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Determination of the fatty acid profile of neutral lipids, free fatty acids and phospholipids in human plasma

Abstract

Background: Knowledge of the fatty acid composition of lipid classes in human plasma is an important factor in the investigation of human metabolism. Therefore, a method for the analysis of neutral lipid (NL), phospholipid (PL) and free fatty acids (FFA) in human plasma has been developed and validated.

Methods: Separation of lipid classes was carried out by solid phase extraction of the lipid extract. The fractions were transesterified and the resulting fatty acid methyl esters were determined by GC/FID. For the method to be validated, precision, detection and quantification limits, as well as recovery, were determined for combined lipid extraction, solid phase extraction and GC analysis.

Results: The lipid extraction was miniaturized and simplified by application of an ultrasound 'Sonotrode'. The resolution of lipid classes was optimized with appropriate standards added to a representative plasma sample. In addition, a rapid derivatization procedure using trimethylsulfoniumhydroxide was established. Low determination limits (1.5, 0.2 and 1.3 μg/g plasma for NL, PL and FFA, respectively) indicate that the method's sensitivity is sufficient to quantify even minor components. Furthermore, recovery for NL and PL fatty acids was found to range from 80% to 110%. The results were similar for FFA apart from more polar free fatty acids due to their higher solubility in water. Repetitive measurements showed very good precision apart from the long chain PUFA for which the coefficients of variation were significantly higher.

Conclusions: The present method is applicable to the quantitation of fatty acids in lipid classes of human plasma including several minor components.

Keywords: fatty acids; gas chromatography; lipid separation; miniaturized ultrasonication; plasma lipids, solid phase extraction; TMSH; validation.

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Introduction

Determination of the fatty acid composition of plasma lipid classes is essential for a wide range of studies in human and animal physiology. Lipids are involved in many different vital biological processes. Moreover, lifestyle as well as nutrition challenges often are mirrored by the fatty acid pattern. Therefore, a detailed knowledge of the blood fatty acid composition allows conclusions on human nutrition and health conditions, particularly as certain blood lipids are associated with a disposition to various diseases. For example, high plasma levels of triacylglycerols (TG) are associated with coronary heart disease [1]. Furthermore, individual free fatty acids (FFA) are related to diabetes [2] and cardiovascular risk [3], and the levels of some phospholipid (PL) fatty acids may be connected to increased cancer risk [4]. Moreover, certain fatty acids in the diet may have a positive [5] or negative [6] impact on such diseases.

Several methods to separate lipid classes have been reported. Most of them are based on the method of Kaluzny et al. [7], who were able to separate 10 different lipid classes using solid phase extraction (SPE) with aminopropyl bonded silica sorbent. They gained satisfactory recovery and purity, which were confirmed with thin layer chromatography (TLC). Subsequently, many authors have published different methods for the separation of lipid classes in plasma and whole blood with SPE on aminopropyl cartridges. The separation of major o-ester lipid classes including a very simple extraction method using methyl *tert*-butyl ether (MTBE) was accomplished by Ichihara [8], and Kim and Salem [9] achieved the separation of neutral

and acidic PL. Agren et al. [10] separated cholesteryl esters (CE), TG, FFA and PL on a single SPE column and reported recoveries exceeding 98% for all lipid classes. Thereafter, Burdge et al. [11] developed a method to separate the same lipid classes. The latter authors estimated the recovery for the whole procedure, including extraction of total lipids, using an internal standard (ISTD) for each fraction and comparing with a recovery reference standard. Their recoveries, in particular for FFA and PL, were lower than those estimated by Agren et al. [10] due to incomplete isolation or partial retention of the polar lipids on the SPE column.

The esterification procedures used in the literature are rather tedious as they need time for incubation and further extraction steps with n-hexane after methylation with BX_3 [10, 12, 13] or acidified methanol [11, 14]. The application of trimethylsulfoniumhydroxide (TMSH) as an alkaline-based methylating reagent appears to be a suitable alternative, as it is an extremely rapid one-step method, which does not require any further extraction. Moreover, the International Organization for Standardization (ISO) recommended TMSH for the preparation of fatty acid methyl esters (FAME, [15]), and TMSH already was used by Taylor et al. [16] for derivatization of fatty acids in the PL fraction of human plasma and by Akoto et al. [17] for total plasma lipids with satisfactory results. Ishida et al. [18] underlined that methylation with TMSH is superior compared to other alkaline-based reagents because of less isomerization or degradation of PUFA. Ishida et al. [19] and El-Hamdy and Christie [20] depicted the dependency of TMSH on the lipid class, which becomes apparent in variable conversion rates between lipid classes, and once again on the degree of unsaturation.

However, these methods have never been thoroughly validated. In the present study, the analytical steps of the lipid extraction, SPE fractionation and derivatization were revised and optimized. Additionally, the recoveries of the complete extraction procedure for several important fatty acids per fraction were measured. Moreover, determination (DL) and quantification limits (QL) were determined along with repeatability and intermediate precision.

Materials and methods

Materials and chemicals

Aminopropyl bonded silica sorbent, polytetrafluoroethylene (PTFE) frits and glass columns were purchased from J.T. Baker (Phillipsburg, NJ, USA). The following chemicals were obtained commercially from the sources given in parentheses: MTBE, acetic acid (96%), chloroform, n-hexane, methanol, potassium hydroxide, sodium chloride and sodium hydrogen sulfate hydrate (Merck, Darmstadt, Germany); TMSH (Machery and Nagel, Düren, Germany); 3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT), lauric acid, tridecanoic acid, myristic acid, palmitic acid, heptadecanoic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, docosahexaenoic acid, tritridecanoin, trimyristoin, tripentadecanoin, tripalmitoin, tripalmitolein, triolein, trilinolein, trinonadecanoin, cholesteryl linoleate and tridecanoic acid methyl ester (Sigma, Taufkirchen, Germany); non adecanoic acid, tristearin, trilinolenin, triarachidonin and tridocosahexadecenoin (LGC Standards, Wesel, Germany); and di-tridecanoyl-phosphocholine (PC), di-myristoyl-PC, di-pentadecanoyl-PC, di-palmitoyl-PC, di-stearoyl-PC, di-oleoyl-PC, di-linoleoyl-PC, di-linolenoyl-PC, di-non adecanoyl-PC, di-arachidonyl-PC and di-docosahexaenoyl-PC (Avanti Polar Lipids, Alabaster, AL, USA).

Extraction of total plasma lipids

Blood samples were collected using lithium heparin as anticoagulant. The blood was centrifuged for 10 min at 2000 *g* and the plasma stored at −60°C until use. For validation, pooled plasma from blood collections of healthy young women was used (n=2). For the application to a plasma sample, pooled plasma from blood collections of healthy men and women was used (n=9, 3 male, 6 female). Total plasma lipid extraction was performed by a modification of the method of Folch et al. [21]. Tritridecanoin (TG-13:0, 50 µg in chloroform), heptadecanoic acid (17:0, 10 µg in chloroform) and di-pentadecanoyl-PC (PC-15:0, 100 µg in chloroform) were added as ISTD to 0.5 g of plasma. PC and TG were chosen as representatives for PL and neutral lipid (NL) fractions, respectively, the latter of which includes both TG and esterified cholesterol as PC and TG are most abundant in these fractions of human plasma. Freshly prepared chloroform/methanol (2:1, v/v) containing 0.01% BHT was added (8 mL) and processed using an ultrasound 'Sonotrode' (type UW 2070, Bandelin, Berlin, Germany) for 1 min at 40 Hz at room temperature. Thereafter, chloroform/methanol (1:1, v/v) was added (8 mL) and processed in the same manner. After centrifugation (4000 *g* for 5 min at 4°C), the supernatant was collected in a separating funnel. The residue was processed again, as detailed before, for its complete extraction. Both supernatants were combined, aqueous sodium chloride (0.1 mol/L, 14 mL) was added and the mixture shaken for 1 min. After separation, the chloroform layer was drained and evaporated at 37°C under vacuum.

To evaluate the robustness of the method, the clean-up was processed at 0, 20 and 50°C and the fatty acids of FFA and PL fractions were measured and compared.

Solid phase extraction of neutral lipids, free fatty acids and phospholipids

Total plasma lipid extracts were dissolved in chloroform (200 µL) and applied to a self-packed aminopropyl silica column (Bakerbond, 3 mL glass cartridges filled with 250 mg aminopropyl silica sorbent and PTFE frits), which had been conditioned with hexane (2×2 mL) and equilibrated with chloroform (2×2 mL). After application of the

extract, residual solvent was pulled through. The NL, FFA and PL were eluted with chloroform (2×2 mL), 2% acetic acid in diethyl ether (2×2 mL) and methanol (3×2 mL), successively. The solvents of the fractions were evaporated under vacuum at 37°C.

Preparation of fatty acid methyl esters using TMSH and GC analysis

NL and PL were dissolved in 100 µL of MTBE and TMSH (50 µL) was added as an alkaline-based methylating reagent. FFA were dissolved in 50 µL of MTBE and methylated with TMSH (25 µL). These mixtures were injected directly into the gas chromatograph. As the esterification takes place directly in the injector, no incubation or previous heating was needed.

FAME were resolved on a Hewlett Packard 6890 GC equipped with an Agilent 7683 Autosampler. A CP 7420 column (coating select FAME 100% bonded cyano-propyl-phase, 100 m×0.25 mm) with 0.25 µm film thickness and flame ionization detection was used (Agilent Technologies, Boeblingen, Germany). The split/splitless injector was used with split 20, split 20 and split 1 for NL, PL and FFA, respectively. The samples were injected at 50°C. Then, the oven temperature was raised by 6°C/min to 150°C and then by 3°C/min to 240°C as the final temperature. Injector and detector temperatures were 260°C and 270°C, respectively. Hydrogen (Westfalen, Muenster, Germany) was used as the carrier gas. Peaks were identified by comparison of retention times with known FAME standards. Response factors for the quantification of individual fatty acids were determined by using corresponding TG, PC and FFA standards in appropriate mixtures with TG-13:0, 17:0 and PC-15:0 as ISTD after applying the derivatization procedure described above.

GC/MS analysis was performed on a Hewlett Packard 6890 GC equipped with an HP 6890 Series Mass Selective Detector. A DB23 column (60 m×0.25 mm, 0.25 µm film thickness) was used (Agilent Technologies, Boeblingen, Germany). The split/splitless injector was utilized with a split ratio of 20. The samples were injected at 50°C. Then, the oven temperature was raised by 4°C/min to 170°C, followed by increasing the temperature by 4°C/min to 250°C and after 45 min to 260°C as the final temperature. Injector and detector temperatures were 270°C and 280°C, respectively. Helium (Westfalen, Muenster, Germany) was used as the carrier gas. Peaks were identified by comparing retention times and mass spectra of FAME reference compounds.

Methylation with potassium hydroxide in methanol

Lipid standards (PC-14:0, –15:0, –18:0, –20:4 and FFA-14:0, –15:0, –18:0, –20:4, 100 µg each) were diluted in 150 µL n-hexane. Ten microliters of methanolic potassium hydroxide solution (2 mol/L) were added and shaken vigorously for 1 min. The mixture was allowed to stand for 5 min. Subsequently, the mixture was mixed with 40 mg sodium hydrogen sulfate hydrate to neutralize the hydroxide. Tridecanoicacid methyl ester was added as ISTD (30 µg). After shaking and phase separation the upper layer was taken off and injected into the GC. Recoveries of individual fatty acids were estimated in relation to the ISTD.

Recovery and response factors of the rapid esterification method using TMSH

Mixtures of TG [14:0, 16:0, 16:1 (9), 18:0, 18:1 (9), 18:2 (9,12), 18:3 (9,12,15), 20:4 (5,8,11,14) and 22:6 (4,7,10,13,16,19)]; FFA [12:0, 13:0, 14:0, 16:0, 18:0, 18:1 (9), 18:2 (9,12), 18:3 (9,12,15), 20:4 (5,8,11,14) and 22:6 (4,7,10,13,16,19)]; and PL [14:0, 16:0, 18:0, 18:1 (9), 18:2 (9,12), 18:3 (9,12,15), 20:4 (5,8,11,14), 22:6 (4,7,10,13,16,19)] in chloroform were prepared in the amounts expected in plasma samples. TG-13:0, FFA-17:0 and PC-15:0 were added as ISTD, respectively. The mixtures were evaporated and esterified as described above. In addition, 50 µg of the CE cholesteryl linoleate were esterified in the presence of tridecanoic acid methyl ester as ISTD. Recoveries of individual fatty acids were determined in relation to the ISTD. Response factors for individual fatty acids in each lipid class were calculated from these mixtures.

Quantification of endogenous amounts of internal standards

TG-19:0 (100 µg in chloroform), FFA-13:0 (10 µg in chloroform) and PC-13:0 (100 µg in chloroform) were added to 0.5 g plasma as the ISTD. Plasma samples were spiked with four increasing amounts of TG-13:0, FFA-17:0 and PC-15:0. Each concentration level was processed in triplicate. Samples were cleaned up as described above and FAME resolved on GC/FID and GC/MS.

Detection and quantification limits

DL and QL were determined according to Vogelgesang and Hädrich [22]. Plasma was spiked with TG-19:0, PC-13:0 and FFA-13:0 as they naturally appear only in negligible traces in human plasma. The spiking was carried out at four different concentration levels (each in triplicate) starting slightly above the estimated DL and covering one order of concentration magnitude. TG-15:0, PC-15:0 and FFA-17:0 were added as ISTD and samples prepared as described above. DL and QL were derived statistically from the data according to the published method [22].

Precision (inter- and intraday precision)

Intraday precision was determined by analyzing one sample of pooled plasma 6-fold within one day. Interday precision was determined by analyzing two samples of pooled plasma in sextuplicate during 4 weeks.

Recovery

Samples of plasma were spiked with TG [14:0, 15:0, 18:0, 19:0 and 18:3 (9,12,15)]; FFA [12:0, 14:0, 16:0, 18:0, 19:0, 18:1 (9), 18:2 (9,12), 18:3 (9,12,15) and 20:4 (5,8,11,14)] and PC [14:0, 16:0, 18:0, 18:1 (9), 18:2 (9,12), 20:4 (5,8,11,14) and 22:6 (4,7,10,13,16,19)] standards in triplicate to approximately double the quantity present in human plasma (except for minor fatty acids, which were used in higher amounts). The recoveries were calculated from the difference of spiked and unspiked plasma as the mean of the addition experiments.

Results and discussion

Implementation and test of an improved extraction procedure

Typically, extraction of total lipid extracts from human plasma and other matrices is based on the method of Folch et al. [21], e.g. 11, 23, 24 or on the modified version of Bligh and Dyer [25], e.g., [16, 26]. Most of them involve extensive and time consuming shaking [11, 23, 24] or vortexing [16, 27] steps. In contrast, the miniaturized sonication by a 'Sonotrode' is extremely feasible as no manual mixing is needed and the extraction time is rather short. Additionally, only small amounts of solvent are needed and losses of lipids can be reduced through the small and even surface of the 'Sonotrode' compared to conventionally used extraction equipment, such as an Ultra Turrax or a Waring Blender.

Regarding the formation of artefacts, Christie [28] states that enzymatic hydrolysis of lipids, catalyzed by enzymes present in the sample may take place during extraction and that precautions must be taken to reduce the risk of lipid hydrolysis and of poly unsaturated fatty acids (PUFA) autoxidation. Consequently, samples have to be extracted at the lowest temperature feasible and antioxidants should be added [28]. To check the need to perform extraction at low temperatures, the extraction was performed at 0, 20 and 50°C, respectively. However, no variation in the PL or in the FFA fraction was observed (Figure 1). Even highly unsaturated fatty acids, like arachidonic acid, were stable under these conditions. This might be due to the use of BHT, which prevents autoxidation of PUFA. Accordingly, the procedure seems to be rather robust regarding amounts of PUFA and hydrolysis of PL, and it makes it possible to work at room temperature without the need for any cumbersome cooling.

Finally, we recognized that it is very important to use freshly prepared solvent mixtures when BHT is enclosed, because it tends to create further peaks, in particular in the NL chromatogram, when in contact with solvent overnight.

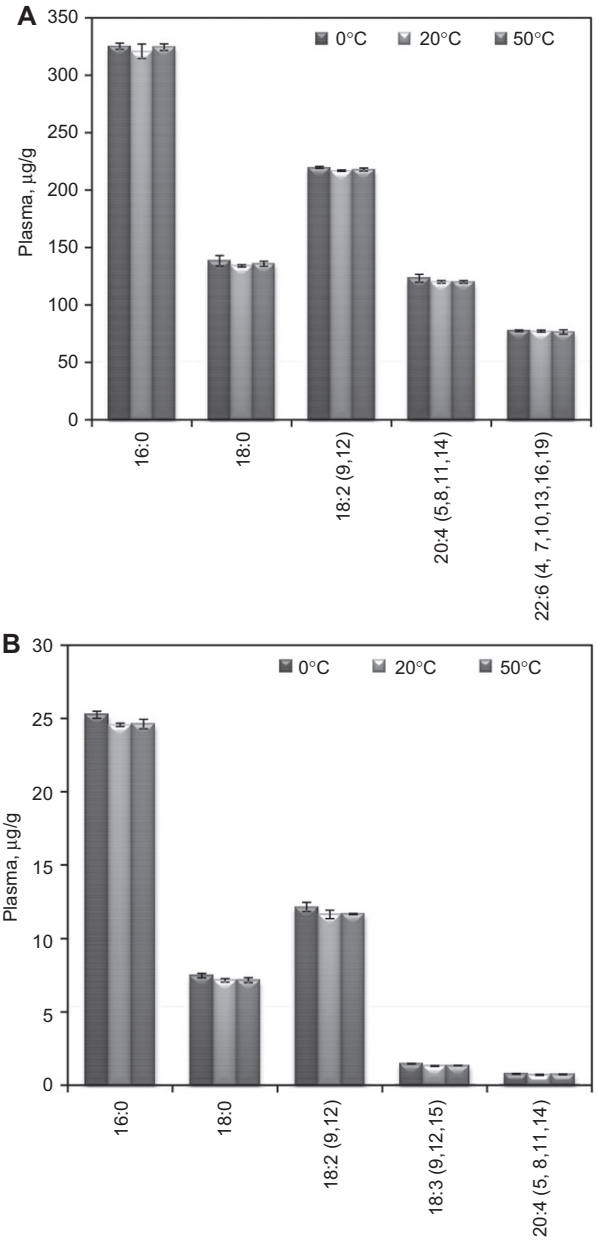

Figure 1 Extraction of plasma samples at 0, 20 and 50°C, respectively. (A) Stability of PL during extraction and clean-up. Amount of fatty acids in the PL fraction after methylation is displayed when clean-up was processed at 0, 20 and 50°C. (B) Content of FFA after extracting plasma samples at different temperatures. Amount of fatty acids in FFA fraction is displayed when clean-up was processed at 0, 20 and 50°C.

Efficiency of separating lipid classes by solid phase extraction

GC analysis of fatty acids of fractions obtained by SPE of mixtures of TG-13:0, PC-15:0 and FFA-17:0 showed no detectable co-elution of lipid classes. Comparable resolution was achieved by the analysis of lipid standards added to plasma. With the conventionally used method of Kaluzny et al. [7], we were not able to achieve their excellent resolution of lipid classes using chloroform/2-propanol (2:1, v/v) as the eluent for NL (Figure 2). Following the elution scheme of the latter authors [7], we evidenced substantial co-elution of PL in the NL fraction. This is easily visible from the ISTD PC-15:0, which is located almost entirely

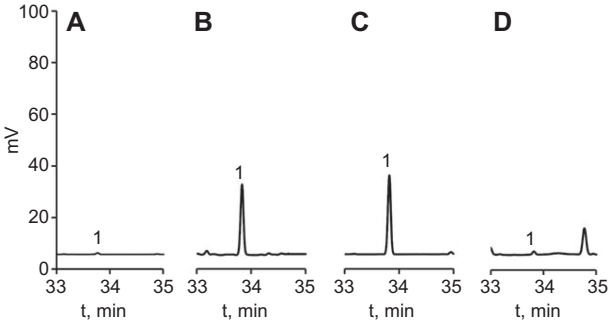

Figure 2 Resolution of lipid classes when using the protocol from the literature (7) and the modified approach presented here. Chromatogram sections of PL (A) and NL (B) fraction eluted with chloroform/isopropanol (2:1, v/v) and of PL (C) and NL (D) fraction eluted with pure chloroform. [1] peak of pentadecanoic acid methyl ester originating from PC-15:0, which is used as ISTD in the PL fraction.

in the NL fraction when being eluted with chloroform/2-propanol (2:1, v/v). This fact was already reported for microbial lipids [29] and muscle foods [30]. Pinkart et al. [29] assumed that the co-elution of PL depended on the sample type and correspondent polarity of PL and should not take place in plasma. However, the latter authors [29] also observed an inadequate separation of NL and PL for standard substances. By reducing the amount of 2-propanol and hence the polarity of the eluent, we were able to avoid this cross contamination, whereas the best results were obtained with pure chloroform (Figure 2). We also realized that PL are eluted fairly slowly from the SPE column with methanol. Therefore, it is important to use adequate amounts of solvent for complete recovery. In our study 3×2 mL methanol was sufficient.

The suitability of this method is illustrated in comparison to a more conventional approach that also uses a modification of Folch et al. [21]. The protocol used by Burdge et al. revealed recoveries of 74%, 74% and 87% for PC, FFA and TG, respectively [11], which includes both extraction and lipid separation efficiency. However, in our approach recoveries of 92%, 84% and 96% for NL, FFA and PL, respectively, were gained.

Furthermore, commercially available pre-packed plastic columns caused contamination in the GC chromatogram due to co-elution of plasticizers as previously reported [10, 16]. For this reason, we used self-packed glass cartridges, equilibrated them with chloroform before use and prevented any contact with plastic material.

Establishing the rapid esterification method

Completeness of the derivatization process was investigated in the present study with mixtures of each TG, FFA and PL standards that were composed of diverse fatty acids, respectively. TG-13:0, 17:0 and PC-15:0 were used as ISTD. Nearly all fatty acids were almost quantitatively recovered from TG, PL and FFA (Table 1), but the results clearly indicate a dependency of the esterification on the degree of unsaturation, since PUFA were only recovered as methyl esters with just above 60% from all lipid classes. This matches the results of Ishida et al. [19], who examined the correlation of the degree of derivatization with particular lipid classes and the degree of unsaturation. They achieved appropriate recoveries for each TG, PL and

Fatty acid	TG		FFA		PL	
	Recovery	COV, %	Recovery	COV, %	Recovery	COV, %
12:0	n.a.	n.a.	101.7	1.0	n.a.	n.a.
13:0	n.a.	n.a.	91.9	1.3	n.a.	n.a.
14:0	103.3	0.3	96.3	0.5	90.1	0.3
16:0	97.6	0.6	91.3	1.1	100.7	0.1
16:1 (9)	86.0	0.3	n.a.	n.a.	n.a.	n.a.
18:0	97.6	3.1	92.8	4.9	92.2	0.7
18:1 (9)	96.9	0.9	95.2	0.9	93.8	0.2
18:2 (9,12)	76.4	0.7	89.9	0.4	92.8	0.6
18:3 (9,12,15)	87.9	1.3	86.8	0.7	91.3	0.6
20:4 (5,8,11,14)	74.8	1.0	74.9	0.9	97.2	1.5
22:6 (4,7,10,13, 16,19)	61.6	2.5	71.7	0.9	78.7	1.6

Table 1 Recoveries (%) of esterified or free fatty acids in various lipid classes throughout the derivatization procedure with TMSH and corresponding COV[a].

[a]Solutions of standard mixtures of each lipid class including the corresponding ISTD (TG-13:0, 17:0, PL-15:0) were evaporated and derivatized with 100 µL MTBE and 50 µL TMSH in triplicate. The recovery of each fatty acid in comparison to the ISTD was calculated. n.a., not analyzed.

FFA (80%–90%) but also observed losses of PUFA in TG and PL fractions with recoveries of approximately 70%. These losses of PUFA obviously have to be adjusted by implementing response factors for quantification, which consider discriminations due to the derivatization procedure. In addition, we tested another rather fast method which is recommended by ISO [15] for the preparation of FAME. The transesterification procedure with methanolic potassium hydroxide solution is described above. It worked acceptably for PL with average recoveries of 100% and showed no discrimination of chain length or double bonds. But overall, the variations between different fatty acids were similarly high as those of the TMSH method. Moreover, a well-known problem occurred for FFA [28]. The peaks in the chromatogram were very small and the calculated recoveries were mostly as low as 10%. Christie [28] recommends the application of acidified methanol for FFA which is a fairly time-consuming procedure. Hence, the described procedure was still laborious and did not seem to be beneficial compared to the TMSH technique.

Furthermore, Ishida et al. [19] observed that CE were not derivatized with TMSH due to their larger steric hindrance. In another study, El-Hamdy and Christie [20] reported that CE are methylated much slower and to a lesser extent than other lipid classes. They suggested using higher amounts of reagent and higher temperatures. However, in the present method, derivatization of CE proved to be complete. This might be due to different methylation conditions, especially the solvent in which the derivatization takes place. We found that chloroform residues can interfere with methylation. Only MTBE, which the derivatization reagent solution was added to, led to optimal results. Thus, even cold on-column injection led to complete derivatization of FFA, despite temperatures of 200°C supposedly being necessary for derivatization [15].

Quantification of endogenous amounts of the internal standards

Generally, fatty acids with unevenly numbered carbons are chosen as ISTD because they only occur in traces in human plasma. For example, Bondia-Pons et al. [24] used PC-15:0 and other authors [10, 11, 16] applied PC-17:0 as ISTD for the PL fraction of human plasma. Agren et al. [10] used 15:0 and in other studies [13, 31, 32] 17:0 was applied for the FFA fraction. For the NL or TG fraction or total plasma lipid extracts, TG-13:0 was often used [12, 33, 34]. As depicted in Figure 3, the methyl esters of tridecanoic, heptadecanoic and pentadecanoic acid showed the smallest peaks in the chromatograms and, therefore, we chose TG-13:0, 17:0 and PC-15:0 as ISTD. However, traces of these fatty acids are still detectable. To estimate the overall precision of the method, it appeared essential to determine the exact quantity of the fatty acids that are used as ISTD. The amount of natural TG-13:0 is below 1% of the amount that is added as ISTD to the plasma sample (0.7 µg/g endogenous plasma amount, 100 µg used as ISTD). However, the naturally occurring amounts of 17:0 and PC-15:0 were in the range between 2% and 3% (0.5 µg/g endogenous plasma amount of 17:0, 20 µg used as ISTD; 5.9 µg/g endogenous plasma amount of PC-15:0,

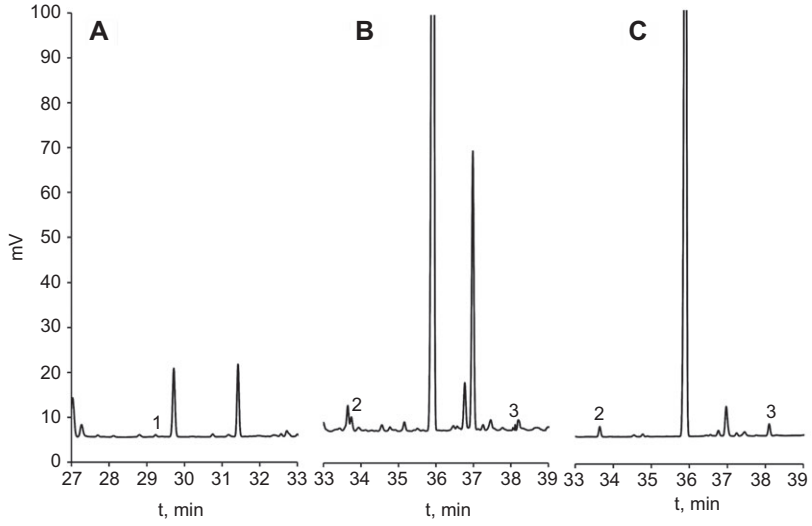

Figure 3 Chromatogram sections of the NL (A), FFA (B), and PL (C) fraction of human plasma showing the endogenously occurring traces of the fatty acids (1) 13:0, (2) 15:0, (3) 17:0.

Fatty acid	Intraday precision[b]			Interday precision[c]		
	NL	FFA	PL	NL	FFA	PL
14:0	1.02	5.34	1.90	3.51	7.53	3.92
16:0	0.99	2.63	0.76	3.44	6.56	2.07
16:1 (9)	0.82	1.06	0.96	3.44	3.16	2.07
18:0	1.31	5.53	0.68	4.08	10.09	2.58
18:1 (9)	0.93	1.11	0.65	3.77	4.88	2.76
18:1 (11)	2.37	1.50	0.69	2.08	5.48	4.44
18:2 (9,12)	0.78	1.91	0.86	4.68	11.30	2.66
18:3 (9,12,15)	0.81	2.08	3.79	5.56	24.52	6.55
20:4 (5,8,11,14)	0.54	10.71	2.90	5.62	25.19	4.95
20:5 (5,8,11,14,17)	0.52	n.d.	2.90	8.45	n.d.	7.47
22:6 (4,7,10,13,16,19)	1.25	7.85	2.44	5.80	17.27	8.61
SFA	1.11	4.50	1.11	3.67	8.06	2.85
MUFA	1.37	1.22	0.77	3.10	4.51	3.09
PUFA	0.66	2.00	2.58	6.08	19.57	6.05

Table 2 Inter- and intraday precision of NL, FFA and PL fatty acids[a].
MUFA, mono unsaturated fatty acids; n.d., below DL; PUFA, poly unsaturated fatty acids; SFA, saturated fatty acids. [a]COV (%) of fatty acid content in a pooled plasma sample, [b]6-fold determination of the sample at 1 day, [c]6-fold determination at 2 days during 4 weeks.

200 µg used as ISTD). We repeated the quantification on the GC/MS (m/z 74) additionally. Here, contents of TG-13:0, 17:0 and PC-15:0 were 0.2, 0.1 and 1.7 µg/g plasma (0.4, 0.8 and 1.7% of the amount that is added as ISTD to the plasma sample), respectively. Due to the lower specificity, GC/FID shows higher amounts of these fatty acids. In conclusion, for routine analyses, two approaches with slightly varying accuracy can be pursued. If an inaccuracy of at most 3% in the FFA or PL fraction is acceptable, the naturally occurring traces of the ISTD need not be considered. If a more accurate result is necessary, this endogenous amount has to be determined in a preceding experiment without addition of ISTD.

Method validation

Limits of detection and quantification

DL and QL were determined following the procedure detailed by Vogelgesang and Hädrich [22]. Accordingly, QL of 1.6 and 1.3 µg/g plasma were determined for TG-13:0 and 19:0, respectively (DL of 0.5 and 0.4 µg/g plasma, respectively). A QL of 0.2 µg/g plasma for both 13:0 and 19:0 was obtained for FFA (DL of 0.1 µg/g plasma). In the PL fraction, a QL of 1.3 µg/g plasma was obtained for both 13:0 and 19:0 (DL of 0.4 µg/g plasma). To the best of our knowledge, DL and QL have never been determined for the whole extraction

Fatty acid	NL		FFA		PL	
	Recovery	COV, %	Recovery	COV, %	Recovery	COV, %
12:0	n.a.	n.a.	56.8	9.9	n.a.	n.a.
14:0	95.6	2.8	62.2	3.8	111.4	0.7
15:0	83.1	2.4	n.a.	n.a.	n.a.	n.a.
16:0	n.a.	n.a.	89.4	6.4	103.4	2.0
18:0	90.2	2.4	89.2	8.3	94.8	3.4
19:0	94.0	0.9	106.6	6.8	n.a.	n.a.
18:1 (9)	n.a.	n.a.	99.3	4.9	99.1	7.0
18:2 (9,12)	n.a.	n.a.	96.5	5.4	89.3	2.3
18:3 (9,12,15)	97.9	1.2	94.0	2.8	84.3	0.9
20:4 (5,8,11,14)	n.a.	n.a.	66.1	8.3	80.5	0.6
22:6 (4,7,10,13,16,19)	n.a.	n.a.	n.a.	n.a.	109.5	5.1

Table 3 Recoveries (%) of fatty acids of lipid standards in spiked human plasma samples (n=3)[a].
[a]Determined in spiking experiments at plasma concentration of the respective fatty acids in triplicate as described in the Materials and method section. n.a., not analyzed.

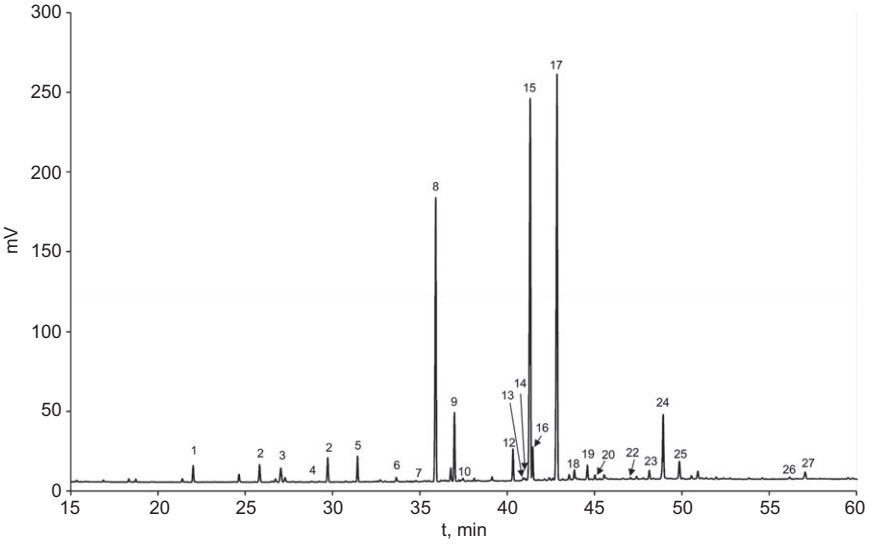

Figure 4 Typical chromatogram of NL fatty acids as FAME.
(1) TMSH, (2) BHT, derivatized or underivatized, (3) lauric acid, (4) tridecanoic acid, (5) myristic acid, (6) pentadecanoic acid, (7) iso-hexadecanoic acid, (8) palmitic acid, (9) palmitoleic acid, (10) anteiso-heptadecanoic acid, (11) heptadecanoic acid, (12) stearic acid, (13) elaidic acid, (14) trans-vaccenic acid, (15) oleic acid, (16) vaccenic acid, (17) linoleic acid, (18) γ-linoleinic acid, (19) α-linolenic acid, (20) CLA c9 t11, (21) CLA t10 c12, (22) eicosadienoic acid, (23) eicosatrienoic acid, (24) arachidonic acid, (25) eicosapentadecanoic acid, (26) docosapentadecanoic acid, and (27) docosahexadecanoic acid.

and separation method in human plasma. Persson et al. [35] estimated DL and QL from the signal to noise ratio (3 and 10, respectively) for NL, PL and FFA in human intestinal fluids. In another study, Bondia-Pons et al. [12] determined the DL and QL for the extraction of total lipid extracts of plasma according to the USP criteria without presenting data for single lipid classes. Taylor et al. [16] measured the DL and QL via spiking experiments in human plasma PL. The latter authors added three different linearly increasing amounts of linolenic acid to plasma in sextuplicate. Their calculations resulted in a QL of 0.8 μg/g plasma. However, in the present study, the DL and QL were determined for the whole process in spiking experiments at four concentration levels, each in triplicate, for two fatty acids in each lipid class.

Precision (inter- and intraday precision)

Inter- and intraday precision is presented in Table 2. Precision was very good, since for intraday precision,

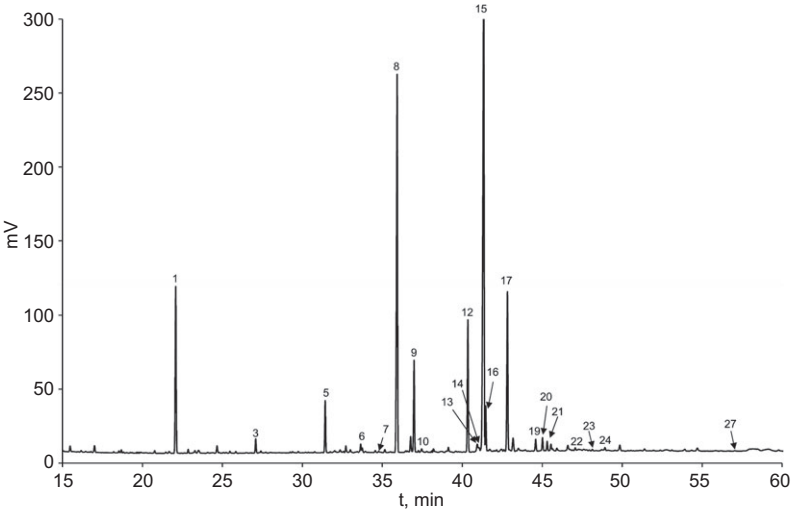

Figure 5 Typical chromatogram of FFA as FAME.
For numbering of components see caption in Figure 4.

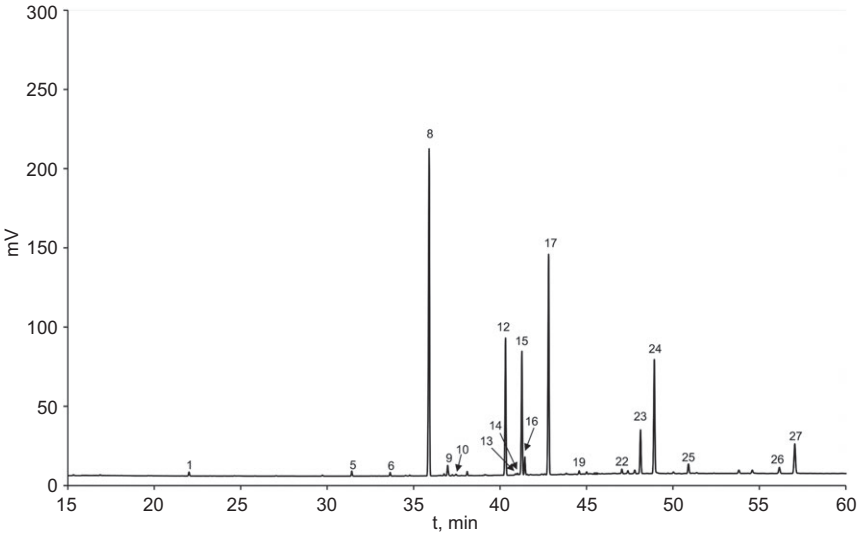

Figure 6 Typical chromatogram of PL fatty acids as FAME.
For numbering of components see caption in Figure 4.

coefficients of variation (COV) of <5% and for interday precision, COV of generally <10%, were obtained. In particular, COV for NL and PL fatty acids were outstanding. Differences between saturated fatty acids and PUFA were only evident for intraday precision, which leads to the conclusion that storage is a delicate factor and that autoxidation might take place even at –35°C, as our samples had been stored for shorter periods at this temperature. Therefore, long-time storage should be held at –60°C or lower as recommended by Christie [28]. Precision of FFA was slightly poorer. Short chain and PUFA in the FFA fraction showed higher COV of up to 25% for interday precision. The individual properties, like polarity, of different fatty acids are of higher significance for FFA than for mixed molecules like TG or PL and have a higher impact, e.g., during solvent extraction and retention on the SPE column. For this reason, FFA are generally more susceptible to all kinds of variation in the procedure. As the intraday precision is satisfactory, even for PUFA in the FFA fraction, it seems to be very important to keep all conditions, such as exact solvent compositions, temperatures, and equipment used, constant from day to day to prevent variations of sensitive fatty acids.

Recovery of plasma neutral lipids

Recoveries of NL fatty acids were found to be from 80% to almost 100% with COV below 3% (Table 3). Expectedly, there was no dependency on chain length or double bonds of fatty acids since NL generally do not contain only one type of fatty acid, which flattens all possible differences.

Recovery of plasma free fatty acids

Recoveries of FFA were subject to higher fluctuations as shown in Table 3. The majority of fatty acids revealed recoveries from 90% to 100% with COV below 10%. But in particular short chain fatty acids, like lauric and myristic acid, showed very low recoveries from 60% to 70%. This may be due to an incomplete extraction of polar FFA because of their higher solubility in water, which was also assumed by Meng et al. [36]. Another reason might be the partial retention of polar lipids on the SPE column [11]. Since recoveries of polar fatty acids were consistently <100%, it is important to take this into account for quantification.

Recovery of plasma phospholipids

Recoveries of PL fatty acids exceeded 80% (Table 3) and COV were below 7%. As expected, there was again no dependency on chain length or double bonds, since PLs are generally not composed of only one type of fatty acid either.

Application to plasma sample

The described method was applied to a pooled plasma sample (n=9) and processed 6-fold. Typical chromatograms of NL, FFA and PL fractions are shown in Figures 4–6. Peaks are mostly baseline separated and even 18:1 isomers, which are often hard to separate, could be separated and evaluated satisfactorily. Identity of fatty acids

Fatty acid	NL			FFA			PL		
	Mean	SD	COV	Mean	SD	COV	Mean	SD	COV
8:0	2.6	±0.1	3.5	0.2	±0.0	8.4	n.d.		
10:0	n.q.	±0.0	10.3	0.3	±0.0	10.1	n.d.		
12:0	29.4	±1.3	4.3	1.1	±0.0	6.6	n.d.		
13:0	n.d.			n.q.	±0.0	11.6	n.d.		
14:0	28.2	±0.4	1.4	3.6	±0.1	5.0	6.0	±0.1	1.4
14:1 (9)	n.d.			0.5	±0.0	9.4	n.q.	±0.0	8.2
15:0 (iso)	n.q.	±0.1	19.4	n.d.			n.d.		
15:0	3.6	±0.1	2.3	0.2	±0.0	12.2	n.d.		
15:0 (anteiso)	n.d.			0.5	±0.0	9.4	n.d.		
16:0 (iso)	n.q.	±0.1	6.6	n.q.	±0.0	6.8	n.d.		
16:0	346.0	±5.2	1.5	22.6	±1.0	4.9	421.7	±7.1	1.7
16:1 (trans 9)	n.q.	±0.0	6.9	n.q.	±0.0	7.1	n.q.	±0.0	4.8
16:1 (9)	85.2	±1.3	1.5	4.9	±0.3	6.8	14.4	±0.3	2.1
17:0 (anteiso)	4.3	±0.2	3.6	0.2	±0.0	8.0	2.1	±0.1	2.5
18:0	35.9	±0.6	1.7	6.5	±0.6	9.7	177.1	±3.0	1.7
18:1 (trans 9)	3.8	±0.2	5.1	0.6	±0.0	6.2	1.7	±0.2	11.2
18:1 (trans 11)	2.7	±0.1	4.8	0.2	±0.0	8.5	2.3	±0.1	2.4
18:1 (9)	548.1	±8.0	1.5	33.2	±0.6	1.9	159.5	±3.3	2.0
18:1 (11)	36.8	±0.5	1.3	2.2	±0.1	6.3	22.3	±0.6	2.5
18:1 (12)	n.q.	±0.0	5.7	n.q.	±0.0	6.7	n.q.	±0.0	3.5
18:2 (9, 12)	451.2	±6.6	1.5	11.1	±0.3	2.6	283.8	±5.4	1.9
18:3 (6, 9, 12)	13.0	±0.2	1.7	n.d.			n.d.		
18:3 (9, 12, 15)	17.5	±0.4	2.1	1.7	±0.1	3.6	4.0	±0.1	2.8
CLA (9c, 11t)	6.3	±0.2	3.4	0.9	±0.1	9.2	n.d.		
CLA (t10, c12)	1.0	±0.1	5.6	0.6	±0.1	12.7	n.d.		
20:1 (11)	5.7	±0.2	2.8	0.5	±0.0	7.0	n.d.		
20:2 (11, 14)	2.4	±0.2	9.8	0.3	±0.0	5.8	6.0	±0.2	2.8
20:3 (8, 11, 14)	14.0	±0.2	1.3	0.4	±0.0	16.7	54.8	±1.3	2.3
20:4 (5, 8, 11, 14)	127.7	±1.6	1.2	1.3	±0.1	11.3	158.8	±3.9	2.5
20:5 (5, 8, 11, 14, 17)	15.3	±0.4	2.7	n.d.			17.9	±0.4	2.5
22:5 (7, 10, 13, 16, 19)	4.9	±0.1	2.1	n.d.			19.6	±0.5	2.6
22:6 (4, 7, 10, 13, 16, 19)	21.4	±0.3	1.3	0.3	±0.0	11.0	102.5	±4.0	3.9
Total amount, µg	1810.2 (54% [a])			94.2 (3% [a])			1456.3 (43%[a])		

Table 4 Fatty acid content (µg/g plasma) in NL, FFA and PL fractions and their COV (%) obtained from analyses of six 0.5 mL aliquots of a pooled plasma sample (n=9).
n.d., below DL; n.q., below QL. [a]of total plasma lipid extract.

was verified by GC/MS in comparison with mass spectra of known FAME standards. In Table 4, the results of the quantification of all identified fatty acids are listed, but some minor fatty acids are below QL and cannot be accurately quantified with this method. However, it is possible to separate and quantify more than 19 fatty acids per fraction including many iso and anteiso minor fatty acids, several 18:1 isomers, two different conjugated linoleic acids (CLA) and numerous PUFA with high precision and accuracy. In conclusion, this method proved to be fast, sensitive and precise. A limitation of the method is the determination of FFA as, in particular, short chain fatty acids and long chain PUFA showed lower recovery and precision, which have to be considered for quantification.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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