Effects of Diets Enriched With Two Different Plant Stanol Ester Mixtures on Plasma Ubiquinol-10 and Fat-Soluble Antioxidant Concentrations

Jogchum Plat and Ronald P. Mensink

Plant stanols lower intestinal cholesterol absorption. This causes a decrease in serum low-density lipoprotein (LDL)-cholesterol, despite a compensatory increase in cholesterol synthesis. We therefore hypothesized that plant stanols also change LDL-cholesterol–standardized concentrations of ubiquinol-10 (a side product of the cholesterol synthesis cascade) and of those fat-soluble antioxidants that are mainly carried by LDL. To examine this, 112 nonhypercholesterolemic subjects consumed low erucic acid rapeseed oil (LEAR)-based margarine and shortening for 4 weeks. For the next 8 weeks, 42 subjects consumed the same products, while the other subjects received products with vegetable oil–based stanols (2.6 g sitostanol plus 1.2 g campestanol daily, n = 36) or wood-based stanols (3.7 g sitostanol plus 0.3 g campestanol daily, n = 34). Consumption of both plant stanol ester mixtures increased cholesterol synthesis and lowered cholesterol absorption, as indicated by increased serum cholesterol-standardized lathosterol and decreased plant sterol concentrations, respectively. Compared with the control group, absolute plasma ubiquinol-10 concentrations were lowered by 12.3% ± 18.9% (−0.14 μg/mL *v* the control group; *P* = .004; 95% confidence interval [CI] for the difference in changes, −0.05 to −0.22 μg/mL) in the vegetable oil–based group and by 15.4% ± 13.0% (−0.17 μg/mL *v* the control group; *P* < .001; 95% CI for the difference, −0.08 to −0.27 μg/mL) in the wood-based group. Changes in LDL-cholesterol–standardized ubiquinol-10 concentrations were not significantly changed. The most lipophylic antioxidants, the hydrocarbon carotenoids (β-carotene, α-carotene, and lycopene), decreased most, followed by the less lipophylic oxygenated carotenoids (lutein/zeaxanthin and β-cryptoxanthin) and the tocopherols. These reductions were related to the reduction in LDL, which carry most of these antioxidants. The decrease in the hydrocarbon carotenoids, however, was also significantly associated with a decrease in cholesterol absorption. LDL-cholesterol–standardized antioxidant concentrations were not changed, except for β-carotene, which was still, although not significantly, lowered by about 10%. We conclude that the increase in endogenous cholesterol synthesis during plant stanol ester consumption does not result in increased LDL-cholesterol–standardized concentrations of ubiquinol-10, a side product of the cholesterol synthesis cascade. Furthermore, decreases in absolute fat-soluble antioxidant concentrations are related to decreases in LDL-cholesterol. However, for the most lipophylic carotenoids, some of the reduction was also related to the decrease in cholesterol absorption.

UBIQUINONE-10 (coenzyme Q10) is well known for its role as an electron transporter in the mitochondrial respiratory chain.[1] It is also present in plasma, where it is almost entirely transported by low-density lipoproteins (LDL),[2,3] mainly in its reduced form (ubiquinol-10).[4] Ubiquinol-10 is a powerful antioxidant,[1,5] even stronger than α-tocopherol.[6] In contrast to other fat-soluble antioxidants, ubiquinone is hardly derived from the diet,[7] but is endogenously synthesized as a side product of the cholesterol synthesis cascade (Fig 1). This may suggest that inhibition of hepatic hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme for cholesterol synthesis, reduces ubiquinone plasma levels, which indeed has been demonstrated after statin treatment.[8,9]

Plant stanols are useful hypocholesterolemic agents and daily intakes of 2 to 3 g reduce serum LDL-cholesterol concentrations by 10% to 15%.[10] This effect is at least partly caused by a decrease in intestinal cholesterol absorption, which induces an increase in endogenous cholesterol synthesis,[11] possibly due to higher HMG-CoA reductase activity.[12] Therefore, consumption of plant stanols may also increase ubiquinol synthesis. On the other hand, plant stanols may lower not only LDL-cholesterol concentrations, but also other lipophylic compounds. Indeed, lipid-standardized plasma concentrations of α- and β-carotene[13-16] and of lycopene[14] were reduced after consumption of plant stanol or plant sterol esters. Lipid-standardized α-tocopherol levels, however, were hardly changed.[13-15]

Information on plant stanols and plasma levels of other carotenoids and tocopherols, which also exert important biologic functions,[17,18] is missing. We therefore decided to study the effects of two different plant stanol ester mixtures on plasma concentrations of ubiquinol-10 and of several carotenoids and tocopherol isomers. In addition, we sought to determine whether changes in these antioxidants were related to the reduced cholesterol absorption, as caused by plant stanols. Serum concentrations of plant sterols and cholesterol precursors were analyzed as markers for cholesterol absorption and cholesterol synthesis.

MATERIALS AND METHODS

Subjects

Characteristics of the population have been described in detail elsewhere.[19] Briefly, 112 participants, 41 males and 71 females, from Maastricht and surrounding municipals completed the study. Their ages were 33 ± 16 years (mean ± SD), body mass index (BMI) 23 ± 2.8 kg/m^2, serum total cholesterol concentrations 4.97 ± 0.73 mmol/L, and serum triacylglycerol concentrations 0.88 ± 0.37 mmol/L. Subjects were normotensive, had no proteinuria or glucosuria, had no history of coronary heart disease, and did not use prescribed diets or medication known to affect serum lipids. Fifteen males and 8 females smoked, 2 males and 2 females were vegetarians, 38 women used oral contracep-

From the Department of Human Biology, Maastricht University, Maastricht, The Netherlands.

Submitted January 25, 2000; accepted November 14, 2000.

Supported by the RAISIO Group, Raisio, Finland.

Address reprint requests to Prof Dr Ronald P. Mensink, Maastricht University, Department of Human Biology, PO Box 616, 6200 MD Maastricht, The Netherlands.

0026-0495/01/5005-0001$35.00/0

doi:10.1053/meta.2001.22509

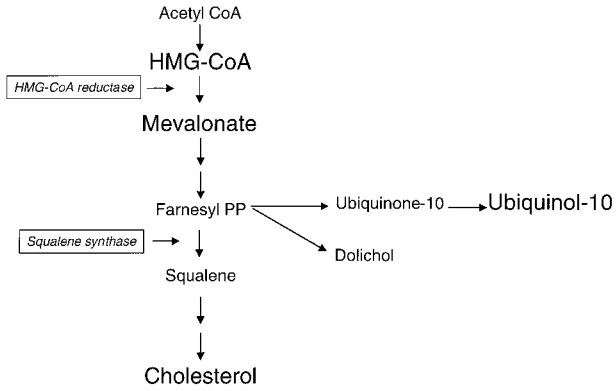

Fig 1. Schematic view of the biosynthesis of cholesterol and nonsterol isoprenes (ubiquinone-10, dolichol). Regulatory proteins are indicated by rectangles.

tives, and 4 women were postmenopausal. All subjects gave their written informed consent before the start of the study. The protocol was approved by the medical ethical committee of Maastricht University.

Diets and Design

Details of this study on the effects of two different plant stanol ester mixtures on various cardiovascular risk markers have been published previously.[19] Briefly, subjects were provided with low erucic acid rapeseed oil (LEAR)-based margarine and shortening to replace their usual margarine and baking fat. The minimal daily intakes were 20 g for margarine and 10 g for shortening. The participants used the margarine at breakfast and lunch, and the shortening at dinner.

During a 4-week run-in period, subjects received a control margarine and shortening to which no plant stanol esters were added. Subjects were then randomly allocated to 1 of 3 treatment groups, stratified for sex and age. The control group continued to use the control LEAR-based margarine and shortening for another 8 weeks, while the second and third groups used the same margarine and shortening with an added vegetable oil– or wood-based plant stanol ester mixture. Both plant stanol ester mixtures were derived by saturation of sitosterol, stigmasterol, and campesterol, followed by transesterification of the obtained free sitostanol and campestanol with rapeseed oil (RAISIO Group, Raisio, Finland). Transesterified stanol esters were subsequently mixed with the margarines and shortenings. The 2 plant stanol ester mixtures differed in their stanol composition. The vegetable oil–based mixture consisted of 70% sitostanol and 30% campestanol, while the wood-based plant stanol mixture contained approximately 93% sitostanol and 7% campestanol. Both the control margarine and shortening, as well as the experimental products, were fortified with normal amounts of vitamin A and D. β-Carotene was used as a coloring agent, while vitamin E was present as a natural compound. The exact compositions of the experimental margarines and shortenings have been presented elsewhere.[20] All products were coded with a color label to blind the subjects and the investigators.

Body weight was recorded every week and did not change during the study. Furthermore, subjects recorded in diaries any signs of illness, medication used, menstrual phase, and any deviations from the protocol. In addition, subjects were asked not to change their habitual diets, levels of physical exercise, smoking habits, or use of alcohol or of oral contraceptives during the study.

To estimate their energy and nutrient intakes, subjects recorded in the last week of the run-in and the intervention period, their food intake during the previous 4 weeks by filling in food frequency lists, consisting of 100 items. These lists were checked immediately in the presence of the subjects by a dietitian, and energy and nutrient intakes were calculated as described.[19]

Blood Sampling and Analyses

Blood sampling. At the end of the run-in period (weeks 3 and 4) and at the end of the intervention period (weeks 11 and 12), blood was sampled after an overnight fast. Subjects abstained from drinking alcohol the day preceding and smoking the morning of blood sampling. After about 13 mL blood was sampled for additional measurements (to be reported elsewhere), blood was drawn in a precooled (4°C) 10-mL EDTA tube (Monoject sterile, Sherwood Medical, Ballymoney, North Ireland) to obtain plasma at a final EDTA concentration of 1 g/L, and in a 10-mL clotting tube to obtain serum. Plasma was obtained directly and serum minimally 1 hour after venipuncture by centrifugation at 2,000 $\times$ *g* for 30 minutes at 4°C.

In weeks 3 and 4 and 11 and 12, a midportion of plasma was snap-frozen and stored directly at −80°C for analysis of antioxidant concentrations. In weeks 4 and 12 only, saccharose and butylated hydroxy toluene (BHT) were added to another midportion of the plasma, at final concentrations of 6 g/L and 250 mg/L, respectively. These samples were directly used to determine plasma ubiquinone and ubiquinol-10 concentrations. Serum aliquots were stored immediately at −80°C for analysis of lipid and lipoprotein, plant sterol, and cholesterol precursor concentrations.

Plant sterols and cholesterol precursors. For analysis of serum cholesterol, serum plant sterols and stanols (sitosterol, sitostanol, campesterol, and campestanol), and cholesterol precursors (lathosterol) by gas chromatography, serum of weeks 3 and 4 and of weeks 11 and 12 was pooled before analyses. Samples of 1 subject were analyzed in the same analytical run to exclude variations between runs. First, 200 μL serum was saponified for 1.5 hours at 70°C with 2 mL of a mixture consisting of a 10-mol/L KOH solution in water and ethanol (1:9% vol/vol). After cooling to room temperature, 1.4 mL water was added, mixed, and then 2 mL hexane added. This mixture was shaken vigorously and centrifuged for 2 minutes at 2,000 $\times$ *g*. The hexane layer was transferred to another tube, and the hexane extraction was repeated once to improve recovery. The hexane extracts were combined and evaporated to dryness at 37°C under a moderate nitrogen flow. The nonsaponifiable serum lipids were silylated for 15 minutes after addition of 200 μL Bis (trimethylsilyl)-trifluoracetamid (BSTFA) (Merck, Darmstadt, Germany) containing 1% trimethylchlorosilane (TMCS) (Sigma-Alderich Chemie BV, Zwijndrecht, The Netherlands), and 100 μL pyridine (Merck). After evaporation under a moderate nitrogen flow at 50°C, the samples were dissolved in 500 μL pentane. Samples were then analyzed with a GC8000 Top gas chromatograph (Carlo Erba, Milan, Italy) fitted with a 25-m AT1701 capillary column with an inner diameter of 0.32 mm and 0.30 μm film thickness (Alltech, Breda, The Netherlands) using cold on-column injection. A 1 μL quantity of the samples was injected into the gas chromatograph. Helium was used as carrier gas. The temperature of the injector was 20°C after secondary cooling. The oven temperature was programmed at 40°C for 1 minute, followed by a rise to 250°C at a rate of 79°C/min and then kept constant for 5 minutes. Next, the temperature increased to 265°C at a rate of 1°C/min and then kept constant for 15 minutes. The flame ionization detector temperature was set at 270°C; 100 μg of 5α-cholestane and 2 μg of 5β-cholestan-3α-ol (Sigma-Alderich Chemie BV, Zwijndrecht, The Netherlands) were added to all serum samples before extraction and used as internal standards for cholesterol and noncholesterol sterols, respectively. A standard mixture was used to identify the sterols by means of the retention times. A serum pool was prepared using blood from 34 healthy volunteers after 4 weeks of plant stanol ester consumption. This pool was stored at −80°C and analyzed twice in each run. Coefficients of variation within runs were 3.8% for cholesterol, 8.4% for sitosterol, 4.8% for campesterol, 10.0% for sitostanol,

11.0% for campestanol, and 6.7% for lathosterol. Sterol concentrations were expressed per millimole of total cholesterol.

Ubiquinone-10 and ubiquinol-10. Plasma ubiquinol-10 concentrations were analyzed in samples before and after in vitro treatment with sodium borohydride ($NaBH_4$) (Fluka Chemica, Neu-Ulm, Switzerland), which reduces ubiquinone-10 into ubiquinol-10. Ubiquinone-10 concentrations were calculated as the difference in ubiquinol-10 concentrations between the treated and the untreated samples. Extractions and analyses were performed in the dark to minimize photochemical decomposition of ubiquinol-10.

Extraction of ubiquinone from plasma was performed as described by Edlund.[2] Briefly, a 300 μL sample was mixed with 25 μL internal standard (ubiquinol-9, final concentration 40 μg/mL in ethanol) and 1 mL isopropanol. After mixing thoroughly, the tubes were placed on melting ice for 10 minutes, while mixing was repeated every 2 minutes. Samples were then centrifuged for 8 minutes at 2,500 $\times$ *g* and 4°C, and 175 μL of the supernatant was pipetted into an amber-colored 2-mL vial, which was placed directly at 4°C. Next, 350 μL of the remaining supernatant was transferred into a precooled glass tube to which 10 μL $NaBH_4$ (0.25% m/vol in methanol) was added and vortexed. The tube was placed for 10 minutes on melting ice, while vortexing was repeated every 2 minutes. Finally, 175 μL of this reduced sample was transferred into a second amber-colored vial.

Ubiquinol-10 plasma concentrations were determined using freshly prepared standard mixtures of ubiquinol-10 and ubiquinol-9, which were made from ubiquinone-9 and ubiquinone-10 (Fluka Chemica) also by treatment with $NaBH_4$. Standards were extracted using a comparable procedure as described for the samples. Briefly, to a 1-mL ubiquinone-10 or ubiquinone-9 stock solution in ethanol (100 μg/mL), 50 μL $NaBH_4$ (0.25% in methanol) was added and incubated for 30 minutes on melting ice in the dark. Next, 1 mL water was added to the tube, vortexed, and mixed with 4 mL hexane. This solution was centrifuged for 4 minutes at 1,000 $\times$ *g* at 4°C. The hexane phase was removed and transferred into another precooled tube. This extraction procedure was repeated once. Both hexane phases were mixed, evaporated to dryness under nitrogen, and dissolved in 2 mL ethanol. The exact concentrations of the standard solutions were measured spectrophotometrically at 290 nm by using the extinction $E_{1\%}$ = 46.6 L $mol^{-1} \cdot cm^{-1}$ for ubiquinol-10 and $E_{1\%}$ = 49.7 L $mol^{-1} \cdot cm^{-1}$ for ubiquinol-9. Four standard solutions with increasing ubiquinol-10 concentrations and constant ubiquinol-9 (40 μg/mL) concentrations, diluted with 30 mmol/L lithiumphosphate in a mixture of methanol:ethanol:isopropanol (22.5:73.6:3.9 vol/vol/vol), were prepared. Since standard solutions and plasma samples all contained the same concentration of ubiquinol-9, the ratio of ubiquinol-10 to ubiquinol-9 could be used to calculate the plasma ubiquinol-10 concentration. Calibration samples were placed at the start and at the end of each run.

The high-performance liquid chromatography (HPLC) system consisted of a cartridge guard column E, a 5-μm Inertsil ODS-2 and a 3-μm Inertsil ODS-3 column, 150 $\times$ 4.0 mm (GL Sciences, Tokyo, Japan). A working electrode against an Ag/AgCl reference electrode in saturated LiCl constituted the electrochemical detection system. Analyses were performed at +0.6V, 30°C, and at a flow rate of 0.5 mL/min. The mobile phase consisted of 30 mmol/L lithiumphosphate in the methanol:ethanol:isopropanol mixture. Peaks were recorded and analyzed with Gynkosoft Chromatography Data System 4.12 (Gynkotek HPLC, Germering, Germany).

Ubiquinone and ubiquinol-10 concentrations were determined in 111 instead of 112 subjects, since one analysis failed. A plasma pool was prepared using blood from 5 healthy volunteers. This pool was stored at −80°C and analyzed twice in each run. Coefficients of variations within runs were 6.1% and between runs 5.4% for ubiquinol-10, and 5.4% and 7.5% for total coenzyme Q10.

Antioxidants. The concentrations of lipophylic antioxidants in plasma samples were determined after storage at −80°C for 3 to 4 months, which does not affect antioxidant concentrations.[21] Tocopherols and carotenoids were determined simultaneously according to a modification of the assay of Hess et al,[22] as described by Oostenbrug et al.[23] Briefly, plasma samples were extracted twice with hexane, while retinylacetate was used as an internal standard. The amounts of lipid-soluble antioxidants were determined by reverse-phase HPLC. Samples from one subject of weeks 3 and 4 as well as of weeks 11 and 12 were pooled before analysis and analyzed in the same analytical run. The mean recovery of retinylacetate was 95.3% ± 1.7%.

Lipids and lipoproteins. Serum lipids and lipoproteins were analyzed as described.[19] The mean LDL cholesterol concentrations from weeks 3 and 4 and from weeks 11 and 12 were averaged to calculate LDL-cholesterol–standardized antioxidant concentrations, whereas LDL cholesterol concentrations from weeks 4 and 12 were used to calculate LDL-cholesterol–standardized ubiquinol-10 concentrations.

Statistics

Data from 112 subjects who completed the study were included in the analyses. Results are expressed as means ± SD. Changes for all parameters were calculated for each subject as the difference between values of the experimental period and run-in period. The differences in changes between both intervention groups and the control group were tested with analysis of variance (ANOVA). When a significant diet effect was found ($P < .05$), the 3 treatments were compared pairwise and corrected for 3 group comparisons ($\alpha = 0.017$) using the Bonferroni multicomparison test. Effects of gender and smoking on the responses were examined by including these factors as well as their interaction terms with treatment into the statistical model. Multiple regression analysis was used to examine the relationship between changes in cholesterol absorption and antioxidant concentrations. All statistical analyses were performed with Statview 4.5.[24]

RESULTS

Serum Lipids and Lipoproteins

Consumption for 8 weeks decreased serum total cholesterol from 4.93 mmol/L to 4.89 mmol/L on the control diet, from 4.89 mmol/L to 4.56 mmol/L on the vegetable oil–based diet ($P < .001$ *v* the control group; 95% CI for the difference in changes between the 2 groups, −0.23 to −0.55 mmol/L), and from 4.98 mmol/L to 4.55 mmol/L on the wood-based diet ($P < .001$ *v* the control group; 95% CI for the difference, −0.20 to −0.56 mmol/L). LDL-cholesterol concentrations changed from 2.96 mmol/L to 2.90 mmol/L on the control diet, from 2.94 mmol/L to 2.51 mmol/L on the vegetable oil–based diet ($P < .001$ *v* the control group; 95% CI for the difference, −0.22 to −0.51 mmol/L), and from 2.94 mmol/L to 2.54 mmol/L on the wood-based diet ($P < .001$ *v* the control group; 95% CI for the difference, −0.18 to −0.51 mmol/L). The decreases in total and LDL-cholesterol between both intervention groups were not significantly different from each other ($P = .925$ and $P = .793$, respectively). HDL-cholesterol and triacylglycerol concentrations were not changed after consumption of the 2 plant stanol ester mixtures.[19]

Plant Sterols and Cholesterol Precursors

Total plant sterol intake, as derived from the experimental products (~155 mg) did not change during the study and was not significantly different among the 3 groups.[19] Compared with the control group, cholesterol-standardized serum plant

sterol concentrations (sitosterol and campesterol) were decreased to the same extent after consumption of the vegetable oil– or wood-based stanol ester mixtures, indicating a reduction in cholesterol absorption (Table 1). Compared with the control and the wood-based group, the cholesterol-standardized serum campestanol concentration was significantly increased in the vegetable oil–based group ($P < .001$ and $P = .003$, respectively), most likely due to the higher dietary campestanol intake. Cholesterol-standardized sitostanol concentrations were increased in both stanol ester groups. This effect was slightly more pronounced in the sitostanol ester–rich wood-based group, although the change did not differ significantly from that in the vegetable oil–based group. These results suggest that plant stanols are absorbed to a small extent and that changes in serum plant stanol concentrations reflect their dietary intakes. However, cholesterol-standardized serum levels remained at very low levels. The cholesterol-standardized concentration of the cholesterol precursor lathosterol increased significantly with 22.2% in the vegetable oil group ($P < .001$) and with 17.8% in the wood-based group ($P < .001$) as compared with a change of 3.1% in the control group, indicating an increased cholesterol synthesis.

Table 1. Effects of Vegetable Oil– and Wood-Based Plant Stanol Esters on Cholesterol-Standardized Serum Plant Sterol and Stanol, and Lathosterol Concentrations During the Study

	Control Group	Vegetable Oil–Based Group	Wood-Based Group
Sitosterol ($10^2 \times \mu$mol/mmol cholesterol)			
Run-in	113.8 ± 32.7	113.9 ± 43.3	117.7 ± 57.8
Test period	105.9 ± 35.4	69.6 ± 24.1	62.9 ± 23.8
Change	−7.8 ± 22.4	−44.3 ± 26.9*	−54.7 ± 41.5†
Campesterol ($10^2 \times \mu$mol/mmol cholesterol)			
Run-in	304.3 ± 76.5	302.2 ± 100.9	303.5 ± 119.3
Test period	298.4 ± 82.3	209.4 ± 53.2	189.5 ± 61.2
Change	−5.9 ± 39.2	−92.8 ± 63.0*	−114.0 ± 77.3†
Sitostanol ($10^2 \times \mu$mol/mmol cholesterol)			
Run-in	7.1 ± 4.2	8.4 ± 4.8	7.3 ± 3.7
Test period	7.6 ± 3.7	15.8 ± 5.8	16.3 ± 6.0
Change	0.4 ± 4.0	7.4 ± 4.3*	9.0 ± 4.6†
Campestanol ($10^2 \times \mu$mol/mmol cholesterol)			
Run-in	10.7 ± 7.7	8.9 ± 4.5	9.5 ± 6.9
Test period	10.1 ± 6.8	17.9 ± 8.3	13.0 ± 7.7
Change	−0.6 ± 7.2	9.0 ± 7.3*,‡	3.5 ± 8.0
Lathosterol ($10^2 \times \mu$mol/mmol cholesterol)			
Run-in	97.0 ± 37.3	95.5 ± 28.7	99.7 ± 34.2
Test period	97.5 ± 33.9	115.3 ± 37.9	114.5 ± 35.6
Change	0.5 ± 19.5	19.8 ± 24.9§	14.8 ± 18.9¶

NOTE. Values are means ± SD. A total of 112 nonhypercholesterolemic men and women consumed LEAR-based margarine and shortening for 4 weeks (run-in), without plant stanol esters. For the next 8 weeks (test period), 42 subjects continued with these products, while the other subjects received products with a vegetable oil–based stanol ester mixture (2.6 g sitostanol and 1.2 g campestanol; n = 36) or a pine wood-based stanol ester mixture (3.7 g sitostanol and 0.3 g campestanol; n = 34). Changes were calculated as the difference between values of the experimental period and run-in period.

*$P < .001$ control group *v* vegetable oil–based group.

†$P < .001$ control group *v* wood-based group.

‡$P < .01$ wood-based group *v* vegetable oil–based group.

§$P < .017$ control group *v* vegetable oil–based group.

¶$P < .017$ control group *v* wood-based group.

Ubiquinone-10 and Ubiquinol-10 Concentrations

Table 2 shows that consumption of the plant stanol ester mixtures lowered the plasma concentrations of ubiquinol-10 and total coenzyme Q10. For ubiquinol-10, this decrease was 0.12 ± 0.18 μg/mL or 12.3% ± 18.9% in the vegetable oil–based group, which was significantly different from the change of 0.01 ± 0.19 μg/mL in the control group ($P = .004$; 95% CI for the difference, 0.05 to 0.22 μg/mL). Consumption of the wood-based stanol ester mixture caused a decrease of 0.16 ± 0.24 μg/mL or 15.4% ± 23.0% ($P < .001$ *v* the control group; 95% CI for the difference, 0.08 to 0.27 μg/mL). Both plant stanol ester mixtures decreased ubiquinol-10 concentrations to the same extent ($P = .473$; 95% CI for the difference, −0.06 to 0.14 μg/mL). Total coenzyme Q10 reductions paralleled changes in ubiquinol-10 concentrations.

Since changes in ubiquinol-10 and total coenzyme Q10 concentrations paralleled changes in LDL-cholesterol concentrations, changes in LDL-cholesterol–standardized ubiquinol-10 and total coenzyme Q10 concentrations did not differ significantly between the groups (Table 2). Effects were not modified by gender or smoking.

Antioxidant Concentrations

Consumption of the 2 plant stanol ester mixtures significantly lowered total carotenoid and total tocopherol concentrations, whereas retinol concentrations were not affected (Table 3). These effects were not modified by gender or smoking. Figure 2 shows the effects of the diets on the various carotenoids, phytofluene, and tocopherol isomers. Antioxidants are presented in order of decreasing lipophylicity. These results suggest that the most lipophylic antioxidants were affected most by the 2 plant stanol ester mixtures. The vegetable oil–based stanol ester mixture decreased β-carotene, δ-tocopherol, and α-tocopherol by, respectively, 0.092 ± 0.085 μmol/L or 26.1% ± 22.6%, 0.037 ± 0.077 or 10.7% ± 38.3%, and 2.029 ± 2.608 μmol/L or 7.8% ± 9.9% (Fig 2). These changes were significantly different from the changes in the control group for β-carotene ($P = .014$; 95% CI for the difference, −0.016 to −0.108 μmol/L), for δ-tocopherol ($P = .008$; 95% CI for the difference, −0.013 to −0.082 μmol/L), and for α-tocopherol ($P < .001$; 95% CI for the difference, −1.352 to −4.377 μmol/L). Consumption of the wood-based stanol ester mixture significantly decreased concentrations of lycopene (−0.147 μmol/L *v* the control group: $P = .002$; 95% CI for the difference, −0.056 to −0.238 μmol/L), as well as phytofluene (−0.205 mV · min/μL; amplification 100 *v* the control group: $P < .001$; 95% CI for the difference, −0.108 to −0.302 mV · min/μL; amplification 100), lutein/zeaxanthin (−0.056 μmol/L *v* the control group: $P < .012$; 95% CI for the difference, −0.016 to −0.096 μmol/L), and α-tocopherol (−3.195 μmol/L *v* the control group: $P < .001$; 95% CI for the difference, −1.601 to −4.789 μmol/L). The decrease of phytofluene in the wood-based group was different from the change in the vegetable oil–based group as well ($P = .001$; 95% CI for the difference, −0.056 to −0.268 mV · min/μL; amplification

Table 2. Effects of Vegetable Oil– and Wood-Based Plant Stanol Esters on Absolute and LDL-Cholesterol–Standardized Plasma Ubiquinol-10 and Total Coenzyme Q10 Concentrations During the Study

	Control Group	Vegetable Oil–Based Group	Wood-Based Group
Ubiquinol-10 (μg/mL)			
Run-in	0.76 ± 0.16	0.85 ± 0.24	0.81 ± 0.29
Test period	0.78 ± 0.24	0.73 ± 0.19	0.65 ± 0.17
Change	0.01 ± 0.19	−0.12 ± 0.18*	−0.16 ± 0.24†
Total coenzyme Q10 (μg/mL)			
Run-in	0.85 ± 0.20	0.95 ± 0.28	0.90 ± 0.31
Test period	0.83 ± 0.24	0.77 ± 0.19	0.70 ± 0.19
Change	−0.02 ± 0.19	−0.18 ± 0.21*	−0.20 ± 0.25†
Ubiquinol-10 (μmol/mmol LDL cholesterol)			
Run-in	0.31 ± 0.09	0.35 ± 0.10	0.32 ± 0.09
Test period	0.32 ± 0.10	0.36 ± 0.15	0.31 ± 0.11
Change	0.01 ± 0.07	0.01 ± 0.10	−0.01 ± 0.09
Total coenzyme Q10 (μmol/mmol LDL cholesterol)			
Run-in	0.34 ± 0.10	0.39 ± 0.11	0.36 ± 0.10
Test period	0.34 ± 0.10	0.38 ± 0.14	0.33 ± 0.11
Change	0.00 ± 0.07	−0.01 ± 0.10	−0.03 ± 0.09

NOTE. See Table 1. Values from 1 subject of the vegetable oil–based group are missing.

*$P < .017$ control group *v* vegetable oil–based group.

†$P < .001$ control group *v* wood-based group.

100). The difference in changes in β-carotene concentrations nearly reached statistical significance compared with the control group ($P = .027$; 95% CI for the difference, −0.002 to −0.110 μmol/L).

After plant stanol ester consumption, changes in LDL-cholesterol–standardized antioxidant concentrations (μmol/mmol LDL-cholesterol) were no longer significant (Fig 3), except for the 16.6% ± 26.9% reduction in phytofluene concentrations (a change of −0.059 ± 0.106 mV · min/nmol LDL-cholesterol; amplification 100 *v* the change of −0.000 ± 0.073 mV · min/nmol LDL-cholesterol; amplification 100 in the control group; $P = .003$; 95% CI for the difference, −0.018 to −0.100 mV · min/nmol LDL-cholesterol; amplification 100). However, changes were in general negative for the hydrocarbon carotenoids (ie, β-carotene, α-carotene, and lycopene), about zero for the oxygenated carotenoids (lutein/zeaxanthin, β-cryptoxanthin), and positive for the tocopherols. When antioxidant concentrations were standardized for total cholesterol or for total cholesterol plus triacylglycerol, conclusions remained essentially similar.

The results of the lipid-standardized concentrations of tocopherols and oxygenated carotenoids, which did not change, suggest that the decreases in absolute oxygenated carotenoid and tocopherol concentrations are mainly related to decreases in serum cholesterol concentrations, as the lipoproteins are the major carrier for lipid-soluble antioxidants. The changes in the lipid-standardized concentrations of the hydrocarbon carotenoids, however, were still negative. This may suggest that reductions in absolute hydrocarbon carotenoid concentrations were associated with the decrease in cholesterol absorption caused by the plant stanols. To examine this issue in more detail, we related changes in serum cholesterol-standardized campesterol concentrations (as a marker for cholesterol absorption[11]) with changes in plasma antioxidant concentrations. Because antioxidants and campesterol are both transported by lipoproteins, changes in total cholesterol were included into the regression model as well. Multiple regression models were calculated for the less polar hydrocarbon carotenoids (the sum of β-carotene, α-carotene, and lycopene concentrations), the more polar oxygenated carotenoids (lutein/zeaxanthin and β-cryptoxanthin), and for the tocopherol isomers (α-tocopherol, δ-tocopherol, and β- and γ-tocopherol) (Table 4). The regression coefficient for total cholesterol predicted that decreasing the total cholesterol concentration by 1 mmol/L will decrease hydrocarbon carotenoid concentrations by 0.162 μmol/L, while the regression coefficient for cholesterol-standardized campesterol predicted that decreasing the cholesterol-standardized campesterol concentration by 100 ($10^2 \times \mu$mol/mmol cholesterol) will decrease β-carotene concentrations by 0.099 μmol/L (Table 4, model A). Thus, using the observed changes of 0.43 mmol/L for total cholesterol and of 103.1 ($10^2 \times \mu$mol/mmol cholesterol) for cholesterol-standardized campesterol concentrations, it can be predicted that the effects of these 2 parameters on total hydrocarbon carotenoid concentrations were −0.069 μmol/L (95% CI, −0.147 to 0.008 μmol/L) and −0.102 μmol/L (95% CI, −0.189 to −0.014 μmol/L), respectively. This indicates that the changes in hydrocarbon carotenoid concentrations were significantly related to the reduced cholesterol absorption. For the oxygenated carotenoids and the tocopherols (Table 4, models B and C) changes were only significantly related to changes in total cholesterol. The ratio of the carrier-associated contribution and the cholesterol absorption-associated contribution to the hydrocarbon carotenoid reduction was 0.6. Compared with the 7.1 ratio for the oxygenated carotenoids and the 9.9 ratio for the tocopherols, this suggests that in particular the lipophylic hydrocarbon carotenoid reductions were associated with the reduced cholesterol absorption. Although the tendency was similar, the relationship between the changes in the individual carotenoids and tocopherols with cholesterol absorption did not reach statistical significance.

Retinol and vitamin E intakes from the margarines and shortenings were comparable among the 3 groups, while β-carotene intake was slightly lower in the vegetable oil–based group (127 ± 21 μg) compared with the control group (150 ± 40 μg) ($P = .008$). This was due to a slightly lower β-carotene content of the vegetable oil–based margarine and shortening.[20] Total dietary vitamin E intake during the experimental period in the control group (13.5 ± 3.0 mg) was also comparable to the intakes in the vegetable oil–based group (13.8 ± 3.2 mg) and the wood-based group (14.5 ± 4.0 mg).

DISCUSSION

As in other studies,[16] we found that plant stanol esters lowered intestinal cholesterol absorption but increased cholesterol synthesis. Reducing cholesterol synthesis with statins has been demonstrated to lower plasma ubiquinol-10 concentrations.[8,9,25] In contrast, squalestatin, a specific squalene synthase inhibitor, significantly decreased the incorporation of [^{3}H]mevalonate into cholesterol, whereas the incorporation into

Table 3. Effects of Vegetable Oil– and Wood-based Plant Stanol Esters on Absolute and LDL-Cholesterol–Standardized Plasma Lipid-Soluble Antioxidant and Retinol Concentrations During the Study

	Control Group	Vegetable Oil–Based Group	Wood-Based Group
Total carotenoids (μmol/L)			
Run-in	1.656 ± 0.548	1.623 ± 0.462	1.666 ± 0.651
Test period	1.568 ± 0.506	1.331 ± 0.445	1.261 ± 0.429
Change	−0.087 ± 0.305	−0.292 ± 0.319¶	−0.405 ± 0.374†
Hydrocarbon carotenoids (μmol/L)			
Run-in	0.961 ± 0.387	0.916 ± 0.311	0.919 ± 0.438
Test period	0.899 ± 0.344	0.717 ± 0.297	0.644 ± 0.262
Change	−0.061 ± 0.216	−0.199 ± 0.243¶	−0.275 ± 0.266†
Oxygenated carotenoids (μmol/L)			
Run-in	0.695 ± 0.216	0.707 ± 0.230	0.747 ± 0.311
Test period	0.669 ± 0.227	0.614 ± 0.257	0.617 ± 0.252
Change	−0.026 ± 0.144	−0.093 ± 0.149	−0.130 ± 0.160§
Total tocopherols (μmol/L)			
Run-in	26.152 ± 6.223	26.688 ± 5.430	27.432 ± 6.460
Test period	26.601 ± 5.247	24.471 ± 4.532	24.756 ± 6.201
Change	0.449 ± 2.591	−2.217 ± 2.630*	−2.676 ± 3.145†
Retinol (μmol/L)			
Run-in	1.896 ± 0.355	1.983 ± 0.413	2.125 ± 0.387§
Test period	1.909 ± 0.373	1.969 ± 0.480	2.097 ± 0.457
Change	0.013 ± 0.225	−0.014 ± 0.208	−0.028 ± 0.277
Total carotenoids (μmol/mmol LDL-cholesterol)			
Run-in	0.581 ± 0.194	0.570 ± 0.159	0.607 ± 0.274
Test period	0.565 ± 0.203	0.551 ± 0.187	0.540 ± 0.242
Change	−0.016 ± 0.130	−0.019 ± 0.117	−0.067 ± 0.127
Hydrocarbon carotenoids (μmol/mmol LDL-cholesterol)			
Run-in	0.337 ± 0.135	0.319 ± 0.099	0.334 ± 0.181
Test period	0.324 ± 0.133	0.294 ± 0.123	0.273 ± 0.139
Change	−0.013 ± 0.091	−0.025 ± 0.091	−0.061 ± 0.088
Oxygenated carotenoids (μmol/mmol LDL-cholesterol)			
Run-in	0.244 ± 0.083	0.251 ± 0.096	0.273 ± 0.124
Test period	0.241 ± 0.096	0.257 ± 0.116	0.267 ± 0.133
Change	−0.003 ± 0.057	0.006 ± 0.054	−0.006 ± 0.060
Total tocopherols (μmol/mmol LDL-cholesterol)			
Run-in	9.055 ± 1.906	9.431 ± 2.139	9.757 ± 2.551
Test period	9.451 ± 1.812	10.194 ± 2.439	10.155 ± 2.507
Change	0.396 ± 1.163	0.763 ± 1.077	0.399 ± 1.326
Retinol (μmol/mmol LDL-cholesterol)			
Run-in	0.680 ± 0.210	0.707 ± 0.193	0.777 ± 0.258
Test period	0.699 ± 0.226	0.821 ± 0.239	0.873 ± 0.252
Change	0.020 ± 0.105	0.114 ± 0.103*	0.095 ± 0.145§

NOTE. Values are means ± SD. For the run-in period, samples from weeks 3 and 4 were averaged, and for the test period, samples from weeks 11 and 12 were averaged. Changes were calculated as the difference between values of the test period and run-in period. Total carotenoids were calculated as the sum of α- and β-carotene, lycopene, lutein/zeaxanthin, and β-cryptoxanthin. Hydrocarbon carotenoids were calculated as the sum of α- and β-carotene and lycopene. Oxygenated carotenoids were calculated as the sum of lutein/zeaxanthin and β-cryptoxanthin. Total tocopherols were calculated as the sum of α-, β-, γ-, and δ-tocopherol

*$P < .001$ control group *v* vegetable oil–based group.

†$P < .001$ control grip *v* wood-based group.

‡$P < .01$ wood based group *v* vegetable oil–based group.

§$P < .017$ control group *v* wood-based group.

¶$P < .017$ control group *v* vegetable oil–based group.

ubiquinone was increased 3- to 4-fold.[26] These findings suggest that a change in the farnesyl-pyrophosphate (PP) pool size, the branching intermediate for cholesterol and ubiquinone synthesis, may be a determinant of ubiquinone-10 synthesis. We therefore hypothesized that an increase in cholesterol production, as induced by plant stanol ester consumption, may increase ubiquinone-10 synthesis and subsequently plasma ubiquinol-10 concentrations. Our study, however, demonstrated for the first time that dietary enrichment with plant stanol esters lowered absolute concentrations of plasma ubiquinol-10. In fact, to our knowledge, this is the first time a dietary intervention has been shown to change plasma ubiquinol-10 concentrations. Changes in LDL-cholesterol–standardized ubiquinol-10 concentrations, however, were similar in all groups. It might therefore be hypothesized that LDL, which is the major carrier of ubiquinol, was already saturated with ubiquinol, and

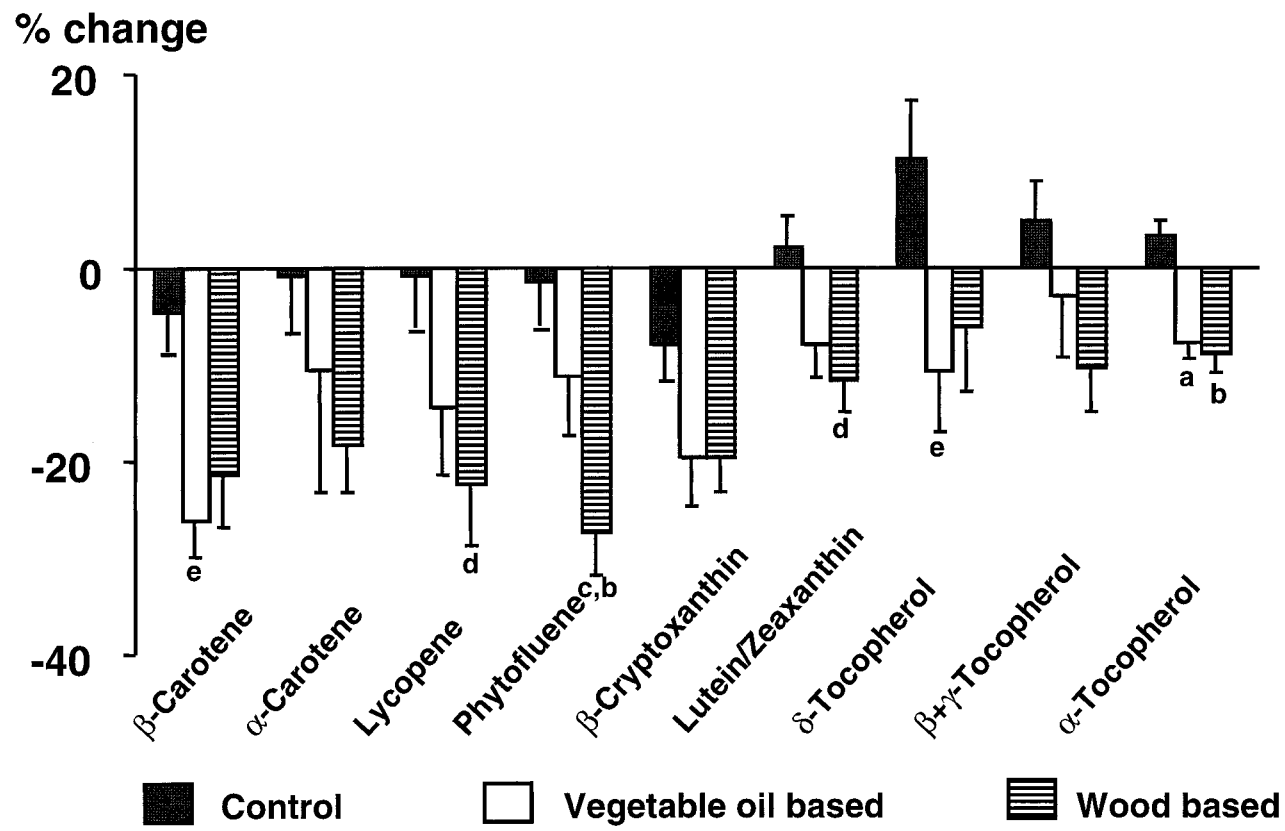

Fig 2. Percent changes of absolute plasma antioxidant concentrations (μmol/L) during the study. Values are means ± SE. For the run-in period, samples from weeks 3 and 4 were averaged, and for the test period, samples from weeks 11 and 12 were averaged. Changes were calculated as the difference between values of the test period and run-in period. Percent changes were calculated *v* the end of the run-in period. [a]*P* < .001 control group *v* vegetable oil–based group; [b]*P* < .001 control group *v* wood-based group; [c]*P* = .001 wood-based group *v* vegetable oil–based group; [d]*P* < .017 control group *v* wood-based group; [e]*P* < .017 control group *v* vegetable oil–based group.

that the reduction in the absolute amount of plasma ubiquinol-10 was simply a carrier effect. However, daily ubiquinone supplementation, which is efficiently reduced to ubiquinol-10 during uptake, increased plasma ubiquinol-10 concentrations 3- to 4-fold.[27,28] After statin treatment, LDL-cholesterol–standardized ubiquinol-10 concentrations were reduced,[25] although not always significantly.[29] This is surprising as, according the flux diversion hypothesis[30]—combined with the higher affinity of the ubiquinone synthesizing enzyme for farnesyl-PP as compared with the affinity of squalene synthase[31]—partial HMG-CoA reductase inhibition by statin treatment is expected to increase the ubiquinol-10 to cholesterol ratio, or at least not to change it. In a study with heterozygous familiar hypercholesterolemia (FH) patients, treatment with cholestyramine, a bile acid sequestrant that increases endogenous cholesterol synthesis, also decreased plasma ubiquinone concentrations insignificantly by 13%, while the ubiquinone to LDL-cholesterol ratio was significantly increased.[29] These findings suggest that changes in ubiquinol-10 synthesis cannot simply be predicted from changes in cholesterol synthesis. For our study, one explanation for the unchanged LDL-cholesterol–standardized concentrations of ubiquinol-10 may be that plant stanol ester consumption increased cholesterol and ubiquinol-10 synthesis to the same extent. In this way, the ubiquinol-10 to cholesterol ratio of very–low-density lipoprotein (VLDL) particles excreted into the circulation by the liver may not have changed during the study. Since VLDL is subsequently metabolized via intermediate-density lipoprotein (IDL) into LDL, and IDL and LDL cholesterol clearance may increase due to a higher LDL-receptor expression,[33] ubiquinol-10 clearance may also increase, resulting in an unchanged ubiquinol-10 to LDL-cholesterol ratio. Of course, other explanations are also possible such as an increased reuse of ubiquinol-10 in the liver or a decreased incorporation of newly synthesized cholesterol into VLDL. Studies with stable isotopes are therefore needed to examine if ubiquinol-10 synthesis is truly affected by plant stanol ester consumption.

Other studies have found that plant stanol esters lowered both plasma absolute and lipid-standardized α- and β-carotene and lycopene concentrations,[14] as well as absolute α-tocopherol concentrations.[33,34] Comparable results have been found for other compounds that interfere with cholesterol, bile, or fat absorption, such as plant sterols,[14] cholestyramine,[35] neomycin,[36] and olestra.[37] We have now demonstrated that plant stanol esters, with daily intakes equivalent to 3.8 to 4.0 g plant stanols, lower plasma absolute levels of other carotenoids and tocopherol isomers as well. In addition, it was demonstrated that effects were not similar for the various carotenoids and

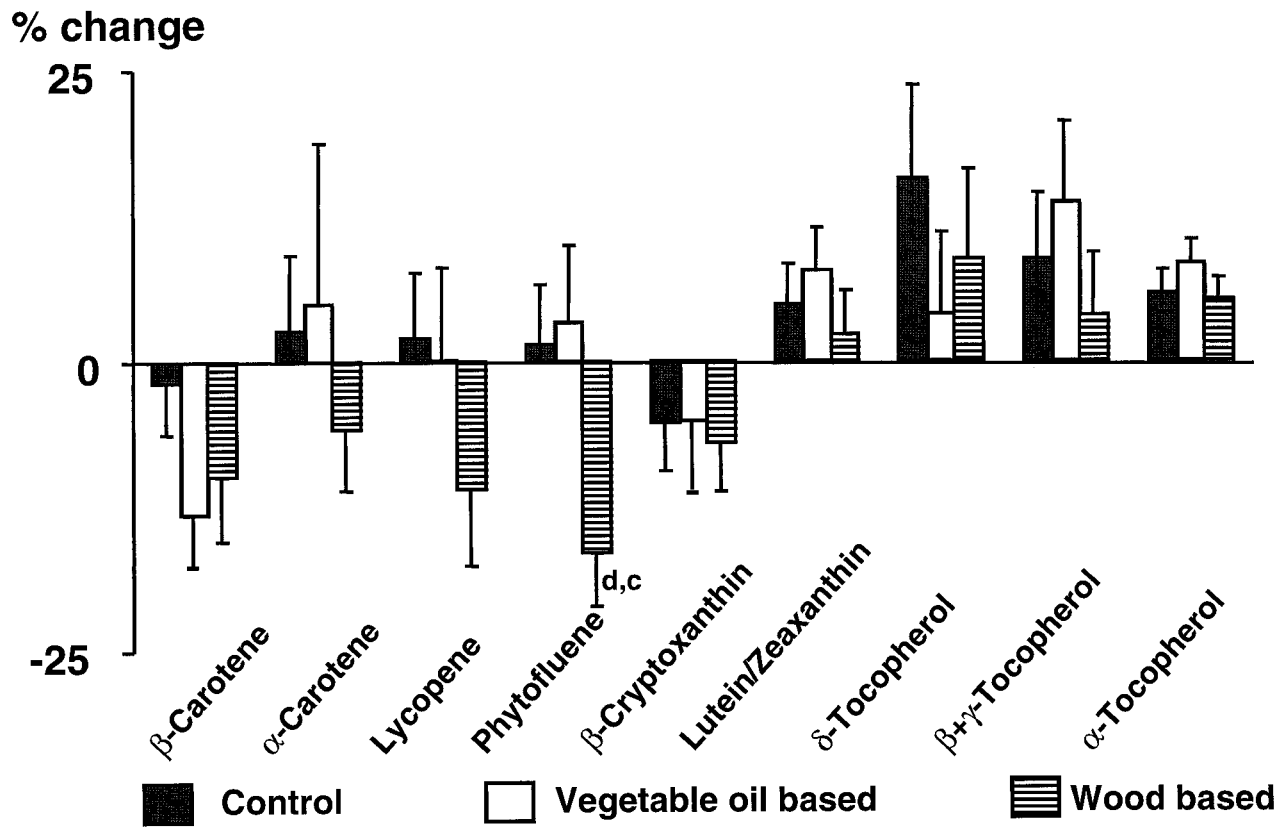

Fig 3. Percent changes of LDL-cholesterol–standardized plasma antioxidant concentrations (μmol/mmol LDL-cholesterol) during the study. Values are means ± SE. For the run-in period, samples from weeks 3 and 4 were averaged, and for the test period, samples from weeks 11 and 12 were averaged. Changes were calculated as the difference between values of the test period and run-in period. Percent changes were calculated *v* the end of the run-in period. [c]*P* = .001 wood based group *v* vegetable oil–based group; [d]*P* < .017 control group *v* wood-based group.

tocopherols. The hydrocarbon carotenoids, ie, α- and β-carotene, and lycopene showed the most pronounced decreases, followed by the oxygenated carotenoids and the tocopherols, while retinol concentrations were hardly changed. This order of changes is comparable to that of the sequence of these compounds in decreasing lipophylicity. Thus, the most lipophylic antioxidants—the hydrocarbon carotenoids—showed the largest reductions. Mucosal uptake of lipophylic compounds requires incorporation into mixed micelles. The amount of carotenoid taken up in the mixed micelle depends on the polarity of the compound.[38] Therefore, it is tempting to suggest that plant stanol esters affect not only the micellar solubility of cholesterol,[39] but also of the most lipophylic antioxidants. This concept is supported by the finding that the reductions in plasma concentrations of the most lipophylic antioxidants, the hydrocarbon carotenoids, were significantly related to the decrease in cholesterol absorption. This association was not found for the oxygenated carotenoids and the tocopherols, which are more polar. It should be noted that absolute plasma retinol concentrations did not change after plant stanol consumption. For humans, β-carotene is the most important dietary provitamin A. The reduction in β-carotene might therefore also be explained by its increased conversion into retinol in the enterocyte. However, only direct measurements of fat-soluble antioxidant absorption can unravel the exact mechanism underlying the antioxidant reductions. However, for all of the antioxidants studied, the major part of the reductions can be explained simply by the decrease in LDL, the major carrier of these antioxidants. In contrast to our speculations, sheanut oil–derived plant sterols, which are 4,4′-dimethylsterols, do significantly lower absolute and lipid standardized α- and β-carotene and lycopene concentrations,[14] but do not change serum cholesterol concentrations. Thus, changes in cholesterol absorption and carotenoid concentrations are not necessarily associated. It therefore seems that the presence of 4,4′-dimethylsterols in the intestinal lumen affects hydrocarbon carotenoid metabolism independent of effects on cholesterol absorption.

In summary, consumption of both vegetable oil– and wood-based plant stanol ester mixtures lower absolute plasma ubiquinol-10 concentrations, but does not change the ratio of ubiquinol-10 to LDL-cholesterol. Absolute plasma carotenoid and vitamin E concentrations were also lowered, although concentrations of β-carotene[40,41] and other antioxidants studied[23] remained within the normal range. These effects were most pronounced for the most lipophylic antioxidants and for the hydrocarbon carotenoids only—although not significantly—also evident after standardization for LDL-cholesterol. These reductions were mainly explained by a reduction in carrier

Table 4. Results of Multiple Linear Regression Analyses to Examine the Association Between Serum Cholesterol-Standardized Campesterol Concentrations and Hydrocarbon Carotenoid, Oxygenated Carotenoid, or Tocopherol Concentrations After Adjustment for Changes in Total Cholesterol Concentrations

Variables			Regression Coefficient		
Model	Dependent	Independent	b	SE (b)	*P* Value
A	Δ Hydrocarbon carotenoids	Δ Total cholesterol	0.162	0.090	.078
		Δ Total cholesterol–standardized campesterol	0.099	0.043	.023
		Intercept	−0.064		
B	Δ Oxygenated carotenoids	Δ Total cholesterol	0.182	0.054	.001
		Δ Total cholesterol–standardized campesterol	0.011	0.025	.673
		Intercept	−0.022		
C	Δ Tocopherols	Δ Total cholesterol	5.076	0.884	<.001
		Δ Total cholesterol–standardized campesterol	0.216	0.416	.605
		Intercept	−0.046		

capacity, although the decrease in the hydrocarbon carotenoids was also related to the reduced cholesterol absorption. An important question is whether these changes in antioxidant concentrations affect health. At this moment, it is only possible to speculate about the clinical relevance of the observed changes in antioxidant concentrations. Compared with large intervention trials with β-carotene or vitamin E supplements,[42] the changes in antioxidant concentrations observed in our study were very small. Furthermore, it is not likely that the changes in antioxidants counterbalance the positive effects of plant stanols on LDL-cholesterol concentrations, and animal studies have indeed demonstrated that plant stanol ester consumption reduces cardiovascular risk.[43] However, modest effects on other health parameters cannot be excluded. Therefore, the in vivo relevance of the present findings and whether the total body antioxidant status is affected needs further study.

ACKNOWLEDGMENT

We are indebted to the members of our technical and dietary staff, especially F.J.J. Cox, M.M.A. van Heugten, R.F.G Offermans, and J.W. Wiertz; and to all of the volunteers for their cooperation and interest.

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