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Methods Paper

Liquid Chromatography/Electrospray Mass Spectrometric Detection of Fatty Acid by Charge Reversal Derivatization with More Than 4-Orders of Magnitude Improvement in Sensitivity

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Abbreviations: ESI-MS/MS, electrospray ionization tandem mass spectrometry; LC/ESI-MS/MS, liquid chromatography coupled to ESI-MS/MS; fatty acyl chains are abbreviated with the number of carbons and double bounds and the double bond positions, i.e. 5,9-18:2 is an 18 carbon fatty acyl chain with 2 double bonds starting at carbons 5 and 9 from the carboxyl end. All double bonds have the *cis* (*Z*) configuation unless stated otherwise.

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Abstract

Quantitative analysis of fatty acids (FAs) is an important area of analytical biochemistry. Ultra high sensitivity FA analysis usually is done with gas chromatography of pentafluorobenzyl esters coupled to an electron capture detector. With the popularity of electrospray ionization mass spectrometers coupled to liquid chromagraphy, it would be convenient to develop a method for ultra high sensitivity FA detection using this equipment. Although fatty acids can be analyzed by electrospray ionization in negative ion mode, this method is not very sensitive. In this study, we demonstrate a new method of FA analysis based on conversion of the carboxylic acid to an amide bearing a permanent positive charge (N-(4-aminomethylphenyl)pyridinium) (AMPP) combined with analysis on a reverse phase liquid chromatography column coupled to an electrospray ionization mass spectrometer operating in positive ion mode. This leads to a ~60,000-fold increase in sensitivity compared to the same method carried out with underivatized FAs. The new method is about 10-fold more sensitive than the existing method of gas chromatography/electron-capture mass spectrometry of fatty acid pentafluorobenzyl esters. Furthermore, significant fragmentation of the precursor ions in the non-tag portion improves analytical specificity. We show that a large number of FA molecular species can be analyzed with this method in complex biological samples such as mouse serum.

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INTRODUCTION

The analysis of fatty acids (FAs) is of considerable importance to both the clinical and biomedical research communities. From the clinical perspective, perturbations of FA metabolism have important physiological implications for a variety of medical conditions such as obesity, cardiovascular disease, and diabetes mellitus (1-3). Attention from the biomedical community is largely derived from the observation that some FAs, in particular the non-esterified fractions of polyunsaturated species such as arachidonic acid (AA) and docosohexaenoic acid (DHA), have distinct roles as precursors to important lipid signaling molecules (4,5). Given their diverse biological roles and implication in a host of pathological conditions, considerable effort is dedicated to the development of methodologies to reliably and accurately assess FA composition and metabolism in a host of biological contexts. To meet these ends, tandem mass spectrometry (MS/MS) has emerged as the premier analytical platform due to its sensitivity, specificity, and ability to be directly coupled to chromatography systems (6).

Early quantitation methods for free FAs typically relied on gas chromatography with flame ionization detection or coupled to a mass spectrometer via electron ionization. The advantages of gas chromatography include high specificity, sensitivity, and good reproducibility (7). Resolution of fatty acids requires prior derivatization to increase their volatility and thermal stability. This has been typically accomplished by esterification to methyl (8) trimethylsilyl (9), or pentafluorobenzyl esters (10). The utility of these methods was greatly enhanced through the development of novel ionization sources and tandem MS/MS instrumentation capable of selected reaction monitoring (SRM) experiments. SRM detects fragmentation products of specific chemical species at the exclusion of potential interference from chemical noise and co-eluting compounds with identical masses. The analytical specificity of these experiments enables the direct quantitative analysis of species from very complex biological mixtures. Although useful, these

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methods are still limited by dynamic range limitations and compound volatility considerations (11). Although electron capture detection of pentafluorobenzyl esters of fatty acids provides exceptionally high sensitivity, there are many laborataories worldwide that now have access to electrospray ionization (ESI) machines rather than electron capture instruments. However, a major obstacle to the ESI technique is that FAs undergo less than ideal fragmentation behavior in negative ion mode via collision-induced dissociation (CID). Under low-energy (< 100 eV) CID conditions typical to most commercial instruments, fragmentation of the featureless backbone of a saturated FA is minimal. Furthermore, the most prominent fragments originate from the loss of CO_2 (−44 Da) and elimination of water (−18 *amu's*) from the carboxylic acid group, neither of which are specific enough for reliable quantitation in complex matrices. Unsaturated FAs do undergo, to some extent, fragmentations that are specific to their structure. However, the abundances of these fragmentations are relatively weak and result in SRM measurements of poor sensitivity. Another major limitation of this approach is related to a FAs relative ionization efficiency and the manner in which the ions are analyzed. For compounds that contain free carboxylates such as FAs, ionization is best achieved in negative ion mode under basic pH conditions where the carboxylate is ionized (12-15). Unfortunately, optimal LC chromatographic resolution is facilitated by acidic pH conditions, to keep the carboxyl group protonated, where ionization of the carboxylate is suppressed. Post-column addition of base could potentially alleviate this problem at the expense of the method's simplicity and sensitivity.

One group recently reported a LC-ESI-MS/MS method of FA analysis in plasma using post-column infusion of a barium ion solution (16). The formation of positively charged adduct ions promotes diagnostic fragmentation reactions of unsaturated FA species with enhanced SRM detection sensitivity. Other cation reagents, including alkaline earth metals and copper ions, also proved suitable for enhanced sensitivity for

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FA analysis in the SRM mode (17,18) An alternative strategy for enhanced sensitivity is to improve the ionization efficiency of FAs via specific derivatization with reagents that introduce either readily chargeable or fixed charge groups such as tertiary or quaternary amines, respectively. Many derivatives of this nature have been reported including: pyrolidides (19-21), picolinyl esters (22,23), dimethyloxazolines (24-28), benzofurazans (29), pyridiniums (30), and cholines (31). The advantages of these derivatives include improved MS sensitivity and reproducible chromatography profiles. A major limitation of these methodologies is the relatively harsh conditions usually required for derivitization, which can result in unwanted oxidation, isomerization, or degradation of some FAs. This limitation could potentially be addressed by the development of robust derivatization procedures that require milder conditions. Another major limitation is the tendency of these derivatives to fragment via CID in immediate proximity to the chargeable/cationic site. Fragmentation in the derivatization tag is undesirable due to the fact that analytes that form isobaric precursor ions and co-elute during LC will not be distinguished in the mass spectrometer if they give rise to the same detected fragment ion, essentially eliminating any advantage a MS/MS experiment has over a MS one. This loss of specificity represents a significant limitation when analyzing complex biological samples.

We recently reported a straightforward LC-ESI-MS/MS derivitization procedure for the targeted lipidomic analysis of eicosanoids via stable isotope dilution (32). The carboxyl group is derivatized with a newly developed reagent N-(4-aminomethylphenyl)pyridinium (AMPP) that results a permanent positive charge (charge reversal). This derivatization results in a 10- to 20-fold improvement in detection sensitivity by LC-ESI-MS/MS (32). Our methodology employed a simple solid-phase extraction procedure of eicosanoids from a variety of biological matrices followed by a mild, quantitative derivatization step with AMPP. The resulting derivatives can be directly submitted to LC-ESI-MS/MS and display robust fragmentations in their analyte

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segments making them attractive candidates for high-sensitivity/specificity SRM experiments. Here we utilize a similar approach, with the exception of an alternative extraction method, to monitor the free FA profiles in complex biological samples. We developed and validated a stable isotope dilution LC-ESI-MS/MS method that is able to detect essentially all saturated and unsaturated FAs in a single chromatographic run. Sensitivity improvement over LC-ESI-MS/MS of underivatized FAs in negative ion mode is ~60,000-fold.

METHODS

Preparation of fatty acid-free glassware and reagents. Low abundant fatty acids such as arachidonic acid are usually not present as a contaminant in glassware and reagents; however, abundant fatty acids such as oleic, palmitic and stearic acids are present as common contaminants. It has not been possible to remove these contaminants to a level below the fatty acid detection limit for the method described in this paper. The procedure described here reduces abundant fatty acid contamination to a level usually below the amounts to be detected in the sample of interest.

All glassware used for extraction and pre-LC-ESI-MS/MS work-up was baked overnight in a high temperature oven at 450 ^{o}C to remove any residual fatty acid contamination. Similarly, isooctane (Sigma Chromasolv Plus Cat. # 650439), DMF (Sigma Cat. # 227056), Milli-Q water, ethanol, and acetonitrile (Fisher Optima Grade Cat. # L-14338) were distilled in-house (DMF distilled under vacuum) with an oven baked (450 ^{o}C, overnight) distillation apparatus into oven baked glass-stoppered flasks. Finally, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDCI) (TCI America Cat. # D1601), 1-Hydroxy-7-azabenzotriazole (HOAt) (Sigma Cat. # 44545-2), and AMPP (32) were triturated with distilled isooctane to remove any residual fatty acid contamination.

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Preparation of fatty acid stock solutions. The following fatty acid standards from Cayman Chemicals were used (d_{14}-palmitoleic acid, d_{14}-α-linolenic acid, d_4-linoleic acid, d_5-eicosapentaenoic acid, d_8-arachidonic acid, d_{17}-oleic acid, d_6-dihomo-γ–Linolenic, d_5-docosohexaenoic acid, stearidonic acid, and arachidonic acid (ω-3). d_{31}-Palmitic acid and d_{35}-stearic acid are from Sigma-Aldrich. GLC-463 standard (Nu-Check Prep. Inc.), containing 52 distinct FA molecular species, was used for the rest of the calibration standards. Stock solutions of fatty acids were prepared at concentration of 25-100 pg/μl in absolute ethanol and stored at -80 °C under Ar in 1.5 mL amber vials (Agilent Cat. # 5182-0716) with PTFE/silicone septum screw caps (Agilent Cat. # 5185-5838). Serial dilutions of the stock solutions were made in absolute ethanol for standard curve and extraction recovery analyses. Internal standards were diluted to a working stock of 100 pg/μl in absolute ethanol.

Preparation of samples and derivatization with AMPP.

Standard curves. Each sample contained 1 ng of each internal standard and various amounts of non-deuterated FAs (added from serial dilutions of the accurate concentration stock solution made from milligram amounts of FA as described above) transferred to a glass auto-sampler vial (Waters Total Recovery Screw Cap Vial cat. # 186002805). Solvent was removed with a stream of nitrogen, and the residue was derivatized with AMPP as described below.

Extraction of FAs from mouse serum. Analysis of endogenous FAs in serum was carried out with commercial mouse serum (Atlantic Biologicals catalog # S18110). A 10 μL aliquot of serum was transferred to a 12 X 75 mm glass culture tube. To each culture tube, 50 μL of absolute ethanol containing 1 ng of each internal standard was added. The sample was adjusted to 125 μL by adding purified water (Milli-Q, Millipore Corp.). Aliquots of 250 μL of methanol (Fisher Optima Grade Cat. # A456-4) and 12.5 μL of 1 N

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HCl were added to each sample. A bi-phasic solution was formed via addition of 750 μL of isooctane. This solution was vortexed for 60 sec, and the phases were separated by centrifugation at 3000 rpm for 60 seconds. The upper isooctane phase was removed via an oven baked glass pasteur pipet and transferred to an oven baked Waters Total Recovery vial. The remaining aqueous phase was extracted once more with an additional 750 μL of isooctane. The combined isooctane phases were evaporated to dryness under a stream of filtered N_2 and derivatized with AMPP as described below.

Derivatization with AMPP. AMPP was synthesized in-house as described previously (32). Subsequent to our lead publication, the AMPP reagent was made commercially available by Cayman Chemical Company (Cat. # 710000) under the product name AMP+ Mass Spectrometry Kit.

To the residue in the oven baked Waters Total Recovery auto-sampler vial was added 10 μL of ice-cold acetonitrile/DMF (4:1, v:v). Ten μL of ice-cold 1 M EDCI in distilled Milli-Q water (freshly prepared daily) was added. The vial was briefly mixed on a vortex mixer and placed on ice while other samples were processed as above. To each vial was added 20 μL of 5 mM HOAt/15 mM AMPP in distilled acetonitrile (stored at -20 ^{o}C and warmed to 65 ^{o}C immediately prior to use). The vials were mixed briefly on a vortex mixer, capped with a split-septum screw cap (Agilent Cat. # 5185-5824), and placed in a 60 °C incubator for 30 min. Samples were analyzed on the same day and kept in the auto-sampler rack at 10 °C while queued for injection.

LC/ESI-MS/MS analysis.

Studies were carried out on a Waters Xevo TQ triple quadrupole mass spectrometer interfaced to an Acquity UPLC. The MassLynx 4.1 software package was used for data collection and analysis. Chromatography was carried out with a C18 reverse-phase column (Waters Acquity UPLC BEH Shield RP18, 2.1 x 100 mm, 1.7 μm,

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Cat. # 186002854). Solvent A is 100% water (Fisher Optima Grade Cat. # L-13780)/0.1% formic acid (Fisher Optima Grade Cat. # A117-50), and solvent B is acetonitrile (Fisher Optima Grade Cat. # L-14338)/0.1% formic acid. The solvent program is (linear gradients): 0-0.5 min, 90% A; 0.5-0.51 min, 90% – 80% A; 0.51-10.0 min, 80% – 30% A; 10.0-10.1 min, 30% – 0% A; 10.1-12.0 min, 0% A; 12.0-12.1 min, 0% – 90% A; 12.1-15.0 min, 90% A. The flow rate is 0.4 mL/min and column temperature is 45 ^{o}C. Supplemental Tables 1 and 2 summarize the auto-sampler and ESI-MS/MS parameters for data collection, respectively.

RESULTS and DISCUSSION:

As noted in the introduction, conversion of the carboxyl group of lipids such as eicosanoids and FAs to the AMPP amide results in an analyte with a permanent positive charge, which can be analyzed by LC-ESI-MS/MS in positive ion mode. This is more sensitive than negative ion mode detection of the underivatized caboxylate anion since ionization of the latter is greatly suppressed by the protonation resulting from the addition of weak organic acid such as acetic or formic acid, which are necessary for optimal LC on reverse-phase columns. As shown in Fig. 1 AMPP amides of fatty acids give rise to spectral signature ions at *m*/*z* 169 and 183 due to collision-induced dissociation of the AMPP tag. However, abundant high molecular weight fragments are also generated. For example, *m*/*z* = 239 for fragmentation between C3 and C4 in most fatty acid species (Fig. 1). AMPP amides of oleic acid and its deuterated analog show an abundant product ion at *m*/*z* = 239 due to cleavage between C7 and C8, thus leaving a relatively stable allylic radical. This ion is not present in the spectrum of the AMPP amide of petroselenic acid (Fig. 1). Likewise, vaccenic AMPP amide shows a major product ion at *m*/*z* = 323, due to cleavage of the C9-C10 bond to generate an allylic radical. This species is not present in the other 18:1 spectra. These high molecular

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weight ion product ions provide for high analytical specificity, which may be important for analysis of FAs in complex biological samples. If a product ion resulting from cleavage of the AMPP tag is used for MS/MS, it would not be possible to distinguish isobaric, AMPP-labeled species that co-elute during LC. High molecular weight product ions were observed for all FAs analyzed. Precursor and product *m*/*z* values for all FAs are given in Table 1, and product ion mass spectra are shown in Fig. 1 and in Supplemental Figure 1.

Isobaric species (i.e. cis/trans isomers or double bond positional isomers) were addressed via LC retention times. This was true for all species with the exception of the 18:1 isomers, which were not completely resolved. Although not applicable to the current study, alternative SRM transitions for the isobaric species could also be used as a method to resolve these species as each isomer has a distinct fragmentation pattern.

The limit-of-quantifications were all on the order of 50-100 femtograms on-column as determined by standard curve analysis. We used accurate concentration fatty acid stock solutions made from milligram amounts of fatty acids and carried out serial dilution to obtain low concentration stock solutions. AMPP derivatization and pre-MS/MS sample clean-up were carried out on fully diluted FA solutions, so the limit-of-quantificaiton we report include any losses due to AMPP derivatization and pre-MS/MS sample clean-up. The limit-of-quantification of FA by gas chromatography/electron-capture mass spectrometry of pentafluorobenzyl esters is reported to be about 10 femtomoles (3000 femtograms) (33). Thus, our method is about 10-fold more sensitive than this previous method of FA analysis.

To gauge the increase in FA detection sensitivity we analyzed various amounts of d_8-20:4 AMPP amide in positive ion mode and various amounts of d_8-20:4 free acid in negative ion mode. For the latter we monitored a major high mass product ion due to the loss of CO_2. We also tuned the instrument to optimize the cone voltage and collision

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energy for this transition in negative ion mode. Results are shown in Figure 2. Injection of 0.78 pg of d_8-20:4 AMPP amide gives rise to a peak area of 22,800 in positive ion mode versus 17,100 for 50 ng of d_8-20:4 free acid in negative ion mode. Thus, the increase in sensitivity for the AMPP derivatization method is 64,000-fold.

Next we analyzed the fatty acids present in mouse serum, and the results are summarized in Table 2. Intra-assay coefficients of variation based on 5 injections of the same sample were typically less than 4%. Inter-assay coefficients of variations based on injections of 6 independently extractions of the same serum were typically less than 6%. Thus, the method is highly reproducible. For these studies we used the m/z = 239 production ion. As noted above, this is present in all of the fatty acids, but its use is adequate in the case of mouse serum. Additional analytical specificity can be obtained by monitoring analyte-specific precursor ions, such as those noted above for the 18:1 species.

It should be mentioned that accurate quantification of the absolute amount of any particular fatty acid species requires a chemically identical, isotopic substituted internal standard. Only in this way can one account for differences in ionization efficiencies in the mass spectrometer source and in differences in precursore-to-product ion generation for the different fatty acid molecular species. Also, if a deuterated fatty acid is used as in internal standard, deuterium should not be present at a site that leads to an isotope effect on the amount of product ion generated. Another option is to use a limited number of heavy atom substituted FA internal standards and to determine the relative MS/MS signal intensities of each FA molecular species by using standard curves for the appropriate species. This is not as accurate as an internal standard for absolute quantification.

Background contamination of solvents was particularly bad for the saturated series of chains 12-18 carbons in length as well as for the monounsaturated 18 carbon

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series. Baking of glassware and trituration of reagents improved background levels significantly.

Large amounts of derivatization reagents relative to FAs are used to ensure quantitative conversion to AMPP amides. All reagents and their products elute in the void volume of the LC run and do not enter the ESI-MS/MS source because a diversion value is used to direct LC output to waste during the initial part of the run. Thus, the method does not lead to excessive loading of the ESI-MS/MS source.

In summary, we have developed a new FA quantitative analysis using readily available LC/ESI-MS/MS equipment that provides a sensitivity close to that of the most sensitive FA method so far developed (gas chromatography of pentafluorobenzyl esters with electron capture detection). Although LC does not provide the resolving power of capillary gas chromatography, the use of unqiue MS/MS channels is usually sufficient to resolve isobaric species that co-elute during LC. The new method should find widespread use given the relatively large number of ESI-MS/MS instruments available in modern analytical laboratories.

Financial disclosure. The AMPP derivatization reagent is commercial available from Cayman Chemicals under the name AMP+ Mass Spectrometry Kit (Cat. 710000). The University of Washington derives royalty revenue from the net sales of this product.

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Table 1: Liquid chromatography retention times and tandem mass spectrometry parameters for analysis of fatty acid AMPP amide molecular species.

Fatty Acid Molecular Species	LC retention time (min)[1]	Retention Window[2]	Internal Standard	Precursor ion[3] (*m/z*)	Product ion[3] (*m/z*)	Cone Voltage[4] (V)	Collision Energy[4] (eV)
Dodecenoic (*11*-**12:1**)	4.37	**1**	**A**	365	239	56	42
Lauric (**12:0**)	4.96	**1**	**A**	367	239	60	44
Myristoleic (*9Z*-**14:1**)	5.35	**1**	**A**	393	239	58	44
Myristic (**14:0**)	6.04	**1**	**A**	395	239	62	47
Palmitoleic (*9Z*-**16:1**)	6.34	**1**	**A**	421	239	60	47
Palmitoleic (9*E*-**16:1**)	6.5	**1**	**A**	421	239	60	47
Palmitic (**16:0**)	7.02	**1**	**B**	423	239	65	49
Stearidonic (*6Z,9Z,12Z,15Z*-**18:4**)	5.8	**1**	**A**	443	239	64	42
α-Linolenic (*9Z,12Z,15Z*-**18:3**)	6.21	**1**	**C**	445	239	64	46
					337	64	38
γ-Linolenic (*6Z,9Z,12Z*-**18:3**)	6.29	**1**	**C**	445	239	64	45
					347	64	38
Linoleic (*9Z,12Z*-**18:2**)	6.7	**1**	**D**	447	239	65	48
Linoleic (*9E,12E*-**18:2**)	6.96	**1**	**D**	447	239	65	48
Oleic (*9Z*-**18:1**)	7.26	**1**	**E**	449	239	64	45
Petroselinic (*6Z*-**18:1**)	7.26	**1**	**E**	449	239	64	45
Vaccenic (*11Z*-**18:1**)	7.26	**1**	**E**	449	239	64	45
Stearic (**18:0**)	7.91	**2**	**G**	451	239	66	45
Eicosapentaenoic (*5Z,8Z,11Z,14Z,17Z*-**20:5**)	6.26	**1**	**F**	469	239	62	42
Arachidonic (*5Z,8Z,11Z,14Z*-**20:4**)	6.73	**1**	**H**	471	239	70	50
					373	70	40
ω3-Arachidonic (*8Z,11Z,14Z,17Z*-**20:4**)	6.6	**1**	**H**	471	239	70	50
					363	70	40
Eicosatrienoic (*11Z,14Z,17Z*-**20:3**)	7.07	**1**	**I**	473	239	70	50
					365	70	40
Dihomo-γ-Linolenic (*8Z,11Z,14Z*-**20:3**)	7.07	**1**	**I**	473	239	70	50
					375	70	38
Eicosadienoic (*11Z,14Z*-**20:2**)	7.53	**1**	**E**	475	239	65	52
5-Eicosenoic (*5Z*-**20:1**)	8.29	**2**	**G**	477	239	70	50
8-Eicosenoic (*8Z*-**20:1**)	8.15	**2**	**G**	477	239	70	50
11-Eicosenoic (*11Z*-**20:1**)	8.08	**2**	**G**	477	239	70	50
Arachidic (**20:0**)	8.74	**2**	**G**	479	239	66	55
Docosohexaenoic (4Z,7Z,10Z,13Z,16Z,19Z-**22:6**)	6.74	**1**	**J**	495	239	64	36
Docosopentaenoic (*7Z,10Z,13Z,16Z,19Z*-**22:5**)	6.98	**1**	**J**	497	239	65	55
Docosopentaenoic (*4Z,7Z,10Z,13Z,16Z*-**22:5**)	6.98	**1**	**J**	497	239	65	55
Docosotetraenoic (*7Z,10Z,13Z,16Z*-**22:4**)	7.41	**1**	**J**	499	239	65	55
Docosotrienoic (*13Z,16Z,19Z*-**22:3**)	7.88	**2**	**G**	501	239	65	55
Docosodienoic (*13Z,16Z*-**22:2**)	8.33	**2**	**G**	503	239	65	55
Erucic (*13Z*-**22:1**)	8.85	**2**	**G**	505	239	65	55

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Fatty Acid Molecular Species	LC retention time (min)[1]	Retention Window[2]	Internal Standard	Precursor ion[3] (*m/z*)	Product ion[3] (*m/z*)	Cone Voltage[4] (V)	Collision Energy[4] (eV)
Behenic (**22:0**)	9.51	**2**	**G**	507	239	65	55
Nervonic (*15Z*-**24:1**)	9.59	**2**	**G**	533	239	70	60
Lignoceric (**24:0**)	10.22	**2**	**G**	535	239	65	55
Internal Standards							
(A) D14 Palmitoleic (*9Z*-**16:1**)	6.3	**1**	**A**	435	242	60	47
(B) D31 Palmitic (**16:0**)	6.92	**1**	**B**	455	242	65	55
(C) D14 a-Linolenic (*9Z,12Z,15Z*-**18:3**)	6.17	**1**	**C**	459	242	64	48
(D) D4 Linoleic (*9Z,12Z*-**18:2**)	6.75	**1**	**D**	451	239	68	44
(E) D17 Oleic (*9Z*-**18:1**)	7.28	**1**	**E**	466	239	68	48
(F) D5 Eicosapentaenoic (*5Z,8Z,11Z,14Z,17Z*-**20:5**)	6.31	**1**	**F**	474	239	62	43
(G) D35 Stearic (**18:0**)	7.81	**2**	**G**	487	242	65	58
(H) D8 Arachidonic (*5Z,8Z,11Z,14Z*-**20:4**)	6.77	**1**	**H**	479	239	65	45
(I) D6 Dihomo-g-Linolenic (*8Z,11Z,14Z*-**20:3**)	7.04	**1**	**I**	479	239	70	46
(J) D5 Docosohexaenoic (*4Z,7Z,10Z,13Z,16Z,19Z*-**22:6**)	6.73	**1**	**J**	501	239	64	36

[1]Retention times listed are derived from the LC protocol detailed in the Materials/Methods section.
[2]Data for retention window 1 was collected from minutes 4.0 to 7.65. Data for retention window 2 was collected from minutes 7.65 to 10.
[3]*m/z* Values listed are calculated monoisotopic values. The actual center mass values used are derived from instrument tuning, which is instrument dependent.
[4]Cone voltages and collision energies were optimized for each analyte. These numbers are instrument dependent.

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Table 2. Calculated concentrations (pg/µl) and coefficients of variation (%) for LC/ESI-MS/MS analysis of fatty acid AMPP amides in commercial mouse serum.[1]

	FA (pg/µL)	%CV Intra-sample (10 µL of Serum)[2]	%CV Inter-sample (10 µL of Serum)[3]
Lauric	143.2	3.1	7.6
Myristoleic	12.9	1.2	5.8
Myristic	299.4	2.2	3.0
Palmitoleic	295.8	0.8	2.6
Palmitic	1286.9	1.2	5.3
Linolenic	185.2	1.6	2.7
Linoleic	462.0	0.6	3.2
Oleic	2066.1	1.1	4.3
Stearic	1820.4	1.8	6.1
Eicosapentaenoic	72.0	0.9	3.7
Arachidonic	873.0	1.7	5.2
ω3-Arachidonic	24.2	4.8	5.9
Eicosatrienoic	147.0	2.4	1.5
Eicosadienoic	53.9	1.7	3.8
Eicosenoic Acid	98.7	1.4	2.4
Arachidic	21.4	2.9	8.1
Docosohexaenoic	605.6	4.4	6.9
Docosopentaenoic	148.2	3.9	7.9
Docosotertaenoic	66.9	3.5	9.0
Docosotrienoic	14.5	2.4	2.7
Docosodienoic	0.8	3.1	2.7
Erucic	8.3	3.0	10.4
Behenic	7.6	1.7	5.3
Nervonic	20.6	3.5	6.0
Lignoceric	18.6	1.9	7.8

[1]A trace amount of 12:1 fatty acid was seen but variability was high due to its low level.
[2]Coefficient of variation for the analysis of the same sample of extracted and derivatized serum injected 5 times onto the LC/ESI-MS/MS.
[3]Coefficient of variation for the analysis of 6 independently extracted and derivatized serum samples.

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Figure Legends

Figure 1. Product ion mass spectra for petraselenic acid AMPP amide (first panel), oleic acid AMPP amide (second panel), d_8-oleic acid AMPP amide (third panel) and vacenic acid AMPP amide (fourth panel).

Figure 2. Selected-ion trace for (top panel) d_8-20:4 AMPP amide in positive ion mode (0.78 pg injected, 471>239 transition) and (bottom panel) d_8-20:4 free acid in negative ion mode (50 ng injection, 311>267 transition). Both peaks integrate to similar area (22,800 for d_8-20:4 AMPP amide and 17,100 for d_8-20:4 free acid).

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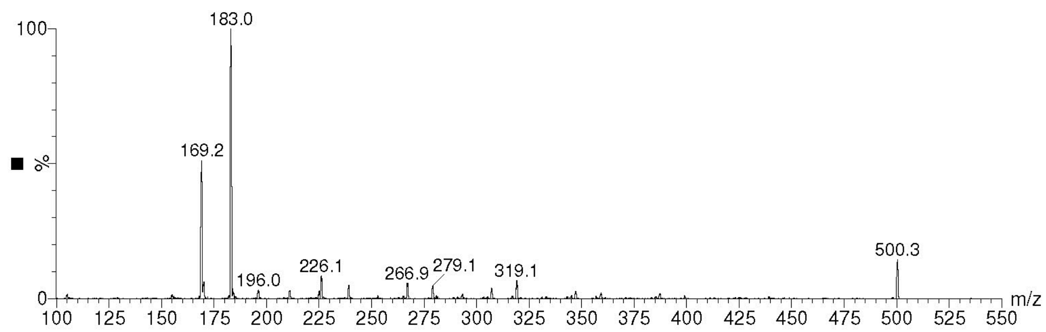

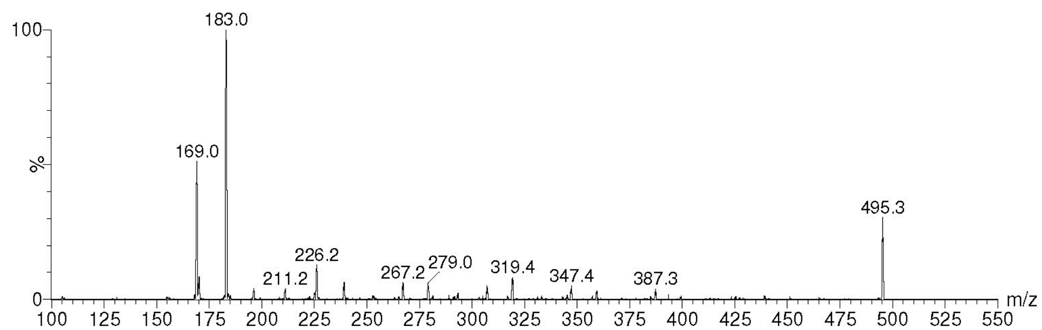

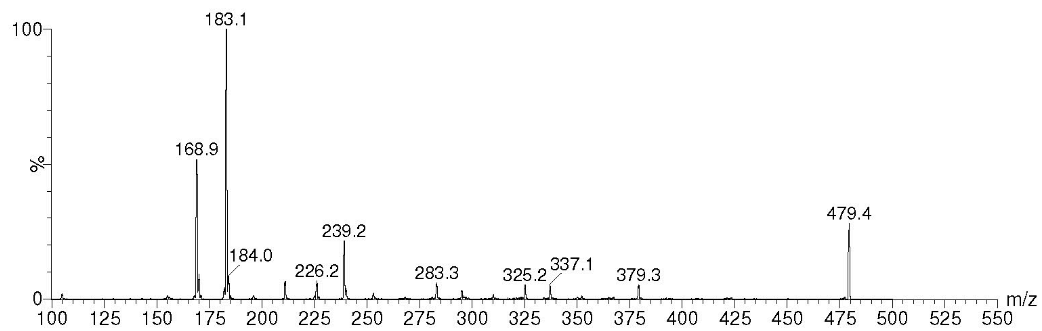

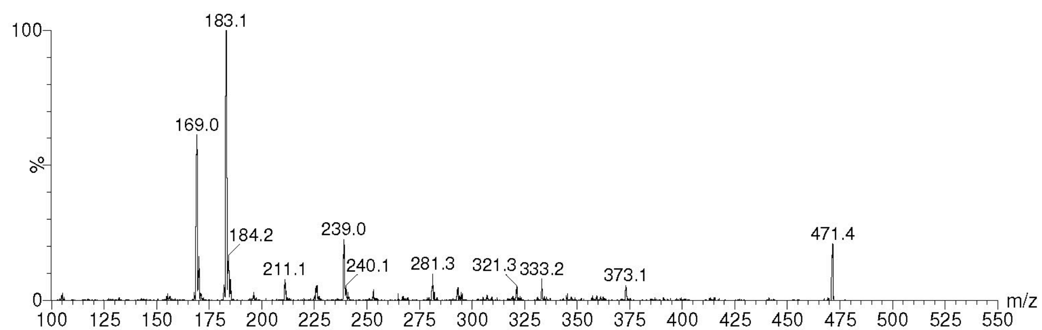

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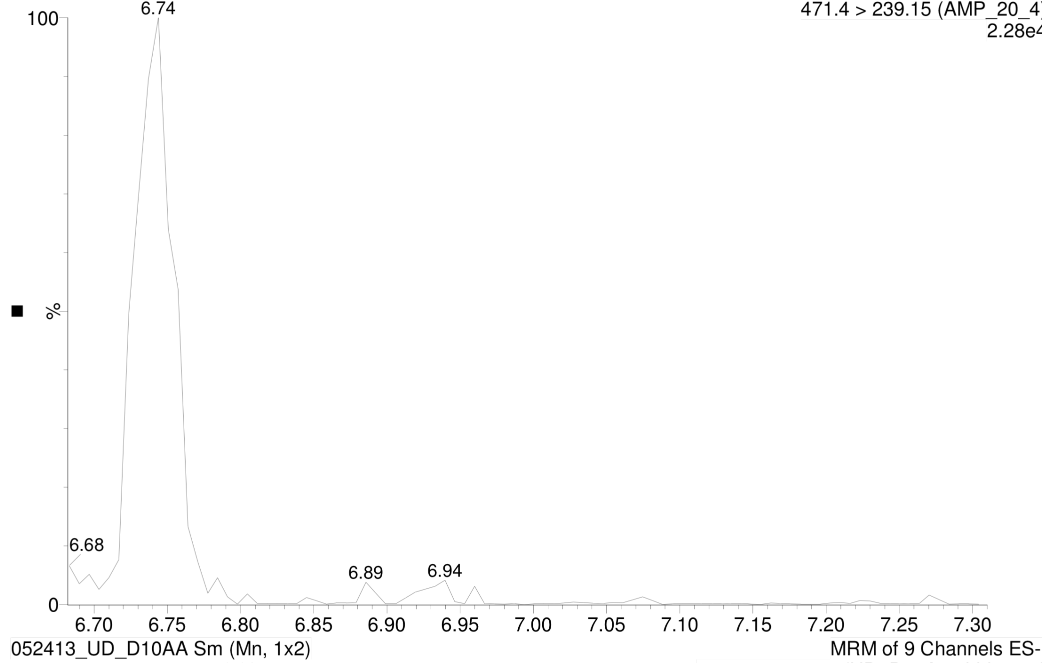

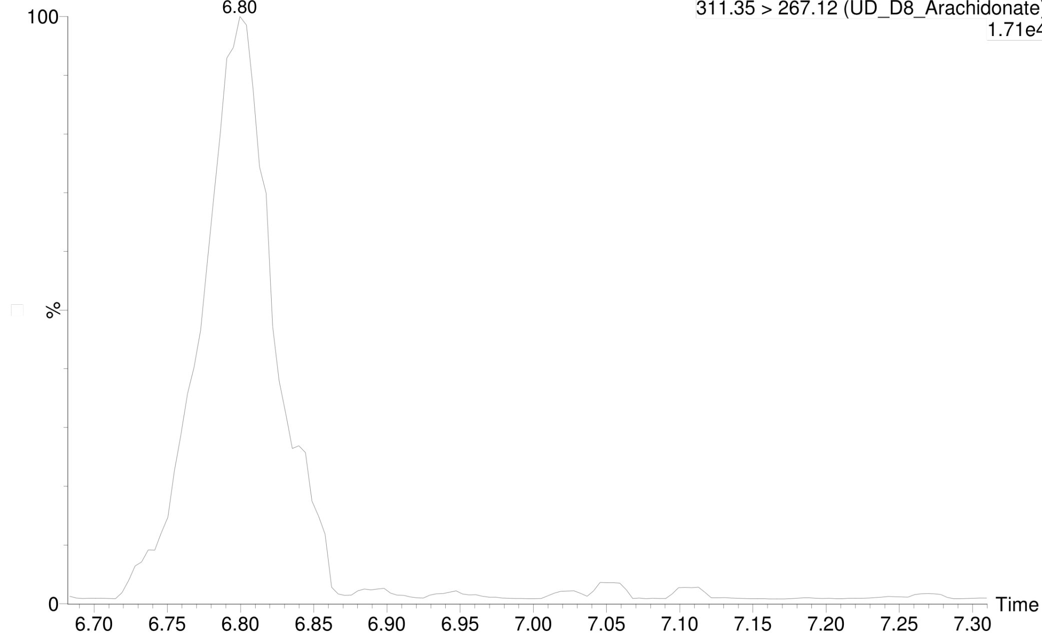

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