Novel methodologies for assessing omega-3 fatty acid status – a systematic review

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Abstract

Over the last few decades n-3 long chain polyunsaturated fatty acid status became of special interest for scientists. Biochemical measures on the n-3 fatty acid status vary depending on body compartment assessed and measures chosen. Plasma phospholipids and red blood cell membrane phospholipids are mainly used as n-3 fatty acid status marker. The conventional analysis of phospholipid fatty acids involves lipid extraction and consecutive chromatographic separation of phospholipids from other lipid fractions, which is time-consuming and costly. In recent years, different investigators have tried to overcome these limitations by using other biological markers or by modifying the analytical procedures used to assess n-3 fatty acid status. The aim of this systematic review was to provide an overview on these novel analytical methods developed for the fatty acid quantification by gas chromatography, highlights the methodological limitations, and discusses advantages or disadvantages of the biological markers used. Seventeen papers were identified that fulfilled the inclusion criteria. New opportunities arise from sensitive and precise high-throughput methodologies for assessment of plasma total lipid and plasma glycerophospholipid fatty acids, as well as cheek cell fatty acid composition.

Key words: Docosahexaenoic acid: eicosapentaenoic acid: omega-3 status: gas chromatography: biological marker: FAME preparation

Diet and metabolic turnover determine an individual's fatty acid status[1]. Studies on the dietary fatty acid intake often rely on food frequency questionnaires. The data obtained provide information about the type of diet consumed, but in general, they are improper as accurate measurement of the individual fatty acid status. Various biological compartments, such as whole blood, blood cells, plasma, and adipose tissue have been used to determine the individual fatty acid status reflecting the dietary fat intake[2;3]. The fatty acid status can also be used as marker for malnutrition, for example, a deficiency in linoleic acid can be assessed by comparing the ratio between mead acid (C20:3n-9) and arachidonic acid (ARA) levels[4].

Alterations in fatty acid status are also found in certain diseases, such as cystic fibrosis or gestational diabetes mellitus[5–7].

Over the last few decades the n-3 long chain polyunsaturated fatty acid (LC-PUFA) status became of special interest for scientists, focusing on eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids have been investigated extensively in cross-sectional or longitudinal observation studies and intervention trials to determine their effect on growth and development of pre-term and full-term infants[8–11], the outcome of pregnancy[12–14], and the prevention or treatment of diseases, such as attention deficit hyperactivity disorder[15], cardiovascular diseases[16;17], cancer[18–20], depression[21–23] and post-partum depression[24], Crohn's disease[25], asthma[26], cystic fibrosis[27], dementia[28] and Alzheimer disease[29].

All these studies relate analysed n-3 fatty acids levels to defined clinical outcome parameters, however, there is no agreed standard procedure for fatty acid analysis. Various analytical methods are published to determine the n-3 fatty acid status of an individual. Most of these methods are time-consuming, require a great amount of handling and are costly. Moreover, the applied techniques often use invasive sampling, which may limit their use in studies involving infants and young children, or subjects with inaccessible veins. This will impede the recruitment of volunteers for studies, especially if multiple blood sampling is necessary.

In recent years, researchers have tried to overcome these limitations by using different biological markers or by adapting the analytical procedures to assess the n-3 fatty acid status. This systematic review was conducted to identify

methods, which exclude traditional lipid extraction and chromatographic lipid class separation. It will highlight the advantages and disadvantages of these novel analytical methods developed for the fatty acid quantification by gas chromatography.

Methods

A systematic literature research of articles from 1990 onwards was conducted in April 2011. The search comprised the databases Medline and EmBASE. Further studies were identified through hand searching of original articles on subjects found by the computerised search.

Search terms

The following search terms were used to identify relevant scientific articles in the Medline and EmBASE databases: analysis, biological marker, blood, blood drop, blood spot, cheek cell OR cheek cells, direct, dried blood, EPA + DHA, fatty acid OR fatty acids, fatty acid status, gas chromatography, glycerophospholipids, high-throughput, omega-3 fatty acids, rapid method, total lipids, transesterification, and whole blood. Studies were limited to those executed with humans and written in English from January 1990 and April 2011, with the exception of the study of Lepage and Roy conducted in 1986[30].

Selection of studies

Articles reporting total fatty acid contents in blood compartments or cheek cells were included in the survey. Exclusion criteria were lipid extraction from specimens, the chromatographic separation of lipids, i.e. thin layer chromatography or solid phase extraction, and multiple steps for the esterification of fatty acids. Furthermore, articles which did not fully describe the method but referred to other authors were also excluded.

The initial screening of the articles was based on the title and the abstract. Abstracts which did not meet the inclusion criteria were rejected. Articles that were thought to be adequate for this review or whose eligibility was unclear were obtained as full text. The selected articles were evaluated according the inclusion and exclusion criteria. In addition, these articles were hand searched for further relevant publications.

Results

A total of 419 abstracts were identified from the database search. After careful screening, 61 full text articles were obtained, reviewed and analysed. Of these, only 17 met the inclusion criteria (Fig. 1). Two authors used the original method of Lepage and Roy, thus 15 different methods were evaluated and summarised in Table 1. In this table relevant aspects regarding sampling procedure, storage of fresh samples, methodological procedure, validation, and pro and cons of the method were described.

Findings

In general, the analysis of the fatty acid profile in biological samples often includes the extraction of crude lipids[31–33],

the separation of lipid fractions by using thin layer chromatography[34;35] or solid phase extraction[36–39], the fatty acid methyl ester (FAME) synthesis[40] and the chromatographic quantification[41] (Fig. 2A). These procedures are time-consuming, costly and require a great amount of handling. The following section reviews novel approaches for the fatty acid status marker assessment. The presented methods exclude traditional lipid extraction and chromatographic lipid class separation. Most of these methods have been validated in comparison to different reference methods or have been applied in various studies.

Plasma Total Lipids

In 1986, Lepage and Roy developed a rapid method for the fatty acid analysis of total lipids in plasma (Fig. 2 B)[30]. Solvents (methanol-benzene 4:1) and internal standard were directly added to a small plasma aliquot. Acetyl chloride was used for FAME synthesis (100°C, 60 min). The sample was neutralised with potash and the upper benzene phase was directly injected into GC for fatty acid analysis. Later, the method was applied to determine an essential fatty acid deficiency in patients with cystic fibrosis[42], or for the monitoring of DHA plasma contents in patients with a disorder of peroxisome biogenesis[43]. The method was also be applied for other biological samples, i.e. breast milk[44] or red blood cells (RBC) and fibroblasts[43;45].

Recently, this method was further optimised for the robotics-amenable analysis of plasma fatty acids[46]. In this process, benzene was replaced by methanol and toluene. A stock solution was prepared containing these solvents, internal standard and acetyl chloride. The solution was added to plasma and heated to 80°C in an open tube for 120 min. Every 25 min a second stock solution without internal standard was added to replace the evaporated solvents. FAME were extracted with hexane without neutralising the reaction. The robotic variant was validated against the method of Lepage and Roy. Low coefficients of variation (CV) were indicated for all fatty acids of both procedures. The authors claimed that fatty acid concentrations were similar irrespective of the method applied, although no statistical analysis was provided. In a further publication the semi-automated method was described in more detail[47]. The analysis of up to 200 samples per day is predicted, but limitations of data analysis have to be overcome before applying the method in large clinical trials.

The modified method of Lepage and Roy was used by another author for the determination of changes in plasma total fatty acids after fish oil supplementation[48].

Plasma total lipids have also been analysed with an on-column methylation procedure, which was recently described by Akoto *et al.*[49]. A small plasma volume was placed in an insert inside a capped liner. The plasma sample was dried for 3 min before trimethylsulfonium hydroxide was added. The mixture was incubated for 15 min and dried under vacuum. After adding an internal standard the liner was heated from 40°C to 280°C to effect thermally assisted methylation. The procedure indicated a good repeatability and was also tested for whole blood fatty acids. However, a comparison
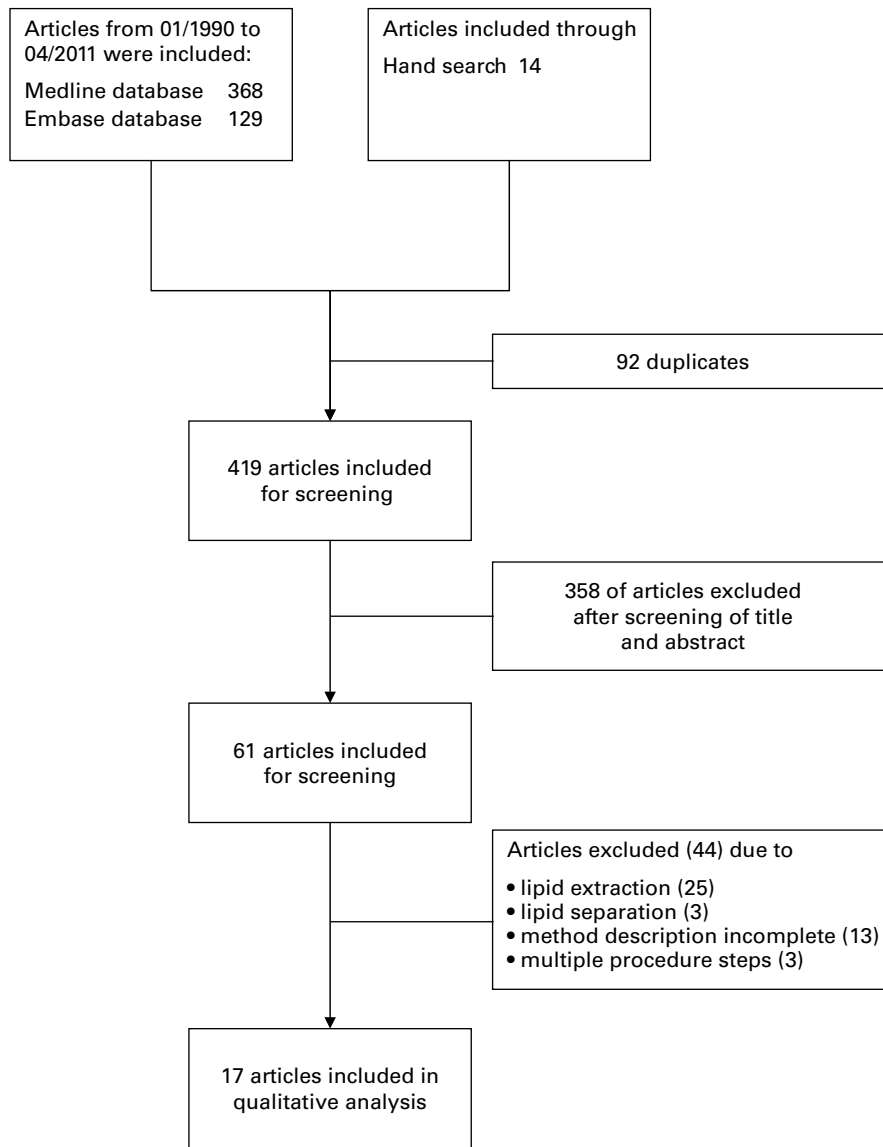
Fig. 1. Flowchart of articles

with a conventional method indicated different percentages for almost all fatty acids, particularly *n*-3 fatty acids, although the authors claim that saturated fatty acids (SFA), monounsaturated fatty acids, and PUFA contents are similar.

A simplified in-situ derivatisation method was published by Glaser *et al.* for a high-throughput fatty acid analysis of total plasma lipids[50]. Internal standard and methanolic HCl (+butylhydroxytoluene; BHT) were added to plasma aliquots and incubated for 45 min at 85°C. After cooling of the samples, FAME were extracted by hexane without neutralisation step. Intra-assay reproducibility was determined by repeated aliquot measurements. Low CVs were observed for fatty acids contributing more than 1% to total fatty acids. Storage of FAME derivatives at −20°C for 4 weeks did not affect the fatty acid pattern. Compared to an established laboratory standard procedure, the in-situ transesterification method revealed higher plasma total fatty acid concentrations. Although the

differences were significant, both methods indicated similar FA proportions.

A similar method was published by Takemoto *et al.* for the screening of peroxisomal disorders[51]. The settings for the FAME synthesis with HCl were different (100°C, 120 min), but the resulting methyl ester were also extracted with hexane without neutralisation step. The intra-assay precision for most of the analysed fatty acids was very good, except for very long saturated fatty acids > 24 C-atoms.

Whole Blood Total lipids

The fatty acid status of whole blood total lipids is rarely described in literature. Recently, the fatty acid analysis of dried blood on filter paper obtained by finger prick was introduced[52–54]. Marangoni *et al.* developed a simple method, which abandoned the separation of blood cells and
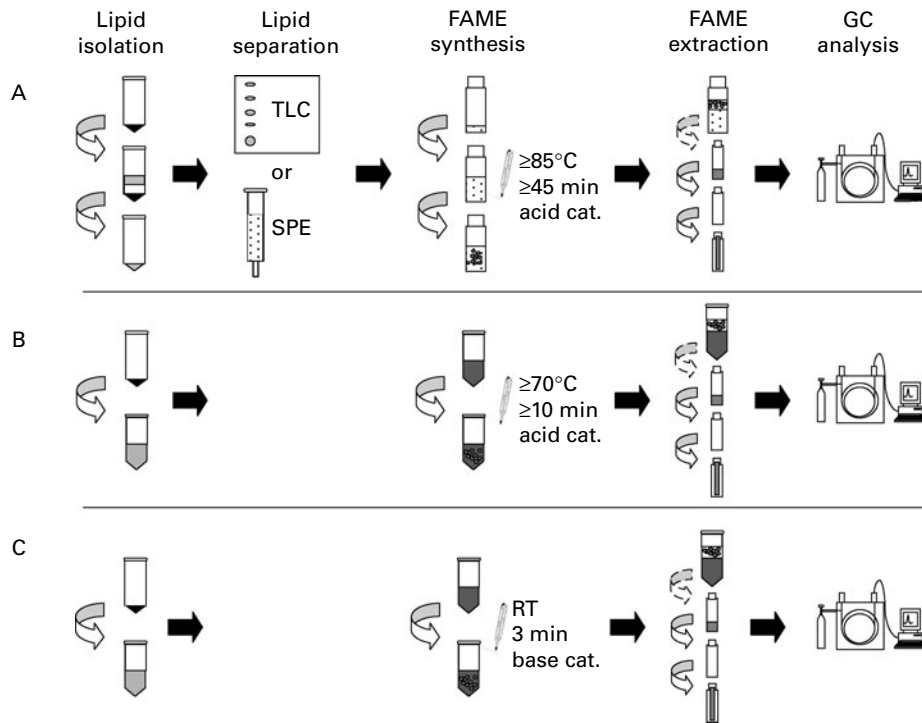
Fig. 2. Schematic overview of analytical procedures for gas chromatographic fatty acid quantification. A: conventional procedure for the analysis of individual lipid fractions, B: procedure for whole blood, plasma or RBC total lipids, C: procedure for plasma or cheek cell glycerophospholipids. GC: gas chromatographic, TLC: thin layer chromatography, SPE: solid phase extraction, cat.: catalysed, RT: room temperature

plasma[54]. Capillary blood was obtained by a finger prick and collected on filter paper. The paper was treated with BHT, if the storage period exceeded more than 14 days. For FAME synthesis a strip of filter paper (1 cm²), containing approximately 15 to 75µl whole blood, was submerged directly in methanolic HCl. Subsequently, water and KCl were added before extracting FAME with hexane. The results have not been compared with a reference method, but repeated analyses of samples indicated a high reproducibility. The method was validated in a study with volunteers categorised in low vs. high fish consumer or low vs. high meat consumers. Outcome of this study was that the different dietary habits were reflected in whole blood DHA or ARA contents. This method was later applied in various clinical studies to assess the fatty acid status of infants in relation to intrauterine growth or maternal smoking habits and changes in the fatty acid status in patient with cystic fibrosis[7,55,56].

The group of Bailey-Hall *et al.* also used dried blood samples for the evaluation of PUFA levels in humans[53]. Filter paper was treated with BHT prior to blood collection. Blood spots were dried over night and stored for 1 day at −80°C before further processing. Samples of the filter paper and internal standard were transferred into glass tubes, saponified with NaOH and subsequently transesterified with methanolic BF₃. Details for FAME extraction were not provided. The results of capillary blood obtained by finger prick were compared against plasma PL and RBC total lipids. The majority of fatty acids (mol %) from plasma PL, RBC, and capillary whole blood was different with significantly lower DHA and ARA contents in the latter compartment. Correlation analysis

showed strong relationships for DHA, EPA and docosapentae-noic acid (DPA) and relatively low, but still significant relationships for ARA in all 3 compartments. This method was used in an intervention trial to evaluate the effect of DHA supplements on cognitive functions in healthy pre-school children[57].

A further procedure for the evaluation of capillary whole blood fatty acids was provided by Armstrong *et al.*[52]. Capillary blood collected by puncturing the fingertip was dropped on chromatography paper. The treatment of the paper with antioxidants was abandoned because of the immediate sample preparation after collection. For the transesterification procedure of whole blood fatty acids, a strip of 0·5 x 1 cm was submerged in methanolic BF₃ plus hexane containing BHT, and placed in a commercial microwave oven for 45 sec. Additional hexane was added to extract the FAME. The results of the microwave treatment were compared to a conventional method including lipid extraction and acid catalysed FAME derivatisation (BF₃/MeOH, 90°C, 60 min). It was shown that the microwave supported transesterification process resulted in 2-fold higher stearic acid levels (mol %) and significantly lower oleic acid and ARA levels. Significant differences between *n*-3 fatty acids were not observed. The authors concluded that the use of microwave heating for FAME synthesis has potential, but additional modifications and testing are required. Further applications of the described method are not known.

A further method for the analysis of whole blood total lipids was described by Bicalho *et al.*[58]. Blood (35-60 µg) was collected with a capillary containing citric acid, transferred in a reaction vial and directly transesterified with acetyl chloride

Table 1. Overview on novel methodological approaches for the assessment of n-3 fatty acid status

	Storage[1]	Sampling	Transesterification reagents and settings	FAME extraction	Validation	Pros[2]	Cons
Whole blood total lipids							
Armstrong et al.(2008)	processed immediately	Volume n/d. blood obtained from finger prick, dried blood on chromatography paper	BF$_3$ in methanol (14 %) + hexane short term microwave heating for 45 sec	hexane	comparison with reference method applied to venous blood, (Folch extraction, BF$_3$)	Advantages of blood sampling by finger prick[3], very short processing time	loss of n-3 during storage[4], depending on postprandial state[5], significantly lower estimates of MUFA and PUFA compared to conventional method
Akoto et al. (2008)	processed immediately or stored at 4°C + BHT in ethanol	2 μl whole blood or plasma, sampling n/d	trimethylsulfonium hydroxide solution in methanol (5 mM), 15 min incubation; vacuum for 45 sec, heated from 40°–280°C at 16°C/sec	n/a	comparison with reference method applied to whole blood and plasma (sodium methylate, BF$_3$)	on column transesterification	DHA + EPA values differed to reference-method
Bailey-Hall et al. (2007)	−80°C for 1 d, filter treated with BHT	Volume n/d. blood obtained from finger prick, dried blood on chromatography paper	NaOH + BF$_3$ in methanol (14 %); heated to 100°C for 30 min	hexane	comparison with reference method applied to RBC and plasma, (Bligh & Dyer or Folch extraction, BF$_3$)	Advantages of blood sampling by finger prick[3]	loss of n-3 during storage[4], depending on postprandial state[5]
Bicalho et al. (2008)	processed immediately	35-60 μl blood from finger prick, collected in capillary coated with citric acid /methanol	acetyl chloride in methanol (5 %); heated at 90°C for 30 min	hexane	n/d, method applied to create a methyl ester database for lipid profiling	Advantages of blood sampling by finger prick[3]	depending on postprandial state[5]
Marangoni et al. (2004)	4°C, filter treated with BHT (50 μg in 10 μl methanol)	15-75 μl blood obtained from finger prick, dried blood on chromatography paper	3 N methanolic HCl; heated at 90°C for 60 min	hexane	Inter-Assay < 3 % for all analysed FA Intra-Assay < 4 % for all analysed FA	Advantages of blood sampling by finger prick[3], method indicates a good repeatability	loss of n-3 during storage[4], depending on postprandial state[5]
Min et al. (2011)	4°C, filter treated with 80 μl BHT solution	20-40 μl blood obtained from finger prick, dried blood on filter paper (Fluka dipstick)	acetyl chloride in methanol (15 %); heated at 70°C for 180 min	sodium chloride (5 %) + petrol spirit (2:1)	comparison with reference method applied to venous blood, (Folch extraction, acetyl chloride)	Advantages of blood sampling by finger prick[3]	loss of n-3 during storage[4], depending on postprandial state[5], time of FAME synthesis
RBC total lipids							
Araujo et al. (2008)	n/d	sampling not described, 50 μl RBC used for analysis	BF$_3$ in methanol (20 %) or BCl$_3$ in methanol (14 %); heated to 100°C for 60 min	hexane	total variance in average 3·2 %, comparison with reference method	BF$_3$ or BCl$_3$ indicate no differences in transesterification efficiency	
Block et al. (2008)	n/d	sampling not described, 250 μl RBC membrane used for analysis	BF$_3$ in methanol (14 %), heated to 100°C for 10 min	water-hexane (1:1)	CV for EPA + DHA <5 %, method applied to measure the omega-3 index in humans	specific for GPL and non-esterified fatty acids	strict derivatisation procedure has to be followed as FAME synthesis of lipid classes is time dependent, only limited information available to calculate the omega-3 index
Plasma total lipids							
Garg et al. (2006)	n/d	sampling not described, 100 μl plasma used for analysis	methanol-toluene (4:1) + acetyl chloride; heated to 100°C for 60 min, neutralised with potassium carbonate	toluene	n/d, method applied to analyse plasma total lipids of heart patients	benzene replaced by the less toxic toluene	depending on postprandial state[5]

Table 1. Continued

	Storage[1]	Sampling	Transesterification reagents and settings	FAME extraction	Validation	Pros[2]	Cons
Glaser et al. (2010 a)	n/d	sampling not described, 100 μl plasma used for analysis	3 N methanolic HCl; heated at 85°C for 45 min	hexane	Intra-Assay < 8 % for all analysed FA, comparison with reference method applied to plasma (Folch extraction, HCl)	method indicates a good repeatability	depending on postprandial state[5]
Lepage and Roy (1986)	processed immediately	sampling not described, 100 μl plasma used for analysis	methanol-benzene (4:1) + acetyl chloride; heated to 100°C for 60 min, neutralised with potassium carbonate	benzene	recovery rates of standards, comparison with reference method (Folch extraction)	method applicable for differentbiological samples,	depending on postprandial state[5]toxicity of benzene
Masood et al. (2005), Masood et al. (2008)	-80°C, plasma aliquots	venipuncture, 50 μl plasma used for analysis	methanol + acetyl chloride; heated to 100°C for 60 min	hexane	Intra-Assay < 8 % for all analysed FA, comparison with the method of Lepage & Roy	semi-automated analysis of FA, high-throughput of samples,	depending on postprandial state[5]
Takemoto et al. (2003)	-20°C, plasma or serum	sampling not described, 100 μl plasma or serum used for analysis	methanolic HCL (5 %); heated at 100°C for 120 min	hexane	Intra-Assay < 4·3 % for all analysed FA, except for FA with > 24 C-atoms	method indicates a good repeatability	depending on postprandial state[5]
Plasma glycerophospholipids							
Glaser et al. (2010 b)	-20°C	sampling not described, 100 μl plasma used for analysis	methanol + sodium methoxide in methanol (25 %); incubated at RT for 3 min	hexane	Intra-Assay < 4 % for all analysed FAIntra-Assay <11 % for all analysed FA, comparison with reference method for PL (Folch extraction, TLC, HCl)	specific for GPL FA acids, FAME synthesis at room temperature short processing time high-throughput of samples possibility for automated FA analysis	SP or SFA with > 20 C-atoms are not detected with this method
Cheek cell glycerophospholipids							
Klingler et al. (2011)	processed immediately	swab of the inner side of both cheeks, 100,000–850,000 cells used for analysis	methanol + sodium methoxide in methanol (25 %); incubated at RT for 4 min	hexane	Intra-Assay < 10 % for all analysed FAInter-Assay <14·2 % for all analysed FA, comparison with plasma GPL	non-invasive sampling specific for GPL FA acids, FAME synthesis at room temperature short processing time high-throughput of samples possibility for automated FA analysis	SP or SFA with > 20 C-atoms are not detected with this method, sample size varies,samples might be lost if cell number is too low

n/d: not described, FA: fatty acids, MUFA: monounsaturated FA

1 Storage of samples prior to analysis

2 Advantages such as time and cost savings, reduced solvent and equipment usage, enhanced through-put of samples and low sample volume needed for analysis apply to all the methods presented in this table

3 Advantages of blood sampling by finger prick: minimal invasive sampling; no venipuncture required for blood sampling; samples can be taken independently to location of a laboratory; sample preparation, storage and shipment save costs; sample storage requires much less space

4 Losses of n-3 LC-PUFA observed during storage at room temperature or at 4°C, although filter paper was treated with BHT

5 TAG and CE lipid levels in blood are rapidly affected after a meal. Strict sampling conditions such as overnight fasting are required (not applicable in infants)

in methanol (90°C, 30 min). The resulting FAME were extracted with hexane. The methyl ester were analysed by mass spectrometry, thus more than 80 fatty acids were detected. The aim of the study was to create a FAME database for lipid profiling, thus data on methodological precision or accuracy are not presented.

Another study group used a similar method for the synthesis of FAME in dried blood on filter paper. A higher concentration of acetyl chloride in methanol was chosen for the methylation of fatty acids with different incubation parameters (70°C, 180 min)[59]. After the derivatisation FAME were extracted with petrol spirit. Precision and accuracy data are not presented in this paper, but data were compared to a conventional method (Lipid extraction by Folch, same derivatisation procedure). The data indicate that slightly higher proportions of SFA and monounsaturated fatty acids and lower proportions of n-3 and n-6 PUFA are obtained, although no statistical analysed was conducted.

RBC Total Lipids

One of the most recognised findings in terms of n-3 LC-PUFA research over the last decade was the invention of the 'omega-3-index'[60]. The omega-3-index basically describes the proportional EPA+DHA content of total fatty acids in RBC, although the methodological approach does not capture the total fatty acid profile of all RBC lipid fractions[61]. A detailed description of the method was not provided in the article of Harris and von Schacky[60], however a similar method for the analysis of RBC fatty acids was published by Block et al., which also is described in a patent[61;62]. Hydrolysed erythrocyte membranes were separated by high-speed centrifugation, the supernatant discarded and the remaining cell pellet was transferred into a tube and resuspended in methanolic BF_3. The tube was sealed and heated to 100°C for 10 min. Under these conditions FAME were mainly generated from membrane glycerophospholipids (GPL), which were subsequently extracted with equal amounts of hexane and water. A CV was provided for the sum of EPA and DHA (omega-3-index) only. Further data describing the accuracy or repeatability of the method or other individual fatty acid contents were not provided. The method was applied in clinical trials and claims were made that the omega-3-index is inversely correlated with the risk for death from coronary heart disease[60] or the odds-ratio for an acute coronary syndrome[61].

Araujo et al. evaluated whether BF_3 or BCl_3 is more suitable to methylate total fatty acids of RBC or serum lipids. Biological samples were mixed with both of the boron trihalides individually and FAME synthesis was generated at 100°C for 60 min. The resulting methyl esters were extracted with hexane. Differences in concentrations or fatty acid proportions between both methods were not observed. The repeatability of the method for individual fatty acids was not shown, but an average percentage of total variance of 3·2 % was reported. Furthermore, the effect of adding hexane before or after FAME synthesis was investigated. Significant differences were found in samples with low or high (triacylglycerides) triacylglycerides (TAG) concentrations. Thus, the authors

advised to add hexane subsequently to the methylation process.

Plasma GPL

Glaser et al. published and patented a method for the analysis of GPL fatty acids in plasma[63;64]. The sample preparation was reduced to a 2-step procedure (Fig. 2C). In a first step, plasma proteins were precipitated with methanol and removed by centrifugation together with TAG and cholesteryl ester (CE). In a second step, GPL fatty acids were selectively transmethylated by base catalysis (sodium methoxide) at room temperature. CE, non-esterified fatty acids (NEFA) and sphingomyelins (SP) were not converted to FAME under the conditions applied. FAME were extracted by hexane. Testing intra- and inter assay reproducibility indicated low CV for all analysed fatty acids. A comparison to a reference method for plasma PL showed high correlations for all fatty acids contributing more than 1% to total fatty acids. The method was used for the analysis of serum samples from 951 children between 2 and 6 years of age. The results of this study are comparable with published results for plasma and serum PL[65−68] and might be used as reference values[69].

Cheek Cells

McMurchie has proposed cheek cells as biomarker for the dietary fat intake in 1985[70], but its use in studies is limited[70−76]. The main hurdle to overcome is the small sample size, which may limit a proper analysis of fatty acids. In addition, losses of lipid fractions through the chromatographic separation hamper an accurate and reliable fatty acid determination. Recently a method published for the analysis of cheek cell GPL overcame these limitations[77]. Cheek cells were collected with an endocervical brush with or without additional mouth rinse. Cheek cells were separated from the supernatant by centrifugation and the pellet redissolved in methanol. Ultrasound treatment of the cell pellet was necessary to disrupt the cells before eliminating the proteins by centrifugation. GPL fatty acids in the methanolic supernatant were transesterified to FAME by sodium methoxide and hexane used for extraction. Intra- and inter-assay repeatability indicated low CV for all analysed fatty acids in samples with more than 100,000 cells. A comparison of cheek cell sampling with and without mouth rinse showed significant differences in yield, but also without mouth rinse cell numbers were sufficient for analysis. The quantitative and qualitative determination of GPL fatty acids in cheek cells kept over 48 hours at room temperature showed no changes in fatty acid patterns, but losses in fatty acid concentrations. The method was used for the comparison of GPL fatty acid levels between plasma and cheek cell of subjects participating in a healthy lifestyle study. Strong correlations were found for DHA and EPA, and lower correlations for AA, although the n-3 fatty acid proportions in cheek cells were much lower then in plasma. This is in accordance to cheek cell PL data published by Hoffman et al. and Laitinen et al.[72;75].

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Discussion

This systematic review describes novel methods, which exclude lipid extraction and lipid class separation. These procedures offer advantages over the conventional methods, e.g. lower costs and handling, a higher throughput and reduced equipment and solvent usage. However, different biological compartments have been used for the evaluation of the n-3 fatty acid status, such as whole blood, total plasma or RBC lipids or cheek cells. These biological markers may inherent limitations, which are described below. Differences in fatty acid concentrations and composition caused by the choice of specific catalysts[58;78] or the derivatisation settings applied[79] are not evaluated in this review.

In general, the results obtained for whole blood lipids correlate with established fatty acid status markers[53;54]. Moreover, the determination of n-3 fatty acid levels in capillary whole blood lipids provides a series of advantages: (a) minimal invasive sampling; (b) no venipuncture is required for blood sampling (c); samples can be taken independently to location of a laboratory; d) sample preparation, storage and shipment saves costs; and (e) sample storage requires much less space. However, some limitations have to be considered when using this procedure in clinical trials. It was reported that the treatment of the filter paper with BHT is essential to avoid significantly high losses of DHA, which were significant already after 24 hours[80]. Furthermore, the BHT treatment does not prevent losses of n-3 LC-PUFA during the long-term storage at room temperature or at 4°C[59]. It seems to be necessary to keep samples at -80°C, if storage over a long period is required. Furthermore, total plasma lipids represent a mixture of fatty acid moieties, i.e. CE, PL, TAG and NEFA. Hodson et al. estimated that approximately half of all plasma fatty acids can derive from TAG[3]. Thus, the apparent fatty acid status depends on the postprandial state of the subject. Strict sampling conditions such as overnight fasting can reduce the influence of TAG on the overall fatty acid status of whole blood, but this limits the application in infants.

Analysing RBC total lipids has the advantage that RBC are not immediately influenced by the postprandial state of a subject and therefore might be better suited as fatty acid status marker. The method described for the assessment of the omega-3 index in RBC[60;61] relates on the specific FAME synthesis of individual lipid classes. Only free fatty acids, mono- and diglycerides, phosphatidylcholine, -serine, -ethanolamine and -inositol are transesterified within the first 10 min at 100°C[79]. A partial transesterification of fatty acids from other RBC lipid fractions, such as SP, CE, or TAG may take place, if time and temperature settings are changed. Therefore, a strict compliance to the analytical procedure is indispensable for reproducible and accurate data.

The analysis of total plasma lipids has the advantage that process steps can be kept to a minimum. The 'open-tube' transesterification procedure allows a semi-automated fatty acid analysis[46]. This has the potential to save preparation time and labour costs and high quantities of samples can be prepared for GC analysis. This also will simplify the execution of large clinical trials with the emphasis on total plasma lipids

due to the simple and rapid sample preparation. However, restrictions apply similar to those of whole blood mentioned above.

The advantage of the base catalysed transesterification of plasma GPL, which can be carried out at room temperature, is the high-throughput of samples (> 50 per day). The authors claim that the time for the gas chromatographic analysis is the limiting factor for the throughput. In addition, no chromatographic separation is necessary for the specific analysis of plasma GPL. A limitation of the method might be the exclusion of SP, as SFA with > 20 carbon atoms and C24:1n-9 are mainly incorporated in this fraction. This might change the proportion of other fatty acids, but the proportions of these fatty acids in plasma SP are low and the comparison with plasma PL indicated high correlations for the n-3 fatty acids. Based on the FAME synthesis and open-tube application, it seems to be possible to automate this procedure for the specific analysis of plasma GPL. Although sampling requires blood collection, the method is well suitable to determine the fatty acid status of individuals in large clinical studies.

Cheek cell sampling is non-invasive, which can be done without trained clinical personal and independently of the location of a laboratory. The major advantage is the higher willingness of individuals to donate cheek cells rather than blood. In addition, cheek cell fatty acid levels are not dependent on the postprandial state of subjects. A limitation of cheek cell sampling could be the insecurity in collecting sufficient amounts of cell material in contrast to defined volumes taken by blood sampling. The contamination of samples with TAG derived from food residues is possible, which affects the determination of cheek cell total lipids. However, analysing GPL fatty acids strongly reduces this impact, but strict sampling procedures are necessary to collect high-quality samples and sufficient cell amounts. The application of cheek cell PL or GPL as n-3 fatty acid status markers has been demonstrated[70–72;77]. The method is well suitable for large n-3 intervention studies, especially with infants, where invasive sampling is restricted.

Conclusion

Many new approaches have been developed for the n-3 fatty acid status determination. All these methods have strengths and weaknesses and their application need to be carefully considered in order to answer specific questions. It has been shown that the traditional lipid extraction and lipid class separation can be abandoned to save time and costs, which allows analysing larger sample numbers of clinical or epidemiological trials. When successfully validated against dietary intake of n-3 fatty acids, this opens new avenues in the area of lipid research, and may push the clarification of potential health benefits of n-3 fatty acids.

Acknowledgements

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