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LC-MS/MS applications in Targeted Clinical Metabolomics: Method development and validation with focus on sulphur-containing amino acids and nonesterified fatty acids

Dissertation

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1. Introduction

“Targeted clinical metabolomics” focuses on the analysis of pre-defined metabolites in biological samples (1, 2). The quantitative determination of several hundreds of metabolites in clinical trials generates a snapshot of the actual metabolic system permitting conclusions about nutritional habits, metabolic regulations and pathway connections (3, 4). This knowledge supports the understanding of diseases development and permits data-based hypotheses for disease risk factors and biomarkers, e.g. for non-fatty liver disease (5) or obesity (6).

In the wide range of applicable analytical methods, liquid chromatography coupled to tandem mass-spectrometry (LC-MS/MS) provides a powerful tool to determine this wide range of metabolites. Sample are prepared in a short time without extraction or derivatization procedures which are often necessary for other methods like gas chromatography (GC) or ultra-violet detection (UV) (7). Since LC-MS/MS can be used for sensitive analysis of a wide range of analytes (8), a metabolomics platform was established in the Division of Metabolic and Nutritional Medicine at the Dr. von Hauner Children’s Hospital. Utilizing the power of LC-MS/MS, several methods were developed to determine amino acids (AA) (9), glycerophospholipid species (10), creatinine (11) as well as folate metabolites (12) and a shotgun MS/MS method to analyze several lipid species. The LC-MS/MS methods were implemented in addition to GC methods for the analysis of fatty acids (FA) (13).

Within the metaboliomic platform, one objective of the presented dissertation was to expand the metabolomics platform by implementing applications for characterization of further disease risk factors. Precisely, class-specific methods were developed for nonesterified fatty acids (NEFA) and sulphur-containing AA, homocysteine (Hcy), cysteine (Cys) and methionine (Met).

Sulphur-containing AA play a relevant role, both as methyl donators for the synthesis of different biological compounds (e.g. neurotransmitter, DNA) and as precursor for proteins or glutathione, which is essential for detoxication, modulation of the red-ox status and immune-modulation (14, 15). Dysregulation of sulphur amino acids is associated with nutritional aspects and genetic factors (16, 17).

Lower intake of folate and elevated Hcy concentrations are associated with increased cardiovascular disease (CVD) mortality (18, 19), increased incidence of dementia and Alzheimer's disease (20) and further pathological outcomes (21).

Hcy is a potential biomarker for nutritional deficiency or cognitive function and is additionally a potential risk factor for CVD. Thus, Hcy measurement is routinely used in clinical laboratories.

Previous methods used expensive enzyme- and immunoassays (22), HPLC methods using UV, fluorescence, or electrochemical detection (23) and GC- MS methods (24, 25) for Hcy determination. In recent years, LC-MS/MS methods were established for Hcy analysis (26-28) providing more specificity, elevated sensitivity and improved cost-efficiency by decreased run time and less solvent consumption (29). The previous LC-MS/MS methods achieved cycle times about 3-4 minutes. One reason for the very short run time is the almost non-existent or low retention of Hcy on reversed phase columns. But remaining adequate retention is an important factor in LC optimization to separate the analyte from interfering substances, which naturally exist in biological matrices like urine or blood plasma (30). These substances cause matrix effects with lower (ion-suppression) or higher response (co-elutions) as expected by the calibration curve which is normally calculated with aqueous standards (31).

Thus, one aim of the presented dissertation was to establish a robust method not only for Hcy but also for Cys and Met analysis using a special chromatography system to achieve an increase in retention time and avoid matrix effects. An additional determination of Met and Cys supports the identification of causal mechanisms of hyperhomocysteinemia more accurately, as the sulphur amino acids undergo an interrelated metabolism (18). The method will be especially applied to trials studying the effect of folate as well as vitamin B_2 supplementation on Hcy levels and cognitive functions. Thus, the application will support the understanding of Hcy as nutritional marker as well as biomarker.

A further class of metabolites, which have a strong potential as marker for nutrition and as risk factor for the development of diseases, are nonesterified fatty acids (NEFA). NEFA are highly associated with various diseases like obesity (32), insulin resistance (33), diabetes (34), metabolic syndrome (35), adrenoleukodystrophy (36), Alzheimer’s disease or schizophrenia (37). NEFA are involved in the link of obesity, insulin resistance and diabetes contributing to lipotoxicity by affecting insulin sensitivity and ß-cell function (38). Single NEFA species may be more accurate and sensitive to identify biomarker and describing metabolic process than determination of total NEFA (39). Yang et al. identified 10 relevant lipid species as biomarkers of type 2 diabetes (T2DM), including nonesterified oleic acid (18:1), eicosapentaenoic acid (20:5) and docosahexaenoic acid (DHA, 22:6) (40). Furthermore, palmitic acid (16:0), stearic acid (18:0) and oleic acid were found to be suitable to reflect differences between T2DM and control (41). Recently, Novgorodtseva et al. reported an

accumulation of polyunsaturated fatty acids (18:2 n6, 18:3 n3, 22:4 n6) and a lower pool of saturated fatty acids (12:0,14:0, 16:0, 17:0) in plasma of patients with metabolic syndrome (35). To unravel the mechanisms of lipotoxicity and the influence on metabolomic functions, the LIPID MAPS Consortium established a widespread lipidomics platform (42). Regarding this platform, all lipid classes, except NEFA, are measured with LC-MS/MS applications (43). NEFA are traditionally determined with GC methods which are used in routine procedures for analysis of NEFA with excellent chromatographic resolution but utilizing extraction and derivatization procedures to enhance volatility (39, 44).

Considering the simplicity of sample preparation for LC-MS/MS, the lack of methods for determination of NEFA in human plasma by LC-MS/MS is quite remarkable. A problem in MS/MS determination of NEFA is their poor fragmentation (45). Post-column addition of cations (46, 47) or derivatization procedures (48, 49) improve fragmentation behavior, but provide additional preparation steps, which result in prolonged sample preparation procedures. Thus, one objective of the presented dissertation was to develop a comprehensive method for quantification of NEFA in human plasma. This method should avoid extraction and derivatization procedures to enable a cost-effective and fast determination of NEFA in clinical trials and cohort studies.

One reason for the development of the NEFA method is the strong relation of NEFA to adipose tissue (AT) fatty acids, as NEFA are mainly generated by lipolysis of triacylglycerols (TAG) in AT during fasting. AT is an accepted long-term biomarker for the assessment of dietary fatty acid (FA) intake (50, 51). Furthermore, FA composition of AT is of special interest for disease progress and prediction, as alterations in AT fatty acid composition play a crucial role in the development of obesity (50). Different studies have been accomplished to analyze the influence of AT to obesity with inconsistent results (52-54). Hence, further investigations of AT fatty acid composition are required to determine the molecular switch of obesity to insulin resistance manifesting finally in T2DM.

Despite the potential of adipose tissue FA, determination of AT composition is not feasible, as an invasive biopsy is required. This biopsy is not always practicable in large clinical trials. Therefore, samples of AT are often not available, and the implementation of a surrogate marker is needed. A close correlation between the surrogate marker and AT FA composition would enable less-invasive sample collection for patients. This is particular in patients, e.g. children, in whom it is difficult to perform an AT biopsy. This surrogate marker can be provided by plasma NEFA.

Despite the important relationship between NEFA and AT, less comprehensive in-vivo studies were accomplished. Most studies focused on single pathway steps, like release of NEFA from AT (55, 56) or (re-)uptake of NEFA to AT (57-59).

Since, to our knowledge, only Yli-Jama et al. investigated the relation of AT to plasma NEFA in a comprehensive work (60), a further aim of this dissertation was to investigate the association of NEFA composition with AT FA composition to evaluate if NEFA may provide a potential surrogate marker for AT FA. Additionally to previous studies, we focused on the influence of BMI and AT site on the relation of plasma NEFA and AT FA composition.

In this study, TAG of AT were analysed by hydrolysis of TAG species in three FA and glycerol. Afterwards, the released FA were determined with the same analytical method used for NEFA analysis. The FA composition of AT was determined without regarding exact structures of lipid species.

Besides the measurement of the FA composition, other approaches exist to determine TAG and the other acylglycerol classes, diacylglycerols (DAG) and monoacylglycerols (MAG).

Summarized, three different strategies were implemented. First, enzymatic and radiometric assays were implemented for measurement of total TAG and DAG (61). As second approach for acylglycerol determination, GC applications were developed to analyse the fatty acid content in the classes of tri- and diacylglycerol, usually in combination with thin-layer chromatography. The last strategy is the identification of exact acylglycerol species. With this approach, the correct position of FA in the TAG structure was determined by the different chromatographic and fragmentation behaviour of TAG in LC-MS/MS applications.

Especially focusing on obesity, insulin resistance and diabetes, all of the three presented strategies have been used to analyse acylglycerols, because excess availability of TAG (34) and DAG (62) to insulin-sensitive tissue, like the liver, may be a key predictor for the development of insulin resistance. Correlation between total TAG and type 2 diabetes (61, 63), obesity and insulin resistance (34) has widely been studied. More detailed applications look for relations between FA content and fasting insulin and glucose levels (64). In recent works, TAG species were found as more precise markers for insulin resistance compared to total TAG (65). This importance of exact acylglycerol species is highlighted by other studies (66, 67). But this approach is solvent- and time-consuming. The last objective of the presented dissertation was to review the advantages and disadvantages of the different strategies for acylglycerol analysis and to assess the need to develop an LC-MS/MS application for the DAG and TAG regarding the already established GC method for measurement of FA content (13).

The key objectives of this work are as follows:

- Development of a LC-MS/MS method for high-throughput analysis of sulfur-containing AA in plasma.
- Development of a LC-MS/MS method for high-throughput analysis of NEFA concentrations and composition in plasma.
- Evaluation of the relation of plasma NEFA and adipose tissue fatty acid composition.
- Review of analytical methods for determination of acylglycerols.

This dissertation is based on the following published or submitted articles:

1. **Research Article**

   Hellmuth C, Koletzko B, Peissner W. Aqueous normal phase chromatography improves quantification and qualification of homocysteine, cysteine and methionine by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2011 Jan 1;879(1):83-9

2. **Review**

   Hellmuth C, Uhl O, Segura-Moreno M, Demmelmair H, Koletzko B. Determination of acylglycerols from biological samples with chromatography-based methods. J Sep Sci. 2011 Dec;34(24):3470-83.

3. **Research Article**

   Hellmuth C, Weber M, Koletzko B, Peissner W. Nonesterified fatty acid determination for functional lipidomics: comprehensive ultrahigh performance liquid chromatography-tandem mass spectrometry quantitation, qualification, and parameter prediction. Anal Chem. 2012 Feb 7;84(3):1483-90.

4. **Research Article (submitted)**

   Hellmuth C, Demmelmair H, Schmitt I, Peissner W, Blüher M, Koletzko B. Association between plasma nonesterified fatty acids species and adipose tissue fatty acid composition. Submitted to PLOS One.

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2. Publication 1: Aqueous normal phase chromatography improves quantification and qualification of homocysteine, cysteine and methionine by liquid chromatography–tandem mass spectrometry

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Aqueous normal phase chromatography improves quantification and qualification of homocysteine, cysteine and methionine by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Elevation of plasma homocysteine concentration is recognized as an independent predictor of cardiovascular disease risk. Therefore, quantification of homocysteine and related sulphur amino acids cysteine and methionine from plasma samples is routinely performed in clinical laboratories. Due to the highly hydrophilic character of these amino acids, previously reported LC–MS methods often suffered from very short chromatographic retention resulting in inadequate separation from matrix background and possible co-eluents. In the present method, aqueous normal phase (ANP) chromatography was introduced to improve chromatographic separation for liquid chromatography–electrospray ionization tandem mass spectrometry. Selective qualification of analytes and internal standards was achieved by qualifier ion monitoring. Using this enhanced selectivity, spurious co-eluents were identified and separated from the analyte signal by optimization of chromatographic conditions. Method validation proved high precision and accuracy (intra-assay reproducibility 1.2–4.3% CV, inter-assay reproducibility 3.4–6.1% CV, accuracy 91.3–105.9%). Total cycle time of 7 min and low costs per sample allow high-throughput application in clinical diagnostics and research trials.

1. Introduction

Aqueous normal phase (ANP) chromatography represents an important new technology for the separation of endogenous metabolites in biological matrices. It enables high-resolution separations of hydrophilic compounds which are not adequately retained and separated on conventionally used stationary phases in reversed-phase operation mode [1–3]. In contrast to hydrophilic interaction chromatography (HILIC) and ion-pair reversed phase chromatography, which have also been proposed for the separation of hydrophilic molecules [1], ANP chromatography relies neither on the use of higher buffer concentrations nor of ion-pair reagents. Eluent systems suitable for ANP chromatography utilize acetonitrile (or acetone) as the weak and water as the strong elution solvent, including minor amounts of volatile buffers or acids as modifiers. The high content of organic solvent in the eluent system is both favorable for compatibility with mass spectrometry detection and also enables straightforward sample preparation protocols using organic solvent protein precipitation without subsequent solvent evaporation [1–5].

The ANP chromatography method presented here is useful for accurate und selective quantitation of the hydrophilic sulphur amino acids homocysteine (Hcy), cysteine (Cys) and methionine (Met). Several observational studies have confirmed that elevated plasma Hcy concentration might be an independent predictor of cardiovascular disease risk [6–8]. There is considerable interest in quantitative methods for the measurement of plasma Hcy both for clinical diagnosis and for epidemiological research. The biochemistry of Hcy is closely linked to Met and Cys. The essential amino acid Met is first activated to S-adenosylmethionine, a universal methyl donor for transmethylation reactions, including synthesis of neurotransmitters and methylation of DNA and RNA [6,9]. After donating its methyl group, S-adenosylmethionine is converted to S-adenosylhomocysteine, which is subsequently hydrolyzed to Hcy and adenosine. Hcy is remethylated to Met or irreversibly converted to Cys by transsulfuration [10]. Hcy can also be converted to homocysteine-thiolactone by methionyl-tRNAse in an error-editing reaction [11] or S-nitroso-homocysteine [12]. Hyperhomocysteinemia has also been related to nutritional deficiency (folate or cobalamin) [13], disease (diabetes) or genetic factors [9,14]. In addition to increased cardiovascular disease risk [6,7], hyperhomocysteinemia is also a risk factor for stroke, tumor [15], Alzheimer's disease and dementia [16].

Interest in Hcy as a diagnostic and predictive biomarker has furthered development of several quantitative analytical methods.

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Hcy can be assessed with expensive enzyme- and immunoassays [17]. Various high performance liquid chromatography (HPLC) methods were developed using ultraviolet, fluorescence, or electrochemical detection [18]. Gas chromatography–mass spectrometry methods [19,20] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) were established for the analysis of Hcy or its metabolites, including Hcy-thiolactone [12] and N-homocysteinylation at albumin lysine-525 [21]. LC–MS/MS methods afford several advantages including high specificity by multiple reaction monitoring (MRM), elevated sensitivity and decreased run time. Methods for LC–MS/MS analysis of total homocysteine (tHcy) were published first by Magera in 1999 [22] and subsequently by others [23,24]. More recently, tHcy was measured together with related metabolites including total Cysteine (tCys) [25,26], Met [25] and methylmalonic acid [27]. Previously published LC–MS/MS methods achieved cycle times of about 3–4 min by using C_{18}-, C_8- or CN-columns [23,24,28]. Common to these proposed methods is that very short cycle time was achieved by sacrificing adequate chromatographic retention and separation of analytes, which eluted almost immediately after the column void volume. In LC–MS/MS analysis however, inadequate separation from complex biological matrices may result in ion-suppression effects and spurious signals due to co-eluting isobaric components [29].

The reported application utilizes ANP chromatography to optimize separation of the hydrophilic amino acids Hcy, Cys and Met. Occurrence of co-elution bias was systematically assessed and excluded using qualifier ion monitoring.

2. Experimental

2.1. Chemicals and reagents

Methionine and dithiothreitol were supplied from Sigma (Taufkirchen, Germany). Acetonitrile, water, water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid (all LC–MS grade) were purchased from J.T.Baker (Griesheim, Germany). Homocysteine, cysteine and formic acid (LC–MS grade) were supplied by Fluka (Taufkirchen, Germany). Plasma controls (ClinChek® Plasma Control, Lyophilized, for Amino Acids, Levels I and II; ClinChek® Plasma Control, Lyophilized, for Homocysteine, Levels I and II, ClinCal® Calibrator for Hcy) and ClinMass® d8-Homocystine were obtained from Recipe (Munich, Germany).

2.2. ESI-MS/MS operating conditions

A hybride triple quadrupole mass spectrometer (4000 QTRAP®, AB Sciex, Darmstadt, Germany) with a Turbolon source operating in positive electrospray ionization mode (+ESI) was used. System operation and data acquisition were controlled using Analyst™ software 1.5 (AB Sciex, Darmstadt, Germany). Multiple reaction monitoring (MRM) mode was utilized. Ion spray voltage was set to 5.5 kV and source temperature (TEM) to 700 °C. Collision activated dissociation gas (CAD) was set at 4 psi and nitrogen was used as collision gas. Nebulizer gas (GS1) was set to 80 psi just like auxiliary gas (GS2). Curtain gas (CUR) was set to 50 psi. Compound dependent operating parameters (declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)) were optimized by injecting pure solutions of Hcy, Cys and Met into the ion source with a syringe pump in manual tuning mode. Full product spectra with varying collision energy were recorded and the three highest intensity fragments were used for quantification and qualification (see Section 3.1 and Table 1).

Table 1
Instrument settings. MRM transitions and optimized ion path parameters of analytes and internal standard for the ABSciex 4000Qtrap. Qualifier ions are marked with an asterisk (*).

	MRM transition	DP [V]	EP [V]	CE [V]	CXP [V]
Hcy.01	136.0/90.0	41	10	17	6
Hcy.02*	136.0/56.1	41	10	27	10
Hcy.03*	136.0/118.1	41	10	11	8
Hcy-d4.01	140.1/94.0	41	10	17	6
Hcy-d4.02*	140.1/59.1	41	10	27	10
Hcy-d4.03*	140.1/122.0	41	10	11	8
Met.01	150.1/104.0	41	10	15	6
Met.02*	150.1/133.0	41	10	15	8
Met.03*	150.1/61.0	41	10	31	10
Cys.01	122.0/59.0	41	10	33	8
Cys.02*	122.0/76.0	41	10	20	8
Cys.03*	122.0/105.0	41	10	14	8

2.3. LC–ESI-MS/MS analysis

An Agilent 1200 SL series HPLC system (Waldbronn, Germany) was used, combining a G1379B degasser, a G1312B binary pump, a G1367C autosampler and a G1316B column oven. A silica hydride-based stationary phase (Cogent Diamond Hydride, 15.0 cm × 2.1 mm, 4 μm particle size, MicroSolv Technology Corporation, Eatontown, USA) was used with a Phenomenex® Luna HILIC SecurityGuard® pre-column (4.0 mm × 2.0 mm, Phenomenex®, Aschaffenburg, Germany). Autosampler injection volume was set to 2 μl, eluent flow rate to 500 μl/min. After 3 min of equilibration time, the gradient of the mobile phase started with 10% eluent A (water with 0.1% formic acid) and 90% of eluent B (acetonitrile with 0.1% formic acid), linearly increasing to 70% of eluent A at the end of the run after 4 min.

The HPLC system was coupled via a 2-position Valco switching valve (VICI AG International, Schenkon, Switzerland) to a 4000 QTrap mass spectrometer operating in triple quadrupole mode with settings detailed in Section 2.2. Analytes and internal standard (IS), d_4-homocysteine (d_4-Hcy), were detected with MRM mass transitions shown in Table 1.

2.4. Collection and handling of plasma samples

For method development and evaluation, 227 plasma samples from volunteers examined for their folic acid status were used. The samples were collected into Li-Heparin containing tubes, centrifuged and the plasma stored at −80 °C. The vials were unfrozen at room temperature and sample preparation was started immediately after thawing. Additional plasma samples of two in-house volunteers were collected in the same way and used as quality control plasma for the assessment of inter-batch reproducibility. Lyophilized RECIPE® control plasma samples were dissolved in 3.0 ml water according to the manufacturer's instructions.

2.5. Preparation of aqueous standards

Aqueous calibration samples were freshly prepared before sample preparation. The stock solution contained 9.72 mM Hcy, 9.96 mM Met, 39.7 mM Cys and dithiothreitol (77 mg/ml) in water. The stock solution was diluted with water to yield a series of 10 standard samples with approximate ranges of 0.1–100 μM (Hcy, Met) or 0.4–400 μM (Cys), covering the range of physiological concentrations.

2.6. Sample preparation

Sample preparation was done by a liquid handler GILSON® GX-271 (Middleton, WI, USA). 20 μl of plasma or aqueous calibration

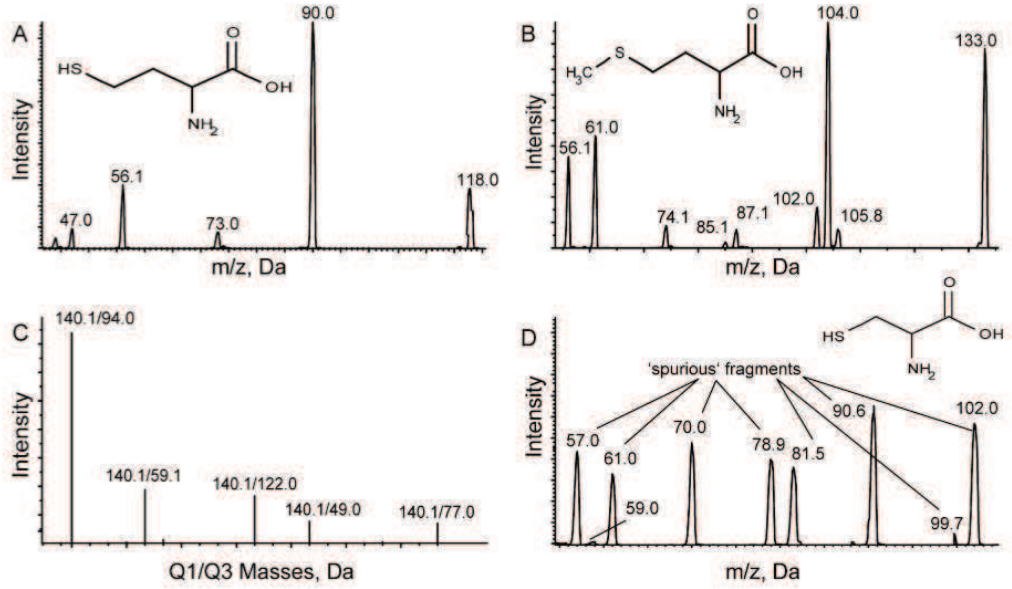

Fig. 1. Product scans. (A) Final products scan of Hcy after compound optimization. (B) Final products scan of Met after compound optimization. (C) MRM transitions of d_4-Hcy. (D) Final products scan of Cys after compound optimization with spurious fragments.

samples, respectively, was mixed with 20 μl of an aqueous IS solution containing 5 μg/ml d_8-homocystine (d_4-homocysteine after reduction) in a Riplate® 1.2 ml 96-deepwell plate (Ritter, Schwabmuenchen, Germany). Thereafter, 20 μl of the reduction reagent dithiothreitol (77 mg/ml) [10] was added and the plate was mixed for 5 min on a neoLab® thermo shaker (Heidelberg, Germany) with 600 rpm at room temperature. After incubation for 15 min at room temperature, 100 μl of the precipitation reagent (0.1% formic acid in acetonitrile) was added. The solution was mixed again for 5 min and then incubated for 30 min at 4 °C. After centrifugation for 10 min at 500 × g at room temperature, 2 μl of the supernatant was injected into the HPLC–MS/MS system, effectively loading an equivalent volume of 0.25 μl plasma. Blank and zero samples were prepared in the same way using water instead of plasma.

As an optional variation of the aforesaid protocol, sample clean-up using filter plates was tested. To this end, 100 μl of supernatants from the centrifugation step in Section 2.6 was transferred to solvent-resistent 96-well filter plates (MultiScreen® Solvinert, 0.45 μm pore size, Millipore, Billerica, MA, USA) with hydrophilic or hydrophobic membranes, respectively. The filtrate was collected in standard 96-wellplates after centrifugation at 500 × g for 5 min at room temperature. This filtration procedure was not found to be necessary and was therefore omitted in the final sample preparation protocol (see Section 3.3).

2.7. Method comparison

The developed LC–MS/MS method for the determination of Hcy was compared with an ADVIA Centaur® XP Immunoassay System (Siemens, Eschborn, Germany), a fully automated competitive chemiluminescence immunoassay for Hcy. 20 EDTA plasma samples from healthy male volunteers were analyzed in comparison. For determining the Hcy concentration using the immunoassay, the blood samples were analyzed shortly after collection. Aliquots of blood samples were transferred to Eppendorf vials, centrifuged at 2500 rpm for 5 min and the plasma stored at −80 °C until determination using the reported LC–MS/MS method.

3. Results and discussion

3.1. ESI-MS/MS condition optimization

High signal intensity was observed when the pure solutions of Hcy and Met were analyzed in positive ESI mode. The $+Q_1$ full scan mass spectra showed predominately protonated molecular ions at m/z 136.0 for Hcy and m/z 150.1 for Met. Major product ion of Hcy was m/z 90.0 (loss of 46 Da). Further Hcy fragments 56.1 and 110.1 showed similar intensity after optimization of ion path conditions (Fig. 1). Met was fragmented into product ions m/z 104.0, 133.0 and 61.0 with descending order of intensity (Fig. 1). During continuous infusion analysis, we noticed 'spurious' fragments in the product ion scan for Cys, which were not reported in previously published work and suppressed the primary product ions of Cys (Fig. 1). We decided to scan for the three major product ions of Cys (m/z 59.0, 76.0 and 105.0) found in previously published articles [25,26] and publicly available databases [30,31] and to optimize compound dependent parameters in manual tuning mode. All collected mass transitions were subsequently validated by chromatographically separating the analytes from isobaric solvent interferences. As mentioned above, this procedure was especially necessary in the case of Cys for sorting out 'spurious' product ions probably resulting from solvent impurities.

For each analyte the highest intensity product ion was utilized for quantification and the next two as qualifier ions. Qualifier ions permit accurate identification since the ratio between product ions of a given precursor is known to be a consistent property of the analyte molecule in the absence of co-eluents. Observed qualifier ratios were approximately normally distributed with standard deviation of 2% of the mean (Fig. 2). Based on the precision of the qualifier ion ratio determined from aqueous standards, we used cut-off limits outside of 91–109% of the normalized qualifier ion ratio for the exclusion of suspect samples. To our knowledge qualifier ions were not used for Hcy, Met and Cys analysis before. Using this approach, reliable analyte qualification in complex biological matrices is easily achieved (see also Section 3.2).

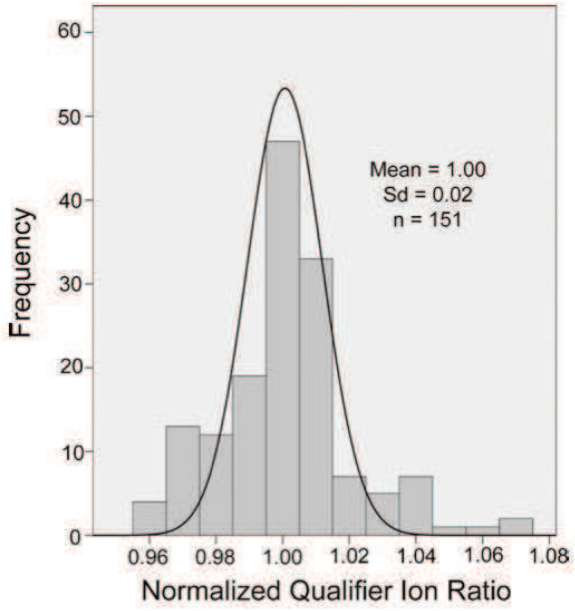

Fig. 2. Qualifier Ions. Histogram with density curve for the ratio of the first qualifier ion for Hcy (136.0/56.1) to the main Hcy transition (136.0/90.0). 151 plasma samples were analyzed during method development.

3.2. Chromatography

As mentioned above, the major aim of this method development was to achieve better chromatographic separation for simultaneously quantification of Hcy, Met and Cys. Best peak shape and resolution were observed using the silica hydride column in aqueous normal phase (ANP) mode. ANP is a chromatographic method using aprotic organic solvents (mostly acetonitrile, or sometimes acetone) at concentrations above 50–70% as the weak and water (optionally together with methanol) as the strong elution solvent. The surface of silica hydride columns is composed primarily of Si-H moieties with low-carbon content [3,5]. Retention mechanism of ANP chromatography on silica hydride stationary phases is currently not completely understood [1,5]. It is presumed that, in addition to partitioning effects similar to HILIC chromatography, analytes may also interact directly with the stationary phase surface [5,32]. The water layer on the stationary phase seems to be weaker and less dense in comparison to HILIC, permitting rapid equilibration [4,5]. In contrast to HILIC, high buffer concentrations, which often result in significant ion suppression during mass spectrometric detection, are not necessary to obtain good peak resolution. Therefore, ANP chromatography with minor amounts of volatile acidic modifiers provides very MS-friendly conditions for selective and sensitive quantitation of positively charged polar compounds.

Utilizing these advantages of ANP chromatography, separation of Hcy, Met and Cys from matrix components was considerably improved compared to previous methods [25–28]. Retention times (RT) of Hcy (3.3 min), Cys (3.35 min) and Met (3.3 min) were acceptably short, with good separation from the column void volume (1.1 min). In favor of improved retention, chromatographic run time was prolonged (4 min gradient time plus 3 min equilibration time) compared to previously published methods which used run times of only 2–4 min. In these methods, sufficient separation of analytes from matrix components was sacrificed for very short cycle time. Elution of Hcy directly following the column void volume was however potentially not sufficient to separate analytes and IS from interfering matrix constituents.

The need for chromatographic separation was underscored in this work by analyzing 227 plasma samples from healthy male volunteers participating in a clinical trial with folic acid supplementation. Co-eluents with transitions *m/z* 140.1/122.0 (d_4-Hcy_03) and *m/z* 150.1/133.0 (Met_02) were found, see Fig. 3. These co-eluents only emerged in plasma of some of the subjects studied. To our knowledge these co-eluents were not described in previous studies nor appeared in published chromatograms. Interfering signals (Fig. 3) of co-eluents would lead to biased ratios of qualifier ions and therewith to an imprecise qualification when separated insufficiently from the internal standard or analyte. This case demonstrates impressively the advantages of proper chromatographic separation, especially when dealing with complex biological matrices whose composition is supposed to change between samples. Co-eluents may also influence the quantification ion and therefore bias the calculated quantitation result. The use of adequate chromatographic separation together with continuous qualifier ion monitoring therefore represents an important improvement towards accurate and unbiased quantification of endogenous metabolites by LC–MS/MS.

Retention times of d_4-Hcy, Hcy, Met and Cys were very similar, thus we assumed that ion suppression affecting the intensity of each analyte and the IS were also comparable. In this case it is possible to use only one IS (d_4-Hcy) for all analytes to improve the cost-effectiveness of the assay. Chromatographic RT was very stable over at least 88 samples of one batch with coefficients of variation of 0.34% (Hcy), 0.51% (Met) and 0.35% (Cys). Variation of retention times between batches was 3.08–3.49 min (Hcy), 3.07–3.31 min (Met) and 3.15–3.37 min (Cys) with about 300 plasma samples analyzed in total. All qualifier ion ratios were determined within defined acceptance limits (see Section 3.1), so that further co-eluents could be excluded (Fig. 2).

3.3. Sample preparation

For protein removal different methods were tested. Initially, filters with a hydrophobic polytetrafluoroethylene (PTFE)/polypropylene (PP) membrane were used. It was observed that in several wells a liquid residue remained in the filter plate after centrifugation and the filtered amount of liquid phase exhibited unacceptably high variation. This unwanted effect is probably associated with the size of the aqueous fraction in the mixture. If the hydrophilic fraction forms a layer between the hydrophobic membrane and the liquid supernatant, the repellent interactions between polar and non-polar phases will not allow the fluid phase to pass through the filter. To avoid this problem, hydrophilic filters can be used or the hydrophobic filters have to be pre-wet with organic solvent.

As pre-treatment of filter plates adds an additional working step and enhances the total analysis time, we compared a preparation method utilizing hydrophilic filter with modified PTFE/PP membrane and preparation without any filter plate (see Section 2.6) using 33 plasma samples. Measured Hcy, Met and Cys concentrations showed very low deviation between the two preparation methods (Table 2). Both reproducibility and accuracy exhibited no significant differences between protocols using hydrophilic filter plates or no filtration at all (intra-day reproducibility 6.0% CV vs. 4.1% CV, accuracy 100.41% vs. 101.04%). Since both methods gave nearly identical results, sample preparation without any filter was clearly the more cost-effective and straightforward alternative and was therefore used in the final protocol.

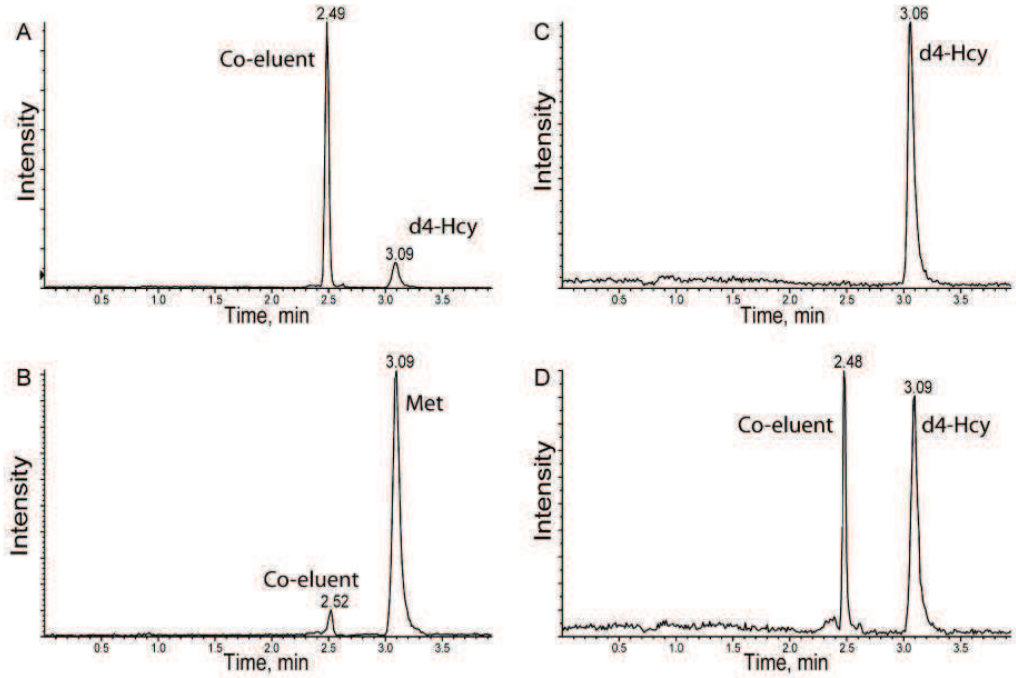

Fig. 3. Separation of interfering co-elutions. (A), (C), (D) Extracted chromatograms of ion transition 140.1/122.0 (d4-Hcy) in plasma samples of 3 different subjects. (B) Extracted chromatogram of ion transition 150.1/133.0 (Met) in plasma.

Table 2
Comparison of sample preparation with and without hydrophilic protein filtration. Measured plasma concentration and deviations of Hcy, Cys and Met of 33 plasma samples.

	Mean without filter [μM]	Mean with hydrophilic filter [μM]	Deviation [μM]	Rel. deviation [%]
Hcy	12.4	12.3	−0.1	−1.0%
Met	32.7	33.0	0.3	1.0%
Cys	244.6	240.3	−4.3	−1.7%

3.4. Linearity, accuracy, recovery and precision

For the determination of limits of detection (LOD) signal-to-noise ratio (S/N) had to be >3, for lower limits of quantification (LLOQ) S/N > 10 was necessary [33]. To identify these limits, aqueous standard samples were used (Table 3). As the standard solution with the lowest concentration was below the LLOQ of Hcy, Met and Cys, this standard was excluded from calibration. The remaining 9 aqueous standards showed good linearity for Hcy, Met and Cys. Fig. 4 illustrates the linearity of Hcy calibration. Calibration equations were $y = 0.0317x + 0.0084$ ($R^2 = 0.9992$, Hcy), $y = 0.0267x + 0.0139$ ($R^2 = 0.9991$, Met) and $y = 0.0059x + 0.0145$ ($R^2 = 0.9993$, Cys). As the calibration equation was created with water samples, the accuracy for plasma samples had to be proven. Accuracy of quantification in plasma samples for Hcy and Met was checked using commercially available lyophilized control plasma. Results are summarized in Table 4. No control plasma was available for Cys, so recovery of Cys was tested by spiking 3 different plasma samples with low and high concentration aqueous solutions (Table 5). Recovery was determined by subtracting the calculated concentration of the unspiked sample from those of the spiked sample. The result of the precision research is shown in Table 3. Three different plasma samples were prepared and analyzed at 5 different dates for the evaluation of inter-batch reproducibility. Accuracy, recovery, precision and LODs/LLOQs were similar to previously reported results [10,14].

Table 3
Precision. Average precision of 4 replicates of 3 plasma samples each. LOD and LLOQ for each analyte.

Analyte	Average intra-batch CV [%]	Inter-batch CV [%]	LOD [μM]	LLOQ [μM]
Hcy	1.66%	4.89%	0.1	1
Met	2.46%	4.80%	0.1	1
Cys	1.97%	5.05%	0.4	4

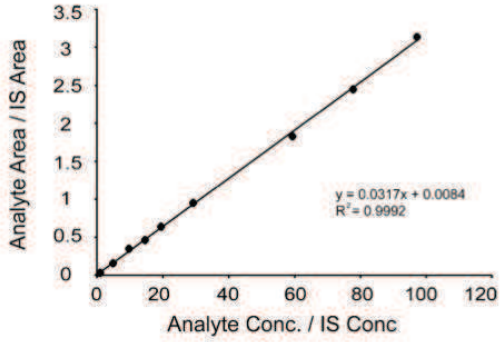

Fig. 4. Calibration curve. Peak area ratio vs. concentration ratio for Hcy with d_4-Hcy as IS.

Table 4
Accuracy and precision of Met and Hcy determination in control plasma. Accuracy is calculated as the quotient of detected and expected concentration. Precision is given as the CV. Each plasma sample was independently processed 5 times.

	Plasma level	Set value [μM]	Mean [μM]	SD [μM]	CV	Accuracy [μM]
Hcy	Low	10.1	9.86	0.72	7.26%	97.65%
	Medium	14.4	15.25	0.40	2.65%	105.90%
	High	23.5	23.40	0.72	3.06%	99.57%
Met	Low	39	38.60	1.13	2.93%	98.97%
	High	68.2	62.25	0.78	1.25%	91.28%

Table 5
Recovery of Cys in spiked plasma ($n = 3$). Recovery is the quotient of the measured difference to unspiked plasma and spiked amount.

Spike level	Spike [μM]	Mean [μM]	Mean recovery
Plasma	0	241.71	–
Low	21	265.21	112%
High	63	307.83	105%

3.5. Method comparison

The developed method was compared with a chemiluminescence immunoassay, a standard method for Hcy determination routinely used in the clinical laboratory. Hcy concentrations of 20 plasma samples analyzed with both methods showed good correlation ($y = 0.9703$, $x - 0.7953$, $R^2 = 0.9881$, y = LC–MS/MS, x = immunoassay) (Fig. 5). In addition, both methods showed favorable distribution of concentration-dependent deviation from the mean of the two determinations, as shown by a Bland–Altman plot (Fig. 5). Even with high concentrations of Hcy, the difference of measured values was in the limits of agreement of the Bland–Altman plot (±1.96 SD). Hcy concentrations determined by LC–MS/MS tended to be little lower compared to the immunoassay. Mean difference was −1.12 μM, which may be explained by differences in sample storage, since the samples for the immunoassay were longer stored at room temperature. Kuhn et al. found an increase of Hcy in serum samples compared to plasma Hcy, accusing the additional time until coagulation as the reason for release of Hcy from erythrocytes [24]. Therefore it seems advisable to remove plasma from erythrocytes as soon as possible after sample collection [14].

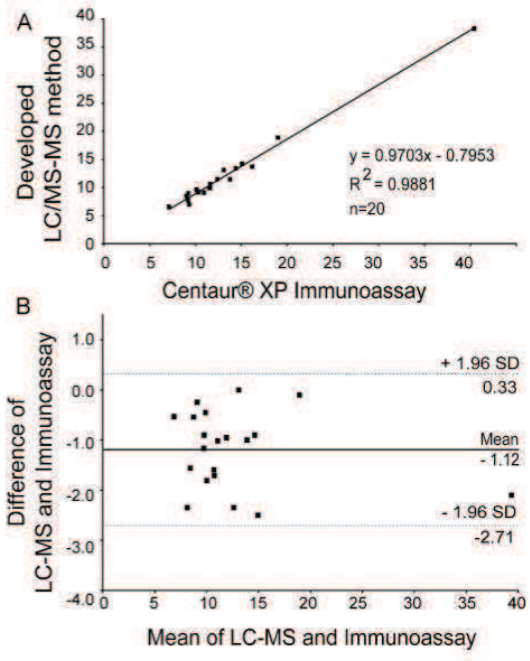

Fig. 5. Method comparison. (A) Linear regression to the comparison of the LC–MS/MS method and Centaur® XP Immunoassay. (B) Bland–Altman plot of the difference vs. the mean value of the LC–MS/MS method and Centaur® XP Immunoassay for 20 plasma samples.

4. Conclusions

The developed LC–MS/MS method permits efficient quantification of Hcy, Cys and Met. Reproducibility and accuracy of the method are comparable to previously published protocols. Improved chromatographic separation and continuous qualifier ion monitoring assure unbiased determination. The benefits of using ANP chromatography pre-separation for LC–MS/MS coupling were exemplified in this report using an expensive 4000Qtrap mass spectrometry system (as this was available in the authors' laboratory). The relatively high concentrations of Hcy, Met and Cys in plasma samples, however, permit seamless implementation of the proposed method using standard entry-level LC–MS/MS equipment. Straightforward sample preparation, short run time and low costs per sample allow analysis of large numbers of sample, e.g. from epidemiological studies.

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3. Publication 2: Nonesterified Fatty Acid Determination for Functional Lipidomics: Comprehensive Ultrahigh Performance Liquid Chromatography−Tandem Mass Spectrometry Quantitation, Qualification, and Parameter Prediction

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Nonesterified Fatty Acid Determination for Functional Lipidomics: Comprehensive Ultrahigh Performance Liquid Chromatography−Tandem Mass Spectrometry Quantitation, Qualification, and Parameter Prediction

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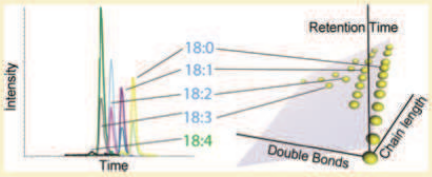
Supporting Information

ABSTRACT: Despite their central importance for lipid metabolism, straightforward quantitative methods for determination of nonesterified fatty acid (NEFA) species are still missing. The protocol presented here provides unbiased quantitation of plasma NEFA species by liquid chromatography−tandem mass spectrometry (LC−MS/MS). Simple deproteination of plasma in organic solvent solution yields high accuracy, including both the unbound and initially protein-bound fractions, while avoiding interferences from hydrolysis of esterified fatty acids from other lipid classes. Sample preparation is fast and nonexpensive, hence well suited for automation and high-throughput applications. Separation of isotopologic NEFA is achieved using ultrahigh-performance liquid chromatography (UPLC) coupled to triple quadrupole LC−MS/MS detection. In combination with automated liquid handling, total assay time per sample is less than 15 min. The analytical spectrum extends beyond readily available NEFA standard compounds by a regression model predicting all the relevant analytical parameters (retention time, ion path settings, and response factor) of NEFA species based on chain length and number of double bonds. Detection of 50 NEFA species and accurate quantification of 36 NEFA species in human plasma is described, the highest numbers ever reported for a LC−MS application. Accuracy and precision are within widely accepted limits. The use of qualifier ions supports unequivocal analyte verification.



Alterations of nonesterified fatty acids (NEFA) metabolism have been observed with numerous disorders like obesity,[1] insulin resistance,[2] diabetes,[3] or metabolic syndrome.[4] Increased NEFA release from lipid tissues is among the most important pathogenic factors in the progression of metabolic syndrome and type 2 diabetes (T2DM).[5] Elevated levels of circulating NEFA negatively affect both peripheral insulin sensitivity and β-cell functioning, contributing to the vicious circle of lipotoxicity.[6] Specific patterns of the plasma NEFA profile discriminating healthy controls from patients with T2DM[7,8] or metabolic syndrome[4] have been reported by several groups. In addition to static profiles, dynamic changes of NEFA concentrations during food challenge testing provide important insight about the functional health of the metabolic system.[9]

The analytical protocol described here was developed for direct and unbiased quantification of NEFA species in plasma and serum. To be useful for large-scale functional lipidomics approaches, the procedure had to meet the following requirements of (a) quantitative recovery of plasma NEFA, (b) mild sample preparation conditions to avoid bias from hydrolysis of esterified fatty acids of other lipid classes, (c) reliable separation, quantification, and qualification of molecular species, (d) elimination of time-consuming extraction, preseparation, or derivatization procedures, and (e) favorable time- and cost-efficiency.

In an attempt to compile a set of analytical standard methods for the rapidly growing field of lipidomics,[10] the LIPID MAPS consortium has established a widespread analytical platform.[11] According to this specification, all lipid classes, such as triacylglycerols or phospholipids, are measured by liquid chromatography−mass spectrometry (LC−MS) except NEFA[12] which are determined using gas chromatography (GC).[13]

GC has traditionally been used as the routine procedure for analysis of NEFA[8] providing very high chromatographic resolution for FA analysis, especially regarding separation of structural isomers differing only in positions of double bonds (DB). Nevertheless, FA have to be derivatized to enhance the volatility for GC,[14] which complicates direct determination of NEFA and warrants special extraction procedures for NEFA[15] or extraction of lipids[16] and subsequent separation of lipid classes, e.g., by solid phase extraction.[17]

Several protocols of precolumn FA derivatization for HPLC or LC−MS detection have been proposed, including formation

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of FA dimethylaminoethyl esters,[18] trimethylaminoethyl ester iodides,[19] and picolylamids,[20] some of them providing resolution comparable to GC[18] methods and excellent sensitivity.[19] Again, laborious extraction and/or preseparation procedures are required prior to derivatization to avoid hydrolysis of esterified FA from other lipid classes.

An approach to avoid derivatization and to improve the unsatisfactory fragmentation behavior of FA in ESI-MS/MS uses postcolumn addition of metal cations, like lithium[21] or barium.[22] Another recently published LC−MS/MS method used "pseudo-molecular" multiple reaction monitoring (MRM) with optimized collision cell parameters to avoid strong fragmentation while using the same ions as the precursor and (unfragmented) product ions.[23] Sufficient sensitivity was achieved, but NEFA species were just identified by retention time (RT) and coelution bias was not appropriately addressed.

Since none of the previously available protocols met all of the cited criteria for unbiased high-throughput quantification of NEFA species, we developed a new straightforward and cost-effective protocol for LC−MS/MS. The concept of "pseudo-molecular" MRM transitions was developed further to "differential-energy" MRM qualifier ion monitoring, providing an additional qualification parameter for unequivocal FA identification in addition to chromatographic RT. Furthermore, a regression model was established to predict relevant analytical parameters of any given NEFA species from its chain length and number of double bonds. This enabled us to comprehensively determine the NEFA profile in human plasma, beyond the limits of FA standards availability.

EXPERIMENTAL SECTION

Chemicals and Reagents. Information for chemical and reagents are provided online as Supporting Information (section S-1A).

Preparation of Internal and External Standards. A total of 100 mg of GLC-85 standard was dissolved in 10 mL of methanol. A volume of 5 mL of this solution was diluted with 45 mL of methanol to obtain a concentration of 0.606 g/L for C16:0 palmitic acid and 0.303 g/L of 31 free FA (see section S-1B in the Supporting Information). The stock solution was diluted with water/acetonitrile (50/50) to yield a series of 12 calibration samples with approximate ranges of 0.01−100 μM covering the range of physiological concentrations in human plasma.

For the internal standard (IS) solution, 2 mg of uniformly ^{13}C-labeled palmitic acid was dissolved in 100 mL of acetonitrile, aliquoted in 1 mL cryovials, and stored at −80 °C. A volume of 316 μL of the aliquoted IS solution was diluted with 25 mL of isopropanol to yield the precipitation reagent for sample preparation.

ESI-MS/MS Operating Conditions. A hybrid triple quadrupole mass spectrometer (4000 QTRAP, AB Sciex, Darmstadt, Germany) with a TurboIon source operating in negative ESI mode was used. MRM and scheduled MRM modes with a detection window of 60 s were utilized. System operation and data acquisition were controlled using Analyst software 1.5.1 (AB Sciex, Darmstadt, Germany). Ion path parameter optimization was achieved in the manual tuning mode of Analyst software. Source parameters were optimized using flow injection analysis of the GLC-85 solution. Accordingly, the ion spray voltage was set to −4 kV and source temperature (TEM) to 650 °C. The collision activated dissociation gas (CAD) was set at 5 psi, and nitrogen was used as the collision gas. The nebulizer gas (GS1) was set to 60 psi, the auxiliary gas (GS2) to 90 psi, and the curtain gas (CUR) to 30 psi. Compound dependent ion path parameters (declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP)) were optimized using continuous infusion (FIA) of a GLC-85 solution with a syringe pump (section S-2 in the Supporting Information).

Full product spectra with varying CE were recorded for fragmentation research. Q1 and Q3 mass filters were set to the monoisotopic $[M - H]^-$ mass of the FA analyte, resulting in so-called "pseudo-molecular" MRM transitions.[23] MRM experiments with low CE were used for NEFA quantification to gain high sensitivity. A second MRM experiment was implemented for every FA using the same mass transition but increased CE. Thus, two transitions were measured for 26 NEFA species whereby the transition with higher intensity was used for quantification and the ratio of both transitions was used for qualification.

Chromatographic Conditions. An Agilent 1200 SL series HPLC system (Waldbronn, Germany) was used, combining a G1379B degasser, a G1312B binary pump, a G1367C autosampler, and a G1316B column oven. Chromatographic separation was performed with a UPLC diphenyl column (Pursuit UPS Diphenyl, particle size 1.9 μm, 100 mm × 3.0 mm; Varian, Darmstadt, Germany). The oven temperature was set to 40 °C to protect the thermally labile polyunsaturated FA.[24] The autosampler injection volume was set to 10 μL and the eluent flow rate to 700 μL/min. After 2.5 min of equilibration with 45% eluent A (water containing 5 mM ammonium acetate, 2.1 mM acetic acid) and 55% of eluent B (acetonitrile with 20% isopropanol), eluent B was linearly increased to 95% during 4 min and was held constant for 3 min for rinsing the LC−MS equipment. The HPLC system was coupled to a 4000 QTrap mass spectrometer with settings detailed above.

Collection and Handling of Plasma Samples. For method development and evaluation, plasma samples taken from eight subjects during oral glucose tolerance testing (OGTT) were used. Overnight fasted subjects ingested 300 mL of a standardized carbohydrate solution (equivalent to 75 g of glucose, Accu-Check Dextro O.G-T., Roche Diagnostics, Mannheim, Germany). Blood samples were taken 5 min before intake and 1 or 2 h after the challenge test. Samples were collected into EDTA containing tubes and centrifuged, and the plasma was stored at −80 °C.

Sample Preparation. Sample preparation was automated using a GILSON GX-271 liquid handler (Middleton, WI). A volume of 20 μL of plasma or calibration samples, respectively, was mixed with 200 μL of IS solution in a 96-deepwell plate to precipitate proteins. Thereafter, the plate was shaken for 30 min and centrifuged for 10 min at 2300g at room temperature. A volume of 50 μL of the supernatant was transferred into a shallow 96-well plate. Blank samples were prepared in the same way using water/acetonitrile (50/50) instead of plasma.

Validation Procedure. A 12-point dilution series was prepared from the GLC-85 standard solution (see above) for external calibration. For each of the NEFA species, at least four of the 12 calibrator solutions were chosen for calibration according to the range of physiological concentrations. Calibration curves were linearly fitted with a weighting factor of $1/x^2$. For determination of precision, three plasma samples of one individual with different NEFA concentrations were used (see Collection and Handling of Plasma Samples). The

samples were measured five times on the same day for intra-assay precision and twice on four days each for interassay precision. Accuracy was analyzed by spiking the 12 standard solutions into one low-concentrated plasma sample collected 1 h after the OGTT challenge. The difference of unspiked versus spiked plasma concentrations was compared to the spiking concentration. For each NEFA, at least 4 of the 12 prepared spiked concentrations were used for determination of accuracy, covering the physiological range. Five standard solutions with a concentration range of 0.001−10 μM were prepared to assess the LOD (limit of detection) and LLOQ (lower limit of quantification). The LOD was determined as the lowest concentration resulting in S/N > 3, while LLOQ was determined measuring each concentration five times and subsequently calculating precision (<20% CV) and accuracy (80−120%). Recovery was determined using NEFA standards in albumin solutions with four different concentrations (30, 40, 50, and 60 g/L). Aliquots of GLC-85 solution (100 μL), with concentrations of 10 μM, were dried under nitrogen and redissolved with 100 μL of one of the four albumin solutions. The mixture was incubated at 37 °C for 30 min prior to LC−MS/MS analysis.

Additionally, investigations for ion suppression were accomplished. According to Matuszewski et al.,[25] two sets of samples were prepared. Set 1 was pure standard solutions with concentrations of about 0.1, 1, 5, and 10 μM. For set 2, plasma samples of three different individuals with low NEFA concentrations (120 min after OGTT challenge) were prepared as mentioned above. After transfer into a 96-well plate, the solvent was removed under nitrogen and the sample redissolved with 50 μL of the four standard solutions of set 1. The extent of ion suppression was then calculated as

$$\text{Ion suppression} = 1 - \frac{\text{Analyte peak area}_{\text{set2}}}{\text{Analyte peak area}_{\text{set1}}}$$

Prediction Model. A prediction model was implemented to facilitate detection and quantification of NEFA species not included in the standard solution. Empirical results determined from NEFA species of the GLC-85 standard were used as the training data set for fitting prediction models of analytical parameters of unavailable NEFA species, such as RT to identify peaks, response factors (RF) for calibration functions as well as MS specific parameters (DP, CE) enabling the detection of analytes. As all NEFA species share a very similar overall structure, DB number and chain length (CL) alone were sufficient predictors to model all of the above-mentioned variables. Sets of linear models with linear to cubic polynomial transformations with and without interactions of the independent variables were evaluated using the "glm" function of "R: A language and environment for statistical computing".[26] Mean prediction errors were estimated using leave-one-out cross-validation provided in the R package "boot"[27] (section S-3 in the Supporting Information).

RESULTS AND DISCUSSION

ESI-MS/MS. The most abundant peak in full scan ESI-MS spectra was the deprotonated molecular ion $[M - H]^-$ for all NEFA. The product ion spectrum of $[M - H]^-$ was also dominated by the unfragmented deprotonated molecular ion. The nearly nonexistent production of detectable fragments of underivatized FA in ESI-MS/MS has similarly been described in previously published reports.[28] Enhanced fragmentation with increasing DB number was observed, which is also consistent with prior observations.[29] FA containing three or more DB showed more intense fragment ions, e.g., fragments formed by loss of carbon dioxide $[M - H - 44]^-$ were found for C22:6 and C20:4, but the product spectra were still dominated by $[M - H]^-$. Because of this, "pseudo-molecular" MRM transitions, as described by Schiesel,[23] were used for quantification (see section S-2 in the Supporting Information).

When plasma was investigated with these parameters, saturation of detection for physiologically higher concentrated NEFA was observed. Thus, compound dependent parameters were adjusted to achieve linear calibration in the physiological range and normal peak shape. CE values for C14:0, C16:0, C16:1, C18:0, C18:1, and C18:2 were increased until calibration curves showed linearity.

Differential Energy Qualifier Ion Monitoring. Qualifier ion monitoring is a valuable technique in LC−MS/MS analysis which permits reliable verification of the analyte simultaneously with quantification.[30] The qualifier ion ratio (QIR) is usually calculated as the ratio of the respective signal intensities of two alternative fragmentation routes of the same molecule. The QIR is a constant property of the analyte given fixed ion path conditions, which is equal for MRM spectra of flow injection analysis and LC−MS/MS applications as well as for neat standard solutions and biological samples. The QIR of each sample has to be in the range of 75−125% of a pure reference sample, further deviation of the QIR being a useful indicator of biased determination due to coelution.[31] QIR monitoring has been successfully implemented for residue analysis,[32] forensic medicine,[33] as well as metabolomic analysis of amino acids[34,35] but, to our knowledge, not yet established for FA qualification.

In collisionally induced dissociation (CID) experiments, there are no detectable fragment ions of saturated FA, although the intensity of the $[M - H]^-$ parent ion is reduced with increasing CE. As a possible explanation of this phenomenon, the authors speculate that FA predominantly fragment by losing (parts of) the carboxyl headgroup with resulting charged fragments being too small for detection in quadrupole mass spectrometers. Given this special property of FA, the usual QIR concept was not applicable here. Instead, as a molecular fingerprint for FA qualification in MS/MS analysis, the uniquely shaped relation of observable signal intensity to CID collision energy was used (Figure 1A).

A second MRM experiment was implemented for every FA using the same mass transition but increased CE. The CE of these qualification transitions was adjusted to yield a signal intensity of about 50% compared to the quantifier transition.

This pair of differential-energy MRM transitions yielded a specific ratio of signal intensities for a specific FA but not for a (potentially) coeluting isobaric interfering compound. "Differential energy QIR" was defined as the ratio of the "pseudo-molecular" and the "pseudo-qualifier" transition. Standard reference QIR and limits of agreement (average ±25%) were determined from 16 pure standard samples with 11 different concentrations (section S-4 in the Supporting Information).

Mean observed QIR of 55 plasma samples from eight individuals, during a challenge test, were favorably compared to standard reference QIR and all plasma QIR were within limits of agreement (section S-4 in the Supporting Information). As previously noted,[30] QIR supports analyte confirmation only if the signal of both transitions is recorded with sufficient intensity and precision. Usefulness of the QIR concept is

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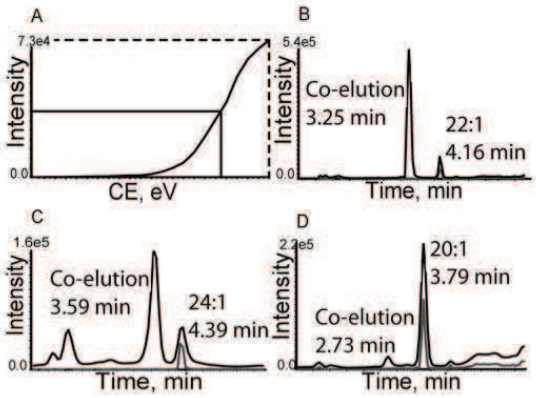

Figure 1. Qualifier ion monitoring. (A) collision energy (CE) dependent signal intensity of the "pseudo-molecular" MRM transition, shown here for FA 22:6 (327.1/327.1) with CE for quantification (dashed line) and qualification transition (solid line). (B,C,D) 22:1 (B), 24:1 (C), and 20:1 (D) showed coeluting peaks in the "pseudomolecular" (quantifier) transition in several plasma samples. To identify the correct peak for the respective fatty acid, the qualifier ion ratio (QIR) of quantifier and "pseudoqualifier" transition was determined.

further demonstrated by identification and proper separation of coeluting compounds (Figure 1B−D).

The "pseudo-molecular" transition of C22:1 showed two peaks in the chromatogram of a plasma sample. For the larger, earlier eluting peak, the qualifier transition was completely missing, while the QIR of the smaller second peak was within limits of agreement. Hence, the second peak could reliably be identified as C22:1. For C20:1 and C24:1, the "pseudo-qualifier" transitions occurred also in the coeluent peaks, but the QIR of coeluents were clearly outside limits of agreement. As underivatized NEFA lack in specific detectable CID fragments and are widely determined by only monitoring the intact deprotonated molecule, progress was needed to leverage the power of MS/MS for detection of coeluted and only partly resolved peaks. Differential energy QIR monitoring provides a valuable tool for reliable confirmation in LC−MS/MS determination of NEFA.

Liquid Chromatography and Sample Preparation. Liquid chromatography and sample preparations conditions were optimized to achieve short run-time while still providing adequate retention to avoid matrix effects and the need for isotope correction. Separation was performed on a diphenyl stationary phase suitable for ultrahigh-performance LC (UPLC). This relatively uncommon reversed-phase material was found largely superior when compared to C8, C18, and phenyl-hexyl by Lee et al.[36] As the weaker elution solvent, water was utilized to facilitate retention of medium chained NEFA such as C10:0 and C12:0. Methanol and acetonitrile were tested as major solvents for the strong eluent with varying addition of 2-propanol to increase elution power for longer fatty acids as well as for other matrix compounds like triacylglycerols.[37] A 2-propanol concentration of 20% was found to be a suitable compromise between backpressure elevation and elution strength. The acetonitrile/isopropanol combination was finally favored as it allowed for higher flow rate, shorter run-time, and better resolution (Figure 2). The time corrected calibrated normalized resolution product (TC-CNRP) was used as optimization criterion for separation of NEFA, which was calculated as follows,

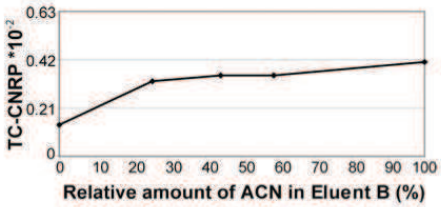

Figure 2. Time corrected calibrated normalized resolution product (TC-CNRP). The average TC-CNRP of 11 fatty acids was determined in different mobile phase compositions. Eluent A, 5 mM ammonium acetate; Eluent B, 20% isopropanol, with varying amounts of methanol and acetonitrile (ACN).

$$\text{TC-CNRP} = \sqrt[n]{\prod_{i=0}^{n}\left(\text{Rs}_{(i,i+1)}/\overline{\text{Rs}}\right)} \Big/ t_{\text{ne}}$$

with $\text{Rs}_{(i,i+1)}$ representing the resolution of two adjacent peaks, $\overline{\text{Rs}}$ the average resolution, and t_{ne} the time correction factor.[38] The TC-CNRP of 11 NEFA was tested including C18:3, C18:2, C18:1, and C 18:0. While the isopropanol fraction of 20% was kept constant, the remaining parts of the strong eluent were composed of different combinations of methanol and acetonitrile ranging from 100% methanol to 100% acetonitrile.

Several HPLC additives were tested. Tributylamine and dibutylamine provided less or equal resolution compared to ammonium acetate. A concentration of 5 mM ammonium acetate was used in the final protocol, providing sufficient resolution without relevant buffer-induced ion suppression. Adjusting the pH value to 5 by addition of acetic acid was found to further improve chromatographic resolution. The final mobile phase was very similar to other published methods using derivatization,[20] postcolumn addition[22] or intact acids.[28,39] The highest applicable flow rate for this mobile phase composition was 700 μL/min (with respect to the 600 bar pressure limit of the HPLC system). This flow rate enabled LC run times of less than 10 min per sample, including equilibration and column flushing. The optimized LC−MS/MS procedure was able to separate NEFA differing by only one DB (mass difference of 2 Da), avoiding potential interference of stable isotopologues (Figure 3).

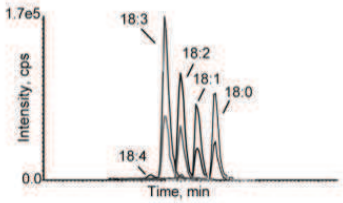

Figure 3. Separation of C18 fatty acids in human plasma by LC−MS/MS.

As the RT of all NEFA remained constant (maximum coefficient of variation (CV) was 1.30%) over 90 measured samples, the scheduled MRM mode was used to improve the number and duration of scan events per peak. In summary, a stable LC procedure was developed with sufficient retention in

only 9.5 min. This run time is very short regarding the number of FA analyzed. Methods with shorter run-times were often developed for a limited number of FA such as saturated FA,[37] polyunsaturated FA,[40] or free FA released from phospholipid formulations.[28] More comprehensive LC−MS methods determining more than 10 FA operated with higher RT, ranging from 15 min[22] to 45 min.[41]

A further approach to decrease total run-time was the implementation of a simple, automated and rapid sample preparation procedure. The optimized sample preparation procedure demanded only the addition of one solvent, with subsequent mixing, centrifugation, and transfer of supernatants.

Thus, total analysis time for a single, separately processed sample was decreased to 55 min. Because of the absence of critical steps like phase separation, the precipitation method was easily implemented with an automated liquid handler. So, total processing time (including sample preparation and LC−MS/MS determination) could be further reduced to 15 min per sample. Compared to previously published LC−MS methods[23,39,42] and even GC applications,[43] the presented method provides an important progress in the development of high-throughput methods, enabling time efficient processing of samples in large-scale clinical or epidemiological studies.

Ion Suppression. As the simple preparation procedure did not exclude matrix components except proteins, a further aim of the LC separation was to minimize ion suppression. Ion suppression of C11:0, C 13:0, C15:1, C22:2, and the uniformly ^{13}C-labeled C16:0 was determined. Measured ion suppression was ranging from 3% (13:0) to 7% (C15:1) with ion suppression of the IS of 6% independent of spiking concentration. The absence of ion suppression was previously reported for LC−ESI-MS.[23] Trufelli et al. justified the use of LC−direct-EI-MS in a recently presented method by absent matrix effects[39] with this ionization source. In exchange, they had to accept a higher LLOQ and to sacrifice comprehensive determination of NEFA, as only FA with a carbon number <22 were accessible with LC−direct-EI-MS. These limitations were not observed with our presented method.

Validation. Linearity was achieved for all NEFA in their physiological range. For example, the regression equation of NEFA C20:0 was $y = 0.398x + 0.0151$ ($r = 0.9915$), where y was the ratio of analyte peak area divided by the internal standard peak area and x was the calculated concentration in μM. The LOD for all NEFA was below 1 nM and not further determined because LLOQ was regarded as the more important limitation. The LLOQ was in the range of 10 to 100 nM for most NEFA (Table 1).

C16:0 and C18:1, the two most abundant NEFA, had a LLOQ of 1 μM due to the elevated CE used to achieve a linear detector response in the range of physiological concentrations. All determined recoveries were within the acceptance range between 80% and 120% of the spiking concentration. The intra-assay and interassay precision of the 22 NEFA in fasted plasma were in the range 0.5−5.3% and 2.3−11.4%, respectively. In addition, precision was also determined in postprandial plasma with low NEFA concentrations. The precision for these samples was still below 20%.

LLOQ of the new method was slightly above the limits previously reported for LC−MS methods with derivatization steps.[36,41] This is not surprising, as derivatization procedures had explicitly been optimized to increase sensitivity. Compared to the LLOQ of recently proposed methods without complex preparation, the presented method affords higher sensitivity with 2-times[23] to up to a 100-times[39] lower LLOQ. Neither precision nor sensitivity was sacrificed for the straightforward and comprehensive analysis.

Table 1. Validation Parameters[a]

	precision (%)				
	intra-assay	interassay	accuracy (%)	range (μM)	LLOQ (μM)
10:0	5.3	8.4	80.3−116.1	0.1−5	0.01
12:0	2.5	2.3	86.5−114.8	0.25−25	0.1
14:0	4.1	7.1	80.5−117.6	0.5−50	0.1
14:1	1.4	5.8	83.1−109.1	0.1−10	0.01
15:0	2.2	10.8	87.6−113.9	0.1−10	0.1
16:0	3.1	9.7	92.7−99.6	10−100	1
16:1	3.4	11.4	84.7−118.7	1−50	0.1
17:0	4.2	11.1	80.2−115.7	0.25−10	0.1
17:1	2.9	8.2	82.3−117.2	0.1−10	0.01
18:0	3.3	10.1	90.8−102.3	5−100	0.1
18:1	0.5	7.7	98.9−106.2	10−100	1
18:2	3.2	8.1	87.7−117.0	5−50	0.1
18:3	3.1	9.2	85.2−117.6	0.5−50	0.01
20:0	4.8	8.7	84.1−118.8	0.025−1	0.1
20:1	0.7	7.6	82.2−112.2	0.1−10	0.1
20:2	2.8	8.1	84.4−103.9	0.1−5	0.1
20:3	1.8	9.0	86.9−109.1	0.1−5	0.01
20:4	2.5	4.8	95.7−111.8	1−25	0.1
22:1	4.8	5.2	88.0−103.2	0.025−0.5	0.1
22:2	3.4	6.1	80.6−96.6	0.025−0.25	0.01
22:6	1.2	9.8	87.0−117.4	0.25−10	0.01
24:1	4.2	8.1	77.5−95.4	0.05−1	0.01

[a]The intra-assay precision was determined by measuring fasted plasma samples with different concentrations five times in one batch. For inter-assay precision, these samples were measured twice on 4 days. For accuracy assessment, aliquots of one low-concentrated plasma sample were spiked with 12 different concentrations, which were determined three times each. The range of the accuracy determination was adapted to physiological concentration. The LLOQ was determined using standard solutions, each analyzed five times with precision limits of less than 20% CV and accuracy between 80 and 120%.

In plasma, NEFA are soluble in concentrations up to about 1 μM. Further increasing NEFA concentrations accordingly increase the plasma albumin-bound NEFA fraction.[44] Therefore, the recoveries from GLC-85 NEFA standard solutions with concentrations of 10 μM were investigated in the presence of different albumin concentrations to ensure accurate quantification independent of albumin concentration (Figure 4).

NEFA recovery was constant over the examined concentration range and independent of the albumin concentration. All determined recoveries were above the acceptable limit of 80%.

Prediction Model. Analyte coverage of FA and NEFA quantitation has traditionally been limited by availability of pure standard compounds. To overcome this limitation, regression models were implemented to predict the analytical parameters of the NEFA species without an available standard (see the Experimental Section). Model specifications, mean prediction errors estimated by leave-one-out cross-validation, and adjusted coefficients of determination (R^2) for the finally selected models are given in Table 2.

Linear models including CL and DB with interaction but without polynomial transformation gave the best results for

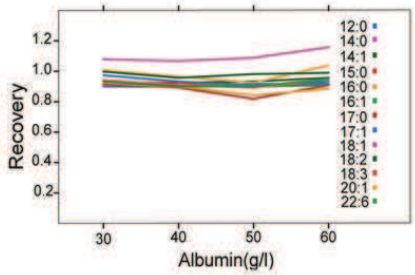

Figure 4. Albumin concentration-dependent NEFA recovery: Standard solutions with high NEFA concentration (10 μM) and varying albumin concentrations were investigated. NEFA recovery was constant over the examined concentration range, independent of albumin concentration and always above 80%.

Table 2. Prediction of Analytical Parameters from NEFA Chain Length and Number of Double Bonds[a]

	degree of polynomial			
	CL	DB	relative prediction error	adjusted R^2
RT	2	1	0.000 13	0.9995
DP	1	1	0.254 97	0.9135
CE	1	1	0.052 51	0.9508
RF	1	1	0.004 99	0.9661

[a]Retention time (RT), declustering potential (DP), collision energy (CE), or response factor (RF), respectively, were modelled by linear models of polynomial transformations of chain length (CL) and double bond (DB) number with consideration of interaction. Mean prediction errors estimated by leave-one-out cross-validation were used as model selection criteria. Adjusted coefficients of determination are given as a goodness of fit statistic.

dependent variables DP, CE, and RF. For the dependent variable RT, the lowest prediction errors were observed including a quadratic polynomial of CL. Most cubic models were inappropriate due to rank-deficiency and were not further pursued, since the simpler models provided excellent goodness of fit, documented by very high coefficients of determination (R^2) and low estimated mean prediction errors (Table 2).

Pooled plasma samples were utilized to search for predicted NEFA species in human plasma using the predicted MS-parameters (section S-5 in the Supporting Information). Observed peaks were assigned to predicted NEFA species only when observed RT deviated less than 5% from predicted RT. Furthermore, appropriate QIR was required for peak assignment. All newly identified NEFA were measured in eight human plasma samples in addition to the NEFA known from the GLC-85 standard (Table 3).

A total of 50 NEFA species were identified in fasted plasma according to RT and QIR, with 26 of them covered by the GLC-85 standard solution and 24 predicted NEFA species. In total, 36 (22/14) of the detected NEFA could be quantified with required precision and accuracy. This is the highest number ever reported for NEFA quantification using LC−MS/MS. In the postprandial state after OGTT challenge, the number of quantifiable NEFA decreased to 26 due to the halting release of NEFA from adipose tissues.

Table 3. Nonesterified Fatty Acid (NEFA) Concentrations (μM, Mean ± Standard Deviation) in Fasting and Postprandial Individuals (n = 8)[a]

NEFA species	fasting	120 min after OGTT challenge
10:0	1.32 ± 0.83	0.17 ± 0.11
11:0	<LOQ	<LOQ
12:0	3.72 ± 2.96	0.41 ± 0.26
12:1	1.3 ± 1.02	0.13 ± 0.09
13:0	<LOQ	<LOQ
13:1	0.31 ± 0.17	<LOQ
14:0	12.12 ± 8.48	1.15 ± 0.5
14:1	2.91 ± 2.12	0.25 ± 0.15
14:2	0.61 ± 0.46	<LOQ
15:0	2.12 ± 1.33	0.36 ± 0.14
15:1	<LOQ	<LOQ
16:0	83.18 ± 52.32	12.2 ± 4.48
16:1	18.83 ± 14.41	1.59 ± 0.72
16:2	0.24 ± 0.14	<LOQ
16:3	0.16 ± 0.08	<LOQ
16:4	0.06 ± 0.05	<LOQ
17:0	1.95 ± 0.94	0.46 ± 0.16
17:1	1.48 ± 0.83	0.24 ± 0.09
18:0	31.38 ± 17.72	5.99 ± 1.72
18:1	149.26 ± 95.27	19.17 ± 10.91
18:2	42.83 ± 25.33	6.12 ± 2.68
18:3	5.56 ± 2.92	0.67 ± 0.31
18:4	0.11 ± 0.05	<LOQ
19:0	0.21 ± 0.09	0.09 ± 0.03
19:1	0.55 ± 0.34	0.09 ± 0.04
19:2	<LOQ	<LOQ
20:0	0.29 ± 0.21	0.09 ± 0.04
20:1	1.69 ± 1.15	0.29 ± 0.12
20:2	1.01 ± 0.59	0.22 ± 0.07
20:3	1.47 ± 0.59	0.41 ± 0.09
20:4	3.66 ± 1.64	0.96 ± 0.25
20:5	0.66 ± 0.31	0.16 ± 0.07
22:0	<LOQ	<LOQ
22:1	0.19 ± 0.13	<LOQ
22:2	0.03 ± 0.02	<LOQ
22:3	<LOQ	<LOQ
22:4	0.5 ± 0.22	0.13 ± 0.03
22:5	1.27 ± 0.55	0.27 ± 0.09
22:6	3.58 ± 1.9	0.91 ± 0.36
24:0	0.24 ± 0.07	0.18 ± 0.05
24:1	0.16 ± 0.06	<LOQ
24:2	<LOQ	<LOQ
24:3	<LOQ	<LOQ
24:4	<LOQ	<LOQ
24:5	<LOQ	<LOQ
24:6	<LOQ	<LOQ
26:1	0.1 ± 0.03	<LOQ
26:2	<LOQ	<LOQ
26:3	<LOQ	<LOQ
26:4	<LOQ	<LOQ

[a]NEFA marked with "<LOQ" could be detected with S/N > 3 but not quantified.

CONCLUSIONS

The new protocol represents important progress of analytical methods for the determination of the NEFA profile in human plasma. The number of quantifiable NEFA species is the highest reported to date for a LC−MS/MS application. Key

benefits of the method are simple sample preparation without laborious extraction or preseparation, rapid analysis time, and consequently low costs per sample. There are clear advantages compared to traditionally used GC analysis which enable quantitative NEFA analysis in large-scale trials.

The achieved improvements in NEFA determination did not compromise precision or accuracy, which are comparable to previously published protocols. Quite contrary, the implementation of differential-energy qualifier ions improved qualification and unbiased quantification. Further improvements of sensitivity can be accomplished, for example, for assessment of very long chained NEFA in peroxisomal disorders or for exact quantitation of low-abundance NEFA in the postprandial state. For these applications, the extraction procedure detailed in the Supporting Information can be utilized to enhance sensitivity of the overall protocol (section S-6 in the Supporting Information). The method is applicable to studies in adults as well as in children and infants, since only 20 μL of plasma volumes are required.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare to have no conflict of interest.

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NEFA determination for functional lipidomics: comprehensive UPLC-MS/MS quantitation, qualification and parameter prediction

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S-1A Chemicals and Reagents

Water, acetonitrile and isopropanol (all LC-MS reagent grade) were supplied from J.T.Baker (Griesheim, Germany). Ammonium acetate ("UPLC-MS optigrade") were supplied by LGC Standards (Wesel, Germany). Methanol ("Chromasolv", LC-MS grade), heptane ("Chromasolv plus", for HPLC) and tributylamine (puriss. plus, ≥ 99.5% (GC)) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetic acid (puriss., additive for LC-MS) was supplied by Fluka (Taufkirchen, Germany). Dibutylammonium acetate (ca. 0.5mol/L in Water, Ion-Pair Reagent for LC-MS) was purchased by TCI Europe (Zwijndrecht, Belgium). Ortho-phosphoric acid 85% (extra pure) was supplied by Merck (Darmstadt, Germany). GLC-85 standard (Nu-Check Prep. Inc., Elysian, MN, USA), containing 32 NEFA species, was used for external calibration. Uniformly labelled palmitic acid (U-13C_{16}, 98 %) was purchased from Euriso-Top (Saarbrücken, Germany) and used as internal standard.

S-1B Composition of GLC-85 solution

C 16:0 palmitic acid

0.606g/l

C4:0 butyric acid
C6:0 hexanoic acid
C8:0 octanoic acid
C10:0 decanoic acid
C11:0 undecanoic acid
C12:0 lauric acid
C13:0 tridecanoic acid
C14:0 myristic acid
C14:1 myristoleic acid
C15:0 pentadecanoic acid
C15:1 pentadecenoic acid
C16:1 palmitoleic acid
C17:0 heptadecanoic acid
C17:1 heptadecenoic acid
C18:0 stearic acid

C18:1 oleic acid
C18:1T elaidic acid
C18:2 linoleic acid
C18:3 alpha-linolenic acid
C18:3 gamma-linolenic acid
C20:0 eicosanoic acid
C20:1 11-eicosenoic acid
C20:2 11-14-eicosadienoic acid
C22:0 behenic acid
C22:1 erucic acid
C20:3 11-14-17-eicosatrienoic acid
C20:3 hommogamma linoleic acidC20:4 arachidonic acid
C24:1 nervonic acid
C22:2 docosadienoic acid
C22:6 docosahexanoic acid

0.303g/l, respectively

S-3

S-2 Instrument settings MRM transitions and optimized ion path parameters of 29 NEFA and internal standard (IS) for the AB Sciex 4000Qtrap. "Pseudo-qualifier" ions are marked with an asterisk (*). ([1]Q1 mass [M-H]$^-$[2]Q3 mass [M-H]$^-$[3]declustering potential[4]entrance potential[5]collision energy[6]collision cell exit potential[7]retention time)

NEFA species	Q1[1] Da	Q3[2] Da	DP[3] V	EP[4] V	CE[5] V	CXP[6] V
4:0	86.9	86.9	-40	-10	-6	-13
4:0*	86.9	87.0	-40	-10	-13	-13
6:0	114.9	114.9	-43	-10	-8	-9
6:0*	114.9	115.0	-43	-10	-15	-9
8:0	142.9	142.9	-50	-10	-8	-9
8:0*	142.9	143.0	-50	-10	-17	-9
10:0	171.0	170.9	-60	-10	-8	-13
10:0*	171.0	171.0	-60	-10	-18	-13
11:0	185.0	185.0	-65	-10	-8	-11
11:0*	185.0	185.0	-65	-10	-20	-11
12:0	199.0	199.0	-68	-10	-8	-11
12:0*	199.0	199.1	-68	-10	-21	-11
13:0	213.0	213.0	-70	-10	-8	-17
13:0*	213.0	213.1	-70	-10	-22	-17
14:0	227.0	227.0	-75	-10	-25	-13
14:0*	227.0	227.1	-75	-10	-30	-13
14:1	225.0	225.0	-75	-10	-8	-13
14:1*	225.0	225.1	-75	-10	-23	-13
15:0	241.0	241.2	-75	-10	-8	-7
15:0*	241.0	241.1	-75	-10	-25	-7
15:1	239.0	239.0	-75	-10	-10	-7
15:1*	239.0	239.1	-75	-10	-25	-7
16:0	255.0	255.0	-80	-10	-35	-13
16:0*	255.0	255.1	-80	-10	-37	-13
16:1	253.0	253.0	-78	-10	-30	-13
16:1*	253.0	253.1	-78	-10	-34	-13
17:0	269.0	269.1	-85	-10	-12	-7
17:0*	269.0	269.0	-85	-10	-28	-7
17:1	267.0	267.2	-75	-10	-10	-7
17:1*	267.0	267.1	-75	-10	-26	-7
18:0	283.1	283.1	-87	-10	-35	-7
18:0*	283.1	283.2	-87	-10	-37	-7
18:1	281.1	281.1	-80	-10	-37	-7
18:1*	281.1	281.0	-80	-10	-39	-7
18:2	279.0	279.1	-80	-10	-32	-7
18:2*	279.0	279.0	-80	-10	-34	-7
18:3	277.1	277.1	-75	-10	-10	-7
18:3*	277.1	277.0	-75	-10	-23	-7
20:0	311.1	311.2	-95	-10	-8	-13
20:0*	311.1	311.1	-95	-10	-32	-13
20:1	309.1	309.1	-90	-10	-8	-9

20:1*	309.1	309.0	-90	-10	-30	-9
20:2	307.1	307.2	-85	-10	-8	-9
20:2*	307.1	307.1	-85	-10	-29	-9
20:3	305.1	305.1	-80	-10	-8	-9
20:3*	305.1	305.0	-80	-10	-26	-9
20:4	303.1	303.1	-70	-10	-10	-9
20:4*	303.1	303.0	-70	-10	-21	-9
22:0	339.1	339.1	-100	-10	-14	-11
22:0*	339.1	339.2	-100	-10	-33	-11
22:1	337.1	337.1	-80	-10	-8	-9
22:1*	337.1	337.0	-80	-10	-30	-9
22:2	335.1	335.1	-80	-10	-8	-11
22:2*	335.1	335.0	-80	-10	-30	-11
22:6	327.1	327.0	-40	-10	-6	-13
22:6*	327.1	327.1	-40	-10	-15	-13
24:1	365.1	365.1	-97	-10	-8	-11
24:1*	365.1	365.0	-97	-10	-33	-11
IS	271.0	271.0	-80	-10	-10	-7
IS *	271.0	271.1	-80	-10	-27	-7

S-3 R code for prediction model

Predictionof retention times with available standards (Complete_set) for other NEFA (New_set)

```
complete.set <- read.csv("Complete_set.csv",sep=";")
New.set <- read.csv("New_set.csv",sep=";")
names(complete.set) <- c("CL","DB","RT")
names(New.set) <- c("CL","DB","RT")

library(boot)
rt.glm.1 <- glm(RT ~ poly(CL,3) + poly(DB,3) + I(DB*CL) + I(DB/CL) + sqrt(DB) + sqrt(CL)+
exp(CL) + log(CL),data=complete.set,family="gaussian")
cv.err.1 <- cv.glm(complete.set,rt.glm.1,K=nrow(complete.set))

rt.glm.2 <- glm( RT ~ poly(CL,3)*poly(DB,3),data=complete.set,family="gaussian")
cv.err.2 <- cv.glm(complete.set,rt.glm.2,K=nrow(complete.set))

rt.glm.3 <- glm( RT ~ poly(CL,3)*poly(DB,2),data=complete.set,family="gaussian")
cv.err.3 <- cv.glm(complete.set,rt.glm.3,K=nrow(complete.set))

rt.glm.4 <- glm(RT ~ poly(CL,3)*DBdata = complete.set,family="gaussian")
cv.err.4 <- cv.glm(complete.set,rt.glm.4,K=nrow(complete.set))

rt.glm.5 <- glm( RT ~ poly(CL,2)*poly(DB,3),data=complete.set,family="gaussian")
cv.err.5 <- cv.glm(complete.set,rt.glm.5,K=nrow(complete.set))

rt.glm.6 <- glm( RT ~ poly(CL,2)*poly(DB,2),data=complete.set,family="gaussian")
cv.err.6 <- cv.glm(complete.set,rt.glm.6,K=nrow(complete.set))

rt.glm.7 <- glm( RT ~ poly(CL,2)*DB,data=complete.set,family="gaussian")
cv.err.7 <- cv.glm(complete.set,rt.glm.7,K=nrow(complete.set))

rt.glm.8 <- glm( RT ~ CL*poly(DB,3),data=complete.set,family="gaussian")
cv.err.8 <- cv.glm(complete.set,rt.glm.8,K=nrow(complete.set))

rt.glm.9 <- glm( RT ~ CL*poly(DB,2),data=complete.set,family="gaussian")
cv.err.9 <- cv.glm(complete.set,rt.glm.9,K=nrow(complete.set))

rt.glm.10 <- glm(RT~CL*DB,data=complete.set,family="gaussian")
cv.err.10 <- cv.glm(complete.set,rt.glm.10,K=nrow(complete.set))

cv.err.1$delta
cv.err.2$delta
cv.err.3$delta
cv.err.4$delta
cv.err.5$delta
cv.err.6$delta
```

```
cv.err.7$delta
cv.err.8$delta
cv.err.9$delta
cv.err.10$delta

###Smallest prediction error: rt.glm.7

rt.lm.new <- lm(RT ~ poly(CL,2)*DB,data=complete.set)
summary(rt.lm.new)
Retention_time <- predict(rt.lm.new,New.set)
Retention_time
Table_RT <- cbind(New.set$CL,New.set$DB,Retention_time)

library(xlsReadWrite)
write.xls(table_RT,"RT_ausgabe.xls",colNames=TRUE,sheet=1,from=1,rowNames=FALSE,naStri
ngs="")
```

Complete_set.csv

Chain_length	Double_Bonds	RT
11	0	1.98
13	0	2.5
14	1	2.52
14	0	2.76
16	1	2.99
15	0	2.99
17	1	3.22
16	0	3.22
18	2	3.23
22	6	3.35
17	0	3.44
20	3	3.46
18	1	3.46
18	0	3.63
20	2	3.63
20	1	3.8
20	0	4
22	1	4.12
22	0	4.31
10	0	1.74
12	0	2.26
15	1	2.75
18	3	3.06
20	4	3.32
22	2	3.97
24	1	4.4

S-4 Qualifier ion ratios (QIR) Standard reference QIR was determined as average of 16 pure standard samples for each fatty acid (FA). 55 plasma samples were measured for plasma QIR.

FA	Standard Reference QIR	Limits of Agreement (AV +/- 25%)	$QIR_{Plasma;Av}$
10:0	2.12	1.59 - 2.65	2.07
12:0	2.44	1.83 - 3.05	2.39
13:0	3.10	2.32 - 3.87	2.87
14:0	5.13	3.85 - 6.41	5.26
14:1	2.65	1.99 - 3.31	2.87
15:0	2.72	2.04 - 3.40	2.69
15:1	1.59	1.19 - 1.99	1.70
16:0	2.78	2.09 - 3.48	2.88
16:1	5.65	4.24 - 7.06	5.69
17:0	3.08	2.31 - 3.85	3.05
17:1	2.41	1.81 - 3.02	2.41
18:0	2.28	1.71 - 2.85	2.35
18:1	2.55	1.91 - 3.19	2.55
18:2	2.28	1.71 - 2.86	2.22
18:3	2.59	1.94 - 3.24	2.44
20:0	3.52	2.64 - 4.40	3.53
20:1	3.11	2.33 - 3.89	3.21
20:2	2.67	2.00 - 3.34	2.83
20:3	2.60	1.95 - 3.25	2.92
20:4	2.45	1.84 - 3.06	2.51
22:0	1.81	1.36 - 2.27	1.81
22:1	1.96	1.47 - 2.45	1.91
22:2	2.27	1.70 - 2.84	2.27
22:6	1.81	1.36 - 2.26	1.92
24:1	2.51	1.88 - 3.14	2.50
IS	2.91	2.18 - 3.64	2.93

S-5 TC-CNRP calculation

TC-CNRP was calculated as follows,

$$TC-CNRP = \sqrt[n]{\prod_{i=0}^{n} (Rs_{(i,i+1)} / \overline{Rs})} / t_{ne}$$

with $Rs_{(i,i+1)}$ representing the resolution of two adjacent peaks, $\overline{Rs}$ the average resolution and t_{ne} the time correction factor.

S-6 Analytical parameters predicted by regression modeling Retention time (RT)declustering potential (DP)collision energy (CE) for the qualifier ion and response factor (RF) for fatty acids (FA) from 10 to 26 carbon atoms with maximum of 6 double bonds.

NEFA species	RT min	DP V	CE V	RF
10:1	1.36	-68	-20	0.039
12:1	1.96	-72	-22	0.119
12:2	1.66	-77	-22	0.091
12:3	1.36	-83	-23	0.063
13:1	2.24	-74	-23	0.159
13:2	1.97	-78	-23	0.140
13:3	1.69	-82	-23	0.121
14:2	2.25	-78	-23	0.189
14:3	2.00	-80	-23	0.180
14:4	1.75	-83	-23	0.171
15:2	2.53	-78	-24	0.239
15:3	2.29	-79	-23	0.239
15:4	2.06	-80	-23	0.239
15:5	1.82	-81	-22	0.239
16:2	2.78	-79	-25	0.288
16:3	2.56	-78	-24	0.298
16:4	2.35	-78	-23	0.307
16:5	2.13	-77	-22	0.317
16:6	1.91	-77	-21	0.326
17:2	3.02	-79	-25	0.337
17:3	2.82	-77	-24	0.356
17:4	2.62	-75	-23	0.375
17:5	2.41	-73	-21	0.394
17:6	2.21	-71	-20	0.413
18:4	2.86	-72	-22	0.443
18:5	2.68	-69	-21	0.472
18:6	2.49	-65	-19	0.500
19:0	3.81	-90	-30	0.360
19:1	3.63	-85	-28	0.398
19:2	3.45	-80	-26	0.436
19:3	3.27	-75	-24	0.474
19:4	3.09	-70	-22	0.511
19:5	2.92	-65	-20	0.549
19:6	2.74	-60	-18	0.587
20:5	3.13	-61	-20	0.627
20:6	2.96	-54	-17	0.674
21:0	4.14	-96	-33	0.421
21:1	3.98	-88	-30	0.478
21:2	3.82	-80	-28	0.534
21:3	3.66	-73	-25	0.591
21:4	3.49	-65	-22	0.648
21:5	3.33	-57	-19	0.704
21:6	3.17	-49	-17	0.761
22:3	3.82	-71	-25	0.650
22:4	3.66	-62	-22	0.716

22:5	3.50	-53	-19	0.782
23:0	4.43	-103	-36	0.482
23:1	4.28	-92	-32	0.557
23:2	4.12	-81	-29	0.633
23:3	3.96	-70	-25	0.708
23:4	3.81	-59	-22	0.784
23:5	3.65	-49	-18	0.859
23:6	3.50	-38	-15	0.935
24:0	4.56	-106	-37	0.512
24:2	4.25	-81	-29	0.682
24:3	4.09	-69	-25	0.767
24:4	3.94	-57	-22	0.852
24:5	3.78	-44	-18	0.937
24:6	3.62	-32	-14	1.022
25:0	4.68	-109	-38	0.543
25:1	4.52	-96	-34	0.637
25:2	4.36	-82	-30	0.731
25:3	4.20	-68	-26	0.826
25:4	4.04	-54	-21	0.920
25:5	3.89	-40	-17	1.014
25:6	3.73	-27	-13	1.109
26:0	4.78	-113	-40	0.573
26:1	4.62	-97	-35	0.677
26:2	4.46	-82	-31	0.781
26:3	4.29	-67	-26	0.884
26:4	4.13	-52	-21	0.988
26:5	3.97	-36	-17	1.092
26:6	3.81	-21	-12	1.196

S-7 Optional extraction procedure

As an optional variation of the aforesaid protocolan extraction method according to Dole was optimized in preliminary experiments. 50 µl of sample were mixed with 250 µl precipitation reagents (acetonitrile/1M H_3PO_4 (40/1) containing 0.71 µM $^{13}C16$ palmitic acid). After shaking for 5 minutes400µl heptane and 150ml water were added for extraction. Afterwardsthe mixture was shaken for 30 minutes and centrifuged for 10 min at 2300 x g at room temperature. 300 µl of the supernatants were transferredsolvent was removed under N_2-stream and the residue was redissolved with 40 µl water/acetonitrile (50/50).

4. Publication 3: Association between plasma nonesterified fatty acids species and adipose tissue fatty acid composition

Association between plasma nonesterified fatty acids species and adipose tissue fatty acid composition

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Synopsis

Fatty acid composition of adipose tissue (AT) is an established long-term biomarker for fatty acid (FA) intake and status, but AT samples are not easily available. Nonesterified FA composition in plasma (pNEFA) may be a good indicator of AT FA composition, because pNEFA are mainly generated by AT lipolysis. We investigated the correlation of 42 pNEFA and subcutaneous as well as visceral AT FA in 27 non-diabetic women.
Close correlations of pNEFA and AT FA were found for odd-chain FA ($C_{15:0}$ r=0.838 and 0.862 for subcutaneous and visceral AT, respectively) and omega-3 FA ($C_{22:6}$ r=0.719/0.535), while no significant or low correlations were found for other FA including 18:1 and 20:4.
The lower correlation for some pNEFA species with AT FA indicates that the variation of most pNEFA is significantly affected by other FA sources and flux of FA to tissue, in addition to release from AT. A relevant influence of BMI on the level of correlation was shown for saturated FA.
NEFA analysis in fasted plasma can serve as a virtual AT biopsy for some FA, and as a biomarker for intake of dairy products and sea fish.

Keywords:
Nonesterified fatty acids (NEFA)
Adipose tissue
Obesity
Dairy fat
Saturated very long-chain fatty acid (VLCFA)
Odd-chain FA

Short title:
Correlation between nonesterified fatty acids and adipose tissue

Abbreviations used: AT, adipose tissue; CHCl3:MeOH, chloroform:methanol ; CV, cross-validation; FA, fatty acids; MLR, multiple linear regression; MUFA, monounsaturated FA; pNEFA, nonesterified fatty acids in plasma; PUFA, polyunsaturated FA; r^2, R-squared; sAT, subcutaneous; SCD-1, stearoyl-CoA desaturase-1; SFA, saturated FA; TAG, triacylglycerols; vAT, visceral adipose tissue; VLCFA, very long-chain fatty acids (VLCFA)

Introduction

Fatty acid (FA) composition of adipose tissue (AT) is a well-accepted biomarker for the assessment of long-term dietary FA intake, considered to be superior to dietary records and food frequency questionnaires [1]. Percentage contributions of FA in AT, representing intake of dairy products, fish or fish oil are highly correlated to dietary intake [2,3]. While essential polyunsaturated FA (PUFA) show a close relationship between dietary intake and AT content, saturated FA (SFA) and monounsaturated FA (MUFA) are less closely correlated [4], presumably because these FA are derived from both diet and endogenous synthesis [5]. Nevertheless, SFA and MUFA in AT are of importance as biomarkers for various disease risks [3].

Alterations of AT fatty acid composition appear to play a crucial role in the development of insulin resistance and diabetes. Many studies have investigated relations between FA in AT and body weight, but results are inconsistent. Garaulet et al. found lower amounts of MUFA in subcutaneous AT (sAT) of obese patients compared to overweight subjects [6]. In contrast, Caron-Jobin et al. showed an inverse correlation between sAT $C_{18:0}$ and BMI and a positive correlation between stearoyl-CoA desaturase-1 (SCD-1) and body fat mass, both reflecting an increase of MUFA with obesity [7]. $C_{22:6n-3}$ was found higher in sAT of morbidly obese subjects (BMI > 40 kg/m²) than in obese or overweight subjects [6], but lower in phospholipids of obese adolescents than in lean controls [8], while Desci et al. reported a close correlation between $C_{22:6}$ in plasma phospholipids and body weight [9].

Although AT is a biomarker for the intake of FA and reflects FA metabolism, routine determination of AT composition is not practical due to the invasive nature of sample collection via biopsies, particular in larger clinical trials or in vulnerable populations such as children. During fasting, AT lipolysis releases nonesterified fatty acids into plasma (pNEFA). Thus, pNEFA could provide a valuable surrogate marker for AT FA composition. Some studies indicate a close correlation between pNEFA and AT FA content for some FA [1]. To our knowledge, only Yli-Jama et al. investigated the relationship of AT and pNEFA for a large number of 27 FA in a sizable group of patients with myocardial infarction and of controls [10]. They reported widely differing coefficients of correlation between AT and pNEFA for individual FA. Furthermore, compositional differences have been reported between different AT sites, which might relevant particularly in obese subjects [11].

So far, most studies of the relationship between pNEFA and AT focused on specific metabolic steps, e.g. mobilization [12] or (re-)uptake of pNEFA by adipocytes [13]. Mobilization studies were mostly performed in-vitro [14], in animals with induced lipolysis [15] or using venous-arterial differences of human AT, which indicated a preferential mobilization of PUFA [16,17]. These approaches do not reflect the relation of AT and pNEFA FA percentages, because the pNEFA pool is affected by a complex interaction of AT lipolysis, reincorporation of NEFA into AT triacylglycerols (TAG), uptake of pNEFA by peripheral tissues, oxidation rates of individual FA, intracellular metabolism and contribution of NEFA derived from plasma TAG or phospholipid hydrolysis.

Given, there is limited information on relationship between pNEFA and AT FA composition, the objective of this study is to explore the relationship of fatty acid composition of pNEFA, visceral AT and subcutaneous AT in subjects with differing BMI. We used a sensitive and precise LC-MS/MS method [18] enabling the quantification of more than 40 FA, including very long-chain fatty acids (VLCFA), saturated and unsaturated odd-chain fatty acids and C24 intermediates of the endogenous DHA and n-6 DPA synthesis.

Experimental

Subjects

Participants were recruited at the University of Leipzig (Department for Internal Medicine, Division for Endocrinology and Nephrology). Fatty acid composition was investigated in 27 donors of paired visceral omental (vAT) and subcutaneous abdominal adipose tissue (sAT) samples, who underwent abdominal surgery for weight reduction (sleeve gastrectomy or Roux en Y gastric bypass). All subjects had a stable weight, defined as the absence of fluctuations of >2% of body weight for at least 3 months before surgery. Adipose tissue was taken during surgery and immediately frozen in liquid nitrogen. Plasma samples were taken after prolonged fasting of more than 12 h on the day of surgery before anaesthetization. Blood samples were collected into refrigerated EDTA containing tubes and centrifuged; subsequently the plasma was aliquoted and stored at −80 °C.
All subjects were Caucasian, female and non-diabetic with a mean age of 55±14 years (M±SD). Diabetes was excluded by fasted glucose and HbA1c analysis. The BMI of the study participants was not normally distributed and ranged from normal weight to extremely obese with a median of 36 (25.Prz: 25; 75.Prz: 49).
The study protocol was approved by the Ethical Committee of the University of Leipzig Medical Center. Written informed consent was obtained from all subjects.

Methods

All used chemicals were obtained from usual suppliers in highest purity available (Merck, Darmstadt (Germany); Promochem, Wesel (Germany); Fluka Sigma-Aldrich Chemistry GmbH, Steinheim (Germany)).

Plasma samples
Sample preparation for pNEFA analysis was performed as previously reported [18]. Briefly, 20 µl of plasma were mixed with 200 µl isopropanol (containing 2 mg/100 ml uniformly ^{13}C-labelled palmitic acid) in a 96-deepwell plate. After centrifugation the supernatant was transferred into a 96-well plate for LC-MS/MS analysis.

Adipose tissue samples
Homogenisation of adipose tissue samples (about 50 mg) was performed in 2 ml Biozym Cryovials (Biozym Scientific GmbH, Oldendorf, Germany) containing glass pellets and 1000 µl chloroform:methanol ($CHCl_3$:MeOH 2:1, + 5g/l butylated hydroxytoluene) for 2 x 30 seconds at 7134 x g with a MagNA Lyser Instrument (Roche Diagnostics, Mannheim, Germany).
After cell lysis the vials were centrifuged for 10 min at 2330 x g (room temperature). 10 µl of the supernatant were used for hydrolysis of total lipids according to Petrinella et al. [19]. The aliquot was dissolved in 850 µl $CHCl_3$:MeOH (1:8) and after the addition of 150 µl KOH (40%) hydrolysis was performed in a nitrogen atmosphere at 60°C for 30 min. After cooling to room temperature, 700 µl phosphate buffer (pH=7.4, 50 mM) and 300 µl HCl (2.5 mM) were added. Extraction was performed with 2 x 2000 µl hexane:diethylether (1:1, v/v). The upper layer was taken off, dried under nitrogen flow and FA redissolved in 500 µl isopropanol for LC/MS-MS.

In agreement with previous observations [1,20], separation of dissolved AT lipids by TLC and subsequent quantification by GLC revealed that TAG contributed more than 97% to total lipids. Therefore, extracts were directly hydrolysed without further purification.

Liquid Chromatography and ESI-MS/MS
LC-MS/MS analysis was performed as previously reported[18]. Briefly, an UPLC diphenyl column (Pursuit UPS Diphenyl, 1.9 µm, 100 mm × 3.0 mm; Varian, Darmstadt, Germany) was used for chromatographic separation at 40°C with an Agilent 1200 SL series HPLC system (Waldbronn, Germany). The injection volume was set to 10 µL for plasma samples and to 2 µl for AT samples with an eluent flow rate of 700 µL/min. Equilibration was performed for 2.5 min with 45% of eluent A (water containing 5 mM ammonium acetate, 2.1 mM acetic acid) and 55% of eluent B (acetonitrile with 20% isopropanol), eluent B was linearly increased to 95% at a duration of 4 min and was held constant for 3 min.
A hybrid triple quadrupole mass spectrometer (4000 QTRAP, AB Sciex, Darmstadt, Germany) operating in negative ESI mode was coupled to the HPLC system [18]. Collision energy was optimized for each fatty acid individually to obtain signals well above the quantification limit, but within the linear response range.

Statistical analysis
Data analysis was performed with Microsoft Excel 2010 (Microsoft Inc., Redmond, WA) and "R: A language and environment for statistical computing" [21]. FA levels in pNEFA, sAT and vAT are presented as molar percentages of total FA analysed unless explicitly stated otherwise. Most fatty acid percentages were not normally distributed according to histograms, QQ plots and Anderson-Darling tests, results are given as median and interquartile ranges. Spearman's rho statistics were used to estimate rank-based correlation coefficients (r). Wilcoxon signed rank tests were performed with a limit for statistical significance of < 0.001068 due to multiple testing. The corrected p-value was calculated according to the Sidak correction [22]

$$\propto_{local} = 1 - \left(1 - \propto_{global}\right)^{1/n}$$

With a global significance level $\propto_{global}$ of 0.05 for n = 48 (42 FA + 6 sum parameters).
To evaluate influence of BMI on the correlation of AT and pNEFA, two quantile regression models were implemented to describe variance of AT FA percentages with pNEFA only or with BMI and pNEFA using the R package "quantreg" [23]. The two models were compared by calculated R-squared (r^2) and ANOVA.
To predict the FA percentages of 15:0, 17:0 and 22:6 which were strongly correlated between compartments and were normally distributed in the 3 compartments, the "glm" function with multiple linear regression (MLR) and leave-one-out cross-validation (CV) of the R package "boot" was utilized [24]. Model 1 was established using pNEFA and BMI for AT FA prediction, while model 2 used pNEFA only.
The model comparisons were performed with ANOVA. Absolute prediction error and adjusted r^2 were estimated to compare different models. Absolute prediction error for every FA was calculated as the mean absolute differences of analysed and predicted values after CV validation.

Results
42 fatty acids were analysed in sAT, vAT and plasma samples with good precision (CV<20%).

Fatty acid composition

Both adipose tissues and pNEFA contained high concentrations of MUFA, dominated by $C_{18:1}$, followed by SFA and PUFA (Table 1).

The most abundant FA in the studied compartments were $C_{18:1}$, $C_{16:0}$ and $C_{18:2}$, followed by $C_{18:0}$ in plasma and $C_{16:1}$ in AT.

Significant differences in FA composition between both sites of AT were mainly found for VLCFA which were higher in vAT, and some PUFA with significantly higher contribution to sAT (Table 1).

Plotting the ratio of sAT to vAT percentages versus carbon CL and number of DB indicated a clear trend for preferential incorporation of highly unsaturated FA into sAT, while longer carbon chain FA tended to be higher in vAT (Figure 1).

No significant difference between sAT and vAT was found for medium- and long-chain SFA ($C_{14:0}$,$C_{16:0}$,$C_{18:0}$), MUFA ($C_{14:1}$,$C_{16:1}$,$C_{18:1}$,$C_{20:1}$) and the sums of SFA and MUFA as well as odd-chain fatty acids ($C_{15:0}$,$C_{17:0}$,$C_{17:1}$,$C_{17:2}$,$C_{19:0}$,$C_{19:1}$,$C_{19:2}$) and some PUFA.

While most FA were not significantly different between sAT and vAT, most pNEFA differed significantly from sAT, vAT or both tissues. The abundant FA $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ were significantly higher in pNEFA, compared to sAT and vAT ($p<1E-07$). As a consequence of the relative excess of these FA in pNEFA, percentages of most other FA were lower in pNEFA compared to AT, with two exceptions.

$C_{22:6}$ had a significantly higher content in pNEFA compared to both AT, and some VLCFA ($C_{24:1}$,$C_{24:2}$,$C_{24:3}$,$C_{24:4}$,$C_{26:2}$) were higher in pNEFA compared to sAT, although not statistically significant compared to vAT.

$C_{20:4}$ was significantly lower in pNEFA than in sAT ($p=2.09E-4$), but tended to be higher than in vAT. No significant difference was found for $C_{18:2}$, $C_{24:0}$, $C_{26:1}$ and the sum of all MUFA in pNEFA in comparison to both AT.

To identify tendencies for the lower concentrated FA, the calculation of molar percentages was repeated for all compartments without the 4 dominating FA whose excess in pNEFA resulted in lower percentages of other FA in this compartment (Table S1). As expected, the comparison of sAT and vAT was hardly affected, but the comparison of pNEFA and AT yielded different results. VLCFA ($C_{24:0}$,$C_{24:1}$,$C_{24:2}$,$C_{24:3}$,$C_{24:4}$,$C_{26:2}$) were significantly higher in pNEFA compared to both AT. Furthermore, odd-chain FA ($C_{17:0}$,$C_{17:1}$,$C_{17:2}$,$C_{19:0}$,$C_{19:1}$,$C_{19:2}$), $C_{20:4}$, $C_{18:3}$ and sum of n3- dominated species as well as sum of all PUFA became significantly higher in pNEFA compared to both AT. Medium-chain FA ($C_{12:0}$,$C_{12:1}$,$C_{14:0}$,$C_{14:1}$) remained significantly lower compared to both AT.

Correlation of fatty acid compositions

Most FA were closely correlated ($r > 0.7$) between sAT and vAT, except some lower concentrated FA and $C_{18:1}$ which showed the lowest correlation coefficient ($r = 0.554$) (Figure 2).

While all FA correlated significantly between both AT, the correlation of pNEFA and AT fatty acid percentages varied widely between FA.

$C_{16:0}$ showed close correlations between pNEFA and the AT fatty acid percentages, while $C_{16:1}$, $C_{18:0}$, $C_{18:2}$ and $C_{18:3}$ had less close correlations. For the most highly concentrated 18:1 the correlation coefficient of pNEFA with sAT was only 0.35 and not significant with vAT.

Odd-chain fatty acids $C_{15:0}$, $C_{17:0}$, $C_{17:1}$, $C_{19:0}$, $C_{19:1}$ and $C_{19:2}$ showed close correlation coefficients above 0.7. Also $C_{22:6}$ (r=0.719/0.535) and $C_{24:6}$ (r=0.694/0.693) had close correlation coefficients between pNEFA and sAT as well as vAT. In contrast, medium-chain FA correlated less or not significantly between NEFA and both AT

sites. Most VLCFA showed no significant correlation between plasma and adipose tissue fatty acid percentages. R-values tended to increase with unsaturation, as $C_{24:5}$ (r=0.447/0.496) and $C_{24:6}$ (r=0.694/0.693) were the only pNEFA with more than 22 carbon atoms correlating significantly with sAT and vAT.
There was a general trend for a closer correlation of PUFA compared to their saturated and monounsaturated counterparts, except for $C_{20:4}$ (r=0.386/ns).

Influence of BMI on correlation of fatty acid compositions

Quantile regression models were established for all FA analysed to determine the influence of BMI on the relation of AT fatty acid composition and pNEFA (Table 2). For some SFA, regression models including BMI in addition to pNEFA explained significantly more variance as well as in sAT and vAT. The saturated VLCFA $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ showed the highest relative improvement of r^2 between models with and without BMI.
A better regression for both AT, including BMI in the regression model also appears for $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{20:0}$, $C_{20:4}$ and $C_{24:1}$. Significantly more variance of vAT was explained for $C_{14:1}$, $C_{18:0}$, $C_{18:1}$, $C_{19:0}$, $C_{22:1}$, $C_{22:2}$, $C_{24:2}$, $C_{24:3}$, $C_{24:5}$ and $C_{26:1}$ by inclusion of BMI into the model. For $C_{12:1}$, $C_{17:1}$, $C_{19:1}$ and $C_{20:3}$, the inclusion of BMI improved r^2 in sAT.
Regarding the r^2 values for both models, the model with pNEFA only explained more than 50% of variance in both AT for $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:2}$, $C_{19:1}$, $C_{22:4}$ and $C_{24:6}$, in sAT for $C_{22:6}$ and in vAT for $C_{22:6}$. The inclusion of BMI additionally explained more than 50% of variance in both AT for $C_{20:0}$, $C_{20:3}$ and $C_{24:0}$, in sAT for $C_{17:1}$, $C_{18:4}$ and $C_{22:0}$ and in vAT for $C_{16:2}$, $C_{19:0}$ and $C_{24:5}$.
For the closely correlated odd-chain FA $C_{15:0}$ and $C_{17:0}$ and $C_{22:6}$, two MLR models were tested to estimate sAT and vAT fatty acid composition from pNEFA (Table 3). Both CV prediction error and adjusted r squared were similar between the models. Prediction quality was improved by including BMI only for $C_{15:0}$. ANOVA analysis showed no significant difference between the two models for $C_{17:0}$ and $C_{22:6}$, but MLR model 1 (including pNEFA & BMI) was significantly better compared to model 2 for $C_{15:0}$. However, regarding the slight difference of prediction error and adjusted R squared for $C_{15:0}$, it is also possible the establish model 2 for this FA.
The close correlation of these FA percentages in pNEFA and AT was apparent from scatter plots of AT fatty acid percentages predicted from pNEFA and actually analysed values in AT (Figure 3). The values were close to the bisecting line without influence of the MLR model. This was true for correlations with sAT and vAT.

Discussion

The present study shows a wide variation of the coefficients of correlation between fatty acid percentages of adipose tissue and nonesterified fatty acids in plasma.
Of importance, close correlations between pNEFA and AT were found for FA known to be markers of specific dietary intakes, i.e. odd-chain FA (markers for dairy fat intake) and omega3 (n-3) LC-PUFA (markers for fish intake).
The content of $C_{15:0}$ in TAG of AT is a marker for the intake of dairy products in women [25] and men [2]. Similarly, total $C_{15:0}$ in plasma and erythrocytes is used as a biomarker for dairy fat intake [26]. Nonesterified FA were not previously analysed in these or other studies on serum markers [26,27]. Considering the close correlation of $C_{15:0}$ and other odd-chain FA percentages in AT and pNEFA, we assume that pNEFA

reflect dairy intake similar to other serum markers. Hence, $C_{15:0}$ in pNEFA could provide an alternative biomarker for habitual dairy food intake in epidemiological studies.

A close correlation between dietary intake and FA percentages in AT has been well established for n-3 FA [3]. Our observation of a high correlation coefficient of 0.719 for the n-3 FA $C_{22:6}$ between pNEFA and sAT is in line with previous findings of Leaf et al (r=0.87) [28] and Yli-Jama et al. (r=0.572) [10] and is further underlined by the good validation parameters of the MLR models for these FA including only pNEFA percentage as independent parameter. The similarly high correlation coefficients of $C_{20:5}$ and $C_{24:6}$ indicate a generally close correlation of n-3 FA between AT and pNEFA. Long chain n-3 FA $C_{20:5}$ and $C_{22:6}$ share a low rate of ß-oxidation in peroxisomes [29,30]; therefore, pNEFA concentrations of individual subjects are only slightly affected by fatty acid oxidation. This agrees with the higher percentages of $C_{22:6}$ in pNEFA compared to AT. Noteworthy, $C_{22:6}$ was found more suitable as marker for n-3 FA dietary intake compared to $C_{20:5}$ [3], and a closer correlation between pNEFA and AT was found for $C_{22:6}$ than for $C_{20:5}$ in the present study. This higher correlation of $C_{22:6}$ may be due to the relatively lower conversion for $C_{22:6}$ towards docosanoids compared to the conversion of $C_{20:5}$ to eicosanoids, because $C_{20:5}$ can function as substrate for COX-1, COX-2 and 5-LOX while $C_{22:6}$ is only metabolized by COX-2 [31].

The good correlation of $C_{22:6}$ and other PUFA was related to higher AT mobilization rates by Yli-Jama et al. [10]. PUFA are preferentially mobilized from AT in in-vitro studies with adipocytes [14], in in-vivo studies in animals [15] and in humans [17]. Raclot et al. explained this observation with a better solubility of TAG containing PUFA, because PUFA increase the polarity of TAG at the lipid/water-interface, where TAG lipolysis occurs [12,32]. Relative mobilization rates were characterized as ratio between pNEFA and AT percentages and were found to increase with increasing number of double bonds and decreasing chain-length. Thus, the mobilization rate decreased in the order: $C_{20:5n3}$, $C_{20:4n6}$, $C_{18:3n3}$, $C_{18:2n6}$, $C_{22:6n3}$ [12]. The correlation coefficients found in this study showed a practically reversed order with the best correlation for $C_{22:6}$ and the lowest correlation for $C_{20:4}$. Furthermore, mobilisation rate for $C_{18:2}$ was 0.99 [14], which is agreement with no significant differences of $C_{18:2}$ between pNEFA and AT found in the present study, but $C_{18:2}$ correlates only weakly (0.54 and 0.61). This indicates that correlations are not directly related to mobilization rates. Since only about 36% of the $C_{18:2}$ variation in pNEFA is explained by the amount of $C_{18:2}$ in AT, the intensity of further metabolic pathways affects pNEFA significantly. Nevertheless, there seems to be a general trend towards relatively high correlation coefficients for PUFA in accordance with their higher mobilization rates.

In contrast to other PUFA, pNEFA $C_{20:4}$ showed a low correlation with sAT (r=0.386) and was even not significantly correlated to vAT. This confirms the observation of Yli-Jama [10]. Low correlations for $C_{20:4}$ have also been observed between diet and AT [3,4] as well as between glycerophospholipids from cheek cells, erythrocytes and plasma [33]. Moreover, correlation between sAT and vAT is lower for $C_{20:4}$ than for most other FA (Table 1). Although a high mobilization rate of 1.6 has been found for $C_{20:4}$ [14], its plasma concentration might be even stronger affected by its conversion to eicosanoids, as this fatty acid is typically the major precursor for eicosanoids [34] and $C_{20:4}$-dervied eicosanoids are formed at a faster rate than $C_{20:5}$-derived eicosanoids [35]. The actual amount of $C_{20:4}$ in tissues and plasma could strongly depend on eicosanoid synthesis which is highly variable in time and differs according to inflammation status [36] and diet [34]. Thus, $C_{20:4}$ metabolism may be different between individuals and thus prevent the establishment of clear correlations between

compartments. It seems challenging to determine the most suitable marker for $C_{20:4}$ status. AT might be useful to assess long-term storage and chronic effects, while nonesterified $C_{20:4}$ in plasma reflects the amount actually available to peripheral tissues.

Another trend, contributing to the variety of the correlation pattern, was found in physicochemical properties. Increasing chain-length and increasing saturation negatively impact correlation coefficients. In previous studies, this has been ascribed to lower mobilization as well as lower clearance of SFA and MUFA, especially of $C_{18:0}$ [17]. The latter is in agreement with the higher pNEFA concentrations of $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, but seems to contradict with the lower pNEFA percentages of $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{20:0}$, $C_{20:1}$ and $C_{22:1}$. These FA are also significantly lower in pNEFA when percentages are calculated excluding the four dominating FA $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$. Exclusion of these FA hardly affected observed correlation coefficients, but comparisons between FA percentages of pNEFA and AT sites led to different results. Most PUFA were higher in pNEFA in accordance with the postulated higher mobilization rates. Additionally, VLCFA and odd-chain FA were found higher in pNEFA. This may be due to lower ß-oxidation of these FA and, consequently, a lower flux to peripheral tissues which is, in agreement with the hypothesis of Halliwell et al. explaining higher amounts of $C_{18:0}$ in pNEFA [17]. This hypothesis is further underlined by the significantly lower percentages of medium-chain FA $C_{12:0}$, $C_{12:1}$, $C_{14:0}$ and $C_{14:1}$, as these FA have a higher flux to tissue due to direct transport through cell membrane and rapid mitochondrial oxidation independently of the carnitine transport system [37].

Many metabolic processes besides AT TAG lipolysis affect pNEFA variation, such as lipolysis of lipoprotein bound TAG by LPL [38] and release or exchange of fatty acids of lipoprotein or membrane phospholipids [39]. Thus, the contribution by non AT lipolysis and cellular FA uptake for beta-oxidation and synthesis of prostaglandins may be regulated differently between FA species and individuals. As a result, the observed correlation between pNEFA and AT decreases. AT is the dominating source for pNEFA generation and mainly determines concentration as well as percentages of pNEFA, as evidenced by the similarity of molar percentages of highly concentrated FA. Variations in pNEFA are also significantly affected by fatty acid flux to tissue and further sources contributing to pNEFA.

In contrast to the wide variation of correlation coefficients between pNEFA and AT, correlation between sAT and vAT FA was strong for the majority of FA species, although significant differences in FA composition between the two sites of AT were found. This demonstrates that close correlations do not necessarily depend on very similar concentrations, but stable pools with slow relative turnover rates support the establishment of close correlations between tissues.

$C_{18:1}$ showed the lowest correlation between the AT sites, which might be due to the high concentrations of this FA in AT, resulting in some kind of saturation in the tissues, which prevents establishment of proportionality between sAT and vAT.

The higher amount of PUFA in sAT is in contrast to a previous study finding no significant differences of PUFA between AT sites [40]. Nevertheless, it agrees with the observation that subcutaneous fat is softer than deeper fat and that vAT contains more SFA compared to sAT [40].

Different expression of fatty acid transfer proteins in vAT and sAT has not been described so far [41], thus slight differences of intracellular processes may cause the difference between sAT and vAT. Since enhanced incorporation of PUFA decreases the viscosity of fat, the different FA pattern may be required to ensure sufficient

fluidity of sAT at slightly lower temperatures than experienced by vAT, which is in the inner body and exposed to less mechanical stress than sAT.
In previous studies sAT [42,43] or vAT [40] have been claimed to be the major source of pNEFA, while our correlation analyses did not point towards a clearly higher contribution of sAT or vAT. It seems that both AT sites contribute similarly to pNEFA, because pNEFA percentages for saturated and mono-unsaturated VLCFA and PUFA reflect a mixture of vAT and sAT percentages. There was no trend for better correlations of pNEFA with vAT, sAT or the AT containing the higher percentage of the respective fatty acid. Nevertheless, the finding that $C_{22:6}$ percentages could only be reliably estimated for sAT from pNEFA may be due to the higher amount of $C_{22:6}$ in sAT and its strong influence on pNEFA compared to vAT.
Remarkably, BMI has a FA specific influence on correlation. Explanation of variance of FA percentages in AT was significantly improved by including BMI additionally to pNEFA into the quantile regression models only for SFA and $C_{20:4}$. Thus, with increasing BMI and increasing fat mass the correlation of SFA content between pNEFA and AT increased which may be a result of a higher contribution of FA release from AT to the pNEFA pool as well as a relatively lower flux of these pNEFA to tissue.
Eventually, the lower flux to tissue is the result of decreased ß-oxidation. In obese mice lower hepatic carnitine levels were found compared to controls [44] resulting in an insufficient ß-oxidation. Additionally, the lower flux to tissue and elevated pNEFA levels may result in an increased (re-)uptake of pNEFA to AT which strengthen the correlation of SFA between pNEFA and AT in obese subjects [45].
Thus, in highly obese patients a relation between SFA in pNEFA and AT FA composition can be supposed, while there is a less close relationship in normal weight subject. As a consequence for biomarker research, an obesity biomarker found in pNEFA is not automatically discriminative in AT FA when trials compare obese, overweight and control groups.
A limitation of our study is that no information about habitual diet of the probands was available, as fat content of the diet may influence the specific uptake into sAT or vAT [46] and the fatty acid composition of the diet, e.g. n3/n6 ratio, affects metabolic fatty acid deposition and AT metabolism [12]. Since none of the participants followed a specific diet and they varied widely in respect to age and BMI, we can assume a wide variation of dietary preferences.
We only studied abdominal sAT, but FA uptake and composition in sAT have been found to differ between sites of sAT [11]. Abdominal sAT is most frequently collected in clinical trials and upper-body fat contributes significantly to pNEFA [16], thus, our results should be of relevance and reflect the major effects. For a more detailed quantification of the contribution of individual kinds of AT to pNEFA a more detailed determination of body composition would have been required, because BMI does not adequately reflect the ratio between sAT and vAT. Since our study was accomplished to determine the suitability of pNEFA as surrogate markers for AT composition, we point out that only women were studied and considering the influence of gender on sAT and vAT metabolism [47], further studies are needed to expand our results to males or infants.

Conclusion

Plasma nonesterified fatty acids are derived mainly from adipose tissue TAG hydrolysis, but additional processes, such as oxidation or prostaglandin synthesis seem to affect their flux and consequently the variation of pNEFA.

We demonstrate that nonesterified odd-chain FA in plasma reflect AT contents very well and thus seem a suitable alternative to AT biopsies for the estimation of long-term status and dietary habits. Fish intake and n-3 status of adipose tissue are reflected by pNEFA $C_{22:6}$.

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Tables and Figures

Table 1 Fatty acid (FA) composition (mol%) of plasma nonesterified fatty acids (pNEFA), subcutaneous adipose tissue (sAT) and visceral adipose tissue (vAT). Percentage concentrations are given as median and interquartile range. *- significantly higher in vAT compared to sAT, +- significantly lower in vAT compared to sAT;Wilcoxon test adjusted for multiple testing

FA	pNEFA	sAT	vAT
12:0*	0.312% [0.263%,0.450%]	1.076% [0.812%,1.402%]	1.258% [1.027%,1.642%]
12:1*	0.065% [0.051%,0.083%]	0.207% [0.137%,0.251%]	0.236% [0.206%,0.339%]
14:0	1.934% [1.710%,2.198%]	5.442% [4.894%,6.646%]	6.020% [5.134%,6.447%]
14:1	0.226% [0.181%,0.334%]	0.785% [0.681%,0.862%]	0.837% [0.742%,0.924%]
15:0	0.400% [0.339%,0.519%]	0.846% [0.673%,0.963%]	0.808% [0.675%,0.956%]
16:0	26.659% [24.985%,28.172%]	22.152% [21.326%,22.797%]	21.548% [21.029%,22.588%]
16:1	5.504% [4.584%,6.559%]	8.740% [7.281%,10.514%]	9.378% [7.895%,10.089%]
16:2	0.050% [0.040%,0.060%]	0.109% [0.100%,0.122%]	0.110% [0.100%,0.121%]
17:0	0.445% [0.403%,0.503%]	0.545% [0.469%,0.609%]	0.528% [0.427%,0.625%]
17:1	0.383% [0.347%,0.407%]	0.459% [0.434%,0.508%]	0.471% [0.438%,0.493%]
17:2	0.009% [0.008%,0.012%]	0.021% [0.020%,0.023%]	0.021% [0.019%,0.023%]
18:0	7.819% [6.967%,8.823%]	5.264% [4.478%,6.403%]	5.428% [4.706%,6.108%]
18:1	40.367% [39.445%,41.434%]	36.294% [35.178%,36.809%]	35.949% [35.556%,36.500%]
18:2	9.959% [9.333%,11.329%]	10.649% [9.791%,10.874%]	10.437% [9.543%,11.006%]
18:3	1.392% [1.148%,1.769%]	1.717% [1.567%,2.021%]	1.683% [1.425%,1.968%]
18:4	0.017% [0.013%,0.022%]	0.038% [0.033%,0.047%]	0.044% [0.039%,0.056%]
19:0	0.040% [0.037%,0.051%]	0.05% [0.041%,0.059%]	0.062% [0.045%,0.071%]
19:1	0.166% [0.149%,0.193%]	0.263% [0.223%,0.296%]	0.277% [0.231%,0.302%]
19:2	0.014% [0.012%,0.016%]	0.021% [0.019%,0.022%]	0.019% [0.018%,0.022%]
20:0*	0.044% [0.038%,0.058%]	0.193% [0.152%,0.276%]	0.258% [0.240%,0.350%]
20:1	0.478% [0.409%,0.509%]	1.364% [1.125%,1.556%]	1.399% [1.230%,1.583%]
20:2+	0.220% [0.197%,0.231%]	0.500% [0.452%,0.533%]	0.444% [0.400%,0.512%]
20:3+	0.230% [0.205%,0.262%]	0.576% [0.444%,0.629%]	0.396% [0.358%,0.511%]
20:4+	0.682% [0.534%,0.729%]	0.868% [0.703%,0.907%]	0.654% [0.557%,0.714%]
20:5+	0.107% [0.078%,0.160%]	0.178% [0.120%,0.218%]	0.130% [0.094%,0.161%]
22:0*	0.015% [0.011%,0.023%]	0.021% [0.012%,0.031%]	0.033% [0.028%,0.048%]
22:1*	0.042% [0.025%,0.049%]	0.129% [0.076%,0.186%]	0.193% [0.127%,0.253%]
22:2*	0.008% [0.006%,0.009%]	0.015% [0.012%,0.018%]	0.018% [0.016%,0.020%]
22:3	0.010% [0.009%,0.013%]	0.030% [0.023%,0.039%]	0.031% [0.024%,0.039%]
22:4+	0.124% [0.108%,0.134%]	0.361% [0.250%,0.395%]	0.266% [0.225%,0.316%]
22:5+	0.222% [0.189%,0.254%]	0.450% [0.362%,0.505%]	0.350% [0.295%,0.405%]
22:6	0.498% [0.350%,0.598%]	0.392% [0.285%,0.458%]	0.317% [0.234%,0.386%]
24:0*	0.020% [0.016%,0.027%]	0.012% [0.008%,0.019%]	0.021% [0.015%,0.029%]
24:1*	0.045% [0.037%,0.054%]	0.016% [0.011%,0.031%]	0.031% [0.021%,0.045%]
24:2*	0.005% [0.004%,0.006%]	0.003% [0.002%,0.004%]	0.004% [0.003%,0.006%]
24:3*	0.001% [0.001%,0.001%]	0.001% [0.000%,0.001%]	0.001% [0.001%,0.001%]
24:4*	0.005% [0.004%,0.006%]	0.003% [0.002%,0.003%]	0.004% [0.003%,0.005%]
24:5*	0.006% [0.005%,0.007%]	0.008% [0.006%,0.009%]	0.010% [0.007%,0.013%]
24:6*	0.004% [0.003%,0.005%]	0.006% [0.005%,0.009%]	0.008% [0.006%,0.011%]

26:0*	0.002% [0.001%,0.003%]	0.003% [0.002%,0.004%]	0.005% [0.003%,0.006%]
26:1*	0.003% [0.003%,0.005%]	0.002% [0.001%,0.004%]	0.003% [0.002%,0.006%]
26:2*	0.002% [0.002%,0.002%]	0.001% [0.001%,0.001%]	0.001% [0.001%,0.002%]
SFA	37.559% [35.820%,39.735%]	36.429% [33.455%,38.296%]	36.591% [34.518%,38.109%]
MUFA	47.405% [46.673%,49.373%]	48.300% [46.207%,49.412%]	48.638% [47.261%,50.094%]
PUFA+	13.697% [12.640%,15.359%]	15.952% [14.694%,16.552%]	14.804% [13.923%,16.606%]
n3+	2.237% [1.898%,2.656%]	2.812% [2.618%,3.048%]	2.593% [2.448%,2.788%]
n6+	11.298% [10.627%,13.205%]	13.125% [12.244%,13.813%]	12.463% [11.835%,13.511%]
(n6 - 18:2)+	1.504% [1.302%,1.636%]	2.790% [2.273%,3.001%]	2.136% [1.952%,2.475%]

Table 2 Quantile regression models to depict visceral (vAT) and subcutanesous adipose tissue (sAT) by plasma nonsterfied fatty acids (pNEFA) ± BMI. For model comparison, ANOVA was performed whereas presented p-values <0.05 indicate significant more explanation of variance with model containing pNEFA and BMI. Bold: FA with p-values < 0.05 when comparing models for vAT and sAT.

FA	*vz*			*sc*		
	~ NEFA (r^2)	*~ NEFA+BMI (r^2)*	*p_value*	*~ NEFA (r^2)*	*~ NEFA+BMI (r^2)*	*p_value*
12:0	0.232	0.401	0.035	0.201	0.356	0.021
12:1	0.303	0.348	0.710	0.247	0.414	0.036
14:0	0.157	0.468	0.006	0.150	0.397	0.006
14:1	0.437	0.492	0.013	0.221	0.316	0.229
15:0	0.612	0.676	0.066	0.609	0.657	0.002
16:0	0.571	0.613	0.437	0.531	0.544	0.191
16:1	0.382	0.402	0.858	0.331	0.338	0.352
16:2	0.426	0.504	0.176	0.291	0.285	0.555
17:0	0.763	0.772	0.578	0.798	0.788	0.567
17:1	0.533	0.560	0.165	0.483	0.518	0.034
17:2	0.258	0.377	0.069	0.161	0.263	0.003
18:0	0.304	0.528	0.085	0.203	0.289	0.246
18:1	0.190	0.460	0.039	0.198	0.280	0.386
18:2	0.700	0.721	0.128	0.604	0.649	0.219
18:3	0.288	0.282	0.820	0.249	0.258	0.553
18:4	0.320	0.490	0.191	0.453	0.527	0.246
19:0	0.486	0.622	0.091	0.396	0.476	0.137
19:1	0.610	0.598	0.454	0.663	0.650	0.012
19:2	0.312	0.298	0.318	0.471	0.489	0.811
20:0	-0.034	0.605	0.001	-0.036	0.541	0.000
20:1	0.237	0.274	0.398	0.166	0.288	0.281
20:2	0.386	0.284	0.576	0.465	0.449	0.891
20:3	0.463	0.528	0.108	0.333	0.569	0.027
20:4	-0.011	0.056	0.048	0.038	0.357	0.003
20:5	0.225	0.242	0.147	0.230	0.351	0.182
22:0	-0.017	0.468	0.000	-0.003	0.507	0.004
22:1	0.280	0.462	0.000	0.244	0.383	0.168
22:2	-0.013	0.207	0.088	0.007	0.121	0.242
22:3	0.241	0.186	0.891	0.268	0.256	0.885

22:4	0.661	0.677	0.550	0.634	0.625	0.669
22:5	0.288	0.292	0.787	0.102	0.088	0.563
22:6	0.416	0.438	0.441	0.534	0.536	0.695
24:0	0.029	0.499	0.000	0.015	0.526	0.023
24:1	-0.041	0.378	0.002	-0.067	0.327	0.019
24:2	0.001	0.333	0.006	-0.015	0.197	0.318
24:3	0.005	0.216	0.008	-0.082	-0.018	0.466
24:4	0.087	0.247	0.174	0.143	0.221	0.643
24:5	0.448	0.554	0.015	0.371	0.455	0.238
24:6	0.624	0.682	0.090	0.601	0.622	0.006
26:0	-0.001	0.466	0.000	0.000	0.461	0.002
26:1	0.010	0.386	0.002	-0.008	0.296	0.115
26:2	0.000	0.387	0.009	-0.011	0.303	0.073

Table 3 Multiple linear regression models (MLR) to estimate fatty acid composition of subcutaneous (sAT) and visceral adipose tissue (vAT) from plasma nonesterified fatty acids (pNEFA) and/or BMI.
Validation was performed using leave-one-out cross-validation with absolute prediction errors, adjusted r squared and model comparison with ANOVA of model 1 and 2.

	vAT			sAT		
	prediction error	p (model comparison)	Adjusted R-squared	prediction error	p (model comparison)	Adjusted R-squared
			MLR model 1 (pNEFA,BMI)			
15:0	1.06E-03	0.046	0.652	9.84E-04	0.073	0.629
17:0	5.79E-04	0.318	0.754	4.95E-04	0.718	0.786
22:6	8.10E-04	0.378	0.403	6.44E-04	0.706	0.498
			MLR model 2 (pNEFA)			
15:0	1.15E-03	-	0.610	1.08E-03	-	0.595
17:0	5.56E-04	-	0.753	4.70E-04	-	0.793
22:6	7.95E-04	-	0.407	6.19E-04	-	0.516

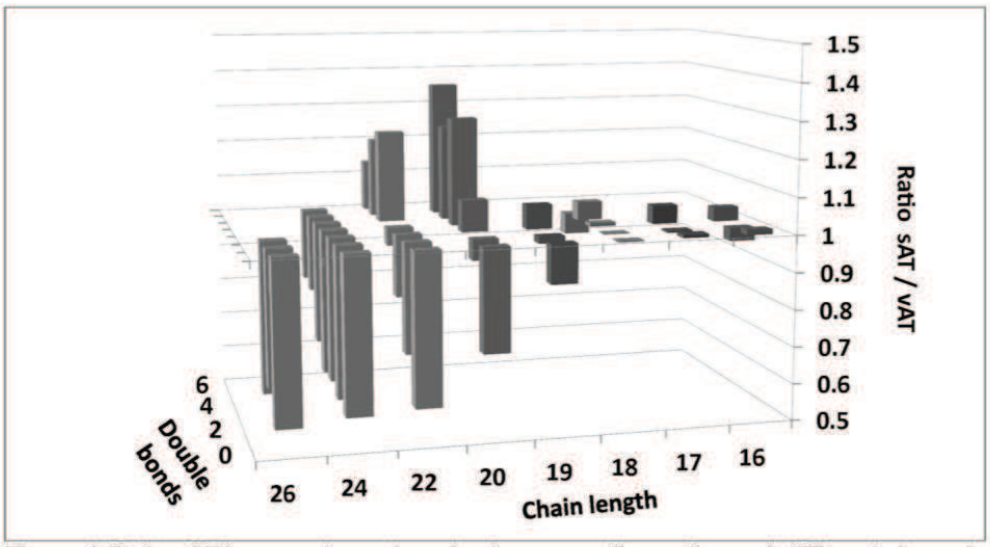

Figure 1 Ratio of FA percentages in subcutaneous adipose tissue (sAT) and visceral adipose tissue (vAT) by chain length and number of double bonds.

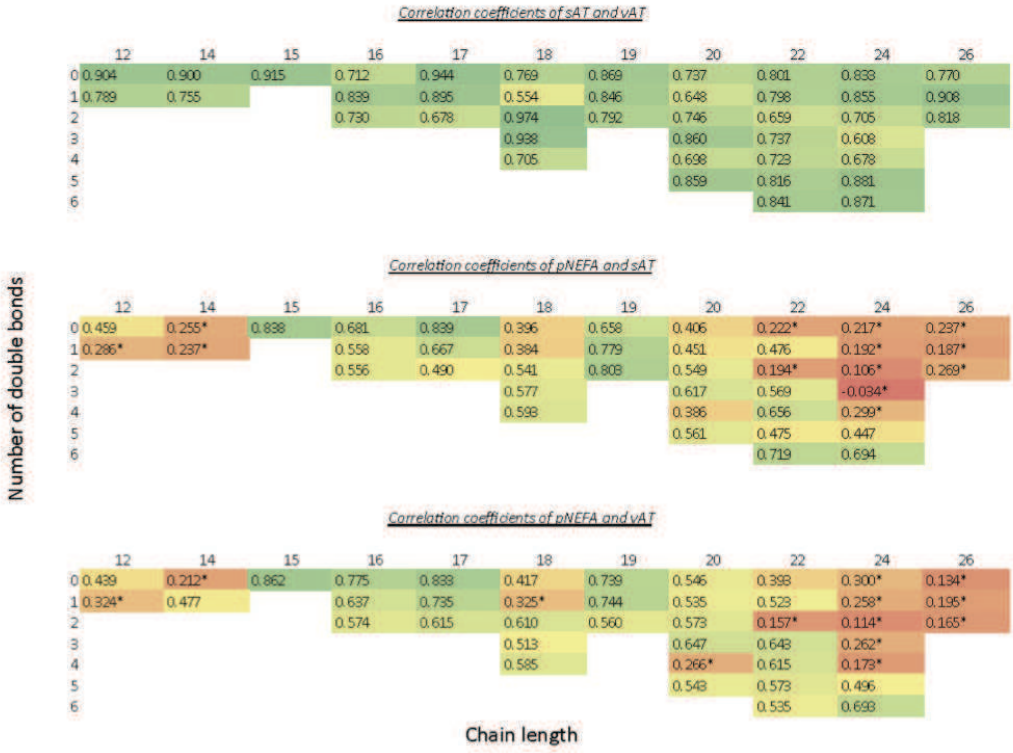

Figure 2 Correlation coefficients (r) of plasma nonesterified fatty acids (pNEFA), subcutaneous adipose tissue (sAT) and visceral adipose tissue (vAT). FA are

arranged by chain length and double bond number. Colour gradient with red (r=0), yellow (r=0.5) and green (r=1). * not significant correlation (p>0.05)

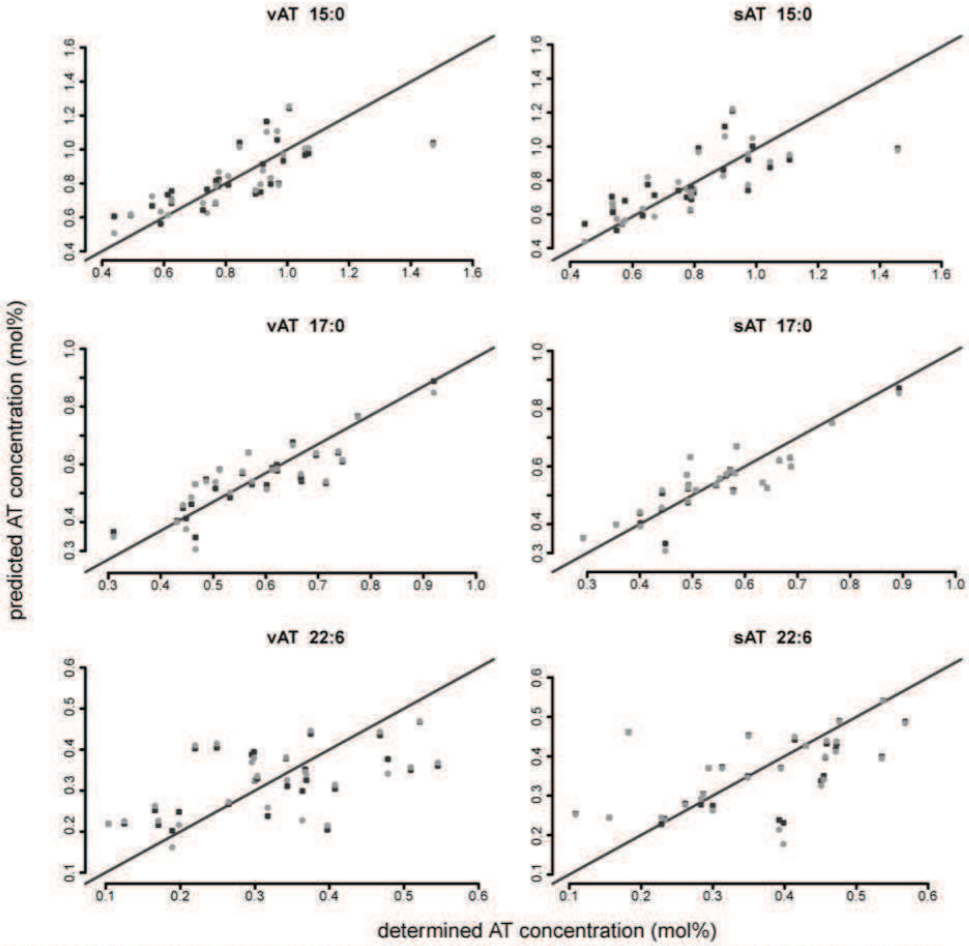

Figure 3 Comparison of analysed subcutaneous (sAT) and visceral adipose tissue (vAT) fatty acid composition and predicted AT fatty acid composition by using different multiple linear regression models with pNEFA (squares) or pNEFA+BMI (circles).

SUPPLEMENTARY ONLINE DATA

Association between plasma nonesterified fatty acids species and adipose tissue fatty acid composition

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Table S1 Fatty acid (FA) composition excluding 18:0, 16:0, 18:1 and 18:2 of plasma nonesterified fatty acids (pNEFA), subcutaneous adipose tissue (sAT), visceral adipose tissue (vAT). Percentage concentrations > 0.01% are given with median

FA	pNEFA	sAT	vAT	FA	pNEFA	sAT	vAT
12:0	2.22%	4.26%	4.79%	22:0	0.11%	0.09%	0.13%
12:1	0.45%	0.78%	0.91%	22:1	0.28%	0.47%	0.69%
14:0	13.41%	21.67%	22.49%	22:2	0.05%	0.06%	0.07%
14:1	1.59%	3.03%	3.17%	22:3	0.07%	0.12%	0.12%
15:0	2.93%	3.21%	3.14%	22:4	0.91%	1.36%	1.06%
16:1	39.65%	33.74%	35.11%	22:5	1.57%	1.69%	1.28%
16:2	0.36%	0.42%	0.41%	22:6	3.6%	1.49%	1.26%
17:0	3.33%	2.11%	1.97%	24:0	0.16%	0.05%	0.08%
17:1	2.61%	1.82%	1.78%	24:1	0.32%	0.06%	0.12%
17:2	0.07%	0.08%	0.08%	24:2	0.03%	0.01%	0.02%
18:3	8.85%	6.66%	6.38%	24:3	0.01%	0%	0%
18:4	0.11%	0.16%	0.17%	24:4	0.04%	0.01%	0.02%
19:0	0.32%	0.19%	0.22%	24:5	0.04%	0.03%	0.04%
19:1	1.15%	1.01%	1.04%	24:6	0.03%	0.02%	0.03%
19:2	0.09%	0.08%	0.07%	26:0	0.02%	0.01%	0.02%
20:0	0.34%	0.78%	1.05%	26:1	0.02%	0.01%	0.01%
20:1	3.24%	5.26%	5.33%	26:2	0.01%	0%	0%
20:2	1.55%	1.97%	1.72%	SFA	23.27%	32.63%	34.43%
20:3	1.82%	2.17%	1.57%	MUFA	49.24%	46.85%	48.65%
20:4	4.34%	3.11%	2.49%	PUFA	25.11%	20.62%	18.05%
20:5	0.81%	0.67%	0.48%	n3	15.66%	11.09%	9.68%
				n6	9.07%	9.02%	7.11%

5. Publication 4: Determination of acylglycerols from biological samples with chromatography based methods

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Review Article

Determination of acylglycerols from biological samples with chromatography-based methods

Lipids are the most diverse class of metabolites in mammalian physiology and dysregulation of lipid metabolism is linked to various diseases. Alterations in acylglycerols, a major class of lipids in plasma and adipose tissue, are involved in the pathogenesis of obesity and type 2 diabetes. Therefore, determination of acylglycerols is important to depict and unravel cellular mechanisms related to pathological outcomes, and specific molecular species of acylglycerols might be promising biomarker candidates. The variety of acylglycerols can be characterized in different ways. Enzymatic assays enable the determination of total tri- or diacylglycerols showing a possible relation to diseases, but they do not allow clarification of molecular mechanism. While gas chromatography can provide an overview of the fatty acid composition of total or separated lipids, a very detailed description of the individual molecular acylglycerol species is possible via liquid chromatography, particularly when combined with mass spectrometry. This review describes the determination of acylglycerols considering recent developments, with a focus on mammalian serum/plasma and tissue.

Keywords: Diacylglycerols / GC / HPLC / MS / Triacylglycerols
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1 Introduction

During the last decade, metabolomics and especially lipidomics have been rapidly expanding areas of biomedical research. Compared with other metabolite groups such as amino acids, organic acids or carbohydrates, lipid metabolites show an extraordinary diversity in their structure, comprised of numerous fatty acids (FAs) and multifaceted backbones [1]. More than one thousand identified lipid species are involved in various metabolic pathways and biological functions. It is remarkable that dysregulation of their metabolism has been associated with type 2 diabetes mellitus [2], obesity [3], cardiovascular disease [4] and numerous other diseases. Understanding the role of lipids in pathophysiological pathways and identification of lipids as biomarkers for disease risk are major focuses of current biomedical research [5].

Circulating and intracellular triacylglycerols (TAGs) might be good markers for the relationships between FA intake, lipid flux as well as metabolism and physiological outcomes. Endogenous TAG are a major source of circulating FA, as lipids are mainly stored as TAG in the adipose tissue and dietary FA are transported as TAG in the circulation after integration in very low-density lipoproteins (VLDL) or chylomicrons, respectively. Acylglycerols are based on a glycerol backbone whose hydroxyl groups are esterified with one, two or three FAs, yielding monoacylglycerols (MAGs), diacylglycerols (DAGs) or TAGs, respectively. Acylglycerols are one of the major lipid classes and comprise up to 10–15% or more of the total human plasma lipids. Quehenberger et al. reported a fasted plasma TAG concentration of about 900 mg/L while DAG were found in concentration of about 30 mg/L [1]. TAG comprise the bulk of storage fat in animal tissues, but they also function as a mediator in processes of metabolism and disease. In placental tissue, TAG represent approximately 4% of the total lipids. In contrast, adipose tissue is very rich in TAG. In tissue and plasma, FAs are released from TAG

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Abbreviations: **APCI**, atmospheric pressure chemical ionization; **APPI**, atmospheric pressure photoionization; **CE**, cholesterol esters; **CN**, number of total carbon atoms in all fatty acyl chains; **CoE**, collision energy; **DAG**, diacylglycerol; **DB**, number of double bonds in all fatty acyl chains; **ECN**, equivalent carbon number; **ELSD**, evaporative light scattering detection; **FA**, fatty acid; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein; **MAG**, monoacylglycerol; **NP**, normal phase; **RID**, refractive index detector; **TAG**, triacylglycerol; **VLDL**, very low-density lipoproteins

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by different (tissue-specific) lipases, and liberated FA are taken up and metabolised in the adjacent tissue [6]. Hence, TAGs are important components of lipid metabolism and related pathologies in specific tissues as well as in plasma. An imbalance of energy intake and consumption leads to an excess of available FA and TAG which may cause insulin resistance if accumulating in non-adipose tissues, such as muscle or liver cells [7]. The negative effect of excessive TAG accumulation on insulin sensitivity might be mediated by DAG [8], intermediates of the intracellular synthesis of TAG and glycerophospholipids (Fig. 1).

Increase of intracellular FA, which exceeds mitochondrial oxidation capacity leads to an increase of the FA metabolites. The importance of acylglycerol determination has been recognized and mainly three different strategies of quantification have been applied:

(i) Measurement of the total acylglycerols.
(ii) Isolation of lipid classes (e.g. TAG, DAG) and quantification by analyzing the corresponding FA.
(iii) Determination of acylglycerolipid molecular species.

Enzymatic and radiometric assays have been implemented for measurement of total TAG and DAG in tissue extracts and plasma [9]. These rapid and specific assays are able to demonstrate correlations between total TAG and type 2 diabetes and insulin resistance [10, 11]. However, determination of total acylgylcerols is less helpful for the understanding of metabolic pathways and for the identification of biomarkers than the analysis of molecular species or contributing FA, depicting the wide heterogeneity of TAG and DAG in more detail. The molecular structure of acylglycerol species is defined by the FA moieties that differ in the chain length and number, position and configuration of the double bonds (DBs) (Table 1). Therefore, GC applications were developed to quantify the FA esterified into TAG and DAG as well as in other lipid classes such as glycerophospholipids.

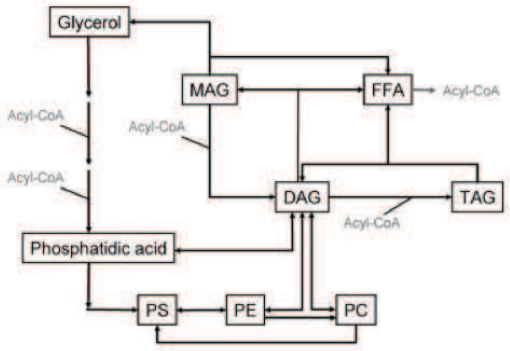

Figure 1. Diacylglycerols (DAG) as intermediates of TAGs and phospholipid (PL) synthesis. In several steps, glycerol and two Acyl-CoA form phosphatidic acid which is mainly converted into DAG. DAGs are primarily converted into TAG or phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Degradation of acylglycerols to free FAs (FFAs) can occur either intracellularly or extracellularly. Intracellular FFAs are converted into Acyl-CoA undergoing β-oxidation or sphingomyelin synthesis as well as contributing to the DAG/TAG/PL pathway. MAG: monoacylglycerol; PS: phosphatidylserine.

Recently published GC-MS applications explored relations between FA content and pathological outcomes. For example, Trembley et al. detected significant correlations between different FA of TAG and fasting insulin and glucose levels [12], but this was not confirmed in other studies [13]. Heterogeneity of the acylglycerols is further caused by the *sn* positioning of the FA in the molecule. Separation by LC-MS/MS has the ability to identify and quantify exact acylglycerol species by their different retentions in the chromatography, their specific molecular mass and the individual fragmentation of the molecules. Kotronen et al. [14] recently measured exact lipid species by LC-MS and found a positive correlation between TAG species containing palmitic acid (P), palmitoleic acid (Po) and/or oleic acid (O) and hepatic insulin resistance, while TAG species containing linoleic acid (L) was inversely correlated. These findings and those in other studies [9, 15] emphasize the importance of a more detailed analysis of TAG beyond the determination of total TAG.

The aim of this review is to describe advantages and disadvantages of different strategies for the analysis of acylglycerol content and composition from biological samples, based on lipid extraction and chromatography. Very sophisticated methods were developed for the analysis of acylglycerols in edible oils and other industrial fat. Therefore, we discuss the general method development regardless of the analyzed nature of samples. Regarding applications and special instrumentation, we will focus on the analysis of acylglycerols from mammalian tissue or serum/plasma (Table 2).

2 Extraction methods

Extraction of lipids is avoided by some analytical procedures, which rely on protein precipitation only or perform an *in situ* transfer of FA into their methyl esters [16]. However, these approaches have not been found suitable for the detailed analysis of neutral lipids, where a prior isolation of lipids is required. The selection of an adequate extraction method is fundamental for the success of the subsequent analyses.

Biological tissues and body liquids contain mixtures of different neutral and polar lipids [17]. Therefore, extraction yields depend critically on the extraction solvent chosen. In tissue or plasma samples, lipids have to be extracted from a matrix, with a considerable water content, even in the case of adipose tissue. Thus, solvent mixtures have to be applied which ensure an intensive contact between the extraction medium and the lipids.

One of the most frequently applied procedures for lipid extraction from biological samples is the method developed

Table 1. FAs commonly bound into TAGs in human tissue and plasma (%)

FA	Common Name	Abbr.	VLDL (Perona 2004) [101]	Plasma (Hodson 2008) [161]	Adipose tissue (Hodson 2008) [161]	Placenta (Klingler 2003) [31]
14:0	Myristic acid	M	0.1	3.3	2.8	1.5
14:1*n*−5	Myristoleic acid	Mo	0.2			
16:0	Palmitic acid	P	30.0	29.5	21.5	27.0
16:1*n*−7	Palmitoleic acid	Po	1.9	5.1	7.2	1.3
16:1*n*−9	Hypogeic acid		0.8			
18:0	Stearic acid	S	8.7	4.5	3.4	11.3
18:1*n*−9	Oleic acid	O	29.4	37.7	43.5	16.3
18:1*n*−7	Vaccenic acid		2.0			
18:2*n*−6	Linoleic acid	L	20.0	15.0	13.9	9.3
18:3*n*−3	α-Linolenic acid	Ln	0.6	0.9	0.8	0.3
18:3*n*−6	γ-Linolenic acid		0.9			
20:2*n*−6	Eicosadienoic acid		0.8			
20:3*n*−6	Dihomo-γ-linolenic acid		0.3	0.2	0.2	6.2
20:4*n*−6	Arachidonic acid	A	2.2	0.8	0.3	15.2
20:5*n*−3	Eicosapentaenoic acid			0.1		0.3
22:4*n*−6	Docosatetraenoic acid				0.1	
22:5*n*−3	Docosapentaenoic acid				0.1	1.0
22:6*n*−3	Docosahexaenoic acid			0.4	0.1	4.5

FA composition of the samples was determined with GC. Abbreviations (Abbr.) were given in the mentioned publications.

by Folch et al. [18]. Using roughly a 20-fold excess of a mixture of chloroform/methanol (2:1), high extraction yields can be obtained. Nevertheless, for some polar lipids, e.g. phosphaditylinositol, <80% may be recovered with a single extraction and up to four replications are required for a quantitative extraction [19]. Bligh and Dyer proposed another well-known extraction method for lipids based on the chloroform–methanol, but with 2 volumes of methanol and 1 volume of chloroform and subsequent addition of 1 volume of chloroform and 1 volume of water [20]. Adherence to the given proportions is of importance, as this ensures the initial establishment of a monophasic system, which supports solubilisation of the lipids. Phase separation with partitioning of the lipids into the chloroform dominated phase only occurs after the further addition of chloroform [21]. A comparison of the extraction efficiency of both methods revealed that very similar results were obtained for a fat content up to 2% of the wet weight, but with higher contents the Bligh and Dyer procedure significantly underestimated the fat content [22]. This is of importance for the determination of acylglycerols, because the higher fat content in the studied fish tissues was mainly contributed by TAG. In addition, a large number of adaptations of the chloroform–methanol extraction as well as some other solvent systems have been successfully applied. As a less toxic alternative, ethyl acetate–ethanol has been evaluated [23]. Dichloromethane–methanol has been applied for total FA analysis and specifically for TAG and DAG analyses [24]. Furthermore, extractions with hexane–isopropanol [25] and methyl-*tert*-butyl ether–methanol [26, 27] have been evaluated favourably against chloroform–methanol according to Folch and have found wide application. These mixtures provide the additional benefit that the solvents containing extracted lipids form the upper layer after phase separation. This makes further handling much easier compared with the bottom layer with lipids in Folch's procedure. From matrices with a low water content extraction with pure hexane has successfully been used for the analysis of acylglycerols [28]. Isooctane–ethyl acetate seems to be an alternative to the classical procedures. Extraction efficiency similar to Bligh and Dyer was reported for acylglycerols, while phospholipids, which might interfere with later mass spectrometry, were discriminated [29]. To avoid phospholipid interference, a further possibility is chromatographic separation of the determined analytes from the phospholipid fraction [30]. Compared with phospholipids, particularly TAG show a different chromatographic behaviour and nearly all published methods work with sufficient retention and long run time, which allows separation of phospholipids and acylglycerols without coelution. Therefore, LC-MS/MS methods do not require elimination of phospholipids from the samples prior to liquid chromatography. While the available procedures work reproducibly, none of them seems able to extract lipids quantitatively. Therefore, the inclusion of internal standards into the extraction and analysis procedure is used to compensate for losses [31, 32].

3 GC

At high temperatures TAG can be separated by GC, but usually GC is applied to analyse the individual FA liberated from TAG and other lipids. Acid- or base-catalysed reactions

Table 2. Applications for the determination of acylglycerols in mammalian tissue by chromatographic methods

Subject	Tissue	Analytes	Chromatography	Detection	Ref.
Dog	Liver	Lipid classes	NP	UV	[54]
Human	Cerumen	TAG	RP	MS	[141]
Human	Cutaneous sebum	TAG, DAG, WE, FFA, CE	RP	MS	[124]
Human	LDL	Lipid classes	NP	MS	[77]
Human	LDL	MAG, DAG, TAG	RP	ELSD	[103]
Human	Muscle	Lipidomics	RP	MS	[154]
Human	Plasma	Lipid classes	NP	ELSD	[74]
Human	Plasma	Lipid classes	NP	ELSD	[17]
Human	Plasma	TAG	GC	FID	[12]
Human	Plasma	TAG	RP	RID	[91]
Human	Plasma	TAG	RP	UV	[92]
Human	TRL	TAG	GC	FID	[98–100]
Human	TRL	TAG	RP	ELSD	[98–100]
Human	TRL	Lipid classes	NP	ELSD	[66]
Human	VLDL	TAG	GC	FID	[101]
Human	VLDL	TAG	RP	ELSD	[101]
Human	VLDL, IDL, LDL, HDL_2, HDL_3	Lipidomics	RP	MS	[14]
Human	VLDL, LDL, HDL	TAG	RP	RID	[81]
Human	VLDL, LDL, HDL_2, HDL_3, chylomicrons	TAG	RP	UV	[92]
Human	Plasma	Lipidomics	RP	MS	[154, 157, 158]
Mouse	Adipose tissue	Lipidomics	RP	MS	[155]
Mouse	Liver	Lipidomics	RP	MS	[156]
Mouse	Muscle	Lipidomics	RP	MS	[155]
Mouse	Heart	Lipid classes	NP	ELSD	[74]
Mouse	Liver	Lipid classes	NP	RID	[55]
Mouse	Liver	Lipid classes	NP	ELSD	[74]
Mouse	Plasma	Lipid classes	NP	ELSD	[74]
Rat	Adipose tissue	TAG	GC	FID	[97]
Rat	Adipose tissue	TAG	RP	ELSD	[97]
Rat	Brain	Lipid classes	NP	ELSD	[63]
Rat	Chylomicrons	TAG	Chiral	UV	[114]
Rat	Epididymal fat pads	TAG	RP	UV	[93]
Rat	Erythrocytes	Lipid classes	NP	ELSD	[56]
Rat	Heart	Lipid classes	NP	ELSD	[63]
Rat	Heart	Lipid classes	NP	ELSD	[56]
Rat	Kidney	Lipid classes	NP	ELSD	[63]
Rat	Liver	DAG	RP	MS	[94, 115]
Rat	Liver	Lipid classes	NP	ELSD	[63]
Rat	Liver	Lipid classes	NP	ELSD	[56]
Rat	Liver	TAG	Chiral	UV	[94, 115]
Rat	Liver	TAG	GC	FID	[96]
Rat	Liver	TAG	RP	ELSD	[94–96, 115]
Rat	Liver	TAG	RP	MS	[94, 115]
Rat	Liver	TAG	RP	UV	[93]
Rat	Lymph	TAG	RP	MS	[140]
Rat	Plasma	Lipid classes	NP	ELSD	[56]
Rat	Plasma	TAG	NP	MS	[128]
Rat	VLDL	DAG	RP	MS	[94, 115]
Rat	VLDL	TAG	Chiral	UV	[94, 115]
Rat	VLDL	TAG	NP	MS	[128]
Rat	VLDL	TAG	RP	ELSD	[94, 115]
Rat	VLDL	TAG	RP	MS	[94, 115]

CE, cholesterol esters; DAG, diacylglycerols; ELSD, evaporative light scattering detector; FFA, free fatty acid; FID, flame ionization detector; HDL, high-density lipoproteins; LDL, low-density lipoproteins; NP, normal phase; RID, refractive index detector; TAG, triacylglycerols; TRL, triacylglycerol-rich lipoproteins; VLDL, very low-density lipoproteins; WE, wax esters.

of the lipids with methanol, where FA are transesterified into fatty acid methyl esters (FAME), have become the methods of choice for derivatisation, while the use of other derivatives is limited to special applications, e.g. the synthesis of pentafluorobenzyl esters in combination with detection by negative-ion chemical ionization MS for highly sensitive detection [33]. Recent methodological developments in the separation of lipid classes and the GC analysis of their FA have been reviewed by Ruiz-Rodriguez et al. [34]. With available techniques, high-throughput analyses have become possible. The use of appropriate GC columns allows for the quantification of individual positional and configurational isomers, e.g. of *trans*-octadecenoic acid or conjugated linoleic acids [34]. Combinations of procedures are necessary for the determination of FA content of acylglycerols. Lipids have to be extracted from tissue, lipid classes are separated and the FAME are gained by derivatisation of acylglycerols. GC analyses and data acquisition complete the labour-intensive determination. Usually, lipid classes are separated by TLC on silica gel plates or by SPE columns, which both separate neutral and polar lipid fractions reliably [27], with an increased risk of oxidation of polyunsaturated FA during TLC [35]. Methodological comparisons show that subtle differences between results obtained with different methods can be expected [27, 36], but established methods usually show excellent reproducibility for tissue [31] and plasma analyses [37].

As FA are transesterified from the lipids, no information about the molecular species becomes available, but individual FA occurring at low level can be determined. Taking advantage of this, Mozzafarian et al. could show an association between circulating *trans*-palmitoleic acid (C16:1*n*−7t) and lower insulin resistance in a cohort of more than 3000 adults [38]. *Trans*-palmitoleic acid is not endogenously produced, but occurs in ruminant-derived food products and was found to contribute only 0.02–0.55% to plasma phospholipids in the studied subjects [38]. GC FA analysis is a versatile tool, suitable for the identification of relationships between individual adipose tissue FA and disease risks in cross-sectional studies [39] as well as the specific quantification of *n*-3-long chain polyunsaturated FA in primary cultures of mammalian cells [40].

A rarely used technique is high-temperature GC analysis, which allows the determination of intact TAG without previous separation of lipid classes and derivatization of the FA moieties. TAGs are introduced into the GC via cold on-column or split/splitless injection. In comparison to the analysis of FA derivatives, the molecular mass of TAG and boiling points of TAG are significantly higher. The challenge was to develop stationary phases which tolerate temperatures up to 400°C, and therefore only a few columns are commercially available. Initially, non-polar dimethyl polysiloxane phases were used, but these phases allowed separation largely only according to the number of carbon atoms (CN) and not according to different numbers of DBs in all fatty acyl chains [41]. Nevertheless, TAG can be analyzed together with trimethylsilylated DAG and MAG and even further lipid classes, such as CE, free FA and others, within one single run on the same capillary column [42]. In combination with MS the molecular species can be well identified [43]. For further separation, more polar phases are necessary. Consequently the assembly of phenyl- and diphenyl-groups was established, but these phases are even more sensitive to high temperatures. Columns coated with up to 75% diphenyl and 25% dimethyl-polysiloxane enabled determination of TAG differing in one DB [44, 45]. General advantages of this technique are the detection of molecular species and a fast sample preparation, because laborious fractionation and derivatization steps are avoided.

4 LC

HPLC is commonly used for lipid separation. LC-MS/MS presents a powerful tool for analyzing molecular lipid species. Therefore, nearly all lipid classes (except non-esterified FA) are analyzed by LC-MS applications in the protocols of the LIPID-MAPS Consortium [46]. The determination of acylglycerols by LC-MS is described below, hence this section will focus on HPLC with traditional detectors. Several hundred methods have been published for lipid analysis by HPLC with evaporative light scattering (ELSD), UV or refractive index detection (RID) [41]. When detection is performed with RID, functionality of RID does not allow eluent gradients. This restriction to isocratic methods limits the separation [47]. Although UV overcomes the lower sensitivity of RID, applications of UV are also restricted in lipid analysis. The absorbance of the chromophore of the ester bond ranges from 200 to 230 nm. Lipids only have low absorbance in this range, hence the choice of solvents absorbing strongly between 200 and 230 nm is not feasible [48]. Therefore acetone, ethylacetate, toluene or chloroform cannot be used in contrast to methanol, acetonitrile and isopropanol. During ELSD solvents are evaporated, so ELSD is not affected by the mobile phase and no baseline drift occurs [49]. Traditional HPLC methods are mostly coupled to ELSD operating with non-linear calibration curves. To overcome the limitation of non-linear response, cholesterol can be added post-column [50]. Traditional detectors provide no additional separation step like MS. The separation of lipid classes and species depends only on their chromatographic behaviour. Two major approaches were established. In normal-phase (NP) chromatography lipids are separated by the characteristics of the headgroup, while reversed-phase (RP) separates lipids according to chain length and degree of unsaturation of FA moieties of the molecule. There are only a few methods determining species of acylglycerols by NP-LC [51–53]. Although NP has not the power of RP to separate acylglycerol species, it is a good possibility to separate lipid classes. TAG, DAG and MAG can be separated from each other and from other lipid classes such as phospholipids, free FAs or cholesterol esters (CE). Greenspan et al. implemented lipid class separation by LC with isocratic

conditions and UV [54] and RID [55], respectivly. Christie established a method for lipid class separation with LC-ELSD in 1985 [56]. Total amounts of lipid classes (including TAG and DAG) in rat heart, liver, plasma and erythrocytes were measured with a silica column and a ternary gradient starting with isooctane/tetrahydrofuran and ending with water/isopropanol. This became the method of choice for lipid class separation [57–59] with some later modifications in mobile phase composition [17, 60–62]. Homan and Anderson used acetone instead of isopropanol to achieve better separation of DAG from cholesterol [63]. Furthermore, different kinds of columns were used like cyanopropyl [62], diol [64–66], polyvinyl alcohol [61, 62, 67], alumina [68] and, more recently, a monolithic silica column [69]. To simplify the chromatographic conditions, binary gradients were implemented [61, 70, 71]. 1,2-DAG could be resolved from 1,3-DAG by a binary gradient system [72]. Rocha et al. used a binary gradient system for separation of neutral lipid classes within 130 min coupled to MS for the identification of species [73]. Ultra performance liquid chromatography (UPLC) was recently introduced in lipid class separation by McLaren et al. [74]. This group achieved a total cycle time of 11 minutes by optimizing a quaternary gradient for the analysis of 20 µl human plasma. They used ELSD, arguing that MS is not the appropriate technique when the aim is just to identify the lipid content based on classes, because of the diversity in ionization efficiency of lipids. Besides measuring total amounts of lipid classes, NP could be used as a pre-fractionation step coupled to other applications which afterwards separate each class in molecular lipid species, e.g. GC [75] or RP-LC [76, 77]. Recently, hydrophilic interaction liquid chromatography (HILIC) was used for pre-fractionation of lipid classes [78]. HILIC provides similar separation as NP, but works with solvents also used in RP. Thus, cleaning steps are not necessary when using both appoaches on the same equipment. Even without prior fractionation, RP is well established for analyzing acylglycerols, especially TAG in oils and fats [41]. As traditional detectors are less specific for lipids, acylglycerols have been separated from other lipid classes prior to LC. TLC or SPE are widely used for this purpose. After separation from other lipid classes, acylglycerols with short, unsaturated fatty acyl chains elute prior to their longer or more saturated counterparts. As TAG consist of three FA moieties, it is a challenge to separate "critical pairs". "Critical pairs" have nearly the same chromatographic behaviour, despite having a different CN and/or a different configuration as well as number of DBs [79]. They are defined by having an equivalent carbon number (ECN). ECN is a retention index calculated as

$$ECN = CN - 2*n$$

with CN as the total number of carbon atoms and n as the number of double bounds in all fatty acyl chains. This means that each DB reduces the retention time by the equivalent of two carbon atoms. As the influence of one DB is not exactly that of two methylene groups, El-Hamdy and Perkins introduced another retention index, the theoretical carbon number [79]. Critical pairs, except regioisomers, are nowadays separable; therefore the retention indices are used for prediction of the retention order of the acylglycerols. ECN was implemented for TAG analysis by Plattner et al. in 1977 [80]. Although limited to isocratic runs by using a refractometer, Plattner et al. depicted some general properties and difficulties of TAG analysis with RP. Besides separating TAG by their ECN and not separating "critical pairs", he described a positive linear correlation between chain length and log retention volume as well as a negative linear correlation between DB and log retention volume. After LC, Plattner et al. collected the eluent volume corresponding to peaks of each ECN and separated the FA of each ECN group after transesterification by GC. This peak collection was also done by other groups [81–83] to ensure the correct identification of the peaks in the LC chromatogram. Furthermore, Plattner et al. used a C18 column which is still commonly used. The final mobile phase was composed of acetonitrile and acetone (2:1). This composition is limited by insufficient solubility of tristearin and longer chained saturated TAG which do not elute and remain on the column. Subsequent applications improved chromatographic separation with a mobile phase composed of acetonitrile or methanol [84] and modifiers for elevating separation or solubility of acylglycerols. Different modifiers were used, such as isopropanol [82], chlorinated solvents [83, 85, 86], tetrahydrofurane [87, 88], methyl-*tert*-butyl ether [89] and, most widely, higher amounts of acetone compared with Plattner's method. To increase the solubility of TAG, most methods avoid water in the mobile phase. As this is non-typical for RP, these special solvent system is called nonaqueous reversed phase (NARP). TAG mixtures of natural fats are less complex than those of biological tissues like muscle and liver cells. Perkins et al. [81] adapted TAG measurement to human lipoprotein classes. They adequately separated critical pairs containing linolenic (Ln), L, O and P isocratically with a mixture of acetone/acetonitrile (63.6:36.4) and two C18 columns in series within 45 min.

Though many studies were published for dietary fat sources detecting up to 115 peaks [90] with ELSD, less improvements were made for measuring acylglycerol species in human or animal tissue and plasma by RP in the following years, while GC was still the established standard method for lipid analysis in many laboratories. The gradient of Perkins was also used for the analysis of plasma TAG composition in a Spanish population one decade later [91]. Another isocratic method was published with isopropanol/acetonitrile (75:25) for the determination of TAG in human lipoprotein classes [92]. Acetone was substituted by isopropanol due to the use of an UV detector. But isopropanol was also used as a modifier in applications working with ELSD, especially for determining TAG in rat adipose tissue [93] and liver [94]. To improve separation, chloroform was used for the separation of critical pairs like triarachidonin and trilaurin in rat liver [95]. In this method, fractions of each peak were collected to accomplish GC for determination of the FA content of each peak. GC was also used by the group

of Perona, Abia and Ruiz-Gutierrez. They determined TAG in rat liver [96], rat adipose tissue [97], TAG-rich lipoproteins [98–100] and VLDL [101] of humans. Beside separation of molecular species with different acetone/acetonitrile gradients on a C18 column with ELSD, an aliquot of the TAG fraction, isolated with TLC, was measured with GC. The obtained FA content was subsequently used to predict possible TAG species and their theoretical ECN which were calculated with a modified formula:

$$ECN = CN - \alpha 1 * DB - \alpha 2 * NUFA$$

where NUFA is the number of unsaturated FA of the TAG molecule. The values of the constants $\alpha 1$ and $\alpha 2$ were calculated by multiple linear regression analysis according to Takahashi et al. [102]. This theoretical ECN was compared with ECN achieved from the chromatogram.

By then fewer papers were published on the determination of DAG and MAG [84] with traditional LC methods compared with TAG. In 2003, Perona et al. separated and quantified 24 TAGs, 18 DAGs and 4 MAGs in human VLDL [103]. After SPE the acylglycerol species were separated by an acetone/acetonitrile gradient. MAG eluted after 8 min, DAG separated into regioisomers after 25 min and TAG thereafter. In spite of separating 1,2- and 1,3-regiosisomers of DAG, positional isomers of TAG could not be differentiated with traditional RP methods.

Chiral stationary phases and silver-ion chromatography present special LC variants for separation of enantiomers and regioisomers. Silver-ion chromatography is especially used in FA and TAG analyses. Silica columns are pre-treated with silver nitrate [104] or, most common, ion-exchange columns are loaded with silver-ions [105, 106]. Silver-ions form weak charge transfer complexes with the π electrons of the DBs. According to the different rules of silver-ion chromatography [107], acylglycerols are separated by overall DB, *cis/trans* configuration of DBs, position of double bounds in polyunsaturated FA residues, partition of DBs between acyl chains and chain length, whereby molecules with longer acyl chains elute before molecules with shorter chains [108]. DBs of FA residues at *sn*−1 or *sn*−3 have a greater effect on retention compared with FA residues at *sn*−2. Hence, positional isomers can be separated [109, 110], but separation of saturated positional isomers of TAG is difficult to achieve with silver-ion chromatography. As MS can determine regioisomeric configurations of TAG by ratios of fragment ions, silver-ion chromatography is not mandatory anymore, although combination of both should provide most reliable results [111]. In contrast to silver-ion chromatography, separation on chiral column is less established for acylglycerols. Takagi and Itabashi implemented an isocratic method for the determination of DAG regioisomers as their 3,5-dinitrophenylurethane derivatives. The isomers were separated on a chiral column containing *N*-(*R*)-l-(α-naphthyl)ethylaminocarbonyl-(*S*)-valin [112] or (*R*)-(+)-l-(l-napthyl)ethylamine polymer [113]. Other groups used this method for analyzing TAG composition of rat lymph chylomicrons [114], rat liver and VLDL [94, 115]. In this application, TAGs were randomly degraded with Grignard reagent and the resulting DAG isomers were separated with chiral LC. In 2008, Deng et al. [116] developed a method for the separation of synthesized MAG enantiomers without prior derivatization using Chiralcel OF column (cellulose-tris(4-chlorophenylcarbamate)-impregnated silica). A disadvantage of their method is detection with ELSD, which does not allow complete separation of overlapping peaks containing early eluting *sn*−2 MAG species. The authors concluded that this drawback can be overcome by using MS for detection, which is relevant for all LC methods using traditional detectors. Although the chromatographic conditions are comparable to LC-MS applications, the detectors have different restrictions and lack in sensitivity and specificity compared with MS. Additionally, the complex isomeric configurations of acylglycerols can better be clarified with MS detection. Separation of positional isomers with RP-HPLC is very time-consuming (e.g. isomers of dipalmitoyl-polyalkenoyl-triglycerides were separated in 160 min [88]). Therefore, separation of regioisomers based on their chromatographic behaviour is not suitable for high-throughput LC, e.g. for large-scale clinical trials. Another method for determining FA in *sn*−2 position uses specific pancreatic lipases [99] which presents an additional, complicated preparation step and cannot be compared with the simple use of MS. In summary, the recent developments in MS offer significant improvements over conventional LC detectors.

5 LC-MS

MS provides advantages for LC analysis of acylglycerols compared with traditional detectors. Despite optimization of chromatographic conditions, some TAGs and DAGs with the same ECN still could not be separated by traditional NP/RP-LC applications. DAG containing arachidonic acid (A) could not be resolved from DAG with Ln (LnL/AL, LnO/AO and LnP/AP) [103]. In the same publication five TAG pairs were also not separated (LnLO/ALO, LnOO/AOO, AOP/ALS, LOP/LLS and OOP/LOS). Insufficient resolution of these pairs was also reported in other studies of mammalian tissues [81, 91, 96–99, 101]. This problem can be overcome by MS, as fragmentation of acylglycerols depends on the composition of their FA residues. Thus, overlapping peaks can be resolved by MS and co-eluting species can be identified. Furthermore, RP is not able to separate positional isomers of acylglycerols. MS can distinguish between FA bound at *sn*−1/3 and FA at the *sn*−2 position due to different fragment ion intensities. In 1999, Holcapek et al. compared different detectors coupled to LC [117]. MS showed a better or at least comparable sensitivity compared with UV, while the LOD for ELSD was at least one magnitude higher. Best agreement for measured concentrations was observed between MS and ELSD and reproducibility was comparable between all detectors. Thus, MS showed the best performance with the additional benefit of structural information [118]. Compositions of the mobile

phases, kind of columns and total run time are very similar to the applications mentioned before. Sommer et al. separated lipid classes with NP-MS in 25 min [77] that corresponds to NP methods mentioned in the section above with run times of about 30 min. Coupled to MS, NP can also be used for resolving species [29, 73, 119, 120], as not all species need baseline separation when detected with MS. Nevertheless, MS is mostly coupled to RP-LC for the separation of molecular acylglycerol species. Stationary phases are widely composed of C18, whereas some applications combined two C18 columns [121–123] or two C8 columns [124]. These methods sacrifice a short run time for an improved resolution of acylglycerols, but most applications utilize one column as a compromise between time and resolution for routine analyses. Nowadays, an adequate run time with good resolution can be achieved by recently introduced UPLC columns [125, 126]. Only a few modifications were made for RP mobile-phase conditions. Isopropanol is used more often as the modifier instead of acetone. Isopropanol promotes formation of molecular adducts with ammonium in the ion source due to a lower gas-phase proton affinity compared with acetone [127]. Acetone competes more with ammonia for protons. Furthermore, post-column addition of solvents containing the cations for adduct formation was implemented [77, 121, 128]. Chlorinated solvents were avoided because of their toxicity [129] and to enhance stability of the signal in MS [28]. To increase separation of the more polar MAG and DAG, aqueous gradients were implemented additionally to the common NARP gradients resulting in ternary gradients [28, 117]. Two ion sources are mainly used in LC-MS application for ionization of acylglycerols, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). As a third rarely used alternative, the atmospheric pressure photoionization (APPI) was implemented in analysis of acylglycerols. APPI was developed to cover the more difficult ionization of non-polar analytes in LC/MS analysis. Photons emitted by a krypton discharge UV lamp are able to ionize analytes if their ionization energy is below the photon energy. Cai and Syage showed that the APPI technique is comparable or superior in sensitivity compared with APCI and ESI sources [130–132]. A promising alternative is MALDI, but this ionization technique is not compatible with LC [133–135].

5.1 APCI

APCI provides ionization after converting mobile phase and analytes into the gas phase. After nebulising with nitrogen, mobile phase and analyte molecules are vapourized in a heating zone. These uncharged gaseous molecules are ionised by high voltage at the corona discharge needle. Within the plasma generated analytes are ionised by charge transfer from ionized solvent molecules. Thus, APCI requires polar solvents but no buffers. Owing to the high voltage at the corona needle and the following chemical reactions, fragmentation of analytes can take place in the ion source and therefore APCI is the less soft ionization technique compared with ESI. As a consequence, fragment ions are widely detected in 1-D MS scans and only few APCI-MS/MS applications were published [123]. In 1995, Byrdwell and Emken analyzed monoacid TAG standards with two C18 columns and APCI-MS [136]. They detected two significant fragments in APCI mass spectra, the protonated molecular ion $[M+H]^+$ and the DAG^+ fragment $[M-R_iCOO]^+$. The intensity of $[M+H]^+$ is positively related to the degree of unsaturation. Completely saturated TAGs show no significant $[M+H]^+$ peak [28]. Hence, $[M+H]^+$ is more intense than $[M-R_iCOO]^+$ for TAG(LLL), while the proportion is converse for TAG(OOO). The missing $[M+H]^+$ ion for saturated TAG complicates the identification, as identical DAG^+ fragments can be formed from different co-eluting TAGs. Therefore, LC is still a sensible and necessary step before APCI-MS. The intensity of the three possible DAG^+ fragments of a TAG molecule strongly depends on the positions of the FA in the molecule. The loss of the FA residue in position *sn*−1/3 is preferred [137]. Therefore, the FA, bound to *sn*−2, can be identified by the low abundance of DAG^+ fragments resulting from the *sn* 2 loss which is energetically not preferred, as less stable five-ring compounds are subsequently formed [28] (Fig. 2).

Positional isomers of the form ABA/AAB can be distinguished by the ratio of the DAG^+ fragments $[AA]^+$ and $[AB]^+$ which is significantly higher in AAB [138]. Calibration curves were established by plotting the ratio of the DAG^+ fragment ions versus the amount of ABA and AAB [138]. This ratio is independent of the TAG concentration [28, 139] and slight difference occurs among different kinds of mass analyzers [111]. Positional isomers of TAG species can be separated and quantified by MS, although they are not separated by LC. In contrast, 1,3-DAG and 1,2-DAG can be resolved chromatographically, but DAG also show predominantly $[M+H]^+$ ions and characteristic fragments are formed by the loss of one FA or water [117]. 1,3-DAG provide higher ratios of $[M+H]^+$ or $[M+H-H_2O]^+$ to $[M+H-FA]^+$ than 1,2 DAG [117]. $[M+H]^+$ ions of MAG dissociate preferentially in $[M+H-H_2O]^+$ ions [117]. Further APCI fragments of MAG are $[R_iCO]^+$ and $[R_iCO-H_2O]^+$. Thus, LC-APCI-MS provides identification and quantification of acylglycerols by resolving molecules with same masses by LC, predicting the ECN by retention time, calculating the molecular mass and composition by comparing molecular ions and fragment ions as well as separating positional isomers due to the relative abundance of DAG^+ fragments. Mu and Hoy resolved TAG of rat lymph with RP-APCI-MS in accordance to the formed ammonium adducts and DAG^+ fragments [140]. Recently, Stransky et al. identified 200 TAG species of human cerumen with RP-APCI-MS [141]. The limitation of RP-APCI-MS, that acylglycerols differing only in the position of DBs in individual FA cannot be resolved [28], can be overcome by silver-ion LC. Laakso and Voutilainen separated TAG isomers with α- or γ-linolenic acid in one position

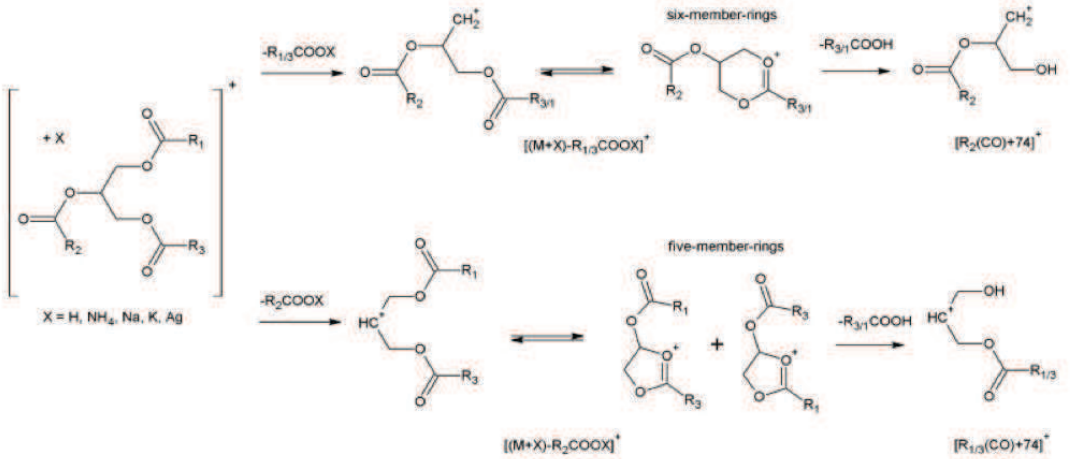

Figure 2. Fragmentation pattern of TAGs ionised by APCI or ESI. The formation of a less stable five-member ring by loss of FAs from position *sn*-2 causes the less determination of these ions compared with the more stable 6-member ring by loss of FA of position *sn*-1 or *sn*-3 (Fig. 1 in [28]). Hence, the determination of the position of the FA at the glycerol backbone is possible by the ratio of resulting ions. Additionally, the actually visible ions depend on specific MS conditions.

chromatographically with an ion exchange column loaded with silver within 90 min [142]. Furthermore, *sn*-positional isomers containing at least one polyunsaturated FA can be partly resolved [127]. Despite separating ω-isomers, silver-ion chromatography lacks in the identification of positional isomers of saturated TAG which can neither be separated by silver-ion chromatography nor by APCI-MS via $[M+H]^+$ fragments [127]. Dugo et al. combined RP, silver-ion chromatography and APCI-MS in a 2-D LC application to identify nearly 60 TAG species in donkey milk fat [143]. The major progress of this method was the online coupling of a silver-ion column and an RP column eliminating cumbersome fraction collection [144]. TAGs were first separated with a silver-ion exchange column within 155 min. Post-column, a specific volume was collected in two 20 μL loops by a switching valve for 2 min. This fraction was subsequently chromatographically separated with RP within 2 min while the next volume was collected. A similar online 2-D LC application was developed by Van der Klift et al. [118]. Retention window of the second dimension is limited by the cycle time for volume collection of the first dimension [145]. Thus, offline coupled methods provide an enhanced separation, but are time- and solvent-consuming and therefore not applicable for routine analyses. Although 2-D methods yield easier to interpret chromatograms, often special software, such as *Triglyc*APCI [146] or MZmine [147], is required to interpret RP-APCI-MS data.

5.2 ESI

Ionisation in ESI occurs within the liquid phase. ESI is more useful for polar analytes that readily form protonated molecular ions $[M+H]^+$ or other charged adducts like $[M+NH_4]^+$ or $[M+Na]^+$. The solvent is nebulised by a stream of an inert gas like nitrogen in the heated spray chamber. As the droplets get smaller due to vaporisation of solvent, the charges per droplet are getting higher until the coulomb explosion lays off ionized analytes. Despite showing less protonated molecular ions or fragments, ESI has a potential advantage: adduct ions created in the ion source are very stable due to a "softer" ionization. The intensities of these adduct ions are higher especially for saturated TAG compared with $[M+H]^+$ in APCI spectra and in-source fragmentation comparable to APCI only occurs at high capillary exit voltages [128]. Structural information can be obtained by MS/MS that provides an advantage due to an exact assignment of fragment ions to molecular ions which is not possible in the 1-D APCI-MS spectra. This may lead to an incorrect determination of co-eluting TAG with the same DAG^+ fragments in APCI [127]. In 1991, Duffin and Henion measured synthetic mixtures of MAG, DAG and TAG dissolved in chloroform/methanol with direct infusion ESI [148]. They tested formic acid, ammonium acetate and sodium acetate as modifiers. Acylglycerols showed only weak intensities of protonated molecular ions in the acidic solvent, while sodium and ammonium additives yielded adduct ions of $[M+Na]^+$ and $[M+NH_4]^+$ with nearly equal intensity. The addition of sodium acetate led to clusters of $Na_nCH_3COO_{n-1}$ at a concentration above 0.1 mmol/L [149]. These spurious background ions were not detected with ammonium acetate. Owing to impurities of the solvent the adduct ions of sodium and ammonium were even detected in the formic acid solvent with higher intensities compared with $[M+H]^+$. Thus, sodium adducts of acylglycerol molecules can be detected without previous addition of sodium compounds. Actually, Herrera et al. argued that the addition of sodium salts provides no significant increase in

the intensity of $[M+Na]^+$ over the range of concentrations studied [150]. Further molecular adducts were measured. Herrera et al. found only small differences (<5%) in the intensity of Li^+, NH_4^+, Ag^+ and Na^+ adducts [150]. The ion response for all adducts depends on the polarity of the molecule. The abundance of $[M+X]^+$ in MS spectra decreases with increasing saturation and shorter chain length of the FA moieties. Adduct ion formation increases in the sequence TAG, DAG, MAG [148]. As no structure information is obtained from in-source fragmentation, ESI is often used in MS/MS applications. ESI-MS/MS analysis of acylglycerols was established by Duffin and Henion [148]. Regarding the investigated adduct ions, $[M+NH_4]^+$ are the most labile ones [149, 150]. As sodium adducts are more stable, a higher value of collision energy (CoE) is necessary to obtain fragments in the MS/MS spectra. Duffin and Henion detected structural-informative fragments of sodium adduct ions with less abundance [148]. Some groups preferred sodium adducts, as the high cone voltage used by these groups completely degraded the ammonium adducts in the ion source and no molecular mass could be obtained [149]. Duffin and Henion determined the fragmentation of $[M+NH_4]^+$ with increasing CoE [148]. The loss of FA residues leads to the most abundant fragments in the spectra at 50 eV, while degradation of carbon–carbon bonds was achieved with higher CoE resulting in fingerprint spectra of the molecules. TAG dissociated mainly into DAG^+ fragments comparable to APCI. The abundance of DAG^+ also depends on the position of the lost FA acyl chain with a favoured loss of FA residues bound to *sn*−1 or *sn*−3 [151]. Thus, calibration curves can be set up, which relate DAG^+ fragment distribution to the ratio of positional isomers (AAB/ABA) [150]. Herrera et al. depicted sodium adducts to be most suitable for the measurement of positional isomers due to the highest and most consistent slopes of the calibration curves. Further fragments of different acylglycerol adducts were found in the ESI spectra. As fragmentation depends on the formed adduct ions, fragments for some molecular adduct ions are discussed in the following section.

Ammonium adducts of TAG dissociate preferentially into $[(M+NH_4)-NH_3-FA]^+$ [71, 119, 150] and $[(M+NH_4)-NH_3]^+$ [119]. In spectra of saturated TAG only fragments formed by the loss of FA residues are observed [119]. These DAG^+ fragments are the most abundant ions in ESI-MS/MS spectra of all monoacid TAG, while the intensity of $[(M+NH_4)-NH_3]^+$ increases with unsaturation. As DAGs are chromatographically separated according to their configuration (1,2-DAG/1,3-DAG) with RP-LC, the different regioisomers can be separately considered. The major ion of 1,3-DAG in ESI-MS spectra is $[M+NH_4-NH_3-H_2O]^+$ [73, 119], while the ammonium adduct is less abundant [119]. $[M+NH_4-NH_3-H_2O]^+$ is also the most intense peak in the ESI-MS/MS spectra of the fragile ammonium adduct of 1,3-DAG [73, 119], while $[Acyl]^+$ ions dominate the MS/MS spectra of $[M+NH_4-NH_3-H_2O]^+$ [73, 119]. The later eluting 1,2-DAG show high abundance of the ammonium adduct which disassociates in the MS/MS spectra into $[M+NH_4-NH_3-H_2O]^+$ and $[M+NH_4-NH_3]^+$ [73, 119]. Hutchins et al. reported an in-source fragmentation of the DAG molecular ion $[M+NH_4]^+$ corresponding to the loss of water [29]. In the ESI-MS spectra of MAG, $[M+NH_4]^+$ [73, 119] and $[M+NH_4-NH_3]^+$ [119] are the predominant peaks, while $[M+NH_4-NH_3-H_2O]^+$ is found only for saturated MAG with less abundance [73, 119]. $[M+NH_4-NH_3]^+$ and $[M+NH_4-NH_3-H_2O]^+$ also dominate the MS/MS spectra of the MAG ammonium adducts [119].

Na^+, Li^+ and Ag^+ adduct ions showed a different fragmentation behaviour compared with NH_4^+ adducts. The favoured fragment ions of the molecular adducts are $[M+X-FA]^+$ and $[M+X-R_iCOOX]^+$ [152]. Silver-ion adducts prefer the formation of $[M+X-FA]^+$, while lithium and sodium favour $[M+X-R_iCOOX]^+$ fragments [150]. Another possibility to overcome the limited structural information provided by ESI-MS is more complex chromatography, which is accompanied by more complex sample separation or equipment conditions. Agren and Kuksis separated TAG after Grignard degradation and derivatization of the generated DAG isomers with NP gradient coupled to ESI [128]. They achieved separation especially for later eluting TAG with CN <36 of rat liver and VLDL within 115 min. Simple RP-ESI-MS equipment was used by Leveque et al., who determined regioisomers by post-column addition of silver nitrate and MS^5 mass spectrometry [153].

5.3 Lipidomics

In the rapidly growing field of lipidomics, LC-MS is often utilized for the determination of numerous lipid species from different classes. The group of Oresic et al. determined TAG and DAG along with other lipid classes in a lipidomics platform composed of RP-ESI-QTOF equipment and MZmine software for data acquisition. They described the differences in the profile of lipid species from plasma and muscles of subjects taking different statins [154]. One of the main differences between simvastatin and atorvastatin was the upregulation of long-chain FA containing TAG in the simvastatin group. Furthermore, they depicted a decrease of short-chain TAG in muscle of mice lacking peroxisome proliferator-activated receptor γ2 [155]. Yetukuri et al. reported an upregulation of TAG and DAG in obese mice and associations of short- and medium-chained TAG with ceramides [156]. Sandra et al. measured different lipid species in human plasma including MAG, DAG and TAG [157]. They used QTOF and a Jetstream ESI source coupled to RP-LC with a run time of 89 min. Camera et al. identified 95 TAG and 29 DAG species in human cutaneous sebum when analyzing these in parallel to CE and other lipids [124]. In a recent trial, Rhee et al. depicted a positive correlation between lipids with a lower CN and lower DB and an increased risk for diabetes with RP-ESI-QTRAP [158]. The observed effect was strongest for TAG.

In "shotgun lipidomics", the time-consuming LC separation is avoided and the sample is directly injected to

the MS. Separation of lipids is only achieved by mass differences. Han and Gross developed a procedure where the full lipidome is analyzed in three injections differing in the ionizing conditions [159]. The problematic ion suppression was reduced by so-called "in-source separation", in which specific adducts are selected for a group of lipids that are characterized by polarization. TAG are measured in positive-ion mode with additional LiOH to form $[M+Li]^+$ ions. Quantification of TAG is realized by the calculation of a correction factor for each single species related to the internal standard. This factor depends on CN and DB. Given that this review focuses on chromtographic applications, the reader is refered to Stahlman et al. [160] for more detailed information about shotgun lipidomics.

6 Concluding remarks

Detailed acylglycerol analysis with sophisticated techniques is widely used for the description of animal fats and plant oils. In the recent years, available techniques have been considerably improved, especially in respect to LC-MS applications. Despite providing the power of structural analysis of acylglycerols, LC-MS/MS is so far rarely used for molecular species determination in mammalian tissues and serum. TAGs are mainly resolved by CN and DB in lipidomics platforms. LC-MS allows a much less demanding sample preparation, while time-consuming preparation steps, such as TLC or SPE and derivatization are required prior to GC analysis of FA from acylglycerols or other lipid classes. LC-MS sample preparation can be automated, because only extraction of lipids from tissue is necessary. Nevertheless, FA composition determination by GC provides the opportunity to quantify individual FA unambiguously, even if they differ only in DB positioning from other FA and as FA bound to different TAG species are combined, less-abundant FA can be determined. While FA composition measured by GC just provides the aggregated FA from all species, concentrations of intact lipid species are obtained by LC measurements. Although intact TAG molecules can be measured with GC, these applications are not widely used because suitable columns are hardly available. LC-MS provides a powerful possibility to identify and quantify exact molecular acylglycerol species in biological samples, and applications of these methods allow more detailed investigations of biochemical processes with involvement of acylglycerols. This seems important to unravel molecular mechanisms of pathologies as insulin resistance or the metabolic link between obesity and type 2 diabetes. However, the choice of technique depends strongly on the research questions.

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6. Summary

Objectives

For “Targeted clinical metabolomics” several metabolites are determined in large cohort trials or intervention studies to describe metabolic pathways and unravel metabolic disorders aiming at the identification of biomarkers and disease risk factors. For this purpose, laboratory methodology is needed, which provides fast and less cost-expensive analysis. Liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS) technology meets these expectations and additionally facilitates analysis with high specificity and sensitivity. Thus, a LC-MS/MS metabolomics platform was established at the division of metabolic and nutritional medicine of the Dr. von Hauner Children’s Hospital of the LMU Munich, measuring phospholipids, acylcarnitines, amino acids (AA) and folate metabolites in addition to routinely used of fatty acids (FA) by gas chromatography (GC).

The objective of this work was to expand this platform and to develop class-specific high throughput methods which enable simple, precise and inexpensive analysis of nonesterified fatty acids (NEFA) and sulphur-containing AA. While the AA are related to nutritional folate deficiency, cognitive dysfunction and cardiovascular risk, NEFA are key metabolites in the development of obesity and diabetes. Furthermore, the relation of NEFA and adipose tissue (AT) composition was determined to investigate the potential of NEFA to act as surrogate marker for AT FA to avoid invasive biopsies.

Different strategies for acylglycerol analysis were reviewed to assess the need to develop a LC-MS/MS application for the di- and triacylglycerols (TAG) regarding the already established GC method for measurement of FA composition.

Methods

LC-MS/MS analysis of sulphur-containing AA was accomplished utilizing simple protein precipitation, including reduction of all homocysteine forms to free homocysteine, as well as aqueous normal phase (ANP) chromatography to increase the retention time aiming for a better separation from matrix and co-eluents.

For NEFA analysis, a sample preparation was utilized avoiding cumbersome and solvent-consuming extraction or derivatization procedures. UPLC was implemented

to achieve short run time combined to good resolution, coupled to LC-MS/MS detection.
Additionally, the implementation of qualifier ions supported the correct determination of NEFA and sulphur-containing AA.
Relation of NEFA and AT FA composition was investigated in 27 donors of paired visceral (vAT) and subcutaneous abdominal AT (sAT) samples as well as plasma samples, who underwent abdominal surgery for weight reduction. Spearman correlation coefficients were estimated to describe the relation between NEFA and AT.

Results
The developed application for sulphur-containing AA allowed sensitive quantification of Hcy, Met and Cys.
The new developed method for comprehensive NEFA analysis facilitates accurate quantification of 36 NEFA species in healthy human plasma.
Both method validations showed high precision (CV <10%,<15%) and accuracy (90-110%,80-120%) for determination of sulphur AA and NEFA, respectively.
The use of qualifier ions supported unequivocal analyte verification, whereby co-eluents were identified for both classes which was assisted, for sulphur AA, by the enhanced retention of using ANP chromatography.
For both applications, sample preparation requires small sample volume (20µl) and short analysis time (<15 min per sample).
Relations between AT FA and NEFA were studied for 42 FA. The correlations of NEFA and AT FA percentages varied widely between FA. Strong correlations between NEFA and sAT/vAT were found for odd-chain FA 15:0 (r=0.838/0.862), 17:0 (0.839/0.833), 17:1 (0.667/0.735), 19:0 (0.658/0.739), 19:1 (0.779/0.744) and 19:2 (0.803/0.560) as well as for omega-3 FA 22:6 (0.719/0.535).

Conclusion
The developed LC-MS/MS methods permit an efficient quantification of Hcy, Cys and Met as well as a comprehensive determination of NEFA in human plasma. The reproducibility and accuracy of both methods are comparable to previous methods, but the establishment of qualifier ions assured correct determination of these metabolites.

With the smaller sample volume and reduced total analysis time, compared to previous methods, the methods are well suited for high-throughput analysis. This enables application in clinical trials with high sample number which have to be analysed in short time and with low costs. In addition, the usage of 20µl plasma facilitates application in studies with children or adolescents, when blood is obtained by finger sticks or heel pricks.

The relevance of the developed comprehensive NEFA application was shown in a study investigating the relation between NEFA in plasma and AT FA. In this study, NEFA groups like odd-chain or very long-chain FA, which were rarely analysed previously, showed different behaviour compared to other NEFA. For the FA content of TAG in adipose tissue, odd-chain FA and long-chain omega-3 FA in the NEFA fraction could serve a surrogate marker in fasted plasma enabling a virtual adipose tissue biopsy for these FA. Furthermore, these NEFA might act as biomarker for long-term dietary intake of dairy products and sea fish or fish oil. The lower correlation for some NEFA species with adipose tissue FA indicates that adipose tissue is not the only quantitatively important compartment affecting the concentration and composition of NEFA in the fasted state.

For TAG analysis, LC-MS/MS application also provides a powerful possibility for identification and quantification of exact molecular acylglycerol species which is important to unravel molecular mechanisms. Nevertheless, the choice of technique strongly depends on the research questions, as FA composition, determined by GC, enables analysis of ω-FA and less abundant FA. Thus, LC-MS/MS applications are not necessarily superior to GC and time- and cost-consumption should be weighed up against possible advantages when developing a new LC-MS/MS approach for acylglycerol analysis.

7. Zusammenfassung

Ziele

Unter „Targeted clinical metabolomics“ versteht man die Bestimmung verschiedener Stoffwechselmetabolite in Blutplasma oder Urin klinischer Studien. Das Ziel dabei ist es, Stoffwechselwege zu beschreiben und Störungen im Stoffwechsel aufzuklären, um Biomarker bzw. Risikofaktoren zu identifizieren.

Für dieses Vorgehen werden Verfahren benötigt, die eine schnelle und kostengünstige Analyse ermöglichen. Liquid Chromatographie gekoppelt mit massenspektrometrischer Detektion (LC-MS/MS) erfüllt diese Kriterien und liefert zusätzlich Ergebnisse mit hoher Spezifität und Selektivität. Aus diesem Grund wurde in der „Abteilung für Stoffwechsel- und Ernährungmedizin“ im Dr. von Haunerschen Kinderspital eine LC-MS/MS-Metabolomics-Plattform installiert. Hierbei werden Phospholipide, Acylcarnitine, Aminosäuren (AA) und Folatmetabolite gemessen, zusätzlich zu den routinemäßig per Gaschromatographie (GC) bestimmten Fettsäuren (FA).

Ziel der vorliegenden Dissertation war es diese Plattform zu erweitern und weitere Methoden zu entwickeln, die die Bestimmung von anderen Metabolitengruppen ermöglichen. Deshalb sollten einfache, präzise und günstige Methoden zur Messung von nicht-veresterten Fettsäuren (NEFA) und Sulfur-haltigen AA entwickelt und validiert werden. Sulfur-AA stehen in Zusammenhang mit mangelhafter Folataufnahmen, kognitiver Fehlfunktion und kardiovaskulärem Risiko, während NEFA eine Schlüsselrolle in der Entwicklung von Adipositas und Diabetes einnehmen.

Des Weiteren wurde die Korrelation zwischen der Fettgewebszusammensetzung und NEFA in Plasma bestimmt, um die Rolle von NEFA als Ersatzmarker für das Fettgewebe (AT) zu untersuchen, so dass aufwändige Fettgewebsbiopsien vermieden werden können.

Als letzter Punkt wurden verschiedene Möglichkeiten zur Bestimmung von Glycerolen diskutiert, um den Nutzen der Neuentwicklung einer LC-MS/MS Methoden gegenüber der bereits eingesetzten GC Methode zu bewerten.

Methoden

Die Analyse von Sulfur-AA per LC-MS/MS wurde mit Hilfe von einfacher Probenvorbereitung und Aqueous Normal Phase (ANP) Chromatographie durchgeführt. ANP Chromatographie ermöglicht einen Anstieg der Retentionszeit und damit verbunden eine Abtrennung des Homocystein von Co-Eluenten und anderen Matrixkomponenten.

Für die Analyse von NEFA wurde auf die sonst nötigen bzw. üblichen Extraktions- und Derivatisierungsverfahren verzichtet, um Zeit und Lösungsmittel zu sparen. Ultra-performance LC (UPLC) wurde verwendet, um kurze Laufzeiten in Kombination mit guter Auflösung zu erhalten.

Zusätzlich wurden Qualifier Ions eingeführt, um die korrekte Bestimmung der NEFA und der Sulfurhaltigen-AA zu unterstützen.

Der Zusammenhang zwischen Fettgewebszusammensetzung und NEFA wurde mit Hilfe von Probentripplets von 27 Probanden bestimmt, die sich einer abdominalen Operation zur Gewichtsreduktion unterzogen. Von diesen Probanden wurde subkutanes Fettgewebe (sAT), abdominales Fettgewebe (vAT) und Blutplasma entnommen. Um den Zusammenhang zwischen AT und NEFA zu beschreiben, wurden Spearmans Rangkorrelationskoeffizienten bestimmt.

Ergebnisse

Die neu entwickelte Methode zur Bestimmung von Suflur-haltigen AA erlaubt die Quantifizierung von Homocystein, Methionin und Cystein.

Die neu entwickelte Methode zur NEFA Messung ermöglicht es, 36 verschiedene NEFA Spezies zu quantifizieren.

Beide Methoden (Sulfur-AA/NEFA) zeigten in der Validierung eine hohe Präzision (CV <10%/<15%) und Genauigkeit (90-110%/80-120%).

Mit Hilfe von Qualifier Ions wurde die Analytenidentifizierung verbessert und es wurden Co-Eluenten sowohl für Sulfur-AA als auch NEFA identifiziert. Bei der Bestimmung der Sulfur-AA wurde die Identifikation von Co-Eluenten durch die verlängerte Retention der Analyten in der ANP Chromatographie unterstützt.

Beide entwickelten Methoden benötigen nur geringe Mengen an Probenvolumen (20µl) und eine kurze Probenvorbereitungszeit.

Der Zusammenhang zwischen AT und NEFA wurde für 42 FA untersucht. Starke Korrelationen zwischen NEFA und sAT/vAT wurden für die ungeradkettigen FA 15:0

(r=0.838/0.862), 17:0 (0.839/0.833), 17:1 (0.667/0.735), 19.0 (0.658/0.739), 19:1 (0.779/0.744) und 19:2 (0.803/0.560) sowie omega-3 FA 22:6 (0.719/0.535) gefunden, während andere FA niedrigere Korrelationen zeigten.

Schlussfolgerungen

Die entwickelten LC-MS/MS Methoden ermöglichen eine effiziente Quantifizierung von Homocystein, Methionin und Cystein sowie eine umfassende Bestimmung von NEFA in menschlichem Blutplasma. Die Reproduzierbarkeit und die Richtigkeit beider Methoden sind vergleichbar mit älteren Methoden. Die Einführung von Qualifier Ions verbesserte allerdings die korrekte Identifizierung der analysierten Metaboliten im MS.

Da weniger Probenvolumen benötigt wird und die gesamte Analysendauer im Vergleich zu vorherigen Methoden reduziert wurde, eignen sich beide Methoden für high-throughput Analysen. Dadurch ist eine Anwendung in größeren klinischen Studien möglich, in denen eine große Anzahl an Proben in kürzester Zeit und mit wenig Kosten analysiert werden müssen. Des Weiteren können die entwickelten Methoden aufgrund des geringen Probenvolumens auch in klinischen Studien angewendet werden, in denen Blut mit Hilfe von Finger- oder Fersenstiche gewonnen wird.

Die Anwendung der neu entwickelten NEFA Methode für die Untersuchung zum Zusammenhang zwischen AT FA und NEFA im Plasma unterstrich die Bedeutung einen umfassenden Bestimmung von NEFA im Plasma, da NEFA Gruppen, wie ungeradkettige oder sehr langkettige FA, die in anderen Methoden selten bestimmt werden, ein andere Verhalten zeigten als andere NEFA Gruppen.

Für den Gehalt von ungeradkettigen FA und langkettigen omega-3 FA im Fettgewebe, können NEFA in Nüchternplasma als Ersatzmarker herangezogen. Des Weiteren können diese NEFA als Marker für die Nahrungsaufnahme von Milchprodukten und Seefisch sowie Fischöl genutzt werden. Die niedrige Korrelation von einigen FA zwischen NEFA im Nüchternplasma und FA im AT lässt darauf schließen, dass nicht nur das Fettgewebe die Zusammensetzung der NEFA im Plasma bestimmt, sondern auch andere Gewebe und Faktoren einen Einfluss auf die Variation von NEFA im Nüchternzustand haben.

Für die Anaylse von Acylglycerolen stellen LC-MS/MS Metthoden ebenfalls eine leistungsfähige Alternative dar, wobei die genaue molekulare Zusammensetzung der

Acylglycerole bestimmt werden kann, was bei der Aufklärung von molekularen Stoffwechselvorgängen von Bedeutung ist. Allerdings hängt die Auswahl des benutzten Analysesystems stark von der Fragestellung der jeweils vorliegenden Studie ab. GC ermöglicht die Bestimmung von sehr niedrig konzentrierten FA in Acylglycerolen und die Trennung von omega FA, im Gegensatz zu LC-MS/MS. Daher sind LC-MS/MS Methoden nicht zwangsläufig den GC Methoden überlegen und der Aufwand von Lösungsmitteleinsatz und Entwicklungszeit für eine neue LC-MS/MS Methode sollte sorgfältig gegen die Vorteile der Bestimmung der Aclyglycerole durch LC-MS/MS abgewogen werden.

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9. Publications and Presentations

Publications

Hellmuth C, Koletzko B, Peissner W. Aqueous normal phase chromatography improves quantification and qualification of homocysteine, cysteine and methionine by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2011 Jan 1;879(1):83-9

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Hellmuth C, Demmelmair H, Schmitt I, Peissner W, Blüher M, Koletzko B. Association between plasma nonesterified fatty acids species and adipose tissue fatty acid composition. Submitted to PLOS One.

Presentations

Hellmuth C, Rauh-Pfeiffer A, Handel U, Demmelmair H, Peissner W, Koletzko B. The effect of folate supplementation on homocysteine level in childhood. ESPGHAN (European Society for Pediatric Gastroenterology, Hepatology and Nutrition) Update Meeting 2012; Stockholm, 27 – 28 April 2012. (poster)

Hellmuth C, Weber M, Koletzko B, Peissner W. NEFA determination for functional lipidomics: comprehensive UPLC-MS/MS quantitation , qualification and parameter prediction. ESPGHAN (European Society for Pediatric Gastroenterology, Hepatology and Nutrition) Update Meeting 2012; Stockholm, 27 – 28 April 2012. (poster)

Koletzko B, **Hellmuth C**, Weber M, Peissner W. NEFA determination in human plasma: comprehensive UPLC-MS/MS quantitation. 10th Congress of the International Society for Study Of Fatty Acids and Lipids (ISSFAL); Vancouver, 26 – 30 May 2012. (poster)

Hellmuth C, Weber M, Koletzko B, Peissner W. NEFA determination for functional lipidomics: comprehensive UPLC-MS/MS quantitation, qualification and parameter prediction. Jahrestagung 2012 der Deutschen Pharmazeutischen Gesellschaft (DPhG); Greifswald, 10 – 13 October 2012. (poster)

Hellmuth C, Schmitt I, Demmelmair J, Blüher M, Koletzko B. Plasma nonesterified fatty acids as non-invasive biomarker for adipose tissue fatty acid composition. 46th Annual Meeting of ESPGHAN (the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition); London, 8 – 11 May 2013. (poster of distinction)