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PROFILING OF PLASMA CHOLESTEROL ESTER AND TRIGLYCERIDE FATTY ACIDS AS THEIR METHYL ESTERS BY CAPILLARY GAS CHROMATOGRAPHY, PRECEDED BY A RAPID AMINOPROPYL-SILICA COLUMN CHROMATOGRAPHIC SEPARATION OF LIPID CLASSES

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SUMMARY

A rapid procedure for the isolation of plasma cholesterol ester and triglyceride fractions with aminopropyl-silica columns, followed by analysis of their fatty acid compositions by capillary gas chromatography with flame ionization detection, is described. Within-series and long-term (six months) series-to-series precision were investigated. The isolation procedure caused minimal cross-over between the two lipid classes. Reference values for 57 apparently healthy Dutch adults were established and compared with data reported from other countries. Feeding of rats with four diets differing in their fatty acid compositions showed the relationship between the composition of the fatty acids in the diet and those esterified to cholesterol in plasma. The method is of potential usefulness to the establishment of the compliance of dietary interventions and basic dietary experiments.

INTRODUCTION

In Western countries, coronary atherosclerotic heart disease (CAHD) is the major cause of death. A high level of serum cholesterol, notably located in the low-density lipoprotein fraction [1], is a serious risk factor for the development

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of CAHD. A decrease of serum cholesterol levels by diet and drugs can arrest and even reverse the atherosclerotic process [2,3].

Lowering of serum cholesterol can be accomplished by diets high in polyunsaturated fatty acids (PUFA), such as those of the ω6 series (mainly 18:2ω6* from vegetable oils) [2,4] or those of the ω3 series (mainly 20:5ω3 and 22:6ω3 from fish oils) [5,6]. Other clinical reasons for prescribing diets with "deviant" fatty acid (FA) compositions are: lowering of serum triglyceride (TG) levels to reduce the risk of pancreatitis [4], changing the mutual ratio between the different series of eicosanoids to suppress chronic inflammatory processes [7] and alteration of the FA composition of phospholipids to influence membrane deformability [8].

For the establishment of patient compliance and dietary experiments it is of importance to monitor the intake of the prescribed diet and its influence on the FA composition of the body. Registration by dietary inquiry is time-consuming and liable to subjective information. The FA composition of the plasma (or serum) cholesterol ester (CE) fraction is considered to be a reliable reflection of the dietary FA composition of the preceding weeks [9–11]. Its measurement seems to offer a more objective means to accomplish the goal.

About 75% of total plasma cholesterol is esterified to long-chain FA (LCFA) [12,13]. In plasma, CE are synthesized by the enzyme lecithin:cholesterol acyl transferase (LCAT; EC 2.3.1.43), which catalyses the transfer of a fatty acyl from the *sn*-2 position of phosphatidyl choline (PC) to free cholesterol. The FA composition of CE synthesized in plasma is therefore an indirect measure of that of the *sn*-2 position of one of the most important structural phospholipids of cell membranes. LCAT has preference for PC species containing 18:2ω6 at their *sn*-2 positions and that are located in high-density lipoproteins [14,15]. Most tissues, including intestinal mucosa and liver, have the ability to produce CE by the enzyme acyl-CoA:cholesterol O-acyltransferase (ACAT; EC 2.3.1.26), which transfers a fatty acyl from an acyl-CoA to cholesterol. It is as yet unclear to what extent intestinal and liver CE, located in chylomicrons and very-low-density lipoproteins, respectively [14], contribute to the circulating plasma CE pool. The higher content of CE 18:1ω9 and the lower content of CE 18:2ω6 in very-low-density lipoproteins, when compared with low- and high-density lipoproteins [16], and the high proportion of 16:0 and 18:1ω9 and low proportion of 18:2ω6 in the plasma CE fraction of patients with LCAT deficiency [14] may point to the secretion of CE synthesized by ACAT in the liver.

We have developed a rapid prepurification method for the profiling of both plasma CE-FA and TG-FA as their methyl esters (FAME) by gas chromatography with flame ionization detection. As a control for its usefulness we measured the plasma CE-FA composition of rats that were fed with four diets, differing in their FA compositions.

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*Fatty acids are abbreviated as a:$b\omega x$, in which a is the number of straight-chain carbon atoms, b the number of methylene interrupted double bonds in the *cis* configuration and x the number of carbon atoms from the last double bond to the terminal methyl group.

EXPERIMENTAL

Materials

CE and TG standards were obtained from Sigma (Brunschwig Chemie, Amsterdam, The Netherlands). Bond Elut aminopropyl disposable columns (500 mg) with stainless-steel frits were purchased from Analytichem (Bètron Scientific, Rotterdam, The Netherlands). Butylated hydroxytoluene (BHT) was obtained from Fluka (Oud-Beijerland, The Netherlands). High-performance thin-layer chromatographic (HPTLC) silica gel 60 plates (10 cm × 10 cm; without fluorescent indicator) and all other reagents were from Merck (Merck Nederland, Amsterdam, The Netherlands).

Standard and internal standard solutions

Two standard solutions were used: (1) a mixture of CE standards (12:0, 14:0, 16:0, 16:1ω7, 18:0, 18:1ω9, 18:2ω6, 20:0 and 20:4ω6), containing 250 mg of each standard and BHT per litre of hexane, and (2) a mixture of TG standards (12:0, 14:0, 16:0, 18:0, 18:1ω9, 18:2ω6 and 20:0), containing 40 mg of each standard and 80 mg of BHT per litre of hexane. Two internal standard solutions were made, the first containing 502 mg of CE (17:0) and 500 mg of BHT per litre of hexane and the second 198 mg of TG (19:0) and 250 mg of BHT per litre of hexane. In addition we prepared a stock solution of 5 g BHT per litre of methanol.

Samples

EDTA-anticoagulated blood samples of 10 ml volume were obtained from 57 apparently healthy adults (28 women aged 20–49 years and 29 men aged 22–55 years) after a 12-h fast. None of these persons was on a restricted diet. Samples were put on ice and processed within 30 min. Thrombocyte-rich plasma was obtained by centrifuging the blood at 800 *g* for 10 min. The thrombocyte-rich plasma fraction was subsequently centrifuged for 10 min at 1500 *g*. The thrombocyte-poor EDTA-plasma was stored at −20°C until the analyses of the CE-FA and TG-FA compositions.

Preparation of a total lipid extract from plasma

Total lipid extracts were prepared essentially as described by Folch et al. [17]. Aliquots (100 μl) of the CE (17:0) and TG (19:0) internal standard solutions were pipetted into a 15-ml glass tube and evaporated to dryness at 40°C under a stream of nitrogen. A 2.5-ml portion of a chloroform–methanol solution (2:1, v/v) was pipetted into the tube, and 100 μl of the thrombocyte-poor EDTA-plasma was added under vigorous mixing. After centrifugation for 10 min at 1500 *g* the supernatant was collected and evaporated to dryness at 40°C under a stream of nitrogen. The lipids were redissolved in 1 ml of hexane. When the final total lipid extracts were to be stored at −20°C until further processing, 100 μl of the BHT stock solution was added to the internal standard solutions before evaporation.

Isolation of the cholesterol ester and triglyceride fractions

A shortened version of the method of Kaluzny et al. [18] was used. Aminopropyl columns were previously washed twice under vacuum with 2 ml of chloroform–methanol (2:1, v/v) and four 2-ml portions of hexane. Immediately after washing the columns were used for the isolation of lipid classes.

Cholesterol ester fraction. A 400-μl aliquot of the total lipid extract in hexane (see above) was applied to the column, and the hexane was drawn through. The eluate was collected in a 15-ml Sovirel glass tube containing 100 μl of the BHT stock solution. The column was eluted with two 2-ml portions of hexane, and the eluates were collected in the same glass tube. These combined eluates contain the CE fraction.

Triglyceride fraction. The column was subsequently eluted with three 2-ml portions of a mixture containing 1% diethyl ether and 10% dichloromethane (v/v) in hexane. The eluate was collected in a 15-ml Sovirel glass tube containing 100 μl of the BHT stock solution.

The CE and TG fractions were evaporated to dryness at 40°C under a stream of nitrogen and redissolved in 0.5 ml of chloroform. For a check on the completeness of the separation, 10-μl aliquots of the lipid fractions were occasionally spotted on an HPTLC plate, which was subsequently developed and visualized according to Kaluzny et al. [18].

Preparation of fatty acid methyl esters

For transesterification [19], 2 ml of methanol–water–hydrochloric acid (five volumes of methanol added to one volume of 6 *M* hydrochloric acid) were added. The tubes were tightly capped and heated at 90°C for 4 h. After cooling, the samples were extracted twice with 2-ml portions of hexane. The combined hexane layers were evaporated to dryness at 40°C under a stream of nitrogen and redissolved in 100 μl of hexane.

Gas chromatographic profiling of fatty acid methyl esters

Portions of 2 μl of the hexane extracts were automatically injected into a Hewlett-Packard Model 5880 gas chromatograph equipped with a Model 7672 A automatic injection system and a 50 m$\times$0.2 mm I.D. (apolar) Ultra 1 cross-linked methyl silicone-coated (film thickness 0.11 μm) column (Hewlett-Packard, Amstelveen, The Netherlands). The flow-rate of helium was 0.52 ml/min, the split ratio 1:20, the flame ionization detector temperature 300°C and the injector temperature 280°C. The oven temperature programme was: 2 min at 160°C; 2°C/min to 240°C; 10°C/min to 290°C; 20 min at 290°C.

Identification, quantification and quality control

FAME were identified by comparison of retention times with those of known standards. Peak areas were calculated using a Nelson Analytical 3000 data system (Cupertino, CA, U.S.A.).

The concentrations of FA in each fraction were calculated by comparing the peak areas of each FAME with that of 17:0 (for the CE fraction) or 19:0 (for the TG fraction), and assuming that equal mass amounts of long-chain FAME give rise to equal GC peak areas [20]. The addition of 50.2 μg of CE (17:0) and 19.8

μg TG (19:0) to 100 μl of plasma fortifies its final transmethylated CE and TG fractions with 21.24 μg of 17:0 and 18.99 μg of 19:0, respectively. The raw data, expressed in μg per 100 μl of plasma, were transformed to mmol/l by dividing by their corresponding molecular masses, summed and normalized to mol per 100 mol.

The cross-overs of FA from the TG fraction into the CE fraction, and vice versa, were estimated by calculating the peak-area ratio 19:0/17:0 in the CE fraction and 17:0/19:0 in the TG fraction, respectively. Assuming that endogenous CE and TG contain hardly any 17:0 or 19:0, a 1% cross-over of TG-FA into the CE-FA fraction would lead to a 19:0/17:0 peak-area ratio of 0.00894, whereas a 1% cross-over of CE-FA into the TG-FA fraction would result in a 17:0/19:0 peak-area ratio of 0.01118.

Within- and between-series precision were determined by analysing six 100-μl aliquots of pooled plasma within one series and a 100-μl aliquot of the same pooled plasma every month for six months, respectively.

Recoveries were calculated by analysing 100 μl of pooled plasma and 100 μl of the same pooled plasma enriched with 200 μl of (previously evaporated) CE and 500 μl of TG standard solutions. The FA peak-area differences between the subsequently isolated unspiked and spiked CE and TG fractions were compared with the data of separately transmethylated CE and TG standards, using 17:0 and 19:0 as internal standards, respectively. Within-series ($n=6$) and between-series ($n=8$) recoveries were calculated.

Direct gas chromatographic profiling of the cholesterol ester fraction

CE fractions, as obtained from aminopropyl-silica columns, were evaporated to dryness at 40 °C under a stream of nitrogen and redissolved in 200 μl of hexane. Aliquots of 2 μl were injected into a Varian Model 3700 gas chromatograph (Varian Benelux, Amsterdam, The Netherlands) equipped with a 25 m × 0.25 mm I.D. (medium-polar) 50% phenyl–50% methyl silicone-coated (film thickness 0.10 μm) WCOT-TAP column (Chrompack Nederland, Middelburg, The Netherlands). The flow-rate of helium was 1.15 ml/min, the split ratio 1:20, the flame ionization detector temperature 350 °C and the injector temperature 350 °C. The oven temperature programme was: initial temperature, 330 °C; 2 °C/min to 350 °C; hold for 15 min.

Animal experiments

Male brown Norwegian rats, six weeks old, were fed for nine weeks with the following four diets (Hope Farms, Woerden, The Netherlands): the standard rat chow (containing 6.5% fat by mass) and a standard fat-free diet supplemented with either 10% hydrogenated coconut fat, a 10% sunflower–soybean oil mixture or 10% fish oil. To avoid any deficiency of FA of the ω6-series, the coconut fat and fish oil diets were fortified with 1.0 g/100 g sunflower–soybean oil mixture. The α-tocopherol content of the diets amounted to 90 mg/kg (standard rat chow) and 200–300 mg/kg (other diets).

Each diet group consisted of two rats. After nine weeks on their respective diets, the rats were anaesthesized with diethyl ether in an undefined metabolic

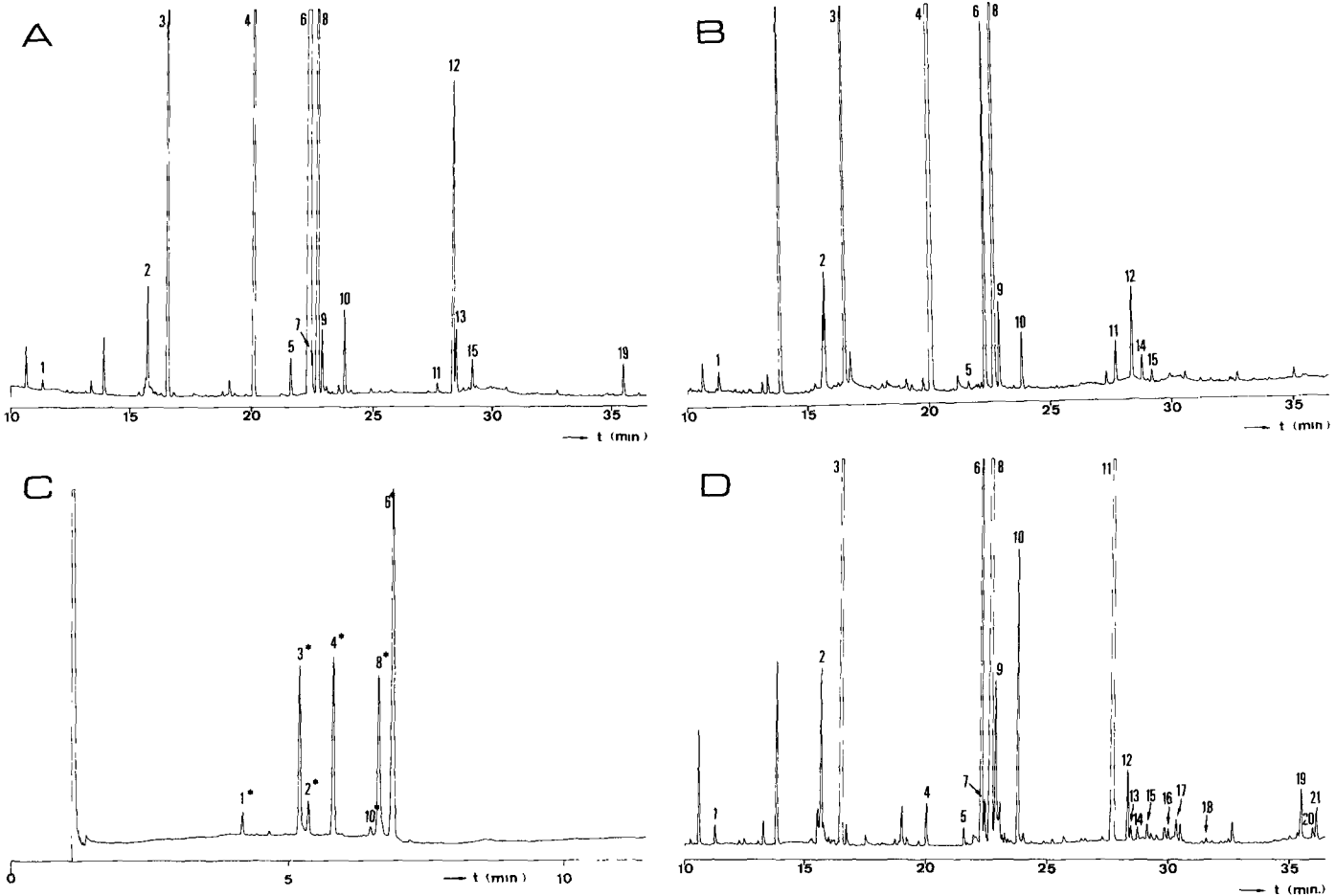

Fig. 1. GC profiles of plasma lipid fractions of an apparently healthy adult (A, C and D) and of a patient with essential FA deficiency due to abetalipoproteinaemia (B). (A) FAME profile of the plasma CE fraction of the healthy adult; (B) FAME profile of the plasma CE fraction of the patient with EFA deficiency; (C) intact CE profile of the healthy adult; (D) FAME profile of the plasma TG fraction of the healthy adult. Peaks (asterisks indicate intact CE): 1 = 14:0; 2 = 16:1ω7; 3 = 16:0; 4 = 17:0; 5 = 18:3ω6; 6 = 18:2ω6; 7 = 18:3ω3; 8 = 18:1ω9; 9 = 18:1ω7; 10 = 18:0; 11 = 19:0; 12 = 20:4ω6; 13 = 20:5ω3; 14 = 20:3ω9; 15 = 20:3ω6; 16 = 20:2ω6; 17 = 20:1ω9; 18 = 20:0; 19 = 22:6ω3; 20 = 22:4ω6; 21 = 22:5ω3.

state. EDTA-anticoagulated blood samples were collected from the aorta and processed as described above. The FA compositions of the diets, including the medium-chain FA, were determined as previously described [21]. The thrombocyte-poor EDTA-plasma CE-FA and TG-FA compositions were assayed as outlined above.

Statistics

Mean reference values and their 95% confidence intervals for the FA compositions of the CE and TG fractions isolated from the plasma of 57 apparently healthy Dutch controls were determined after a two-stage log-power transformation of the individual data, according to the IFCC recommendation [22].

RESULTS AND DISCUSSION

Fig. 1 shows part of the gas chromatograms of FAME prepared from the CE (A) and TG (D) fractions isolated from plasma of an apparently healthy adult. Remarkable differences can be observed between plasma CE-FA profiles of the healthy adult (A) and a paediatric patient with essential FA deficiency due to abetalipoproteinaemia (B). The latter contains much lower relative amounts of FA of the ω6 series, especially 18:2ω6 (peak 6), and additionally contains a peak identified as 20:3ω9 (peak 14) [23–25]. Fig. 1C shows a chromatogram of the intact CE fraction of the same healthy person, isolated by the present method. As may be noted, GC profiling of the intact CE fraction leads to considerably fewer peaks. With this method, which necessitates relatively high column temperatures, the CE of 20:4ω6 remains undetected because of its heat lability.

Quality control

Table I shows the within- and long-term (six months) between-series quality control data for the endogenous CE-FA and TG-FA compositions of pooled plasma. The use of different FA internal standards for the CE and TG fractions allows the estimation of the cross-overs between these fractions for each individual sample, which may be of importance to samples containing abnormally high amounts of these lipid classes. For the pooled plasma sample the mean cross-overs were estimated to be below 3%. There was good precision for the quantitatively most important FA, whereas, as might have been expected, the reproducibility for the minor ones was considerably less favourable. Similar results were obtained by Wang and Peter [26], who used silica Sep-Pak cartridges to fractionate lipid classes and GC analysis on a polar packed column. The high between-series coefficients of variation (C.V.) for 14:0 are caused by the relatively low boiling point of its methyl ester, which results in split injection related discrimination [20,21], whereas the relatively poor reproducibility encountered for 16:1ω7 is due to its poor separation from neighbouring compounds of unknown identity on an apolar stationary phase (see Fig. 1). The calculation of the total CE (SUM CE-FA) and TG (SUM TG-FA/3) concentration of plasma from the sum of all FA in each fraction was found to result in relatively high series-to-series coefficients of vari-

TABLE I

WITHIN-SERIES AND LONG-TERM (SIX MONTHS) BETWEEN-SERIES PRECISION FOR THE FA COMPOSITIONS OF THE CE AND TG FRACTIONS ISOLATED FROM A POOLED PLASMA

Aliquots of 500 μl of pooled plasma were kept frozen at $-20\,^{\circ}C$ until analysis. Each analysis made use of a single aliquot of pooled plasma. Abbreviations: N.D. = not detectable; SAFA = sum of saturated FA; MUFA = sum of monounsaturated FA; PUFA = sum of polyunsaturated FA; SUMω3 = sum of ω3 FA; SUMω6 = sum of ω6 FA; SUMω7 = sum of ω7 FA; SUMω9 = sum of ω9 FA; DBI = double bond index (mean number of double bonds per FA); MCL = mean chain length (mean number of carbon atoms per FA); 19:0/17:0 = measure for the cross-over of the TG fraction into the CE fraction (peak area 19:0/peak area 17:0 × 100%); 17:0/19:0 = measure for the cross-over of the CE fraction into the TG fraction (peak area 17:0/peak area 19:0 × 100%); SUM CE-FA = total concentration of CE (mmol/l) as derived from the sum of the concentrations of the individual FA in the CE fraction assayed by the present method; SUM TG-FA/3 = total concentration of TG (mmol/l) calculated by taking one third of the sum of the concentrations of the individual FA in the TG fraction assayed by the present method; P/S = PUFA/SAFA.

Analyte	Cholesterol esters ($n=6$)				Triglycerides ($n=6$)			
	Within		Between		Within		Between	
	Mean ± S.D. (mol/100 mol)	C.V. (%)	Mean ± S.D. (mol/100 mol)	C.V. (%)	Mean ± S.D. (mol/100 mol)	C.V. (%)	Mean ± S.D. (mol/100 mol)	C.V. (%)
14:0	1.15 ± 0.09	7.5	0.97 ± 0.21	21.1	4.03 ± 0.12	2.9	3.29 ± 0.89	27.1
16:1ω7	3.80 ± 0.15	3.9	2.93 ± 0.59	20.0	3.41 ± 0.43	12.5	3.35 ± 0.62	18.5
16:0	13.01 ± 0.37	2.8	13.52 ± 1.45	10.7	29.89 ± 0.94	3.1	29.77 ± 2.32	7.8
18:3ω6	0.72 ± 0.02	3.2	0.71 ± 0.03	3.8	0.30 ± 0.01	4.6	0.29 ± 0.02	7.2
18:2ω6	54.47 ± 0.58	1.1	54.04 ± 0.98	1.8	18.18 ± 0.47	2.6	17.57 ± 0.90	5.1
18:3ω3	N.D.		0.54 ± 0.03	6.3	1.43 ± 0.05	3.8	1.38 ± 0.22	15.7
18:1ω9	17.24 ± 0.06	0.4	17.69 ± 0.33	1.8	30.57 ± 0.54	1.8	30.86 ± 1.05	3.4
18:1ω7	1.09 ± 0.03	2.9	1.17 ± 0.08	7.1	3.73 ± 0.19	5.1	3.90 ± 0.31	7.8
18:0	1.48 ± 0.23	15.5	1.49 ± 0.22	14.6	5.72 ± 0.19	3.2	6.11 ± 0.81	13.2
20:4ω6	5.68 ± 0.06	1.0	5.53 ± 0.27	4.9	1.07 ± 0.08	7.0	1.16 ± 0.20	16.9
20:5ω3	0.57 ± 0.02	2.7	0.56 ± 0.06	10.1	N.D.		0.19 ± 0.06	29.3
20:3ω9	N.D.		N.D.		N.D.		0.15 ± 0.03	17.4
20:3ω6	0.52 ± 0.02	3.2	0.56 ± 0.02	4.1	0.23 ± 0.02	8.9	0.25 ± 0.03	11.0
20:2ω6	N.D.		N.D.		0.24 ± 0.02	7.9	0.23 ± 0.03	14.4
20:1ω9	N.D.		N.D.		0.34 ± 0.03	8.8	0.47 ± 0.08	17.7
20:0	N.D.		N.D.		0.21 ± 0.01	4.9	0.27 ± 0.05	16.8
22:6ω3	0.29 ± 0.04	12.4	0.30 ± 0.06	19.7	0.43 ± 0.02	4.6	0.42 ± 0.04	10.1
22:4ω6	N.D.		N.D.		N.D.		0.12 ± 0.02	18.8
22:5ω3	N.D.		N.D.		0.23 ± 0.01	4.8	0.24 ± 0.04	15.3
SAFA	15.64 ± 0.68	4.3	15.99 ± 1.66	10.4	39.84 ± 1.21	3.0	39.44 ± 2.88	7.3
MUFA	22.12 ± 0.14	0.7	21.78 ± 0.66	3.0	38.05 ± 0.72	1.9	38.57 ± 1.76	4.6
PUFA	62.24 ± 0.70	1.1	62.23 ± 1.10	1.8	22.10 ± 0.52	2.3	21.99 ± 1.25	5.7
SUMω3	0.86 ± 0.05	5.9	1.40 ± 0.12	8.2	2.09 ± 0.06	2.8	2.22 ± 0.25	11.2
SUMω6	61.38 ± 0.66	1.1	60.84 ± 1.06	1.7	20.02 ± 0.48	2.4	19.62 ± 1.00	5.1
SUMω7	4.88 ± 0.14	2.9	4.09 ± 0.63	15.5	7.14 ± 0.32	4.5	7.24 ± 0.80	11.0
SUMω9	17.24 ± 0.06	0.4	17.69 ± 0.33	1.8	30.91 ± 0.54	1.8	31.48 ± 1.03	3.3
P/S	3.99 ± 0.21	5.1	3.93 ± 0.43	11.1	0.56 ± 0.03	5.3	0.56 ± 0.07	12.5
DBI	1.62 ± 0.02	1.1	1.62 ± 0.03	1.9	0.89 ± 0.02	2.0	0.90 ± 0.05	5.0
MCL	17.77 ± 0.01	0.1	17.78 ± 0.03	0.2	17.24 ± 0.02	0.1	17.29 ± 0.05	0.3
19:0/17:0	1.39 ± 0.26	18.5	1.43 ± 1.40	97.7				
17:0/19:0					3.32 ± 0.26	7.8	2.94 ± 0.55	18.7
SUM CE-FA	3.17 ± 0.08	2.6	2.91 ± 0.43	14.8				
SUM TG-FA/3					1.09 ± 0.06	5.3	0.97 ± 0.22	22.2

TABLE II

WITHIN-SERIES AND LONG-TERM (SIX MONTHS) BETWEEN-SERIES RELATIVE RECOVERIES OF CE AND TG STANDARDS ADDED TO POOLED PLASMA

Recoveries were calculated by comparing FA peak-area differences between the spiked and unspiked pooled plasmas with the corresponding peak areas of separately transmethylated CE and TG standards, using the peak areas of 17:0 and 19:0 as internal standards, respectively. Spiked samples were prepared by fortifying 100 μl of pooled plasma with 50 μg of indicated CE standards and 20 μg of indicated TG standards

Analyte	Within-series ($n=6$)				Between-series ($n=8$)			
	CE		TG		CE		TG	
	Mean (%)	C.V. (%)	Mean (%)	C.V. (%)	Mean (%)	C.V. (%)	Mean (%)	C.V. (%)
14:0	104.3	4.0	88.8	6.9	83.7	19.8	93.8	5.6
16:1ω7	107.4	2.0	–	–	100.6	5.8	–	–
16:0	99.4	2.6	101.1	10.2	97.2	3.3	94.0	9.7
18:2ω6	105.8	8.2	97.0	7.8	100.4	13.2	97.9	4.2
18:1ω9	103.2	3.5	91.9	14.0	101.1	4.2	97.8	14.5
18:0	101.5	1.3	102.3	3.4	103.1	2.9	97.6	2.4
20:4ω6	103.0	3.7	–	–	102.7	3.9	–	–
20:0	103.5	1.2	100.6	0.4	105.4	3.8	99.4	2.0

ation and therefore does not seem to offer a reasonable alternative for the direct measurement of these analytes by other methods.

Table II presents the relative recoveries of added synthetic CE and TG standards. The recoveries relative to their internal standards were close to 100%, with reasonable long-term between-series coefficients of variation. Absolute recoveries as established by Kaluzny et al. [18] for CE(18:1ω9) and TG(18:1ω9) and by Wang and Peter [26] for CE(18:1ω9) and CE(18:2ω6), using similar prepurification methods, amounted to 90–100%.

Reference values

In Table III the reference values of plasma CE-FA and TG-FA for 57 apparently healthy Dutch adults (aged 20–55 years) are listed. When compared with the between-series cross-over of the TG fraction into the CE fraction for a single plasma sample (mean+2S.D. for the 19:0/17:0 ratio=4.23%; Table I),we found a much higher upper limit of this parameter for individual samples (95% upper confidence limit 19:0/17:0 ratio=8.99; Table III). A 19:0/17:0 percentage of 8.99 would imply a cross-over of TG-FA into the CE-FA of ca. 10%, which could not be confirmed on the basis of visual inspection of isolated fractions on HPTLC plates. On the other hand, there was not much difference between the cross-over data for the CE fraction, when the between-series results (mean+2S.D. for the 17:0/19:0 ratio=4.04; Table I) and those obtained from the reference value analysis (95% upper confidence limit for the 17:0/19:0 ratio=4.43%; Table III) were compared. The apparently variable cross-overs of the TG fraction into the CE

TABLE III

FA COMPOSITIONS OF THE CE AND TG FRACTIONS ISOLATED FROM THE PLASMA OF 57 HEALTHY DUTCH ADULTS (AGED 20-55 YEARS) IN THE FASTING STATE

The group was composed of 28 women (aged 20-49 years) and 29 men (aged 22-55 years). Means and 95% confidence intervals were determined from two-stage log-power transformed data. Abbreviations: total chol = concentration of total cholesterol (mmol/l) as determined by the SMA-C; TG = concentration of total triglycerides (mmol/l) as determined by the SMA-C; for other abbreviations see Table I.

Analyte	Cholesterol esters			Triglycerides		
	Mean (mol/100 mol)	95% Confidence interval		Mean (mol/100 mol)	95% Confidence interval	
		2.5%	97.5%		2.5%	97.5%
14:0	0.85	0.31	1.62	2.07	0.61	5.37
16:1ω7	2.59	0.91	4.96	3.26	1.44	6.75
16:0	12.90	10.93	14.86	28.29	21.73	37.12
18:3ω6	0.59	0.20	1.15	0.29	0.05	0.58
18:2ω6	55.85	48.29	63.59	18.44	11.21	33.05
18:3ω3	0.58	0.27	0.96	1.23	0.71	2.47
18:1ω9	16.16	12.49	20.32	32.25	24.93	39.57
18:1ω7	1.23	0.86	1.60	3.73	2.11	6.62
18:0	1.73	1.15	2.90	4.43	2.91	7.27
20:4ω6	5.30	3.83	8.63	1.22	0.69	2.34
20:5ω3	0.51	0.21	1.34	0.17	0.06	0.62
20:3ω9	N.D.			0.19	0.08	0.31
20:3ω6	0.63	0.41	0.97	0.29	0.17	0.51
20:2ω6	N.D.			0.27	0.13	0.64
20:1ω9	N.D.			0.41	0.23	0.80
20:0	N.D.			0.13	0.03	0.29
22:6ω3	0.41	0.26	0.87	0.53	0.18	1.72
22:4ω6	N.D.			0.14	0.07	0.24
22:5ω3	N.D.			0.32	0.12	0.58
SAFA	15.57	13.03	18.11	35.13	27.34	47.02
MUFA	19.99	14.84	26.22	40.02	30.41	49.63
PUFA	64.30	57.38	71.21	23.43	15.32	39.95
SUMω3	1.56	0.91	2.48	2.42	1.42	4.33
SUMω6	62.67	55.50	69.85	20.72	12.88	36.98
SUMω7	3.82	2.10	6.25	7.35	3.87	11.00
SUMω9	16.16	12.49	20.32	32.88	25.51	40.27
P/S	4.13	3.26	5.34	0.66	0.35	1.27
DBI	1.65	1.55	1.77	0.97	0.78	1.26
MCL	17.80	17.70	17.92	17.37	17.12	17.63
19:0/17:0	1.16	0.76	8.99			
17:0/19:0				2.80	1.79	4.43
Total chol	5.16	4.00	6.79			
Sum CE-FA	3.67	2.60	4.75			
TG				0.89	0.49	1.83
SUM TG-FA/3				0.75	0.31	1.71

fraction for individual samples may be caused by a variable occurrence of 19:0 or compounds eluting with a GC retention time of 19:0 in endogenous CE, or non-representative cross-over of TG(19:0) into the CE fraction. In this respect it is important to note that TG(19:0), because of its apolarity, has a higher R_F value in the standard silica HPTLC system than TG from endogenous origin, which would favour contamination that does not accurately reflect cross-over of endogenous TG into the CE-fraction during the isolation of the lipid classes on the aminopropyl-silica columns. The estimation of cross-overs by the measurement of the 17:0/19:0 and 19:0/17:0 ratios should therefore be interpreted with caution.

Comparison of 54 results calculated from the sum of all FA in the CE fraction (SUM CE-FA by the present method; y axis) with those obtained for total serum cholesterol by the SMA-C (x axis) revealed a regression line $y=0.684x+0.07$ (mmol/l), with a correlation coefficient of 0.909 ($p<0.0001$) and a residual S.D. in the y direction of 0.24 mmol/l. The calculated slope of 0.68 is in reasonable agreement with the ca. 70–80% esterified form of plasma cholesterol in healthy subjects [12,13]. A similar correlation study between 54 results calculated from the sum of all FA in the TG fraction times 1/3 (SUM TG-FA/3 by the present method; y axis) with those obtained for serum TG by the SMA-C (x axis) showed a regression line $y=0.913x+0.08$ (mmol/l), with a correlation coefficient of 0.937 ($p<0.0001$) and a residual S.D. in the y direction of 0.12 mmol/l. The ca. 9% lower values obtained by the present method may be related to the presence of ca. 10% mono- and diglycerides in plasma [27,28], which are additionally measured by methods based on the assay of hydrolysable glycerol, such as performed by the SMA-C.

Generally the reference values for the FA composition of the plasma CE and TG fractions as determined by us fit well within those reported by others [9,29–31], the most striking difference being the relatively higher levels for 18:2ω6 and the relatively lower levels for 18:1ω9 and FA of the ω3 series. This will certainly be due to the relatively high consumption of vegetable oils by the Dutch population and the relatively low intake of fish oils [32], respectively. There is much more inter-individual variance in the FA composition of circulating TG in the fasting state (almost exclusively located in very-low-density lipoproteins, originating from the liver) than in the CE-FA composition.

Plasma cholesterol ester fatty acid composition of rats fed with different diets

Table IV lists the results of the experiment in which rats were fed for nine weeks with four diets of different FA compositions. Selected results are depicted in Fig. 2.

In contrast to humans (Table III) the major rat plasma CE-PUFA is 20:4ω6, which may predominantly be caused by the higher rate of 20:4ω6 transfer than 18:2ω6 by rat LCAT [14,33]. However, as in humans [9,34], there is a non-linear relationship between the relative amounts of PUFA in the plasma CE fraction and that in the diet. In all cases relative PUFA levels in the plasma CE exceeded those in the diet, reflecting the preference of PC to accumulate PUFA on the *sn*-2 position and/or of LCAT to transfer PUFA. Large differences in the dietary PUFA content (range 3.60–62.32%; Table IV) resulted in much smaller differ-

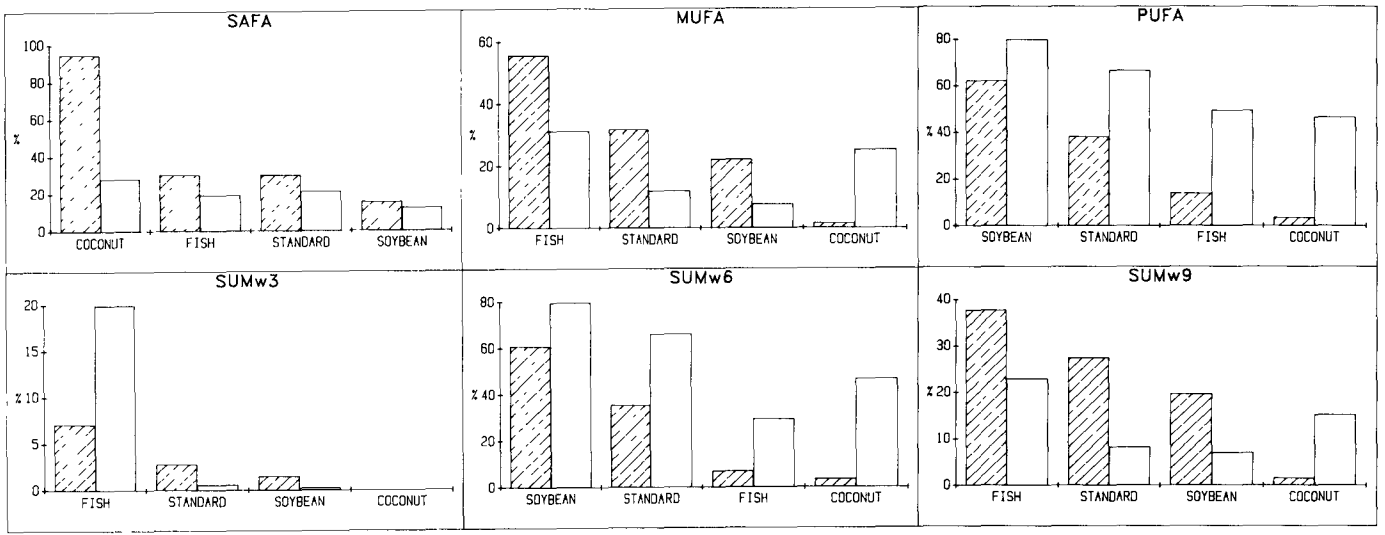

Fig. 2. Comparison between the molar percentages of summed FA in the diet with those in the plasma CE fraction of four groups of rats fed with diets of different FA compositions. For the detailed FA compositions of the diets see Table IV. Hatched bars indicate the summed FA in the diets, and open bars the summed FA in the plasma CE fractions.

TABLE IV

EFFECT OF THE DIETARY FA COMPOSITION ON THE PLASMA CE-FA PROFILE OF RATS

Male brown Norwegian rats (six weeks old) were fed four different diets for nine weeks. The fat contents were: 6.5% (standard chow), 10% (sunflower-soybean oil mixture) and 11% (hydrogenated coconut fat and fish oil). The indicated plasma CE-FA compositions represent the mean of the results obtained from two individual rats. – = Not determined. For other abbreviations see Table I.

Analyte	Standard chow		Coconut fat		Soybean oil		Fish oil	
	Diet	Plasma CE	Diet	Plasma CE	Diet	Plasma CE	Diet	Plasma CE
8:0	N.D.	–	2.93	–	N.D.	–	N.D.	–
10:0	N.D.	–	5.85	–	N.D.	–	N.D.	–
12:0	N.D.	–	51.28	–	N.D.	–	N.D.	–
14:0	1.12	0.70	17.50	3.44	N.D.	N.D.	10.37	N.D.
16:1ω7	1.50	3.29	N.D.	7.61	N.D.	N.D.	12.65	6.35
16:0	20.65	15.05	8.62	20.34	11.17	9.44	17.51	15.33
18:3ω6	N.D.	N.D.	N.D.	N.D.	N.D.	0.76	2.48	N.D.
18:2ω6	35.44	13.50	3.60	10.33	60.81	18.02	4.47	7.09
18:3ω3	2.81	N.D.	N.D.	N.D.	1.51	N.D.	N.D.	N.D.
18:1ω9	27.46	8.26	1.56	15.19	19.67	7.04	17.78	22.93
18:1ω7	2.80	0.49	N.D.	2.38	2.45	0.67	5.24	1.97
18:0	8.23	4.85	8.65	4.31	4.39	2.92	2.38	3.98
20:4ω6	N.D.	52.60	N.D.	36.40	N.D.	60.90	N.D.	22.41
20:5ω3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.90	16.75
20:1ω9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	20.01	N.D.
22:6ω3	N.D.	0.58	N.D.	N.D.	N.D.	0.26	2.21	3.19
24:0	N.D.	0.69	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
SAFA	30.00	21.29	94.83	28.09	15.56	12.36	30.26	19.32
MUFA	31.75	12.04	1.56	25.18	22.12	7.71	55.68	31.25
PUFA	38.25	66.68	3.60	46.73	62.32	79.94	14.06	49.44
SUMω3	2.81	0.58	N.D.	N.D.	1.51	0.26	7.11	19.94
SUMω6	35.44	66.10	3.60	46.73	60.81	79.68	6.95	29.50
SUMω7	4.30	3.78	N.D.	9.99	2.45	0.67	17.89	8.32
SUMω9	27.46	8.26	1.56	15.19	19.67	7.04	37.79	22.93

ences in the plasma CE-PUFA content (range 46.73–79.94%; Table IV), indicating that the latter is a relatively insensitive measure of the former. When rats were fed a fish oil diet, an enrichment of PUFA of the ω3 series in the CE fraction took place, which competed with the accumulation of PUFA of the ω6 series.

The coconut diet, rich in medium-chain saturated FA (MCSAFA), led to enrichment of the plasma CE fraction with endogenously synthesized monounsaturated FA (MUFA), notably 18:1ω9. As in humans [34], the SAFA content of the diet did not seem to influence the CE-SAFA content to a large extent. This may be caused by the small amount of SAFA on the *sn*-2 position of circulating PC, as may be derived from the FA combinations of PC species in human erythrocytes [33,35], and the low affinity of human-LCAT for PC containing a SAFA on the *sn*-2 position [14]. It remains to be established whether the presence of relatively high amounts of certain FA (such as 16:0) esterified to plasma cholesterol, which do not occur in appreciable amounts on the *sn*-2 position of high-

density lipoprotein-PC and, if present on this position, are not preferably transferred by LCAT, originate from intracellular ACAT activity or result from a relatively long plasma half-life.

CONCLUSIONS

The present method for the isolation of plasma CE and TG allows rapid simultaneous processing of large series and results in a reasonable long-term quality control with minimal cross-overs between the two lipid classes. For the subsequent routine GC analysis of their FA compositions, transmethylation to FAME seems at present to be the method of choice, notably because of the as yet poorly solved difficulties associated with the thermal instability of naturally occurring CE and TG and the unavailability of reference TG standards. As illustrated by an intervention experiment with four different diets in rats, the determination of the plasma CE-FA composition gives a reliable estimate of the FA composition of the diet consumed during the preceding weeks. Its measurement may be of importance to the establishment of the compliance of dietary interventions and basic dietary experiments.

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