

High-throughput analysis of fatty acid composition of plasma glycerophospholipids^S

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Abstract Plasma FA composition, a marker of FA status and dietary intake, is associated with health outcomes on a short- and long-term basis. Detailed investigation of the relationships between plasma FA composition and health requires the analysis of large numbers of samples, but manual sample preparation is very cumbersome and time consuming. We developed a high-throughput method for the analysis of FAs in plasma glycerophospholipids (GPs) with increased sensitivity. Sample preparation requires two simple steps: protein precipitation and subsequent base catalyzed methyl ester synthesis. Analysis of GP FAs is performed by gas chromatography. Coefficients of variation for FAs contributing more than 1% to total FAs are below 4%. Compared with the established reference method, results of the new method show good agreement and very good correlations ($r > 0.9$). The new method reduces the manual workload to about 10% of the reference method. Only 100 μ l plasma volume is needed, which allows for the analysis of samples from infants. The method is well suitable for application in large clinical trials and epidemiological studies.—Glaser, C., H. Demmelmair, and B. Koletzko. High-throughput analysis of fatty acid composition of plasma glycerophospholipids. *J. Lipid Res.* 2010. 51: 216–221.

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The FA composition of cellular and plasma lipids is of major importance for many biological functions. Although limited by different turnover rates and widely differing contribution of individual lipid classes to total pools, there is generally a reasonable correlation between FA composition of cellular and of plasma lipids (1). In clinical studies and epidemiological observations, only blood is easily accessible for analysis and can be assessed in large numbers of subjects (2). Many studies have aimed at investigat-

ing the relationship between FA status and cardiovascular disease (3). Furthermore, the availability of n-3 long-chain polyunsaturated fatty acids (LC-PUFAs) has been related to mental development in infants (4) as well as to the attenuation of the decline of mental performance in the elderly (5). The n-6 LC-PUFA dihomo- γ -linolenic acid and arachidonic acid and the n-3 LC-PUFA eicosapentaenoic acid are precursors of eicosanoids with different biological effects (6). The determination of FA status has a pivotal role in clinical trials and cross-sectional studies (7–9) and is a valuable biomarker for the quality of consumed dietary fat (10).

Conventional methods for analyzing the FA composition in biological samples consist of several analytical steps and are, therefore, cumbersome and time consuming. Typically, a skilled chemist requires 2 days for analyzing 10–20 samples (11). In the last years, different approaches are employed to overcome this disadvantage. Fast gas chromatographic techniques were introduced and optimized for fatty acid methyl ester (FAME) analyses and quantification (11, 12), but sample preparation has been the time- and cost-expensive factor. Therefore, new approaches focus on simplifying and reducing sample preparation steps like lipid extraction, lipid separation, and FAME synthesis. Masood and Salem (11) developed a robotic transesterification method for the analysis of FAs from total plasma lipids. Akoto et al. (13) used direct thermal desorption to analyze the FA composition of whole blood and total plasma lipids. Methods for analysis of FAs composition of total plasma or whole blood lipids provide valuable information and are cost-effective alternatives to the complex and time-consuming analysis of individual lipid fractions (1, 14–16). However, analyses of FA composition of individual lipid fractions are more suitable for the sensitive detection of intervention effects or metabolic relationships (17).

Abbreviations: CE, cholesteryl ester; CV, coefficient of variation; FAME, fatty acid methyl ester; GC, gas chromatography; GP, glycerophospholipid; LC-PUFA, long-chain PUFA; PhL, phospholipid; TAG, triacylglycerol.

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We aimed to develop a method that is both sensitive, especially for the measurement of changes in plasma LC-PUFA content, and cost effective. For studies focusing on LC-PUFAs, it seems most promising to analyze plasma phospholipids (PhLs), because they contain higher percentages of LC-PUFAs and are less sensitive to short term variation than plasma triacylglycerols (TAGs) and cholesteryl esters (CEs) (18, 19). For the determination of PhL FA in biological samples, Bondia-Pons et al. (12) presented an optimized method for lipid extraction and lipid separation using solid phase extraction instead of TLC. Their sample preparation technique is easier than those of established methods, but it is still very time consuming.

The term phospholipid is used for all lipids with a phosphorus-containing polar head group. PhLs can be further subdivided in glycerophospholipids (GPs) and SMs, based on their glycerole or sphingoid base backbone, respectively. The major PhL fractions in plasma are glycerophosphocholines and SMs (20, 21). Although LC-PUFA percentages are high in glycerophosphocholines and other GPs, LC-PUFAs contribute less than 5% to the FAs esterified to sphingosine in SMs (20, 22). The contribution of SMs to total plasma PhL varies widely in healthy controls and in patients suffering from coronary artery disease (21). Thus, a further source of variation in LC-PUFA percentages can be eliminated by determining selectively the FA composition of GPs while excluding SMs from FA analysis.

To the best of our knowledge, no method has been described for determination of plasma GP FAs, avoiding cumbersome lipid extraction steps and preparative chromatographic isolation of lipid fractions. We developed a high-throughput method for the determination of the FA composition of plasma GPs by eliminating labor intensive procedures and compared our newly developed method with an established reference method.

MATERIALS AND METHODS

Reagents and samples

Solvents were obtained in the highest available purity from Merck KGaA (Darmstadt, Germany). Methanolic HCl (3 N) and sodium methoxide (25 wt. % in methanol) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Two internal standards were applied. For internal standard A, pentadecanoic acid, cholesteryl pentadecanoate, tripentadecanoin, and 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (Sigma-Aldrich) were dissolved in methanol/chloroform (35:15). The internal standard B consisted of 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol. For the determination of the efficiency of base catalyzed transesterification octadecane (Sigma-Aldrich) dissolved in methanol was used as internal standard. To prevent FA oxidation, 2 g/l 2,6-di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, BHT, Sigma-Aldrich) was added to each internal standard. GLC-85, containing 32 fatty acid methyl esters, (Nu-Check Prep, Inc., Elysian, MN) was applied as external standard. SM (chicken egg yolk, $\geq 98\%$ TLC) was purchased from Sigma-Aldrich. A mixture of sodium carbonate, sodium hydrogen carbonate, and sodium sulfate (1:2:2, Merck KGaA) was applied as buffer for neutralization after acid catalyzed transesterification. Thirty-three blood samples from healthy volunteers (fasting and

nonfasting) were collected in EDTA-containing vacutainers (Sarstedt AG and Co., Nümbrecht, Germany). The plasma was separated by centrifugation (900 g, 5 min) and stored at -20°C until analysis.

New method

A total of 100 μl of plasma, 100 μl of internal standard B, and 0.6 ml methanol (precooled to 5°C) were combined in glass tubes and shaken for 30 s. The precipitated proteins were separated from the methanolic phase by centrifugation at 900 g for 5 min. The methanolic supernatant, which contained mainly polar lipids, was transferred into another glass tube. Twenty-five μl sodium methoxide solution were added to the supernatant, then the tubes were shaken while selective synthesis of methyl esters from GP FAs proceeded at room temperature. The reaction was stopped after 3 min by adding 75 μl methanolic HCl. FAMES were extracted by adding 300 μl hexane and shaking the tubes for 30 s. The upper hexane phase, which contains the extracted GP FAMES, was transferred into a 2 ml vial. The extraction was repeated and combined extracts were dried under nitrogen flow at room temperature. The dry residue was taken up in 50 μl hexane (containing 2 g/l BHT) for GC analysis.

To evaluate lipid compositions in the methanolic supernatant after plasma protein precipitation and to compare the recovery of PhLs in the methanolic supernatant with the recovery of PhLs in Folch extracts (reference method), the supernatant was deposited on a TLC plate. Lipid classes were separated by TLC and FAs bound in the different lipids were converted to FAMES by acid catalyzed transesterification (see reference method).

To optimize base catalyzed transesterification and FAME extraction, a model sample containing 100 μl water (representing plasma), 100 μl internal standard B, and 100 μl octadecane standard (not participating in the reactions) was applied. The ratio of the peak areas of methyl pentadecanoate to octadecane was used as indicator for transesterification as well as for extraction efficiency.

Reference method

Folch extraction. To 250 μl of plasma, 100 μl of internal standard A was added, the lipids were extracted according to a modified Folch method (23, 24) using chloroform/methanol (2:1, v/v), and the extracts were washed two times with NaCl solution (2% in water). The extracts were dried at 30°C under reduced pressure and taken up in 400 μl chloroform/methanol (1:1) for application on the TLC plate.

Lipid fraction separation by TLC, acid catalyzed transesterification. N-heptane, diisopropyl ether, and acetic acid (60:40:3) were used as mobile phase for the separation of PhLs, NEFAs, TAGs, and CEs (24). The corresponding bands were scraped from the TLC plate, transferred into glass tubes and 1.5 ml methanolic HCl was added. The closed tubes were shaken for 30 s and heated to 85°C for FAME synthesis (45 min). After cooling to room temperature, samples were neutralized with carbonate buffer. For methyl ester extraction, 1 ml hexane was added. After centrifugation at 900 g for 5 min, the upper hexane phase was transferred into a further glass tube. The extraction was repeated and combined extracts were taken to dryness under nitrogen flow at room temperature. The dry residue was taken up in 50 μl hexane (containing 2 g/l BHT) for GC analysis.

Chromatography

Individual FAMES were quantified by GC with flame ionization detection. GC analysis was carried out on a BPX 70 column (25 m \times 0.22 mm, 0.25 μm film, SGE, Weiterstadt, Germany) using an Agilent 5890 series II gas chromatograph (Agilent, Waldbronn,

Germany) with an optimized temperature program starting at 150°C. Without initial hold, temperature was increased by 2.5°C per min to 180°C and then with 1.5°C per min to 200°C followed by an 1-min isothermal period, allowing a total run time of only 26.33 min. The pressure program (carrier gas He) started with an initial column head pressure of 0.9 bar, which was increased by 0.02 bar per min to 1.2 bar, with 0.05 bar per min to 1.5 bar and 0.1 bar per min to the final pressure of 2.0 bar.

Data quantitation

Individual FAMES were identified by comparison with authentic standards. For each FAME, the response relative to pentadecanoic acid methyl ester (internal standard) was determined using GLC-85 as external standard. EZChrom Elite version 3.1.7 (Agilent) was used for peak integration.

Statistical analysis

For FAs with a chain length between 14 and 24 carbon atoms, the results were expressed as absolute concentrations (mg/l plasma) and as percentages (% wt/wt). The FA data were presented as mean \pm SD. As a measure of analytical precision, coefficients of variation (CV) expressed as percentages were used. Correlations were evaluated using the two-sided Spearman test and paired *t*-tests were used for comparisons between mean values (*P* < 0.05 was considered statistically significant). All statistical analyses were performed with SPSS for Windows, Version 15.0.1 (SPSS Inc., Chicago, IL).

RESULTS

Individual FAMES were analyzed by GC. For specific analyses of GP FA compositions, a new method was developed, which allowed the selective formation of FAMES from GPs. This was achieved by two simple steps (Fig. 1): 1) Protein precipitation with methanol: separation of polar lipids [PhLs (GPs + SMs) and NEFAs], which were dissolved in the methanolic supernatant, from non polar lipids [TAGs and CEs], which were almost quantitatively

precipitated with the protein; and 2) base catalyzed transesterification at room temperature: FAMES were formed from GPs and the very small quantities of TAGs, (which were not precipitated with the protein), whereas no FAMES were formed from NEFAs or FAs bound in SMs and CEs under these conditions.

To examine the efficiency of the first separation step, we compared the lipid composition of 16 different plasma samples obtained by the reference method with the lipid composition in the methanolic supernatant. The lipid composition was estimated by the sum of FAs determined in the individual lipid fractions. According the reference method, PhLs contributed 37.7%–54.6%, NEFAs 1.3%–3.7%, TAGs 15.4%–35.8%, and CEs 23.6%–32.4% to total extracted lipids. The lipid composition in the methanolic supernatant after protein precipitation and TLC was 90.9%–96.8% PhLs, 1.3%–6.3% NEFAs, 0.9%–2.5% TAGs, and 0.8%–2.0% CEs. The use of 7 vols of methanol to volume of plasma was found to be optimal for dissolving PhLs quantitatively. The recovery of total PhLs (*n* = 16) in the methanolic supernatants was found to be 88.1% \pm 6.6% (mean \pm SD) compared with the reference method (Folch extraction). Direct addition of internal standard B to the plasma enabled correction for the loss of PhLs, thus 101.0% \pm 2.6% of PhLs were correctly determined in the methanolic supernatants.

The total PhL (GP + SM) FA concentration for these 16 samples was, on average, 1317.4 mg/l (1054.2 mg/l–1908.3 mg/l), according to the reference method. The new method identified in total 1229.9 mg/l (970.4 mg/l–1836.3 mg/l) FAs in plasma GPs.

Hydrolysis of methyl esters was a concern, as water (from plasma sample) was present during base catalyzed transesterification. Therefore, we studied reaction yields in a methanolic solution containing 100 μ l of water and 100 μ l of internal standard B. Reaction times between 3 min and 10 min ensured complete transesterification of GP FAs. The recovery of the internal standard B was 99.1% \pm 0.8% (mean \pm SD) relative to the octadecane standard in eight independent analyses.

After base catalyzed transesterification, FAMES were extracted twice with 300 μ l hexane. To evaluate extraction efficiency, samples were reextracted with 1 ml hexane. These extracts contained less than 1% of the total FAMES recovered with the previous extractions.

Storage of the GC ready derivatives for one month at –20°C revealed no significant alterations in FA concentrations (data not shown).

Intra-assay reproducibilities (*n* = 8) obtained by the analysis of PhLs with the reference method were compared with those obtained by the analysis of GPs with the new method. FA concentrations (mg/l) and compositions (% wt/wt) were comparable, but concentrations of the saturated FAs C20:0, C22:0, and C24:0 and of the monounsaturated FA C24:1n-9 were below the quantification level in GPs (Table 1). GP total FA concentration was about 10% lower than in PhLs, whereas some individual FAs showed higher concentrations. For GPs, CVs for all FAs were found to be below 4%; C18:3n-3, contributing only 0.21% to total FAs, had the highest CV (3.8%).

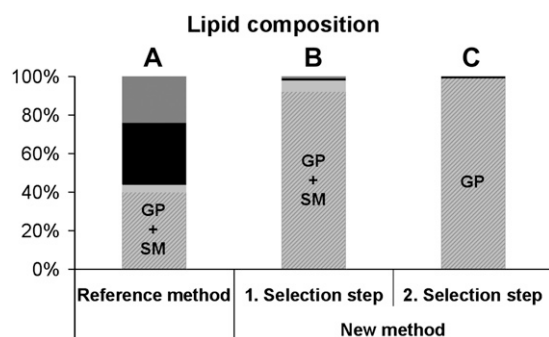


Fig. 1. Lipids recovered from a plasma sample (estimated from total FAs in corresponding fractions) with different analysis methods or at different steps of the method, respectively. A: Lipid composition of a plasma extract obtained by the reference method [solid dark gray, CEs (cholesteryl esters); striped gray, GPs (glycerophospholipids) (+ SM); light gray, NEFAs, and black, TAGs (triacylglycerols)]; all lipid fractions were extracted. B: Lipid composition after the first step of the new method (protein precipitation with methanol), mainly polar lipids were dissolved in the methanolic supernatant. C: Lipid composition after the second step of the new method (base catalyzed transesterification), only GP and TAG fatty acids were transesterified to fatty acid methyl esters.

TABLE 1. Intra-assay reproducibility ($n = 8$) of fatty acid concentration (mg/l) and composition (%) in phospholipids (PL) obtained by the reference method and in glycerophospholipids (GP) obtained by the new method, mean (CV)

FA	FA Concentration (mg/l)		FA Composition (% wt./wt.)	
	PhL	GP	PhL	GP
Saturated FA				
C14:0	5.24 (2.4)	7.95 (3.2)	0.40 (3.0)	0.65 (3.4)
C16:0	368.36 (0.8)	354.78 (0.7)	27.96 (0.3)	28.85 (0.6)
C17:0	5.37 (1.3)	4.93 (1.4)	0.41 (0.7)	0.40 (0.6)
C18:0	185.00 (1.5)	168.46 (1.1)	14.04 (0.9)	13.70 (0.7)
C20:0	6.67 (1.4)	ND	0.51 (1.1)	ND
C22:0	15.64 (1.5)	ND	1.19 (1.4)	ND
C24:0	14.11 (2.7)	ND	1.07 (2.6)	ND
Monounsaturated FA				
C16:1n-7	9.85 (1.5)	14.05 (2.0)	0.75 (1.7)	1.14 (1.8)
C18:1n-7	19.88 (1.4)	20.69 (1.0)	1.51 (0.6)	1.68 (0.6)
C18:1n-9	143.35 (1.0)	156.59 (1.3)	10.88 (0.5)	12.73 (0.7)
C20:1n-9	2.35 (1.5)	2.26 (2.3)	0.18 (1.6)	0.18 (2.6)
C24:1n-9	28.89 (1.2)	ND	2.19 (0.8)	ND
n-9 PUFA				
C20:3n-9	2.64 (2.9)	2.76 (1.9)	0.20 (2.3)	0.22 (1.8)
n-6 PUFA				
C18:2n-6	248.15 (1.1)	251.05 (1.4)	18.84 (0.8)	20.41 (0.7)
C18:3n-6	1.73 (8.0)	2.15 (2.5)	0.13 (8.5)	0.17 (2.4)
C20:2n-6	3.78 (1.7)	3.96 (1.9)	0.29 (1.5)	0.32 (1.3)
C20:3n-6	43.25 (1.3)	41.59 (1.3)	3.28 (0.6)	3.38 (0.7)
C20:4n-6	133.09 (1.2)	124.89 (1.3)	10.10 (0.4)	10.15 (0.8)
C22:4n-6	5.41 (1.5)	4.64 (2.6)	0.41 (1.4)	0.38 (2.0)
C22:5n-6	4.08 (3.2)	3.70 (2.5)	0.31 (3.2)	0.30 (2.1)
n-3 PUFA				
C18:3n-3	1.92 (2.5)	2.59 (3.8)	0.15 (2.1)	0.21 (3.7)
C20:5n-3	10.50 (1.3)	9.99 (1.6)	0.80 (0.6)	0.81 (0.7)
C22:5n-3	12.00 (1.1)	10.69 (1.5)	0.91 (0.6)	0.87 (0.9)
C22:6n-3	46.22 (1.9)	42.15 (1.4)	3.51 (1.1)	3.43 (0.9)
Total FA	1317.42 (0.9)	1229.85 (0.9)		

Inter-assay reproducibility (Table 2) of the new method was obtained by analyzing 49 aliquots of one plasma sample over a period of 4 months. CVs for all FAs contributing more than 1% to total FAs were found to be below 4%. Values of C18:3n-6, contributing 0.10% to total FAs, showed the highest CV (10.9%). The FA contents remained constant over 4 months.

FA concentrations and percentage contributions in PhLs obtained by the reference method were correlated with those in GPs obtained with the new method (see supplementary Table I). For concentrations of all analyzed FAs (except C14:0 and C18:3n-6) in PhLs and GPs, correlation coefficients higher than 0.9 ($P < 0.0001$) were observed. Both C14:0 and C18:3n-6 showed very low concentrations, and their contribution to total FAs in PhLs and GPs was below 1%, respectively. Percentage contributions of most FAs in PhLs and in GPs correlated with $r > 0.9$ ($P < 0.0001$). Only for C14:0, C20:1n-9, C22:4n-9, and C18:3n-3, lower r -values, between 0.76 and 0.89 ($P \leq 0.001$), were observed.

DISCUSSION

The new method enabled rapid, precise, and reproducible analysis of FA composition of plasma GPs

Plasma proteins were precipitated by adding methanol to plasma. TAGs and CEs dissolve poorly in polar solvents such as methanol. NEFAs showed better solubility in meth-

anol. Due to their amphiphilic character, PhLs were easily soluble in methanol. The advantage of methanol precipitation combined with subsequent base catalyzed transesterification was that TAGs and CEs were dissolved in methanol only in negligible amounts, contributing less than 2.5% to the total FA in the methanolic supernatant, and NEFAs, SM FAs, as well as CE FAs were not converted to FAMES by reaction with sodium methoxide at room temperature, as previously shown (25–27). Thus, only two simple steps were required to convert selectively GP FAs to FAMES for GC analysis.

In an alkaline milieu, hydrolysis of synthesized methyl esters must be inhibited by neutralization of the reaction mixture, thus, the reaction was stopped after 3 min by adding methanolic HCl to the mixture. A reaction time of 3 min was sufficient for complete transesterification of GP FAs, but also a reaction time of 10 min yielded complete transesterification without saponification. This was in agreement with previous observations, which showed base catalyzed transesterification to be about 1,500 times faster than saponification (25, 26).

Results showed that C20:0, C22:0, C24:0, and C24:1n-6 were present in PhLs but not in GPs, as these FAs were mainly bound in SMs and, therefore, not transesterified to FAMES. This was in agreement with the previously shown high abundance of these FAs in SMs, as about 95% of the SM FAs were saturated or monounsaturated (22). High percentages of LC-PUFAs were found in GPs, especially glycer-

TABLE 2. Inter-assay reproducibility ($n = 49$) of fatty acid concentrations (mg/l) and compositions (% wt./wt.) in glycerophospholipids analyzed over a time period of 4 months with at least two measurements per week with the new method


FA	FA Concentration		FA Composition	
	Mean	CV	Mean	CV
Saturated FA				
C14:0	7.29	3.5	0.60	3.2
C16:0	378.91	2.1	31.32	1.5
C17:0	4.63	3.3	0.38	2.6
C18:0	167.85	4.0	13.87	2.7
C20:0	ND	ND	ND	ND
C22:0	ND	ND	ND	ND
C24:0	ND	ND	ND	ND
Monounsaturated FA				
C16:1n-7	12.18	2.9	1.01	2.3
C18:1n-7	21.46	3.0	1.77	1.7
C18:1n-9	152.91	3.0	12.64	1.2
C20:1n-9	2.16	6.8	0.18	5.4
C24:1n-9	ND	ND	ND	ND
n-9 PUFA				
C20:3n-9	2.39	7.8	0.20	7.2
n-6 PUFA				
C18:2n-6	244.26	2.8	20.19	1.0
C18:3n-6	1.26	10.9	0.10	10.5
C20:2n-6	4.48	4.4	0.37	3.3
C20:3n-6	38.68	4.0	3.20	2.4
C20:4n-6	108.31	4.3	8.95	3.0
C22:4n-6	3.68	9.5	0.30	8.7
C22:5n-6	4.14	7.6	0.34	6.7
n-3 PUFA				
C18:3n-3	2.73	4.2	0.23	3.3
C20:5n-3	7.19	7.6	0.59	6.6
C22:5n-3	7.90	7.1	0.65	6.1
C22:6n-3	37.56	3.8	3.10	2.8
Total FA	1221.34	2.5		

phosphoethanolamine and glycerophosphoserine but also in glycerophosphocholine (22, 28). Total GP FA concentration was about 10% lower than PhL FA concentrations because SM FAs were not transesterified and, therefore, were not assessed with the new method. Some individual FAs showed higher concentrations in GPs, which could be due to small contaminations with TAGs. Furthermore, Folch extraction has been shown to be nonquantitative in extracting lipids from plasma (29).

Results for GP FAs could not be directly compared with results for PhL FAs because PhL FAs contained additional SM FAs and so differing FA compositions were obtained. Nevertheless, comparison of results for PhL FAs obtained by the reference method with results for GP FAs obtained by the new method showed high correlations.

The LC-PUFA content of plasma glycerophosphocholine, which was by far the most abundant plasma GP, has been found to be similar to red blood cell glycerophosphocholine LC-PUFA content (17). Thus, it can be expected that GP FA composition was representative for the FA status in a variety of physiological and pathological conditions. As shown by the very good correlation between the FA composition of PhLs obtained by the reference method and the FA composition of the GP obtained by the new method, the small amount of TAGs contributing to the analyzed FAs did not disturb the results. Furthermore, the percentage contribution of individual FAs to total FAs

in TAGs and PhLs correlated and, thus, the influence of those residues on compositional results was limited (30).

Our new sample preparation technique enables the analysis of FA composition of plasma glycerophospholipids from more than 50 samples per day. Throughput is limited by the running time of the GC, which takes 26.33 min for each sample. It provides results equivalent to the analysis of PhL FAs by the reference method and is especially suitable for the determination of long-chain unsaturated fatty acids, which are mainly bound in glycerophospholipids. The easy handling allows reproducible and robust analyses and enables the use of FA concentrations as biomarkers in large clinical and epidemiological studies. 

The new method for the analysis of glycerophospholipid fatty acid composition in plasma described in this article is in patent-pending status. The presented data are part of a PhD thesis accomplished by Claudia Glaser at the Medical Faculty of the Ludwig-Maximilians-University of Munich.

REFERENCES

1. Baylin, A., M. K. Kim, A. Donovan-Palmer, X. Siles, L. Dougherty, P. Tocco, and H. Campos. 2005. Fasting whole blood as a biomarker of essential fatty acid intake in epidemiologic studies: comparison with adipose tissue and plasma. *Am. J. Epidemiol.* **162**: 373–381.

2. Hodge A. M., D. R. English, K. O'Dea, A. J. Sinclair, M. Makrides, R. A. Gibson, and G. G. Giles. 2007. Plasma phospholipid and dietary fatty acids as predictors of type 2 diabetes: interpreting the role of linoleic acid. *Am. J. Clin. Nutr.* **86**: 189–197.
3. Getz, G. S., and C. A. Reardon. 2007. Nutrition and cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* **27**: 2499–2506.
4. Fleith, M., and M. T. Clandinin. 2005. Dietary PUFA for preterm and term infants: review of clinical studies. *Crit. Rev. Food Sci. Nutr.* **45**: 205–229.
5. Cole, G. M., G. P. Lim, F. Yang, B. Teter, A. Begum, Q. Ma, M. E. Harris-White, and S. A. Frautschy. 2005. Prevention of Alzheimer's disease: omega-3 fatty acid and phenolic anti-oxidant interventions. *Neurobiol. Aging*. **26**(Suppl 1): 133–136.
6. Schmitz, G., and J. Ecker. 2008. The opposing effects of n-3 and n-6 fatty acids. *Prog. Lipid Res.* **47**: 147–155.
7. Chavarro, J. E., M. J. Stampfer, H. Li, H. Campos, T. Kurth, and J. Ma. 2007. A prospective study of polyunsaturated fatty acid levels in blood and prostate cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **16**: 1364–1370.
8. Albert, C. M., H. Campos, M. J. Stampfer, P. M. Ridker, J. E. Manson, W. C. Willett, and J. Ma. 2002. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *N. Engl. J. Med.* **346**: 1113–1118.
9. von Schacky, C. 2007. Omega-3 fatty acids and cardiovascular disease. *Curr. Opin. Clin. Nutr. Metab. Care.* **10**: 129–135.
10. Baylin, A., and H. Campos. 2006. The use of fatty acid biomarkers to reflect dietary intake. *Curr. Opin. Lipidol.* **17**: 22–27.
11. Masood, M. A., and N. Salem, Jr. 2008. High-throughput analysis of plasma fatty acid methyl esters employing robotic transesterification and fast gas chromatography. *Lipids*. **43**: 171–180.
12. Bondia-Pons, I., S. Morera-Pons, A. I. Castellote, and M. C. Lopez-Sabater. 2006. Determination of phospholipid fatty acids in biological samples by solid-phase extraction and fast gas chromatography. *J. Chromatogr. A*. **1116**: 204–208.
13. Akoto, L., R. J. Vreuls, H. Irth, R. Pel, and F. Stellaard. 2007. Fatty acid profiling of raw human plasma and whole blood using direct thermal desorption combined with gas chromatography-mass spectrometry. *J. Chromatogr. A*. **1186**: 365–371.
14. Bailey-Hall, E., E. B. Nelson, and A. S. Ryan. 2008. Validation of a rapid measure of blood PUFA levels in humans. *Lipids*. **43**: 181–186.
15. Harris, W. S., and C. von Schacky. 2004. The Omega-3 Index: a new risk factor for death from coronary heart disease? *Prev. Med.* **39**: 212–220.
16. Uauy, R., P. Mena, B. Wegher, S. Nieto, and N. Salem, Jr. 2000. Long chain polyunsaturated fatty acid formation in neonates: effect of gestational age and intrauterine growth. *Pediatr. Res.* **47**: 127–135.
17. Rise, P., S. Eligini, S. Ghezzi, S. Colli, and C. Galli. 2007. Fatty acid composition of plasma, blood cells and whole blood: relevance for the assessment of the fatty acid status in humans. *Prostaglandins Leukot. Essent. Fatty Acids*. **76**: 363–369.
18. Callow, J., L. K. Summers, H. Bradshaw, and K. N. Frayn. 2002. Changes in LDL particle composition after the consumption of meals containing different amounts and types of fat. *Am. J. Clin. Nutr.* **76**: 345–350.
19. Skeaff, C. M., L. Hodson, and J. E. McKenzie. 2006. Dietary-induced changes in fatty acid composition of human plasma, platelet, and erythrocyte lipids follow a similar time course. *J. Nutr.* **136**: 565–569.
20. Dougherty, R. M., C. Galli, A. Ferro-Luzzi, and J. M. Iacono. 1987. Lipid and phospholipid fatty acid composition of plasma, red blood cells, and platelets and how they are affected by dietary lipids: a study of normal subjects from Italy, Finland, and the USA. *Am. J. Clin. Nutr.* **45**: 443–455.
21. Jiang, X. C., F. Paultre, T. A. Pearson, R. G. Reed, C. K. Francis, M. Lin, L. Berglund, and A. R. Tall. 2000. Plasma sphingomyelin level as a risk factor for coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* **20**: 2614–2618.
22. Phillips, G. B., and J. T. Dodge. 1967. Composition of phospholipids and of phospholipid fatty acids of human plasma. *J. Lipid Res.* **8**: 676–681.
23. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**: 497–509.
24. Carnielli, V. P., F. Pederzini, R. Vittorangi, I. H. Luijendijk, W. E. Boomaars, D. Pedrotti, and P. J. Sauer. 1996. Plasma and red blood cell fatty acid of very low birth weight infants fed exclusively with expressed preterm human milk. *Pediatr. Res.* **39**: 671–679.
25. Carrapiso, A. I., and C. Garcia. 2000. Development in lipid analysis: some new extraction techniques and in situ transesterification. *Lipids*. **35**: 1167–1177.
26. Suter, B., K. Grob, and B. Pacciarelli. 1997. Determination of fat content and fatty acid composition through 1-min transesterification in the food sample; principles. *Z. Lebensm. Unters. Forsch.* **204**: 252–258.
27. Christie, W. W. 1982. A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. *J. Lipid Res.* **23**: 1072–1075.
28. Trebble, T., N. K. Arden, M. A. Stroud, S. A. Wootton, G. C. Burdge, E. A. Miles, A. B. Ballinger, R. L. Thompson, and P. C. Calder. 2003. Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. *Br. J. Nutr.* **90**: 405–412.
29. Lepage, G., and C. C. Roy. 1986. Direct transesterification of all classes of lipids in a one-step reaction. *J. Lipid Res.* **27**: 114–120.
30. Geppert, J., H. Demmelmair, G. Hornstra, and B. Koletzko. 2008. Co-supplementation of healthy women with fish oil and evening primrose oil increases plasma docosahexaenoic acid, gamma-linolenic acid and dihomo-gamma-linolenic acid levels without reducing arachidonic acid concentrations. *Br. J. Nutr.* **99**: 360–369.