Scientific Committee on Food

General view of the Scientific Committee on Food on the long-term effects of the intake of elevated levels of phytosterols from multiple dietary sources, with particular attention to the effects on β-carotene

(expressed on 26 September 2002)

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1.   TERMS OF REFERENCE

The Scientific Committee on Food was required, on the basis of the information submitted to the Commission, to comment on the possible effects on plasma β-carotene levels of the intake, also on the long term, of the elevated levels of phytosterols from multiple dietary sources.

2.   INTRODUCTION

Phytosterols or plant sterols are lypophilic naturally occurring compounds that are structurally related to cholesterol, but they differ in their side chain substitutions at the C24 position. Over 40 plant sterols have been identified so far in nature. The major phytosterols are β-sitosterol, campesterol, stigmasterol and avenasterol. Rapeseed oil contains a small amount of brassicasterol. Other phytosterols are the so called stanols (plant stanols or phytostanols), which are saturated phytosterols that are less abundant in nature but they can be produced by 5-alpha hydrogenation of the corresponding phytosterols (e.g. sitostanol and campestanol) (Gurr, 1996).

Sterols in plants are found in the free form or esterified to fatty acids or as steryl glycosides. Plant sterols are present in Western diets in amounts similar to those of dietary cholesterol (150 to 400 mg/day), with vegetarian diets containing about 50% higher amounts. The main dietary source of plant sterols is vegetable oils such as corn, sunflower, soybean and rapeseed oils (Ling and Jones, 1995). Phytosterols are not endogenously synthesized in humans and are derived solely from diet. There is no known role for phytosterols in human nutrition.

Increased blood cholesterol concentration is a well-known risk factor for development of coronary heart disease (CHD) and other diseases related to atherosclerosis. The degree of lowering blood cholesterol levels is directly related to the reduction of risk (Law *et al.*, 1994). It has been recently reported that phytosterols that are naturally present in commercial corn oil significantly reduce cholesterol absorption in humans (Ostlund *et al.*, 2002a) and there is some evidence that naturally-occurring plant sterols might reduce blood cholesterol to a small degree (Gurr, 1996). However, for an effective reduction higher doses are required.

In the 1980s, the cholesterol lowering effects of the use of plant sterols to fortify foods were well recognised. Margarines and butter appear as ideal vehicles for plant sterols because of their strong lypophilic nature (Mattson *et al.*, 1982). In Europe, the average consumption of butter or margarine is about 25 g per person per day and, according to the previous opinion of the Committee on the safety for the use of phytosterol esters in yellow fat spreads (SCF, 2000a), the sterol-enriched margarines may contain up to 2 g of plant sterols or stanols per daily portion. Actually, the first food fortified with phytosterols was a margarine, Benecol,

already in 1995; stanols were added because the evidence available suggested that they had a greater blood cholesterol-lowering potential than sterols, and because the amount of stanols absorbed from the gut was very low (Law, 2000). However, cream cheese, mayonnaises, salad dressings, yoghurts and other foods are also intended as phytosterol-enriched foods. Esterification of phytosterols with long chain fatty acids increases their lipid solubility and facilitates their incorporation into these foods.

However, the consumption of high doses of plant sterols significantly reduces the blood levels of carotenoids and, to a lesser extent, of other essential fat-soluble nutrients. This problem has to be considered in the context of the current tendency of the food industry to extend the enrichment with plant sterols to a number of different foods. Thus, in addition to paying attention to every individual application (a process that is under development), the overall consequences should be considered by the Committee, bearing the consequences of all possible applications combined in a common perspective, with a view on the long-term effects of the intake of elevated levels of phytosterols from multiple dietary sources. Particular attention has to be paid to the effects on the blood levels of β-carotene and related fat-soluble nutrients.

3. BENEFITS OF PHYTOSTEROLS BY LOWERING BLOOD LDL-CHOLESTEROL

The cholesterol-lowering effect of plant sterol and stanol esters has been widely documented and reviewed (Law, 2000; Plat *et al.*, 2000; Tammi *et al.*, 2000). It was recognised in the 1950s (Pollak, 1953) that plant sterols lower blood concentrations of cholesterol, by inhibiting intestinal absorption of cholesterol, probably (see below) by competing for the cholesterol space in mixed micelles, which are the form of lipid delivery for absorption into the mucosal cells (Law, 2000; Jones *et al.*, 1997).

A daily dose of 1-3 g plant sterols lowers LDL-cholesterol (LDLc) levels by about 5-15% (Hendriks *et al.*, 1999; Maki *et al.*, 2001; Stalenhoef *et al.*, 2001) in different populations, ages and conditions (hyper- and non-hypercholesterolaemic), including children (Becker *et al.*, 1993; Tammi *et al.*, 2000; Amundsen *et al.*, 2002) and people under hypocholesterolaemic drug treatment (Blair *et al.*, 2000; Neil *et al.*, 2001; Nigon *et al.*, 2001). However, the precise dose-response relationship for various phytosterol-enriched products has not been established. No additional benefit is derived from an intake of phytosterols above the range of 1-3 g per day.

Very recently, it was shown that a daily intake of 1.5 g of plant sterols (as plant sterol-enriched spread) in children with familial hypercholesterolaemia leads to a reduction in LDLc of about 10%, without reported adverse effects (Amundsen *et al.*, 2002), although decreases in alpha- and β-carotene (not significant) and lycopene (statistically significant) were observed (Amundsen *et al.*, 2001).

Based on a number of studies, 2-3 g/day plant stanols from margarines or mayonnaises, as part of a moderately rich- or high-fat diet, significantly reduces total cholesterol (Tc) and LDLc blood concentrations, without affecting HDL-cholesterol (HDLc) or triglyceride blood concentrations (Gylling and Miettinen, 1994; Gylling *et al.*, 1995; Hallikainen and Uusitupa, 1999; Hallikainen *et al.*, 2000a, 2000b).

Lees *et al.* (1977) evaluated the efficacy of plant sterol preparations from two different sources and in two different physical forms in lowering the blood cholesterol of a total of 46 patients with type II hyperlipoproteinaemia when given in addition to appropriate diet therapy. As reported by Raulio *et al.* (2001) the study included different groups and doses, including subjects receiving up to 18 g of sterols per day and the average duration of the test diet was 10 months. There were no outward signs of any side effects. The maximal mean cholesterol-lowering effect in response to any preparation was 12%, although it was much greater in some individual patients. Sterol balance data showed that plant sterols inhibit cholesterol absorption with maximal negative cholesterol balance in adults at a dose of 3 g/day of a tall oil sterol suspension. In this study nutrition markers were not measured (Raulio *et al.*, 2001).

A recent report has shown that the improvements of LDLc, HDLc, Tc, apolipoprotein B concentrations, and LDL/HDL cholesterol ratio during the daily consumption of a phytosterol ester-enriched margarine were most marked in those subjects with a high dietary intake of cholesterol, energy, total fat, and saturated fatty acids and with high baseline absorption (Mussner *et al.*, 2002).

The results of several randomised, double-blind trials in human adults that compared the ability of foods (thirteen polyunsaturated margarines, five mayonnaises, one olive oil, and one butter) with and without added plant sterols to lower cholesterol have been recently reviewed and summarised (Law, 2000). Fourteen trials, with average daily doses between 0.8 and 4.0 g/day, showed significant reductions in Tc and LDLc levels, with little change in blood concentrations of HDLc or triglyceride. There appeared to be a greater response with intakes of about 2 g/day as compared to 1 g/day, while no further increase was noted at intakes above 2 g. Based on the benefits of the observed decreases in blood cholesterol, it was claimed that consumption of margarines enriched with plant sterols or stanols is expected to reduce the risk of heart disease by 25% (Law, 2000). However, there are no studies to show effects of phytosterol intake on rates of cardiovascular disease.

3.1   Efficacy of plant stanol esters *versus* plant sterol esters

First studies indicated that sitostanol was more effective than sitosterol in displacing cholesterol from micelles *in vivo* (Ikeda and Sugano, 1983; Ikeda *et al.*, 1989) and in reducing blood Tc and LDLc levels (Heinemann *et al.*, 1986). This has also been observed more recently (Jones *et al.*, 2000) but not in other recent studies in humans (Weststrate and Meijer, 1998; Hallikainen *et al.*, 2000a; Normen *et al.*, 2000).

One study (Weststrate and Meijer, 1998) included normolipidaemic subjects following their habitual diet except that their habitual spreads were replaced by test margarines-containing plant stanols (Benecol), soybean oil distillates sterols (Henkel Corporation, LaGrange, USA), rice bran sterols (Tsuno, Wakayama, Japan) or sheanut sterols (Loders Croklaan, Wormerveer, The Netherlands). It was shown that unhydrogenated soy sterols were as effective as a stanol ester margarine in lowering blood cholesterol concentrations. Rice bran and sheanut sterols (mainly 4,4-dimethyl sterols) did not lower blood cholesterol levels, but they lowered blood carotenoid levels, even more pronouncedly than the other sterols tested (Weststrate and Meijer, 1998). In this study, the measured daily intake was somewhat higher for sterols (3.2 g) than for stanols (2.7 g), and there were some differences in the fatty acid composition of the various margarines tested. Also, very recently, it has been reported that 4,4'-dimethylsterol esters (of both sterols and stanols) caused a weaker cholesterol-lowering effect compared with the 4-desmehtylsterols (Trautwein *et al.*, 2002).

A recent study in patients after ileostomy showed that addition of 1.5 g of sterol or stanol esters to a high cholesterol diet similarly reduced by about one third the intestinal uptake of cholesterol (Normen *et al.*, 2000).

Stanol ester- and sterol ester- (daily amounts of about 2 g) enriched margarines significantly and similarly reduced blood Tc (by 9.2% and 7.3%, respectively) and LDLc concentrations (by 12.7% and 10.4%, respectively) in hypercholesterolaemic subjects on a low-fat diet, in a 12-week study distributed in three consecutive periods of 4 weeks (Hallikainen *et al.*, 2000a). The sterol and stanol ester spreads used were prepared in the same laboratory, following the same procedure and esterification process. In this study, both types of margarines significantly lowered blood β-carotene concentration. Blood γ-tocopherol was not changed but α-tocopherol levels were significantly lowered. There were no significant changes in the blood concentration of 25-hydroxy-vitamin-D_3 and retinal, or in the blood concentrations of lycopene, α-carotene, β-carotene and tocopherol related to the blood Tc concentration.

Different studies in animals showed enhanced cholesterol-reducing efficacy with plant sterol blends-containing increasing levels of plant stanols (Ntanios and Jones, 1998; Plat, 2001).

From the published results, it can be concluded that the blood Tc- and LDLc-lowering effect of sterols and stanols is quite similar, although the hydrogenated sterol esters may be somehow more efficient in reducing the intestinal absorption of cholesterol (Relas *et al.*, 2001), depending on specific factors which still have not been elucidated.

3.2   Phytosterol esters when used in conjunction with cholesterol-lowering drugs

In principle, plant sterols may be a useful additive therapy in the treatment of hypercholesterolaemic patients (Blair *et al.*, 2000; Neil *et al.*, 2001; Nigon *et al.*, 2001). Two recent studies have been done on the effect of spreads containing phytosterol esters when used in conjunction with cholesterol-lowering drugs i.e. statins and fibrates (Neil *et al.*, 2001; Nigon *et al.*, 2001).

A randomised, double-blind, placebo-controlled crossover trial with two consecutive periods of 8 weeks was conducted (Neil *et al.*, 2001). Thirty patients with heterozygous familial hypercholesterolaemia treated concurrently with an HMG-CoA reductase inhibitor (statin) and 32 patients with type IIa primary hypercholesterolaemia with a Tc concentration >6.5 mmol/L not taking lipid-lowering drug therapy were recruited from a hospital lipid clinic. The active treatment was a fortified fat spread (25 g/day) providing 2.5 g of plant sterols. After 4 weeks, LDLc had decreased 15.0%, there was a small but statistically significant increase in apolipoprotein AI and a decrease in apolipoprotein B in the active treatment group. HDLc and triglyceride concentrations were unchanged. There was no difference in response between patients with statin-treated familial hypercholesterolaemia and patients with type IIa hyperlipoproteinaemia. The conclusion was that a fortified fat spread enriched with vegetable oil sterols reduces LDLc by 10-15% with no difference in response between hypercholesterolaemic patients prescribed statins and those not taking lipid-lowering drug therapy. The purpose of this study was to determine the effect of a fat spread enriched with vegetable oil sterols on blood lipid, lipoprotein and apolipoprotein concentrations.

It has been studied whether patients receiving a lipid-lowering drug (fibrate) might differ in their response to plant sterols (Nigon *et al.*, 2001). The study was a randomized, double-blind, placebo-controlled two-period crossover trial with two treatments and three periods. Fifty

three hypercholesterolaemic patients (31 females and 22 males) completed the study. Both treatment periods lasted 2 months, with a washout period (2 months) between them. Fortified fat spread provided 1.6 g/day of plant sterols derived from edible vegetable oils and fatty acids from sunflower seed oil. The plant sterol content consisted of sitosterol esters (50%), campesterol esters (25%), stigmasterol esters (20%) and 10% of other esters. No adverse side effects of the diet were reported. Blood Tc and LDLc concentrations were significantly reduced by 6.4% and 8.8%, respectively. No effect on HDLc and lipoprotein(a) concentrations was detected. Spread enriched with plant sterol esters significantly lowered blood total and LDLc levels without affecting HDLc concentration, in a hypercholesterolaemic population following a strict low-cholesterol diet. In addition, a combination of fibrate treatment and plant sterol ester-supplemented spread offered a safe and effective measure to significantly decrease abnormally high cholesterol levels. The conclusion was that phytosterol-enriched spread is a useful adjunctive therapy for hypercholesterolaemic patients.

Results suggest that phytosterol esters can be used safely to provide "additional" cholesterol-lowering effect.

4. RISKS OF PHYTOSTEROLS

4.1 Previous evaluation by the Committee

On 6 April 2000, the Committee expressed its opinion on the safety of use of phytosterol esters in yellow fat spreads as a novel food (SCF, 2000a) and concluded that its use at levels up to 8% free phytosterols per 100 g spread is safe for human use. The toxicological information available comprised data from studies on absorption, distribution, metabolism and excretion and on subchronic toxicity, genotoxicity, reproductive toxicity, potential estrogenic activity and from human studies. The Committee concluded that no safety concerns from these specific phytosterols were apparent. The safety in use of phytosterols has been demonstrated for mixtures of predominantly β-sitosterol, campesterol and stigmasterol and/or their esters with fatty acids, to which the specification of the new product should be restricted. For these mixtures, a profile of 30-65% β-sitosterol, 10-40% campesterol, 6-30% stigmasterol and a total of 5% other phytosterols, based on total sterol content (w/w), was considered acceptable by the Committee (SCF, 2000a).

4.2 Intestinal absorption of plant sterols *versus* stanols

Like cholesterol, plant sterols are potentially atherogenic, but only small amounts of plant sterols are absorbed (ranging from less than 1% of dietary stanols to about 5% of β-sitosterol and 15% of campesterol) (Heinemann *et al.*, 1993; Lutjohann *et al.*, 1995; Jones *et al.*, 1997), except in the genetic disorder of sitosterolaemia (see section 2.4). Actually, the particular interest posed on stanols is because they have been considered practically unabsorbable (Lutjohann *et al.*, 1995), or very poorly absorbed (Heinemann *et al.*, 1993; Gylling and Miettinen, 1999; Gylling *et al.*, 1999a). In a clinical study, where plant sterol esters (1.1 and 2.2 g/day) were shown to reduce cholesterol absorption and lower circulating blood cholesterol concentrations when incorporated into the habitual diet, serum plant sterol concentrations increased from baseline (0.48% of total sterols by weight to 0.64 and 0.71% by weight for the low- and high-sterol groups, respectively) (Maki *et al.*, 2001).

In general, it is assumed that increasing the length of the side-chain of cholesterol decreases the absorbability of the resulting sterol, and that hydrogenation of the nucleus double-bond of a sterol causes a decrease of absorbability, as demonstrated for cholesterol/cholestanol and sitosterol/sitostanol pairs. This latter assumption was not shown in one study, also in humans, where campestanol was more absorbed (12%) than campesterol (9.5%) (Heinemann *et al.*, 1993). However, a very recent study in humans showed that absorption from 600 mg of soy sterols given with a standard test breakfast was 0.5% for sitosterol and 1.9% for campesterol, and 0.04% for sitostanol and 0.12% for campestanol (Ostlund *et al.*, 2002b). Thus, reduction of the double-bond at position 5 decreased absorption by around 90%, and the authors' conclusion was that the efficiency of phytosterol absorption was lower than that previously reported.

Recently, the relative extent of intestinal absorption and subsequent tissue distribution of β-sitosterol, β-sitostanol, campesterol, campestanol and stigmasterol has been studied in the rat (Sanders *et al.*, 2000). Campesterol (13%) was more absorbed than β-sitosterol and stigmasterol (both 4%) and than sitostanol and campestanol (1-2%).

4.3   Safety studies

Specifications and safety studies on phytosterol esters as ingredients for a particular novel food have already been considered by the Committee (SCF, 2000a).

A phytosterol profile of 30-65% β-sitosterol, 10-40% campesterol, 6-30% stigmasterol and a total of 5% other phytosterols, based on total sterols content (w/w), was considered acceptable by the Committee. However, the potential sources of phytosterols are much more diverse. For example, based on variability in sourcing/seasonal variation of the plant sterols, a more expanded sterol profile has already been proposed by the industry for its use in yellow fat spreads (SCF, 2000a).

Very recently a petitioner reported (Unilever, 2002a) its intention to change specifications, as during the last five years their analytical techniques have become more precise, identification of sterol profiles improved and they now have much greater knowledge of batch to batch and supplier to supplier variability. These data are under current evaluation by the Committee.

The safety and tolerability of esterified phytosterols administered in reduced-fat spreads and/or in salad dressings to healthy adult men and women during 8 weeks have been studied (Davidson *et al.*, 2001). Eighty-four subjects consumed reduced-fat spread and salad dressing providing 0, 3, 6 or 9 g/day of phytosterols. The only laboratory abnormalities detected were elevations in CK, the levels of which generally experience important fluctuations in response to external factors. Significant reductions were observed in alpha- and β-carotene in the group receiving 9 g phytosterols/day.

A one-year follow-up study on the use of low fat spread enriched with plant sterol esters (Hendriks *et al.*, 2001) showed no adverse effects after daily consumption of 1.6 g phytosterols, but a significant reduction of blood alpha- and β-carotene concentrations, while levels of the fat-soluble vitamins A, K_1, D and E did not change.

It can be added that a recently developed Post Launch Monitoring (PLM) of "yellow fat spreads with added phytosterol esters" did not showed occurrence of adverse health effects from the current intake of marketed spreads containing phytosterol esters (Unilever, 2002b; SCF, 2002).

As previously revised by the Committee (SCF, 2000a), several animal studies indicated that, when used at high levels or when administered subcutaneously, plant sterols, especially sitosterol, might have estrogenic activity. Clear estrogenic effects were found in fish (MacLatchy and van der Kraak, 1995; Mellanen *et al.*, 1996). There were also some controversial data in the rat on orally administered β-sitosterol which were not confirmed by other studies in the same species. The Committee stated that the revised studies, including a two-generation reproductive study in rats, provided sufficient reassurance of the absence of endocrine effects via the oral route.

More recently, an increase in plasma estradiol and thyroid hormones as well as some alterations in intermediary metabolism at doses of about 5 mg/kg body weight/day suggest that phytosterols act as endocrine disruptors in the mustelid European polecat (Nieminen *et al.*, 2002). The endocrine effects were however different from those previously described in fish. Interestingly, in the polecat sterols did not trigger a decrease of blood cholesterol, rather it increased significantly, which suggests important differences with respect to the situation in humans. In any case, none of the observed effects of phytosterols in the polecat seemed harmful or useful in risk assessment by itself.

4.3.1 Safety studies on stanols

The former evaluation of the Committee of yellow fat spreads with added phytosterols (SCF, 2000a) did not assess phytostanols in deep. There are however a number of available studies on safety of stanols. In a 13-week oral toxicity study with stanol esters in rats (Turnbull *et al.*, 1999a) no toxicity was observed after ingestion of wood- or vegetable oil-derived stanol esters at dietary concentrations from 0.2% up to 1% (expressed as free stanols; equivalent to about 0.5 g stanols/kg body weight/day). At dietary levels of 5%, subchronic ingestion of these substances resulted in decreased plasma levels of the fat-soluble vitamins E and K and, to a lesser extent, vitamin D. The same can be said for hepatic vitamin levels, except that vitamin K was not measured.

Plant stanol esters from wood and vegetable oil sources were not genotoxic at doses up to the limit of solubility, with or without the addition of an Aroclor-induced rat liver microsome metabolic activation system. The plant stanol esters were negative in bacterial (*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537) and mammalian cell (L5178Y) gene mutation assays and in a mammalian cell (CHO cells) chromosome aberration assay Turnbull *et al.*, 1999b).

In a developmental toxicity study in rats, no adverse treatment-related maternal or foetal developmental effects were produced following ingestion of a diet containing up to 8.76% plant stanol fatty acid esters. This diet provided up to 5% plant stanols equivalent to 2.4-3.5 g/stanols/kg body weight/day (Slesinski *et al.*, 1999). The sample was reported to contain 57.1% total stanols/100 g fat (68% sitostanol, 30% campestanol and 2% unsaturated sterols), 42% fatty acids and 2% unsaturated sterols and unknowns.

A two-generation reproductive toxicity study of plant stanol esters in rats, that used a commercially manufactured vegetable oil-derived stanol ester mixture containing mainly sitostanol and campestanol, showed no adverse effects on reproduction, pup mortality or pup body weight, at dietary concentrations up to 4.38% plant stanol esters (equivalent to 2.5% total stanols in the diet) (Whittaker *et al.*, 1999).

In an *in vitro* study on potential estrogenic activity, four samples of vegetable oil-derived stanol mixtures (58.3-67.1% sitostanol, 29.3-31.6% campestanol, 0.7-2.6% sitosterol, 0.2-1.1% campesterol, 0.4-8.7% other sterol compounds) did not induce proliferation of estrogen-responsive human breast adenocarcinoma cells (MCF-7) (Turnbull *et al.*, 1999c). In an uterotrophic assay with immature female rats vegetable- and wood-derived stanol esters did not induce significant changes of uterus weights when fed at concentrations of 8.3% in the diet for 4 days.

4.4 Phytosterolaemia

Although in mixed diets plant sterols may contribute nearly as much as cholesterol to total sterols intake, there is a subtle mechanism by which our body distinguishes between cholesterol and non-cholesterol sterols, so that we absorb and retain about 50% of dietary cholesterol but less than 5% of dietary non-cholesterol sterols. However, in sitosterolaemia (also known as phytosterolaemia), an infrequent autosomal recessive disorder of which the precise prevalence rate is not known, affected individuals hyper-absorb and retain not only cholesterol but also all other (plant, sea fish, etc.) sterols. Consequently, patients with this disease have higher blood levels of plant sterols and develop tendon and tuberous xanthomas, arthralgias and arthritis, accelerated atherosclerosis and premature coronary artery disease (Lee *et al.*, 2001a, 2001b). The hallmark of sitosterolaemia is elevated blood levels of plant sterols that are not well catabolised in human metabolism and thus are deposited in tissues, including the vascular intima.

The identification of the defective genes in sitosterolaemia support the hypothesis that a specific molecular mechanism regulates cholesterol entry into and out of the body, and that this mechanism allows for exquisite differentiation between sterol species that are very similar in their chemical structure and in many physicochemical properties. Mutations in two "half" adenosine triphosphate binding cassette (ABC) transporter genes cause this disease (Lee *et al.*, 2001b). Less than 200 bases separate these two genes, that encode sterolins 1 and 2, respectively, two proteins that are critical in the regulation of dietary-sterol absorption and excretion. Interestingly, to cause sitosterolaemia both copies of only one gene have to be defective. It has been hypothesized that sterolins 1 and 2 function as heterodimers, regulating the excretion of non-cholesterol sterols out of the mucosal cell (Lee *et al.*, 2001b; Lu *et al.*, 2002; Plat and Mensink, 2002).

4.5 Effect of phytosterols in lowering blood levels of carotenoids and other nutrients

Reduced absorption of some fat-soluble vitamins, which accompanies the consumption of foods enriched in sterol esters, appears to be the main concern. The Committee considered that ingestion of 20 g per day for one year of products containing 8% free phytosterols reduced blood β-carotene concentration by 20% (SCF, 2000a). Although the β-carotene concentration was still within the normal range and within normal seasonal variation, such a reduction in blood β-carotene levels might become relevant in subjects with a non-optimal vitamin A status. In addition, some other potential benefits of carotenoids not directly related to vitamin A formation can also become compromised.

Since these vitamins or pro-vitamins have some role in protecting LDL from oxidation, it has become a common practice in the literature to evaluate the degree of reduction in their circulating levels relative to the degree of reduction of LDLc levels. In that case, a significant phytosterol-elicited reduction can be observed only for β-carotene, and not for other carotenoids. This standardization makes it difficult to know the absolute figures of carotenoid

and fat-soluble vitamin levels, and their changes. Therefore, such standardization can be useful to assess those aspects derived from or directly related to LDL particles but not in a wider perspective. There are also recent studies showing no effect (after adjusting for total cholesterol reduction) of consumption of esterified plant sterols or stanols on serum fat-soluble vitamins or carotenoid concentration compared with a control diet (Raeini-Sarjaz *et al.*, 2002).

Several randomised trials have shown a lowering effect of phytosterols on blood levels of β-carotene of about 25%, and a smaller, 5-15%, lowering effect on α-carotene, lycopene, vitamin E, and α-tocopherol blood levels, although the magnitude of the effects may vary depending on several factors.

By standardising data available in the literature, it has been calculated that decreases in blood carotenoids plateaued when doses of sterols or stanols reached 2.2 g/day (Plat *et al.*, 2000).

In a very recent study (Mensink *et al.*, 2002) consumption of plant stanol esters (3 g/day) lowered the blood concentrations of several carotenes (α-carotene, β-carotene, lycopene) and xantophyles (lutein/zeaxanthin and β-cryptoxanthin). In this study, the expected reduction in absolute blood tocopherol concentrations, reported in various other studies, was not observed. Actually, the LDL particles were enriched in tocopherols. After standardization for LDLc, levels of the various tocopherols were significantly increased, those of various carotenoids were unchanged and those of β-carotene were decreased. This may suggest that changes in antioxidant concentrations cannot be simply explained by a decrease in the number of circulating LDL particles (Mensink *et al.*, 2002).

4.5.1   *Bioavailability of fat-soluble vitamins and carotenoids*

The common feature of fat-soluble vitamins is that they are all non-polar lipids with extremely low solubilities in aqueous media. Thus, it is generally accepted that absorption of dietary fat-soluble vitamins is, despite individual peculiarities, very much dependent on their incorporation into lipid-mixed micelles formed in the small intestine during food digestion. Bile salts are of major importance in this process by enabling these hydrophobic vitamins, together with cholesterol, carotenoids and other lipids, to become solubilised as micelles within the aqueous content of the intestine, thus allowing their absorption by the enterocytes. Hydrolysis of cholesterol and fat-soluble vitamin esters must occur before absorption can take place.

The efficiency of absorption of these compounds varies. It can be about 40% for cholesterol and vitamins D, E and K, and higher than 50%-75% for retinol (Olson, 1987b; Blomhoff *et al.*, 1991). β-carotene absorption is particularly variable, from 10% to 90% depending on several specific conditions (reviewed by Woutersen *et al.*, 1999; SCF, 2000b, 2000c); it has been described that it can even be as low as 2.5% (O'Neill and Thurnham, 1998). In general, absorption of β-carotene in humans tends to be more or less linear up to intakes of 20-30 mg, and it becomes saturated at higher intakes (SCF, 2000b).

Following their intestinal uptake, vitamins D, E and K remain largely unchanged, cholesterol and retinol are mainly esterified with fatty acids, and β-carotene can partly remain intact and partly cleaved to two molecules of retinal that are then reduced to vitamin A (retinol) in the intestine of humans. Both free and esterified forms of fat-soluble vitamins are incorporated into chylomicrons and reach the bloodstream via the lymphatic pathway. They are taken up by the liver, where they can be stored or released back to the blood. Liver and adipose tissue

are the main site of carotenoid deposition. In the fasted state, about 75% of the β-carotene is bound to LDL and about 25% to HDL and VLDL (SCF, 2000b), a distribution that highly correlates to that of cholesterol. Vitamin E is also transported by blood lipoproteins, whereas retinol and vitamin D have their own specific binding proteins.

Blood levels of β-carotene reflect roughly intake and absorption of this compound, rising dramatically in response to dietary supplementation. In contrast, blood levels of vitamin A seem to be much more stable. The blood depletion half-life of vitamin A is ten times longer than β-carotene's, presumably reflecting the much larger body store of vitamin A (Olson, 1987a).

Other main factors affecting the bioavailability of carotenoids are: the meal contents of dietary fibre and fat, the food matrix, the cooking procedure and cholesterol-lowering medication. It has been reported that, following the intake of a meal containing them, the increase of blood levels of β-carotene, lycopene and lutein, but not those of canthaxantin or α-tocopherol, were significantly reduced (by 30 to 70%) when the meal was enriched with pectin, guar, alginate, cellulose or wheat bran (0.15 g/kg body weight) (WHO, 1998). Also, high doses of pectin (12 g in a single meal with 25 mg β-carotene) reduced by 50% the peak at 30 hours in blood β-carotene (Rock and Swendseid, 1992). In addition, various animal studies have confirmed that pectins can reduce β-carotene bioavailability.

Cholesterol-lowering medication has also been reported to reduce (30-40%) the levels of circulating β-carotene (Probstfield *et al.*, 1985; Yoshida *et al.*, 1995).

4.5.2   *Cholesterol-lowering mechanisms*

The exact mechanism by which phytosterols elicit a decrease of Tc and particularly of LDLc is not known. It has been observed that the concentration of micelles containing phytosterols in the jejunum is key in reducing the uptake of cholesterol by the intestinal cells (Ikeda *et al.*, 1989). It was also shown that both sitosterol and sitostanol competitively decrease the incorporation of cholesterol into mixed micelles, both *in vitro* and *in vivo* (Ikeda and Sugano, 1983).

However, only recent research has directly addressed the effect of phytosterols on the absorption of fat-soluble vitamins in humans (Relas *et al.*, 2001). This study was performed in ten healthy adult men who acutely received a fat load test of margarine (8 g) with or without 1 g stanyl esters. The stanyl ester mixture consisted mainly of sitostanyl ester (92%) and two to three different dosages of vitamin A, E and β-carotene, all given in the 8 g margarine load, were tested. The results showed that 1 g dietary stanyl esters in margarine did not detectably interfere with the absorption (measured levels in serum and lipoproteins) of simultaneously-ingested cholesterol, triacylglycerol, α-tocopherol, β-carotene, retinol and retinyl palmitate during a 24 h follow-up, whereas it lowered the campesterol/cholesterol blood concentration ratio, reflecting reduced sterol absorption efficiency. As postprandial cholesterol was not altered apparently in this study, the authors suggested that reduction of blood cholesterol and β-carotene levels by dietary stanyl esters was probably not an acute effect, but a result of chronic stanyl ester consumption. For cholesterol, the mechanism could be related to a diminished intestinal pool of cholesterol and a compensatory alteration in whole-body cholesterol metabolism (Relas *et al.*, 2001).

The recent discovery of the involvement of ATP binding cassette (ABC) transporters in cholesterol absorption was a lead to further explore the hypocholesterolaemic mechanism of plant stanols. Plat and Mensink (2002) found that mixed micelles enriched with sitostanol or with cholesterol plus sitostanol were potent inducers of ABCA1 expression in caco-2 cells, an accepted model to study human intestinal lipoprotein metabolism. Based on these findings, authors hypothesize that plant stanols -and possibly plant sterols- increase ABCA1-mediated cholesterol efflux back into the intestinal lumen.

Interestingly, recent studies suggest that phytosterols are equally effective when incorporated into low-fat or high-fat novel foods. Thus, low-fat (0.7%) yoghurt enriched with plant stanol esters (3 g) lowered LDLc by 14%, the same extent as oil-based-enriched products, with effects already maximal after one week (Mensink *et al.*, 2002). In agreement with many other studies, in this study reduction of β-carotene levels was also apparent and not limited to the LDL fraction. Blood HDLc levels were not affected. This study suggests that the ability of plant stanol esters to block intestinal cholesterol absorption is not substantially impaired by a low-fat matrix and, together with previous reports, it suggests that, to trigger the cholesterol-lowering effect, the food matrix or the background diet is of more importance for free plant stanols than for esterified plant stanols.

In conclusion, the mechanism of action of phytosterols appears to be related mainly to their physicochemical properties, which enable them to compete with cholesterol for a common space in mixed micelles, interfering with the passive absorption of lipids and with the specific mechanisms controlling the uptake of cholesterol *versus* other sterols. It is becoming clear that a very refined mechanism has evolved (see section 2.5) that allows cells to exquisitely differentiate between sterol species that are very similar in their chemical structure, thus avoiding non-cholesterol sterol absorption. Further understanding of the specific molecular mechanism regulating cholesterol entry into and out of the cells will also help an understanding of the effects of phytosterols on the bioavailability and blood levels of cholesterol, carotenoids and other related compounds.

5. ANTICIPATED INTAKE/EXTENT IN THE EU AND CONSEQUENCES OF USE

With normal consumption of the already authorised yellow fat spreads being 20-30 g/day, the intake of phytosterols will increase to about 1.6-2.4 g/day, which represents a 8-12 fold increase of the current daily intake from traditional products (SCF, 2000a). However, it should be kept in mind that new available data suggest that the potential benefits of phytosterols are not limited to their use in fat spreads or even in high-fat food.

Plant sterol-enriched food (Benecol margarine, containing stanols esterified with fatty acids of rapeseed oil) was first retailed in 1995 in Finland. Later on, other Benecol products (fresh cheese, snack bars, salad dressing and yoghurt) were launched on the market in Finland, Benelux, UK, Ireland, Sweden, Denmark and the USA. The recommended intake is based on 2 g/day of plant stanols.

Nowadays, more than 125 million daily portions have been sold in Finland, and about 200,000 Finnish people eat Benecol products every day.

Unilever launched its plant sterol-enriched margarine and salad dressing TakeControl in 1999, in the USA. The same margarine (Flora/Becel pro.activ) was marketed in Australia, Brazil,

New Zealand and Switzerland in 1999 and in the EU in 2000. The recommended daily intake was 28 g, corresponding to 2.2 g of plant sterols.

Recently, studies on the prevailing food and nutrient intake in different EU countries such as Germany (Hermann-Kunz and Thamm, 1999) and Spain (Serra-Majem *et al.*, 1999) have been published. The mean daily intake by the consumers in the highest quartiles of fat intake of some foods susceptible to be enriched with phytosterols are as follows: 150 to 235 g of total dairy foods (including 90 g of yoghurts and 40-45 g cheese), 145 g fruit juices, 120-300 g soft drinks, 5-75 g butter and margarines, 60 g biscuits plus cakes and pies, 30 g olive oils. Probably, other foods are also suitable for enrichment with plant sterols. The point is that, if each novel food is designed to deliver the effective cholesterol-lowering daily dose of phytosterols, additional measures are needed to avoid an excessive daily intake of these compounds.

A petitioner (Diminicol) suggested the possibility to reach the effective daily dose of phytosterols either through the consumption of a single Diminicol novel food or through the consumption of two or three portions of different Diminicol foods. Both these alternatives lead to the 1.5 g/day intake of sterols that is considered to be effective. According to this petitioner, the anticipated daily intakes of the different novel foods they included in their application are: yoghurt 240 g, fresh cheese 30 g, margarine 20 g, and fruit-milk drink 450 g.

The sterol-enriched foods are expected to replace the ordinary foods in the diet of cholesterol conscious consumers. If the novel foods are equivalent in composition (other than in plant sterols) to their traditional counterparts, no further nutritional imbalances should in principle be expected from their consumption.

5.1   Results of post launch monitoring

As part of the Commission Decision 2000/500/EC on authorising the placing on the market of "yellow fat spreads with added phytosterols" as a novel food or novel food ingredient under Regulation (EC) No 258/97, the applicant was obliged to collect data in order to estimate the extent to which the product is reaching its target group, i.e., people who try to control their elevated blood cholesterol, and to estimate exposures to phytosterols from this source in other population groups.

The petitioner has developed a sort of post marketing surveillance study (Unilever, 2002b), a so called Post Launch Monitoring (PLM) to obtain data on consumption of phytosterol esters in yellow fat spreads (pro.activ). The study was aimed mainly to investigate whether or not the use of the novel food (pro.activ) was as predicted/recommended, and if the effects and side effects were as predicted. Also studies aimed to detect if the product induced unknown side effects have been done.

From this study it can be first outlined that the use of the product was lower than it was anticipated as the regular consumers were buying the product with median intakes of 15-18 g for regular consumers, which was less than the 20-30 g/day anticipated when the original submission was made (SCF, 2002). Also the 95th upper intakes were 50% or less than those previously anticipated. The use of the product was predominantly by one person per household. Although cholesterolaemia was not directly measured in a sample of the target group of consumers results obtained are compatible with the product being bought by the target population and much less, if any, by other population groups.

This study also showed that some pro.activ consumers were also using products containing phytostanol esters, where these were available. It highlights the need to introduce some sort of management control to prevent over-consumption of phytosterols in a free market where many different products enriched with plant sterols are available.

6.   CONSIDERATIONS ON THE EFFECTS OF LONG-TERM INTAKES

In general, a tendency is apparent from the literature that the longer the duration of phytosterol consumption the larger the decrease in blood carotenoid concentration. However, definite conclusions at this respect cannot be drawn from the available data.

In a study of 1 year's consumption of sitostanol ester margarine (Gylling *et al.*, 1999b), the decrease *versus* controls of β-carotene was 33.3% and that of α-carotene 19.5%; the decreases *versus* the baseline home diet were 25% and 10%, respectively. A significant decrease was still observed when β-carotene concentration was expressed in relation to cholesterol concentration. In this study, no significant changes were found for vitamin D and retinol concentrations, or for the ratios of α-tocopherol to cholesterol and of α-carotene to cholesterol. This study also shows that the blood levels of α-tocopherol and carotenes (but not those of retinol and vitamin D) were tightly associated with indicators of cholesterol absorption.

A long-term (52 weeks) follow-up study on the use of a spread enriched with plant sterols (Ntanios, 2001) gave similar results for carotenoids but a LDLc-lowering effect (6% reduction) about half of that obtained in the stanol ester study described above (Gylling *et al.*, 1999b).

Apart from the carotenoid-lowering effect, no other nutritionally-relevant changes nor other abnormalities were evident in several randomised trials of plant sterol or stanol margarines in humans (Ntanios, 2001), some of which lasted for one year (Miettinen *et al.*, 1995).

A long-term study on safety and efficacy of phytosterol esters in 185 volunteers following controlled intake of 20 g/day of a spread-containing phytosterol esters (equivalent to 1.6 g sterols/day) over a one-year period was described (Hendriks *et al.*, 2001). Results are in agreement with a number of other clinical studies and consistently demonstrated a beneficial reduction in the blood Tc and LDLc levels. The only side effect observed has been a reduction in the absorption of the most lypophilic carotenoids (e.g. beta-carotene) (Hendriks *et al.*, 2001).

Apart from the carotenoid-lowering effect, no other adverse effects were observed in humans with doses as high as 3 g/day for three years (Law, 2000). Also, it is of note that stanol margarines have been sold for five years in Finland without overt evidence of hazards.

It is repeatedly argued in reports that the phytosterol-induced decrease of blood β-carotene concentration is of no substantial concern because the levels of β-carotene have always been found to remain within the "normal" range. Indeed, there is a wide range of baseline or control values for blood β-carotene, from 0.2 to 1.7 μmol/L, among the different intervention studies revised here. However, it is probably unacceptable to take such a wide variation as a reference when considering the changes of β-carotene concentration experienced by a given individual. In fact, the general assumption that individuals who have higher levels of blood

β-carotene have a lower risk for cancer and cardiovascular diseases (SCF, 2000b) is established by analysing a smaller range of variation, irrespective that β-carotene levels may be only a marker of the intake of other beneficial substances in fruits and vegetables, or perhaps a marker of other life-style habits.

The value of using blood analytes as nutritional biomarkers depends on an appropriate understanding of the physiological and life-style factors that influence their circulating concentrations and their relationship with health. The latter is not the case for β-carotene. A recent study addressing the factors that influence the absorption and distribution of carotenoids and other nutrients in an adolescent population in the USA showed an important variability in blood levels of β-carotene (10th percentile, 0.11 μmol/L; 90th percentile, 0.41 μmol/L) and lycopene (10th percentile, 0.25 μmol/L; 90th percentile, 0.78 μmol/L) (Neuhouser *et al.*, 2001). Although the relationship with food habits was clear, no direct relationship could be drawn between the levels of carotenoids and any physiological function or health marker.

Some dietary carotenoids, particularly β-carotene, serve as an important source of vitamin A. This is the major known function of carotenoids in humans. Because preformed vitamin A is only present in animal products (e.g. liver, eggs, milk products), in countries where the intake of animal products is low, carotenoids have to meet the vitamin A requirements (i.e. by 80% or more in Asia and Africa). Even in developed countries, carotenoids may usually contribute to vitamin A supply by more than 40%. It seems unlikely that the phytosterol-induced changes in β-carotene (as a vitamin A precursor) pose any serious problem except in countries where vitamin A deficiency is common, or perhaps in situations where vitamin A requirements are greater than normal (i.e. pregnancy, lactation, infancy). This is further supported by the fact that, in the phytosterol-enriched food trials, blood retinol levels remained unchanged despite the consistently observed decreases in β-carotene.

It has been suggested that, apart from their pro-vitamin A function, carotenoids can serve several other functions, such as radical quenching, antioxidant and anti-carcinogenic activities as well as regulators of other cellular functions (SCF, 2000b). All these functions are considered very important on a long-term basis. However, there is no consistent evidence that a decrease of up to one third of β-carotene levels, or of other nutrients which are lowered by phytosterol over-consumption, affects these functions. More research is required before definite conclusions can be drawn.

In view of the existence of two reports on harmful effects of β-carotene supplementation (ATBC, 1994; Omenn *et al.*, 1996) and the fact that blood retinol concentration was unaffected, the β-carotene reduction was not felt an essential concern in a trial of a sitostanol ester margarine (Gylling *et al.*, 1999b). However, the harmful effects were observed in studies using synthetic all-*trans* β-carotene, not natural sources of β-carotene, at doses of 20-30 mg/person/day, which largely exceeds the usual dietary daily dose. Natural dietary sources may contribute in Europe about 3-7 mg/day (up to 10 mg/day, depending on seasonal and regional variations) of β-carotene (SCF, 2000b), and it is quite well established that these levels of β-carotene may confer health benefits when derived from fruit and vegetables (the main natural dietary sources). There may be a very small difference between the levels of β-carotene that may produce adverse effects in smokers (20 mg/day in the ATBC study) and those that may confer health benefits to the general population (up to 10 mg/day) mainly from natural sources, according to previous statements of the Committee (SCF, 2000b, 2000c).

In this context, during long-term over-consumption of phytosterol-enriched foods, it seems convenient to recommend the intake of natural sources of β-carotene, i.e. carotenoid-rich vegetables and fruits, to counterbalance the expected reduction of the levels of β-carotene and of other fat-soluble nutrients.

Interestingly, very recently it has been shown that, when consuming plant sterol esters (2.3 g/day) or stanol esters (2.5 g/day) in spreads, a moderate increase of dietary carotenoids (an additional daily serving of high-carotenoid vegetables or fruits) may be effective in maintaining blood carotenoid concentrations (Noakes *et al.*, 2002). This was observed in a 3-weeks study of forty-six hypercholesterolaemic subjects that completed 3-way, double-blind, randomised crossover comparisons in a free living state. The dietary advice resulted in a 13% increase of β-carotene in subjects who consumed the sterol-free control spread. LDLc decreased by 7.7% and 9.5% after consumption of sterol ester- and stanol ester-enriched spreads, respectively, and there were no significant differences in the blood β-carotene concentrations (standardised by Tc plus triglycerides) of control, stanol ester and sterol ester groups.

7.   CONCLUDING REMARKS

The benefits of using phytosterol-enriched foods with the purpose of helping hypercholesterolaemic individuals reduce their LDLc blood levels are well supported by the available literature. A daily intake in the range of 1-3 g plant sterols lowers LDL-c levels by about 5-15%, in different populations, ages and conditions, but the precise dose-response relationships for various phytosterol-enriched products have not been established. No additional effect on cholesterol levels is derived from an intake of phytosterols above the range of 1-3 g per day.

However, plant sterols and stanols interfere with the absorption of carotenoids as deduced from the reduction of carotenoid blood levels. Other fat-soluble vitamins, such as vitamin E and tocopherols, may also be affected, although to a lesser extent than β-carotene. This problem, the observed effects and the likely mechanisms implicated, has to be considered for an appropriate assessment of the risks associated with consumption of phytosterol-enriched products, particularly in a long-term perspective. The decreases in blood carotenoids appear to plateau when doses of sterols or stanols reached 2.2 g/day and amounted to a reduction of 33% after one-year consumption of an enriched margarine providing 3 g/day. The consequences of such a persistent decrease of blood concentrations of β-carotene on human health are largely unknown. The mechanisms regulating the bioavailability of β-carotene admit a wide range of variation in the uptake and circulating levels of carotenoids. No serious concern can be deduced regarding the role of β-carotene as a vitamin A precursor, except in situations where vitamin A requirements are greater than normal as in pregnancy, lactation or infancy. No definitive conclusions can be drawn regarding other specific physiological roles and benefits of β-carotene, because they remain to be definitively established.

It is generally accepted that doses of up to 10 mg/day of β-carotene from carotenoid-rich fruits and vegetables, which trigger a significant increase of blood concentration of β-carotene, may confer health benefits. In this context, the Committee recommends the use of natural sources of β-carotene, i.e. carotenoid-rich vegetables and fruits, to counterbalance the expected reduction of blood β-carotene and other fat-soluble nutrients levels caused by long-term consumption of phytosterols in enriched foods. First experimental results indicate that

dietary advice in this direction can prove successful in avoiding the decrease of β-carotene and of other fat-soluble related nutrients.

The absorption of plant sterols is much lower than that of cholesterol. However, consumption of phytosterols leads to a small but dose-related increase of their plasma concentrations in short-term studies. Very high plasma levels of phytosterols in individuals with an autosomal recessive disease, sitosterolaemia, leads to severe and premature atherosclerosis. While the studies available provide no evidence of adverse effects associated with a small increase of plasma phytosterols, more information on possible effects of long-term exposure to higher intakes of plant sterols is needed.

The available data do not provide a basis for setting a numerical upper level of total daily intake of phytosterols. In consideration of the dosages found to be effective for cholesterol-lowering, without evidence of additional benefits at higher intakes and the possibility that high intakes might induce undesirable effects, it is prudent to avoid plant sterol intakes exceeding a range of 1-3 g/day. Since a number of foods appear as potential candidates to be enriched with plant sterols, additional management measures may be needed to avoid excessive intakes.

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