



# A Fast and Accurate Way to Determine Short Chain Fatty Acids in Human Serum by GC–MS and Their Distribution in Children with Digestive Diseases

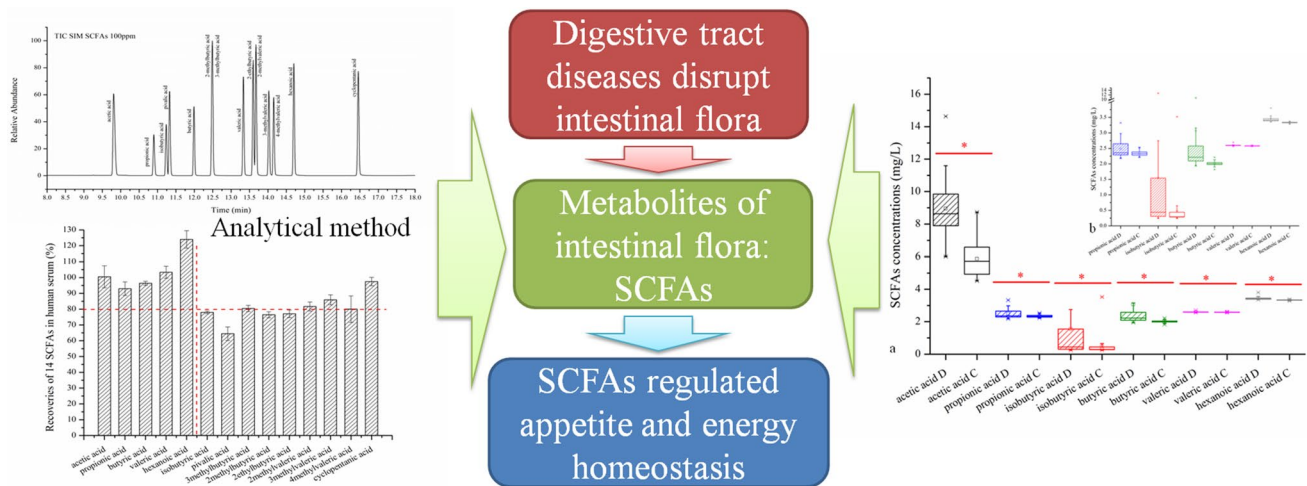
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## Abstract

Short-Chain Fatty Acids (SCFAs) represent the largest group of metabolic nutrients produced by gut microbiota, and can be considered potential biomarkers for digestive tract diseases. A fast, cost-effective, and reproducible analytical method for the analysis of SCFAs is highly required. This work developed a one-step extraction of 14 SCFAs using a mixture of ethanol and *n*-hexane in human serum samples by GC–MS without derivatization. The recoveries of the 14 SCFAs ranged from 62.4% to 129.2% in human serum samples. The LODs and LOQs of the 14 SCFAs were 0.12–0.48 mg/L and 0.40–1.61 mg/L, respectively. The application of this method was carried out by comparing SCFAs in serum samples from children with health and digestive tract diseases, and the results demonstrated that SCFAs in the serum of children with digestive disorders were significantly higher than those of healthy children, implying that digestive tract diseases affect gut microbiota and their metabolisms.

## Graphic abstract



**Keywords** Gas chromatography–mass spectrometry · Short-chain fatty acids (SCFAs) · Human serum · Digestive tract diseases

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Extended author information available on the last page of the article

## Introduction

Short-Chain Fatty Acids (SCFAs) are carboxylic organic acids with aliphatic tails that contain one to six carbon atoms, which mainly include acetate (C<sub>2</sub>), propionate (C<sub>3</sub>) and butyrate (C<sub>4</sub>) [1]. SCFAs represent the largest group of metabolic nutrients produced by anaerobic fermentative gut microbiota [2]. In the intestinal tract, straight-chain SCFAs, such as acetate, propionate, and butyrate, are predominantly from bacterial fermentation of dietary fibers and resistant starch, which can cross the gut epithelium and enter the peripheral blood; branched-chain SCFAs, such as isobutyric acid, isovaleric acid, and 2-methylbutyric acid, are mostly derived from gut microbiota by metabolizing branched-chain amino acids (such as valine, leucine and isoleucine) [1, 3]. Because the luminal pH is in the range of 5.5–6.5, with a pK<sub>a</sub> of about 4.8, more than 90% of the SCFAs in the gut are found in their dissociated states. To travel to tissues and organs beyond the colon, SCFAs are taken up from the intestinal lumen, mainly by passive diffusion (non-ionic) and active transport (SCFA<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange, MCT and SMCT1 transporters) [4].

SCFAs can not only provide ca. 10% of the daily energy requirements in humans, but also play important roles in appetite regulation and energy homeostasis [5, 6], as well as the metabolism of glucose, lipids, and cholesterol in tissues via multiple mechanisms [7, 8]. Acetic acid can traverse the blood brain barrier and regulate appetite; acetic and propionic acids can also directly activate G-protein-coupled Free Fatty Acid Receptors (FFAR); butyric acid is a crucial energy resource for enterocytes, which can inhibit the activity of histone deacetylases and thus reduce the risk of colon cancer and colitis [8]. Recent evidence has shown a direct link between SCFAs and digestive diseases, including Inflammatory Bowel Disease (IBD), Irritable Bowel Syndrome (IBS), diarrhea, and colon cancer [6]. Digestive diseases often cause intestinal flora disorders, and SCFAs are the main products of intestinal flora metabolism [9]; thus, SCFAs can be used as potential biomarkers to detect digestive diseases. At present, qualitative and quantitative measurements of SCFAs in several biological samples, such as feces, urine, blood, and colonic contents, have been developed to increase our understanding of the complex interplay between diet, gut microbiota, and host metabolism homeostasis [10]. Several analytical techniques have been used to analyze SCFAs in biological samples using Gas Chromatography (GC) and Liquid Chromatography (LC) coupled with various kinds of detectors, such as Ultraviolet (UV), Flame Ionization Detector (FID), and Mass Spectrometry (MS) [2].

In general, these small volatile molecules are detected mainly by GC and GC-MS either directly or after

derivatization [11–13]. For example, acidified water extraction—direct injection GC-FID was used to detect the high levels of SCFAs in human and rat fecal, colonic samples, as well as in other environmental samples [14, 15]; other researchers have used derivatization, mostly silylation, in similar studies [2, 16–18]. In the case of GC-MS, water extraction was not indicated, due to its negative effect on MS determination. When LC is employed for SCFAs detection, the SCFAs cannot be effectively separated because they have no typical photo absorption groups. Therefore, in the case of LC-MS or LC-UV, the derivatization provides chromophores to improve the SCFAs' sensitivity and enhance the degree of separation [19–22]. However, it is difficult to separate some derivatives of SCFAs using LC; this process is also time-consuming, expensive, and introduces new deviations to the analysis [1]. Additionally, care should be paid to prevent evaporation of more volatile derivatives during the pre-treatment procedure, as potential losses of SCFAs may occur.

Generally speaking, approximately 95% of the SCFAs are absorbed from the colon; therefore, the SCFAs profiles in feces do not necessarily represent colonic SCFAs levels, meaning that blood analyses may be better alternatives [23]. In fact, the content of SCFAs in the serum is much lower than that in the feces, further increasing the difficulty of analysis. Previous studies on SCFAs analysis in the serum by LC/GC with derivatization have been relatively complex, and unable to simultaneously detect all SCFAs, especially the branched-chain SCFAs, which are critical biomarkers in biological studies. Several organic solvents, such as chloroform, ether, and ethyl acetate, have been tested for extracting SCFAs, but the results have been unsatisfactory [2]. Therefore, a fast and accurate way to detect all SCFAs in the serum is urgently needed. In this work, we established a one-step extraction of 14 SCFAs from human serum samples, using a mixture of ethanol and *n*-hexane, coupled to subsequent analysis by GC-MS without derivatization; and compared differences in recovered SCFAs profiles between healthy children and children with digestive diseases, to evaluate SCFAs as potential digestive disease biomarkers.

## Materials and Methods

### Reagents and Materials

Standards of 14 SCFAs, including acetic acid (CAS No. 64-19-7, ≥ 99.7%), propionic acid (CAS No. 79-09-4, ≥ 99.5%), isobutyric acid (CAS No. 79-31-2, ≥ 99.5%), pivalic acid (CAS No. 75-98-9, ≥ 99.0%), butyric acid (CAS No. 107-92-6, ≥ 99.8%), 2-methylbutyric acid (CAS No. 116-53-0, ≥ 99.0%), 3-methylbutyric acid

(isovaleric acid, CAS No. 503-74-2,  $\geq 99.5\%$ ), valeric acid (CAS No. 109-52-4,  $\geq 99.0\%$ ), 2-ethylbutyric acid (CAS No. 88-09-5,  $\geq 98.0\%$ ), 2-methylvaleric acid (CAS No. 97-61-0,  $\geq 98.0\%$ ), 3-methylvaleric acid (CAS No. 105-43-1,  $\geq 98.0\%$ ), 4-methylvaleric acid (CAS No. 646-07-1,  $\geq 97.0\%$ ), hexanoic acid (CAS No. 142-62-1,  $\geq 99.0\%$ ), and cyclopentanoic acid (CAS No. 3400-45-1,  $\geq 98.0\%$ ), were all purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tokyo Chemical Industry Co., Ltd (TCI). Acetonitrile, methanol and ethanol (HPLC grade) were obtained from Fisher Scientific (Houston, TX, USA). Analytical grade hydrochloric acid (HCl) and other chemicals were all purchased from Beijing Chemical Reagents Company (Beijing, China). Ultra pure water was supplied by a Millipore Milli-Q system.

Individual stock standards were made in ethanol, and all stock solutions were made at a concentration of 100 mg L<sup>-1</sup> and stored at  $-20\text{ }^{\circ}\text{C}$ .

### Sample Collection

Serum samples of 45 healthy children (21 boys, 24 girls), aged from 0 to 18 years old (average age of 9 years old) were obtained from medical examinations by Beijing Center for Diseases Prevention and Control (Beijing CDC). Serum samples of 49 children with digestive tract diseases (28 boys, 21 girls), aged from 0 to 18 years old (average age of 7 years old) and mainly including inflammatory bowel diseases (30), and diarrheal disorders (10), ulcerative bowel disease (4) and food allergies (5), were obtained from the National Center for Children's Health, China, Beijing Children's Hospital. Serum samples used in the development of the method were randomly collected from 3 out of 49 children with digestive tract diseases. The study was carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. All subjects' parents were informed of information and signed a written consent form. Ethical committee approval from Beijing Children's Hospital to Beijing CDC was also obtained.

### Determination of Different Solvents on Extraction Efficiency

Different solvents have strong effects on the efficiency of SCFAs extraction. In this section, four different organic solvents, including methanol, ethanol, acetonitrile, and ethanol + acetonitrile (v:v = 1:1), were tested to determine the optimal extraction solvent. After that, *n*-hexane was added to determine whether interferences from the serum could be eliminated.

### Sample Preparation

A simple and cost-effective extraction procedure was optimized for human serum. 100  $\mu\text{L}$  samples of human serum were mixed with 3 times the volume of ethanol, and placed in 2 mL centrifuge tubes. 800  $\mu\text{L}$  of *n*-hexane was then added into each tube to remove other interferences by vortexing for 3 min and centrifuging at 13,000 rpm for 8 min at  $4\text{ }^{\circ}\text{C}$ . After this, 100  $\mu\text{L}$  aliquots of the lower ethanol extracts were transferred into glass-lined pipes in the vials, and 2  $\mu\text{L}$  of 10% HCl was added to each, to adjust the pH value to around 4.0. Samples were then mixed vigorously prior to GC–MS analysis.

### Instrument Acquisition Methods

The SCFAs were identified by comparing retention times and mass spectra of targeted analytes to those of the reference standards. The mixed standard solutions were diluted to concentrations of 0.5, 1, 5, 10, 25, 50, 100 mg/L and 5 replicates were made for each concentration. The analysis of the SCFAs was performed using an Agilent 7890B GC system coupled to an Agilent 5977A mass spectrometric detector (GC/MSD, Agilent Technologies, St Clara, CA, USA). Sample extracts were separated by a DB-FFAP column (50 m  $\times$  0.32 mm, 0.5  $\mu\text{m}$ , Agilent Technologies). The injection volume was 1  $\mu\text{L}$ . Helium (99.99% purity) was used as a carrier gas at a flow rate of 1 mL/min. The injector and transfer line temperatures were set at  $250\text{ }^{\circ}\text{C}$  and  $230\text{ }^{\circ}\text{C}$ , respectively. In general, the SCFAs extracts in the fecal samples were injected in a split mode due to their higher concentrations [11]. The serum samples were injected using a split-less mode due to their relatively low concentrations. The oven program was set at an initial temperature of  $70\text{ }^{\circ}\text{C}$ , then increased to  $180\text{ }^{\circ}\text{C}$  at a rate of  $10\text{ }^{\circ}\text{C}/\text{min}$ , and finally increased to  $200\text{ }^{\circ}\text{C}$  at a rate of  $5\text{ }^{\circ}\text{C}/\text{min}$ . The total run time was 22 min. The solvent delay was set at 5.5 min.

MS detection was first operated in full-scan mode to determine the retention times of the targets, where the settings were as follows: EI at 70 eV, ion source temperature at  $230\text{ }^{\circ}\text{C}$ , *m/z* values ranging from 40 to 300 Da and acquisition scan time of 0.2 s. After determining the retention time of the analytes, the SIM mode was applied for the analysis of the SCFAs to improve the sensitivity; the full parameters were detailed in the Table 1 and Table S1 (Electronic Supplementary Material).

### Method Development and Validation

Calibration curves were constructed using least-square regression by plotting the peak areas of the quantitation ions of the standards over a range of concentrations. Verification of the standard curve was carried out by comparing the

**Table 1** GC–MS analytical parameters of SCFAs

Compounds	Precursor ion ( <i>m/z</i> )	Retention time (min)	Fragment ion ( <i>m/z</i> )			Linear range (mg/L)	<i>R</i> <sup>2</sup>
Acetic acid	60.02	9.82	43 <sup>a</sup>	45	60	0.5–50	0.9992
Propionic acid	74.04	10.90	74 <sup>a</sup>	45	57	0.5–50	0.9998
Isobutyric acid	88.05	11.25	43 <sup>a</sup>	73	88	0.5–50	0.9994
Pivalic acid	102.06	11.33	57 <sup>a</sup>	41	87	0.5–50	0.9998
Butyric acid	88.05	12.00	60 <sup>a</sup>	73	88	0.5–50	0.9996
3-Methylbutyric acid	102.06	12.50	60 <sup>a</sup>	41	87	0.5–50	0.9995
2-Methylbutyric acid	102.06	12.50	74 <sup>a</sup>	57	87	0.5–50	0.9998
Valeric acid	102.06	13.34	60 <sup>a</sup>	73	55	0.5–50	0.9996
2-Ethylbutyric acid	116.08	13.60	88 <sup>a</sup>	73	43	0.5–50	0.9998
2-Methylvaleric acid	116.08	13.68	74 <sup>a</sup>	43	87	0.5–50	0.9994
3-Methylvaleric acid	116.08	14.03	60 <sup>a</sup>	41	57	1–50	0.9994
4-Methylvaleric acid	116.08	14.16	57 <sup>a</sup>	41	55	0.5–50	0.9991
Hexanoic acid	116.08	14.71	60 <sup>a</sup>	73	87	0.5–50	0.9989
Cyclopentanoic acid	114.06	16.46	73 <sup>a</sup>	41	86	0.5–50	0.9991

<sup>a</sup>Quantitation ion

relative deviation value between the theoretical value and the actual measured value obtained from the standard curve, which was required to fall below 20%.

Precision was determined by the intra-assay and inter-assay variations of replicate analyses of Quality Control (QC) samples spiked at low (2.5 mg/L), medium (10 mg/L) and high (25 mg/L) concentrations 3 times per day, for 3 consecutive days. The QC samples were stored at – 80 °C before determinations.

Human serum samples spiked at three different concentrations (low: 2.5 mg/L; medium: 10 mg/L; high: 25 mg/L) were used to assess the recovery of the method. Four sets of standards were prepared as follows: the first set (Set 1) was prepared in ethanol, as a solvent standard; the second set (Set 2) was prepared in the control human serum after the extraction procedure, as a post-extraction spiked; the third set (Set 3) was prepared in the control human serum before the extraction procedure, as a pre-extraction spiked; and the amounts of analytes in the control serum without standard spiking were also determined as the fourth set (Set 4), which referred to non-spiked. Three replicates were performed for each analysis. The recovery was determined according to the following equation (Eq. 1):

$$\text{Recovery (\%)} = (\text{pre - extraction spiked} - \text{non - spiked}) / (\text{post - extraction spiked} - \text{non - spiked}) \times 100 \quad (1)$$

Method matrix effects were evaluated by comparison of the ratio of the slope between the matrix-matched standard calibration curve and the free-matrix standard calibration curve. In this way, a slope ratio close to 1 indicates that the matrix effect is weaker, but values over or below 1 reflect matrix enhancement or suppression, respectively.

Considering complex biological matrices, especially the serum matrix, six serum samples from different patients with digestive diseases were used to test absolute and relative matrix effects and evaluate the total matrix effects. Absolute matrix effect was calculated to be the average value of the matrix effects from all 6 samples, while the Relative Standard Deviation (RSD) of the matrix effects in all 6 serum samples was expressed as the relative matrix effect, which reflected the variation of matrix effects from different serum samples.

## Statistics

Raw data were extracted and aligned by MassHunter qualitative analysis software (Agilent Technologies) with a tolerance window for *m/z* values set to 20 ppm. One-way Analysis of Variance (ANOVA) was performed to compare the means in different groups using SPSS version 13.0 for statistical analysis. Difference with *p* < 0.05 was considered to be statistically significant. Boxplots and other figures were charted by Origin 8 software.

## Results and discussion

### Optimization of GC–MS

In this study, a rapid and cost-effective GC–MS method was developed to accurately analyze SCFAs in human serum. To

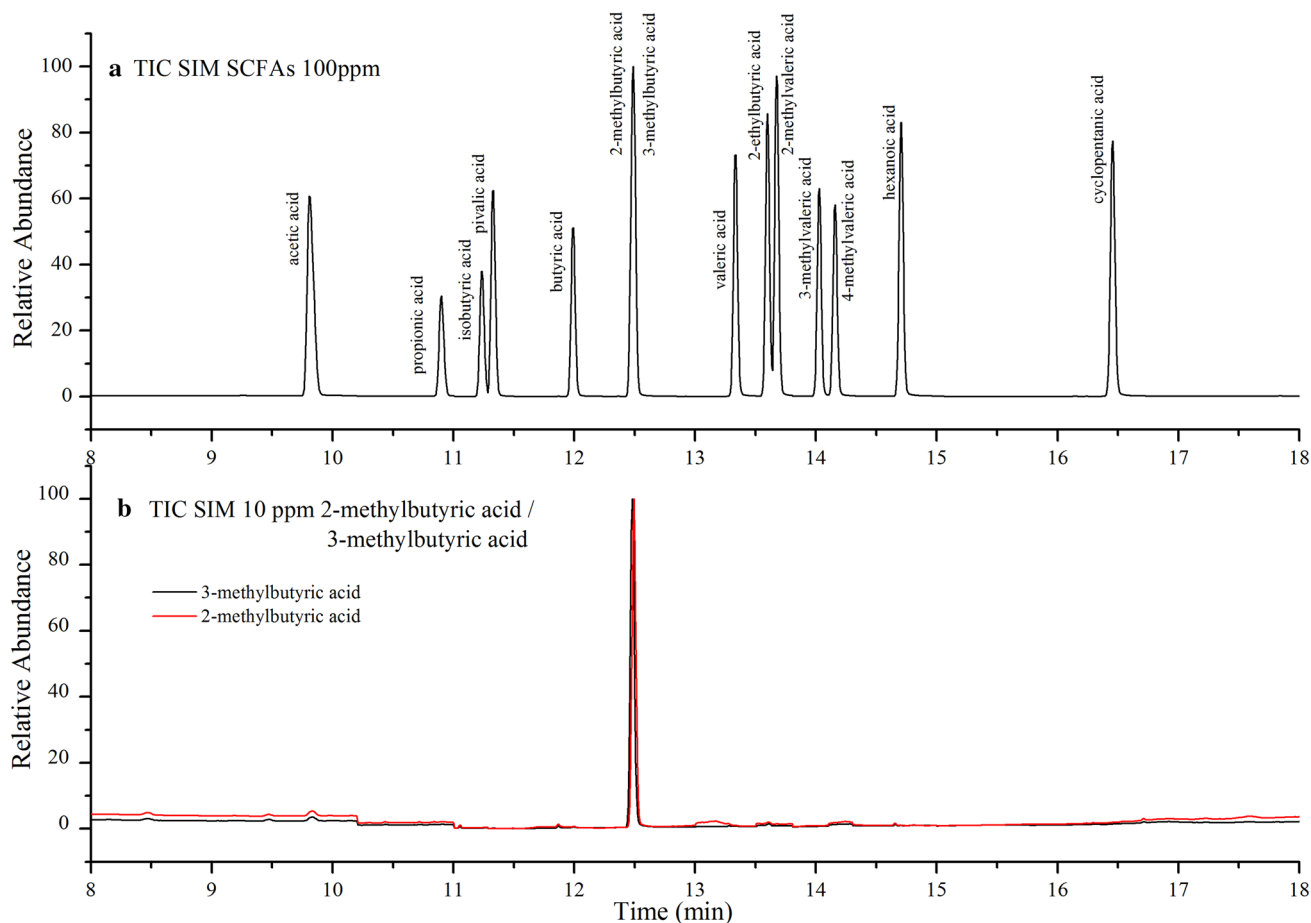
be cost-effective and fast, derivatization steps in the sample preparation were avoided, and the GC–MS method was used to provide effective chromatographic separation and selective quantitative ions with enhanced sensitivity to simultaneously analyze 14 SCFAs, which account for almost all SCFAs and their isomers. Free Fatty Acid Phase (FFAP) columns are Polysiloxanes and Polyethylene Glycol types modified with terephthalic acid (pH modification), which are ideal for free fatty acids separation without any derivatization. In this study, a FFAP column was selected to separate 14 SCFAs using a temperature program ramping from 70 to 200 °C. The assay included two, four and five isomers of C4, C5 and C6 SCFAs, respectively. The TIC profile of the standards was shown in Fig. 1, and a baseline separation was achieved for most of the targets. Complete baseline separation for partial compounds, such as isobutyric acid and pivalic acid, 2-ethylbutyric acid and 2-methylvaleric acid could not be achieved, but their different quantitation ions proved enough to distinguish them (Table 1, Electronic Supplementary Material Figure S1). 2-Methylbutyric acid and 3-methylbutyric acid were eluted completely as one

peak (Fig. 1b), but their quantitation ions were 60 and 74, respectively (Table 1, Electronic Supplementary Material Figure S1).

## Optimization of Liquid–Liquid Extraction

### Efficiency of Different Extraction Solvents

In this work, four extraction solvents, including methanol, ethanol, acetonitrile, and a mixture of acetonitrile and ethanol (v:v = 1:1) were tested to determine the most suitable conditions for detecting and quantifying all 14 SCFAs in the serum by GC–MS simultaneously. The recovery results of the human serum samples spiked at two levels (10 mg/L and 25 mg/L) were shown in Table 2 (and Figure S2). Among the four assays, the recoveries of the straight-chain SCFAs were evidently higher than those of the branched-chain SCFAs, where the average recoveries of the straight-chain SCFAs ranged from 67.5 to 119.5%, and those of the branched-chain SCFAs ranged from 47.9 to 84.9%. Because of its special structure, the solubility of pivalic acid in methanol, ethanol



**Fig. 1** The TIC of standards of 14 SCFAs by GC–MS (a the TIC of mixed standards of 14 SCFAs in the SIM mode; b the TIC of single standards of 2-methylbutyric acid and 3-methylbutyric acid in the SIM mode)

**Table 2** The recovery of SCFAs in human serum spiked at two levels (10 and 25 mg/L) and extracted by methanol, ethanol, acetonitrile and ethanol + acetonitrile

	Methanol	Ethanol	Acetonitrile	Ethanol + acetonitrile
Acetic acid	73.0 ± 19.8	80.0 ± 2.4	80.0 ± 2.8	52.2 ± 3.0
Propionic acid	67.5 ± 6.9	88.8 ± 3.6	75.3 ± 3.7	73.8 ± 9.2
Isobutyric acid	50.2 ± 2.0	75.6 ± 2.7	58.9 ± 3.5	62.8 ± 7.5
Pivalic acid	47.9 ± 1.6	65.3 ± 1.4	49.9 ± 1.9	54.0 ± 5.7
Butyric acid	69.1 ± 6.4	93.4 ± 3.8	75.5 ± 5.1	79.9 ± 11.3
3-Methylbutyric acid	59.4 ± 4.7	79.0 ± 2.9	62.5 ± 4.1	67.3 ± 8.1
2-Methylbutyric acid	56.2 ± 3.9	75.7 ± 2.9	57.2 ± 3.4	64.5 ± 8.0
Valeric acid	72.2 ± 6.3	99.6 ± 3.9	82.6 ± 5.2	84.3 ± 10.5
2-Ethylbutyric acid	56.7 ± 4.1	76.5 ± 2.7	54.3 ± 2.8	65.7 ± 8.1
2-Methylvaleric acid	59.0 ± 3.6	80.6 ± 2.4	61.1 ± 3.1	68.8 ± 7.9
3-Methylvaleric acid	62.8 ± 4.3	84.9 ± 3.3	50.8 ± 5.5	71.8 ± 8.4
4-Methylvaleric acid	54.5 ± 2.1	80.6 ± 1.2	57.6 ± 4.5	66.7 ± 5.8
Hexanoic acid	87.7 ± 8.0	119.5 ± 4.9	99.6 ± 6.4	101.4 ± 12.8
Cyclopentanoic acid	68.8 ± 5.6	94.2 ± 4.5	77.1 ± 4.1	78.9 ± 9.6
Average	63.2 ± 5.7	85.3 ± 3.0	66.5 ± 4.0	67.6 ± 8.3

and acetonitrile was relatively lower. However, pivalic acid was not detected in any human serum samples. It was obvious that ethanol was the best solvent for the 14 SCFAs, as the average recovery in ethanol reached up to 85.3%, much higher than the recoveries in the other solvents (methanol: 63.2%; acetonitrile: 66.5%; acetonitrile + ethanol: 67.6%).

For the fecal samples, water was often used as a solvent to extract SCFAs. As a result, water extracts of biological samples contained many non-volatile compounds, which frequently contaminated the inlet and ion source, affecting MS determination. For serum samples, protein removal was the very first step in the analysis of metabolites, such as SCFAs. Previous study has shown that methanol, ethanol and acetonitrile have commonly been used as protein removal solvents in serum, and that a solvent volume of 3 times the serum volume achieves the best results [24]. In this study, methanol, ethanol and acetonitrile were added at 3 times the serum volume, and the efficiency and efficacy of SCFAs extraction was evaluated. SCFAs, particularly the straight-chain SCFAs (mainly including acetic acid, propionic acid, butyric acid) are able to ionize in polar solvents, such as methanol, which reduces the amount of SCFAs present in the vaporization chamber. The polarity of ethanol is much weaker than that of methanol, which can effectively inhibit the ionization of SCFAs during the process of sample preparation, and thus increase the recoveries of SCFAs. Therefore, ethanol was selected as the extraction solvent for further analysis.

The efficiency and efficacy of several solvents, such as diethyl ether, dichloromethane and ethyl acetate were explored and discussed in extracting SCFAs from fecal samples by García-Villalba [25]. Dichloromethane led to gradual deterioration of chromatographic signals, and to deficient and distorted peak shapes, with significant tailing and loss of

sensitivity; diethyl ether frequently introduced bubbles into the syringe due to its low boiling point and extremely flammable. SCFAs recoveries were as high as 80% and matrix effects were almost negligible using acidified ethyl acetate, but ethyl acetate can be hydrolyzed in strong acid or basic conditions, releasing acetic acid. Therefore, we concluded that these three solvents were not suitable for extracting SCFAs in the biological samples. In this study, all of these solvents were avoided and ethanol was selected to remove proteins and extract SCFAs.

#### Elimination of Interferences by *n*-Hexane

Although the efficiency and efficacy of ethanol-based SCFAs extraction was relatively high, there were still some non-volatile impurities in human serum which contaminated the inlet, chromatographic column, ion source of GC-MS, overloading the system, and resulting in a shorter column life span. *n*-hexane is commonly used to extract non-polar lipids and other lipid-soluble compounds due to its highly non-polar characteristics [26]. In this study, 800 µL of *n*-hexane was added to the serum samples after ethanol to remove some non-volatile interferences. After centrifugation, the protein was deposited at the bottom, while the *n*-hexane and the ethanol-serum mixture were stratified. The *n*-hexane layer (supernatant) was discarded, 100 µL of each ethanol-serum mixture was used for further SCFAs analysis by GC-MS. The human serum samples extracted with/without *n*-hexane were used to compare the contaminations of the GC inlet tube by continuous injected 50 needles. The results showed that there were obvious blank carbonized particles in the inner liner of the inlet with the serum extracted without *n*-hexane, but there was no such phenomenon when using the serum extracted

with *n*-hexane (Electronic Supplementary Material Fig. S3). This demonstrated that *n*-hexane was able to effectively remove some non-volatile interferences and reduce the contaminations of the GC inlet.

To further prove the functionality of *n*-hexane, the Signal-to-Noise Ratio (SNR) of the targets extracted with or without *n*-hexane in human serum samples spiked with 10 mg/L SCFAs were measured and calculated. The results showed that the SNRs of SCFAs extracted with *n*-hexane were almost 1.5 times higher than those extracted without *n*-hexane (Table 3). This demonstrated that *n*-hexane was able to effectively improve signals and reduce noises during the pretreatment of human serum.

The discarded *n*-hexane layer was transferred to another 1.5 mL tube, dried by nitrogen, and redissolved with 100  $\mu$ L methanol to further analysis by UHPLC-HRMS (Supporting Information). The results showed that a lot of lipid-soluble compounds in the *n*-hexane layer of the human serum were eluted and detected by UHPLC-HRMS (Electronic Supplementary Material Fig. S4). It was therefore once again proved, from another perspective, that *n*-hexane was able to effectively remove these non-volatile compounds from the serum samples.

**Table 3** Comparing S/N of 14 SCFAs in human serums spiked 10 mg/L with/without *n*-hexane purification

Compound	S/N of human serum without <i>n</i> -hexane	S/N of human serum with <i>n</i> -hexane	Increased multiples
	* $p=0.004521 < 0.05$		
Acetic acid	130.8	186.2	0.42
Propionic acid	58.1	81.0	0.39
Isobutyric acid	85.6	124.8	0.46
Pivalic acid	99.7	141.7	0.42
Butyric acid	84.3	124.7	0.48
3-Methylbutyric acid	162.4	238.7	0.47
2-Methylbutyric acid	162.4	238.7	0.47
Valeric acid	125.4	186.9	0.49
2-Ethylbutyric acid	154.4	224.7	0.46
2-Methylvaleric acid	174.0	254.3	0.46
3-Methylvaleric acid	105.4	154.3	0.46
4-Methylvaleric acid	95.2	141.4	0.49
Hexanoic acid	126.9	188.3	0.48
Cyclopentanic acid	121.4	177.8	0.46

Correlation is significant at the 0.05 level ( $p < 0.05$ )\*

## Effects of *n*-Hexane on the Recoveries and Matrix

It was also important to determine whether *n*-hexane affected the recoveries of the target compounds while removing matrix interference. Comparing the recoveries of human serum spiked with a 25 mg/L mixture of SCFAs standards, the results showed that *n*-hexane was able to effectively increase the recoveries of SCFAs from human serum, which ranged from 83.0 to 92.7% (Fig. 2a). Since SCFAs are volatile compounds, they volatilize during the extraction process. When *n*-hexane is added, stratification occurs and the *n*-hexane layer is in the upper layer, which effectively prevents loss due to volatilization. Thus, recoveries of all the targets were improved.

The matrix effects of human serum were almost negligible for ethanol extraction with the average value of 83.2%. As for acetic acid and propionic acid, the matrix effects were almost 100% (105.6% and 107.2%, respectively) (Fig. 2b). *N*-hexane therefore slightly reduced matrix inhibition on the target compounds in human serum.

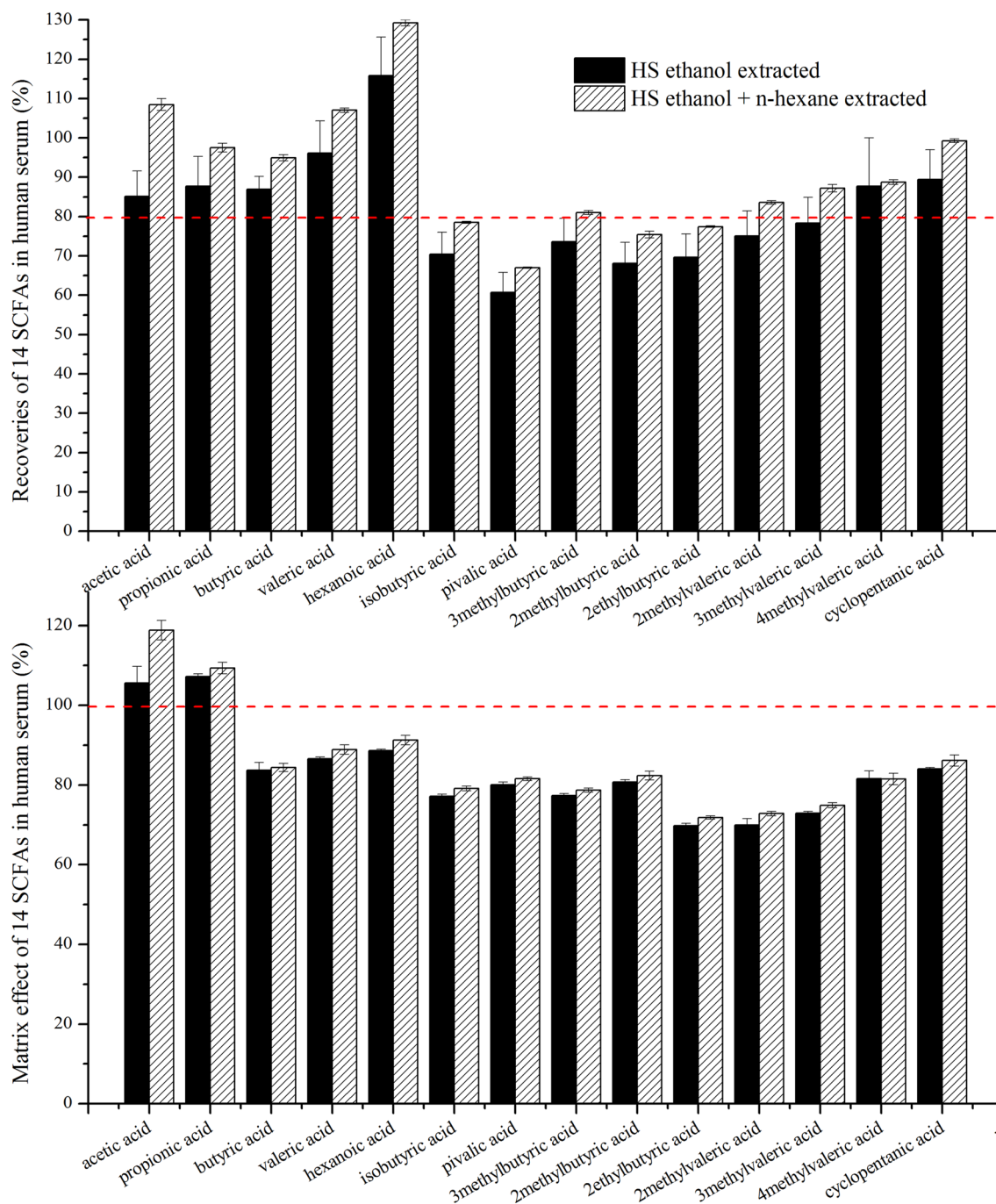
## Method Validation

Calibration curves were constructed using the peak areas of the 14 SCFAs standards using quantitation ion peaks, and the linear range was shown in Table 1. The linearity was acceptable for all the SCFAs over the whole range of concentrations, as proved by the correlation coefficients ( $R$ ) being over 0.99 for all curves.

Limit of Detection (LOD), limit of Quantification (LOQ) were assessed over the detection ranges of the analytical method. The LODs and the LOQs of the SCFAs were 0.12–0.48 mg/L and 0.40–1.61 mg/L, respectively, which were sensitive enough to detect all the SCFAs in the serum samples. As expected, all solvent blanks were below the LODs of all analytes. Overall, the recoveries at three different concentrations (2.5 mg/L, 10 mg/L, 25 mg/L) ranged from 62.4 to 129.2% in the human serum samples (Fig. 3 and Table 4), which indicated that the analysis of the 14 SCFAs was accurate and reliable.

The precision were assessed using QC samples at low (2.5 mg/L), medium (10 mg/L) and high (25 mg/L) levels, and the results are summarized in Table 4. Precision was calculated as the Relative Standard Deviation (RSD) of triplicate injections in 1 day or three consecutive days. All intra-day and inter-day variations were less than 10%, with intra-day being 7.2–9.1% and inter-day being 8.2–9.8%.

Based on the evaluation method of matrix effects, Table 4 showed that a weak and tolerable absolute matrix effect of the 14 SCFAs was observed in the 6 different serum samples with the average slope ratio value ranging from 0.72 to 0.96. The relative matrix effect is often evaluated for complex bio-analytical matrices, and is required



**Fig. 2** Effect of *n*-hexane addition on recoveries and matrix effects of 14 SCFAs in human serum (a the recoveries of 14 SCFAs in human serums spiked 25 mg/L; b the matrix effects of 14 SCFAs in human serums spiked 25 mg/L)

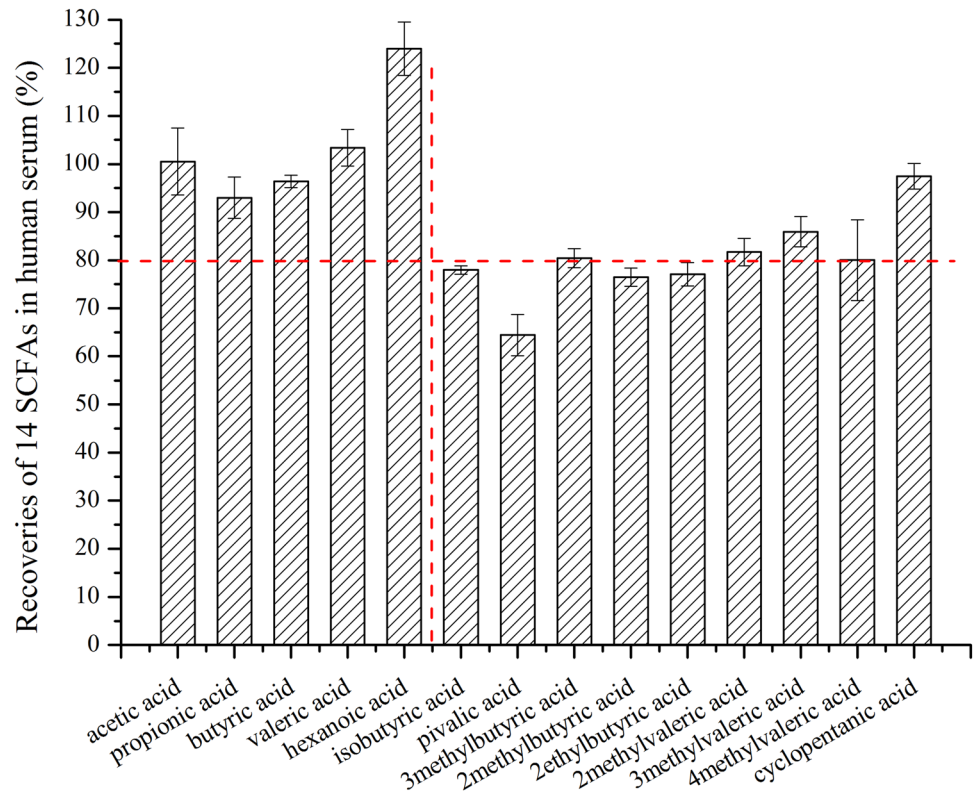
to be no greater than 15%. In this study, a weak relative matrix effect was also found in the 6 serum samples with standard deviations of absolute matrix effects of ranging from 1.0 to 3.2%, indicating that this method could be applied to human serum matrices using a free-matrix standard calibration curve even if no one-to-one internal standard was available.

### Method Comparison

Compared to previously studies for the SCFAs analysis, the methods with derivatization are able to achieve lower LOD and LOQ (LOQ for acetic acid is 0.013  $\mu\text{g/L}$ ) [27]. However, additional steps prolong the time needed for analysis; they may lead to potential SCFAs losses and increase



**Fig. 3** The average recoveries of 14 SCFAs in human serum spiked at three different concentrations (low: 2.5 mg/L, middle: 10 mg/L, and high: 25 mg/L; HS represents human serum) extracted with ethanol + *n*-hexane



**Table 4** Validation GC–MS analytical method. Recoveries, intra-day and inter-day precision (% CV) at low (2.5 mg/L), medium (10 mg/L), and high (25 mg/L) levels used for the validation, matrix effect at medium level

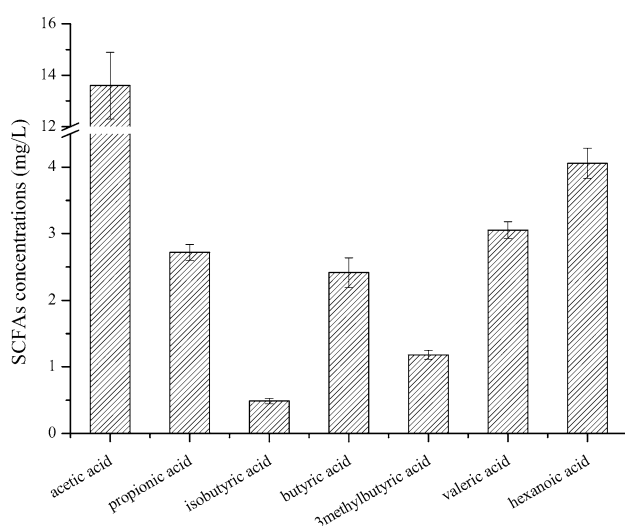
Compounds	LOD (mg/L)	LOQ (mg/L)	Matrix effects (ME)		Recoveries % (n=3)			Intra-day CV % n=3 Low/medium/ high	Inter-day CV % n=3 Low/medium/ high
			Absolute ME	Relative ME %	Low	Medium	High		
Acetic acid	0.18	0.60	0.87	2.2	95.5±5.1	97.6±8.1	108.4±6.7	7.5/7.2/7.9	8.7/8.6/8.9
Propionic acid	0.22	0.75	0.83	1.6	89.0±0.0	92.4±7.8	97.5±9.8	8.1/8.1/8.7	8.5/8.7/9.0
Isobutyric acid	0.33	1.09	0.83	1.2	76.9±1.9	78.4±5.6	78.5±8.0	8.4/7.9/7.5	8.8/8.9/8.9
Pivalic acid	0.19	0.65	0.72	1.0	62.4±2.1	66.7±4.4	67.0±6.8	8.9/8.5/8.7	8.9/8.8/9.1
Butyric acid	0.14	0.47	0.84	1.3	96.9±1.5	97.3±8.8	94.9±9.7	7.5/7.7/7.2	8.4/8.8/8.9
3-Methylbutyric acid	0.19	0.64	0.82	1.5	78.2±2.2	82.0±5.8	81.0±8.5	8.5/8.7/8.2	8.8/8.7/9.0
2-Methylbutyric acid	0.19	0.64	0.82	1.4	75.2±2.0	78.6±5.5	75.4±8.0	8.9/8.3/8.8	9.0/9.1/8.6
Valeric acid	0.12	0.40	0.89	1.7	99.4±1.5	103.6±8.2	107.0±10.4	7.9/7.4/8.1	8.2/8.5/8.9
2-Ethylbutyric acid	0.18	0.60	0.83	1.3	74.4±1.8	79.3±6.3	77.4±8.0	9.1/8.6/8.8	9.0/9.1/8.7
2-Methylvaleric acid	0.22	0.74	0.86	1.2	78.4±1.5	83.0±6.3	83.5±9.8	9.1/8.9/8.3	9.8/9.3/9.6
3-Methylvaleric acid	0.48	1.61	0.87	1.5	82.3±1.9	88.2±6.8	87.2±8.8	8.2/7.9/8.3	8.4/8.8/8.7
4-Methylvaleric acid	0.16	0.52	0.84	3.2	71.9±5.8	79.3±4.2	88.7±6.0	8.0/7.7/7.5	8.5/8.3/8.8
Hexanoic acid	0.16	0.53	0.93	2.3	118.1±2.1	124.5±10.6	129.2±12.7	7.9/8.2/7.7	8.3/8.6/8.7
Cyclopentanic acid	0.45	1.52	0.96	2.1	94.3±2.1	98.7±8.3	99.2±9.9	8.0/8.3/7.7	8.4/8.7/8.5

consumption. In contrast, LOD and LOQ are relatively high in analytical methods without derivatization, e.g., LOD and LOQ of acetic acid are 0.26 and 0.86 mg/L, respectively [14]. In our study, LOD and LOQ of the SCFAs are relatively lower (LOD and LOQ of acetic acid are 0.18 and 0.60 mg/L, respectively) and are sensitive enough to determine the several detected SCFAs in serum samples. Therefore, this method gives comparable analytical results to previously reported techniques and can afford relatively high sensitivity and selectivity, good quantification extraction efficiency, and reduce operation procedure to the other techniques.

## Application of the SCFAs Method

### Application in Human Serum

The SCFAs analytical method was used to detect these metabolites in human serum samples. The TIC of the SCFAs in human serum samples were shown in Electronic Supplementary Material Fig. S5. By comparison with the corresponding standards, 14 SCFAs were determined, and only 7 SCFAs, including acetic acid, propionic acid, isobutyric acid, butyric acid, 3-methylbutyric acid (isovaleric acid), valeric acid, and hexanoic acid, were detected in the human serum samples. Figure 4 showed that the concentrations of the straight-chain SCFAs were evidently higher than those of the branched-chain SCFAs in the human serum samples. The concentrations of acetic acid were the highest, reaching 13.6 mg/L in the human serum samples. As bacterial fermentation end product concentrations depend greatly on the gut microbiota profile, they have high individual variance and are strongly influenced by diet. According to



**Fig. 4** The concentrations of the detected SCFAs in human serum samples

the literature, the serum from different donors appears to have different concentrations of SCFAs (about 80  $\mu\text{mol/L}$ , 4.8 mg/L), which seems to be characteristic for each person and only varies slightly in different samples collected from the same individual at different time points [28].

The SCFAs analytical method was applied to probe the changes of these metabolites in serum samples among healthy children, which contained 21 boys and 24 girls aged from 0 to 18, the results of which were shown in Fig. 5. Among these detected SCFAs, the concentrations of acetic acid were the highest, ranging from 4.5 to 8.7 mg/L. The second highest were for propionic acid, at concentrations ranging from 2.2 to 2.5 mg/L. The average values of butyric acid, valeric acid and hexanoic acid were 2.0, 2.5, and 3.3 mg/L, respectively. The concentrations of the two branched-chain fatty acids, including isobutyric acid and isovaleric acid, were the lowest, at 0.4 and 0.8 mg/L, respectively.

One interesting observation was that the concentrations of acetic acid, propionic acid decreased with age, and that the acetic acid and propionic acid concentrations in the serum samples of girls were considerably higher than those of boys at the same ages (Fig. 5). Acetic acid is the main product of intestinal flora fermentation and reflects microbial activity, after transporting across the gut epithelium; it can be detected in portal, hepatic and peripheral blood. Normally, about 70% of the acetic acid absorbed from the gut reaches the systemic circulation, where it is used throughout the body as an energy source, as a substrate for cholesterol biosynthesis and during protein acetylating, an important epigenetic process. Propionic acid is largely cleared by the liver, and the majority of butyric acid is used as an energy source by the intestinal mucosa [29, 30].

### Application in Serum of Digestive Diseases Patients

Finally, the SCFAs analytical method was applied to assess changes in SCFAs between children with digestive diseases, mainly including inflammatory bowel diseases and diarrheal disorders, and healthy volunteers. The results of the box diagram (all samples were counted regardless of age) showed that the concentrations of SCFAs in the serums of diseased children were significantly higher than those of healthy children (Fig. 6). Comparing by age group, SCFAs in the serums of diseased children were also significantly higher than those of healthy children in the same age group, especially acetic acid (healthy girls: average value 6.5 mg/L; healthy boys: 5.1 mg/L; digestive diseases girls: 9.2 mg/L; digestive diseases boys: 8.6 mg/L) and propionic acid (healthy girls: 2.3 mg/L; healthy boys: 2.2 mg/L; digestive diseases girls: 2.4 mg/L; digestive diseases boys: 2.4 mg/L) (Fig. 5).

Digestive diseases are usually caused by intestinal flora disorders, and lead to changes in their products, such as SCFAs. Bian et al. reported that SCFAs in the serums of

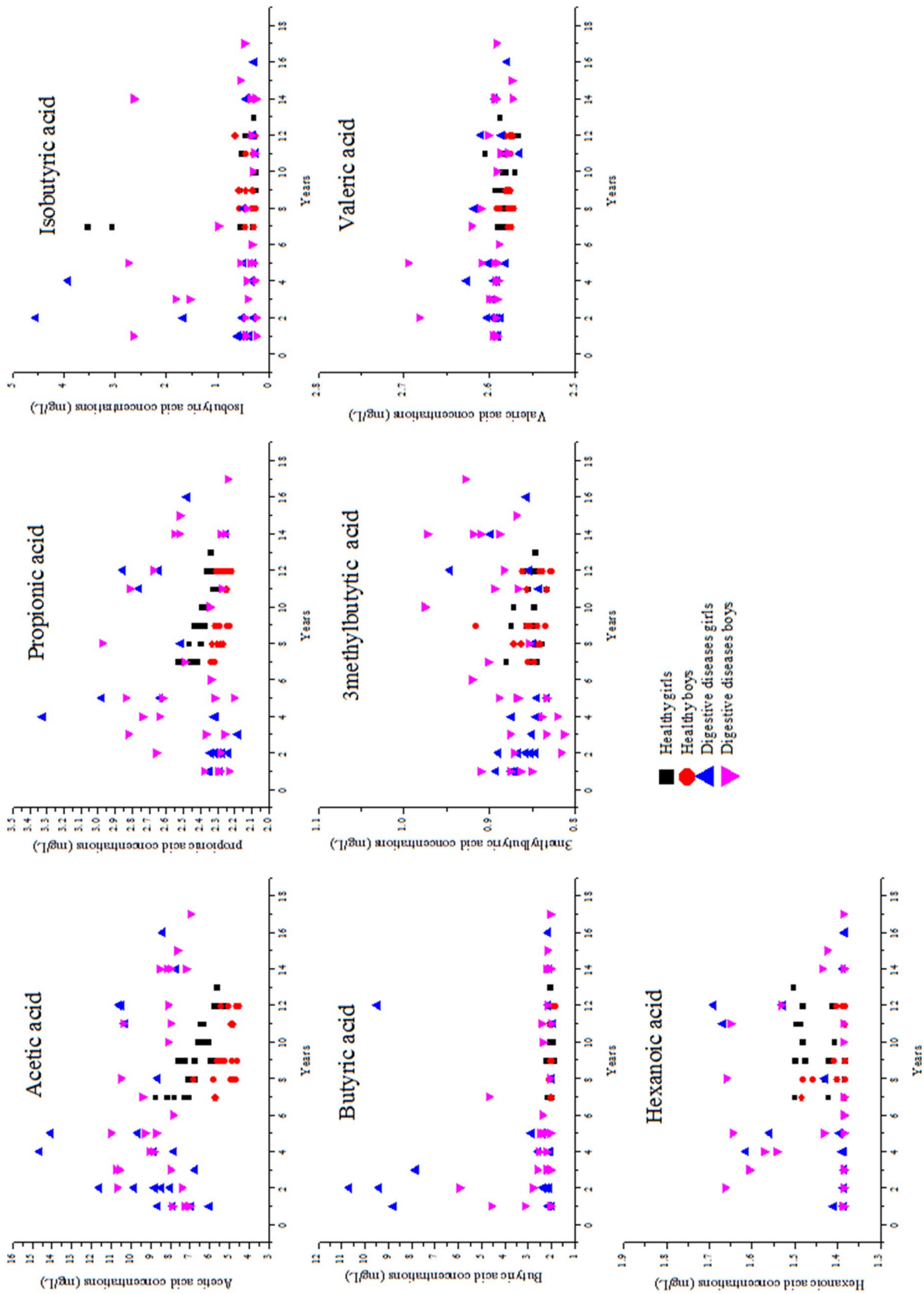
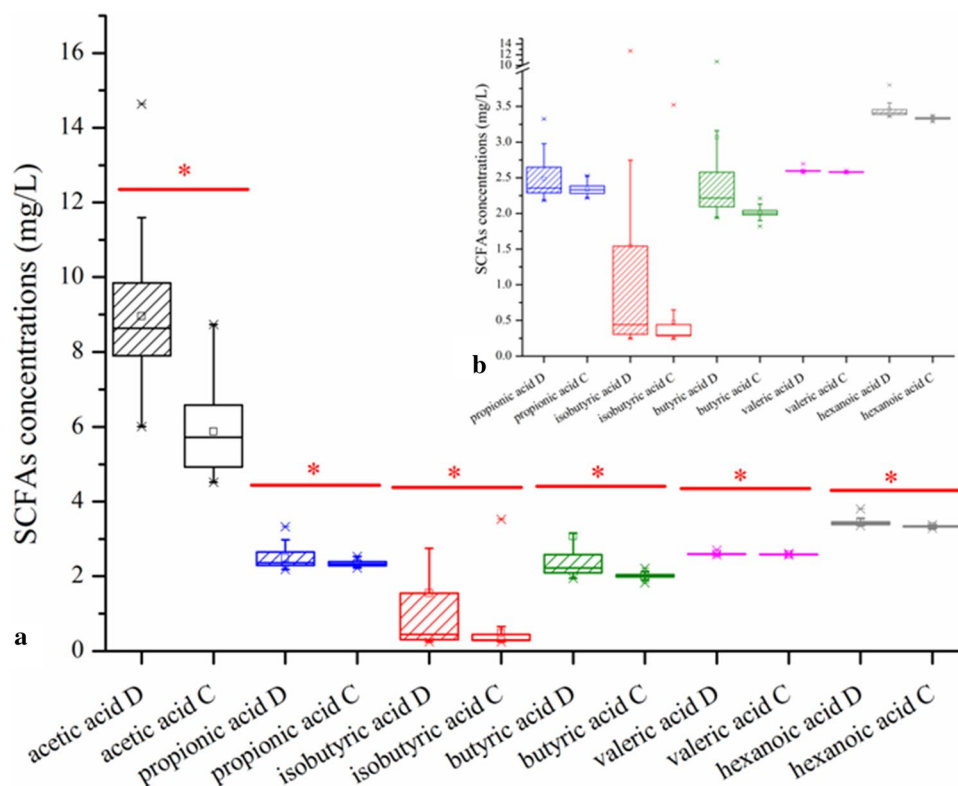


Fig. 5 Distribution of SCFAs with age in serum of children with health and digestive diseases (scatter plot)

**Fig. 6** Comparison of SCFAs in serum of children with health and digestive diseases (*D* represents digestive diseases assay, and *C* represents control assay, asterisk represents significant differences between the digestive diseases assay (*D*) and the control assay (*C*), which  $p$  value  $\leq 0.05$ . Among the box plots, box ranges from 25 to 75%, and – represents the max and min values,  $\times$  represents the 99% and 1% values, open square represents the average value)



colorectal cancer patients were evidently lower compared to normal controls [31]. In this study, SCFAs in the serums of patients suffering from digestive diseases were significantly higher than those of normal controls. These results demonstrated that SCFAs may be potential biomarkers in the diagnosis of some diseases. Several studies have indicated that SCFAs might play key roles in the prevention and treatment of metabolic syndromes, such as bowel disorders and certain types of cancer. In clinical studies, administration of SCFAs has positively influenced the treatment of ulcerative colitis, Crohn's disease, and antibiotic-associated diarrhea [6, 32]. However, the confirmation of these potential biomarkers and the underlying reasons for this relationship needs to be further studied by increasing sample sizes and by carrying out animal experiments.

## Conclusion

This work has established a fast and accurate way to determine 14 SCFAs in human serum samples by GC–MS. A mixture of ethanol and *n*-hexane was selected as the most suitable extraction solvent, with recoveries ranging from 62.4 to 129.2% in human serum samples. The LODs and the LOQs of the SCFAs were 0.12–0.48 mg/L and 0.40–1.61 mg/L, respectively, which were sensitive enough to detect all the SCFAs in the serum samples. The weak and tolerable absolute and relative matrix effects on the 14

SCFAs were observed and characterized in different serum samples. The application of this method was carried out by comparing SCFAs in the serums of healthy children to those of children suffering from digestive diseases by age group, and the results demonstrated that the concentrations of acetic acid were the highest (about 13.6 mg/L) in human serum samples. The concentrations of acetic acid and propionic acid in the serums of girls were significantly higher than those of boys at the same age. Additionally, the concentrations of SCFAs in the serums of diseased children were significantly higher than those of healthy children, the reasons for which merit further study.

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## Compliance with Ethical Standards

**Conflict of interest** All authors have been informed of the full content and approved the publication of this manuscript, and all authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in the study involving human participants were in accordance with the ethical standards of Beijing Children's Hospital and Beijing CDC research committee and

with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards (case number: 2016-10).

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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