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Sensitive analysis of fatty acid esters of hydroxy fatty acids in biological lipid extracts by shotgun lipidomics after one-step derivatization

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Abstract

Branched fatty acid esters of hydroxy fatty acids (FAHFAs) are an important family of endogenous lipids, possessing antidiabetic and anti-inflammatory functions. Therefore, analysis of FAHFAs in biological samples obtained under healthy and disease states can uncover underlying mechanisms of various relevant disorders (e.g., diabetes and autoimmune diseases). Up to now, due to their extremely low abundance, the determination of the changed levels of these species is still a huge challenge, even though great efforts have been made by utilizing liquid chromatography-tandem mass spectrometry with or without derivatization. Herein, we described a novel method for analysis of FAHFAs present in lipid extracts of biological examples after solid-phase extraction and chemical derivatization with one authentic FAHFA specie as an internal standard based on the principles of multi-dimensional mass spectrometry-based shotgun lipidomics. The approach possessed marked sensitivity, high specificity, and broad linear dynamic range of over 3 orders without obvious matrix effects. Moreover, after chemical derivatization, the molecular masses of FAHFAs shift from an overlapped region with ceramide species to a new region without overlaps, removing these contaminating signals from ceramides, and thereby reducing the false results of FAHFAs. Finally, this novel method was successfully applied for determining FAHFAs levels in varieties of representative biological samples, including plasma from lean and overweight/obese individuals of normoglycemia, and tissue samples (such as liver and white adipose tissue from diabetic (*db/db*) mice). We revealed significant alterations of FAHFAs in samples under patho(physio)logical conditions compared to their respective controls. Taken together, the developed method could greatly contribute to studying altered FAHFA levels under a variety of

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

biological/biomedical conditions, and facilitate the understanding of these lipid species in the patho(physio)logical process.

Graphical abstract

Keywords

FAHFAs; Diabetes; Multi-dimensional mass spectrometry; Shotgun lipidomics; Solid-phase extraction

1. Introduction

Nowadays, overweight/obesity has become an epidemic issue worldwide. This condition is strongly associated with a series of fatal complications, such as cardiac diseases, atherosclerosis, nonalcoholic fatty liver disease, and especially type-2 diabetes mellitus (T2DM). Epidemiological studies show that obesity is one of the major risk factors for T2DM as ~60% of T2DM patients are overweight/obese [1]. It is well-known that chronic low-grade inflammation and insulin resistance play crucial roles in the pathogenesis of T2DM. Thus, it is logical to discover some compounds that possess antidiabetic and anti-inflammatory properties to reduce the risks of T2DM in overweight/obese individuals.

Recently, a new category of endogenous lipids known as branched fatty acid esters of hydroxy fatty acids (FAHFAs) formed by esterification of long-chain fatty acids (FAs) with hydroxy fatty acids (HFAs) was discovered in tissues [2]. The FAs and HFAs found in FAHFAs were predominantly saturated/monounsaturated C18 and C16 fatty acyl chains, such as palmitic acid (PA), palmitoleic acid (PoA), oleic acid (OA), stearic acid (SA) and their hydroxylated counterparts [3]. Some previous studies demonstrated that FAHFAs could improve glucose uptake from the blood, enhance insulin secretion, and relieve obesity-associated inflammation in mammals, exhibiting antidiabetic and anti-inflammatory effects [2, 4]. Despite the recognition of these important functions in biological processes, much more physiological functions and molecular regulation mechanisms of FAHFAs remain unknown and are desirable. The concentrations of FAHFAs that exist in serum and all kinds

of tissues are extremely low (i.e., pmol/mL for serum or fmol/mg for tissue) [2, 4], resulting in the absence of a simple and sensitive enough method for analysis of FAHFAs, and thereby restraining the relevant studies. Although many liquid chromatography-tandem mass spectrometry-based approaches for determination of FAHFAs have been established, these methods have limitations. First, most of the methods are insensitive, requiring large amounts of biological samples [3, 5–8]. For example, the amount of tissue samples (e.g., the liver) and the volume of body fluids (e.g., plasma) suggested for FAHFAs analysis is ~100 mg and ~200 μ L, respectively [7, 8]. Second, some methods suffer solid-phase extraction (SPE) background issues and contaminating signals from ceramides when measuring FAHFAs with multiple reaction monitoring (MRM) in the negative ionization mode. For instance, C16:0 ceramide shares all of the major MRM transitions with palmitic acid-hydroxy stearic acid (PAHSA) [7]. It is difficult to separate the PAHSA and the ceramide peak in practical analysis of biological samples. Thus, this problem could lead to falsely positive results of FAHFA species. Last, but not the least, the majority of these methods need a series of authentic FAHFAs used as internal/external standards, but most of these FAHFA standards are not commercially available [3, 5–8].

Multi-dimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) technology is one of the most useful and powerful approaches in the global analysis of cellular lipidomes with high accuracy/precision [9]. With the development of many strategies, such as modifier addition, prefractionation, charge feature utilization, and especially chemical derivatization, MDMS-SL could identify and quantify thousands of individual lipid species of over 50 lipid classes (including those poorly ionized lipids present in very low abundance) in an automated and relatively high-throughput manner [10, 11]. For comprehensive analysis of all FA species by ESI-MS, a fixed charge site (such as tertiary amine) introduced through derivatization is needed. This allows for charge-remote fragmentation in a way that is effective for dissociation of FA species from long aliphatic chains [12, 13]. After the reaction, not only is the ionization efficiency of all FA species selectively enhanced, but also the molecular masses of FAs shift from an overlapped region to a new region without overlaps. Additionally, lipid species in lipid extracts of biological samples are very complicated, inevitably resulting in ion suppression in lipidomics analysis [14]. SPE technique is a useful tool for enrichment of analyte(s) of interest. Following this line of reasoning, an MDMS-SL method coupled with an SPE step and chemical derivatization for sensitively qualitative and quantitative analysis of FAHFAs with only one authentic FAHFA specie as an internal standard (IS) was developed. Furthermore, this method was applied to determine FAHFA levels of a variety of biological samples. We found that significant alterations of FAHFA species in plasma from overweight/obese individuals of normoglycemia, and tissues, including liver and white adipose tissue (i.e., subcutaneous adipose and perigonadal adipose samples) from diabetic (*db/db*) mice are present when compared to their respective controls. In conclusion, the developed method extended the MDMS-SL platform as a powerful tool for analysis of FAHFA species.

2. Materials and methods

2.1 Materials

The derivative reagent *N*-[4-(aminomethyl)phenyl]pyridinium (AMPP) was synthesized as described previously [13]. Briefly, certain amounts of both pyridine dissolved in ethanol and 1-chloro-2,4-dinitrobenzene were added as substrates to a reaction vessel. The mixture was heated and recrystallized to obtain the intermediate product *N*-2,4-dinitrophenyl pyridium chloride. The intermediate product was used to react with 4-(*N*-Boc)-aminomethyl aniline in ethanol-pyridine solution. After filtration to remove solid 2,4-dinitroaniline, the residual solution was evaporated to obtain the product as brown oil. This oil was subjected to deprotection of Boc with trifluoroacetic acid to yield the final product AMPP. AMPP was brown solid and its structure was further confirmed with ¹H NMR.

The FAHFA d4–16:0 ester of 12-OH-18:0 (12-d4 PAHSA), which was used as IS for quantification of FAHFAs, was synthesized in house using a previously described protocol [15]. Other chemicals were bought from Sigma-Aldrich (St. Louis, MO).

2.2 Animal experiments

Homozygous diabetic (*db/db*) and wild type (WT) male mice (C57BL/6, 4 animals per group) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at a temperature- (20–22 °C) and lighting-controlled house (12 h of light and dark), and were fed with a standard rodent chow diet and water *ad libitum*. At 3 months age, the mice were euthanized by asphyxiation with CO₂ followed by decapitation. All tissues were collected as described previously [16] and stored at –80 °C until lipid extraction and analysis. All animal procedures conducted in the study in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council of National Academies) were approved by the UT Health SA Institutional Animal Care and Use Committee (IACUC).

2.3 Human plasma

The participating individuals were selected from among >200 healthy volunteers of normoglycemia. The study protocol was approved by the Ethic Committee of Zhejiang Chinese Medical University. Written informed consents were obtained from the participants. According to body mass index (BMI), the 40 individuals were divided into two groups: the lean group (BMI 18.5 Kg/m²) and the overweight/obese group (BMI ≥ 25 Kg/m²) (Table S1 in the Supporting Information). All individuals were sex- and age-matched between 20 and 40 years old and none of them were pregnant or took lipid-lowering medicine during the period of the study. Almost all of the individuals never smoke or smoke rarely. Fasted blood samples were collected in the early morning after the individuals fasted for at least 12 h. Plasma was obtained through centrifugal separation using a standardized procedure, and stored at –80 °C prior to use [17].

2.4 Preparation of lipid extracts

An individual sample (i.e., ~100 µL for plasma or equivalent to ~1 mg of tissue protein content determined with a BCA assay kit (Pierce, Rockford, IL) for liver sample) was accurately transferred into a disposable glass culture test tube. A modified Bligh and Dyer

procedure [18] was conducted to extract the lipids as described previously [19] in the presence of an IS (12-d4 PAHSA). Each lipid extract was resuspended in a certain volume of $\text{CHCl}_3/\text{MeOH}$ (1:1, v/v) (e.g., 2000 $\mu\text{L}/\text{mL}$ plasma and 100 $\mu\text{L}/\text{mg}$ of tissue protein), flushed with N_2 , capped, and stored at -20°C .

2.5 Solid-phase extraction for enrichment of FAHFAs

SPE was performed according to the previously reported method with slightly modified [7]. Briefly, SPE was conducted with a HyperSep silica SPE cartridge (200 mg bed weight, 3 mL, Thermo Scientific, cat. no. 60108–410) at room temperature. The SPE cartridge was firstly conditioned with 15 mL of hexane. Individual aforementioned lipid extract dried with N_2 stream was reconstituted in 200 μL of chloroform, and then loaded onto the cartridge. Neutral lipids (i.e., triglyceride, and cholesterol and its esters) were eluted with 15 mL of 5% ethyl acetate in hexane, followed by elution of FAHFAs with 10 mL of ethyl acetate. Afterward, the FAHFAs eluent collected in a disposable conical tube was totally dried under a stream of N_2 , and stored at -80°C prior to use.

2.6 Derivatization of FAHFAs with AMPP

Derivatization of FAHFAs with AMPP was proceeded as described previously [13]. Specifically, 10 μL of ice-cold acetonitrile/*N,N*-dimethylformamide (4:1, v/v) was added into the conical tube containing the FAHFA residue, then 10 μL of ice-cold 640 mM [3-(dimethylamino)propyl]-ethylcarbodiimide hydrochloride aqueous solution was added. After being vortexed for 10 s, 10 μL of ice-cold 10 mM *N*-hydroxybenzotriazole and 10 μL of 30 mM AMPP, both dissolved in acetonitrile, were added. *N*-hydroxybenzotriazole serves the role as condensation agent in the reaction. The tube was vortexed thoroughly, filled with N_2 , capped, and incubated at 68°C for 1.5 h. After incubation, the derivatives were extracted with 4.5 mL of $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (1:1:1, v/v/v). The bottom layer was collected and dried under a gentle N_2 stream. The residue was resuspended in 50 μL of $\text{CHCl}_3/\text{MeOH}$ (1:1, v/v), and stored at -80°C prior to MS analysis.

2.7 MS analysis of FAHFA derivatives

MS and tandem MS analyses of derivatized FAHFAs in the positive-ion mode were performed in a QqQ mass spectrometer (TSQ Quantiva, Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray device (Advion Bioscience, Ithaca, NY), as previously described [11, 20]. Prior to direct infusion, individual FAHFA derivative solutions were diluted in $\text{CHCl}_3/\text{MeOH}/\text{isopropyl alcohol}$ (1:2:4, v/v/v) to avoid possible lipid aggregation. Product-ion analysis of a diluted lipid solution was conducted under fixed collision gas pressure of 1.0 mTorr while the collision energy was varying to achieve optimized conditions. The optimized collision-induced dissociation (CID) condition was also employed for neutral-loss scanning (NLS) for quantification of FAHFA species.

All mass spectral data were acquired by a customized sequence subroutine operated under Xcalibur software [11]. Data processing was conducted according to the previously published protocol [11]. All data were presented as mean $\pm$ SD unless otherwise indicated. Statistical significance between the groups was determined by a two-tailed Student *t*-test relative to the control, where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results and discussion

It has been reported that detection sensitivity of carboxylic acid compounds (i.e., nonesterified FAs) could be drastically enhanced (~2500 fold) in ESI-MS analysis through introduction of a fixed charge site (e.g., a tertiary amine) [21]. Moreover, the ionization efficiencies of these derivatives of carboxylic acid species are nearly identical after correction for differential ^{13}C isotope distribution in the low-concentration region, greatly improving the quantification accuracy of these weak anionic lipid species [13, 21]. With respect to the hydrophobicity, charge strength, and derivatization yields (>90%), AMPP, one of the most representative derivative reagents for introduction of a tertiary amine to carboxylic acid molecules through an amidation reaction, has been successfully exploited for fatty acidomics [13]. Following this line of reasoning, we utilized AMPP as a derivative reagent for the enhancement of ionization efficiency to achieve the analysis of FAHFA species.

Product-ion MS spectral analysis of the $[12\text{-d}_4\text{ PAHSA}+\text{AMPP}]^+$ ion demonstrated informative fragment ions for structural determination (Figure 1). Like the fragmentation pattern of FA species derivatized with AMPP, the product-ion MS of derivatives of FAHFA with AMPP also displayed a series of low-abundance fragment ions with the mass difference of 14 Da, representing the sequential loss of a methylene group from product ions $[(\text{FA}_2\text{-2H})+\text{AMPP}]^+$. The double bond generated after the loss of OH in the product ion $[(\text{FA}_2\text{-2H})+\text{AMPP}]^+$ could be on either side, leading to some characteristic ions (i.e., m/z 351, 363, 377 and 391 in Figure 1). The product ions (e.g., m/z 379 and 393 in Figure 1) might be yielded due to dihydrogen transfer after CID. Among them, the relative abundance fragment ion was at m/z 239, corresponding to the loss of alkanes containing $m-3$ carbon atoms from the aliphatic chain of HFA (“ m ” means the total number of carbon atoms in HFA species), generating a conjugated stable product ion with a carbonyl group (e.g., $[\text{CH}_2=\text{CH-CO-NH}+\text{AMPP}]^+$). However, it is difficult to structurally characterize FA_2 using these informative fragment ions in practical analysis of complex biological samples due to their low abundance. There also existed fragment ions yielding from the derivative reagent with or without the nitrogen of the amide bond (e.g., m/z 169 and 183) in the product-ion MS spectrum (Figure 1). In fatty acidomics analysis, these two fragment ions were used for effectively determining the presence of a carboxyl moiety through precursor-ion scanning of these ions since they were the most abundant in the product-ion MS spectra of $[\text{FA}+\text{AMPP}]$ ions [13].

However, the most abundant fragment ion in the product-ion MS spectrum of FAHFA species derivatized with AMPP were $[(\text{FA}_2\text{-2H})+\text{AMPP}]^+$ ions generated from the neutral losses of side aliphatic chains from the molecular ion, suggesting that the side aliphatic chain of FAHFAs is prone to lose as a free FA (Figure 1). Thus, this characteristic fragmentation pattern could be theoretically used for identification of FAHFA species through NLS of the possible FA chains (such as NLS 254 for 16:1, NLS 256 for 16:0, NLS 282 for 18:1, etc.) and for sensitively determining the concentration of the derivatized FAHFA species in comparison with the selected IS(s) (see below).

It is well known that MS/MS analysis of individual species within a lipid class is a process, depending on the structure of each lipid species [9]. Usually, at least two representative species are needed as IS for quantification of individual species within a lipid class when a tandem MS approach is developed [22]. Certainly, ramping collision energy at fixed collision pressure could also be employed to minimize the differential effects of the process on different molecular species of a lipid class, but it is very time-consuming. Therefore, using a representative collision energy that could balance the differential effects is frequently employed when numerous species in the lipid class are available [23–25]. Unfortunately, many FAHFA species have not been commercially available. Therefore, an alternative method through trial and error in combination with ramping collision energy in analysis of biological samples with one IS was employed to determine the appropriate collision energy to balance the differential fragmentation effects as previously described [26, 27].

Specifically, mouse liver extracts prepared as described above without addition of IS species were equally divided into more than 8 fractions. Different amount of IS species was added to each of the fractions. After chemical derivatization with AMPP, different NLS used for quantification of different FAHFAs were acquired from each of the solutions prepared above at the fixed collision pressure of 1 mTorr with varied collision energy from 10 to 50 at 5 eV per step for first ramping of determination. Then the acquired data from the series of solutions at different collision energy were plotted as peak intensity ratios (I_{IS}/I_X) of liver FAHFA species (e.g., 16:0H16:0) and IS vs. their molar ratio (C_{IS}/C_X). The slope of the obtained linear plot meant the effects of collision energy on instrument response factor of the FAHFA of interest. A set of optimal collision energy values with the slope of nearly 1 for each species of FAHFA could be obtained in this step. Then these determined collision energy values were averaged and a certain range (± 10 eV) of this average value was ramped again with a fine step of 2 eV. The most abundance of ion intensity was used to estimate the content of individual species again. An optimized collision energy 40 eV was selected until the determined content of individual species was within a very small difference (i.e., 5 %) compared to that of the previously determined one.

To examine the optimized condition and determine the dynamic range for quantitation of FAHFAs after AMPP derivatization in the presence of other lipids, we spiked nearly identical amounts of lipid extracts from mouse liver with various amounts of IS 12-d4 PAHSA. Each mixture was subjected to derivatization with AMPP and then diluted to prevent potential aggregates from forming prior to direct infusion for MS analysis with the optimized CID condition. The linearity of the peak intensity ratios of the standard to individual FAHFA species after ^{13}C deisotoping versus their corresponding molar ratios was analyzed by linear regression of logarithmic plots, as previously described [16] (Figure 2D). A logarithmic plot was adopted in order to guarantee an equal contribution of individual data point to the fitting of a straight line. An essentially identical linear correlation was well observed for all the FAHFA species present in mouse liver with the optimized collision energy, further suggesting that the ionization efficiencies of the derivatives were virtually identical and the optimized collision energy was suitable in this method. This result also indicated that quantitation of all FAHFA species present in biological samples could be acquired with only one FAHFA species as IS under the conditions. Moreover, the results

demonstrated a broad linear dynamic range of over 3 orders for quantitative analysis of FAHFA species.

Many lipid classes are extractable with the classical extraction methods, such as the modified method of Bligh and Dyer, the Folch method, or other solvent systems. Even though the ionization efficiency of FAHFA species was greatly enhanced after derivatization, the analysis of these species in biological samples still posed a big challenge. This holds especially true in adipose tissues and plasma/serum (mass spectra not shown) due to their extremely low abundance and inherent ion suppression resulting from the abundance of nonpolar lipids (i.e., triglycerides, and cholesterol and its esters). Furthermore, a large amount of these nonpolar lipids could significantly reduce the upper limit concentration of the lipid mixture in the infusion solution, destabilize the ionization current, and change the efficiency of droplet formation, thereby reducing the ionization efficiency of the analyte of interest [14]. In order to overcome this problem, HyperSep silica SPE was employed to enrich FAHFAs present in biological samples [7]. The nonpolar lipids were firstly eluted with 5% ethyl acetate in hexane, and then FAHFA species were eluted with ethyl acetate and collected accordingly. The FAHFA eluent was subjected to derivatization with AMPP and analyzed by MS. The signal-to-noise ratio of spectra was greatly improved and FAHFA peaks were predominantly displayed in the spectra acquired through NLS of acyl chains with the optimized collision energy.

Based on these facts, a strategy for quantitative analysis of individual FAHFA species present in biological samples based on MDMS-SL was formulated. First, FAHFAs present in a variety of biological samples were extracted with a classical extraction method in the presence of an IS. Second, the lipid extract was subjected to silica SPE to enrich FAHFAs by removing nonpolar lipids. Then the FAHFA eluent was derivatized with AMPP reagent. Finally, NLS of the possible FA chains of the AMPP derivatized FAHFAs was acquired for identification and quantification of individual FAHFA species.

To determine the capability and utility of the developed strategy, the method was applied for analysis of FAHFA species present in plasma from lean and overweight/obese individuals, as well as tissue samples (such as liver and white adipose tissue) from *db/db* and wild type mice.

The results of MDMS-SL analysis showed that the mass levels of FAHFA individual species in human plasma ranged from 0.5 – 25 pmol/mL (Figure 3A), in accordance with what has been previously reported [2]. Although the total amount of FAHFAs in plasma of the two groups is not significantly different (106.10 ± 5.14 and 124.31 ± 10.06 pmol/mL for the lean and the overweight/obese groups, respectively) (Figure 3B), some FAHFA species in the overweight/obese group showed an obvious increase in comparison to that of the lean group, such as 16:0H18:0 and 18:0H18:0, both of which are major FAHFA species in human plasma ($p < 0.05$, Figure 3A). This may indicate that the increased FAHFA levels in overweight/obese individuals of normoglycemia might be a kind of stress response that protects the body from chronic inflammation induced being overweight or obesity. Therefore, the results could reflect the anti-inflammatory effect of FAHFAs to some extent.

However, the levels of FAHFAs were significantly reduced in insulin-resistant mice. Specifically, MDMS-SL analysis of hepatic FAHFA species demonstrated that the total amount of all FAHFAs in *db/db* mice substantially reduced to 0.65 ± 0.17 pmol/mg protein from 1.83 ± 0.21 pmol/mg protein in controls (a reduction of about 65%, $p < 0.001$, Figure 3C) (detail information for each FAHFA species shown in Table S2 in the Supporting Information). Similarly, the mass level of FAHFA species present in subcutaneous and perigonadal adipose tissues from *db/db* group also displayed reductions to some extent in comparison to that in the control group (Table 1), although there were no significant differences between the two groups ($p > 0.05$). The different extents of alteration of FAHFAs in these tissues might result from the fact that the change of FAHFAs in different tissue is time-dependent, or that a portion of FAHFA species secreted by the white adipose tissue can alter the reduction of FAHFAs in the tissues [2]. Certainly, additional experiments should be performed to further demonstrate these possibilities.

4. Conclusion

An MDMS-SL approach coupled with an SPE step and chemical derivatization with AMPP for sensitively qualitative and quantitative analysis of FAHFAs in crude lipid extracts of biological samples with one authentic FAHFA specie used as IS was developed. The developed method effectively overcomes the issue of the contaminating signals from ceramide species present in previous method and reduces the amount of individual biological sample needed for analysis of FAHFA species through chemical derivatization. The utility of this novel method was demonstrated by revealing the different alterations of FAHFAs in human plasma from overweight/obese individuals of normoglycemia, as well as *db/db* mouse liver and white adipose tissues compared to that in the controls. It is anticipated that the development of this new method should greatly facilitate the studies on the role of FAHFA species in biological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMPP	<i>N</i> -[4-(aminomethyl)phenyl]pyridinium
BMI	body mass index
CID	collision-induced dissociation
T2DM	type-2 diabetes mellitus

FAHFAs	branched fatty acid esters of hydroxy fatty acids
FAs	fatty acids
HFAs	hydroxy fatty acids
IS	internal standard
MDMS-SL	multi-dimensional mass spectrometry-based shotgun lipidomics
MRM	multiple reaction monitoring
NLS	neutral-loss scanning
OA	oleic acid
PA	palmitic acid
PAHSA	palmitic acid-hydroxy stearic acid
PoA	palmitoleic acid
SA	stearic acid
SPE	solid-phase extraction

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Highlights

1. Developed a multi-dimensional mass spectrometry-based shotgun lipidomics approach coupled with solid-phase extraction and chemical derivatization for qualitative and quantitative analysis of FAHFA species with one authentic FAHFA specie used as an internal standard.
2. This method effectively solved the issue of contaminating signals from ceramides through chemical derivatization.
3. Compared with previous methods, the amount of individual biological sample suggested for FAHFAs analysis using this novel method is greatly reduced.
4. Successfully applied the developed method for determining FAHFA levels in representative biological samples and revealed their alterations in patho(physio)logical states.

Figure 1.

Representative product-ion MS spectrum of FAHFA species after derivatization with AMPP. Product-ion MS spectrum of $[12-d_4 \text{ PAHSA}+AMPP]$ ion was acquired at collision energy of 40 eV and collision pressure of 1 mTorr, as described in the section of Material and methods. The insets displayed a series of fragment ions, representing the sequential losses of a methylene group from the HFA chain.

Figure 2.

Determination of linear dynamic range of the method with optimized collision energy for quantification of FAHFA species in the lipid extract from mouse liver. A fixed amount of lipid extract from mouse liver was spiked with different amounts of 12-d4 PAHSA as an IS for quantification of FAHFA species present in mouse liver lipid extracts. Representative MS/MS spectra of NLS 260 (i.e., 12-d4 16:0 FA, panel A), NLS 254 (i.e., 16:1 FA, panel B) and NLS 282 (i.e., 18:1 FA, panel C) were acquired at collision energy of 40 eV as described in Material and methods. Linear regression (panel D) of peak intensity ratios (I_{IS}/I_x) of liver FAHFA species (e.g., 16:0H16:0 and 18:1H18:0) and IS vs. their molar ratio (C_{IS}/C_x) was performed to demonstrate the true linearity of the data as previously described [26]. A logarithmic plot was adopted in order to guarantee an equal contribution of individual data point to the fitting of a straight line.

Figure 3.

Comparison of the levels of FAHFA species in lipid extracts. The levels of individual FAHFA species (A) in lipid extracts of human plasma from lean (open bar) and overweight/obese (solid bar) subjects of normoglycemia and the total amount of FAHFAs in human plasma (B) and in liver lipid extract from control and *db/db* mice (C) were determined with the developed method, respectively. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to respective controls.

Table 1.

Comparison of mass levels of FAHFA species in lipid extracts of different adipose tissues between *db/db* and wild type mice at 3 months of age (pmol/mg tissue) *

FAHFA species	ScAT		PgAT	
	Wild type	<i>db/db</i>	Wild type	<i>db/db</i>
16:1H16:1	0.03±0.01	0.02±0.00	0.02±0.01	0.01±0.01
16:1H16:0	0.83±0.26	0.48±0.10	0.52±0.18	0.29±0.09
16:0H16:1	0.04±0.02	0.04±0.01	0.03±0.01	0.02±0.01
16:0H16:0	0.24±0.08	0.19±0.04	0.13±0.02	0.11±0.03
16:1H18:2	0.25±0.08	0.17±0.02	0.14±0.05	0.10±0.03
18:2H16:1	0.02±0.01	0.02±0.00	0.01±0.01	0.01±0.00
16:1H18:1	4.56±1.53	2.86±0.46	2.30±0.85	1.47±0.62
16:0H18:2	0.14±0.06	0.11±0.03	0.06±0.01	0.05±0.02
18:2H16:0	0.04±0.01	0.05±0.01	0.02±0.01	0.02±0.01
18:1H16:1	0.04±0.01	0.03±0.01	0.04±0.01	0.03±0.01
16:1H18:0	7.99±2.33	5.46±0.97	4.45±1.49	2.73±1.13
16:0H18:1	0.93±0.34	0.63±0.12	0.45±0.12	0.31±0.11
18:1H16:0	0.11±0.07	0.10±0.06	0.07±0.03	0.08±0.02
18:0H16:1	0.01±0.00	0.01±0.00	0.01±0.01	0.01±0.00
16:0H18:0	1.46±0.48	1.03±0.18	0.81±0.23	0.55±0.21
18:0H16:0	0.04±0.02	0.03±0.02	0.04±0.01	0.03±0.01
18:2H18:2	0.05±0.02	0.05±0.02	0.02±0.01	0.02±0.01
18:2H18:1	0.16±0.07	0.12±0.04	0.07±0.01	0.06±0.02
18:1H18:2	0.10±0.06	0.07±0.04	0.04±0.01	0.04±0.01
18:2H18:0	0.18±0.08	0.14±0.06	0.10±0.02	0.08±0.03
18:1H18:1	0.51±0.48	0.38±0.36	0.25±0.17	0.29±0.11
18:0H18:2	0.02±0.02	0.02±0.01	0.01±0.01	0.01±0.00
18:1H18:0	0.67±0.65	0.54±0.55	0.37±0.26	0.45±0.18
18:0H18:1	0.20±0.22	0.15±0.15	0.11±0.07	0.12±0.05
18:0H18:0	0.34±0.32	0.27±0.28	0.20±0.12	0.22±0.08
Total	18.97±6.96	12.96±2.60	10.31±3.22	7.13±2.77

* The data present means ± SD (n = 4 for each group). ScAT and PgAT denote subcutaneous adipose tissue and perigonadal adipose tissue, respectively.