A straightforward LC-MS/MS analysis to study serum profile of short and medium chain fatty acids

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Abstract

Short and medium fatty acids derived from either dietary sources, gut microbiota, and liver production might play a role in the modulation of metabolism and inflammation. The outcome of different autoimmune or inflammatory diseases could be related to microbiota composition and consequently fatty acids production. Their analytical detection, historically completed by GC, was herein investigated using a sensitive approach of LC-MS/MS with straightforward chemical derivatization, using 3-NPH, to the respective acylhydrazines. An isopropanol protein precipitation coupled to LC-MS/MS analysis allowed to separate and quantify butyric, valeric, hexanoic acid and their branched forms. The serum physiological ranges of short and medium chain fatty acids were determined in a heterogeneous healthy population (*n*=54) from 18-85 years finding a concentration of 935.6 ± 246.5 (butyric), 698.8 ± 204.7 (isobutyric), 62.9 ± 15.3 (valeric), 1155.0 ± 490.4 (isovaleric) and 468.7 ± 377.5 (hexanoic) ng/mL respectively (mean ± SD). As expected, the biological levels in human serum are reasonably wide-ranging depending on several factors such as body-weight, gut microbiome dysbiosis, gut permeability, cardiometabolic dysregulation, and diet.

Keyword: short chain fatty acid, medium chain fatty acid, LC-MS/MS, organic acids; derivatization;

Introduction

Fatty acids are a class of carboxylic acid with an aliphatic chain, linear or branched, composed from 2 to 36 carbon atoms. By the number of carbon atoms they can be divided into three main classes: short (C2-C4), medium (C5-C10) and long chain fatty acids (C>10). Short chain acids are acetic acid, propionic acid, and butyric acid whereas medium chain acids are valeric (C5), hexanoic or caproic

(C6), caprylic (C8) and capric acid (C10). Gut anaerobic metabolism of dietary fiber and undigested saccharides, by intestinal microbiota, generates high concentrations of short-chain fatty acids whereas medium-chain fatty acids derive mostly from the diet. Human liver can also contribute to their production as the end-products of peroxisomal beta-oxidation of long chain fatty acids. Short- and medium chain fatty acids (SMCFAs) modulate 1) carbohydrate and lipid tissue metabolism constraining glycolysis and lipogenesis or gluconeogenesis stimulation 2) the production of mitochondrial energy by reducing the respiratory chain equivalents and partially the efficacy of ATP synthesis [1] 3) inflammatory responses and metabolic disorders through activation of G protein-coupled receptors [2] 4) the GLP-1 secretion which can reduce liver steatosis, lowering plasma glucose, improving cardiac function and protecting from kidney failure [3] and 5) the maintenance of immune homeostasis [4]. Microbiota composition and consequently fatty acids production might modulate immune responses and, thus, could be used to ameliorate the outcomes of different pathologies [5]. Several GC-MS methods [6–10] were applied to measure SMCFAs in different matrices, but most of these are not sensitive enough and require time-consuming sample preparation and derivatization [11], which can influence their recovery drastically. This article was focused on the development of a LC-MS/MS technique with an effortless derivatization step [12–17] to detect SMCFAs physiological range in the serum of healthy volunteers and to shed light on the difficulty to correctly quantify their levels, by LC-MS, without chemical derivatization [18].

2. Materials and methods

2.1 Reagent and chemicals

The chemicals were all analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions were prepared using purified water at a Milli-Q grade (Burlington, MA, USA). Butyric acid, isobutyric acid, 4-chloro butyric acid, 2-fluoro propionic acid, 2-isobutoxyacetic acid, and 2-ethyl butyric acid were purchased by Sigma-Aldrich (St. Louis, MO, USA). Valeric, isovaleric and hexanoic acid were purchased by Alfa Aesar (Karlsruhe, Germany). The solutions were prepared in acetonitrile and stored at -20 °C. For derivatization 3-Nitrophenylhydrazine hydrochloride (3-NPH), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and pyridine were purchased by Sigma-Aldrich (St. Louis, MO, USA) and each solution was made in water:methanol (3:7, v/v) and stored (for maximum 3 days) at -20 °C.

2.2 Serum sample from healthy volunteers

Participants included female and male individuals (total *n*=54, male *n*= 22 and female *n*=32), healthy, with normal weight (BMI 18.5-25.0) who voluntarily accepted to join the study. All volunteers were enrolled from IRCCS S. Maria Nascente, Fondazione Don Gnocchi and provided their informed consent, signing a letter of agreement. The recruitment criteria were 1) aged 18–85 years and 2) any pathological conditions. Venous whole blood was gathered in the fasting state and left undisturbed at room temperature (30 min) to allow clotting. Then the clot was removed by centrifugation (2500 RPM, 15 min) to obtain serum which was stored at -80°C until analysis. All the procedures adopted in the present study were in respect to the ethical standards in the Helsinki Declaration.

2.3 Extraction and derivatization of short-fatty acid

Serum (50 μL) was deproteinized with 100 μL of cold-isopropanol and added with 10 μL of IS (2-isobutoxyacetic acid, 2.5 μg/mL). After centrifugation (13400 RPM, 5 min) 100 μL of supernatant was separated in a glass vial for the derivatization step. To the latter were consequently added 50 μL of 50 mM of 3-NPH, 50 μL of 50 mM of EDC, 50 μL of pyridine (7%) in methanol. Derivatization (Figure 1) was performed in an incubator at 37°C for 30 min. The solution was diluted with 250 μL 0.5% of formic acid in water and directly injected.

2.4 LC-MS/MS analysis

The analytical system consists of an HPLC Dionex 3000 UltiMate system (Thermo Fisher Scientific, MA, USA) coupled to a tandem mass spectrometer AB Sciex 3200 QTRAP (Sciex, Milan, Italy) operated under negative ESI mode (Figure S1). The instrument parameters were: CUR 30, GS1 40, GS2 40, capillary voltage -4,5 kV and source temperature 400 °C. The 3-NPH derivatives of SMCFAs yielded in MS/MS the prominent ion *m/z* 137 which corresponded to the loss of ammonia from 3-NPH and a neutral loss of 43 Da [14] (Figure 2). Chromatographic separation was achieved on a Restek Raptor C18 2.7 μm 2.1x100 mm (Bellefonte, PA, USA) using as mobile phase (A) water + 0.1% formic acid and (B) acetonitrile. The elution program (%B) was 0-2.5 min 10%, 2.5-16 min 10-50%, 16-16.2 50-10% maintained until 18 min. The flow rate was 0.4 mL/min, the column and the autosampler temperature were 35 °C and 15 °C. The analytical data comprehensive of both chromatograms and spectra were processed using Analyst software (v.1.2, Sciex). Quantitative analysis was performed by semi-automatic area integration using MultiQuant (v.2.1, Sciex). LC-MS/MS analysis conditions were summarized in Table 1. In order to avoid error in identification,

between isomers, relative retention times (RR_t, calculated against the retention time of the internal standard) were monitored (acceptable values were below ± 0.01).

Table 1. Chromatographic and mass spectrometry characteristics of 3-NPH derivatives of SMCFAs.

Analytes	R_t (min)	RR_t	MRM transition	DP (eV)	CE (eV)
Butyric-NPH	9.9	0.70	222 > 137	-50	-26
Isobutyric-NPH	9.6	0.67	222 > 137	-50	-26
Valeric-NPH	12.6	0.86	236 > 137	-55	-28
Isovaleric-NPH	12.3	0.83	236 > 137	-55	-28
Hexanoic-NPH	14.5	1.01	250 > 137	-65	-30
IS	14.3	-	266 > 137	-60	-30

Rt: retention times, RRt: relative (to IS) retention times, MRM: multiple reaction monitoring, DP: declustering potential, CE: collision energy.

2.5 Extraction and GC-MS/TOF analysis of SMCFAs as methyl esters derivatives

Serum (500 μL) was diluted with 100 μL of water; 100 μL of 4-chloro butyric acid (20 μg/mL, 2 μg) were added. Samples were acidified by adding 5 μL of HCl 2 M (pH<3), saturated with NaCl (salting-out) and extracted with 2 mL of acetonitrile. The organic phase was withdrawn after centrifugation (15 min at 4000 RPM) and anhydrification was achieved by Na_2SO_4. Acetonitrile was evaporated to reach 100 μL of volume which was derivatized at 58°C for 2 h with 500 μL of methanolic HCl 3 M to get the methyl-esters of fatty acid. The derivatized solution was back-extracted with hexane which was centrifuged, withdrawn and evaporated until 250 μL. Three microliters of clean supernatant were directly injected in GC-MS/TOF. GC-MS/TOF analysis was performed by Dani Master GC system couplet to a Dani Master TOF Plus detector (Dani Instruments, Cologno Monzese, Italy) operated in electron ionization (EI) mode (70 eV). The GC was equipped with a HB-5ms capillary colomn (20mx0.18mmx0.18 um, Agilent Technologies). The GC/MS conditions employed were: pulsed splitless for 1 min at 1.2 bar, helium flow rate 0.6 mL/min, injection temperature 250°C, solvent delay 135s, quadrupole 150°C, ion source 200°C and transfer line 250 °C. The initial column temperature was 40 and held 4 min, ramped to 280 at 20 °C/min and kept at this temperature for 3 min with a total run time of 20 min. The MS data were acquired in full scan mode from *m/z* 35-120 with an acquisition frequency at 5 scans/s. Instrument was managed by the software Masterlab (v.4.1.5.0) and data were further analysed using Chromazing (v.1.50.105). Chromatographic separation and some specifications can be found in Figure S2 and Table S1. Quantification was achieved extrapolating the *m/z* 74 of the methyl esters (ME) of SMCFAs except using the *m/z* 102 for isobutyric acid ME (Figure S3).

2.7 Extraction and LC-MS analysis of SMCFAs without chemical derivatization

Serum (50 µL) was deproteinized and acidified with 50 µL methanol 0.05% formic acid, diluted with 140 µL of deionised water, added with 10 µl of IS (2-isobuthoxyacetic acid 10 µg/mL, 100 ng) and incubated in ice for 10 min to facilitate protein precipitation. Liquid-liquid extraction was performed with 400 µl of ethyl acetate-diethyl ether (1:1, v/v) by mechanical shaking for 5 min at 50 oscillations/min. After centrifugation (13400 RPM, 5 min) organic phase was separated, evaporated under nitrogen and reconstituted with 100 ul of a mixture water-acetonitrile (8:2, v/v) with 2 mM of ammonia; 10 µL was directly injected in LC-MS. The analytical system consists of an HPLC Dionex 3000 UltiMate system (Thermo Fisher Scientific, MA, USA) coupled to a tandem mass spectrometer AB Sciex 3200 QTRAP (Sciex, Milan, Italy) operating under ESI negative conditions. The instrument parameters were: CUR 30, GS1 40, GS2 55, capillary voltage -4,5 kV and source temperature 550 °C. SMCFAs, since does not undergo CID fragmentation, were analysed by monitoring the parent transition $[MH]^-$ m/z 87>87 for butyric acid, m/z 101>101 valeric acid, m/z 115>115 hexanoic acid with declustering potential and collision energy fixed to -30 eV and -5 eV. In contrast, 2-isobuthoxyacetic acid (IS) was analysed by the transition m/z 131>75 with declustering potential and collision energy fixed to -35 eV and -16 eV. Chromatographic separation was achieved on a Synergy Polar RP column, 2.5x100 mm 2.5 um (Phenomenex, Torrance, CA, USA); (A) water + 0.1% formic acid and (B) acetonitrile. The flow rate was 400 µL/min, the column and the autosampler temperature were 35 °C and 15 °C. The isocratic elution was for 5 min at 20% (B). Retention times for 2-isobuthoxyacetic acid (IS), butyric acid, valeric acid and hexanoic acid were 0.60, 0.70, 0.90, 1.20 min respectively (Figure S4).

2.7 Experimental details on the evaluation of analytical performances in LC-MS/MS

The LOD and LOQ were defined as the concentration which yield a measure peak with a signal/noise S/N>3 and S/N>10, respectively. The precision was evaluated as the coefficient of variation (CV%) that is CV%= standard deviation/ average *100. It was determined by different extraction of the same pool of serum in the same day (CV% intra-day) and between different days (CV% inter-day). Accuracy was measured as percent of the nominal value (E%, |experimental value - theorical value|/ theorical value or |experimental value GC/MS - theorical value LC-MS/MS|/ theorical value LC-MS/MS) on serum samples analyzed with a GC/MS technique.

2.8 Data analysis

Graphs, and statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, CA).

3. Results and discussion

3.1 The straightforward analysis of SMCFAs as 3-NPH derivatives

The first choice was GC-MS, but it was discharged for 1) the need of methylation (2h) and back-extraction in hexane which made the sample preparation very tedious and long-timing and 2) it required an appreciable amount of serum (500 μL, Figure S2) which was not always available. The GC-MS/TOF was found to be a robust method to detect SMCFAs but in here its applicability was limited to a small number of samples, used for the determination of accuracy of the LC-MS/MS technique. The approach of LC-MS was driven by a shorter time of both analysis and sample preparation which theoretically can bypass the derivatization and the back-extraction steps used by other authors in either GC or LC [6,7,12,13]. Hence, a first approach was the detection of SMCFAs without derivatization [18]. Nonetheless, their analysis in LC-MS and the extraction of free fatty acids was very challenging (Note S1, Table S2, Figure S5) and the results, in our hands, were unreliable (Figure S6-7). The use of LC-MS by selected ion monitoring, forced by an inappreciable MS/MS fragmentation, leads to many interferences brought by both solvent (especially ketones and ethers) and biological matrices (Figure S6). Moreover, the use of ammonia, as a negative ionization enhancer, cause the co-elution of linear and branched forms. Since their volatile nature, the extracts should be immediately injected, and a delayed analysis can be undesired. This experimental evidence can be overcome with a 3-NPH derivatization step which consequently led to a confident quantification.

A protein precipitation procedure was set-up evaluating different precipitating solvents such as methanol, ethanol, isopropanol, acetonitrile, zinc sulfate (15 mg/mL) in methanol, methanol/isopropanol (1:1, v/v), acetonitrile/isopropanol (1:1, v/v) and methanol/diethyl ether (1:1, v/v). The best extraction recovery (>75% for each analyte) and chromatographic traces were found with pure isopropanol. The derivatization conditions were optimized in order to 1) avoid nitrogen drying which can cause a loss in volatile SMCFAs, 2) warranty a mild reaction condition (<50 °C) in the shortest possible time and 3) allow the derivatization in a water based solution. Temperature from 25-40°C and derivatization time from 30-120 min did not impact dramatically on the peak area of analytes. Thus, derivatization was performed in dark-incubation at 37°C for 30 min.

3.2 Internal standards

Labeled analogues of SMCFAs were very expensive due to an inherent difficulty in their chemical synthesis, thus affordable options were herein evaluated such as 2-ethyl butyric acid, 2,2-dimethyl butyric acid, 2-isobutoxyacetic acid, 4-chloro butyric acid and 2-fluoro propionic acid. Derivatized 2-ethyl butyric acid and 2,2-dimethyl butyric acid shared the MS/MS transitions (250>137) with hexanoic acid, and even if they were base-to-base separated to the latter, they interfere with unknown matrix interferences. 4-chloro butyric acid demonstrated a completely different behavior in LC-MS/MS: 1) a high value of DP (-180 eV), 2) the absence of the *m/z* 137 replaced by *m/z* 147 and 3) poor chromatographic retention (0.7 min). The 2-fluoro propionic acid was found to be not chromatographic compatible demonstrating, in these conditions, a broad peak. 2-isobutoxy acetic acid fulfilled our expectation since it shared both the chromatographic and mass spectrometry conditions with the studied analytes.

3.3 LC-MS/MS analytical performances

Analytical performances (Table 2) were evaluated studying parameters such as specificity, precision, linearity, the limit of quantification (LOQ) and detection (LOD). The analysis in serum was found to be specific for the determination of butyric, valeric, hexanoic acid, and their branched isomers (isobutyric acid and isovaleric acid) (Figure 3). The LOD and LOQ were confirmed at 3 and 10 ng/mL, respectively. To establish the linearity an eight-point calibration curve was studied in a surrogate matrix (0.2% BSA in PBS) since SMCFAs were physiological in serum. The linearity, in the range between 10-2500 ng/mL, was proven according to the regression line by the method of least squares and expressed by the coefficient of correlation (R^2>0.99). The precision evaluated as the coefficient of variation (CV%) was determined by different extraction of the same pool of serum resulting in an intra-day and inter-day CV% < 15%. Accuracy was measured as percent of the nominal value (E%) on serum samples (*n*=5) analyzed by LC-MS/MS vs GC/MS accounting inferior than 15% (Table S3).

Table 2. Analytical performances results of SMCFAs analysed in LC-MS/MS after 3-NPH derivatization.

Analytes	**Equation**	**R^2**	**CV% intra-day** (n=10)	**CV% inter-day** (n=20)	**E (%)** (n=5)
Butyric acid	y=0.00238x-0.02555	0.9993	13.0	12.1	12.3
Isobutyric acid	y=0.00303x-0.05273	0.9998	10.9	10.0	14.1
Valeric acid	y=0.00234x-0.01887	0.9993	13.3	13.8	10.1
Isovaleric acid	y=0.00231x-0.03858	0.9996	8.0	9.8	9.8
Hexanoic	y=0.00245x-0.06811	0.9970	13.7	12.1	8.4

R^2: r-squared, CV%: coefficient of percentage variation, E(%): relative percentage error.

3.4 Short- and medium- fatty acids serum profile

The production and thus the serum concentration of SMCFAs is strictly linked to diet and the gut microbiome [6], in particular, short chains are mostly generated by colonic bacteria whereas medium chains arise mostly from dietary triglycerides (milk and dairy products) [19]. The concentration in peripheral blood is 1000-fold lower than in the large intestine [4], but in the same way, it depends on several factors such as body-weight, gut microbiome dysbiosis, gut permeability, cardiometabolic dysregulation, central obesity, hypertension and diet [20]. Accordingly, their physiological levels in human serum extracted from literature data are reasonably wide-ranging (Table 3). The serum physiological ranges of short and medium chain fatty acids determined in a heterogeneous healthy population (n=54) from 18-85 years showed a concentration of 935.6 ± 246.5 (butyric), 698.8 ± 204.7 (isobutyric), 62.9 ± 15.3 (valeric), 1155.0 ± 490.4 (isovaleric) and 468.7 ± 377.5 (hexanoic) ng /mL respectively (mean ± SD). Results obtained in this study are illustrated in Figure 4 and are in accordance with previous data reported in the literature (Figure S8). Any statistical difference was found among gender and classes of age (Figure 4).

Table 3. Blood, serum or plasma concentration of fatty acids (C4-C6) both linear and branched.

Analytes	Matrix	Concentration	References
Butyric acid	Blood	0.3-1.5 μM (30-140 ng/mL)	[21]
	Blood from radial artery	1.8-8.3 μM (160-700 ng/mL)	[18]
	Serum	9.2-29.1 μM (800-2550 ng/mL)	[22]
	Serum	9-17 μM (800-1400 ng/mL)	[23]
	Venous blood	2 μM (200 ng/mL)	[24]
	Serum	0.6 μM (50 ng/mL)	[25]
	Serum	< 10 μM (1000 ng/mL)	[7]
	Plasma	1.3 μM (100 ng/mL)	[8]
Isobutyric acid	Blood	0.7-4.4 μM (60-400 ng/mL)	[21]
	Serum	15 μM (1300 ng/mL)	[22]
	Serum	6-15 μM (500-1300 ng/mL)	[23]
	Venous blood	13 μM (1100 ng/mL)	[24]
	Serum	0.3 μM (30 ng/mL)	[25]
	Serum	< 10 μM (1000 ng/mL)	[7]
Valeric acid	Blood	0.3-1.2 μM (30-120 ng/mL)	[21]
	Serum	0.2-0.4 μM (20-40 ng/mL)	[22]
	Serum	1.4-2 μM (140- 200 ng/mL)	[23]
	Venous blood	1.3 μM (130 ng/mL)	[24]
	Serum	0.4 μM (40 ng/mL)	[25]
Isovaleric acid	Blood	0.3-2.7 μM (30-270 ng/mL)	[21]
	Blood (children)	4.9 μM (500 ng/mL)	[26]
	Serum	11.2-44.4 μM (1100-4400 ng/mL)	[22]
	Serum	38-88 μM (3800-9000 ng/mL)	[23]
	Serum	40 μM (4000 ng/mL)	[24]
	Serum	0.6 μM (60 ng/mL)	[25]
Hexanoic acid	Serum	1.4-9.7 μM (160-1100 ng/mL)	[22]
	Blood	0-105 μM (0-1700 ng/mL)	[27]

Analytes	Matrix	Concentration	References
	Serum	2 μM (230 ng/mL)	[25]

4. Conclusions

The LC-MS employing SIM method for the determination of butyric, valeric and hexanoic acid in serum was found to be unspecific and the quantification unreliable. The GC-MS/TOF was found to be a robust method to detect SMCFAs but it requires a long time in pre-analytical procedures. An easy and quick derivatization step employing 3-NPH was indeed implemented and optimized in order to allow the extraction and analysis of five SMCFAs from many samples in a day (*n*=100). The method was successfully applied determining physiological ranges of fatty acids with carbon-chain from C4 to C6, including two branched forms. Our work implements the reference healthy population (*n*= 54) to establish a range of physiological concentration in serum.

Abbreviations

SMCFAs short-medium chain fatty acids; GC-MS gas chromatography coupled to mass spectrometry; LC-MS liquid chromatography coupled to mass spectrometry; TFA trifluoroacetic acid; TCA trichloroacetic acid; ESI electrospray ionization; IS internal standard; CID collision induced dissociation; LOQ limit of quantification; LOD limit of detection; MW molecular weight; BSA bovine serum albumin; PBS phosphate buffer saline;

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Conflict of interest none

Fundings none

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Figure captions

Figure 1. Acylhydrazine synthesis by the reaction of 3-NPH with the carboxylic function of SMCFAs. EDC is used as a carboxyl activating agent for the coupling with primary amines to yield acylhydrazine, this reaction was catalyzed by pyridine. The reaction was allowed in the dark for 30 min at 37°C.

Figure 2. MS/MS spectra of 3-NPH derivatives of butyric and isobutyric acid (**A**), valeric and isovaleric acid (**B**), hexanoic acid (**C**) and 2-isobutoxyacetic acid (**D**). In the spectra of SMCFAs (**A, B, C**) can be recognized the neutral loss of 43 Da and the common *m/z* ions 106, 121, 137 and 152 which are diagnostic of 3-NPH.

Figure 3. LC-MS/MS analysis in a serum sample. The chromatogram showed isobutyric acid (**A**), butyric acid (**B**), 2-methyl butyric acid (**C**, not quantified), isovaleric acid (**D**), valeric acid (**E**), 2-isobutoxyacetic acid (**IS**) and hexanoic acid (**F**) as 3-NPH derivatives.

Figure 4. Serum concentrations (*n*=54) of butyric acid (**A**, min to max, 416.9-2071 ng/mL), isobutyric acid (**B**, 400.1-1117 ng/mL), valeric acid (**C**, 36.0-106.6 ng/mL), isovaleric acid (**D**, 474.4-2312 ng/mL) and hexanoic acid (**E**, 92.9-1429 ng/mL). Visualization by box and whiskers plot: the box always extends from the 25th to 75th percentiles, the line in the middle of the box is plotted at the median, whiskers are drawn down to the 10th percentile and up to the 90th whereas points below and above the whiskers are drawn as individual points. The graphs below show the serum distribution of SMCFAs among gender (blue) and classes of age (pink) in the same order as reported in the upper panel: butyric acid (**A**), isobutyric acid (**B**), valeric acid (**C**), isovaleric acid (**D**) and hexanoic acid (**E**). Any statistical differences were found in these sub-populations.