Effects of a Diet High in Plant Sterols, Vegetable Proteins, and Viscous Fibers (Dietary Portfolio) on Circulating Sterol Levels and Red Cell Fragility in Hypercholesterolemic Subjects

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ABSTRACT: Plant sterols, soy proteins, viscous fibers, and nuts are advised for cholesterol reduction, but their combined effect on plant sterol absorption has never been tested. We assessed their combined action on serum sterols in hyperlipidemic subjects who were following low-saturated fat diets before starting the study and who returned to these diets post-test. The 1-mon test (combination) diet was high in plant sterols (1 g/1,000 kcal), soy protein (23 g/1,000 kcal), viscous fiber (9 g/1,000 kcal), and almonds (14 g/1000 kcal). Fasting blood was obtained for serum lipids and sterols, and erythrocytes were obtained for fragility prior to and at 2-wk intervals during the study. The combination diet raised serum campesterol concentrations by 50% and β-sitosterol by 27%, although these changes were not significant after Bonferroni correction; near-maximal rises were found by the end of the first week, but no change was found in red cell fragility despite a 29% reduction in the LDL cholesterol level. No significant associations were observed between changes in red cell fragility and blood lipids or sterols. We conclude that plant sterols had a minimal impact on serum sterol concentrations or red cell fragility in hyperlipidemic subjects on diets that greatly reduced their serum lipids.

Paper no. L9398 in *Lipids 40*, 169–174 (February 2005).

Plant sterols have been shown to lower serum LDL cholesterol (1–3) by 8–12% in the absence of other dietary modifications (e.g., vs. low-fat diets) in a meta-analysis by Law (4). Despite broad acceptance of the safety of plant sterols by most Western countries, concerns have been raised that absorption of plant sterols may have adverse consequences, possibly by modifying cell membrane fragility resulting from the displacement of cholesterol (5). Few data are available from human studies concerning the influence of dietary sterols on membrane fragility and serum sterols, especially using diets that result in marked reductions in serum cholesterol levels (6,7). We therefore assessed the effect on serum plant sterols of diets high in other cholesterol-lowering dietary components. These components, which included viscous fiber (8–11), soy protein (12,13), and almonds in combination (dietary portfolio), have been shown to produce a marked reduction in serum cholesterol (6,7). The specific objective was to assess whether a plant sterol-enriched diet, combined with other agents known to reduce circulating cholesterol levels, would result in a change in cell membrane fragility and circulating plant sterol and lipid concentrations in hyperlipidemic individuals.

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EXPERIMENTAL PROCEDURES

Subjects. Thirteen subjects (7 men and 6 postmenopausal women), aged (mean ± SE) 65 ± 3 yr (median 64 yr; range 43–84 yr); body mass index 25.6 ± 0.9 kg/m^2 (median 26.1 kg/m^2; range 20.6–30.7 kg/m^2); baseline LDL cholesterol 4.22 ± 0.11 mmol/L (median 4.27 mmol/L; range 3.51–4.99 mmol/L) were recruited from patients attending the Risk Factor Modification Center, St. Michael's Hospital. All subjects had taken part in previous dietary studies, were experienced in following dietary protocols and previously had had raised LDL cholesterol levels (>4.1 mmol/L) (14). At the time of the study, 5 subjects had raised LDL cholesterol levels, one subject had raised TG levels (>2.30 mmol/L, range 0.7–5.1 mmol/L), 3 subjects had both raised LDL cholesterol and TG levels, one subject had a low HDL cholesterol concentration (<0.9 mmol/L), and 3 subjects had blood lipids in the normal range (14). None of the subjects had a history of diabetes, renal disease, or liver disease, and none were taking medications known to influence serum lipids. One subject completed only 3 wk and withdrew because of dyspepsia associated with a *Helicobacter pylori* infection requiring antibiotic therapy.

Dietary advice on low-saturated fat (<7% dietary calories) and low-cholesterol diets (<200 mg/d) had been reinforced on at least two occasions over the previous year, and at entry to the study, 6 subjects had recorded diets with <7% (total energy) saturated fat and 9 subjects had followed diets with <200 mg/d cholesterol.

Study protocol. Subjects were followed on their own low-saturated fat therapeutic diets for 1 wk prior to the start of the study, and for an additional 2 wk after the study on return to their low-saturated fat therapeutic diets. During the middle 4

TABLE 1
Calculated Macronutrient Intakes (mean + SE) During the Run-in, Test, and Run-out Phases of the Portfolio Study

	Run-in (n = 12)	Portfolio diet (mean weeks 2–4, n = 13)	Run-out (week 6, n = 12)
Energy (kcal/d)	1,703 ± 120	1,999 ± 118	1,703 ± 104
Total protein (% of protein)	17.3 ± 0.8	22.4 ± 0.5	18.1 ± 0.8
Vegetable protein	48.7 ± 3.5	96.8 ± 0.2	39.1 ± 2.8
Available carbohydrate (% of energy)	52.9 ± 2.8	50.6 ± 0.6	58.2 ± 1.3
Total dietary fiber (g/1,000 kcal)	17.1 ± 1.9	30.7 ± 1.0	17.8 ± 1.8
Total fat (% of energy)[a]	28.3 ± 2.5	27.0 ± 0.8	22.7 ± 1.5
SFA	7.7 ± 0.7	4.3 ± 0.1	6.2 ± 0.7
MUFA	11.9 ± 1.6	11.8 ± 0.5	9.0 ± 0.7
PUFA	6.0 ± 0.4	9.9 ± 0.2	5.3 ± 0.5
Dietary cholesterol (mg/1,000 kcal)	99 ± 13	10 ± 3	79 ± 9
Alcohol (% of energy)	1.5 ± 0.5	0.2 ± 0.1	1.0 ± 0.4
Satiety (–3 to +3)[b]	1.3 ± 0.2	2.9 ± 0.2	1.3 ± 0.3

[a]SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA.
[b]Satiety: –3, extreme hunger; +3, extremely full.

wk, subjects followed a combination diet in which all foods were provided with the exception of fresh fruit and most vegetables. Blood samples and body weights were obtained after 12-h overnight fasts at weekly intervals and at week 2 of the washout. Seven-day weighed diet histories were obtained for the week prior to and for 2 wk following the combination diet. Completed menu checklists were returned at weekly intervals during the 4-wk combination diet period.

The study was approved by the Ethics Committee of the University of Toronto and St. Michael's Hospital, and informed consent was obtained from the subjects.

Diets. Diets eaten before and after the 4-wk combination diet were the subjects' routine therapeutic low-fat diets, which approximated National Cholesterol Education Program Step 2 guidelines (<7% energy from saturated fat and <200 mg/d dietary cholesterol) (Table 1) (14). Subjects were provided with self-taring electronic scales and asked to weigh all food items consumed during the study period. During the combination diet period, all foods consumed by the subjects were provided at weekly clinic visits with the exception of fruit and low-calorie vegetables (i.e., non-starch-containing vegetables), which subjects were instructed to obtain from their local stores. Subjects were provided with a 7-d rotating menu plan, including specified fruits and vegetables, on which they checked off each item as it was eaten and confirmed the weight of the foods. The same menu plan was used for all subjects but was modified to suit individual preferences, providing the goals for viscous fiber, soy protein, plant sterols, and almond consumption were met. For ease of consumption, where possible, items were prescribed in whole units.

The aim of the combination diet (dietary portfolio) was to provide 1 g of plant sterols per 1,000 kcal as an enriched margarine. The Unilever margarine contained approximately 46% sitosterol, 26% campesterol, 19% stigmasterol, 2.7% brassicasterol, 1.3% sitostanol, 0.8% campestanol, and 0.8% avenasterol, with the remainder made up of various other plant sterols. The Unilever margarine provided 12% plant sterol (w/w). Two grams of plant sterol was contained in 25 g of margarine, for 82 kcal. In addition, the diet supplied 8.2 g of viscous fiber per 1,000 kcal from oats, barley, and psyllium and 22.7 g of soy protein per 1,000 kcal as soy milk or meat analogs. Raw, unblanched almonds also provided vegetable protein (2.9 g/1,000 kcal). Emphasis was placed on eggplant and okra as additional sources of viscous fiber (0.55 g/1,000 kcal and 0.67 g/1,000 kcal, respectively). Thus, 200 g of eggplant and 100 g of okra were prescribed to be eaten on a 2,000-kcal diet each day. Diets were provided at a targeted intake to maintain body weight based on estimated caloric requirements (15).

Compliance was assessed from the completed weekly checklists and from the return of uneaten food items.

Analyses. Serum lipid data were reported previously (6). All samples were stored at –70°C prior to analysis. Sera for plant sterol analysis were unavailable for one subject, and an additional 3 subjects were missing one or both week 1 and week 3 samples. As also mentioned earlier, one subject dropped out at week 3. Plant sterols and cholesterol in serum and membrane were measured by GLC (HP 5890 Series II; Hewlett-Packard, Palo Alto, CA). Briefly, 5α-cholestane was added to each sample as an internal standard. Samples were saponified with 0.5 M methanol-KOH for 1 h at 100°C, and sterols were extracted using petroleum ether and injected into the gas–liquid chromatograph. The column temperature was 285°C. Isothermal running conditions (oven temperature 285°C) were maintained for 42 min. The injector and detector were set at 300 and 310°C, respectively. The carrier gas (helium) flow rate was 1.2 mL/min with the inlet splitter set at 100:1. Individual plant sterols and cholesterol were identified using authentic standards (Sigma-Aldrich Canada Ltd., Oakville, Ontario). The CV in the plant sterol measurement was 4% (16). Serum was analyzed according to the Lipid Research Clinics' protocol (17) for total cholesterol, TG, and HDL cholesterol, after dextran sulfate–magnesium chloride precipitation (18). Levels of LDL cholesterol were calculated (19). Serum apolipoprotein A-l and B were measured

by nephelometry (20). All samples from a given individual were analyzed in the same batch.

Red cell fragility was assessed on fresh red cells collected in vacutainer tubes containing EDTA (Becton Dickinson, Mississauga, Ontario). Packed red cells (0.02 mL) were added to 2 mL unbuffered saline covering the range of sodium chloride concentrations from 0.20 to 0.70 g/L in 0.05 g/L increments. After 1 h, the cells were centrifuged at 1,000 × *g* at room temperature for 5 min and the supernatant was read at 540 nm (21). Data are presented as unadjusted O.D. readings and as adjusted percentages of the maximum O.D. obtained for both tests combined. The adjusted values were used to calculate the saline concentration that corresponded to the 50% hemolysis value. The p50 value for red cell hemolysis (50% hemolysis value) was calculated, assuming a linear response between the two consecutive O.D. readings spanning the half-point of the maximum hemolysis recorded for the subject. For each subject, the maximum O.D. (maximum hemolysis) obtained from both tests combined represented the 100% hemolysis value for that subject. Preliminary data on red cell fragility expressed as 50% hemolysis were reported previously (6).

Diets were analyzed using a program based on USDA data (22) with additional data on foods analyzed in the laboratory for protein, total fat, and dietary fiber using AOAC methods (23). FA were analyzed by GLC (24). Additional dietary fiber values were obtained from the tables of Anderson and Bridges (25).

Statistical analysis. The results were expressed as means ± SE. The significance of the differences between the pretreatment diet, combination diet, and post-treatment diet was assessed by the least squares means test with the Tukey–Kramer adjustment for multiplicity of simultaneous comparisons (PROC MIXED/SAS 8.2) (26). The model used had the treatment value as the response variable and the week and interaction term diet by sex as main effects and a random term corresponding to subject nested within sex. Student's paired *t*-test (two-tailed) was used to assess the significance of the percentage change from pretreatment.

With the present subject numbers for red cell fragility, assuming a SD of effect of 3.4%, a 3% difference could be detected as significant ($P < 0.05$). Likewise, assuming a SD of effect of 0.015 g/100 mL, a difference of 0.013 g/100 mL should be detected as significant.

The concentration required to obtain 50% hemolysis was determined assuming a linear response between consecutive observations. For each subject, the maximal O.D. obtained from both tests combined represented the 100% hemolysis value for that subject. A Pearson correlation analysis was used to assess relations between plant sterol measurements and other measurements. A Bonferroni adjustment was also made to the significance levels to allow for the multiple comparisons (26). Six largely independent primary measures were recognized: change in red cell fragility, serum campesterol and β-sitosterol, and LDL cholesterol, HDL cholesterol, and TG.

RESULTS

Demographics and compliance. Throughout the period of observation, subjects tended to lose weight: [–0.10 ± 0.05 kg/wk ($P = 0.127$) over the combination diet, and –0.2 ± 0.05 kg/wk ($P = 0.001$) during the run-out phase]. In the majority of subjects, compliance in terms of caloric intake was good, with 92.5 ± 2.9% of the calories prescribed being consumed.

Serum plant sterols. Serum plant sterol concentrations tended to increase over the 1-mon combination diet (Fig. 1). Serum campesterol concentrations increased by 50 ± 15% from baseline for weeks 2–4; the respective increase for serum sitosterol was 27 ± 14% (Table 2). For campesterol, the unadjusted rise was significant ($P = 0.007$) but disappeared after Bonferroni correction ($P = 0.139$).

Serum lipids. Full details of the blood lipid responses were reported previously (6). Significant reductions in blood lipids were seen at the end of the combination diet compared with the run-in and run-out periods (Table 2). From baseline, reductions were seen in LDL cholesterol (29.0 ± 2.7%, $P < 0.001$), apolipoprotein B (24.3 ± 2.0%, $P < 0.001$), and the total/HDL cholesterol ratio (19.8 ± 2.9%, $P = 0.004$).

Red cell fragility. No significant difference was seen in red cell fragility between the pretreatment and week 4 of the com-

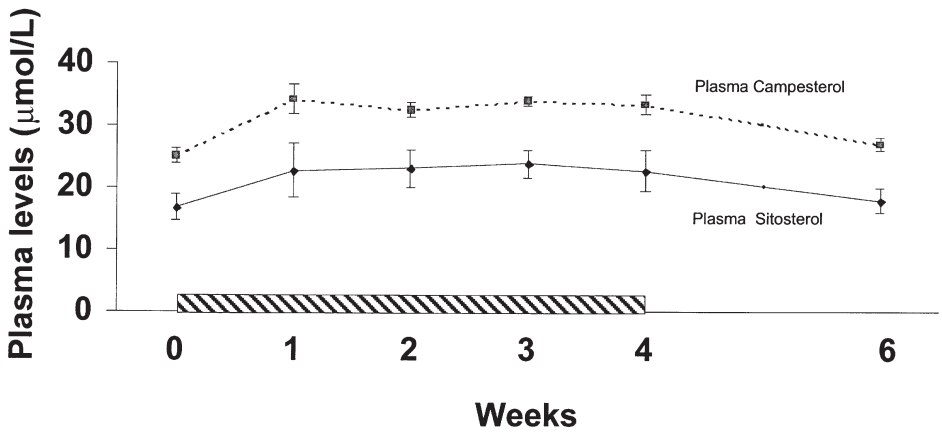

FIG. 1. Mean plasma values for campesterol and sitosterol over 6 wk in 7 subjects. (hatched bar) Administration period of sterols. Plant sterol data were unavailable for one subject, an additional subject dropped out prior to the week 4 sample, and 3 subjects were lacking one or both week 1 and week 3 samples.

TABLE 2
Blood Lipids[a] and Apolipoproteins at Baseline and on the Combination Diet

	Baseline (week 0)	Mean treatment[b] (weeks 2–4)	P^c
Cholesterol[d] (mmol/L)			
Total-C	6.46 ± 0.21	5.01 ± 0.20	<0.001
LDL-C	4.22 ± 0.11	3.01 ± 0.17	<0.001
HDL-C	1.37 ± 0.11	1.34 ± 0.11	0.992
TG[d] (mmol/L)	1.92 ± 0.35	1.45 ± 0.18	0.984
Apolipoproteins[e] (g/L)			
ApoA-1	1.70 ± 0.07	1.61 ± 0.08	0.334
ApoB	1.32 ± 0.05	1.01 ± 0.05	<0.001
Ratios			
Total-C/HDL-C	5.06 ± 0.41	4.00 ± 0.30	0.004
LDL-C/HDL-C	3.31 ± 0.26	2.45 ± 0.24	<0.001
ApoB/apoA-1	0.80 ± 0.05	0.64 ± 0.05	0.004

[a]Blood lipid results have been published in full elsewhere (6). Total-C, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol.
[b]Treatment values represent the mean of weeks 2, 3, and 4.
[c]P values after Bonferroni correction.
[d]To convert cholesterol and TG to mg/dL, multiply by 38.67 and 88.57, respectively.
[e]To convert apolipoprotein A-1 and B values to mg/dL, multiply by 100.

bination diet (saline concentration for 50% hemolysis, 0.436 ± 0.007 g/100 mL vs. 0.441 ± 0.006 g/100 mL, $P = 0.223$, unadjusted, $P = 0.734$ adjusted). No significant differences between mean pre- and postcombination diet values were seen at any time point when expressed as unadjusted O.D. readings at 540 nm (Fig. 2A) or as percent hemolysis, where the highest O.D. value for each individual was taken as 100% for that individual (Fig. 2B).

Relation of plant sterols to other measurements. No significant associations were seen between change in serum sterols and red cell fragility (serum campesterol, $r = –0.09$, $P = 0.803$; sitosterol $r = 0.12$, $P = 0.717$). The negative values indicate the tendency of a reduced fragility (reduced saline concentration for hemolysis) with increasing plant sterols, although no associations were significant.

For serum sterols, an association was seen at baseline between plasma sitosterol and total cholesterol ($r = 0.72$, $P = 0.008$), LDL cholesterol ($r = 0.83$, $P = 0.001$), and apolipoprotein B ($r = 0.91$, $P < 0.001$). No relation was seen between changes in serum sterols and blood lipids.

DISCUSSION

Supplementation with plant sterols in a diet that also contained high levels of viscous fibers, soy protein, and almonds produced large reductions in serum lipids but was associated with only relatively small increases in plasma sterol concentrations. There has been concern that significant increases in plasma sterols may result in modification of membrane lipids with the exclusion of cholesterol and that this change would increase membrane fragility (5). The present data indicate that the small changes in serum lipids do not relate significantly to red cell osmotic fragility.

The absence of effect on red cell fragility indices examined opposes recent findings in rats suggesting that diets high in

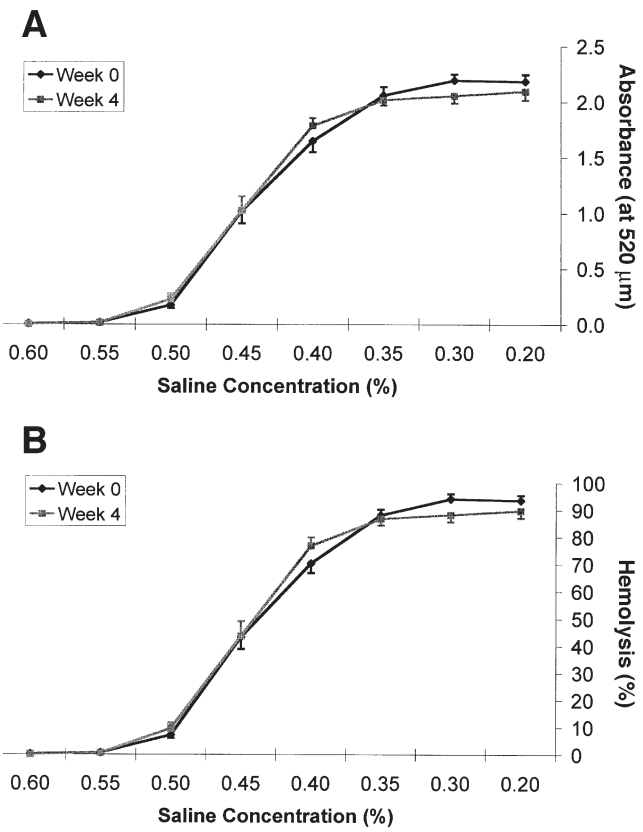

FIG. 2. (A) Osmotic fragility of red blood cells from subjects at baseline (week 0) and week 4 of the combination diet after 6 h of exposure to varying saline concentrations. Data are presented as unadjusted O.D. readings. (B) Osmotic fragility of red blood cells from subjects at baseline (week 0) and week 4 of the combination diet after 6 h of exposure to varying saline concentrations. Data are presented as the percentage of hemolysis, with the maximal O.D. reading obtained for each subject representing 100% hemolysis.

plant sterols may increase the predisposition to hemorrhagic events (5). Clearly, more effort is required to define the real risk of hemorrhagic sequelae in the use of plant sterols as cholesterol-lowering agents at the levels provided in functional foods.

Components used in this combination diet were based on the hypothesis that during the course of human evolution, the diet was likely to have been high in vegetable proteins, dietary fiber, and plant sterols. All these components have now been recognized to lower serum cholesterol (1–3,8–13, 27, 28). Vegetable protein as soy (13), viscous dietary fiber, oats (27), psyllium (10), and, most recently, plant sterols (4) have received FDA approval for health claims for coronary heart disease risk reduction. These components, together with almonds (29,30), which may combine several cholesterol-lowering components, were the focus of the present diet.

In a previous study an attempt was made to reconstruct diets that may have been eaten at an earlier stage in human evolution by using plant foods readily available in the supermarket (31). The plant sterol intake on this "Myocene," or 4–7 million-year-old, diet was 1 g/d for men, indicating that historically high plant sterol intakes may have been associated with diets high in fiber and vegetable protein (31). The cholesterol lowering achieved with this evolutionary model diet was of a magnitude similar to that seen in the present study, in which the diet was high in the same active ingredients.

It is possible that almonds and viscous fiber in the present diet may have protective effects on red cell fragility and that adverse effects of plant sterols may have been masked. However, there are no data on the effects of nuts or viscous fiber on red cell fragility. Equally, it could be argued that nuts, which are good sources of plant sterols, might be expected to add to any adverse effect of the sterol margarine if such existed and that this might be compounded by viscous fiber, which further reduces serum cholesterol levels, possibly increasing the stiffness of the membrane and increasing the risk of hemolysis. Nevertheless, these points are purely speculative since no significant change in hemolysis was observed.

It is also possible that 4 wk was not long enough to demonstrate more subtle changes in red cell hemolysis since the red cell half-life in humans is 25–35 d for a mean life span of 120 d (32). In the rat the red cell half-life is of the order of 19 d with a life span of 60 d (33). In this situation, 1-mon studies might be more appropriate; however, this was the time frame used in rat studies that showed increased hemolysis with plant sterols by 4–5% (5). Based on studies of spontaneously hypertensive rats (5), it has been suggested that increased plant sterol intakes, especially from canola oil, may increase the risk of hemorrhagic stroke (5). This effect is thought to be due to increased cell membrane fragility (5,21) secondary to displacement of cholesterol by plant sterols in the membrane. However, the similar survival of olive oil-fed and corn-oil fed spontaneously hypertensive rats leaves questions remaining as to the role of phytosterol intakes in this process. Also, we found no relation between serum plant sterol levels and red cell osmotic fragility. A good relation, however, has been observed between plasma and membrane plant sterols in a study involving hypercholesterolemic children (34). Furthermore, there was no significant association between the change in concentration of plasma sterols and red cell fragility, although our subject numbers are small. These data add support to the general acceptance of plant sterol-enriched margarine for use by the public. An additional reason why the present study demonstrated no difference in red cell fragility may relate to the lack of sensitivity of the test itself. Pre-incubated erythrocytes are more sensitive indicators of red cell fragility than fresh erythrocytes, especially in situations of pathologically increased fragility (35). However, the rat studies demonstrating the ill effects of plant sterols did not use pre-incubated cells. Our study therefore endeavored to use the same type of analysis to allow direct comparison.

Were plant sterol consumption to be restricted, then the guidelines promoting increased intakes of fruit, vegetables, whole-grain cereals, legumes, and unhydrogenated vegetable oils would need to be revised. Such diets deliver appreciable amounts of plant sterols, and it could be argued that, from the evolutionary perspective, these are the diets to which we have adapted in the context of high-fiber, vegetable protein, and plant sterol intakes. The lack of these plant food components results in unacceptable levels of serum cholesterol that will qualify a significant proportion of the adult population for cholesterol-lowering medications (14).

In conclusion, high plant sterol intakes in the context of high-fiber vegetable protein diets have only a small effect on serum plant sterol concentrations despite large reductions in serum lipids. Moreover, high plant sterol-containing diets do not alter red cell fragility, a finding that should be examined further, given the recently reported promotion of hemorrhagic events by plant sterols in rats.

ACKNOWLEDGMENTS

The authors sincerely thank Loblaw Brands Limited (Toronto, Ontario), Unilever Canada (Toronto, Ontario), the Almond Board of California (Modesto, CA), and Procter & Gamble Canada (Toronto, Ontario) for the generous donation of the foods used in this study. The authors also wish to thank Robert Chenaux and Larry C. Griffin of Loblaw Brands Ltd., Paul Schur of Unilever Canada, and Kathy Galbraith of Natural Temptations Bakery (Burlington, Ontario) for their assistance on this project. This research was supported by Loblaw Brands Ltd., the Almond Board of California, the Canadian Research Chair Endowment, and the Natural Sciences and Engineering Research Council of Canada.

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[Received October 23, 2003, and in revised form December 23, 2004; revision accepted January 16, 2005]