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

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SAMPLE PREPARATION

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 analysed, 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, 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. 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.

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. Pre-purification 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. 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. 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.

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. 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.

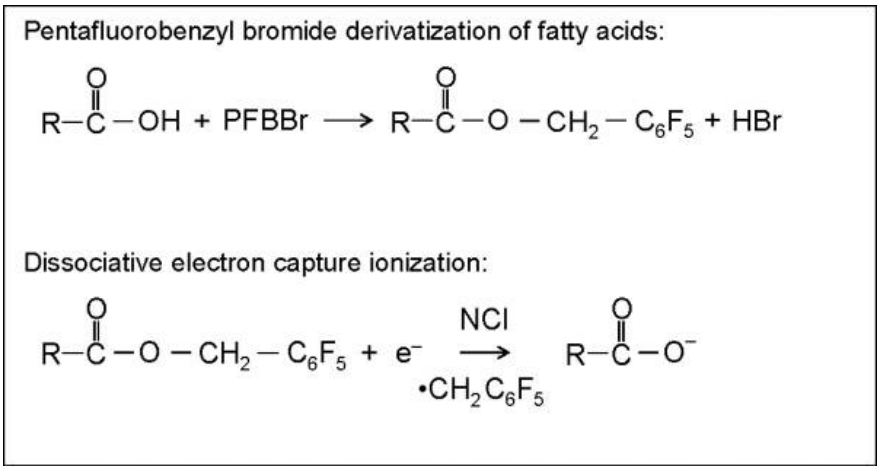
For total fatty acid analysis of blood plasma, 10 µl of plasma are brought to a volume of 200 µl with PBS and extracted as described above. For analysis of free and esterified fatty acids in tissues such as liver or adipocytes, 1 mg of tissue is homogenized (2 min) and sonicated (30 s) on ice in 500 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 and is useful for compositional analysis of glycerophosphatidylcholine and glycophosphatidylinositol. 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.

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.

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

Derivatization of fatty acids

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). 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-labelled fatty acid internal standards with chemical and physical properties similar to those of the target analytes. 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. 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.



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 derivat-

izing agents that increase electron affinity and enhance sensitivity for NCI-MS. Kawahara introduced pentafluorobenzyl (PFB) bromide to derivatize organic carboxylic acids and showed that the resulting halogenated derivatives can be easily monitored by electron capture.

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.

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.

GC/MS analysis of fatty acids

Gas chromatography

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.

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.

Table 1. Experimental conditions and validation data for the GC/MS analysis of fatty acids.

							Precision (RSD)[d]	
Common Name	**Chain Length**	**$[RCO_2]^-$ *m/z***	**t_R[a] (min)**	**INT STD[b]**	**SIM Group**	**LOD[c] (pg)**	**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
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

[a]t_R, retention time.
[b]INT STD, deuterated internal standards. The INT STD are listed 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.

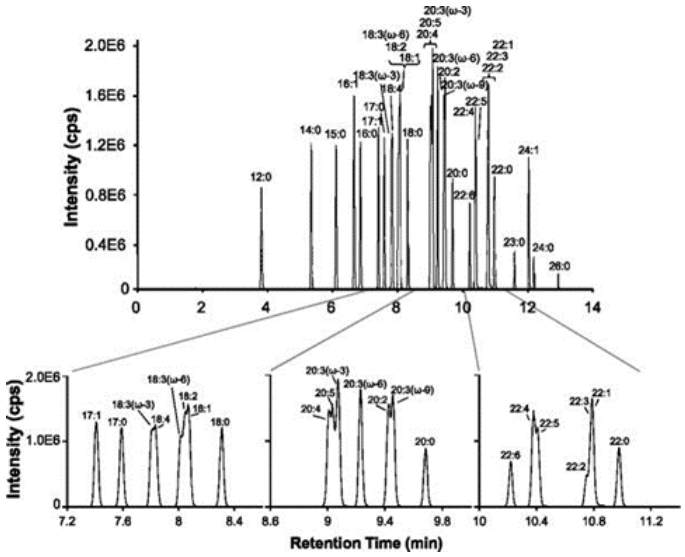


Figure 1. GC/MS chromatogram of a reference mixture of fatty acid PFB esters. The chromatogram for this figure and all subsequent figures were obtained with an Agilent 6890N Gas Chromatograph using Zebron ZB-1 capillary column. The eluting fatty acids were measured with an Agilent 5975 Mass Selective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

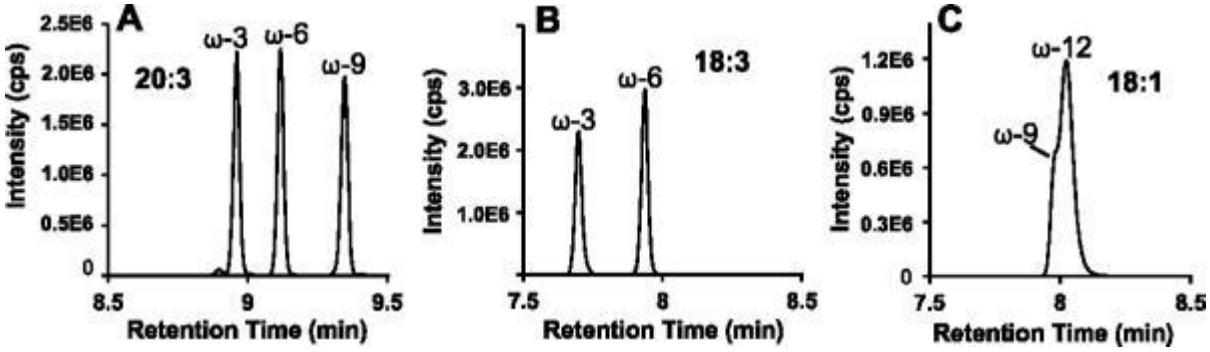


Figure 2. GC elution profiles of positional isomers of unsaturated fatty acids.

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.

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. 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 paper for details).

Mass spectrometry

Electron ionization is often used for fatty acids analysis in combination with GC. 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

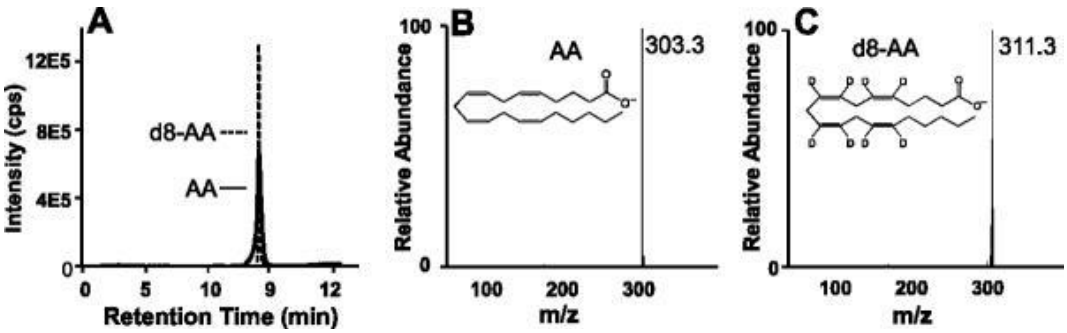


Figure 3 NCI mass spectra of fatty acid PFB esters. **A)** GC chromatogram of pure arachidonic acid (AA) and deuterated arachidonic acid (d8-AA) reference standards. Both standards are chromatographically indistinguishable and co-elute. The chromatogram was recorded with the mass spectrometer operated 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.

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.

Quantitative standards and preparation of standard curves

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.

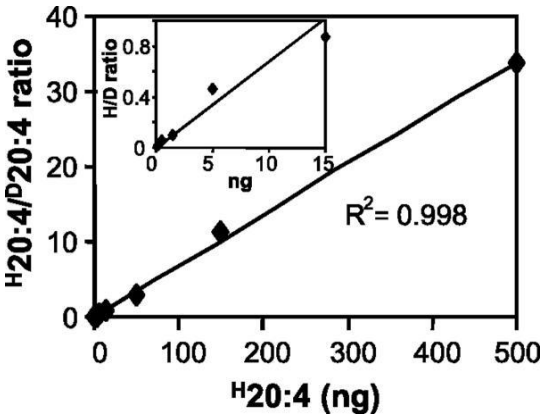


Figure 4 Representative fatty acid calibration curve. The quantitation of fatty acids by the stable isotope dilution method is based on empirically determined standard curves that are prepared by mixing increasing quantities of unlabeled fatty acids with fixed amounts of deuterium-labeled internal standards. 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.

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. 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. 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.

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**.

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. **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.

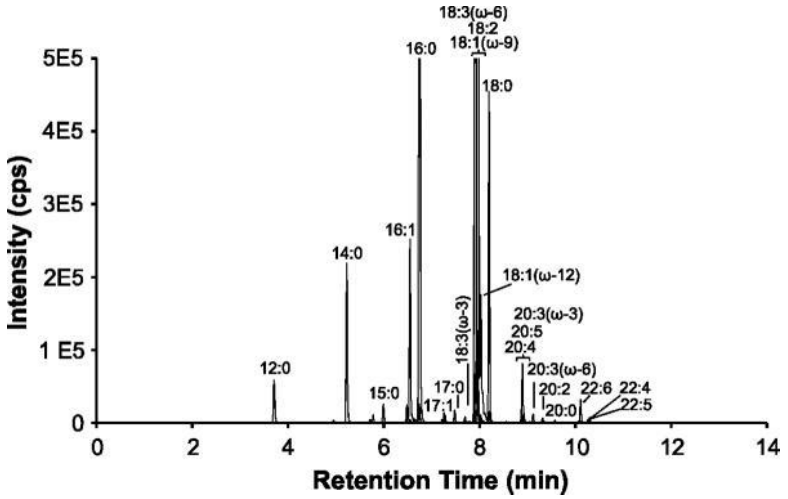


Figure 5. GC/MS chromatogram of free fatty acids in human plasma. The free fatty acids were extracted from pooled human plasma derived from healthy individuals after overnight fasting, derivatized and analyzed by GC/MS. For clear illustration, the figure shows the profile of fatty acids without internal 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.

Table 2. Free (non-esterified) fatty acid composition of human plasma. All measurements were performed in triplicate.

Fatty Acid	Mean (µmol/l)	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.

Fatty Acid	Mean (µmol/l)	RSE (%)	Distribution (%)	Recovery (%)
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