study (Hallikainen & Uusitupa, 1999), low-fat stanol ester margarines, consumed as part of a recommended low-fat, low-cholesterol diet, reduced serum LDL cholesterol by 18.4–23.6% as compared to the high-fat baseline diet, the additional effect of stanol ester margarine being 8.6–10.6%. Sitostanol has been suggested to have greater hypocholesterolaemic activity than sitosterol (Becker *et al*, 1993; Heinemann *et al*, 1986). In one single study with normolipidaemic subjects, a soybean sterol ester margarine, based on unhydrogenated soysterols with an esterification degree of 65%, was found to be as effective as a stanol ester margarine in lowering plasma cholesterol concentrations (Weststrate & Meijer, 1998). In that trial, subjects followed their own habitual diet except that the habitually used spreads were replaced by test margarines. However, efficacy of plant sterol ester margarine in comparison to stanol ester margarine with matching fatty acid compositions, equal esterification degree (>98.5%) and equal daily intake of total

*Correspondence: MA Hallikainen, Department of Clinical Nutrition, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland.
E-mail: Maarit.Hallikainen@uku.fi
Guarantor: MIJ Uusitupa.
Contributors: MAH was responsible for the study design, gave nutrition counselling, analysed and interpreted the data and wrote the manuscript. MIJU and ESS contributed to the planning of study design and diets, interpreting the data and writing the manuscript. HG analysed serum cholesterol precursors and plant sterols, and ATE analysed fatty acid composition of serum lipids and, in addition, both also contributed to interpreting the data and writing the manuscript.

sterols (unsaturated + saturated sterols) has not been studied in connection with a low-fat, cholesterol-lowering diet.

Plant stanols have been suggested to be almost non-absorbable (Hassan & Rampone, 1979; Lütjohann *et al*, 1995), but recent studies indicate that they may be poorly absorbed from the small intestine (Gylling *et al*, 1999a; Gylling & Miettinen, 1999). Plant sterols are absorbed from 5% (sitosterol) to 15% (campesterol) (Heinemann *et al*, 1993; Salen *et al*, 1970). Furthermore, plant sterols and stanols may interfere with the absorption of carotenoids, as indicated by reduced serum carotenoid concentrations (Gylling *et al*, 1999b; Hallikainen *et al*, 1999, 2000; Hallikainen & Uusitupa, 1999; Hendriks *et al*, 1999; Weststrate & Meijer, 1998). This has to be taken into account in long-term use of plant sterol- and stanol-containing products emphasizing the use of a generally recommended diet.

Therefore, in the present study we investigated whether or not the two margarines enriched with plant sterol esters or stanol esters are equal in lowering serum total and LDL cholesterol concentrations and to what extent these two margarines would reduce serum cholesterol concentrations as part of a low-fat diet in reference to the low-fat diet alone. Additionally serum plant sterol and stanol concentrations were studied. As a *post-hoc* analysis we also investigated the effects of the main apolipoprotein E genotypes on lipid responses.

Methods

Subjects

Altogether 42 subjects (20 men/22 women) with mild to moderate hypercholesterolaemia were recruited to the study from the former studies carried out at the Department of Clinical Nutrition, University of Kuopio and from the occupational health care system. In addition, employees of the city of Kuopio were recruited to the study. The main inclusion criteria were as follows: serum total cholesterol 4.8–7.0 mmol/l and total triglycerides below 2.5 mmol/l at screening. Other inclusion criteria were age 30–65 y, normal liver, kidney and thyroid function, no lipid lowering medication, no unstable coronary heart disease, no diabetes, no gastrointestinal diseases, no alcohol abuse (>45 g ethanol/day) and no irregular eating habits. Eight subjects dropped out during the study: one at the beginning of the pre-trial period due to poor compliance; four at the end of the pre-trial period due to personal reasons or poor compliance; and three during the first period due to personal reasons. Altogether 34 subjects completed the study. In the beginning of the study their mean age was 48.8 ± 8.1 y (mean $\pm$ s.d.), their mean body mass index (BMI) was $24.9 \pm 2.4\,kg/m^2$, and their mean systolic and diastolic blood pressure was 124 ± 14 and 81 ± 9 mmHg, respectively. Baseline total-, LDL-, HDL- and VLDL-cholesterol and triglyceride concentrations were 6.24 ± 0.83, 4.43 ± 0.81, 1.60 ± 0.31, 0.22 ± 0.21 and 1.11 ± 0.52 mmol/l, respectively.

One subject used low-oestrogen oral contraceptives, two subjects had hormone-releasing intra-uterine devices (IUD), one subject used hormone replacement therapy, three subjects used postmenopausal oestrogen therapy, one had thyroxin therapy for hypothyroidism, one used a calcium channel blocker, one used a renin–angiotensin system-affecting medication and two used anti-thrombolytic medication. Two subjects were smokers. The subjects were requested to maintain their medication, weight, alcohol consumption, smoking habits and physical activity constant during the study.

The subjects gave written consent for the study and the study protocol was approved by the Ethics Committee of the University of Kuopio.

Study design

The study was carried out from September to December 1998 applying a randomized double-blind repeated measures design with three test spreads (stanol ester (STAEST), sterol ester (STEEST) and control margarine). Each period lasted for 4 weeks. The randomization was made according to the model of Latin square design. Before randomization during the first 2 weeks the subjects followed a standardized low-fat diet and their diet was monitored. The pre-trial period made it possible to evaluate the inclusion criteria and compliance with the study.

Routine laboratory measurements were taken to ensure normal health status at the first and last visits of the study. In addition, previous and present diseases, current medication, alcohol and tobacco consumption, physical activity, use of vitamins or other nutrient supplements were interviewed by a structured questionnaire at the first visit of the study. Alcohol and tobacco consumption and physical activity were reviewed also at the last visit. Furthermore, possible changes in health, medication, use of vitamin or nutrient supplements were recorded during the study. Fasting blood samples were taken at the beginning of the pre-trial period (−2 weeks), at the beginning of the first experimental period (0 weeks), at the middle and the end of each period (2, 4, 6, 8, 10 and 12 weeks). Body weight was recorded at each visit. The possible adverse effects and symptoms were interviewed based on a structured questionnaire at the end of each period.

Diet

The composition of low erucic acid rapeseed oil (LEAR)-based margarines (Raisio Group, Raisio, Finland) is presented in Table 1. The total amount of fat was 80% and 71%, and amount of absorbable fat excluding sterols and stanols was 70% and 71% in test margarines and control margarine, respectively. STAEST margarine was prepared from wood and vegetable sterols (DRT, Les Derives Resiniques & Terpeniques Granel S.A. Dax Cedex, France and Archer Daniels Midland Co, Decatur, IL, respectively) by recrystallization, hydrogenation to form plant stanols, and esterification to produce low erucic acid rapeseed oil based fatty acid esters of plant stanols. In turn STEEST margarine was prepared from vegetable oil based sterols (Archer Daniels Midland Co, Decatur, IL) by recrystallization, and esterification with low erucic acid rapeseed oil based fatty acid esters to produce fatty acid esters of plant sterols. The daily dose of the test margarine was 20 g taken in two to three portions with meals. The theoretical daily amount of total sterols and stanols was 2.02 g (0.10 g total sterols and 1.92 g total stanols) in the STAEST margarine and 2.06 g (1.98 g total sterols and 0.09 g total stanols) in the STEEST margarine. The control margarine and the margarine consumed during the pre-trial period contained naturally small amounts of sterols (about 0.09 g/daily dose of margarine). All three margarines were fortified with vitamin A (870 μg RE/100 g) and vitamin D

Table 1 Composition of daily dosage (20 g) of test margarines

Nutrients (g/20 g margarine)	*Control margarine*	*STAEST margarine*	*STEEST margarine*
Total fat	14.2	16.2	16.0
Absorbable fat[a]	14.2	14.0	14.0
Fatty acids:			
Saturated	3.2	3.3	3.3
Lauric (C 12:0)	0.3	0.4	0.5
Myristic (C 14:0)	0.2	0.2	0.3
Palmitic (C 16:0)	3.3	3.3	3.3
Stearic (18:0)	0.6	0.5	0.5
Monounsaturated	7.1	7.0	6.9
Oleic (C 18:1 *cis*)	10.2	10.1	9.9
Polyunsaturated	3.1	3.1	3.2
Linoleic (C 18:2 *cis*)	3.2	3.3	3.3
Linolenic (C 18:3 *cis*)	1.3	1.3	1.4
Total stanols	0	1.92	0.09
Sitostanol	0	1.43	0.06
Campestanol	0	0.49	0.02
Total sterols	0.09	0.10	1.98
Brassicasterol	0.01	—	0.06
Campesterol	0.03	0.04	0.57
Sitosterol	0.04	0.06	1.00
Stigmasterol	—	—	0.34
Total sterols and stanols	0.09	2.02	2.06

STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Absorbable fat = total fat−total sterol and stanols.

(7 μg/100 g). This kind of fortification of margarines is a normal procedure in Finland.

The subjects received the coded tubs of test margarines when visiting the study unit. To verify the precise daily dose of test spread, 142 g, ie weekly dose of the test and control margarines, was packed into one tub and subjects were advised to mark the fat spread with a knife into seven equal parts in advance. One tub of test spread per each week was delivered and one extra tub for occasions of unexpected loss or damage of test products. The subjects were asked to record the use of test fats daily, and to return the empty and partly empty tubs and the extra tub of test spread to the study unit at the end of each period. The packages and the test spread left over were weighed and recorded.

Subjects followed a low-fat diet (step 1) of the National Cholesterol Education Program (1994) throughout the study. The planned composition of the diet was: <30 of energy percent (E%) from fat including 8–10 E% saturated, 12–14 E% monounsaturated and 5–7 E% polyunsaturated fat, and <300 mg/day dietary cholesterol. The diet was composed of normal Finnish food items. All subjects received individual oral and written instructions on the diet, including the precise amounts and quality of foods as main food groups: fats, dairy products, meat and meat products, cereals, fruits and berries, and vegetables and roots. The diet plan was made for eight energy levels: 6.7–12.6 MJ/day. The energy requirement of a subject was estimated according to the Harris Benedict formula with the energy requirement due to physical activity added (Alpers *et al*, 1986). If necessary the energy intake level was changed in order to ensure unchanged body weight during the study. The feasibility of the diet was improved by providing test margarines, rapeseed oil, salad dressing and low-fat cheese for the participants free of charge.

Adherence to the low-fat diet was monitored by 4-day food records kept at the end of each period, four times altogether during the study. One of the recording days was a weekend day or the person's day off from work. The subjects recorded their food consumption using a portion-size booklet with photos to estimate the portion size (Haapa *et al*, 1985). At study visits the amounts and qualities of foods in the records were checked by the nutritionist for completion, filling in data that were lacking. Fatty acid composition of serum lipids was determined as an objective marker of dietary adherence.

The diet was planned and the nutrients in the food records were calculated using the Micro-Nutrica® dietary analysis program (version 2.0, Finnish Social Insurance Institute, Turku, Finland). The food composition database is based on analyses of the Finnish food and international food composition tables (Rastas *et al*, 1993). In addition, the database was updated for the purposes of the present study.

Laboratory measurements

Systolic and diastolic blood pressure was measured by a mercury sphygmomanometer (Mercurius Stator, Spiedel + Keller, Germany) after subjects had rested for 5–10 min. Two measurements were taken and the mean of them used in the analyses. Body weight was measured with a digital scale. All measurements were done and venous blood samples were obtained after a 12 h overnight fast using standardized methods. Since the phase of the menstrual cycle may have an effect on serum cholesterol concentration (Cullinane *et al*, 1995), the end measurements were performed at days 5–10 of the cycle in those women with the menstrual cycle.

Routine laboratory samples were analysed with standardized methods at the Kuopio University Hospital. Plasma glucose was analysed by enzymatic photometric method using reagent Granutest 100 (Merck, Damstadt, Germany) with a Kone Specific Clinical Analyser (Kone Ltd, Espoo, Finland).

Lipoproteins were separated by ultracentrifugation for 18 h at density 1.006 to remove very low density lipoprotein (VLDL). HDL in the infranatant was separated from LDL by precipitation of LDL with dextran sulphate and magnesium chloride (Penttilä *et al*, 1981). LDL cholesterol was calculated as a difference between the mass of cholesterol in the infranatant and HDL, and VLDL cholesterol was calculated as a difference between the whole serum and the infranatant. Enzymatic photometric methods were used for the determination of cholesterol and triglycerides from whole serum and separated lipoproteins using commercial kits (Monotest® Cholesterol and Triglyceride GPO-PAP, Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) with a Kone Specific Clinical Analyser (Kone Ltd, Espoo, Finland). The coefficient of variance (CV) between measurements for serum total cholesterol was 1.3–1.4%, for triglycerides 1.7–1.9% and for HDL cholesterol 1.1–1.2%.

Serum samples for analysis of apolipoprotein (apo) A-I and B, carotenoids and fat soluble vitamins, cholesterol precursors, plant sterols and cholestanol, and fatty acid composition were stored at −70°C until analysed at the end of the study. Analyses of apolipoproteins were based on the measurement of immunoprecipitation enhanced by polyethylene glycol (PEG) at 340 nm. A Kone Specific Clinical Analyser and apo A-I and apo B reagents from Kone Corporation (Espoo, Finland) were used. Serum carotenoids, retinol and tocopherols were analysed by the high-performance liquid chromatography system (Perkin-Elmer, Norwalk, CT) on a C_{18} column (Waters, Milford, MA)

(Kaplan *et al*, 1987; Parviainen, 1983) using an external standard. Serum 25-hydroxyvitamin D_3 was analysed with a radioimmunoassay method (25-Hydroxyvitamin D I125 RIA KIT, DiaSorin, Stillwater, MN) using an external standard.

Serum cholesterol precursors (Δ8-cholestenol, Δ7-lathosterol, desmosterol and squalene), plant sterols (sitosterol, sitostanol, campesterol, campestanol and avenasterol) and cholestanol, a metabolite of cholesterol, were quantified from nonsaponifiable serum materials by capillary gas–liquid chromatograph (GLC, HP 5890 Series II, Hewlett Packard, Delaware) equipped with a 50 m long Ultra 1 capillary column (methyl-polysiloxane) (Hewlett Packard, USA) for cholestanol, squalene, Δ8-cholestenol, Δ7-lathosterol, campesterol and sitosterol, and equipped with a 50 m long Ultra 2 capillary column (5% phenyl-methyl siloxane, Hewlett Packard, USA) for sitostanol and campestanol (Miettinen 1988, Miettinen & Koivisto 1983). Serum cholesterol precursors, plant sterols and cholestanol were determined in duplicate from the same samples and the mean value of two measurements was used in the statistical analyses.

In the analysis of serum fatty acid composition lipids were extracted with chloroform–methanol (2 : 1) (Ågren *et al*, 1992), and lipid fractions (cholesteryl esters, triglycerides and phospholipids) were separated with an aminopropyl column. Fatty acids were analysed with a gas-chromatograph (Hewlett-Packard 5890 series II, Hewlett-Packard Company, Waldbronn, Germany) equipped with FFAP-column (length 25 m, inner diameter 2 mm and film thickness 0.3 μm). Fatty acids are presented as molar percentage of total fatty acids.

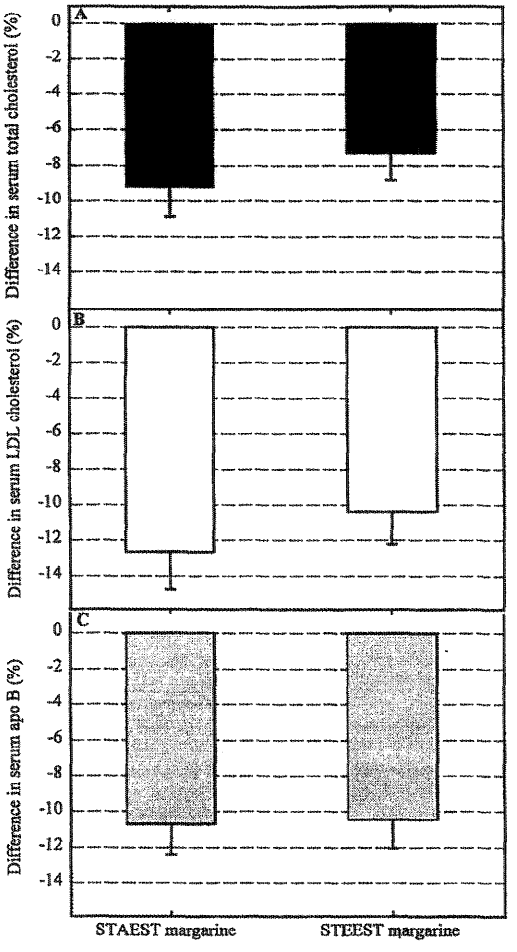


Figure 1 Differences in serum total (A) and LDL cholesterol (B), and apo B (C) concentrations (%) in reference to the control margarine period; $n = 34$, values are means ± s.e.m. There were no significant differences in serum lipid and lipoprotein responses between the two test margarine periods analysed with analysis of variance for repeated measurements (GLM). STAEST = stanol ester margarine and STEEST = sterol ester margarine.

Apolipoprotein E genotypes were determined by polymerase chain reaction (PCR) using primers described previously by Tsukamoto *et al* (1993). PCR amplification of apolipoprotein E polymorphism was conducted in a 16 µl volume containing 50 ng of genomic DNA, 0.4 pmol/l of each primer, 10 mmol/l Tris–HCl (pH 8.8), 50 mmol/l KCl, 1.5 mmol/l of $MgCl_2$, 0.1% Triton X-100, 113 µmol/l dNTPs, 0.7 units of DNA polymerase (DynaZyme DNA polymerase, Finnzymes, Espoo, Finland), and 8.5% glycerol to enhance amplification and annealing of GC-rich primers. Conditions for amplification were denaturation at 96°C for 4 min, followed by 35 cycles of denaturation at 96°C for 45 s, annealing at 64°C for 45 s, and extension at 72°C for 60 s with final extension at 72°C for 4 min. The PCR product was digested with *Hha*I (New England Biolabs, Beverly, MA). The digested DNA fragments were separated on 12% polyacrylamide gel. Finally, separated DNA fragments were visualized by ethidium bromide staining.

Statistical analyses

All statistical analyses were performed with SPSS for Windows 7.5 statistics program (SPSS, Chicago, IL, USA). The results are given as means ± s.d., except in Figure 1, where they are given as means ± s.e.m.

The main comparison was made among the mean values at the end of each experimental period. In the results and discussion sections only the end measurements, and their absolute or percentage changes, are presented. The percentage changes were computed comparing the end measurements of both test margarine periods to the end measurement of the control margarine period. To eliminate the effects of changes in lipoprotein concentrations, serum carotenoid, tocopherol, cholesterol precursor, plant sterol and cholestanol values are given, besides the crude concentrations, also in terms mmol/mol of cholesterol or 10^{-3} mg/mg of cholesterol, which express the ratios to total cholesterol.

Normal distribution was checked with Shapiro Wilks test, and homogeneity of variance was checked with analysis of variance for repeated measurement (GLM) before further analyses. If a variable was not normally distributed the statistical analysis was made after logarithmic transformation. GLM was used to compare the overall changes and the effect of the order of spread consumption periods, carry-over effect and gender on the main end-point variables among the different experimental margarine periods. GLM was also used in the further analyses. Confidence intervals (CI) presented in the text for the percentage changes of total and LDL cholesterol and apo B were not corrected for the above-mentioned factors.

Intake of nutrients and serum fatty acid composition among the experimental periods was analysed with GLM and a paired *t*-test, or if nutrient data or fatty acid data was not normally distributed even after the logarithmic transformation Friedman two-tailed ANOVA test was used. To control the overall α level, Bonferroni adjustment was used. The Wilcoxon matched-paired signed rank test was used to compare the change of alcohol consumption, smoking habits and physical activity. Routine laboratory examinations were tested with paired *t*-test or Wilcoxon matched-paired signed rank test.

The number of subjects recruited for the study was based on an assumption of having 0.2 mmol/l difference in serum LDL cholesterol response among the test spreads significant with an α level of 0.05 ($P<0.05$), and with 0.80 statistical power (Cohen, 1988).

Results

Baseline characteristics

There were no significant changes in BMI (24.9 ± 2.3, 24.8 ± 2.4 and 24.8 ± 2.4 kg/m^2 during the control, STAEST and STEEST margarine periods, respectively) or systolic and diastolic blood pressure during the study. Physical activity, alcohol consumption and smoking habits remained stable. A woman who used low-oestrogen oral contraceptives stopped use at the end of the first study period. Excluding her from the statistical analyses did not affect the results.

To ensure normal health status during the study, samples for routine laboratory measurements were drawn in the beginning (−2 weeks) and at the end (12 weeks) of the study. There was a small but significant decrease in the mean fasting plasma glucose (from 5.53 ± 0.41 to 5.35 ± 0.41 mmol/l) and serum γ-glutamyltransferase (from 25.8 ± 20.3 to 21.3 ± 12.5 U/l) values during the study. The mean serum TSH concentration increased slightly (from 1.7 ± 1.3 to 1.9 ± 1.2 mU/l, $P<0.05$). One subject had a marginally elevated serum TSH concentration (7.3 and 6.1 mU/l, at the beginning and end of the study, respectively). Excluding him from statistical analyses did not affect the results. The changes in routine laboratory measurements cannot be connected with the test margarines. In men the mean blood haemoglobin concentration decreased slightly (from 144.4 ± 8.1 to 141.2 ± 6.3 g/l) but significantly due to the fact that seven of the men also participated in a fat-loading test with many blood samples at the end of all test margarine periods. In women blood haemoglobin concentration remained unchanged during the entire study. Other routine measurements, blood thrombocytes, serum alanine aminotransferase and creatinine, did not change significantly during the study.

Feasibility of the diet

The mean daily consumption of margarine according to the weighed returned tubs was 19.2–19.9 g during the different margarine periods. The actual mean daily intake of total sterols and stanols was 0.09 ± 0.03 g (0.09 ± 0.03 g total sterols and 0 g total stanols), 2.01 ± 0.06 g (0.10 ± 0.03 g total sterols and 1.91 ± 0.05 g total stanols) and 2.04 ± 0.14 (1.96 ± 0.13 g total sterols and 0.09 ± 0.06 g total stanols) during the control, STAEST margarine and STEEST margarine periods, respectively.

The actual composition of the diet during the different test margarine periods is presented in Table 2. The goal for the composition of the low-fat diet was well achieved (Table 2). Generally, there were no significant differences in the nutrient intakes among the periods.

The results of the fatty acid composition of serum cholesteryl esters paralleled with the food records (Table 3). In general, there were no major differences in the fatty acid composition during the different test margarine periods. However, the proportion of oleic acid was slightly, but

Table 2 Actual composition of the diets during the different test margarine periods

	Period			
Nutrients	*Control margarine (n = 34)*	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P-values*[a]
Energy (MJ/day)	8.3 ± 1.9	8.3 ± 1.8	8.1 ± 1.7	0.318
Fat (percentage total energy intake, E%)	30.0 ± 3.7	30.5 ± 3.2	31.1 ± 3.9	0.100
Saturated fatty acids (E%)	8.2 ± 1.9	8.4 ± 1.8	8.8 ± 2.0	0.347
Monounsaturated fatty acids (E%)	12.4 ± 1.6	12.5 ± 1.5	12.7 ± 1.9	0.648
Polyunsaturated fatty acids (E%)	6.7 ± 0.9	6.6 ± 0.8	6.9 ± 0.9	0.069
Proteins (E%)	16.1 ± 2.3	16.9 ± 2.1	17.2 ± 2.7	0.045
Carbohydrates (E%)	49.6 ± 5.4	49.1 ± 5.2	48.3 ± 4.8	0.916
Alcohol (E%)	2.9 ± 4.1	2.1 ± 3.3	2.0 ± 2.5	0.642
Cholesterol (mg/d)	166 ± 88	173 ± 66	179 ± 66	0.098
Cholesterol (mg/MJ)	19.6 ± 7.6	20.5 ± 4.9	22.2 ± 6.6	0.061
Fibre (g/day)	30.3 ± 8.4	30.1 ± 7.5	28.9 ± 9.5	0.443
Fibre (g/MJ)	3.7 ± 1.0	3.7 ± 0.9	3.6 ± 0.9	0.445
Vitamin A (μg RE/day)[b]	1337 ± 878	1188 ± 544	1139 ± 736	0.452
β-carotene (μg/day)	4155 ± 2732	4726 ± 3120	4056 ± 2638	0.413
Vitamin D (μg/day)	4.4 ± 2.7	5.7 ± 3.8	6.1 ± 4.9	0.318
Vitamin E (mg/day)	12.3 ± 3.3	12.5 ± 2.8	12.0 ± 3.2	0.434

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Variables were analysed with Friedman two-way ANOVA test (energy, saturated fatty acid, carbohydrates, alcohol, vitamin A and vitamin D) or analysis of variance for repeated measurements (GLM) and paired *t*-test with Bonferroni correction. In pairwise comparisons after Bonferroni correction there were no significant differences in protein intake between any two periods.
[b]RE = retinol equivalents.

significantly lower at the end of the STEEST margarine period than at the end of the control period. In addition, the proportion of stearic acid was significantly lower at the end of both test margarine periods than at the end of the control period. There were no significant differences in the fatty acid composition of triglycerides or phospolipids during the study (data not shown).

Serum lipids and lipoproteins

The concentrations of serum lipids and lipoproteins at the end of each experimental periods are shown in Table 4. Figure 1 presents the percentage differences in serum total and LDL cholesterol and apo B compared to the control margarine period.

No significant differences were found in the concentrations of serum lipids between genders ($P = 0.095$) or among orders of spread consumption ($P > 0.1$). In addition, no carry-over effect was found ($P > 0.1$).

Serum total and LDL cholesterol concentrations were significantly lower at the end of STAEST and STEEST margarine periods than at the end of the control period (Table 4). Compared to the control period the mean decreases in serum total cholesterol were 9.2 ± 9.7% (CI −12.6, −5.8) and 7.3 ± 8.9% (CI −10.4, −4.2) during the STAEST and STEEST margarine periods, respectively. For LDL cholesterol the mean decreases were 12.7 ± 12.2% (CI −16.9, −8.4) and 10.4 ± 10.6% (CI −14.1, −6.7), respectively. There were no significant differences in the decreases of serum total and LDL cholesterol concentrations between the two test margarine periods (difference 1.9 ± 8.4% and 2.3 ± 11.7%, respectively).

Serum HDL and VLDL cholesterol, and triglyceride concentration did not change significantly during the entire study (Table 4).

There were no significant changes in serum apo AI concentration (Table 4) during the study. The changes in

Table 3 Serum fatty acid composition of cholesteryl esters during the different test margarine periods

	Period			
Fatty acid (mol %)	*Control margarine (n = 34)*	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P values*[a]
Myristic acid 14:0	1.17 ± 0.33	1.14 ± 0.36	1.22 ± 0.42	0.526
Palmitic acid 16:0	12.46 ± 0.87	12.37 ± 0.86	12.45 ± 0.89	0.699
Palmitoleic acid 16:1	3.80 ± 0.88	3.68 ± 0.91	3.58 ± 0.80	0.233
Stearic acid 18:0	0.78 ± 0.15	0.67 ± 0.13*	0.67 ± 0.13*	<0.001
Oleic acid 18:1 n-9 + n-7	21.22 ± 1.50	21.07 ± 1.49	20.66 ± 1.52†	0.007
Linoleic acid 18:2 n-6	51.45 ± 3.21	52.27 ± 3.27	52.20 ± 3.37	0.142
γ-linolenic acid 18:3 n-6	0.65 ± 0.29	0.63 ± 0.32	0.59 ± 0.26	0.313
α-linolenic acid 18:3 n-3	1.07 ± 0.20	1.00 ± 0.16	0.99 ± 0.23	0.282
Dihomo-γ-linolenic acid 20:3 n-6	0.52 ± 0.15	0.49 ± 0.10	0.53 ± 0.17	0.360
Arachidonic acid 20:4 n-6	4.58 ± 0.95	4.50 ± 0.89	4.63 ± 0.82	0.374
Eicosapentanoic acid 20:5 n-3	1.68 ± 1.00	1.57 ± 0.66	1.86 ± 1.04	0.065
Docosahexanoic acid 22:6 n-3	0.63 ± 0.18	0.60 ± 0.19	0.63 ± 0.20	0.486

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]The significance of the differences for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM) or Friedman two-tailed ANOVA (α-linolenic acid and eicosapentanoic acid).
*$P < 0.001$; †$P < 0.01$, significant difference between either of the test margarine periods and control margarine period analysed with paired *t*-test with Bonferroni correction. There were no significant differences in serum fatty acid composition of cholesteryl esters between two test margarine periods.

Table 4 Serum lipids and lipoproteins at the end of the test margarine periods

	Period			
Variables	*Control margarine (n = 34)*	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P-values*[a]
Total cholesterol (mmol/l)	6.10 ± 0.69	5.52 ± 0.75*	5.64 ± 0.71*	<0.001
LDL cholesterol (mmol/l)	4.19 ± 0.61	3.65 ± 0.69*	3.74 ± 0.58*	<0.001
HDL cholesterol (mmol/l)	1.50 ± 0.27	1.50 ± 0.29	1.55 ± 0.31	0.079
VLDL cholesterol (mmol/l)	0.40 ± 0.30	0.37 ± 0.26	0.35 ± 0.14	0.768
Triglycerides (mmol/l)	1.13 ± 0.45	1.10 ± 0.53	1.03 ± 0.33	0.221
Apo AI (g/l)	1.59 ± 0.22	1.55 ± 0.23	1.59 ± 0.27	0.143
Apo B (g/l)	1.01 ± 0.18	0.90 ± 0.18*	0.90 ± 0.13*	<0.001
Apo AI/apo B	1.62 ± 0.41	1.79 ± 0.46*	1.81 ± 0.43*	<0.001

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Significance of the difference for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM).
*$P<0.001$ denotes the significance of the difference between either of the test margarine periods and control period analysed with GLM with Bonferroni correction. There were no significant differences in serum lipid and lipoprotein concentrations between the two test margarine periods.

serum apo B concentration and in apo AI/apo B lipoprotein ratio were parallel with the changes in serum LDL cholesterol concentration (Table 4). The mean decreases in apo B concentrations were 10.7 ± 9.7% (CI −14.1, −7.3) and 10.4 ± 9.3% (CI −13.7, −7.2) during the STAEST and STEEST margarine periods, respectively, compared with the control period.

In a secondary analysis we also examined whether apolipoprotein E genotype group (E4/3, $n=12$ *vs* E3/3, $n=22$) has an effect on the LDL cholesterol response during the STAEST and STEEST margarine periods. This analysis revealed that the overall reduction in LDL cholesterol was greater in subjects with apolipoprotein E4/3 than in those with apolipoprotein E3/3 ($P=0.024$, interaction term of genotype with test margarine periods, GLM). This was entirely due to a greater effect of STAEST margarine on the LDL cholesterol response in subjects having apolipoprotein E4 allele (16.8 ± 13.9% *vs* 10.4 ± 10.8%, apolipoprotein E4/3 *vs* E3/3, $P=0.141$, ANOVA). The LDL cholesterol response was almost the same in the two apolipoprotein E groups (9.6 ± 13.5% *vs* 10.8 ± 8.9%, apolipoprotein E4/3 *vs* E 3/3, $P=0.707$, ANOVA) during the STEEST margarine period.

Cholesterol precursors and plant sterols

The concentrations of serum cholesterol precursors and plant sterols during the different test margarine periods are shown in Table 5.

As expected, serum Δ8-cholestenol and Δ7-lathosterol concentrations, which are indicators of cholesterol synthesis, increased during the STAEST and STEEST margarine periods in reference to the control period (Table 5). When those concentrations were related to serum total cholesterol

Table 5 Serum cholesterol precursors, plant sterols and cholestanol (mg/l), and ratios of serum cholesterol precursors, plant sterols and cholestanol to cholesterol (10^{-3} mg/mg of cholesterol) at the end of the test margarine periods

	Period			
	Control margarine (n = 34)	*STAEST margarine (n = 34)*	*STEEST margarine (n = 34)*	*P-values*[a]
Cholestanol	2.68 ± 0.48	2.36 ± 0.52*	2.28 ± 0.42*	<0.001
Δ8-cholestenol	0.33 ± 0.12	0.36 ± 0.13	0.37 ± 0.12[†]	0.010
Desmosterol	1.22 ± 0.35	1.22 ± 0.29	1.17 ± 0.24	0.850
Δ7-lathosterol	3.28 ± 1.11	3.58 ± 1.21	3.60 ± 1.24[†]	0.008
Campesterol	7.70 ± 1.92	5.09 ± 1.58*	10.46 ± 2.44*[‡]	<0.001
Sitosterol	3.40 ± 0.88	2.21 ± 0.73*	4.23 ± 1.02*[‡]	<0.001
Squalene	0.66 ± 0.14	0.71 ± 0.17	0.70 ± 0.17	0.292
Campestanol	0.06 ± 0.03	0.16 ± 0.04*	0.05 ± 0.02[‡]	<0.001
Sitostanol	0.08 ± 0.04	0.27 ± 0.07*	0.06 ± 0.04[‡]	<0.001
Avenasterol	1.00 ± 0.22	0.70 ± 0.15*	0.83 ± 0.16*[‡]	<0.001
Cholestanol/TC[b]	1.36 ± 0.23	1.30 ± 0.24	1.25 ± 0.24*[§]	<0.001
Δ8-Cholestenol/TC	0.16 ± 0.06	0.20 ± 0.06*	0.20 ± 0.06*	<0.001
Desmosterol/TC	0.61 ± 0.15	0.66 ± 0.13[†]	0.64 ± 0.12[¶]	0.001
Δ7-lathosterol/TC	1.64 ± 0.50	1.95 ± 0.61*	1.95 ± 0.59*	<0.001
Campesterol/TC	3.88 ± 0.91	2.78 ± 0.74*	5.70 ± 1.11*[‡]	<0.001
Sitosterol/TC	1.72 ± 0.43	1.21 ± 0.33*	2.30 ± 0.47*[‡]	<0.001
Squalene/TC	0.33 ± 0.07	0.39 ± 0.10[¶]	0.39 ± 0.10[¶]	0.003
Campestanol/TC	0.03 ± 0.01	0.09 ± 0.02*	0.03 ± 0.01[‡]	<0.001
Sitostanol/TC	0.04 ± 0.02	0.15 ± 0.04*	0.03 ± 0.02[‡]	<0.001
Avenasterol/TC	0.50 ± 0.10	0.39 ± 0.06*	0.45 ± 0.06*[‡]	<0.001

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]Significance of the differences for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM).
[b]TC = total cholesterol.
*$P<0.001$, [†]$P<0.01$, [¶]$P<0.05$, significant difference between the either the test margarine period and the control margarine period; [‡]$P<0.001$, [§]$P<0.05$, significant difference between the STAEST margarine and the STEEST margarine periods analysed with GLM with Bonferroni correction.

concentration the increases were more pronounced (Table 5). Serum cholestanol concentration, which reflects cholesterol absorption, decreased significantly during both test margarine periods (Table 5).

Serum campesterol and sitosterol concentrations decreased significantly during the STAEST margarine period and increased significantly during the STEEST margarine period compared to the control period (Table 5). Furthermore, serum campesterol and sitosterol concentrations were significantly higher at the end of the STEEST margarine period than at the end of the STAEST margarine period. In reference to control period, the reduction in serum campesterol and sitosterol concentrations was 2.60 ± 1.03 mg/l (33.8%) and 1.18 ± 0.47 mg/l (34.8%), respectively, during the STAEST margarine period. During the STEEST margarine period the increase in serum campesterol and sitosterol concentrations was 2.77 ± 1.49 mg/l (38.3%) and 0.83 ± 0.62 mg/l (26.6%), respectively. Changes in the ratios of serum campesterol and sitosterol to serum total cholesterol were parallel with the changes in their absolute concentrations (Table 5).

Serum campestanol (0.11 ± 0.04 and 0.11 ± 0.05 mg/l, change in reference to control and STEEST margarine period) and sitostanol concentrations (0.19 ± 0.07 mg/l and 0.21 ± 0.68 mg/l, change in reference to control and STEEST margarine period) were slightly but significantly higher at the end of STAEST margarine period than at the end of the control and the STEEST margarine periods (Table 5). There were no significant differences in serum campestanol or sitostanol concentrations between the STEEST margarine period and the control margarine period. Serum sitostanol concentration was significantly higher in women than in men at the end of the STAEST margarine period (0.24 ± 0.07 *vs* 0.30 ± 0.05 mg/l men *vs* women), but there were no differences in serum sitostanol concentrations between the genders at the end of the two other periods.

There were no significant differences in percentage changes in either serum cholesterol precursors or plant sterols between two apolipoprotein E genotype groups (3/3 and 4/3) among the different test margarine periods (data not shown).

Carotenoids and fat soluble vitamins

There were no significant changes in serum 25-hydroxyvitamin D_3, retinol, α-carotene or lycopene concentrations nor their ratios to the serum total cholesterol during the study (Table 6). Serum β-carotene concentration was significantly lower at the end of the STAEST and STEEST margarine periods than at the end of the control period. The serum α + β-carotene concentration was significantly lower at the end of the STEEST margarine period as compared to the control period, but the difference between the control and the STAEST margarine periods was not significant. However, there were no significant differences in serum β-carotene/total cholesterol ratio or α + β-carotene/total cholesterol ratio among the periods.

Serum γ-tocopherol concentration did not change significantly during the study, but serum α-tocopherol concentrations was significantly lower at the end of both test margarine periods than at the end of the control period (Table 6). After relating the serum α- and α + γ- tocopherol to the serum total cholesterol there were no significant differences among the different periods.

Discussion

In the present study the STAEST and STEEST margarines reduced significantly serum total (9.2% and 7.3%, respectively) and LDL cholesterol (12.7% and 10.4%, respectively) concentration as part of a low-fat diet compared to a low-fat diet alone in the subjects with mild to moderate hypercholesterolaemia, but the cholesterol-lowering effects of the test margarines did not differ significantly from each other. The decreases of serum apo B were parallel with the decreases of serum LDL cholesterol concentration.

On the basis of the food records the adherence to the low-fat diet was good. The intake of saturated fatty acids achieved the goal of step 1 diet of the National Cholesterol Education Program (1994) (actual mean intake 8–9 E% *vs*

Table 6 Serum carotenoids and fat-soluble vitamins at the end of the test margarine periods

	Period			
	Control margarine (n = 34)[a]	*STAEST margarine (n = 34)*[a]	*STEEST margarine (n = 34)*[a]	*P-values*[b]
Retinol (μmol/l)	2.80 ± 1.00	2.71 ± 1.04	2.70 ± 1.01	0.494
α-Carotene (μmol/l)	0.66 ± 0.43	0.64 ± 0.42	0.61 ± 0.36	0.129
β-Carotene (μmol/l)	1.39 ± 1.03	1.23 ± 0.99*	1.16 ± 0.82*	0.004
α + β-Carotene (μmol/l)	2.05 ± 1.43	1.87 ± 1.38	1.77 ± 1.16*	0.022
Lycopene (μmol/l)	0.73 ± 0.37	0.71 ± 0.37	0.69 ± 0.39	0.677
α-Tocopherol (μmol/l)	43.55 ± 7.56	40.08 ± 6.83†	40.55 ± 7.01†	0.001
γ-Tocopherol (μmol/l)	3.00 ± 0.98	2.98 ± 0.81	2.87 ± 0.89	0.592
α + γ-Tocopherol (μmol/l)	46.55 ± 7.95	43.07 ± 7.15†	43.42 ± 7.41†	0.001
25-Hydroxyvitamin D_3 (nmol/l)	50.33 ± 23.52	49.38 ± 24.?9	52.26 ± 25.18	0.659
α-Carotene/TC	0.11 ± 0.08	0.12 ± 0.08	0.11 ± 0.07	0.161
β-Carotene/TC	0.23 ± 0.17	0.23 ± 0.19	0.21 ± 0.15	0.251
α + β-Carotene/TC	0.34 ± 0.24	0.35 ± 0.26	0.32 ± 0.22	0.563
Lycopene/TC	0.12 ± 0.07	0.13 ± 0.07	0.13 ± 0.08	0.505
α-Tocopherol/TC	7.14 ± 0.89	7.27 ± 0.91	7.21 ± 1.03	0.382
γ-Tocopherol/TC	0.49 ± 0.15	0.55 ± 0.15	0.51 ± 0.15	0.086
α + γ-Tocopherol/TC	7.63 ± 0.91	7.82 ± 0.96	7.72 ± 1.06	0.249

Values are means ± s.d. STAEST = stanol ester margarine and STEEST = sterol ester margarine.
[a]$n = 33$ for results concerning lycopene and lycopene/TC (TC = total cholesterol).
[b]Significance of the difference for overall changes during the test margarine periods analysed with analysis of variance for repeated measurements (GLM).
*$P<0.05$, †$P<0.001$, significant difference between the either test margarine period and control period analysed with GLM with Bonferroni correction. There were no significant differences in serum fat-soluble and carotenoid concentrations between two test margarine periods.

goal 8–10 E%) and the mean intake of fat (30–31 E%) was close to the goal (<30 E%) in all experimental periods. In addition, the mean intake of dietary cholesterol met the goal well. The results of the fatty acid composition of cholesteryl esters paralleled with the results of the food records. There were no major differences in serum fatty acid composition among the experimental periods. Furthermore, the fatty acid composition of the STAEST and STEEST margarine, the fatty acid composition of the sterol and stanol fatty acid esters, the esterification degree of stanols and sterols, and the actual daily intake of total sterols (unsaturated and saturated) in the STAEST and STEEST margarine were identical. No significant changes in BMI, physical activity or other living habits were found during the study. Thus, the differences in lipid responses between two test margarine periods can be ascribed to the stanol and sterol fatty acid esters rather than differences in background diet or other background variables.

In the present study the subjects consumed test margarines in a randomized order according to the model of Latin square design, and each subject worked as his/her own control. The benefit of the present study design is that it eliminates the between-individual variation and the effect of time. In earlier studies it has been shown that plant sterols reduce cholesterol concentrations within 2–3 weeks of the initiation of treatment, and on the other hand, that the serum cholesterol concentration return to initial value within 2–3 weeks, upon cessation the ingestion of plant sterols (Farquhar *et al*, 1956; Heinemann *et al*, 1986; Jones *et al*, 1997; Weststrate & Meijer, 1998). Therefore, the 4 week study period can be considered long enough to demonstrate the cholesterol-lowering effect of the test margarines.

Our findings are parallel with the findings of our earlier study (Hallikainen & Uusitupa, 1999) and strengthen the view that stanol ester margarine decreases serum total and LDL cholesterol concentrations as part of a low-fat diet. Furthermore, our findings are in accordance with the study of Weststrate and Meijer (1998), in which a soybean oil sterol ester margarine based on unhydrogenated soysterols was equally effective as a stanol ester margarine (Benecol®) in lowering plasma total and LDL cholesterol concentration. The finding that plant stanols or sterols can reduce serum cholesterol concentrations even after a markedly low dietary cholesterol intake indicates that plant stanols and sterols must inhibit not only the absorption of dietary but also that of biliary cholesterol. This is supported by the findings of the earlier plant stanols/sterols studies, in which the faecal excretion of neutral sterols was increased despite the constant dietary cholesterol intake (Becker *et al*, 1993; Gylling *et al*, 1997; Gylling & Miettinen 1994).

The subjects with apolipoprotein E4 allele have been found to have a higher cholesterol absorption rate (Kesäniemi *et al*, 1996; Ordovas, 1999) and therefore those subjects might have more benefit from plant stanols and sterols. In a secondary analysis the subjects with apolipoprotein E4/3 genotype had a greater percentage reduction in LDL cholesterol during the STAEST margarine period (16.8%) than during the STEEST margarine period (9.6%). There are some studies in which the effects of sitosterol, sitostanol and sitostanol esters after combining different treatment groups (Miettinen & Vanhanen, 1994), sitostanol esters (Vanhanen *et al*, 1994) or plant stanol esters (Uusitupa & Hallikainen, 1999) on lipid responses in different apolipoprotein E phenotype or genotype groups have been investigated. Miettinen and Vanhanen (1994) and Vanhanen *et al* (1994) found that serum total and LDL cholesterol concentrations were reduced more effectively in subjects with the apolipoprotein E allele 4 than those with allele 2 or 3. However, we did not find this difference with stanol ester-enriched low-fat margarines (Uusitupa & Hallikainen, 1999). As far as we know there are no previous studies in which the effects of plant stanols on serum cholesterol concentrations in different apolipoprotein E genotype groups have been compared to that of plant sterols. Our present results are interesting, but these results need to be confirmed in a prospective study design where equal numbers of subjects with different apolipoprotein E genotypes should be allocated to different diet groups.

Serum plant sterols in very high concentrations have been found to be atherogenic (Glueck *et al*, 1991). Under normal conditions the plant sterol concentration is only on an average 3–17 mg/l in serum being roughly only 1/1000 of serum cholesterol concentration, although typical dietary intake of plant sterols is almost equal to dietary intake of cholesterol (about 160–360 mg/day, Ling & Jones 1995). Furthermore, dietary intake of plant stanols and their serum concentrations are very low compared to that of the plant sterol concentrations. In the present study serum campesterol and sitosterol concentrations were significantly higher at the end of the STEEST margarine period compared to the end of the control and STAEST margarine periods. Respectively, serum sitostanol and campestanol concentrations were significantly higher at the end of the STAEST margarine period than at the end of the control and STEEST margarine periods. However, during the entire study serum plant sterol and stanol concentrations remained very low, indicating that the absorbed amounts were very small in relation to the daily intake of plant sterols or stanols from the test margarines. Although STAEST margarine also contained a small amount of plant sterols, their serum concentrations reduced significantly when that margarine was consumed owing to the ability of the plant stanols to inhibit the absorption of plant sterols. All these findings are in line with the findings of earlier studies (Gylling *et al*, 1999a; Gylling & Miettinen 1999; Hallikainen & Uusitupa, 1999; Hallikainen *et al*, 2000; Jones *et al*, 1997,1999; Weststrate & Meijer, 1998). Besides the negligible absorption of plant stanols, the low serum concentrations could also result from fast and effective clearance of absorbed stanols (Salen *et al*, 1970).

The increased serum Δ7-lathosterol concentration and Δ7-lathosterol/total cholesterol ratio can be ascribed to compensatorily increased endogenous cholesterol synthesis due to cholesterol malabsorption during the test margarine periods. Also, in previous studies the synthesis of Δ7-lathosterol has been found to be stimulated by plant stanol esters (Gylling *et al*, 1995,1997,1999a; Gylling & Miettinen, 1994,1996; Vanhanen *et al*, 1993).

During the test margarine periods there were no significant changes in concentrations of serum 25-hydroxyvitamin D_3, retinol or in concentrations of serum lycopene, α-carotene, β-carotene and tocopherols related to the serum total cholesterol concentration. The findings of the present study are in agreement with the findings of our earlier studies (Hallikainen *et al*, 1999, 2000; Hallikainen & Uusitupa, 1999), in which we found only small effects on serum carotenoid concentrations when changes in serum carotenoids were related to the changes in serum total cholesterol. However, there are plant stanol and plant

sterol studies (Gylling *et al*, 1999b; Gylling & Miettinen 1999; Hendriks *et al*, 1999; Weststrate & Meijer, 1998), in which serum β- or $\alpha+\beta$-carotene concentrations were found to decrease significantly even after relating to the changes in serum lipid concentrations. The differences in the results might be a consequence of variability in composition of background diets. The diets in the studies by Gylling *et al* (1999b), Gylling and Miettinen (1999), Hendriks *et al* (1999) and Weststrate and Meijer (1998) were not standardized the way the diets were in our earlier studies (Hallikainen *et al*, 1999, 2000; Hallikainen & Uusitupa, 1999) and in the present one. In our studies subjects received detailed written and oral instructions about the low-fat diets, specifying the amounts and quality of food by main food groups, including vegetables. According to our findings the effects of plant sterols and stanols on serum carotenoid concentrations are minor.

In conclusion, as part of a low-fat diet the STAEST and STEEST margarines reduce serum total and LDL cholesterol concentrations significantly and there is no significant difference in their cholesterol-lowering efficacy in subjects with mild to moderate hypercholesterolaemia. In a secondary analysis the subjects with apolipoprotein E4 allele seemed to have greater effect on LDL cholesterol response with the STAEST margarine than with the STEEST margarine, but this result should be confirmed in a prospective study. Serum plant stanol and sterol concentrations increased with the STAEST and STEEST margarine, respectively, but their concentrations in serum remained very low. However, our study indicates that both plant stanols and sterols are absorbable in small amounts from the intestine.

Acknowledgements—The authors wish to thank Soili Lehtomäki for nutrition counselling, Virpi Lindi for analysis of apolipoprotein E genotypes, and Leena Kaipiainen, Pia Hoffström, Ritva Nissilä, Irja Lyytikäinen, Erja Kinnunen and Kaija Kettunen for excellent laboratory work. In addition, the authors wish to thank statistician Pirjo Halonen for valuable advice on study planning and data analyses.

References

Ågren JJ, Julkunen A & Penttilä I (1992): Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column. *J. Lipid Res.* **33**, 1871–1876.

Alpers DH, Clouse RE & Stenson WF (1986): Protein and Calorie Requirements. In: *Manual of Nutritional Therapeutics*, pp 131–159. Toronto: Little Brown and Co.

Becker M, Staab D & von Bergmann K (1993): Treatment of severe familial hypercholesterolemia in childhood with sitosterol and sitostanol. *J. Pediatr.* **122**, 292–296.

Cohen J (1988): *Statistical Power Analysis for the Behavioral Sciences*, Vol 2. Hillsdale, NJ: Lawrence Erlbaum.

Cullinane EM, Yurgalevitch SM, Saritelli AL, Herbert PN & Thompson PD (1995): Variations in plasma volume affect total and low-density lipoprotein cholesterol concentrations during the menstrual cycle. *Metabolism* **44**, 965–971.

Farquhar JW, Smith RE & Dempsey ME (1956): The effect of beta sitosterol on the serum lipids of young men with arteriosclerotic heart disease. *Circulation* **XIV**, 77–82.

Glueck CJ, Speirs J, Tracy T, Streicher P, Illig E & Vandegrift J (1991): Relationships of serum plant sterols (phytosterols) and cholesterol in 595 hypercholesterolemic subjects, and familial aggregation of phytosterols, cholesterol, and premature coronary heart disease in hyperphytosterolemic probands and their first-degree relatives. *Metabolism* **40**, 842–848.

Gylling H & Miettinen TA (1994): Serum cholesterol and cholesterol and lipoprotein metabolism in hypercholesterolaemic NIDDM patients before and during sitostanol ester-margarine treatment. *Diabetologia* **37**, 773–780.

Gylling H & Miettinen TA (1996): Effects of inhibiting cholesterol absorption and synthesis on cholesterol and lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic men. *J. Lipid Res.* **37**, 1776–1785.

Gylling H & Miettinen T (1999): Cholesterol reduction by different plant stanol mixtures and with variable fat intake. *Metabolism* **48**, 575–580.

Gylling H, Siimes M & Miettinen TA (1995): Sitostanol ester margarine in dietary treatment of children with familial hypercholesterolemia. *J. Lipid Res.* **36**, 1807–1812.

Gylling H, Radhakrishnan R & Miettinen TA (1997): Reduction of serum cholesterol in postmenopausal women with previous myocardial infarction and cholesterol malabsorption induced by dietary sitostanol ester margarine. Women and dietary sitostanol. *Circulation* **96**, 4226–4231.

Gylling H, Puska P, Vartiainen E & Miettinen TA (1999a): Serum sterols during stanol ester feeding in a mildly hypercholesterolemic population. *J. Lipid Res.* **40**, 593–600.

Gylling H, Puska P, Vartiainen E & Miettinen TA (1999b): Retinol, vitamin D, carotenes, and α-tocopherol in serum of a moderately hypercholesterolemic population consuming sitostanol ester margarine. *Atherosclerosis* **145**, 279–285.

Haapa E, Toponen T, Pietinen P & Räsänen L 1985: *Annoskuvakirja* (in Finnish) [Portion size booklet]. Helsinki: National Public Health Institute.

Hallikainen MA & Uusitupa MIJ (1999): Effects of 2 low-fat stanol ester-containing margarines on serum cholesterol concentrations as part of a low-fat diet in hypercholesterolemic subjects. *Am. J. Clin. Nutr.* **69**, 403–410.

Hallikainen MA, Sarkkinen ES & Uusitupa MIJ (1999): Effects of low-fat stanol ester margarines on concentrations of serum carotenoids in subjects with elevated serum cholesterol concentrations. *Eur. J. Clin. Nutr.* **53**, 966–969.

Hallikainen MA, Sarkkinen ES & Uusitupa MIJ. (2000): Plant stanol esters affect on serum cholesterol concentrations of hypercholesterolemic subjects in a dose-dependent manner. *J. Nutr.* **130**, 767–776.

Hassan AS & Rampone AJ (1979): Intestinal absorption and lymphatic transport of cholesterol and β-sitostanol in rats. *J. Lipid Res.* **20**, 646–653.

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

Heinemann T, Axtmann G & von Bergmann K (1993). Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur. J. Clin. Invest.* **23**, 827–831.

Hendriks HFJ, Weststrate JA, van Vlient T & Meijer GW (1999): Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* **53**, 319–327.

Jones PJH, MacDougall DE, Ntanios F & Vanstone CA (1997). Dietary phytosterols as cholesterol-lowering agents in humans. *Can. J. Physiol. Pharmac.* **75**, 217–227.

Jones PJH, Ntanios FY, Raeini-Sarjaz M & Vanstone CA (1999): Cholesterol-lowering efficacy of a sitostanol-containing phytosterol mixture with a prudent diet in hyperlipidemic men. *Am. J. Clin. Nutr.* **69**, 1144–1150.

Kaplan LA, Miller JA & Stein EA (1987). Simultaneous measurement of serum retinol, tocopherols, carotenes, and carotenoids by high performance liquid chromatography. *J. Clin. Lab. Anal.* **1**, 147–152.

Kesäniemi YA, Savolainen MJ, Uusitupa M, Sarkkinen E, Rantala M & Kervinen K (1996): Apolipoprotein E phenotypes and response to dietary fat and cholesterol. In: *Pennigton Center Nutrition Series. Nutrition, Genetics, and Heart Disease*, Vol 6, GA Bray & DH Ryan, (eds.) pp 379–397. Baton Rouge and London: Lousiana State University Press.

Ling WH & Jones PJH (1995): Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sci.* **57**, 195–206.

Lütjohann D, Björkhem I, Beil UF & von Bergmann K (1995): Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment. *J. Lipid Res.* **36**, 1763–1773.

Miettinen TA (1988): Cholesterol metabolism during ketoconazole treatment in man. *J. Lipid Res.* **29**, 43–51.

Miettinen TA & Koivisto P (1983): Non-cholesterol sterols and bile acid production in hypercholesterolaetnic patients with ileal bypass. In: *Bile Acid and Cholesterol in Health and Disease*, G Paumgartner, A Stiehl & W Gerok (eds.), pp 183–187. Lancaster: MTP Press.

Miettinen TA & Vanhanen HT (1994). Dietary sitostanol related to absorption, synthesis and serum level of cholesterol in different apolipoprotein E phenotypes. *Atherosclerosis* **105**, 217–226.

Miettinen TA, Puska P, Gylling H, Vanhanen H & Vartiainen E (1995). Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *New Engl. J. Med.* **333**, 1308–1312.

National Cholesterol Education Program (1994): Second report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel II). *Circulation* **89**, 1329–1445.

Niinikoski H, Viikari J & Palmu T (1997): Cholesterol-lowering effect and sensory properties of sitostanol ester margarine in normocholesterolemic adults. *Scand. J. Nutr.* **41**, 9–12.

Ordovas JM (1999): The genetics of serum lipid responsiveness to dietary interventions. *Proc. Nutr. Soc.* **58**, 171–187.

Parviainen MT (1983): *The fat-soluble vitamins A, D and E—their metabolism, binding-proteins and determination from human serum.* Doctoral thesis, Vol 159, Acta Universitatis Tamperensis. Ser A. University of Tampere, Tampere.

Penttilä IM, Voutilainen E, Laitinen O & Juutilainen P (1981): Comparison of different analytical and precipitation methods for the direct estimation of high-density lipoprotein cholesterol. *Scand. J. Clin. Lab. Invest.* **41**, 353–360.

Pollak OJ & Kritchevsky D (1981): *Sitosterol. Monographs on Atherosclerosis*, TB Clarkson, D Kritchevsky, OJ Pollak, (eds.) Vol 10. New York: Basel.

Rastas M, Seppänen R, Knuts L-R, Karvetti R-L & Varo P 1993. *Nutrient Composition of Foods*. Helsinki: Social Insurance Institution.

Salen G, Ahrens EH Jr & Grundy SM (1970). Metabolism of β-sitosterol in man. *J. Clin. Invest.* **49**, 952–967.

Tsukamoto K, Watanabe T, Matsushima T, Kinoshita M, Kato H, Hashimoto Y, Kurokawa K & Teramoto T (1993): Determination by PCR-RFLP of apo E genotype in a Japanese population. *J. Lab. Clin. Med.* **121**, 598–602.

Uusitupa MIJ & Hallikainen MA (1999): Efficacy of plant stanol esters in subjects consuming NCEP diets. *Eur. Heart J. Suppl.* **1**(Suppl S), S91–S95.

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

Vanhanen HT, Kajander J, Lehtovirta H & Miettinen TA (1994): Serum levels, absorption efficiency, faecal elimination and synthesis of cholesterol during increasing doses of dietary sitostanol esters in hypercholesterolaemic subjects. *Clin. Sci.* **87**, 61–67.

Weststrate JA & Meijer GW (1998): Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* **52**, 334–343.

Kuopion yliopiston julkaisuja D. Lääketiede
Kuopio University Publications D. Medical Sciences

234. Valve, Raisa. Studies on Genetic Regulation of Basal Metabolic Rate in Obese Subjects. 2001. 82 p. + appendix. Diss. 100 mk

235. Rahkonen, Terhi. Delirium in the non-demented elderly. Causative factors, prognosis and the effects of a support intervention in community care after a delirium episode. 2001. 89 p. + appendix. Diss. 100 mk

236. Huusko, Tiina. Hip fragtures in central Finland and geriatric rehabilitation after hip fracture operation. 2001. 61 p. + appendix. Diss. 100 mk

237. Hyppölä, Harri. Development and Evaluation of Undergraduate Medical Education in Finland. 2001. 85 p. + appendix. Diss. 100 mk

238. Vauhkonen, Ilkka. Early Defects in Glucose Metabolism in Relatives of Patients with Different Phenotypes of Diabetes Mellitus. 2001. 106 p. + appendix. Diss. 100 mk

239. Voutilainen-Kaunisto, Raija. Ocular Manifestations in Type 2 Diabetes. Controlled Ten-Year Follow-Up Study. 2001. 115 p. + appendix. Diss. 100 mk

240. Jakob, Stephan M. Splanchnic Vasoregulation and Metabolism: New Insights into Physiology. 2001. 102 p. + appendix. Diss. 100 mk

241. Loimas, Sami. Herpes simplex virus type 1 thymidine kinase - green fluorescent protein fusion gene as a tool in cancer gene therapy research. 2001. 69 p. + appendix. Diss. 100 mk

242. Karonen, Jari. Diffusion and Perfusion Imaging in Human Acute Ischemic Stroke. 2001. 79 p. + appendix. Diss. 100 mk

243. Huuskonen, Jouni. Osteoporosis in men. 2001. 119 p. + appendix. Diss. 100 mk

244. Böhm, Jan. Prognosis of Differentiated Thyroid Carcinoma; Relation to Clinical Factors and Cell Adhesion. 2001. 102 p. + appendix. Diss. 100 mk

245. Honkalampi, Kirsi. Studies on the Relationship Between Alexithymia and Depression. 2001. 96 p. + appendix. Diss. 100 mk

246. Voutilainen, Sari; Salonen, Jukka T. (Eds.). Third International Conference on Natural Antioxidants and Anticarcinogens in Food, Health and Disease (NAHD 2001), June 6-9, 2001 Helsinki, Finland. 2001. 133 p. 100 mk

247. Viitanen, Laura. Studies on the genetic basis of premature coronary heart disease using candidate gene approach and genome-wide scanning. 2001. 112 p. + appendix. Diss. 100 mk