Development of a sensitive and quantitative method for the identification of two major furan fatty acids in human plasma

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**Abbreviations:** AMMP, 3-acyl-oxymethyl-1-methylpyridinium iodide; AMPP, N-(4-aminomethylphenyl) pyridinium; BMP, 2-bromo-1-methylpyridinium iodide; CMP, 3-carbinol-1-methylpyridinium iodide; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanopropanoic acid; CMPentylF, 3-carboxy-4-methyl-5-pentyl-2-furanopropanoic acid; 11D3, 11-(3,4-dimethyl-5-propylfuran-2-yl)undecanoic acid; 11D3-d5, 11-(3,4-dimethyl-5-(propyl-2,2,3,3,3-d5)furan-2-yl)undecanoic acid; 11D5, 11-(3,4-dimethyl-5-pentylfuran-2-yl)undecanoic acid; 11D5-d5, 11-(3,4-dimethyl-5-(pentyl-4,4,5,5,5-d5)furan-2-yl)undecanoic acid; DHA, docosahexaenoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EI, electron-impact ionization; EPA, eicosapentaenoic acid; ESI, electrospray ionization; FAMEs: fatty acid methyl esters; HOBt: N-hydroxybenzotriazole; IS, internal standards; LC-GC-PID-FID, high-performance liquid chromatography coupled on-line with capillary gas chromatography with a photoionization detector mounted in series with a flame ionization detector; LOD, limit-of-detection; LOQ, limit-of-quantitation; MRM, multiple reaction monitoring; MS, mass spectrometry; MW: molecular weight; PIS: precursor ion scan; RSD, relative standard deviation; RT: retention time; SNR, single-to-noise ratio; T2D, type 2 diabetes; TEA, triethylamine; TIC, total ion chromatogram.
Abstract

This paper focuses on the establishment of an accurate and sensitive quantitation method for analysis of furan fatty acids. Particularly, the sensitivity of GC-MS and UPLC-ESI-MS/MS was compared for the identification and quantification of furan fatty acids. Different methylation methods were tested with respect to GC-MS analysis. Special attention needs to be paid to the methylation of furan fatty acids as acidic catalysts might lead to the degradation of furan ring. GC-MS analysis in full scan mode demonstrated that the limit-of-quantitation (LOQ) was 10 μM. UPLC-ESI-MS/MS in multiple reaction monitoring (MRM) mode displayed a higher detection sensitivity than GC-MS. Moreover, identification of furan fatty acids with charge-reversal derivatization was tested in positive mode with two widely used pyridinium salts. Unexpectedly, significant oxidation was observed using N-(4-aminomethylphenyl)pyridinium (AMPP) as a derivatization agent. The formed 3-acyl-oxymethyl-1-methylpyridinium iodide (AMMP) derivatized by 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide improved the sensitivity more than 2000 fold, compared with non-derivatization in negative mode by UPLC-ESI-MS/MS. This charge reversal derivatization enabled the targeted quantitation of furan fatty acids in human plasma. Thus, it is anticipated that this protocol could greatly contribute to the clarification of pathological mechanisms related to furan fatty acids and their metabolites.

Keywords: Charge-reversal derivatization; Furan fatty acids; Multiple reaction monitoring; UPLC-ESI-MS/MS; Precursor ion scan
Introduction

The current state-of-the-art lipidomics and metabolomics enable the discovery of various lipids associated with the development and progression of type 2 diabetes (T2D) (1-3), cancer (4) and cardiovascular diseases (5, 6). Recent studies showed that a dibasic urofuran acid, 3-carboxy-4-methyl-5-propyl-2-furanopropanoic acid (CMPF), is found in high concentrations in subjects diagnosed with prediabetes, gestational diabetes and T2D (7, 8). The elevated CMPF has been recognized as a potential biomarker in diabetes development. CMPF is believed to increase oxidative stress, impair insulin granule maturation and secretion (9). It has been claimed that CMPF is a metabolite of furan fatty acids (Figure 1), however, it is documented that furan fatty acids have a line of homologs in nature. Intriguingly, very recent human studies demonstrated that CMPF formed as a significant metabolite of fish oil composed of only eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) ethyl esters without any furan fatty acids identified (10-12). Hence, the exact origin of CMPF remains controversial and related metabolic pathways remain elusive.

An accurate and sensitive quantitation method of furan fatty acids in food and biological samples would greatly contribute to a clarification of the ongoing debate. Gas chromatography-flame ion detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) are generally considered as the most preferable methods for furan fatty acid profiling. For this, methylation of the carboxylate group is inevitable (13-15). However, the similar chromatographic behavior of the furan ring with some common monounsaturated fatty acids at high concentrations renders the peak identification of furan fatty acids ambiguous and laborious (16). It has been documented that hydrogenation of furan fatty acid methyl esters to more polar...
tetrahydrofurancarboxylic acid methyl esters could facilitate the chromatographic separations (17). Moreover, some improved analytical methods including multi-dimensional GC-MS and high-performance liquid chromatography coupled on-line with capillary gas chromatography with a photoionization detector mounted in series with a flame ionization detector (LC-GC-PID-FID) contributes greatly to the identification of furan fatty acids (18, 19). Very recently, the quantification of furan fatty acids has also been achieved by $^1$H NMR (20). Accordingly, it has been reported that a variety of food sources especially fish and fish oil contain furan fatty acids in a sub-mg/g level (21, 22). Nonetheless, little evidence for the existence of furan fatty acids in human tissues exists so far and extremely low levels of less than 50 ng/mL have been reported (17, 23, 24). A complicated enrichment from large volumes of plasma/serum (e.g., >20 mL) is imperative before analysis (24). Additionally, lack of commercial furan fatty acid standards and deuterated analogs represents further key limiting factors for the accurate quantitation in biological samples.

Generally, natural furan fatty acids can be divided into two major classes. The first class is represented by a propyl moiety whereas the second is dominated by a pentyl moiety at $\alpha_2$ position of the furan ring (19, 25). In addition to CMPF, 3-carboxy-4-methyl-5-pentyl-2-furanopropanoic acid (CMPentylF) has also been identified in human plasma and urine (10, 26). It has been suggested that the metabolic fate of furan fatty acids is associated with this difference (propyl or pentyl) (27). The most abundant furan fatty acid homologs detected in food matrices and human plasma are 11-(3,4-dimethyl-5-propylfuran-2-yl)undecanoic acid (11D3) and 11-(3,4-dimethyl-5-pentylfuran-2-yl)undecanoic acid (11D5), representing the aforementioned two classes (17, 28). Therefore, a targeted quantitation of these two classes of
furan fatty acids especially 11D3 and 11D5 in human diets and biological tissues represents a promising approach to clarify the relationship of furan fatty acids with CMPF.

Shot-gun lipidomics and liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based lipidomics have also been well developed for fatty acid analysis (29, 30). However, an accurate and sensitive protocol for quantitative analysis of furan fatty acids by shot-gun lipidomics or LC-MS/MS has not been developed yet. Up to now, only one report exists on the qualitative analysis of furan fatty acids in fish lipids by HPLC/ESI-Q-TOF-MS (31).

Herein, we explored an approach for identification and quantitation of furan fatty acids in biological samples by comparing the sensitivity of GC-MS and LC-MS/MS, with a special focus on the levels of 11D3 and 11D5. Special attentions regarding the derivatization of furan fatty acids were proposed for the first time in our investigation. Notably, the abundant fragments derived from the derivatized moiety could be employed for quantitative analysis of individual furan fatty acid species. We believe that this approach could facilitate the clarification of the metabolic precursor of CMPF and contribute to investigate the biological relevance of furan fatty acids.
Experimental methods

Reagents and materials

Acetonitrile and methanol of LC-MS grade were purchased from Merck (Germany). Ammonium acetate, 2-bromopyridine, 3-carbinolpyridine, Diazald®, formic acid and triethylamine (TEA) were purchased from Sigma-Aldrich (USA). 1,2-Dihenarachidoyl-sn-glycero-3-phosphocholine (PC-21:0/21:0) were obtained from Nu-Chek Prep, Inc (USA). Diethylene glycol monoethyl ether was obtained from TCI (Shanghai) development Co., Ltd (China). Iodomethane was purchased from Acros Organics (Belgium). Water was prepared using a Milli-Q system (Millipore, USA).

11-(3,4-dimethyl-5-propylfuran-2-yl)undecanoic acid (purity > 98%) and 11-(3,4-dimethyl-5-pentylfuran-2-yl)undecanoic acid (purity > 98%) were synthesized by Shanghai Medicilon Inc., China. 11-(3,4-dimethyl-5-(propyl-2,2,3,3,3-d₅)furan-2-yl)undecanoic acid (chemical purity > 99.5%, deuterium purity > 99.0%) and 11-(3,4-dimethyl-5-(pentyl-4,4,5,5,5-d₅)furan-2-yl)undecanoic acid (chemical purity > 99.5%, deuterium purity > 99.0%) were synthesized by Syncom, Netherlands. AMP® MaxSpec® Kit (50 tests, Catalog #710000), containing N-(4-aminomethylphenyl) pyridinium (AMPP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride aqueous solution, N-hydroxybenzotriazole (HOBt), and acetonitrile/N,N-dimethylformamide (4:1, v/v) solution, were purchased from Cayman Chemical Co. (Ann Arbor, MI).

Nomenclature
Furan fatty acids are abbreviated according to the simplified “number-letter-number” notation (21). The first number is indicative of the length of carboxyalkyl chain at α₁ position. A letter of “M” or “D” represents one or two methyl moieties at β positions. The second letter donates the length of alkyl chain at α₂ position. Accordingly, 11-(3,4-dimethyl-5-propylfuran-2-yl)undecanoic acid and 11-(3,4-dimethyl-5-pentylfuran-2-yl)undecanoic acid are denoted as 11D3 and 11D5, respectively. The corresponding deuterated analogs, 11-(3,4-dimethyl-5-(propyl-2,2,3,3,3-d₅)furan-2-yl)undecanoic acid and 11-(3,4-dimethyl-5-(pentyl-4,4,5,5,5-d₅)furan-2-yl)undecanoic acid, are abbreviated as 11D3-d₅ and 11D5-d₅, respectively.

Preparation of furan fatty acid mixtures

The stock solutions of furan fatty acids and deuterated internal standards (IS) were prepared in pure acetonitrile with a concentration of 10 μg/mL. The concentration of each stock solution was determined gravimetrically. The mixtures of furan fatty acids were prepared from individual stock solutions.

Preparation of human plasma samples

This study was approved by Ethics Committee of Shanghai General Hospital, affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai, China (Approval Number: Expedited Review [2018] 136). Fasting blood samples were collected from healthy subjects and stored at −80 °C according to a standardized procedure as previously described (32).

Methylation of furan fatty acids
The methylation of furan fatty acids was conducted according to three different methods: ethereal diazomethane, methanolic sulfuric acid (H₂SO₄-MeOH) and boron trifluoride-methanol (BF₃-MeOH).

Firstly, PC-21:0/21:0 (5 μg) was mixed with furan fatty acids standards (11D3 and 11D5) and dried under N₂. The methyl esters were prepared according to the method described by Uchida et al with slight modifications (31). The mixed standards were dissolved in toluene (0.2 mL) and mixed with 0.5 M sodium methoxide in methanol (0.4 mL). The solution was maintained at 50 °C for 10 min. Glacial acetic acid (20 μL) is then added, followed by ddH₂O (1 mL). The required methyl esters and non-esterified free furan fatty acids were extracted into n-hexane (3 × 1 mL) and dried under N₂. The following methylation of free fatty acids was performed by ethereal diazomethane. The final products were resuspended in n-hexane (50 μL), then subjected to GC/MS analysis.

Secondly, furan fatty acid standards (11D3 and 11D5) with PC-21:0/21:0 (5 μg) as IS were derivatized with 2 mL of methanol containing 2% sulfuric acid for 2 h at 80 °C in sealed borosilicate glass tubes. Subsequently, 1 mL of aqueous saturated NaCl solution were added, and the generated furan fatty acid methyl esters were extracted three times using 1 mL of n-hexane. The combined organic layer was dried under N₂ and re-suspended in 50 μL of n-hexane, then subjected to GC/MS analysis.

Thirdly, furan fatty acid standards (11D3 and 11D5) with PC-21:0/21:0 (5 μg) as IS were derivatized with 2 mL of ~15% boron trifluoride in methanol for 1 h at 90 °C in sealed borosilicate glass tubes. Subsequently, 1 mL of aqueous saturated NaCl solution were added, and the generated furan fatty acid methyl esters were extracted three times using 1 mL of n-
hexane. The combined organic layer was dried under N₂ and re-dissolved in 50 μL of n-hexane before subjected to GC/MS analysis.

*Synthesis of 2-bromo-1-methylpyridinium iodide (BMP) and 3-carbinol-1-methylpyridinium iodide (CMP)*

Iodomethane (50 mmol, 3.11 mL) was added to 2-bromopyridine (10 mmol, 0.97 mL) or 3-carbinolpyridine (10 mmol, 0.96 mL) and stirred at room temperature for 1 h. The yellow crystals were washed with cold acetone (5 mL) for three times and dried in vacuum.

*Derivatization of furan fatty acids with AMPP and BMP & CMP*

The derivatization of furan fatty acids with AMPP was carried out according to the manufacturer’s instruction. Briefly, 100 μL of 11D3 and 11D5 (1 μg/mL in acetonitrile) was dried under N₂ and resuspended in 20 μL of cold acetonitrile/DMF (4:1, v/v). Then cold EDC (20 μL) and HOBt (10 μL) were sequentially added to the solution. The vial was briefly mixed on a vortex mixer and placed on ice. Finally, the AMP⁺ solution (30 μL) was added, mixed and heated at 60 °C for 30 min.

The derivatization of furan fatty acids with BMP and CMP was conducted according to the method of Yang et al with slight modifications (33). 10 μL of 11D3 and 11D5 (10 μg/mL in acetone), BMP (7.5 mg/mL in acetonitrile, 20 μL) and CMP (10 mg/mL in acetonitrile, 20 μL) were added and briefly mixed on a vortex mixer. Subsequently, TEA (1 μL) was added to the solution, mixed and heated at 50 °C for 30 min. After derivatization, the solution was dried under N₂ and resuspended in 100 μL of acetonitrile/H₂O (7:3, v/v). The formed 3-acyl-
oxymethyl-1-methylpyridinium iodides were abbreviated as 11D3-AMMP and 11D5-AMMP.

The scheme of furan fatty acid derivatization is shown in Figure S1.

**GC-MS analysis**

Furan fatty acids were analyzed as fatty acid methyl esters (FAMEs), which were separated on an Agilent HP-INNOWax column (30 m × 0.25 mm i.d., 0.25 μm film thickness; USA) and analyzed by gas chromatography triple quadrupole tandem mass spectrometry (GC-TQ/MS, Agilent, Santa Clara, CA) based on an electron-impact (EI) ion source. The EI ionization at 70 eV was performed, with a full scan range of m/z 50-500. All measurements were carried out according to the following oven temperature program: the initial temperature of 60 °C was held for 2 min, and raised to 160 °C with a ramp of 20 °C/min. Then the temperature increased to 240 °C with a ramp of 5 °C/min and was held for 7 min, resulting in a total run time of 30 min.

**UPLC-ESI-MS/MS analysis**

The analysis of free and derivatized furan fatty acids including furan fatty acids-AMPP and furan fatty acids-AMMP was performed in ultra-high performance liquid chromatography-tandem mass spectrometry system (UPLC-MS/MS 8050, Shimadzu, Kyoto, Japan) composed of a Shimadzu 30 AD liquid chromatography system (an LC-30 A binary pump, an SIL-30AC autosampler and a CTO-30AC column oven) and an 8050 triple quadrupole mass spectrometer equipped with a heated electrospray ionization (ESI) source.

Analysis of free furan fatty acids was performed in negative mode. Separation of analytes
was achieved with an Agilent ZORBAX Eclipse Plus C18 column (2.1 mm × 100 mm, i.d. 1.8 μm) at a flow rate of 0.3 mL/min. The mobile phase was consisted of solvent A (H2O with 10 mM CH3COONH4) and solvent B (MeOH with 10 mM CH3COONH4). Samples were applied to the column at 60% B and eluted with a linear increase in B (60%-80% B from 1.0 to 6.5 min) that reached 100% at 7.5 min and was held for 6 min. Then the elution was decreased to 80% in B at 14.0 min and was held for 3.5 min. Finally, the elution was returned to the initial condition at 18.0 min for 2 min of equilibration. The MS parameters were: nebulizing gas (N2) flow rate, 3 L/min; drying gas (N2) flow rate, 15 L/min; desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; interface temperature, 350 °C.

Analysis of derivatized furan fatty acids was performed in positive mode. Separation of analytes was achieved with a Waters ACQUITY UPLC® CSH™ C18 column (2.1 mm × 100 mm, i.d. 1.7 μm) at a flow rate of 0.4 mL/min. The mobile phase was consisted of solvent A (H2O with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). Samples were applied to the column at 10% B, then raised to 30% B from 1.0 to 2.0 min. The elution was increased linearly in B (30%-80% from 2.0 to 5.0 min) that reached 100% at 6.0 min and was held for 1 min before returning to initial conditions for 3 min of equilibration.

**Validation procedure**

The peak area ratios of furan fatty acid standards to corresponding IS against the concentration ratios were plotted to construct the standard curves in pooled plasma. The concentrations of standards are shown in Table S1.

A 7-point dilution series of each furan fatty acid species was prepared from the stock
solution for external calibration. The above calibrator solutions were selected according to the range of physiological levels. Three aliquots of one low-concentrated plasma sample spiked with different furan fatty acid concentrations were used to determined precision and accuracy. The intra-assay and inter-assay precision were evaluated by the relative standard deviation (RSD) of triplicate injections on the same day and on three consecutive days, respectively. The accuracy was assessed according to the following formula:

$$\text{Accuracy} (\%) = \frac{\text{measured concentration} - \text{basal concentration}}{\text{added concentration}} \times 100$$

Additionally, five standard solutions with a concentration range of 0.005-50 ng/mL were prepared to evaluate the limit-of-detection (LOD) and lower limit-of-quantitation (LLOQ). The LOD was determined as the lowest concentration with single-to-noise ratio (SNR) > 3, while LLOQ was determined according to the calculated precision (< 20% CV) and accuracy (80-120%) by measurement of each concentration five times.

Analysis of furan fatty acids in human plasma

The furan fatty acid extraction was carried out by the following protocol. 11D3-d5 and 11D5-d5 (each of 10 ng) and plasma (20 μL) were added to a glass vial. Then the vial was sealed with a Teflon/silicone disk and incubated at 60 °C for 2 h after 1 M KOH in 95% ethanol (500 μL) was mixed with the plasma. Subsequently, pH was adjusted to 3–4 with 1 M HCl when the mixture was cooled to room temperature. Free fatty acids were extracted by 300 μL of n-hexane for three times and dried under N₂. The following derivatization with BMP and CMP was carried out according to the above protocol.
Statistical analysis

Significant differences of furan fatty acid concentrations between female and male subjects were evaluated by student's unpaired t-tests. Data analysis was performed using GraphPad Prism Software 8.0. A value of $p < 0.05$ was considered as statistical significance.
Results and discussion

Comparison of different methylation methods of furan fatty acids for GC-MS analysis

Generally, fatty acids are analyzed by GC-FID or GC-MS as their volatile non-polar derivatives. Methyl esters are the preferred derivates and can be derived by acid- or base-catalyzed methylation or using diazomethane and related reagents (34). Acid- and base-catalyzed methylation are suitable for the most common fatty acids such as straight-chain and branched-chain fatty acids. However, special attention has to be paid to some unusual fatty acids (e.g. fatty acids containing cyclopropene, cyclopropane or epoxy groups) as they are susceptible to chemical attack by acidic catalyst (34, 35).

To compare the efficiency of different methylation protocols for furan fatty acids, 11D3 and 11D5 standards were derivatized with \( H_2SO_4 \)-MeOH, BF\(_3\)-MeOH and ethereal diazomethane using PC-21:0/21:0 as IS. The typical GC-MS chromatograms are shown in Figure 2. While BF\(_3\)-MeOH was inefficient for the methylation of 11D3 and 11D5 (no methyl ester peaks were observed), both \( H_2SO_4 \)-MeOH and CH\(_3\)ONa-MeOH + CH\(_2\)N\(_2\) yielded the desired methyl products at approximately the same levels. Possibly, BF\(_3\) induced hydrolytic degradation of the furan moiety (36). It should be noted that also using \( H_2SO_4 \)-MeOH can lead to degreased yields of furan fatty acid methyl esters if performed at elevated reaction temperatures (> 90 °C) or in the presence of elevated concentrations (> 2%) (data not shown).

These findings cast some doubt on the accuracy of some furan fatty acid determinations in food samples using the BF\(_3\)-MeOH method (21). Overall, our results strongly indicate that basic derivatization protocols should be used for the quantification of furan fatty acids.
The LOQ and LOD for the diazomethane and methanolic sulfuric acid method were 10 μM and 3.33 μM, respectively. The level of furan fatty acids within human blood is less than 0.30 μM (17). As a consequence, tedious isolation and concentration of large volumes of plasma/serum would be necessary, which means that GC-MS in full scan mode is not ideally suited for the identification of furan fatty acids in human plasma or serum.

Analysis of furan fatty acids by UPLC-ESI-MS/MS in the negative mode

LC-MS/MS is widely used in lipidomics due to its high sensitivity and accuracy. We therefore also analyzed free furan fatty acids (11D3 and 11D5, both at 1 μg/mL) with LC-MS/MS in multiple reaction monitoring (MRM) mode (Figure S2). To optimize the detection of 11D3 and 11D5, parameters such as dwell time, Q1 voltage, CE and Q3 voltage were investigated in details (Table S2). The main fragmentations were: 11D3 \( (m/z \ 321 \rightarrow 71, 321 \rightarrow 99, 321 \rightarrow 141) \) and 11D5 \( (m/z \ 349 \rightarrow 71, 349 \rightarrow 127, 349 \rightarrow 141) \) (Figure S2). The most intensive fragments for both 11D3 and 11D5 derived from the cleavage of the alkyl carboxyl chains. It is worth noting that the abundance of fragments in MS\(^2\) spectra was 1000-fold less than the molecular ion in MS\(^1\). The fragmentation of furan fatty acids in the collision cell seemed to be relatively random rather than specific. Results showed that LOQ and LOD were 50 ng/mL and 16.67 ng/mL, respectively.

Analysis of furan fatty acids by UPLC-ESI-MS/MS in the positive mode

It is generally acknowledged that in the electrospray ionization source cations exhibit a better ionization efficiency than anions (37). Therefore, charge reversal derivatization is a
powerful protocol to improve the sensitivity of mass spectrometric detection of fatty acids (29). Particularly, pyridinium salts such as $N$-[4-(aminomethyl)phenyl]pyridinium, $N$-[4-(aminomethyl)benzyl]-2,4,6-trimethylpyridinium, $N$-(benzenemethylamine)-2,4,6-trimethylpyridinium, BMP and CMP can result in remarkable enhancements of the detection sensitivity of fatty acids (29, 33). Amongst these pyridinium salts, AMMP and AMPP showed the highest derivatization efficiency and therefore enhanced the detection sensitivity most efficiently (29, 33). In addition, fragmentation patterns of most of the other pyridinium derivates are more complex (29). Therefore, in the present study, derivatizations of furan fatty acids with AMMP and AMPP were investigated. The chemical structures of the derivatives (-AMPP and -AMMP) are illustrated in Figure 3.

In case of AMPP-derivatization of furan fatty acids, oxidation products ([M + 16]$^{+}$ and [M + 32]$^{+}$) were detected even if the derivates had been stored at $-80 \, ^{\circ}$C overnight. Especially $m/z$ 367 was very prominent in the MS/MS spectra of [11D3-AMPP + 32]$^{+}$ ($m/z$ 521) and [11D5-AMPP + 32]$^{+}$ ($m/z$ 549) (Figure S3). In MS$^{1}$ scanning, the oxidation product peaks exhibit a low abundance and therefore can easily be overseen.

Based on the fragmentation pattern, we hypothesize that the activated methylene groups at the furan moiety are oxidized. [M − 18]$^{+}$ was observed in MS/MS spectra of both 11D3-AMPP and 11D5-AMPP. Presumably, [M − H$_2$O]$^{+}$ stems from the dehydration of the aromatic side chain of the oxidation product. Interestingly, similar hydroxylated furan fatty acids had been tentatively identified at very low concentrations in fish lipids (31). In the light of our findings, this interpretation, however, is questionable and further analyses will be necessary to confirm or disprove the natural occurrence of these products. In any case, we concluded that
AMPP derivatization is not ideally suited for the derivatization of furan fatty acids.

Fortunately, no such artifacts (oxidation of the methylene group) was observed upon derivatization with BMP and CMP. Charge remote fragmentation could be clearly observed in the MS/MS spectra of both 11D3-AMMP and 11D5-AMMP (Figure 4). The fragmentation of the N-pyridylcarbinol moiety yielded product ions with \( m/z \) 107 and \( m/z \) 124. The fragment at \( m/z \) 178 was derived from the heterolytic cleavage between the β- and γ-C in the alkylcarboxyl chain (33). Additionally, a series of fragments at \( m/z \) 192, 206, 220, 234 and 248 were observed in the MS/MS spectra, indicating a difference of —CH$_2$— (14 amu). Transition ion of \( M^+ \rightarrow 124 \) was selected for quantitative analysis of 11D3-AMMP and 11D5-AMMP, with \( M^+ \rightarrow 107 \) and \( M^+ \rightarrow 178 \) as qualitative references. Key parameters such as dwell time, Q1 voltage, CE and Q3 voltage for monitoring the above three transition ions were optimized. The results are shown in Table S3.

Separation efficiency

Obviously, introduction of pyridinium group contributes to the polarity of fatty acids. Fatty acids derivatized with AMMP (from C12:0-AMMP to C20:0-AMMP) could be separated well on a C18 column (Figure 5). Although the peak of 11D3-AMMP (\( m/z \) 428) overlapped to some extend with the peaks of C18:2-AMMP (\( m/z \) 386) and C15:0-AMMP (\( m/z \) 348), the first quadruple (Q1) could discriminate the parent molecular ions clearly due to the different \( m/z \) (differences of mass-to-charge ratio \( \gg 0.7 \)).
The esterification of free fatty acids with AMMP and derivative yields has been studied extensively (33, 38). Mechanistically, the final 3-acyloxymethyl-1-methylpyridinium iodide could be formed via a nucleophilic attack by CMP on the 2-acyloxy1-methylpyridinium iodide (the esterification product of free fatty acid and BMP). Using equimolar amounts of starting materials and a two-fold molar excess of TEA, the desired products have been obtained in good yields (> 80%) (38). Based on these observations, derivatization was carried out in a one-pot strategy by simply mixing 11D3/11D5, BMP, CMP and TEA. The derivatized 11D3 and 11D5 were quantified by UPLC-ESI-MS/MS with deuterium labeled 11D3 and 11D5, respectively, as IS. Standard curves were established based on the peak area ratios of derivatized furan fatty acids and the corresponding IS with transition ions (M⁺→124). As shown in Table 1, good linearities were obtained in dynamic ranges of 0.5 - 320 ng/mL and 2.5 - 1600 ng/mL for 11D3-AMMP and 11D5-AMMP, respectively.

The precision and accuracy of this UPLC-ESI-MS/MS approach were analyzed on seven physiological concentrations covering the range of measured levels in subjects. As can be seen from Table 2 and Table 3, most intra-day and inter-day RSDs were lower than 12%, while the accuracy was between 84% and 115%. Moreover, our results demonstrated that 11D3-AMMP and 11D5-AMMP had an LLOQ of 0.05 ng/mL. The detection sensitivity was 1000-fold higher than in the negative mode of ionization used with free 11D3 and 11D5. Therefore, this derivatization method is accurate for the quantitation of furan fatty acids in plasma or other biological samples.

Targeted quantification of furan fatty acids in human plasma
11D3 and 11D5 were quantified in the plasma of 27 healthy Chinese subjects (18 males and 9 females) using the AMMP derivatization methods. The UPLC-ESI-MS/MS chromatograms of 11D3, 11D5 in human plasma and corresponding IS are shown in Figure 6. Although some unidentified peaks were observed in these samples, the detection and identification of furan fatty acids was not impaired. Therefore, targeted quantitation of 11D3 and 11D5 in human plasma could be achieved. The concentrations of 11D3 and 11D5 in the individuals are shown in Figure 7. The concentration of 11D3 was generally low, usually below 20 ng/mL. In contrast, 11D5 was found at concentrations above 100 ng/mL. Interestingly, in female subjects, the average level of furan fatty acids was significantly ($p < 0.05$) higher than in male subjects. Also, the contents of furan fatty acids in female cohorts showed larger deviations than in male cohorts.

Fatty acid profiles in human plasma have been extensively investigated by GC-MS (39), multidimensional mass-spectrometry-based shotgun lipidomics (29) and LC-MS/MS (33). However, to the best of our knowledge, furan fatty acids have been reported in three publications so far investigating the fatty acid content of German individuals (17, 23, 24). In these studies, unusually high sample volumes (> 20 mL of plasma or 30 mL of whole blood) were used and concentrated prior analysis. This explains, why ever since then, furan fatty acids have not been reported. In addition, the ‘unusual’ fragmentation pattern of furan fatty acids may have prevented their identification.

Comparing our results on furan fatty acids in human blood with the previous study (17), we found parallel levels of 11D3 and significantly higher levels of 11D5. Furthermore, it is interesting to note that the sex-specific differences had not been reported previously. Future
investigations will have to clarify these striking differences. Overall, it can be asserted that the sensitivity of our new UPLC-ESI-MS/MS protocol (not necessitating isolation or enrichment) is approximately 1000-fold higher than the previously established GC-MS protocols.

The metabolic precursor of CMPF yet remains to be identified. According to previous findings, β-oxidation does not occur on the alkyl chain at α₂ position of furan fatty acids (40). Therefore, it was proposed that furan fatty acids with a propyl group at α₂ positions serve as precursors of CMPF. Accordingly, homologs bearing a pentyl group at α₂ position serve as precursors of CMPentylF (27). It is well-documented that the concentrations of CMPF in males is generally higher than in females (7, 10). Our results and previous findings indicate that higher levels of CMPF coincides with lower levels of 11D3. Therefore, this sex-specific difference in 11D3 and CMPF concentrations in human plasma may indicate that 11D3 serves as metabolic precursor for CMPF. This assumption also warrants further specific studies.

Non-targeted analysis of furan fatty acid analogues

The above-described charge reversal derivatization method enables the identification of furan fatty acids (11D3 and 11D5) according to the transition ions of M⁺→107, M⁺→124 and M⁺→178. Several saturated and unsaturated furan fatty acids have previously been identified in other biological samples (27). To investigate the occurrence of 11D3 and 11D5 analogues in human plasma, precursor ion scan (PIS) was used for the identification of furan fatty acids in pooled samples based on the product ions of m/z 107, 124 and 178 at the collision energy of −48, −40 and −52 eV, respectively. The total ion chromatogram (TIC) of PIS (precursor of m/z 124) is shown in Figure 8. Precursors of m/z 107, 124 and 178 were compared for
comprehensive identification of all fatty acids in human plasma (Figure S4 ~ S13). However, some homologs including 7-(3,4-dimethyl-5-pentylfuran-2-yl)heptanoic acid, 9-(3,4-dimethyl-5-propylfuran-2-yl)nonanoic acid, 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and 9-(3,4-dimethyl-5-pentylfuran-2-yl)nonanoic acid (former three, < 0.5 ng/mL; the last, ~ 4.0 ng/mL), which had been identified in German individuals (17), were not found in our subjects. These differences might be related to race, diet and pathophysiologic conditions. Therefore, future investigations will be necessary to confirm this observation.
Conclusions

In the present study, we have demonstrated the superiority of UPLC-ESI-MS/MS over the established GC/MS methods. Acid-catalyzed ring opening of the furan moiety and oxidation of the activated methylene moieties were identified as major side reactions during sample preparation and derivatization. The optimized protocol reported here largely avoid these side reactions thereby allowing for more accurate, quantitative determination of furan fatty acids from biological samples. Moreover, the non-targeted strategy using PIS shows great potential for the detection and identification of furan fatty acid analogues.

To the best of our knowledge, this is the first study using this strategy for characterization and quantification of furan fatty acids in human tissues. We believe that the UPLC-ESI-MS/MS approach will greatly contribute to the illumination of the CMPF precursor and the related metabolic pathway.
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Conflict of interest

The authors declare that they have no conflict of interest.
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Table 1. Linearity range of furan fatty acid derivatives

| Furan fatty acid derivatives | IS           | R^2   | Linearity (ng/mL) |
|-----------------------------|--------------|-------|-------------------|
| 11D3-AMMP                   | 11D3-\(d_5\)-AMMP | 0.9995 | 0.5-320           |
| 11D5-AMMP                   | 11D5-\(d_5\)-AMMP | 0.9991 | 2.5-1600          |
Table 2. Precision of furan fatty acid derivatives

| Furan fatty acid derivatives | STD spiked 1 | STD spiked 2 | STD spiked 3 | STD spiked 4 | STD spiked 5 | STD spiked 6 | STD spiked 7 |
|-----------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
|                             | Intra-assay  | Inter-assay  | Intra-assay  | Inter-assay  | Intra-assay  | Inter-assay  | Intra-assay  |
| 11D3-AMMP                   | 6.31         | 12.01        | 3.49         | 6.17         | 3.19         | 7.61         | 4.35         |
| 11D5-AMMP                   | 1.99         | 7.86         | 2.92         | 4.82         | 1.14         | 6.74         | 3.87         |

* Precision of 11D3: aliquots of one plasma sample with a low level of 5.01 ± 0.17 ng/mL were spiked with 7 different concentrations (No. 1~7: 5, 10, 15, 20, 25, 30, 35 ng/mL);

* Precision of 11D5: aliquots of one plasma sample with a low level of 104.41 ± 0.62 ng/mL were spiked with 7 different concentrations (No. 1~7: 20, 40, 60, 80, 100, 120, 140 ng/mL);

* Data shown as the relative standard deviation (RSD).
Table 3 Accuracy of furan fatty acid derivatives

| Furan fatty acid derivatives | STD spiked 1 (%) | STD spiked 2 (%) | STD spiked 3 (%) | STD spiked 4 (%) | STD spiked 5 (%) | STD spiked 6 (%) | STD spiked 7 (%) | Range   |
|-----------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|---------|
| 11D3-AMMP                   | 102.51 ± 10.98   | 104.11 ± 3.86    | 98.13 ± 4.80     | 102.07 ± 4.72    | 90.34 ± 6.28     | 93.42 ± 2.37     | 84 ~ 115         |
| 11D5-AMMP                   | 104.37 ± 4.27    | 90.72 ± 5.23     | 99.52 ± 3.85     | 102.77 ± 4.47    | 101.25 ± 2.93    | 96.36 ± 2.70     | 86 ~ 108         |

* Accuracy of 11D3: aliquots of one plasma sample with a low level (5.01 ± 0.17 ng/mL) were spiked with 7 different concentrations (No. 1~7: 5, 10, 15, 20, 25, 30, 35 ng/mL);

b Accuracy of 11D5: aliquots of one plasma sample with a low level (104.41 ± 0.62 ng/mL) were spiked with 7 different concentrations (No. 1~7: 20, 40, 60, 80, 100, 120, 140 ng/mL);

c Data shown as mean ± SD.
Figure 1. General structure of furan fatty acids. R = H or CH₃. m = 0~4, n = 2~14.
**Figure 2.** Furan fatty acid methyl esters derivatized by different methylation protocols. IS: PC-21:0/21:0; CH$_2$N$_2$: ethereal diazomethane.
Figure 3. The derivative products of furan fatty acids. (a) 11D3-AMPP; (b) 11D5-AMPP; (c) 11D3-AMMP; (d) 11D5-AMMP.
Figure 4. MS/MS spectra of furan fatty acids-AMMP. (a) [11D3-AMMP]$^+$; (b) [11D5-AMMP]$^+$. 
Figure 5. LC-MS chromatograms of derivatized fatty acid standards. 1) lauric acid (C12:0)-AMMP, RT = 4.79 min, MW = 306; 2) myristoleic acid (C14:1)-AMMP, RT = 4.95 min, MW = 332; 3) 10-pentadecenoic acid (C15:1)-AMMP, RT = 5.22 min, MW = 346; 4) myristic acid (C14:0)-AMMP, RT = 5.35 min, MW = 334; 5) palmitoleic acid (C16:1)-AMMP, RT = 5.47 min, MW = 360; 6) linoleic acid (C18:2)-AMMP, RT = 5.63 min, MW = 386; 7) pentadecanoic acid (C15:0)-AMMP, RT = 5.65 min, MW = 348; 8) 11D3-AMMP, RT = 5.69 min, MW = 428; 9) palmitic acid (C16:0)-AMMP, RT = 5.94 min, MW = 362; 10) oleic acid (C18:1)-AMMP, RT = 6.02 min, MW = 388; 11) 11D5-AMMP, RT = 6.16 min, MW = 456; 12) heptadecanoic acid (C17:0)-AMMP, RT = 6.23 min, MW = 376; 13) stearic acid (C18:0)-AMMP, RT = 6.49 min, MW = 390; 14) eicosanoic acid (C20:0)-AMMP, RT = 6.99 min, MW = 418.
Figure 6. Multiple reaction monitoring chromatography of (a) 11D3, (b) 11D3-\textit{d}_5, (c) 11D5 and (d) 11D5-\textit{d}_5.
Figure 7. The concentration of furan fatty acids in the plasma of healthy subjects.
Figure 8. Total ion chromatogram of precursor ion scan (precursor of $m/z$ 124). 1) RT=4.05 min, MW=278, C10:0; 2) RT=4.56 min, MW=306, C12:0; 3) RT=5.04 min, MW=334, C14:0; 4) RT=5.04 min, MW=384, C18:3; 5) RT=5.38 min, MW=386, C18:2; 6) RT=5.46 min, MW=412, C20:3; 7) RT=5.50 min, MW=362, C16:0; 8) RT=5.64 min, MW=388, C18:1; 9) RT=5.77 min, MW=414, C20:2; 10) RT=6.01 min, MW=390, C18:0; 11) RT=6.08 min, MW=416, C20:1; 12) RT=6.48 min, MW=418, C20:0; 13) RT=6.48 min, MW=444, C22:1; 14) RT=6.89 min, MW=446, C22:0; 15) RT=6.89 min, MW=472, C24:1.