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High Sensitivity Quantitative Lipidomics Analysis of Fatty Acids in Biological Samples by Gas Chromatography-Mass Spectrometry

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Abstract

Historically considered to be simple membrane components serving as structural elements and energy storing entities, fatty acids are now increasingly recognized as potent signaling molecules involved in many metabolic processes. Quantitative determination of fatty acids and exploration of fatty acid profiles have become common place in lipid analysis. We present here a reliable and sensitive method for comprehensive analysis of free fatty acids and fatty acid composition of complex lipids in biological material. The separation and quantitation of fatty acids is achieved by capillary gas chromatography. The analytical method uses pentafluorobenzyl bromide derivatization and negative chemical ionization gas chromatography-mass spectrometry. The chromatographic procedure provides base line separation between saturated and unsaturated fatty acids of different chain lengths as well as between most positional isomers. Fatty acids are extracted in the presence of isotope-labeled internal standards for high quantitation accuracy. Mass spectrometer conditions are optimized for broad detection capacity and sensitivity capable of measuring trace amounts of fatty acids in complex biological samples.

1. INTRODUCTION

Fatty acids have broad metabolic functions and exist in free forms or integrated into more complex lipids [1]. They are present in all organisms and constitute essential structural elements of biological membranes, regulate the activity of enzymes and fulfill important roles as signaling molecules. Because of their highly reduced chemical structure, fatty acids yield more than twice as much energy upon oxidation compared with polysaccharides making fat the most efficient form for living organisms to store excess energy. However, increased cellular concentrations of free fatty acids are toxic. Therefore, they are stored primarily as triacylglycerols and sterol esters in intracellular neutral lipid droplets that

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function as energy reservoirs and as a stockpile of fatty acids and sterols needed for membrane biosynthesis. Lipid droplets were originally found in fat-related tissues such as the adipose tissue, but subsequent research established that lipid droplets constitute a class of organelles that are present in virtually all cell types [2,3]. On demand, fatty acids are released from storage or membrane lipids by enzymatic hydrolysis mediated by lipases [4,5].

Aside from their role in energy homeostasis, fatty acids are also integral components of cellular membranes in the form of amphipathic lipids, predominantly glycerophospholipids. The lipid bilayer of membranes is often seen as an inert hydrophobic phase acting as a barrier between two compartments and as a matrix for transmembrane proteins. However, the bilayer is a highly organized system and a dynamically active environment. The fatty acid composition of membrane lipids regulates the physical properties, general architecture and function of membranes [6,7]. The fatty acid chain length and the number of double bonds as well as the position of these double bonds determine membrane fluidity and other dynamic properties including lateral and transverse movements of molecules within and across membranes [8]. The nature of the acyl chains defines the activities of membrane-associated enzymes and receptors and influences membrane budding, fission and fusion as part of membrane trafficking processes and cell division [9]. The fatty acid composition also affects the organizational arrangement of membrane micro domains and ultimately transmembrane signaling functions [10,11]. Membranes partition animal cells into sub-cellular structures to produce discrete organelles. This compartmentalization provides an active environment that enables the segregation of biochemical reactions for increased specificity and efficacy as well as controlled dispersion of the reaction products. Despite rapid turnover during lipid remodeling, mammalian membranes remain relatively constant in their saturated and monounsatured fatty acid composition, even over a wide range of dietary distributions of fatty acids, suggesting that the membrane lipid composition is a regulated parameter intended to maintain the integrity of membrane functions [12].

Free fatty acids (FFAs) have important functions as potent signaling molecules taking part in many physiological processes and aberrant fatty acid metabolism causing a chronic overabundance of FFAs can have pathological consequences. Chronically elevated levels of plasma FFAs may cause muscle insulin resistance, desensitization of adipocytes to the lipogenic effects of insulin, diabetes and steatosis in the liver [13–15]. Plasma FFAs have also been linked to cystic fibrosis [16], asthma [17], cancer and cancer-induced cachexia [18,19], impairment of general lymphocyte function [20], and sudden cardiac death [21]. However, not all types of fatty acids contribute equally to the pathological outcomes of associated diseases.

Fatty acids are aliphatic monocarboxylic acids with a large diversity in structure ranging from simple saturated carbon chains to more complex unsaturated, branched, cyclic and cis/trans configurations. They can also carry additional functional groups including keto, hydroxyl, peroxy and epoxy groups. Dicarboxylic acids do not occur in appreciable amounts in animal or vegetable lipids but can be produced metabolically from fatty acids and are useful industrial substrates. The fatty acids can be categorized into saturated, monounsaturated and polyunsaturated fatty acids. The polyunsaturated fatty acids (PUFAs) can be further divided into omega-3 (ω-3), omega-6 (ω-6) and omega-9 (ω-9) types. Eukaryotes can synthesize most fatty acids by chain elongation of an acetyl-CoA primer but lack the enzymatic system to introduce double bonds at position ω-6 or lower. These fatty acids or their precursors must be obtained from the diet [22]. For humans, linoleic acid (18:2, ω-6) and alpha-linolenic acid (18:3, ω-3) are considered essential fatty acids. Linoleic acid is the parent molecule for the ω-6 type of fatty acids including arachidonic acid (AA,

20:4), while alpha-linolenic acid can be elongated and desaturated to other ω-3 fatty acids including eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6).

An increase in ω-3 PUFAs has been shown to confer biological benefits. For example, several animal studies and clinical trials in humans have demonstrated that saturated and monounsaturated fatty acids increase the risk for ventricular arrhythmia, whereas ω-3 PUFAs prevented this arrhythmogenic action, with a significant reduction in cardiac death [23,24]. Further, epidemiological studies have indicated that fat rich in saturated fatty acids promotes insulin resistance, while PUFAs, in particular DHA, improved insulin sensitivity [25,26]. Free fatty acids have additional functions as important regulators of inflammatory responses by binding to specific fatty acid receptors, with a binding affinity that is typically in the micromolar range [27,28]. Fatty acids are also precursors of many potent bioactive lipids. Arachidonic acid can be converted to eicosanoids including prostaglandins, thromboxanes and leukotrienes that regulate a large number of pathways that control immune responses [29]. These metabolites act locally, have very limited half-lives and as such have the capacity to very precisely modulate the host response in inflammation [30]. There is a temporal progression of the classes of bioactive lipids that are produced during inflammation. Initially proinflammatory eicosanoids such as prostaglandins and leukotrienes are generated that stimulate, amongst others chemotaxis, leukocyte recruitment and vascular permeability. At a later stage, anti-inflammatory and pro-resolving lipid mediators are formed, which limit the inflammatory response and restore tissue homeostasis [31]. These highly stereospecific metabolites are derived from ω-6 AA, ω-3 EPA and ω-3 DHA and include lipoxins, resolvins and protectins. Pro-resolving mediators are produced locally at sites of inflammation, bind with high affinity to specific receptors and are part of immunoregulatory mechanisms that facilitate the resolution of inflammation [32]. Collectively, these findings establish an important biological network between innate immunity and lipid metabolism [33].

The physiological role of fatty acids in health and disease has gained appreciation and there has been an intense effort to develop suitable methodologies to quantitatively monitor fatty acid composition in biological material in a manner that satisfies the requirements for comprehensiveness, sensitivity and accuracy. The LIPID MAPS Consortium (www.lipidmaps.org) has established detailed procedures for sample extraction, separation and quantitative analysis for all major lipid categories [34] and has introduced a systematic classification system [35]. In this review, we will present quantitative aspects of fatty acid analysis using gas chromatography-mass spectrometry (GC/MS) to establish fatty acid profiles in biological samples and discuss alternative approaches.

2. SAMPLE PREPARATION

2.1. Extraction of free fatty acids (FFAs) from biological samples

The majority of fatty acids exist in the form of esters and amides in lipids and the FFAs constitute only a small portion of the total fatty acids. Therefore, before the FFA content of a biological sample can be analyzed, extraction and separation procedures are required to remove esterified fatty acids contained in all other lipid categories. Typically, lipids are extracted from tissues using solvent mixtures as described by Folch et al. [36] and Bligh and Dyer [37], followed by chromatographic separation techniques such as thin-layer chromatography (TLC) or liquid chromatography (LC) to establish lipid fractions. However, these strategies are time-consuming and oxidation of PUFAs and incomplete recovery are common complications with TLC [38]. Alternatively, the FFA fraction can be isolated by solid-phase extraction with aminopropyl-silica cartridges, however, recoveries may vary between tissue types and need to be determined empirically for each individual sample [39].

As part of the LIPID MAPS Consortium we have developed a rapid protocol for high-throughput extraction and isolation of FFAs from complex mixtures of blood plasma lipids, cultured cells, primary cells and animal tissues [40,41]. Prepurification and enrichment of FFAs are achieved with the application of a bi-phasic solution of acidified methanol and isooctane. A set of deuterated fatty acids is added to samples to serve as internal standards for quantitation and to compensate for any losses during the analytical procedure. For the analysis of the FFA composition of cultured cells, 0.5×10^6 cells are suspended in 250 μl PBS and 100 μl of deuterated internal standards, prepared as described below, are added. The extraction is initiated with 500 μl of methanol and 25 μl of 1 N HCl and a bi-phasic solution is formed by addition of 1.5 ml of isooctane. This solution is vigorously mixed for 30 sec and the phases are separated by centrifugation at 3000 rpm for 2 min. The upper isooctane phase containing the FFA fraction is removed, the extraction is repeated once and the combined extracts are evaporated to dryness. To minimize oxidative damage of PUFAs during prolonged storage, butylated hydroxytoluene (50 μg/ml) can be added to samples, but is not necessary if the samples are processed immediately.

The protocol is optimized for the analysis of mouse macrophage cell lines and primary macrophages but it is applicable to other cell types and tissues with minor modifications [42]. For the analysis of human or murine blood plasma, 100 μl of plasma are supplemented with internal standards and extracted twice with 0.05 N methanolic HCl/isooctane as described above [40]. The analysis of tissue, including adipose and liver tissue, involves a homogenization step as part of the extraction procedure. Adipose tissue is a relatively soft tissue and requires only brief sonication to achieve a fine dispersion suitable for quantitative FFA extraction. Typically, 5–10 mg of adipose tissue are suspended in 1 ml methanolic HCl, supplemented with internal standard, sonicated briefly (30 s) and then extracted twice with isooctane. Liver tissue (about 5 mg) is first homogenized (Omni Tissue Homogenizer) and then sonicated before the identical extraction protocol is applied.

2.2. Extraction of total fatty acids from biological samples

For the analysis of free and matrix bound fatty acid composition, a total lipid extraction is carried out first followed by a saponification step. Of the several lipid extraction procedures that are available, the modified Bligh and Dyer method is well suited for the extraction of lipids from cell cultures and tissues [37]. For the analysis of cell cultures or primary cells, ice-cold methanol (500 μl) and dichloromethane (250 μl) are added to 200 μl cell suspension (0.1×10^6 cells) and vortexed for 2 min. To minimize oxidation, 50 μg of butylated hydroxytoluene are added. A biphasic solution is formed by addition of 250 μl of dichloromethane and 250 μl of water. The phases are separated by centrifugation at 3000 rpm for 3 min and the lower organic layer is collected. The extraction is repeated once, 100 μl of internal standards are added and the combined lipid extracts are taken to dryness under argon.

For total fatty acid analysis of blood plasma, 10 μl of plasma are brought to a volume of 200 μl with PBS and extracted as described above. For analysis of free and esterified fatty acids in tissues such as liver or adipocytes, 1 mg of tissue is homogenized (2 min) and sonicated (30 s) on ice in 500 μl of methanol, spiked with internal standards and lipids are extracted as described above for cells. To ensure complete extraction, the vortexing step is extended to 10 min. Of note, the addition of acetic acid or HCl to the water phase significantly increases the recovery of some glycerophospholipids [43,44] and is particular useful for compositional analysis of glycerophosphatidylcholine and glycophosphatidylinositol [45]. It has also been reported that under certain conditions the Bligh and Dyer procedure may lead to an underestimation of lipids, especially when the total lipid content of the sample is exceedingly high. In that case, the Folch extraction represents a useful alternative [46].

For saponification, the dried lipids are resuspended in 500 μl of methanol:15% KOH (1:1) and incubated at 37 C for 30 min followed by acidification with 1N HCl to a pH<5. The lipid hydrolates are extracted twice with 1.5 ml of isooctane and the combined extracts containing the FFA fraction are taken to dryness under argon.

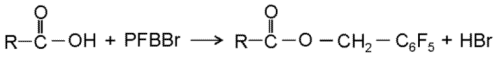
3. GAS CHROMATOGRAPHY OF FATTY ACIDS, INTERNAL STANDARDS AND PREPARATION OF FATTY ACID STANDARD CURVES

Gas chromatography (GC) has become widely adopted as a reliable tool for the quantitative analysis of complex mixtures of fatty acids. The method dates back to the early 1950s with the discovery that short-chain fatty acids can be separated by vapor-phase chromatography [47]. The first critical advancement to the initial protocol followed shortly thereafter when it was shown that converting the fatty acids to methyl esters improved the physical properties of long-chain fatty acids for analysis by GC [48]. Since then, major improvements have been made in both methodology and technology, and GC coupled with mass spectrometry (GC/MS) has become a routine procedure with broad application to biochemical, biomedical, forensic, agricultural, environmental and industrial research.

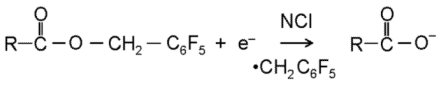
3.1. Derivatization of fatty acids

The analysis of fatty acids by GC is complicated by their polarity and inadequate volatility. To permit analysis by GC, the polar carbonyl groups must first be converted to produce more volatile non-polar derivatives. A wide range of alkylation reagents are available for this purpose and fatty acids are frequently converted to their corresponding fatty acid methyl esters (FAMEs). FAME derivatization has been extensively used for fatty acid analysis, especially in connection with flame ionization detection (FID) [49]. Analytical laboratories frequently apply FID as a method for fatty acid quantitation but certain limitations apply. The absolute quantitation typically depends on the signal strength of a single internal standard, usually heptadecanoic acid, that is applied to all fatty acids in the sample, rather than using mixtures of isotope-labeled fatty acid internal standards with chemical and physical properties similar to those of the target analytes [50]. Humans cannot make fatty acids with odd-numbered chain lengths, including heptadecanoic acid, but they can be taken up through the diet and are found in blood plasma where they contribute to the signal of the internal standard and confound quantitation [40]. Further, FID does not provide any information on molecular mass or other structural characteristics that can be used to discriminate between various fatty acids. Therefore, accurate quantitation by FID hinges on complete chromatographic resolution of all analytes of interest.

Pentafluorobenzyl bromide derivatization of fatty acids:



Dissociative electron capture ionization:



The introduction of mass spectrometry (MS) solved these issues and FAME quantitation has been successfully performed with electron ionization (EI) GC/MS. One has to keep in mind that fatty acids readily undergo fragmentation under hard EI conditions, which may aid structural analysis but at the expense of sensitivity. Soft ionization techniques such as negative chemical ionization (NCI) that leave quantifiable fragments in great supply

substantially improve detection limits. The detection limits can be further boosted by using halogenated derivatizing agents that increase electron affinity and enhance sensitivity for NCI-MS. Kawahara [51] introduced pentafluorobenzyl (PFB) bromide to derivatize organic carboxylic acids and showed that the resulting halogenated derivatives can be easily monitored by electron capture. Subsequently, the method was expanded to fatty acids and demonstrated that PFB esters of fatty acids show superior detection characteristics in NCI-MS, compared with FAME analogues that are typically analyzed by EI in positive ion mode [52]. The PFB ester derivatives display highly efficient electron capture properties. Subsequent fragmentation in the ion source of the mass spectrometer is entirely directed away from the fatty acid molecule. During heterolytic cleavage in the gas phase, a PFB radical is detached, leading to the formation of the stabilized carboxylate anion.

In preparation for GC analysis, fatty acids extracted from samples and the serial dilutions of quantitative standards, prepared as described below, are taken up in 25 μl of 1% diisopropylethylamine in acetonitrile and derivatized with 25 μl 1% PFB bromide in acetonitrile at room temperature for 20 min in capped glass tubes. The solvent is removed by a gentle stream of argon, the residues are dissolved in 50 μl isooctane and 1 μl of the volatile fatty acid PFB esters is analyzed by GC/MS using NCI (see below). The derivatization efficiency was determined on several selected saturated and unsaturated fatty acids. Known amounts of individual fatty acids were derivatized with PFB bromide using routine procedures and the non-derivatized fraction remaining in the reaction mixture was estimated by LC/MS using electrospray ionization in the negative ion mode. Equal amounts of free fatty acids without preceding derivatization were analyzed under identical conditions. The derivatization efficiencies, calculated by comparison of the signal strengths of the corresponding carboxylate anions, were about 80 – 85%. We did not observe any differences between fatty acids of various carbon chain lengths or degree of unsaturation.

The combination of GC and electron capture NCI has also been successfully applied to the identification and highly sensitive quantitation of prostaglandins, leukotrienes and other hydroxylated metabolites of AA, EPA and DHA including lipoxins [53,54]. All eicosanoids and related metabolites possess a terminal carboxyl function that can be readily converted to a PFB ester derivative, facilitating the thermal electron capture in the gas phase [55]. Eicosanoids contain one or more additional functional groups including keto, hydroxyl, peroxy and epoxy groups that require an extra derivatization step to improve the physical properties for GC/MS. Currently, eicosanoids are typically analyzed by LC/MS, which does not require any derivatization [56]. Nevertheless, the GC/MS approach can be further developed to measure the combination of both fatty acids and selected bioactive lipid mediators in the same profile.

3.2. Fatty acid internal standards

Using the GC/MS approach, fatty acids are reliably quantitated by the stable isotope dilution method. This method is based on the principle that each target analyte is compared to a deuterated analogue with similar chemical and structural properties. This technique increases the precision and accuracy of the measurement and compensates for any losses during sample preparation and analysis. The concentrations of the internal standards do not need to be precise, but it is imperative that they are added to all samples as well as to the reference material used for standard curves at exactly the same amounts. Presently, we use 14 deuterated fatty acid internal standards to quantitate 33 fatty acids. In some cases when isotope-labeled internal standards are not commercially available or when deuterated standards cannot be chromatographically resolved from unlabeled target analytes with identical mass, we employ labeled analogues with the closest chemical characteristics. A complete list of all internal standards as well as the corresponding fatty acids for which they are used in quantitative analyses is given in Table 1. For practical reasons, a stock of internal

standards is made in ethanol that contains each isotope-labeled fatty acid at 0.25 ng/ml. All samples and quantitative standards are spiked with 100 μl of the internal standard mixture.

3.3. GC/MS analysis of fatty acids

3.3.1. Gas chromatography—Our predominant goal was to establish a procedure that maximizes the number of analytes that can be accurately quantitated in a single analytical run. The protocol utilizes an Agilent 6890N gas chromatograph equipped with an Agilent 7683 autosampler (Santa Clara, CA). The fatty acid PFB esters dissolved in 50 μl isooctane are injected (1 μl) with a pulsed (25 psi) splitless injection mode with the injector temperature kept at 250°C. The fatty acid analysis demands high chromatographic resolution to separate fatty acids of various chain lengths and to accurately identify geometric and positional isomers of unsaturated fatty acids in complex mixtures. Capillary columns are traditionally used and come with an assortment of stationary phases of various polarities. We extensively use a Zebron ZB-1 column (15 m × 0.25 mm i.d.) with 0.1 μm 100% dimethylpolysiloxane as the stationary phase (Phenomenex, Torrance, CA). This low polarity general purpose column provides excellent separation of long-chain fatty acid PFB esters with low bleed characteristics for increased signal-to-noise ratio. With careful optimization of the temperature gradient program, full resolution over a wide range of fatty acids can be achieved. The GC oven temperature is ramped linearly from 150°C to 270°C at 10°C/min, increased to 310°C at 40°C/min and kept at this temperature for 1 min for column bake out. The sample injector and GC/MS transfer line are kept at 250°C and 280°C, respectively.

3.3.2. Separation of fatty acid isomers—A representative chromatographic separation of long-chain saturated and unsaturated fatty acids (>12 carbons) included in our quantitative standard reference mixture is shown in Figure 1. The data show that the temperature gradient and column selection achieve high peak resolution. Regioisomers are well separated, facilitating accurate peak alignment and identification. As can be seen in Figure 2A and 2B, good baseline separation is accomplished with all positional isomers of PUFAs commonly found in biological samples, including all ω-3 and ω-6 PUFAs.

Positional isomers of monoenoic fatty acids such as oleic acid (18:1; ω-9) and petroselinic acid (18:1; ω-12) are less well separated using this type of chromatographic column (Figure 2C). Similarly, the analysis of geometric isomers is challenging due to the subtlety of their chemical and physical differentiating characteristics that can be chromatographically exploited. Sophisticated application of mass spectrometry can reportedly distinguish between cis and trans isomers of monounsaturated fatty acids based on the relative signal strengths of a specific pair of ion signals [57]. However, this method is only applicable to a pure reference molecule and is not suitable for comprehensive fatty acid profiling studies of complex samples. The identification and separation of geometrical isomers in complex samples is most effectively achieved by GC under appropriate conditions using long capillary columns coated with highly polar cyanopolysiloxane stationary phases (for a discussion of columns see reference [58]). These columns enable the separation of geometric and positional isomers that are not fully resolved on columns with less polar stationary phases [59–61]. Even with optimized chromatographic conditions, overlapping elution of some isomers still occurs, but this can be prevented in part by fine tuning of the chromatographic conditions to target specific isomers of interest. An added complication is that polar columns required for separation of geometric isomers can cause co-elution of some saturated and unsaturated fatty acids, confounding component identification. Some of these concerns can be addressed by two-dimensional GC but this requires a complex technical arrangement [62]. Alternatively, a pre-purification step using silver-ion solid phase

extraction with silver-ion impregnated silica can be employed to fractionate cis and trans isomers of fatty acids prior to capillary GC analysis [63,64].

3.3.3. Mass spectrometry—Electron ionization is often used for fatty acids analysis in combination with GC [65]. This ionization method generates positive ions and extensive fragmentation. It is useful in conjunction with specialized derivatization techniques to obtain structural information, but is less powerful for the quantitative analysis of fatty acids. At high electron energy widespread molecular disruptions occurs with particular high yields of low molecular mass product ions but the fatty acid molecular ions are hardly detectable. Chemical ionization is a more gentle form of ionization with little fragmentation that renders the pseudo-molecular carboxylic acid ion mostly intact. The high ion yield with an m/z value close to that of a singly charged fatty acid molecular ion makes soft chemical ionization techniques highly applicable for quantitative fatty acid analysis. For enhanced sensitivity, we operate an Agilent 5975 mass selective detector in negative ion mode using NCI with methane as the reagent gas, the quadrupole mass filter temperature set at 250°C, the source temperature set at 280°C and with electron energies of 200 eV. With these settings, no fatty acid fragmentation is observed and because the electron capture is dissociative for the fatty acid PFB esters, the carboxylate anion of the fatty acid $[RCO_2]^-$ occurs in high abundance for quantitation (Figure 3). An additional advantage of operating the MS in NCI mode is that during the bombardment of methane with high energy electrons from the filament, no negative reagent gas ions are formed, which reduces background noise and enhances the signal-to-noise ratio. This protocol is optimized for PFB derivatives of fatty acids.

Due to the large number of analytes (33 unlabeled fatty acids and 14 isotope-labeled internal standards) that are included in this protocol, data acquisition must be divided into groups for selected ion monitoring (SIM) to achieve high sensitivity. A full scan provides more information and is useful for determining unknown molecules in a sample. However, in full scan operation the sensitivity of the mass spectrometer decreases due to fewer scans per time unit as each scan covers a wider range of mass ions. Operating the GC/MS in SIM mode can increase sensitivity by a factor of 10 to 100 times that of GC/MS run in full scan mode. Furthermore, careful assignment of the molecular ions to the various SIM groups increases specificity as only data for particular analytes of interest are gathered and matrix interferences are minimized. During method development, we first examined every fatty acid included in the analysis in full scan mode to establish retention time and confirm mass fingerprints. As an example, the GC/MS chromatogram and NCI mass spectrum of arachidonic acid is shown in Figure 3. Each fatty acid is then assigned to one of eight SIM groups based on retention time (Table 1). In each SIM group, individual ion optics, mass scan rate and dwell times (10 ms) are adjusted for optimal specificity and sensitivity. Fine tuning of the chromatographic methods, MS parameters, and group assignments of ions, is essential for selective profiling of fatty acids in biological samples.

3.4. Quantitative standards and preparation of standard curves

3.4.1. Fatty acid quantitation—The quantitative assessment of fatty acids in a sample is achieved by comparison of the mass spectrometric ion signal of the target molecule with that of an identical standard. Fatty acid standard curves are prepared from serial dilutions of a standard mixture of unlabeled quantitative fatty acid standards at precise concentrations. Currently, we are monitoring 33 individual fatty acids for which we have defined quantitative standards (Table 1). Each fatty acid is represented in the dilution sets of quantitative standards in the range of 0.15°500 ng. The standard sets receive exactly 100 μl of the isotope-labeled internal standard mixture, taken to dryness and the PFB derivatives are prepared as described above. A standard curve is generated by linear regression analysis

of the ratio between the ion yields of the quantitative standard and internal standard plotted versus the absolute amounts of the quantitative standard. The fatty acid content in the sample is then calculated from the standard curve using analyte/internal standard ion yield ratios. Figure 4 shows an example of a standard curve for arachidonic acid (AA). Similar standard curves are generated for all 33 fatty acids. The analysis of the regression parameters is also useful to ensure that the instrumental response is linear over the full analytical range. Using this protocol we found that the instrumental response is linear for all fatty acids over a range of 1 pg –10,000 pg injected.

3.4.2. Limits of detection—The dilutions of the composite reference mixture of fatty acids are also useful to determine the lower limit of detection (LOD). The LOD is defined by the signal to noise ratio of 3 and can be calculated from the chromatograms of the standard mixture at various dilutions. Using this procedure, the LODs for most fatty acids range from 0.05 pg to 1.0 pg (Table 1). In general, a higher sensitivity is observed for medium chain-length fatty acids, presumably due to differences in ionization efficiency. The LOD achieved with the method described here is about one order of magnitude lower than that of a procedure recently developed for fatty acid analysis using LC/MS [66]. The sensitivity of our method is comparable to the one achieved in the original study on the semi-quantitative analysis of PFB esters of fatty acids [52]. However, that study used instrument settings that were optimized for analysis of a few selected fatty acids. Such settings are technically not practical for the purpose of generating complete fatty acid profiles requiring simultaneous and unbiased quantitation of a large number of fatty acids. The method described in this review is balanced to achieve broad detection capacity while preserving sensitivity.

3.4.3. Assay validation—Validation assays are performed to determine accuracy and precision of the method. The accuracy of the isotope dilution quantitation approach is largely dependent on the precise knowledge of the concentration of the quantitative standards. To advance the mass spectrometry capabilities, the LIPID MAPS Consortium is actively working with commercial entities to facilitate the formulation of defined standards that meet LIPID MAPS specifications for purity and concentrations and make it available to the research community. The precision of the method is determined empirically using designated calibration controls consisting of a mixture of composite unlabeled standards and deuterated internal standards. The precision assays are carried out with all fatty acids in triplicate using routine sample preparation procedures. The analytical precision for the various fatty acids expressed as relative standard deviation (RSD) is shown in Table 1.

4. APPLICATION OF THE METHOD TO BIOLOGICAL SAMPLES

The method described here is routinely applied to establish complete fatty acid profiles in cultured and primary cells, tissues and blood plasma samples [40,41]. Figure 5 shows a representative chromatogram of free (non-esterified) fatty acids isolated from human plasma. As can be seen, not all fatty acids are fully baseline resolved, but they are distinguished by MS due to differences in *m*/*z*. This illustrates the importance of careful SIM group assignments of analytes and appropriate adjustments of SIM parameters to achieve complete discrimination from other fatty acids with similar retention times. It also illustrates the power of MS detection over FID. A complete list of all free fatty acids found in normal fasting human plasma is shown in Table 2. To estimate extraction recoveries, plasma was spiked with known amounts of fatty acids prior to extraction. Recoveries were calculated by comparison of the fatty acid content of the spiked plasma with the basal fatty acid content of the untreated plasma.

5. CONCLUSION

The collective tools of GC and MS represent a powerful platform for the analysis of fatty acids in complex samples and allow selective detection of saturated fatty acids, unsaturated fatty acids and their positional isomers with exceptional sensitivity. A combination of the chromatographic system with appropriate quantitation procedures facilitates measurement with great accuracy and precision.

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Research Highlights

- We review quantitative analyses of fatty acids in biological material
- Chromatographic separation of fatty acids is achieved by capillary GC
- Mass spectrometry using soft ionization techniques enables sensitive detection
- Isotope dilution method facilitates accurate and precise quantitation

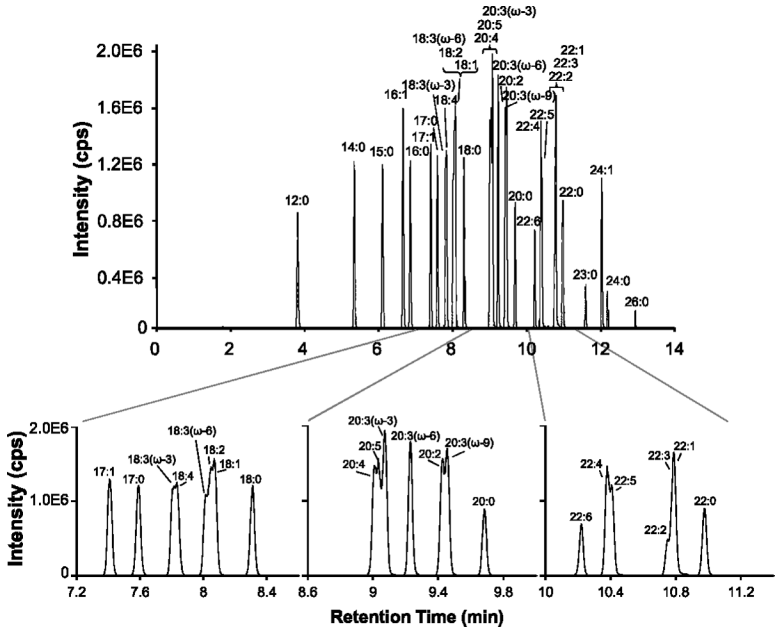

Figure 1.

GC/MS chromatogram of a reference mixture of fatty acid PFB esters.

The chromatogram for this figure and all subsequent figures were obtained with an Agilent 6890N Gas Chromatograph using Zebron ZB-1 capillary column. The eluting fatty acids were measured with an Agilent 5975 Mass Selective Detector using negative chemical ionization (NCI) and operated in negative ion mode and selected ion monitoring (SIM). Adapted from reference [42]. Insets show fatty acid profiles at magnifications of selected elution times.

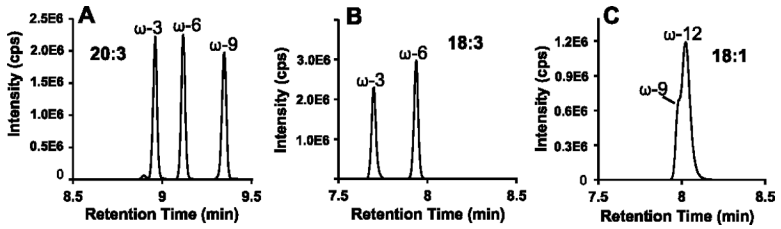

Figure 2.

GC elution profiles of positional isomers of unsaturated fatty acids.

A) Chromatographic separation of a mixture of eicosatrienoic acid isomers consisting of 11,14,17-eicosatrienoic acid (ω-3), bishomo-γ-linolenic acid (ω-6) and 5,8,11-eicosatrienoic acid (ω-9). **B)** Chromatographic separation of a mixture of octadecatrienoic acid isomers consisting of alpha-linolenic acid (ω-3) and gamma-linolenic acid (ω-6). **C)** Chromatogram of a mixture of octadecaenoic acid isomers consisting of oleic acid (ω-9) and petroselinic acid (ω-12). As can be seen, the applied chromatographic conditions facilitate baseline separation of all positional isomers of polyunsaturated fatty acids. Monoenoic fatty acids are less well separated and their complete resolution requires more polar column stationary phases.

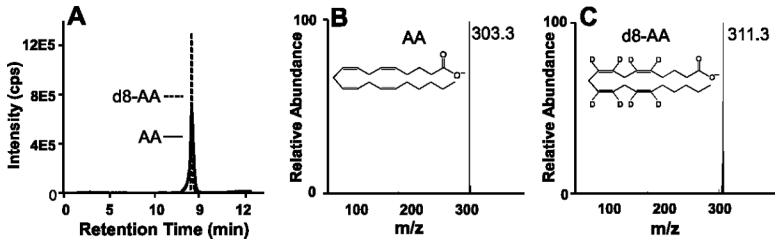

Figure 3.

NCI mass spectra of fatty acid PFB esters.

A) GC chromatogram of pure arachidonic acid (AA) and deuterated arachidonic acid (d8-AA) reference standards. Both standards are chromatographically indistinguishable and co-elute. The chromatogram was recorded with the mass spectrometer operated in negative ion mode and full scan. **B.** Mass spectrum of AA by NCI in negative ion mode. **C.** Mass spectrum of d8-AA by NCI in negative ion mode. No fragmentation is observed under the mass spectrometry conditions described and the fatty acid carboxylate anions are generated with high abundance yielding strong signals for sensitive detection and quantitation. Similar GC chromatograms and mass spectra are collected for all fatty acids to establish accurate ion masses and retention times.

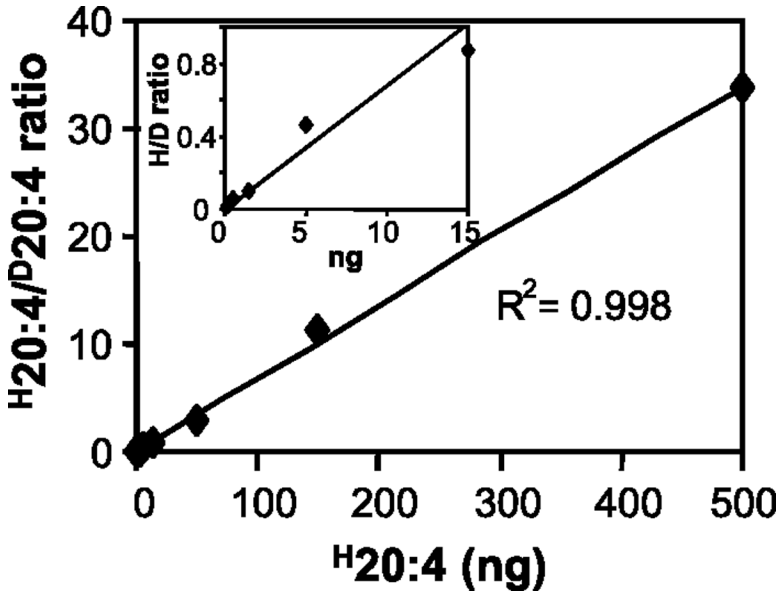

Figure 4.

Representative fatty acid calibration curve.

The quantitation of fatty acids by the stable isotope dilution method is based on empirically determined standard curves that are prepared by mixing increasing quantities of unlabeled fatty acids with fixed amounts of deuterium-labeled internal standards. For linear regression analysis, the signal ratio of unlabeled fatty acid/deuterated internal standard is calculated and plotted against the absolute amount of unlabeled fatty acid. Linear regression parameters are computed and used to convert the signal ratio of target analyte/deuterated internal standard into an absolute quantity. Inset shows an expansion of the calibration curve at low fatty acid concentrations. A linear instrumental response is observed over several orders of magnitude of amounts of analytes (1 pg – 10,000 pg) and is not significantly affected by matrix effects from the samples. Adapted from reference [42].

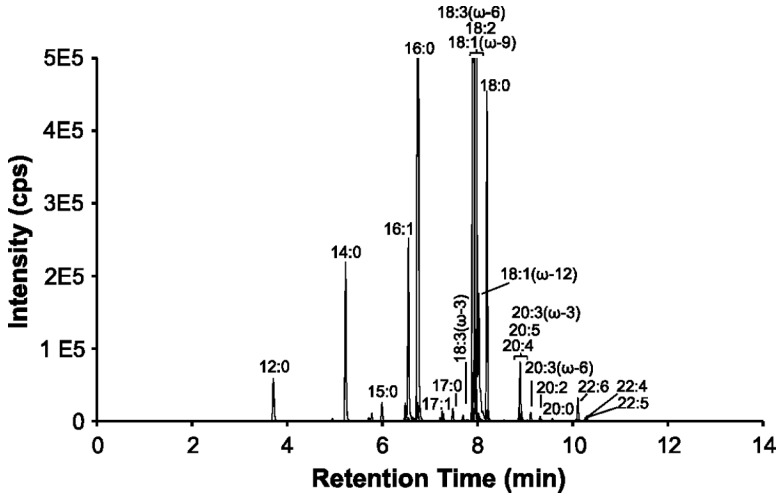

Figure 5.

GC/MS chromatogram of free fatty acids in human plasma.

The free fatty acids were extracted from pooled human plasma derived from healthy individuals after overnight fasting, derivatized and analyzed by GC/MS. For clear illustration, the figure shows the profile of fatty acids without internal standards. For quantification, a mixture of internal standards is added to the plasma sample prior to the extraction step. Each free fatty acid is quantitated from respective standard curves using the ratio between analyte peak area and corresponding internal peak area, which converts the instrumental signal into absolute amounts. Adapted from reference [40].

Table 1

Experimental conditions and validation data for the GC/MS analysis of fatty acids.

Common Name	Chain Length	$[RCO_2]^-$ *m/z*	t_R [a] (min)	INT STD [b]	SIM Group	LOD [c] (pg)	Precision (RSD) [d]	
							Intra-day	Inter-day
Laurie acid	12:0	199	3.75	12:0-d3	1	0.05	12	10
	12:0-d3	202	3.73	-	1	-	-	-
Myristic acid	14:0	227	5.28	14:0-d3	2	0.05	5	9
	14:0-d3	230	5.27	-	2	-	-	-
Pentadecanoic acid	15:0	241	6.04	15:0-d3	2	0.05	10	7
	15:0-d3	244	6.03		2	-	-	-
Palmitic acid	16:0	255	6.80	16:0-d3	3	0.1	8	8
	16:0-d3	258	6.78	-	3	-	-	-
Palmitoleic acid	16:1 (ω-7)	253	6.60	16:0-d3	3	0.5	9	15
Margaric acid	17:0	269	7.54	17:0-d3	3	0.1	9	15
	17:0-d3	272	7.53	-	3	-	-	-
Heptadecaenoic acid	17:1 (ω-7)	267	7.35	17:0-d3	3	5	12	16
Stearic acid	18:0	283	8.25	18:0-d3	4	0.1	7	11
	18:0-d3	286	8.24	-	4	-	-	-
Oleic acid	18:1 (ω-9)	281	8.02	18:1-d2	4	0.05	8	12
	18:1-d2	283	8.00	-	4	-	-	-
Linoleic acid	18:2 (ω-6)	279	7.96	18:2-d4	4	0.05	9	12
α-linolenic acid	18:3 (ω-3)	277	7.75	18:2-d4	4	1	9	10
γ-linolenic acid	18:3 (ω-6)	277	7.99	18:2-d4	4	1	9	7
Stearidonic acid	18:4 (ω-3)	275	7.77	18:2-d4	4	5	6	8
Arachidic acid	20:0	311	9.63	20:0-d3	5	5	8	8
	20:0-d3	314	9.61	-	5	-	-	-
Gondoic acid	20:1 (ω-9)	309	9.42	20:0-d3	5	n/d	n/d	n/d
Eicosadienoic acid	20:2 (ω-6)	307	9.37	20:4-d8	5	5	7	12
Eicosatrienoic acid	20:3 (ω-3)	305	9.01	20:4-d8	5	5	6	10
Bishomo-γ-linolenic acid	20:3 (ω-6)	305	9.17	20:4-d8	5	1	6	13
Eicosatrienoic acid	20:3 (ω-9)	305	9.40	20:4-d8	5	1	5	8

Common Name	Chain Length	$[RCO_2]^-$ *m/z*	t_R [a] (min)	INT STD [b]	SIM Group	LOD [c] (pg)	Precision (RSD) [d]	
							Intra-day	Inter-day
Arachidonic acid	20:4 (ω-6)	303	8.95	20:4-d8	5	0.05	7	12
	20:4-d8	311	8.93	-	5	-	-	-
Eicosapentaenoic acid	20:5 (ω-3)	301	8.97	20:5-d5	5	0.5	7	8
	20:5-d5	306	8.95	-	5	-	-	-
Behenic acid	22:0	339	10.92	22:0-d3	6	5	14	15
	22:0-d3	342	10.90	-	6	-	-	-
Erucic acid	22:1 (ω-9)	337	10.74	22:0-d3	6	0.75	9	9
Docosadienoic acid	22:2 (ω-6)	335	10.70	22:0-d3	6	5	12	15
Docosatrienoic acid	22:3 (ω-3)	333	10.73	22:6-d5	6	10	10	14
Docosatetraenoic acid	22:4 (ω-6)	331	10.32	22:6-d5	6	5	8	15
Docosapentaenoic acid	22:5 (ω-3)	329	10.13	22:6-d5	6	0.5	4	15
Docosapentaenoic acid	22:5 (ω-6)	329	10.35	22:6-d5		n/d	n/d	n/d
Docosahexaenoic acid	22:6 (ω-3)	327	10.16	22:6-d5	6	0.5	7	9
	22:6-d5	332	10.15	-	6	-	-	-
Tricosanoic acid	23:0	353	11.54	24:0-d4	7	0.25	13	9
Lignoceric acid	24:0	367	12.14	24:0-d4	7	1	7	8
	24:0-d4	371	12.13	-	7	-	-	-
Nervonic acid	24:1 (ω-9)	365	11.98	24:0-d4	7	1	11	17
Cerotic acid	26:0	395	12.91	26:0-d4	8	0.5	14	10
	26:0-d4	399	12.90	-	8	-	-	-

[a] t_R, retention time.

[b] INT STD, deuterated internal standards. The INT STD are listed in reference to the corresponding fatty acids for which they were used for quantification purpose.

[c] LOD, The lower limit of detection.

[d] The analytical precision was determined in triplicates for both intra- and inter-day precision and expressed as the relative standard deviation (RSD). N/d, not determined. Adapted from reference [42].

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Table 2

Free (non-esterified) fatty acid composition of human plasma. All measurements were performed in triplicate.

Fatty Acid	Mean (μmoI/I)	RSE (%)	Distribution (%)	Recovery (%)
Lauric acid (12:0)	0.719	4.1	0.3	99
Myristic acid (14:0)	6.06	1.1	2.8	91
Pentadecanoic acid (15:0)	0.653	0.6	0.3	94
Palmitic acid (16:0)	63.8	0.6	29.7	90
Palmitoleic acid (16:1)	14.7	1.2	6.7	95
Margaric acid (17:0)	1.20	0.3	0.6	93
Heptadecenoic acid (17:1 ω-7)	1.03	5.5	0.5	n.d.
Stearic acid (18:0)	22.1	0.2	10.5	95
Oleic acid (18:1)	80.3	11.6	37.6	115
Linoleic acid (18:2)	15.2	2.9	7.1	104
α-linolenic acid (18:3)	0.115	3.8	0.1	89
γ-Linolenic acid (18:3)	1.03	0.4	0.5	97
Stearidonic acid (18:4)	0.016	4.1	0.0	n.d.
Arachidic acid (20:0)	0.238	1.0	0.1	90
Eicosadienoic acid (20:2 ω-6)	0.352	8.2	0.2	n.d.
Eicosatrienoic acid (20:3 ω-3)	0.341	4.6	0.2	n.d.
Bishomo-γ-linolenic Acid (20:3 ω-6)	0.542	0.9	0.3	91
Eicosatrienoic acid (20:3 ω-9)	0.095	12.8	0.0	n.d.
Arachidonic acid (20:4)	2.94	2.0	1.3	101
Eicosapentaenoic acid (20:5 ω-3)	0.435	2.4	0.2	105
Behenic acid (22:0)	0.160	4.3	0.1	78
Erucic acid (22:1)	0.028	7.4	0.0	n.d.
Docosadienoic acid (22:2 ω-6)	0.011	4.8	0.0	101
Docosatrienoic acid (22:3 ω-3)	0.004	21.0	0.0	n.d.
Docosatetraenoic acid (22:4 ω-6)	0.364	1.3	0.2	n.d.
Docosapentaenoic acid (22:5 ω-3)	0.400	1.4	0.2	96
Docosahexaenoic acid (22:6 ω-3)	0.990	0.9	0.4	108
Tricosanoic acid (23:0)	0.033	10.9	0.0	89
Lignoceric acid (24:0)	0.262	5.3	0.1	87
Nervonic acid (24:1)	0.070	8.2	0.0	n.d.
Cerotic acid (26:0)	0.110	5.6	0.1	104
Total	**214.3**		**100**	

RSE: Relative standard error of the mean.

Recovery was calculated by the formula: recovery = [(measured FFA − basal FFA)/added FFA] × 100

Data derived from reference [40].