Effects of partially hydrogenated fish oil, partially hydrogenated soybean oil, and butter on serum lipoproteins and Lp[a] in men

Kari Almendingen,* Odd Jordal,† Peter Kierulf,§ Berit Sandstad, and Jan I. Pedersen[1,*,††]**

Institute for Nutrition Research,* University of Oslo, P.O. Box 1046 Blindern, 0316 Oslo, Norway; A/S Denofa og Lilleborg Fabriker,† P.O. Box 33, 1601 Fredrikstad, Norway; Clinical Chemistry Department,§ Ullevaal Hospital, 0407 Oslo, Norway; Section for Dietary Research,** University of Oslo, P.O. Box 1117 Blindern, 0317 Oslo, Norway; and Akershus College,†† 1340 Bekkestua, Norway

Abstract We have compared the effects of partially hydrogenated fish oil (PHFO-diet), partially hydrogenated soybean oil (PHSO-diet), and butterfat (butter-diet) on serum lipids and lipoprotein[a] in 31 young men. The three test margarines, which contributed 78% of total fat in the diets, were produced from 70% of butterfat, PHSO, or PHFO, each with 30% of soybean oil. Fat provided about 35% of energy, and *trans* fatty acids 0.9%, 8.5%, and 8.0% of energy in the butter-, the PHSO-, and the PHFO-diet, respectively. Dietary cholesterol was balanced by the addition of dried egg powder to the PHSO- and the butter-diet; thus all diets contained 420 mg dietary cholesterol per 10 MJ per day. The subjects consumed all three test diets for 19–21 days in a random order (crossover design). The serum levels of total and LDL-cholesterol were significantly elevated on the PHFO-diet (mean values 5.42 and 3.94 mmol/L, respectively) compared to the PHSO-diet (5.11 and 3.58 mmol/L, respectively) but not different from those on the butter-diet (5.32 and 3.81 mmol/L, respectively). LDL-cholesterol was significantly reduced on the PHSO-diet compared to the butter-diet. The level of HDL-cholesterol was significantly lower on the PHFO-diet (0.98 mmol/L) when compared to the butter-diet (1.05 mmol/L) and with border-line significance compared to the PHSO-diet (1.05 mmol/L). The ratio of LDL- to HDL-cholesterol was significantly higher on the PHFO-diet (4.20) when compared to both other test diets (3.85 and 3.65, respectively). No significant differences in triglyceride values were observed. Lp[a] increased and apoA-I decreased significantly after consumption of both the PHSO-diet and the PHFO-diet, compared to the butter-diet. In conclusion, our results indicate that consumption of PHFO may unfavorably affect lipid risk indicators for coronary heart disease at least to the same extent as butterfat. To what extent the observed effects are due to the content of monoene *trans,* diene *trans,* or to the long chain saturated fatty acids in PHFO remains to be elucidated.—**Almendingen, K., O. Jordal, P. Kierulf, B. Sandstad, and J. I. Pedersen.** Effects of partially hydrogenated fish oil, partially hydrogenated soybean oil, and butter on serum lipoproteins and Lp[a] in men. *J. Lipid Res.* 1995. **36:** 1370–1384.

Supplementary key words saturated fatty acids • cholesterol • lipids • lipoproteins • apolipoproteins • Lp[a] • *trans* fatty acids

Trans fatty acids are produced during industrial hydrogenation of edible oils and fats, but are also naturally present in butterfat and meat from ruminants. In addition to partially hydrogenated vegetable oils (PHVO), partially hydrogenated fish oil (PHFO) is another important source of *trans* fatty acids (1–4). The hydrogenation of both marine and vegetable oils results in a number of geometrical and positional isomers of mono- and di-unsaturated fatty acids, in addition to an increased amount of saturated fatty acids. In PHFO, long chain fatty acids (C20–C24) may constitute up to 40% or even more, of total fatty acid content (1, 3). In PHVO with a melting point of 40/42°C the figure is usually less than 1% (2). Although *trans*-monoenoic acids of chain length C16 to C22 comprise the major proportion of *trans* fatty acids in commercially PHFO, variable quantities of *trans,trans, cis,trans*-, and *trans,cis*-dienoic acids are also present. Geometrical *trans* isomers of polyunsaturated fatty acids (trienoic and higher) may be present as well in minor quantities. It is possible to separate these complex mixtures of isomers to some extent on strongly polar GLC capillary columns. Complete resolution, however, requires other and more advanced combined techniques. Partially hydrogenated fish oils are also more variable in

Abbreviations: apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; CETP, cholesteryl ester transfer protein; GLC, gas–liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein; Lp[a], lipoprotein[a]; PHFO, partially hydrogenated fish oil; PHSO, partially hydrogenated soybean oil; PHVO, partially hydrogenated vegetable oil; TLC, thin-layer chromatography.

[1]To whom correspondence should be addressed at: Institute for Nutrition Research, University of Oslo, P.O. Box 1046 Blindern, 0316 Oslo, Norway.

fatty acid composition and content of *trans* isomers than partially hydrogenated vegetable oils because of the larger variability among the different types of unhydrogenated fish oils (3).

In 1986 the daily per capita consumption of PHFO was reported to be 11 g in Great Britain, 24 g in the Netherlands, and 10 g in West Germany (1). The present intake of PHFO in Norway is 11 g (A/S Denofa og Lilleborg Fabriker, personal communication). Of the partially hydrogenated oils used in Norway, approximately two-thirds are PHFO. The daily per capita intake of PHFO and PHVO is approximately 10% and 5% of total fat intake, respectively (A/S Denofa og Lilleborg Fabriker, personal communication).

Recently it has been shown that *trans* fatty acids from partially hydrogenated vegetable oils increase total (5–10) and LDL-cholesterol (5, 7–10) and decrease HDL-cholesterol (5–10) in plasma in humans relative to other fatty acids. In addition, they have been shown to raise the plasma levels of Lp[a] (6, 11). These findings and results from epidemiological studies have raised the question about possible negative health effects of these acids (12, 13). So far, most studies on the effects of *trans* fatty acids have been conducted with partially hydrogenated vegetable oils. Although PHFO have been widely used for human consumption for three-fourths of this century, particularly in Europe, South America, and in South Africa, their effects on plasma lipids in humans have, to our knowledge, previously been reported from only one dietary study (14). In that study several partially hydrogenated fats of both vegetable, marine mammal, and fish origin as well as mixtures of these fats were tested. The results indicated that butter and hydrogenated whale oil were comparable in their hypercholesterolemic effects.

The aim of this study was to investigate the effects on serum lipids and Lp[a] of PHFO compared to those of PHSO and butterfat. This was done in a controlled dietary study in which a diet containing butterfat was compared to diets containing either PHFO or PHSO. The PHFO and PHSO were taken directly from the ordinary production line and correspond to the raw materials used in commercial margarines.

METHODS

Subjects and their baseline characteristics

The experimental subjects were male students studying domestic and kitchen management, and they were considered highly motivated for participation in a strictly controlled dietary study. They were all in basic good health with no history of coronary heart disease, diabetes, allergy, or intolerance inconsistent with normal dietary habits. Most of the participants were educated as cooks, and had several years of working practice. As we wanted a group of 'normal male students' we had no screening criteria as with regard to smoking habits, age, physical activity, or body weight. The participants were, however, encouraged to maintain their normal lifestyle preferences throughout the study period, and to report in a diary any deviation from the usual behavior. They were asked to abstain from alcohol consumption during the study period. No payment was given, except for the free food in the study and some free food items after completion of the study.

The age of the participants ranged from 21 to 46 years (mean 28 years), and the body mass index ranged from 19 to 34 kg/m^2 (mean 26 kg/m^2). Baseline levels of serum lipids and lipoproteins are given in Table 4A. Thirteen men (39%) smoked, and they smoked on average 14 cigarettes/day. Thirty-nine percent of the men had relatives with a known history of cardiovascular disease, none of which was identified as familiar hypercholesterolemia.

Twenty four percent of the participants used some type of medications, but none of these were considered incompatible with participation. Regular use of medications included drugs against asthma (n = 1), migraine (n = 1), morbus Bechterew (n = 1), whiplash (n = 1), dyspepsia (n = 1), skin disease (n = 1), chronic bronchitis (n = 1), and muscle spasm (n = 1). In addition, some nonprescription medications against minor pain were used.

The protocol and aims of the study were fully explained to the subjects, who gave their written consent before entry into the study. Approval of the study was obtained from the Regional Committee for Ethics in Biomedical Research.

Habitual diet

Before the study began, the participants answered a quantitative food frequency questionnaire. The quantitative food frequency questionnaire was answered by the participants alone, but each questionnaire was checked in cooperation with the participants. This was conducted as a dietary history interview and allowed an estimation of the habitual level of energy and nutrient intake. The average level of total fat in the habitual diet was found to be 35 ± 7% of energy, and the intake of linoleic and linolenic acid was 12 g and 2 g, respectively. The mean intake of dietary cholesterol was 535 ± 248 mg/day. The ratio between the intake of polyunsaturated and saturated fatty acids was estimated to 0.44 ± 0.16. The quantitative food frequency questionnaire has been validated and described elsewhere (15).

Experimental design

The study took place from September 6 to November 22, 1993 during three periods of 3 weeks each. The participants received the three different test diets in a random

order in such a way that each diet was consumed by one-third of the subjects in each period. The study was conducted as an intraindividual comparison of the effects on blood lipids of three test diets differing in such a way that a certain amount of saturated fat in one diet (butter-diet) was exchanged with a certain amount of either partially hydrogenated soybean oil (PHSO-diet) or partially hydrogenated fish oil (PHFO-diet) in the others. In this way, variation due to residual effects of the previous diet or to drift of variables over time could be minimized. At the end of one test period, subjects were crossed over to the next diet with a wash-out period of 1 week. The participants were allowed to return to their normal eating and living habits during this week.

The body weight was monitored twice a week (non-fasting) and at the start and the end of each study period (fasting). The weight was measured, in light clothes on a digital balance (SECA), and read to the nearest 0.1 kg. Body height was measured without shoes, and read to the nearest 0.1 cm. Body Mass Index (BMI) was calculated as: weight (kg)/height (m)^2. Adjustments in energy intake were made in the individual meal plan according to changes in the body weight. Dietary compliance was monitored regularly by interviews and diaries. It turned out that individual compliance to the diets could be improved if the wash-out periods included two weekends instead of one. Most men, therefore, received the diets for 19 days. Other studies (16–18) have demonstrated new stable levels of plasma cholesterol within 14 days on a controlled diet.

Experimental test diets

The three diets followed during the study were based on a 7-day menu. The participants were informed in advance of the study that they would be given meals containing different types of fat and that the type of fat would not be known to them during the study period. All three periods comprised a background diet that contained 22% of the total fat (fat from dairy products, meat, fish, and cereals). Menus for the three experimental diets contained the same basic food items, egg, meat, fish, bread, vegetables, fruit, etc. They were as similar in appearance as possible considering that they contained different test margarines. Monday through Friday of each week lunch was served in a specially prepared dining room. Dinner, supper, and breakfast for the next day were packaged for home consumption and provided to the participants each afternoon. Weekend meals were packaged and provided each Friday afternoon. No foods other than those provided were allowed during the controlled feeding periods. Instant coffee, tea, and mineral water with artificial sweeteners were allowed. Most menu items were weighed out for each individual participant to provide meals with varying energy content. The test diets were served according to four different energy levels, 10 MJ, 13 MJ, 15 MJ, and 17 MJ per day.

The contribution of *trans* fatty acids from the background diet was minor ($\leq$ 0.5%). The served level of the three test margarines was 87 g each day (per 10 MJ), which together with the fat from the background diet (20 g per 10 MJ) gave a total of approximately 35% of energy from fat. The amount of test fats was chosen in order to facilitate detection of statistical significance of possible differences between the two types of hydrogenated fats. The level was chosen in order to be within the range of 7.5 to 11% of energy as *trans* fatty acids, a range within which a dose-response has been established between amount of vegetable *trans* fatty acids and elevation of LDL-cholesterol (9). The test margarines were supplied to the menus as spreads, bakery products, sauces, etc. The background diet provided 90 mg dietary cholesterol. In order to balance the different amounts of cholesterol in the test fats, calculated quantities of dried egg powder (2.25% cholesterol) were added to the PHSO-diet (15.0 g per 10 MJ) and to the butter-diet (9.6 g per 10 MJ). Dried egg powder was used as an additive to vanilla sauce, dressings, milkshakes, etc. Apart from the nutrient contribution from the dried egg powder to the butter-diet and the PHSO-diet, the nutrient content of the background diet was planned to be the same for all three test diets. The nutrient contribution of dried egg powder yielded per 10 MJ: 9 g of fat, 5 g of protein, and 455 kJ (109 kcal) to the PHSO-diet. The resulting small differences in fat content due to the dried egg powder (mostly mono- and polyunsaturated fatty acids) was not corrected for as it was considered not to have any significant effect on the serum lipids (19). The content of oxidized cholesterol products in the dried egg powder was analyzed. We found no significant differences in the levels of important oxidation products in samples collected prior to the study and in samples collected from the last servings.

Test margarines

Only ordinary raw materials for the food industry were used in our study and the partially hydrogenated oils were taken directly from the production line. The three test fats consisted of partially hydrogenated soybean oil (PHSO), partially hydrogenated fish oil (PHFO), and butterfat. The melting point of the PHSO was 40/42°C. The PHFO consisted of 67% partially hydrogenated fish oil with melting point 30/32°C (PHFO 30/32) and 33% partially hydrogenated fish oil with melting point 38/40°C (PHFO 38/40). The raw material of the PHFO was 62% Norwegian capeline oil and 38% Peruvian anchovy oil. The hydrogenation conditions were the following: temperature: 155°C (PHSO) or 155–180°C (PHFO), catalyst: nickel, pressure: 0.5–1.0 bar above atmospheric pressure. Thirty percent of refined soybean oil was added to each of the three test fats to obtain a sufficient and equal content of linoleic and α-linolenic acid in the diets. The special test margarines were produced by the addition of 16% water, vitamin A, vitamin D, emulsifier, and NaCl to each

of the test fats. In addition, color and aroma were added to the PHFO- and PHSO-margarines. No antioxidants were added. The test margarines were produced under identical conditions by A/S Denofa og Lilleborg Fabriker, Fredrikstad, Norway.

Chemical analysis

Duplicate portions of all diets were collected for three imaginary participants with a daily energy intake of 10 MJ at the third week of period 1, the second week of period 2, and the first week of period 3, a total of 21 days from each test diet. The duplicate portions were kept frozen (−20°C) until they were homogenized and analyzed. The seven homogenates from each week were pooled and aliquots for each test-diet from the three weeks were analyzed separately.

The content of total fat in the duplicate portions was determined by chloroform–methanol extraction (20). The metabolizable energy content of the diets was determined as described by Andersson et al. (21). The nitrogen content was determined by the Kjeldahl technique. The factor used for conversion of nitrogen content to protein was 6.25.

The total amount of *trans* isomerism, i.e., the total number of isolated nonconjugated *trans* unsaturated bonds of the fat extracts, was determined by the infrared method (22) using methyl elaidate (9-*trans*-C18:1 methyl ester obtained from Nu-Chek-Prep, Inc., Elysian, MN) as reference standard.

For analysis of fatty acid composition, the lipid fractions of the duplicate portions were isolated by Soxhlet extraction of about 5 g of homogenates from each series. The solvent used was diethyl ether, analytical grade, and the extraction time was 4 h. Quantitatively the lipid recovery was almost the same as extraction with the more polar solvent mixture chloroform–methanol (20). (Very polar phospholipids are not extracted with diethyl ether.) The fatty acids of the respective fat extracts were converted to methyl esters by the BF_3 method (23). Capillary GLC performed on a column coated with polar film (cyanopropyl phase) was the method chosen to analyze the fatty acid composition and separate the *trans*- and *cis*-monoenes (24). The GLC chromatographic conditions were the following: instrument: Perkin-Elmer 8320B Capillary Gas Chromatograph, column: SP-2560 fused silica capillary (100 m × 0.25 mm I.D.), temperature: 175–190°C, iso time: 20–65 min, rampe rate: 2°C/min, injection temperature: 240°C, detection temperature: 260°C, split ratio: 1:75, carrier gas: helium, column pressure: 30 psig. The methyl ester of 9,12-*trans,trans* C18:2 (from Nu-Chek-Prep, Inc.) was used as a reference standard to identify and estimate the content of the *trans* dienes.

In this work the main interest was to quantify the total amount of *trans* monoenes and *trans* dienes for each chain length. To this end, and in order to improve resolution and correct for overlapping peaks, the fatty acid methyl esters were preseparated by thin-layer chromatography (TLC) with plates coated with $AgNO_3$-impregnated silica gel as described by Ulberth and Henninger (25). The two zones corresponding to saturated + *trans* monoenes were scraped off in one fraction as recommended (25), eluted by diethyl ether, and the isolated methyl esters were finally analyzed by GLC.

The GLC-chromatogram of the PHSO with melting point 40/42°C is shown in **Fig. 1A.** *Trans* C18:1 appears with several isomers, some of them overlapping, and the

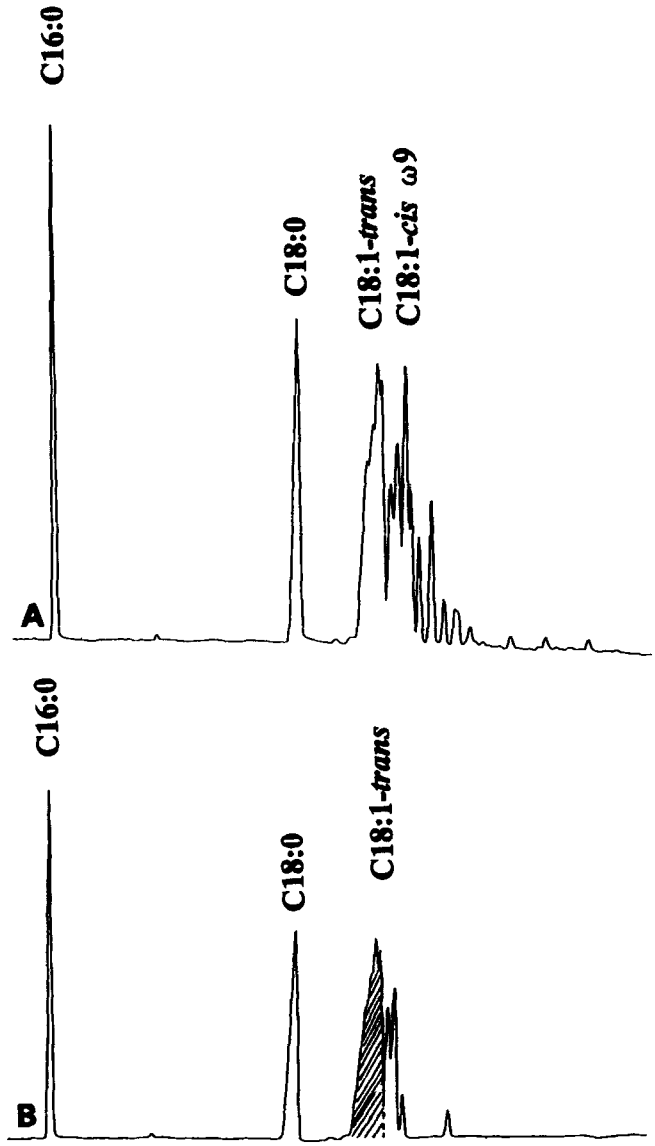

Fig. 1. Capillary GLC chromatogram of fatty acid methyl esters of partially hydrogenated soybean oil before (A) and after (B) preseparation of the *trans* monoene and the saturated fatty acid fraction by thin-layer chromatography. GLC conditions and TLC separation are described in Methods. The shaded area in (B) amounts to 70% of the total *trans* C18:1 area (see text). Retention times: C16:0, 26.77 min; C18:0, 36.36 min; *trans* C18:1 main peak, 39.52 min.

individual positions have been identified earlier and reported for this type of column (24). Figure 1B shows a separation of the total *trans* monoenes and saturated fatty acids after preseparation on TLC as described above. In addition to the main *trans* C18:1 peak, four other peaks that should be included in the *trans*-fraction are seen. It can be calculated that the area of the main peak amounts to 70% of the total *trans* area. To arrive at the total *trans* C18:1 from the chromatogram in Fig. 1A the area of the main *trans* C18:1 peak in this chromatogram was therefore corrected by the factor 100/70, thus minimizing the loss of overlapping peaks.

The GLC-chromatogram of the lipid fraction from the PHFO-margarine is shown in **Fig. 2A,** and in Fig. 2B is shown the corresponding TLC-fractionated *trans* monoenes and saturated fatty acids from PHFO. As

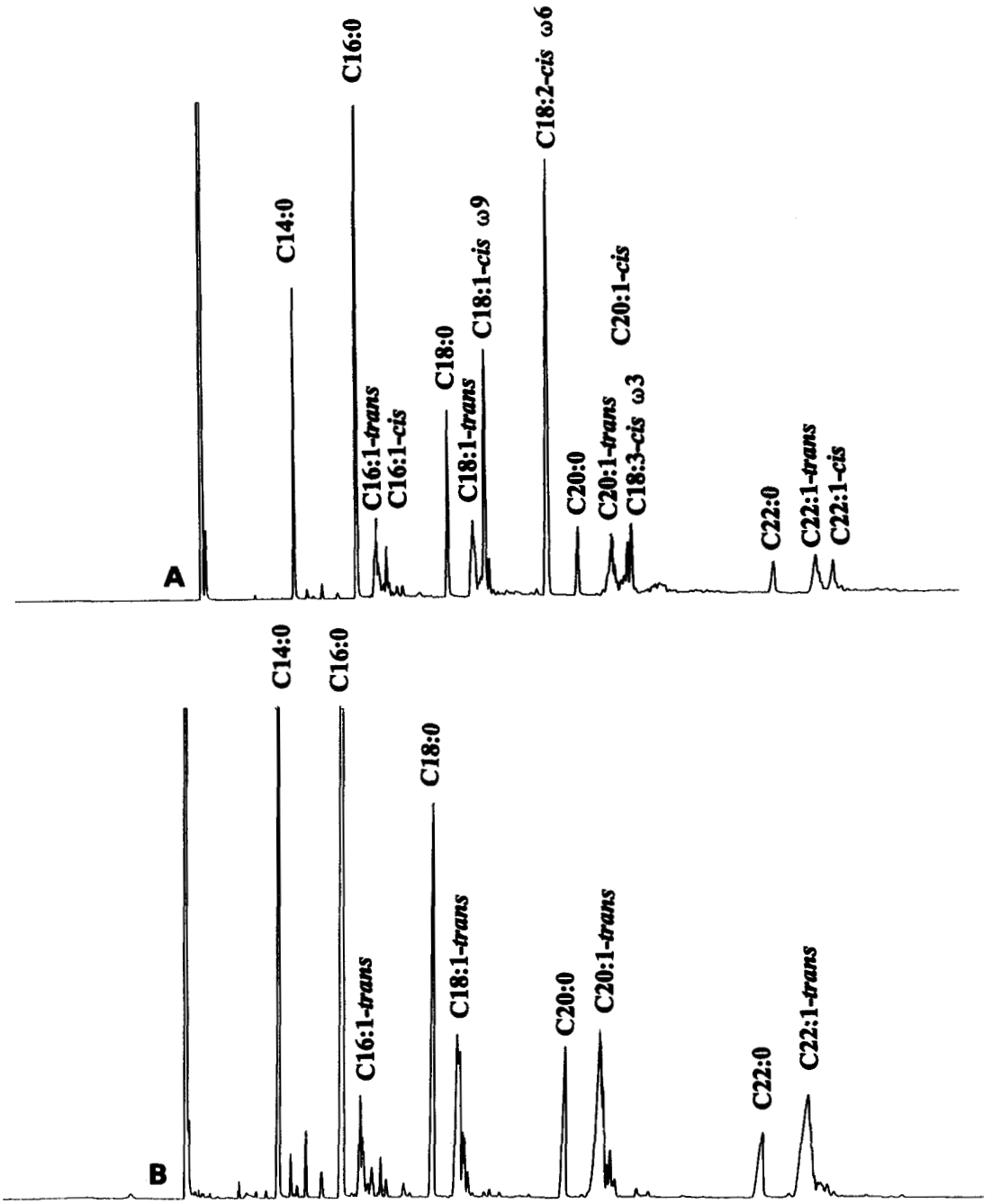

Fig. 2. Capillary GLC chromatogram of fatty acid methyl esters from margarine containing partially hydrogenated fish oil before (A) and after (B) separation of the *trans* monoene and the saturated fatty acid fraction by thin-layer chromatography. GLC conditions and TLC separation are described in Methods. The main areas of *trans* C20:1 and C22:1 in (B) were calculated to be 78% and 93% of total *trans* monoene area, respectively. Retention times (A): C16:0, 26.46 min; *trans* C18:1, 35.38 min; *trans* C20:1, 46.22 min; *trans* C22:1, 62.0 min.

described above for the PHSO, correction factors could be calculated for the sum of *trans* C18:1 (100/70), sum of *trans* C20:1 (100/78), and sum of *trans* C22:1 (100/93). By using these factors total *trans* monoenes of the corresponding chain lengths could be calculated from the chromatogram in Fig. 2A.

The *trans* diene fraction in PHFO was isolated by a similar argentation TLC of the corresponding methyl esters using 9,12-*trans,trans*-C18:2 methyl ester as a reference standard. The marked zone was scraped off, eluted by diethyl ether and the diene acids (*trans,trans; cis,trans; trans,cis*) were further separated on GLC. In **Fig. 3A** is shown a chromatogram of PHFO before, and in Fig. 3B, after preseparation of the *trans* diene fraction. The registered R_t value of the reference standard was used to calculate the corresponding R_t values of homologous

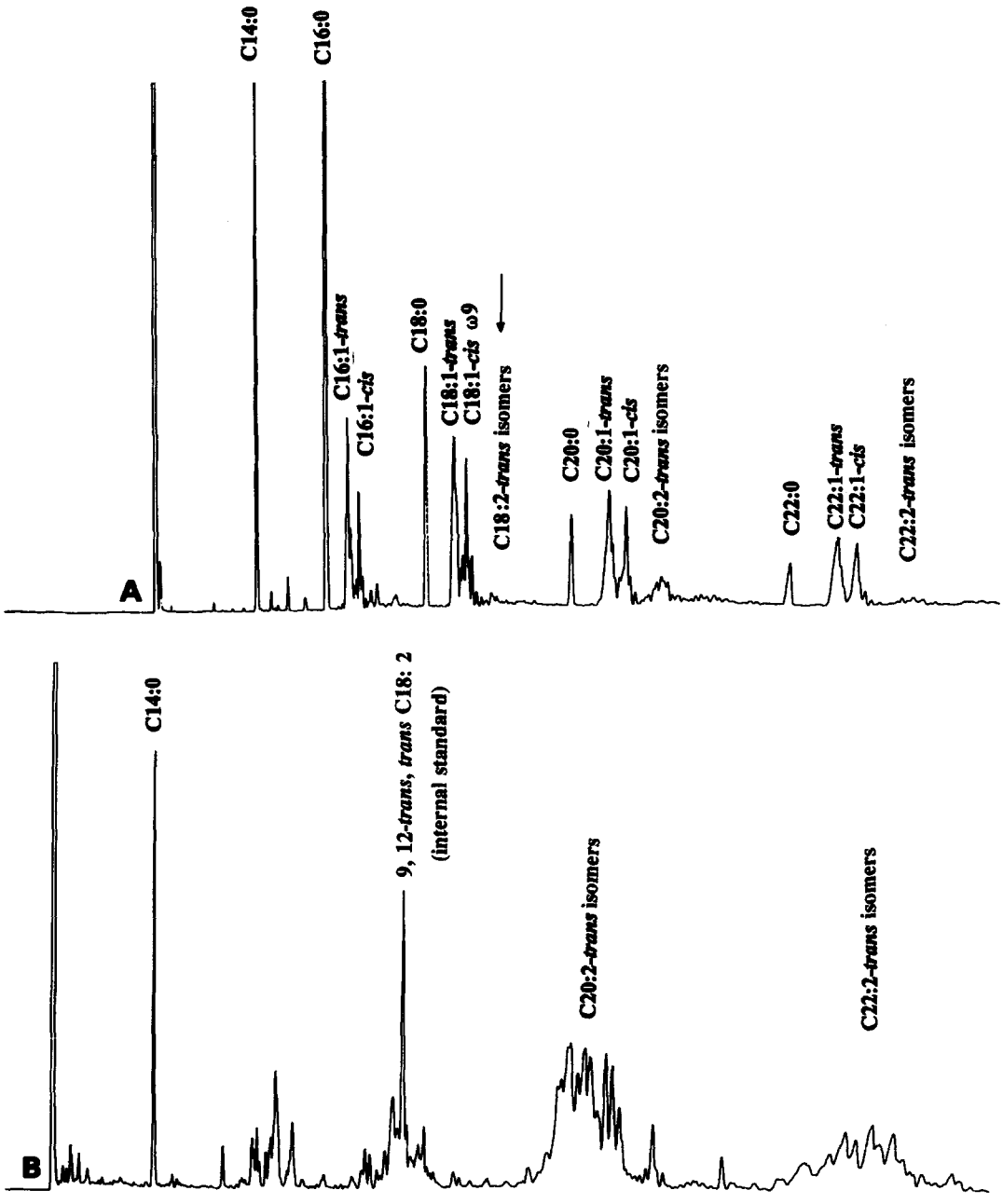

Fig. 3. Capillary GLC chromatogram of fatty acid methyl esters of partially hydrogenated fish oil 30/32 before (A) and after (B) pre-separation by thin-layer chromatography of the *trans* diene fraction. GLC conditions and TLC separation are described in Methods. The arrow in (A) indicates the position of 9,12 *trans,trans* C18:2 used as an internal standard. In (B) the internal standard is seen in the bunch of C18:2 *trans,trans* isomers. All diene *trans* isomer fractions contain *trans,trans; trans,cis* and *cis,trans* isomers (see text). Retention times: C16:0, 26.50 min; C18:0, 33.14; internal standard 9,12 *trans,trans* C18:2, 38.47 min: C20:2 *trans* isomers approximately 50 min, and C22:2 *trans* isomers approximately 72 min.

chain lengths (*trans*-C20:2/-C22:2). As can be seen, several peaks in different positions were obtained for each chain length. The identity of these isomers have so far not been further clarified. According to other investigators (24), the *trans,cis*- and *cis,trans*-isomers will elute just in front of 9,12-*trans,trans*-C18:2. The *trans*-diene fractions of PHFO will therefore probably consist of a mixture of *trans,trans*-, *trans,cis*-, and *cis,trans*-isomers. The GLC fatty acid analysis of the TLC-fractionated *trans* dienes from PHFO was performed without or with the addition of an exact amount of 9,12-*trans,trans* C18:2 methyl ester to the fatty acid methyl ester derivatives of the fat extracts. Thus, the reference standard was identified on the chromatogram in the group of C18 diene peaks (Fig. 3B) and used to estimate the amount of *trans* dienes for each chain length.

The cholesterol content in the test fats and the homogenates was determined on the lipid extract as trimethylsilyl ether derivatives on GLC (26). 5β,3α-Cholestanol was used as internal standard, and the separation between cholesterol, tocopherols, and plant sterols was acceptable on the nonpolar DB-5 capillary column used (30 m × 0.25 mm).

Blood sampling and analyses

Before the trial was started each participant received a random inclusion number that was used for identification and labeling of blood and serum tubes. Blood samples were drawn after an overnight fast immediately before breakfast at the end of each period. Baseline samples were collected during the week before initiation of the controlled feeding and at the start of the first period. All venipunctures of each participant were performed by the same technician and in the same place. Serum was obtained by low-speed centrifugation within 1 h of venipuncture and stored at −70°C until analyzed. All samples were analyzed within the same run within 1 month after the study was finished in order to eliminate variability due to laboratory drift.

Sample treatments were blinded to the analysts and results were blinded to the investigators. Plasma samples were submitted to the analytical laboratory without identification of treatments, and data from the laboratory were not made available to the investigators until after the analytical data were finalized.

Serum cholesterol and serum triglycerides were measured by enzymatic methods (27, 28) using automated analyzer equipment (Hitachi 737, Hitachi Limited, Tokyo, Japan). LDL-cholesterol was calculated using the Friedewald equation (29). Serum HDL-cholesterol was measured essentially by a similar enzymatic technique (27) after precipitation of the LDL-fraction with dextran-sulfate and magnesium (Technicon Reagent T 0.1-2801-56, Tarrytown, NY). Serum apolipoprotein A-I (Orion Diagnostika, Espoo, Finland) and serum apolipoprotein B (Behringwerke Ag, Marburg, Germany) were both quantified immunoturbidometrically using a seven-point standard curve and an automated enzyme analyzer (Cobas Fara, Hofman-La Roche, Basel, Switzerland) essentially according to the manufacturer's instructions. Serum lipoprotein[a] was quantified by a commercial ELISA kit (TINTELIZA Lp[a], Biopool, Umeå, Sweden) according to the manufacturer's instructions (coefficients of variation at: 100 mg/L, 7.7%, at 400 mg/L, 2.7%). All lipid analyses were performed at the Clinical Chemistry Department, Ullevaal Hospital, Oslo.

Statistical methods

Mean values and standard deviations are presented. Two-way analysis of variance was performed to examine whether there were differences between diets. When a significant diet effect was observed ($P \leq 0.05$), pairwise comparisons between the three groups were done by pairwise *t*-tests and appropriate confidence intervals, all adjusted using the Bonferroni method (30). As three comparisons are involved, the confidence interval given is 98.3% (corresponding to a *P* value of 0.017) instead of the usual 95%. All *P*-values are two-tailed. Correlation coefficients (Pearson) between baseline levels of serum lipids and apolipoproteins are presented when suitable. Plasma Lp[a] had a skewed distribution, and pairwise comparisons were done by Wilcoxon signed ranks test, after performing the Quade test on all three diets simultaneously (31). Data analysis was performed using the statistical package Minitab Release 9.

RESULTS

Participants

Of the 33 volunteer students who completed the screening process, all entered the study. After the end of the first period, one withdrew because of reluctance to take the blood tests, and one because he quit school. All analyses are therefore based on the results from the 31 men who completed the trial.

Compliance with the diets was judged by direct observation of consumption of weekday lunch, weight maintenance, and by evaluation of food diaries. Some small deviations from the diets were noted, but it was not considered necessary to exclude any participant from the analysis. During each diet period, two to four subjects occasionally consumed some alcoholic beverages, mostly light beer. One person reported having consumed up to 2 liters of beer during the first and second weekend of each diet period. None of these persons reported any beer consumption during the last week of the three study periods. One participant reported having consumed half a bottle of whisky on one occasion the day before the blood sample was drawn. These deviations from the menus may have

contributed to dilute the significance of any differences in our results.

The initial fasting weights were not significantly different from the fasting weights after completion of the study (n = 31).

Test diets

The analysis of the three test margarines and the duplicate portions of the three test diets revealed that the objectives of the dietary interventions had been achieved as they agreed closely with the initial calculations (**Table 1**). The intake of energy from protein was 15% in all test diets. The addition of dried egg powder to the PHSO-diet (15 g per 10 MJ day) and butter-diet (10 g per 10 MJ per day) was reflected in the slightly higher energy levels (MJ) and energy contribution from fat (%), but all the test diets contained close to 35% of energy from fat as planned. The content of cholesterol was identical in all three test diets, approximately 420 mg cholesterol per 10 MJ. Participants, assuming full compliance, would have consumed 72 g of fatty acids from the test margarines per 10 MJ per day. The test margarines were estimated to contribute to 78% of total fat and 27% of total energy.

The content of identified *trans* fatty acids in partially hydrogenated soybean oil, partially hydrogenated fish oils, butterfat, PHSO-margarine, PHFO-margarine, and butter-based margarine is shown in **Table 2.** The values for total *trans* monoenes determined by GLC and the total content of *trans* bonds determined by the IR spectrophotometric method (22) were in good agreement for the PHSO-margarine, 33.7% and 34.4%, respectively. The PHSO-margarine contained only traces of *trans* dienes (< 1%). The difference between the total *trans* content determined by IR-spectrophotometric method and by GLC was more pronounced for the PHFO-margarine, 41% and 27%, respectively. The IR-absorption of isolated *trans* double bonds is not equivalent, however, to the amount of *trans* fatty acids in the PHFO because of the presence of polyenoic *trans* fatty acids. It has been proven by various investigators that the IR-absorption of *trans* dienes and *trans* trienes increasingly affect the isolated *trans* value because of different absorption coefficients (24, 32). Furthermore, the IR *trans* peak can be enlarged when interfering absorptions occur (conjugated double bonds, etc.). In addition, we probably will have some undetected polyenoic acids as *trans* trienes and *trans* tetraenes in PHFO that also might contribute to the high value of isolated IR *trans* and to some underestimation of the total GLC *trans* isomer level. As a conclusion it may be said that an accurate determination of the total *trans* isomers in PHFO is a difficult task that requires additional investigations with appropriate equipment. In general, the content of *trans* isomers in PHFO will vary within certain limits depending upon the composition of the crude oil used (degree of unsaturation) and furthermore on the hydrogenation conditions.

TABLE 1. The content of energy and nutrients of duplicate portions of the test diets

	Butter-Diet	PHSO-Diet	PHFO-Diet
Energy, MJ	10.0	10.3	9.7
Protein, % of energy	15.4	15.1	15.3
Fat, % of energy	34.8	36.2	33.5
Cholesterol, mg per 10 MJ	420	430	420

TABLE 2. The content of *trans* fatty acids in soybean oil, butterfat, butter-based margarine, partially hydrogenated soybean oil (PHSO 40/42), PHSO-margarine, partially hydrogenated fish oils (PHFO 30/32 and PHFO 38/40), and PHFO-margarine

	Total Monenes[a]	*Trans* Monoenes[a]	*Trans* Dienes[a]	Total *trans*[b]
		% of total fatty acids		
Soybean oil	20.8	0	0	0
Butter-fat				
C16	1.7	0.4		
C18	21.1	1.8		
		2.2	0	3.7
Butter-based margarine				
C16	1.2	0.3		
C18	19.8	1.3		
		1.6	0	2.9
PHSO 40/42				
C18	64.7	48.5	< 1.0[c]	51.0
PHSO-margarine				
C18	51.7	33.7	< 1.0[c]	34.4
PHFO 30/32				
C16	10.3	6.9	-	
C18	13.9	9.9	0.2	
C20	11.5	8.5	3.5	
C22	7.7	4.7	2.3	
C24	0.5[d]	0.2	—	
		30.2	6.0[c]	59.5
PHFO 38/40				
C16	7.6	5.7	-	
C18	11.4	9.3	0.2	
C20	11.7	9.5	3.0	
C22	8.0	4.9	2.0	
C24	0.5[d]	0.3	—	
		29.7	5.2[c]	49.8
PHFO-margarine				
C16	6.7	4.6	-	
C18	15.4	7.1	0.3	
C20	7.7	6.3	2.2	
C22	6.7	4.3	1.8	
C24	0.4[d]	0.2	—	
		22.5	4.3[c]	41.0

[a] Determined by GLC as described in Methods.
[b] Determined by IR-spectrophotometric method as given in Methods.
[c] Includes *trans,trans, cis,trans*-, and *trans,cis*-dienes.
[d] Contains *cis* and *trans* in estimated equal proportions.

The fatty acid composition of the duplicate portions of the diets is shown in **Table 3.** In the butter-diet, 47.0% of the fatty acids were saturated compared to 30.3% for the PHSO-diet, and 33.7% for the PHFO-diet. The PHFO-diet had the highest content of saturated long chain fatty acids, while the butter-diet had the highest content of saturated short chain fatty acids. The content of very long chain (C20–C24) *trans* monoenes and *trans* dienes was highest in the PHFO-diet. The sum of *trans* monoenes and *trans* dienes was 2.6% in the butter-diet, 23.5% in the PHSO-diet, and 24.0% in the PHFO-diet (the IR-spectrophotometric method resulted in higher values for total *trans* fatty acid content: 2.6% in the butter-diet, 27.0% in the PHSO-diet, and 33.1% in the PHFO-diet). The sum of total saturated fatty acids and *trans* fatty acids was highest in the PHFO-diet, and lowest in the butter-diet. The sum of *trans* fatty acids and C12:0, C14:0, and C16:0 was 34% for the butter-diet, 39% for the PHSO-diet, and 46% for the PHFO-diet. The energy intake from *trans* fatty acids was calculated from the GLC analyses to be approximately 0.9% from the butter-diet, 8.5% from the PHSO-diet, and 8.0% from the PHFO-diet. All diets contained ample and almost identical amounts of linoleic and α-linolenic acid (Table 3).

Serum lipids, apolipoproteins and lipoprotein[a]

The levels in serum of total, LDL-, and HDL-cholesterol, apolipoprotein B (apoB) and apolipoprotein A-I (apoA-I), and triglycerides on the three different test diets and the baseline values are given in **Table 4. Table**

TABLE 3. Fatty acid composition of the fat extracts of the duplicate portions of the test diets

	Butter-Diet	PHSO-Diet	PHFO-Diet
		% of total fatty acids	
C4:0	0.9		
C6:0	1.0		
C8:0	0.8	0.2	0.4
C10:0	1.7	0.3	0.6
C12:0	2.1	0.4	0.5
C14:0	6.6	1.0	5.3
C14:1	0.5	0.2	0.2
C15:0	0.6	0.3	0.4
C15:1	0.8	0.1	0.2
C16:0	22.7	14.1	16.3
C16:1-*trans*	0.7	0.4	3.8
C16:1-*cis*	1.6	0.7	1.7
C17:0	0.8	0.3	0.3
C17:1	0.8	0.2	
C18:0	8.7	12.7	6.0
C18:1-*trans*	1.5	22.8	6.5
C18:1-*cis* n-9	20.8	18.8	9.7
C18:1-*cis* n-7[a]	2.2	6.2	2.0
C18:2-*trans*[b]		0.3	0.6
C18:2-*cis* n-6	15.4	15.0	15.7
C18:3-*cis* n-3	2.3	3.0	2.5
C20:0	0.6	0.4	2.2
C20:1-*trans*	0.4		5.4
C20:1-*cis*	0.5	0.3	1.3
C20:2-*trans*[b]			2.4
C20:2-*cis,cis*			2.3
C22:0	0.5	0.6	1.5
C22:1-*trans*			3.5
C22:1-*cis*	0.3		2.4
C22:2-*trans*[b]			1.4
C22:2-*cis,cis*			1.3
C24:0			0.2
C24:1[c]			0.4
Sum saturated fatty acids	47.0	30.3	33.7
Sum *trans* monoenes	2.6	23.2	19.6
Sum *trans* dienes[b]		0.3	4.4
Sum C12:0, C14:0, C16:0 and *trans* isomers	34.0	39.0	46.1
% of energy from *trans* fatty acids	0.9	8.5	8.0

[a] Includes some unidentified *cis* monoenes.
[b] Includes *trans,trans*, *cis,trans* and *trans,cis*.
[c] Contains *cis* and *trans* in estimated equal proportions.

TABLE 4. Serum lipid and lipoprotein levels at baseline and at the end of the dietary test periods

	Baseline	Butter-Diet	PHSO-Diet	PHFO-Diet
Total cholesterol, mmol/L (n = 31)	5.35 ± 1.19	5.32 ± 1.13	5.11 ± 1.13	5.42 ± 1.25
LDL-cholesterol, mmol/L (n = 30)	3.58 ± 1.10	3.81 ± 1.03	3.58 ± 1.08	3.94 ± 1.16
HDL-cholesterol, mmol/L (n = 30)	1.09 ± 0.27	1.05 ± 0.26	1.05 ± 0.25	0.98 ± 0.21
LDL-cholesterol/HDL-cholesterol (n = 30)	3.60 ± 1.83	3.85 ± 1.48	3.65 ± 1.54	4.20 ± 1.59
Triglycerides, mmol/L (n = 31)	1.60 ± 0.93	1.14 ± 0.49	1.23 ± 0.94	1.20 ± 0.69
ApoB, g/L (n = 31)	1.21 ± 0.31	1.22 ± 0.28	1.22 ± 0.32	1.28 ± 0.32
ApoA-I, g/L (n = 31)	1.35 ± 0.16	1.28 ± 0.18	1.24 ± 0.15	1.22 ± 0.18

Values given as mean ± SD.

5 shows the mean differences among diets and their statistical significance probabilities.

Total serum cholesterol levels were significantly lower on the PHSO-diet compared to the PHFO-diet (difference: −0.30 mmol/L; $P \leq 0.01$), and also lower compared to the butter-diet although this difference did not reach significance level ($P = 0.04$). No difference was found for total cholesterol between the PHFO-diet and the butter-diet.

The levels of LDL-cholesterol were significantly lower on the PHSO-diet compared to the PHFO-diet (difference: −0.36 mmol/L; $P \leq 0.01$) and also compared to butter-diet (difference: 0.23 mmol/L; $P = 0.02$). The levels of LDL-cholesterol did not differ between the PHFO-diet and the butter-diet.

HDL-cholesterol levels were significantly lower on the PHFO-diet compared to the butter-diet (difference: 0.07 mmol/L), and also lower, although not significantly, compared to the PHSO-diet (difference: 0.06 mmol/L; $P = 0.03$). No difference was found between the butter-diet and the PHSO-diet.

Compared to the PHFO-diet the ratio of LDL-cholesterol to HDL-cholesterol was reduced both on the PHSO-diet (difference: −0.56) and on the butter-diet (difference: −0.36). The ratio did not differ between the butter-diet and the PHSO-diet.

The levels of apoB were higher on the PHFO-diet when compared to both the butter-diet ($P \leq 0.01$) and PHSO-diet ($P \leq 0.01$). No difference was found between the butter-diet and the PHSO-diet. The Pearson coefficient of

TABLE 5. Differences in serum lipid and lipoprotein levels between the dietary test periods

	Mean Differences	P Value[a]	98.3% Confidence Interval
Total cholesterol, mmol/L (n = 31)			
PHSO-diet − PHFO-diet	−0.30	≤ 0.01	(−0.52, −0.08)
Butter-diet − PHFO-diet	−0.09	0.25	(−0.29, 0.11)
Butter-diet − PHSO-diet	0.21	0.04	(−0.04, 0.46)
LDL-cholesterol, mmol/L (n = 30)			
PHSO-diet − PHFO-diet	−0.36	≤ 0.01	(−0.58, −0.14)
Butter-diet − PHFO-diet	−0.13	0.11	(−0.34, 0.07)
Butter-diet − PHSO-diet	0.23	0.02	(−0.01, 0.46)
HDL-cholesterol, mmol/L (n = 30)			
PHSO-diet − PHFO-diet	0.06	0.03	(−0.01, 0.13)
Butter-diet − PHFO-diet	0.07	≤ 0.01	(0.00, 0.13)
Butter-diet − PHSO-diet	0.00	0.88	(−0.06, 0.07)
LDL-cholesterol/HDL-cholesterol (n = 30)			
PHSO-diet − PHFO-diet	−0.56	≤ 0.01	(−0.91, −0.20)
Butter-diet − PHFO-diet	−0.36	0.02	(−0.70, −0.01)
Butter-diet − PHSO-diet	0.20	0.23	(−0.22, 0.62)
ApoB, g/L (n = 31)			
PHSO-diet − PHFO-diet	−0.06	≤ 0.01	(−0.12, −0.01)
Butter-diet − PHFO-diet	−0.06	≤ 0.01	(−0.12, −0.01)
Butter-diet − PHSO-diet	0.00	1.00	(−0.05, 0.05)
ApoA-I, g/L (n = 31)			
PHSO-diet − PHFO-diet	0.02	0.23	(−0.02, 0.06)
Butter-diet − PHFO-diet	0.05	≤ 0.01	(0.01, 0.10)
Butter-diet − PHSO-diet	0.04	≤ 0.01	(0.01, 0.07)

[a] P values ≤ 0.02 were regarded as significant.

TABLE 6. Lp[a] at baseline and at the end of the dietary test periods

	Baseline	Butter-Diet	PHSO-Diet	PHFO-Diet
		Q1[a], median, Q3[b] (Mean ± SD)		
Lp[a], mg/L (n = 31)	39, 60, 301 (178 ± 186)	47, 91, 350 (194 ± 193)	53, 103, 397 (238 ± 237)	50, 121, 379 (234 ± 227)

[a]Q1 = lower quartile.
[b]Q3 = upper quartile.

correlation between the baseline levels of apoB and LDL-cholesterol at baseline was 0.83.

Relative to the levels on the butter-diet, apoA-I decreased both on the PHFO-diet ($P \leq 0.01$) and on the PHSO-diet ($P \leq 0.01$). No difference was found between the PHFO-diet and the PHSO-diet. The Pearson coefficient of correlation between the baseline levels of apoA-I and HDL-cholesterol at baseline was 0.69.

No differences in triglyceride values were observed between the test-diets.

Both the PHSO-diet and the PHFO-diet resulted in significantly higher levels of Lp[a] compared to the butter-diet (**Table 6** and **Table 7**). No difference was found between the PHFO-diet and the PHSO-diet.

DISCUSSION

In this strictly controlled dietary study we have shown that a diet that contains PHFO has an effect on total and LDL-cholesterol comparable to that of butter but a significantly more cholesterol-increasing effect than a diet where PHFO is exchanged for PHSO. Our findings of a serum cholesterol-increasing effect of PHFO about equal to that of butter is in accordance with an older study (14) where it was found that partially hydrogenated whale oil increased total serum cholesterol to the same extent as butter. The content of long chain saturates and monoene fatty acids in whale oil was considered responsible for this effect. Whale oil has a composition comparable to that of marine fish oil but with about half the content of eicosapentaenoic and docosahexaenoic acid (3). In that study (14) PHFO was also found to increase total serum cholesterol but because 40% of sunflower oil was added to the PHFO diet the results cannot be directly compared to ours.

Several studies have recently shown that partially hydrogenated vegetable oil increases LDL-cholesterol (5, 7–10) and decreases HDL-cholesterol (5–10) and these effects have been attributed primarily to the high content of different isomers of *trans*-C18:1, in particular to elaidic acid, in such fat. With PHFO the situation is more complex because of the large number of isomeric fatty acids of different chain length, both *cis* and *trans,* in addition to very long chain saturated acids. A complete resolution of the individual geometrical position isomers of the fatty acids was not possible in this study and it is therefore not possible to differentiate the effects on serum lipids of individual fatty acids in PHFO. The findings observed in this study should be considered overall effects of PHFO to which the *trans* or *cis* monoene fatty acids of different chain length, the *trans* dienes, or the long chain (C20–C24) saturated fatty acids may have contributed. The sum of the cholesterol-increasing saturated fatty acids C12:0, C14:0, C16:0, and the *trans* fatty acids was highest in the PHFO-diet and lowest in the butter-diet (Table 3). If the effects on serum lipoproteins of C18:1-*trans* in PHSO were comparable to the very long chain *trans* fatty acids of PHFO the higher levels of total and LDL-cholesterol on the PHFO-diet would be expected. The decrease in HDL-cholesterol and apoA-I and the increase in Lp[a] on the PHFO-diet are also similar to previous findings with partially hydrogenated vegetable oils (5, 6) indicating that these effects are shared by both *trans* C18:1 and the very long chain *trans* fatty acids in PHFO.

TABLE 7. Differences in Lp[a] between the dietary test periods

	Median Differences	*P* Value[a]	98.3% Confidence Interval
Lp[a], mg/L (n = 31)			
PHSO-diet − PHFO-diet	−1	0.99	(−16, 23)
Butter-diet − PHFO-diet	−25	≤ 0.01	(−67, −11)
Butter-diet − PHSO-diet	−20	≤ 0.01	(−62, −14)

[a]*P* values ≤ 0.02 were regarded as significant.

Although the sum of saturated and *trans* fatty acids was higher in the PHSO-diet than in the butter-diet, this last diet resulted in significantly higher serum concentrations of total and LDL-cholesterol. This indicates that the saturated fatty acids in butter (C12–C16) have a more potent hypercholesterolemic effect than the *trans* fatty acids in PHSO as has also been found in other studies (5, 8, 10).

Compared to the butter-diet, both PHFO and PHSO reduced apolipoprotein A-I but only PHFO reduced serum HDL-cholesterol. The differential effect on LDL- and HDL-cholesterol of the three diets resulted in a higher ratio of LDL- to HDL-cholesterol on the PHFO-diet than on the two other diets. It has previously been shown that C18:1 *trans* fatty acids have an HDL-cholesterol lowering effect compared to both saturated fat and to oleic acid (5, 10). As a mechanism for this HDL-reducing effect of *trans* fatty acids an increase in activity of cholesteryl ester transfer protein (CETP) has been proposed (33). The relation of CETP to the atherosclerotic process, if any, is not clear. Patients with a genetic lack of this protein have high levels of HDL-cholesterol and may be protected against premature atherosclerosis (34), but arguments for a proatherogenic role of this protein has also been presented (35). The health risk implications at the population level of the HDL-cholesterol lowering effect of *trans* fatty acids may also be difficult to evaluate. Low HDL-cholesterol has been identified as a risk factor in a number of epidemiological studies in high risk populations (36). However, in a meta-analysis of the correlations between coronary heart disease mortality and mean cholesterol levels in 17 different populations, Law and Wald (37) found a strong positive association with LDL-cholesterol but no correlation with HDL-cholesterol. In another large prospective population study of 47,000 middle-aged men and women, it was found that the inverse association between HDL-cholesterol and coronary heart disease mortality in men disappeared when serum total cholesterol level was below 6.5 mmol/L (38). In a study where a low coronary heart disease risk population in Taipei was compared with a high risk population in Framingham (39), the Taipei men had significantly lower levels of LDL-cholesterol and apoB and significantly higher levels of HDL-cholesterol. After adjusting for smoking and body mass index, only differences in total and LDL-cholesterol remained significant. The study supports the hypothesis that differences in the levels of LDL-cholesterol but not in HDL-cholesterol are connected to the twofold difference in coronary heart disease mortality between the two populations.

Epidemiological studies related to the consumption of PHFO and risk of cardiovascular disease are scant and inconclusive. Although a positive association between intake of PHFO and mortality from ischemic heart disease has been hypothesized in one study (40), such association was not confirmed in a later study by the same authors (41). The methodology of this work has also been criticized (1, 42). Animal studies do not support the existence of serious health risks of PHFO. In primates given partially hydrogenated herring oil at levels of greater than 50% of dietary energy, total serum cholesterol increased significantly but no atherosclerotic lesions were found after 30 months of feeding (43). In a life-span study in rats, Duthie et al. (44) showed that PHFO feeding reduced total serum cholesterol compared to either a low erucic acid rapeseed oil- or a PHSO-diet. To interpret the results from rats to humans is difficult, however, because of differences in lipoprotein metabolism. If the effects of PHFO in humans are due to the content of *trans* fatty acids and mediated through cholesteryl ester transfer protein (33), the lack of LDL-cholesterol in the rat makes a major difference.

Our results are in good agreement with earlier dietary studies when comparing the effects of the PHSO-diet to the effects of the butter-diet on total cholesterol (5, 8), LDL-cholesterol (3, 6), triglycerides (5, 6), apoA-I (8), and apoB (8, 9). The levels and source of vegetable *trans* fatty acids and saturated fatty acids in these studies differ, but they support the existence of a dose-response relationship between intake of *trans* fatty acids and lipid levels as suggested by Zock and Katan (9). Nestel et al. (6), however, failed to show an increase in total and LDL-cholesterol with an elaidic acid-rich diet (7% of energy) in 27 mildly hypercholesterolemic men as compared to a lauric–myristic–palmitic acid-rich diet (saturated fatty acid-rich diet) and a palmitic acid-rich diet. However, the elaidic acid-rich diet showed significantly higher levels of total and LDL-cholesterol than an oleic acid-rich diet. HDL-cholesterol was significantly higher with the palmitic acid-rich diet compared with elaidic acid, which in turn was not lower than with oleic acid (6).

High concentrations of Lp[a] have been identified as an independent risk factor for coronary heart disease (45). The level of Lp[a] is considered to be largely under genetic control, but previous studies have indicated that dietary fat may influence Lp[a] concentrations (46, 47). The results of these studies, and the structural similarity with plasminogen, form the basis for the assumption of Lp[a] as a link between thrombosis and atherosclerosis. In our study, the levels of Lp[a] rose with the intake of both PHSO and PHFO, indicating that the effect may be linked to the content of *trans* fatty acids (6, 11). However, the effects are considered dose-dependent (11), and Lichtenstein et al. (7) found no effect of C18:1 *trans* at a low intake of vegetable *trans* fatty acids of 4.2% of energy. Hornstra et al. (47) suggested that palm oil lowered Lp[a] due to a specific component in the palm oil or to displacement of a component present in the habitual diet.

In order to minimize the influence of dietary cholesterol on serum lipids and to balance the differences in dietary cholesterol among the diets, we added dried egg

powder to the butter-diet and to the PHSO-diet. The observed changes in blood lipid levels should, therefore, be due to differences in the content of fatty acids, not to differences in dietary cholesterol. The calculated effect on serum cholesterol of the maximum difference in dietary cholesterol content between the PHFO- and PHSO-margarines, 338 mg per 10 MJ per day, corresponded according to Keys' formula (16) to approximately 0.28 mmol/L and according to Hegsted's formula (48) to approximately 0.38 mmol/l. We therefore assume that the effects on serum total cholesterol of PHFO and butter compared to PHSO would have been significantly more pronounced without the addition of egg powder to the actual diets.

The content of linoleic and linolenic acid did not differ among the diets in our study (Table 3) and corresponded to approximately 5.0% and 1.0% of energy, respectively. The effects of the test margarines on blood lipids can therefore not be explained by deficiencies of essential fatty acids.

The studies by Mensink and Katan (5) and Zock and Katan (9) have been criticized for the use of specially prepared partially hydrogenated fat with a distribution of *trans* monoene isomers different from that of commercially available fats. As opposed to most other controlled dietary studies, we used oils and fats taken directly from the ordinary production line. The results should thus be of direct relevance to the general population. The PHFO-margarine contained approximately 27% *trans* isomers (determined by GLC as described in Methods) and the commercial corresponding margarines contain 21–27%. The ratio of PHFO 30/32 and PHFO 38/40 used in this study corresponds to overall Norwegian sales figures for PHFO. The PHSO-margarine contained approximately 34% *trans* isomers and the corresponding commercial margarines contain 20–25% (GLC). The amounts of PHFO consumed during this study were 4–5 higher than the average intake in Norway. This high level was chosen in order to be within the range of 7.5 to 11% of energy as *trans* fatty acids, a range within which a dose-response has been established between amount of *trans* fatty acids in the diet and elevation of LDL-cholesterol (9). As demonstrated (2, 42), intakes of *trans* fatty acids approaching the level used in our study may be reached by persons who eat large amounts of hardened fats and products prepared with or fried in such fats. At the population level, a daily per capita intake in Norway of 11 g of PHFO may have an effect on serum cholesterol that is not negligible. No firm conclusions can be made, however, with regard to a potential dose-response relationship between dietary intake of PHFO and effect on serum cholesterol.

In conclusion, our results indicate that consumption of PHFO may affect lipid risk indicators for coronary heart disease at least to the same extent as butterfat. The effects of PHSO on total and LDL-cholesterol were less potent when compared to the effects of both PHFO and butterfat. As the level of *trans* fatty acids was not the sole variable between the diets, our results cannot be attributed to specific fatty acids, but to an overall effect of industrially produced partially hydrogenated oils. As hardened margarine is widely used in the food industry and also in home cooking, and often replaces soft margarines in spreads etc., our findings might have important health implications. According to our data it would be prudent to lower the content of PHFO in margarines intended for human consumption. Our data do not support, however, strict limitations on the use of partially hydrogenated vegetable oils in margarine production if the alternative is a replacement by cholesterol-increasing saturated fat.

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