

**Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters by gas chromatography
– A comparison of different derivatization and extraction procedures**

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Analysis of the fatty acid composition in biological samples is commonly carried out by gas liquid chromatography (GC) after transesterification to volatile fatty acid methyl esters (FAME). We compared the efficacy of six frequently used protocols for derivatization of different lipid classes as well as for plasma and tissue samples. Transesterification with trimethylsulfonium hydroxide (TMSH) led to insufficient derivatization efficacies for polyunsaturated fatty acids (PUFA, <50%). Derivatization in presence of potassium hydroxide (KOH) failed at derivatizing free fatty acids (FFA). Boron trifluoride (BF₃) 7% in hexane/MeOH (1:1) was insufficient for the transesterification of cholestol ester (CE) as well as triacylglycerols (TG). In contrast, methanolic hydrochloric acid (HCl) as well as a combination of BF₃ with methanolic sodium hydroxide (NaOH+BF₃) were suitable for the derivatization of FFA, polar lipids, TG and CE (derivatization rate >80% for all tested lipids). Regarding plasma samples, all methods led to an overall similar relative FA pattern. However, significant differences were observed e.g. for the relative amount of EPA+DHA ("n3-index"). Absolute FA plasma concentrations differed considerably among the methods, with low yields for KOH and BF₃. We also demonstrate that lipid extraction with tert-butyl methyl ether/methanol (MTBE/MeOH) is as efficient as the classical method according to Bligh and Dyer making it possible to replace (environmentally) toxic chloroform.

We conclude that HCl catalyzed derivatization in combination with MeOH/MTBE extraction is the most appropriate among the methods tested for the analysis of FA concentrations and FA pattern in small biological samples. A detailed protocol for the analysis of plasma and tissues is included in this article.

Introduction

For half a century fatty acids have been routinely quantified by gas chromatography with flame ionization detection (GC-FID) following transesterification to fatty acid methyl esters (FAME) [1]. This well-established technique is an integral tool in the characterization of authenticity and nutritional value of food. Since food analysis is a highly regulated area several standard methods for the analysis of fatty acids are suggested by food authorities in the United States and European Union [2, 3].

With the finding that the dietary intake of long chain polyunsaturated fatty acids (LC-PUFA) has direct impact on human health, numerous studies aimed to investigate the effects of a modulation of the PUFA pattern in blood and tissues in response to the diet [4-7]. At last, the broad recognition of the physiological importance of LC-n3-PUFA and their oxylipins caused a renaissance in GC-FID FAME analysis in bio analysis labs. In fact, most modern LC-MS based targeted oxylipin metabolomics studies are backed up by classic GC-FID FAME analysis of the precursor FA [8-12].

Compared to the area of food analysis dealing with rather large volumes, sample preparation and derivatization methods for the analysis of small amounts of tissue and low plasma volumes is less standardized. Most commonly acid derivatization with boron trifluoride (BF_3) [5, 7, 10-14] or hydrochloric acid (HCl) [4, 15-17] are employed and have been used to characterize FA pattern in plasma and tissues [8, 15]. Moreover, trimethylsulfonium hydroxide (TMSH) [18-20] has been used in these kind of studies and alkaline derivatization with sodium or potassium methanolate [6] or hydroxide [19] have been proven to be suitable for the analysis of different lipid classes. Moreover, combinations of different reagents, such as BF_3 and methanolic sodium hydroxide have been developed [21-23].

Lipid extraction is generally carried out according to Bligh and Dyer [24] or for samples with high fat content according to Folch [25] [26] albeit several years ago Matyash et al. described an extraction strategy allowing a replacement of halogenated solvents [27]. Most studies start with an extraction of lipids from plasma or (homogenized) tissue while few apply the derivatization agent without any prior sample preparation [15, 28].

Because GC-FID FAME analysis is such a well-established technique only little information on method details and validation data are given in recent articles. As a consequence it is difficult to deduce the most suited sample preparation method for quantitative FAME analysis in small biological samples based on current literature data. In effort to establish GC-FID FAME analysis in our lab we compared the performance of six common derivatization methods. Most techniques led to comparable relative FA patterns. However, absolute concentrations of the different FA and sums of all FA determined in a plasma or tissue sample varied between the different methods. In the present article, we therefore systematically elucidated reasons for these differences by analysis of derivatization efficacy for all relevant classes of lipids and different FA. Furthermore, we compared two different extraction protocols (Bligh and Dyer as well as tert-butyl methyl ether/methanol (MTBE/MeOH)) and tested alkaline hydrolysis as additional sample preparation step to improve tissue homogenization and extraction.

Material and Methods

Chemicals and biological material

Chloroform (uvasolv grade), ethanol (uvasolv grade), ammonium acetate (p.a.), sodium chloride (p.a.) and potassium hydroxide (p.a.) were obtained from Merck (Darmstadt, Germany). *n*-Hexane (hexane in the following) (HPLC grade), sodium hydroxide (p.a.) and MTBE (HPLC grade) were purchased from Carl Roth (Karlsruhe, Germany). TMSH was obtained from CS Chromatographie Service (Langerwehe, Germany). Methanol (HPLC grade) was purchased from J.T. Baker (Deventer, Holland). Methyl tricosanoate used as internal standard (FAME C23:0, >98%) and cholesteryl heptadecanoate (CE C17:0, >98%) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dihenarachidoyl-sn-glycero-3-phosphocholine (PC C21:0, >99%) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Arachidonic acid (C20:4n6), eicosapentaenoic acid (C20:5n3) and docosahexaenoic acid (C22:6n3) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Acetic acid (Acros Organics) was obtained from Fisher Scientific (Nidderau, Germany). Sodium bisulfate (p.a.) was obtained from Riedel de Haën (Seelze, Germany). All other chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany). Rat liver tissues were collected from male Fischer 344 rats (250-300 g bodyweight, Charles River, Sulzfeld, Germany). Pooled human plasma was obtained from healthy male volunteers.

GC-FID analysis

Gas liquid chromatography was carried out on a 30 m x 0.25 mm FAMEWAX capillary column with a 0.25 µm thick polar polyethylene coating (Restek, Bad Homburg, Germany) equipped with a 5 m x 0.25 mm Guard Column (Restek). Separation was performed with the following temperature gradient on a 6890 series GC instrument (Agilent, Weilbronn, Germany): 140°C to 210°C with 10°C/min, 210°C to 230°C with 2°C/min and 230°C for 7 min (total run time 24 min). Helium was used as carrier gas at a constant flow of 1.5 mL/min. 1 µL of the sample was injected into an injector kept at 275°C and the split was set to 1:50. The flame ionization detector was operated at 300°C with 45 mL/min hydrogen flow, 450 mL/min air flow and a make-up flow of 45 mL/min helium. Under these conditions all biologically relevant FA were baseline separated as shown by a 37 FAME standard from Supelco (CRM47885, Sigma Aldrich) supplemented with 22:5n3 and 22:4n6 (Sigma Aldrich) as well as a 20 FAME fish oil mix (35066, Restek). ChemStation B01.03 (Agilent) was used for instrument control and data handling. Quantification of fatty acid methyl esters was carried out based on their peak areas compared to the internal standard (FAME C23:0) peak area by using response factors introduced by Ackman and Sipos [29, 30]. Linear range and the accuracy of the method were validated by the analysis of FAME standards (see above).

Sample preparation

Alkaline Hydrolysis/sample saponification

10 µL internal standard solution (IS; FAME C23:0, in chloroform/ethanol 1:9) and 10 µL of antioxidant solution (0.2 mg/mL ethylenediaminetetraacetic acid, butylated hydroxytoluene and triphenylphosphine in methanol/water 50:50), were added to 35-45 mg liver tissue. Thereafter, 100 µL methanol (MeOH) and 60 µL 10 M aqueous sodium hydroxide were added and the samples were homogenized in a ball mill (20 Hz, 5 min, 22°C, MM 400, Rentsch, Haan, Germany). The crude suspension was then hydrolysed for 30 min at 60°C. Directly after hydrolysis, samples were neutralized (pH 6-7) on ice with 70 µL of 50% acetic acid and extracted as described below.

Lipid Extraction

Following addition of 10 µL IS solution, pooled human plasma (50 µL) and rat liver tissue (35-45 mg) were extracted by the method of Bligh and Dyer [24] and Matyash [27] with slight

modifications. For Bligh and Dyer [24] extraction, plasma was extracted with 750 μL of chloroform/MeOH (1:2) and the liver tissue was homogenized with 750 μL of chloroform/MeOH (1:2) and 50 μL of water in a ball mill (see above). Thereafter, 250 μL of chloroform and 250 μL of water were added, the samples were mixed for 1.5 min and centrifuged for 10 min at 4500 x g at room temperature. Following collection of the lower organic phase the aqueous phase was re-extracted with 250 μL of chloroform. The organic phases were combined and the sample was evaporated in a vacuum centrifuge (1 mbar, 35°C; Christ, Osterode, Germany).

For the extraction according to Matyash [27] plasma was mixed with 300 μL of MeOH and the liver tissue was homogenized with 300 μL of MeOH and 50 μL of water in a ball mill (see above). Thereafter, 600 μL of MTBE was added, the samples were vigorously shaken for 1.5 min and mixed with 300 μL of 0.15 mmol/L ammonium acetate. After centrifugation at 3500 x g at 4°C the upper organic layer was collected and the surface of the residual aqueous phase was washed with another 300 μL MTBE. The organic phases were combined and evaporated in a vacuum centrifuge (see above).

Derivatization

The dried lipid extracts were transesterified to FAME by six different methods as previously described with slight modifications [15, 19, 20, 22, 28]. For TMSH derivatization, the dried lipid extract was dissolved in 100 μL of hexane and 50 μL of methanolic TMSH (0.2 mol/L). The samples were shaken at 400 U/min for 30 min at room temperature [20]. For BF_3 derivatization the dried lipid extract was dissolved in 500 μL BF_3 solution (12-14% in MeOH) and 500 μL of hexane. The solution was heated for 1 h in a tightly closed vial in a metal block kept at 90-95°C. After cooling, 750 μL of water were added. The sample was shaken vigorously for 4 min, centrifuged at 3500 x g for 5 min and the hexane layer was collected [28]. For the HCl derivatization the dried lipid extract was dissolved in 600 μL of acetylchloride in MeOH (1:9) and 400 μL hexane and the sample was heated to 90-95°C for 1 h in a tightly closed vial. After cooling, 750 μL of aqueous potassium carbonate (0.44 mol/L) were added. The sample was shaken vigorously for 4 min, centrifuged at 3500 x g for 5 min and the hexane layer was collected [15]. For KOH derivatization the lipid extract was dissolved in 200 μL of hexane and 100 μL of methanolic potassium hydroxide (2 mol/L). After incubation at room temperature for 5 min, 40 mg of sodium bisulfate was added and the supernatant was collected [19]. For the combined $\text{NaOH}+\text{BF}_3$ protocol, the dried lipid extract was dissolved in 100 μL methanolic sodium hydroxide (0.5 mol/L) and heated for 15 min at 90-95°C in a tightly closed vial. Thereafter, 500 μL BF_3 solution (12-14% in MeOH) was added and the sample was heated for another 30 min at 90-95°C. 750 μL of saturated aqueous sodium chloride and 500 μL of hexane were added after cooling of the sample. The sample was shaken vigorously for 4 min, centrifuged at 3500 x g for 5 min and the organic phase was collected [22].

Following all derivatization protocols, the organic phases were evaporated in a vacuum centrifuge (1 mbar, 35°C; see above), reconstituted in 50 μL of MTBE/MeOH (9:1) and analyzed by GC-FID. Only for direct TMSH derivatization the dried lipid extract was reconstituted in 50 μL of methanolic TMSH (0.2 mol/L) and 50 μL of MTBE and directly subjected to GC analysis [19].

The derivatization efficacies were analyzed by treating 5 μL of a standard solution containing 2 mmol/L of free FA, a phosphatidylcholine, a cholesterol ester and triacylglycerols, respectively with 10 μL IS as lipid extract.

Results

Derivatization efficacy for different lipid classes

The efficacy of the six different protocols for the transesterification of free fatty acids (FFA), a phosphatidylcholine (PC), triacylglycerols (TG) and a cholesterol ester (CE) are shown in Fig. 1. Potassium hydroxide (KOH) led to good derivatization efficacies for PC and TG (>90%) while FFA as well as CE were not derivatized (<3%). Except for polyunsaturated FFA (derivatization $\leq 11\%$), all tested lipid classes were acceptably methylated using TMSH ($\geq 80\%$). Direct injection of the TMSH derivatization mixture into the hot injector (TMSH inj) led to higher but still low derivatization of polyunsaturated FFA ($\leq 50\%$). Moreover, this procedure caused inefficient transesterification efficacies for TG C19:0 (rec=41%). Derivatization in presence of BF_3 led to good efficacies (>95%) for FFA and the PC, however the CE and TG were not efficiently transesterified ($\leq 30\%$). Derivatization rates with hydrochloric acid (HCl) were good for all tested lipid classes (>80%). The protocol combining derivatization in presence of methanolic sodium hydroxide and boron trifluoride ($\text{NaOH}+\text{BF}_3$) also led to a good derivatization efficacy ($\geq 85\%$) for all tested lipid classes.

Derivatization of lipid extracts from plasma

The sums of quantified saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n3- and n6-polyunsaturated fatty acids (PUFA) as well as the sum of all quantified fatty acids in human plasma extracted with MTBE/MeOH utilizing the six different derivatization protocols are shown in Tab. 1. The plasma concentrations of the biologically relevant PUFA α -linolenic acid (18:3n3, ALA), arachidonic acid (20:4n6, AA), eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA) are highlighted in Fig. 2A.

The concentrations of ALA, AA, EPA and DHA were overall in the same range with all six protocols. However, the concentrations of these PUFA obtained with KOH and BF_3 derivatizations were significantly lower compared to HCl ($p < 0.001$, Fig. 2A). The sums of the concentrations of all detected SFA, MUFA and total n6- and n3-PUFA showed a similar trend (Tab. 1). As a consequence, the sum of the concentrations of all fatty acids was dramatically lower for the KOH and BF_3 protocol (Tab. 1). For all other derivatization methods, the sums of SFA, MUFA, PUFA and total FA concentrations were comparable.

The obtained variation of repeated measurements, calculated as standard deviation (SD) of five independent replicates differed considerably between the protocols. The lowest SD and thus the best precision for the sum of all FA was found for HCl and BF_3 with relative SD of $\leq 2\%$ compared to up to 8% for TMSH.

The six different protocols led to distinct differences in the relative pattern of SFA, MUFA and PUFA (Tab. 1). For n6-PUFA for example, the relative amount of the different FA classes ranged from 25% (KOH) to 32% (TMSH and $\text{NaOH}+\text{BF}_3$) and for SFA between 33% ($\text{NaOH}+\text{BF}_3$) and 42% (BF_3). Regarding the relative content of EPA+DHA of all FA ("n3-index" [31], Fig. 2B), the lowest value was calculated based on the data obtained by TMSH derivatization (5.3%) and the highest value resulted from the BF_3 method (8.3%). Derivatization with HCl, KOH and $\text{NaOH}+\text{BF}_3$ led to a consistent index of $7.4 \pm 0.2\%$. No statistical differences were observed between the three protocols (Fig. 2B). However, with 5.3% and 6.5% both TMSH protocols led for the same samples to a significantly lower, and BF_3 with 8.3% to a significantly higher n3-index compared to HCl.

Efficacies of the Bligh and Dyer vs. the MTBE/MeOH extraction

The lipid extraction efficacy for plasma and liver tissue was compared for Bligh and Dyer and the MTBE/MeOH protocol (Tab. 2 and 3). Derivatization of the resulting lipid extracts was carried out with the HCl and the combined $\text{NaOH}+\text{BF}_3$ method.

As shown in Table 2 and 3 both, the determined FA concentrations as well as the relative FA patterns and standard deviations were comparable for both protocols and derivatization techniques.

Saponification of tissue samples prior extraction

In addition to homogenization and extraction of liver, the tissue was heated under harsh alkaline conditions. This led to an almost complete dissolving of the tissue improving the handling of the following extraction. Lipid extraction was conducted by the methods of Bligh and Dyer and MTBE/MeOH, and HCl as well as NaOH+BF₃ were used for derivatization (Tab. 3).

Compared to the direct extraction, the sample saponification caused neither a difference in the absolute concentrations of the fatty acids, nor in the relative pattern of SFA, MUFA and PUFA. However for both extraction protocols, there was a slight trend towards a higher extraction efficacy for the hydrolysed samples.

Discussion

Fatty acid quantification as FAME by means of GC-FID is a well-established technique. Numerous studies describe the sample preparation procedure particularly derivatization to FAME [2, 3, 14, 15, 18, 20, 22, 24, 25, 27, 28]. However, for small biological samples a variety of different methods are used, making it difficult to deduce the most suitable. Therefore, we compared the most established derivatization methods to elucidate which is the most appropriate technique to analyze the PUFA pattern in plasma and tissue samples.

When analyzing standards of FFA, PC, CE and TG our results show that only the derivatization protocols utilizing HCl and NaOH+BF₃ yielded FAME with satisfying efficacy (>80%). The other four protocols using BF₃, KOH and TMSH failed to derivatize at least one of the lipids (Fig. 1), which are all present in plasma [32]. As described previously, KOH treatment does not esterify FFA to FAME [33]. Low efficacies were also observed for the derivatization of unsaturated FFA by TMSH (≤50%) with direct injection (pyrolysis of the salt formed from the FA and TMSH in the injector [34]) or derivatization at room temperature followed by evaporation. Similar observations have been made for TMSH derivatization before, e.g. Firl et al. reported lower recovery rates for PUFA in comparison to MUFA and SFA [19]. Interestingly, CE derivatization has been described to be problematic using TMSH because of long reaction times required [34]. However, our results indicate efficient transesterification. The BF₃ protocol is often used in literature for the derivatization of lipid extracts from biological samples [5, 7, 10-13, 31] or direct conversion of crude samples [28]. In our analysis derivatization with 7% BF₃ in hexane/MeOH inefficiently transesterified CE and TG, two of the major lipid fractions in plasma [32]. However, it should be noted that the transesterification efficacy depends on the solvent composition [35]. Thus, a lower hexane/MeOH ratio could lead to a better conversion of CE and TG. When comparing different BF₃ protocols it also should be noted, that several described “BF₃ protocols” are two steps procedures of an alkaline treatment followed by BF₃ methylation [36], similar to the highly efficient protocol using NaOH+BF₃ described here. Nevertheless, several authors report potential artifact formation by derivatization of PUFA with BF₃, hence giving reasons to prefer other derivatization procedures [37].

In spite of the varying derivatization of the standards (Fig. 1), it is interesting that all derivatization protocols led to an overall similar relative pattern of SFA, MUFA and PUFA in plasma (Fig. 2B, Table 1). Particularly the determined relative amount of EPA and DHA (“n3-index” [31]) was fairly consistent between the methods (Fig. 2B). Hence, our results indicate that all protocols are suitable to detect changes in the relative FA pattern, as carried out in numerous

studies [4-13, 19, 21, 23, 31]. However, due to the low derivatization efficacy for PUFA, TMSH led to an underestimation of the absolute and relative levels of n3-PUFA and thus to a significantly lower “n3-index” (Fig. 2B). Similarly, too low levels for SFA and MUFA in plasma are obtained for BF₃ in hexane/MeOH (1:1), most likely caused by the low derivatization efficacy of CE and TG. In combination with the high transesterification rate of phospholipids by this BF₃ protocol, the lipid fraction that contains most of all n3-PUFA in plasma [32], a significantly higher “n3-index” results (Fig. 2B).

These results demonstrate that great care should be taken when comparing relative FA pattern reported in different studies. Therefore, a comparison only seems possible between analyses using the HCl, KOH, and NaOH+BF₃ derivatization protocols.

Regarding the absolute concentration of FA determined in plasma much more pronounced differences were obtained between the protocols (Fig. 2A, Table 1). Following KOH and BF₃ derivatization the sum of all FA concentrations were with 2000 and 1800 µg/mL almost 50% lower than for the TMSH, HCl and NaOH+BF₃ protocols which led to concentrations of 3000-3400 µg/mL (Table 1). This poor yield seems to be even problematic for the analysis of relative FA pattern, e.g. by sacrificing detection sensitivity of low abundant FA.

Our results compelled us to conclude that among the procedures tested only HCl and NaOH+BF₃ are suitable protocols for the analysis of an overall FA pattern in plasma without discriminating individual classes of lipids. However, KOH derivatization leads to a consistent FA pattern in plasma, probably because of the low FFA content [32]. Thus, each derivatization method tested here might be suitable for specific samples. For example, BF₃ and KOH protocols might be applicable for the analysis of the relative FA pattern in samples rich in polar lipids, e.g. the composition of erythrocyte membranes.

Aside from the derivatization procedure, extraction of the lipids is the crucial sample preparation step for the analysis of biological samples. Utilizing a slightly modified extraction protocol as described by Matyash [27] about 6 years ago, a comparable extraction efficacy for plasma and tissues was found for the MTBE/MeOH extraction compared to the method of Bligh and Dyer [24] (Tab. 2 and 3). Based on the application of two derivatization protocols and the analysis of plasma and liver tissue, our results clearly demonstrate that both, the absolute concentration of FA as well as the relative FA pattern are comparable between the two extraction protocols. Interestingly, the MTBE/MeOH extraction led to slightly higher concentrations of degradation prone PUFA (Tab. 2 and 3). Thus, the extraction conditions seem to be milder in comparison to the traditional method. It should be noted that the results cannot be applied for the analysis of high fat tissues (>2% fat in the homogenate) where extraction to Folch yields better results than extraction according to Bligh and Dyer [26]. However the good comparability of MTBE/MeOH with Bligh and Dyer encourages to further investigate if, for the analysis of high fat tissues, also Folch extraction could be replaced by this halogenated solvent free procedure.

Regardless the method, extraction of tissues is a challenging task requiring labor-intensive and error prone homogenization techniques. In order to improve extraction efficacy we added a saponification step prior to sample preparation. The tissue almost dissolves in the applied sodium hydroxide solution within 30 min at 60°C and the neutralized samples can be extracted as plasma or other liquid samples. As shown in Tab. 3, the extraction efficacy for liver tissue was comparable for samples prepared with or without hydrolysis prior to extraction. Actually, the extraction is slightly, however not significantly, improved with saponification of the sample. Thus, it is concluded that saponification of tissue samples does not negatively influence fatty acid analysis. Therefore this sample preparation strategy is a promising tool particularly for the FAME analysis of tough tissues, such as gut or other organs rich in connection tissue.

Overall, we suggest, that MTBE/MeOH extraction followed by HCl derivatization as sample preparation technique for FAME analysis in small amounts of biological samples such as plasma and tissues. For the analysis of tissues, base hydrolysis prior extraction can be included in the sample preparation making homogenization easier and more reliable without negatively affecting the results. In order to allow the reader to easily establish these methods in their laboratories we included a detailed step by step protocol for the fatty acid analysis of plasma and tissue in the supplementary material. It should be noted that the combined protocol with NaOH+BF₃ is equally suitable, however, requires more working steps and has the risk of artifact formation [37]. The traditional extraction according to Bligh and Dyer also yields excellent results. Nonetheless, our data indicate no scientific need in continuing to use (environmental) toxic chloroform for extraction.

Most importantly, our studies demonstrate that both, the absolute concentrations, but also in part the relative FA pattern, significantly differ between derivatization methods. Therefore, we urge all authors of future articles using FAME analysis to provide details about their methods. Otherwise, a comparison of different studies might not be possible and potentially valuable information about PUFA biology might be lost.

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Tables

Tab. 1: Determined plasma PUFA levels utilizing different derivatization methods. Shown is the sum of the concentration and the relative amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n6-polyunsaturated fatty acids (n6-PUFA) and n3-PUFA quantified in the same pooled human plasma (50 μ L). All results are shown as mean \pm SD (n=5).

Plasma [μ g/mL]	KOH	TMSH	TMSH direct injection	BF ₃	HCl	NaOH+BF ₃
Σ SFA	810 \pm 21	1200 \pm 72	1200 \pm 36	750 \pm 15	1200 \pm 16	1100 \pm 39
Σ MUFA	480 \pm 13	910 \pm 64	760 \pm 57	390 \pm 8.4	860 \pm 14	870 \pm 55
Σ n6-PUFA	500 \pm 12	1100 \pm 100	820 \pm 120	490 \pm 8.1	1000 \pm 24	1100 \pm 120
Σ n3-PUFA	190 \pm 3.3	230 \pm 38	240 \pm 18	180 \pm 5.5	300 \pm 14	300 \pm 15
Σ total FA	2000 \pm 49	3400 \pm 270	3000 \pm 230	1800 \pm 37	3300 \pm 69	3400 \pm 230

Relative distribution [%]

Σ SFA	41 \pm 1.0	35 \pm 2.1	40 \pm 1.2	42 \pm 0.83	35 \pm 0.49	33 \pm 1.2
Σ MUFA	24 \pm 0.66	26 \pm 1.9	25 \pm 1.9	21 \pm 0.47	26 \pm 0.42	26 \pm 1.6
Σ n6-PUFA	25 \pm 0.60	32 \pm 2.9	27 \pm 3.9	27 \pm 0.45	30 \pm 0.74	32 \pm 3.5
Σ n3-PUFA	9.5 \pm 0.17	6.8 \pm 1.1	8.1 \pm 0.59	9.9 \pm 0.31	9.0 \pm 0.42	8.9 \pm 0.46

Tab. 2: Comparison of the lipid extraction efficacy of the protocol according to Bligh and Dyer and MTBE/MeOH using NaOH+BF₃ or HCl derivatization. Shown are the sums of the concentrations of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n6-polyunsaturated fatty acids (n6-PUFA) and n3-PUFA quantified in the same pooled human plasma (50 µL). The relative DHA+EPA content of all detected FA is presented in the bottom panel of the table. All results are shown as mean ± SD (n=5).

Derivatization Plasma [µg/mL]	Bligh & Dyer	MTBE	Bligh & Dyer	MTBE
	NaOH + BF ₃		HCl	
Σ SFA	1200 ± 80	1100 ± 39	1100 ± 33	1200 ± 16
Σ MUFA	900 ± 65	870 ± 55	820 ± 27	860 ± 14
Σ n6-PUFA	1100 ± 94	1100 ± 120	960 ± 37	1000 ± 24
Σ n3-PUFA	290 ± 33	300 ± 15	280 ± 10	300 ± 14
Σ total FA	3500 ± 270	3400 ± 230	3100 ± 110	3300 ± 69
% (EPA+DHA) of all FA	6.9 ± 0.81	7.3 ± 0.40	7.3 ± 0.28	7.4 ± 0.38

Tab. 3: Comparison of the extraction efficacy of the protocol according to Bligh and Dyer and MTBE/MeOH with either NaOH+BF₃ or HCl derivatization with or without alkaline hydrolysis prior extraction of liver samples. Shown are the sums of the concentrations of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n6-polyunsaturated fatty acids (n6-PUFA) and n3-PUFA quantified in rat liver (35-45 mg) of the same animal. The relative DHA+EPA content of all detected FA is presented in the bottom panel of the table. All results are shown as mean \pm SD (n=5).

Derivatization Liver [μ g/mg]	Bligh & Dyer	MTBE	Bligh & Dyer	MTBE	Bligh & Dyer	MTBE	Bligh & Dyer	MTBE
	Direct extraction of the sample				Alkaline hydrolysis prior extraction			
	NaOH + BF ₃		HCl		NaOH + BF ₃		HCl	
Σ SFA	16 \pm 1.9	18 \pm 2.1	15 \pm 0.99	16 \pm 1.6	16 \pm 0.87	16 \pm 1.3	17 \pm 1.2	17 \pm 0.88
Σ MUFA	5.0 \pm 0.96	6.0 \pm 0.77	4.8 \pm 0.46	5.2 \pm 0.74	5.6 \pm 0.86	5.8 \pm 1.4	5.5 \pm 0.49	5.9 \pm 0.37
Σ n6-PUFA	12 \pm 0.42	12 \pm 1.5	12 \pm 0.48	12 \pm 1.3	13 \pm 1.9	14 \pm 2.9	13 \pm 0.65	14 \pm 0.58
Σ n3-PUFA	1.9 \pm 0.36	2.1 \pm 0.27	2.0 \pm 0.25	5.9 \pm 0.24	2.3 \pm 0.36	2.3 \pm 0.5	2.3 \pm 0.17	2.4 \pm 0.17
Σ total FA	34 \pm 3.6	38 \pm 4.6	34 \pm 2.2	35 \pm 3.9	37 \pm 4.0	38 \pm 6.0	38 \pm 2.5	39 \pm 2.0
% (EPA+DHA) of all FA	4.3 \pm 0.91	4.3 \pm 0.56	4.5 \pm 0.61	4.7 \pm 0.42	4.8 \pm 0.81	4.7 \pm 0.98	4.5 \pm 0.27	4.7 \pm 0.29

Figure Captions

Fig. 1: Derivatization efficacy of FAME generation by the different methods: **A:** saturated non-esterified fatty acids (FFA) C17:0 and C21:0, **B:** polyunsaturated FFAs C20:4n6, C20:5n3 and C22:6n3, **C:** phosphatidylcholine C21:0 and cholesterol ester C17:0 and **D:** triacylglycerols C18:1n9 and C19:0. For each lipid the recovery rate of a derivatized standard (200 $\mu\text{mol/L}$) is shown as mean \pm SD (n=5).

Fig. 2: Plasma PUFA levels determined with different derivatization methods. **A:** concentration of α -linolenic acid (C18:3n3, ALA), arachidonic acid (C20:4n6, AA), eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA), **B:** Relative DHA+EPA content of all detected FA ("n3-index"). For all samples the same pooled human plasma was extracted with MTBE/MeOH and analyzed. Significant differences were determined in comparison to the HCl method by independent sample t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. All results are shown as mean \pm SD (n=5).

Fig. 1

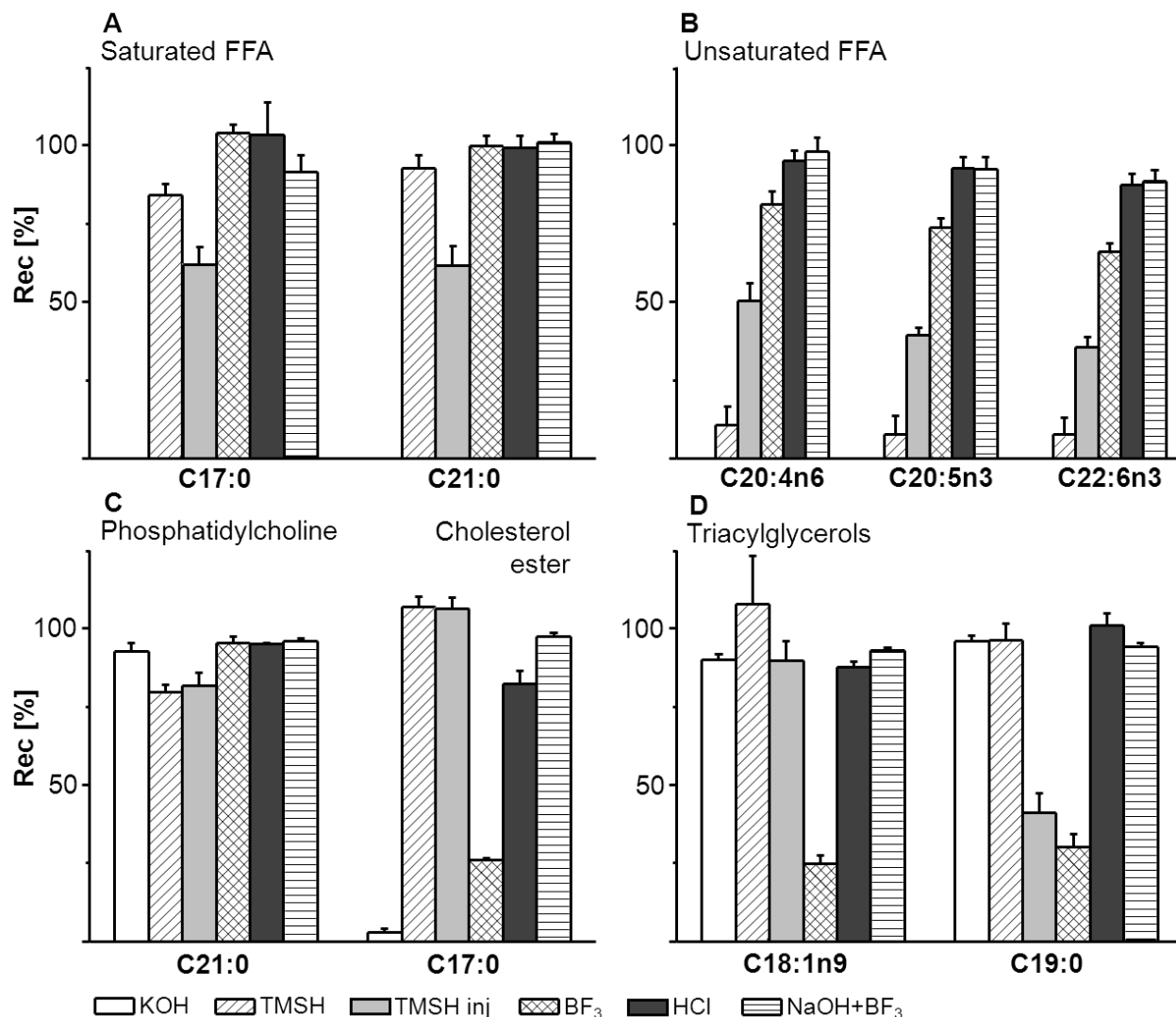
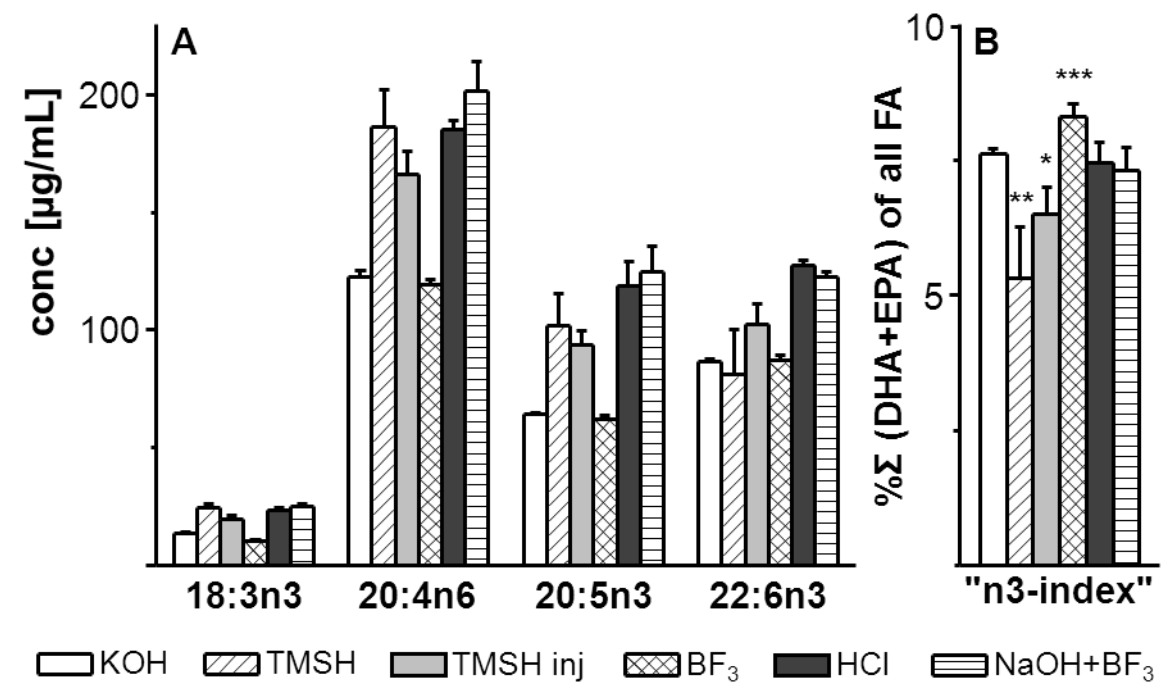


Fig. 2



**Determining the fatty acid composition in plasma and tissues as fatty acid methyl esters by
gas chromatography
– A comparison of different derivatization and extraction procedures**

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Supplementary Data

**Detailed protocol for the extraction and derivatization of lipids in plasma and
tissue**

Alkaline hydrolysis of tissue samples

All steps are conducted on ice, if not noted otherwise

- **Exactly weight** 35-45 mg tissue in a 1.5 ml reaction tube (# 72.706, Sarstedt, Nümbrecht, Germany)
- **Add:**
 - 10 µL IS (75 µmol/L FAME C23:0 in chloroform/ethanol 1:9)
 - 10 µL of antioxidant solution (0.2 mg/mL ethylenediaminetetraacetic acid, butylated hydroxytoluene and triphenylphosphine in methanol/water 50:50)
 - 100 µL methanol
 - 60 µL 10 M aqueous sodium hydroxide
 - two 3 mm stainless steel beads
- **Vortex vigorously** until the tissue detaches from the wall
- **Homogenize in a ball mill** (20 Hz, 5 min; MM 400, Retsch, Haan, Germany) in a precooled sample holder (-20°C)
- **Incubate** under slight shaking **at 60°C for 30 min** in a heating block (Thermo Mixer Comfort, Eppendorf, Hamburg, Germany)
- **Place sample on ice** – (*Note:* precipitation occurs which dissolves in the following)
- **Add** 60 µL of 50% acetic acid
- **Vortex** vigorously until a clear solution results
- **Adjust pH** to 6-7 with 50% acetic acid

→ **Extraction**

Plasma/Tissue extraction with MTBE/MeOH according to Matyash et al. [27]

All steps are conducted on ice, if not noted otherwise

- **Fill 50 µL plasma** in a glass tube (111814004, Labor- und Analystechnik (LAT), Garbsen, Germany)
- **Add**
 - 10 µL of IS (75 µmol/L FAME C23:0 in chloroform/ethanol 1:9)
 - 300 µL of MeOH
- **Seal sample** with a rubber plug (C390, Carl Roth, Karlsruhe, Germany)
- **Vortex** vigorously

- **(Or) Exactly weight** 35-45 mg tissue in a 1.5 mL reaction tube (Sarstedt)
- **Add:**
 - 10 µL of IS (75 µmol/L FAME C23:0 in chloroform/ethanol 1:9)
 - 300 µL of MeOH
 - two 3 mm stainless steel beads
- **Vortex vigorously** until the tissue detaches from the wall
- **Homogenize in a ball mill** (20 Hz, 5 min; Retsch) in a precooled sample holder (-20°C)
- **Transfer** samples into a glass tube (LAT)

- **Add** 600 µL MTBE
- **Seal** glass tube with a rubber plug (Carl Roth)
 - **Vortex vigorously** for 1.5 min (Vortex Genie 2 (#70092) equipped with a sample holder for 12 reaction tubes (#70506), NeoLab, Heidelberg, Germany)

- **Add** 300 µL of 0.15 mmol/L ammonium acetate
- **Vortex** sample
- **Centrifuge** at 3500 x g for 5 min at 4°C

- **Collect upper organic layer** in a new glass tube
- **Wash the surface** of the residual aqueous phase with another 300 µL MTBE by slowly rinsing the solvent down the wall of the glass tube
- Note: If the phases are mixed accidentally, centrifuge the sample again*

- **Combine organic phases**
- **Evaporate** them in a vacuum centrifuge (at 1 mbar, 35°C, about 1.5 h; Christ, Osterode, Germany)

→ Derivatization

Derivatization of the lipid extract with methanolic HCl

- **Add to glass tube** with the dried lipid extract
 - 600 µL of acetylchloride in methanol (1:9)
 - 400 µL n-hexane
- **Vortex** until residue is completely dissolved
- **Transfer** sample to a crimp vial with cap and septum (LC 22.1 and LC29.1, Carl Roth, Karlsruhe, Germany)
- **Seal** the vial tightly
- **Heat** the sample for 1 h in a heating block kept at 90-95°C

Note: The rubber septum from the crimp cap might bend in this step because of the high pressure in the crimp vial. If the vial is sealed tightly, no sample will be lost.
- **Cool** the sample down at room temperature (ca. 5-10 min)
- **Transfer** solution to a 2 mL reaction tube (72.706, Sarstedt) containing 750 µL of aqueous potassium carbonate (0.44 mol/L)
- **Vortex** the sample vigorously for 4 min (Vortex Genie 2, NeoLab)
- **Centrifuge** at 3500 x g for 5 min
- **Transfer** the (upper) hexane layer into a glass tube
- **Evaporate** the solvent in a vacuum centrifuge (at 1 mbar, 35°C, about 10 min; Christ)
- **Reconstitute** sample in 50 µL of MeOH/MTBE (1:9)
- **Transfer in autosampler vial with insert** (vial: IVA70911302; insert: IVA70906500; cap: IVA71509342, IVA-Analysentechnik, Meerbusch, Germany)

→ Analysis by GC-FID