RESEARCH PAPER

A highly sensitive, simple, and fast gas chromatography–mass spectrometry method for the quantification of serum short-chain fatty acids and their potential features in central obesity

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Abstract

Short-chain fatty acids (SCFAs) are the main gut microbe metabolites, which have no more than six carbons. SCFAs are an emerging biomarker in metabolic diseases, including central obesity. Commonly, SCFAs are measured in fecal samples, where they are highly abundant, but here they do not reflect direct interactions with related organs. Serum SCFAs are assumed to be more associated with metabolic disease than fecal SCFAs, albeit at very low concentrations. The aim of the present study is to develop a highly sensitive, simple, and fast method for measuring six SCFAs in the serum by gas chromatography–mass spectrometry (GCMS). The serum is mixed with meta-phosphoric acid and 2,2-dimethylbutyric acid, followed by homogenization and centrifugation. Supernatant is then injected into the fused silica capillary column. The method is linear from 0.12–500 μmol/L for all SCFAs with an accuracy of 90–117%. The total coefficient of variation for precision ranges from 3.8 to 14.1%. A preliminary study is performed with 32 centrally obese subjects and 17 lean subjects. The mean values of all SCFAs, including acetic, propionic, isobutyric, butyric, isovaleric, and valeric acid, in the centrally obese subjects are significantly higher compared with lean subjects. A significant correlation also exists between all SCFAs, with the waist circumference indicating that serum SCFAs have potential features with respect to metabolic diseases, especially central obesity. The validated GCMS method provides highly sensitive, fast, simple, and reliable SCFA quantitation in the serum and demonstrates the potential features of circulating SCFAs in central obesity.

Keywords Short-chain fatty acids · Gas chromatography · Central obesity · Method development · Protein precipitation · Mass spectrometry

Introduction

Short-chain fatty acids (SCFAs) are carboxylic acids which have no more than six carbons [1]. The carbon tail is not only aliphatic chain such as acetic acid, propionic acid, and butyric acid, but also branched chain. Branched short-chain fatty acid mainly contains isobutyric and isovaleric acids [2]. The chemical structure of SCFAs is shown in Fig. 1. SCFAs are the end products of dietary fiber fermentation by gut microbiota and have been shown to exert multiple beneficial effects on the human metabolism [3]. The mechanisms underlying these effects are the subject of intensive research and encompass the complex interplay between diet, gut microbiota, and host energy metabolism [4]. SCFAs also directly modulate host health through a range of tissue-specific mechanisms related to gut barrier function, glucose homeostasis, immunomodulation, and appetite and obesity regulation [5]. There are several ways of changing SCFA bioavailability in the gut, such as probiotics [6], antibiotics [7], and diets with different fiber contents [8]. These methods allow us to investigate the role of SCFAs in certain disease models, especially metabolic diseases such as central obesity.

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Fig. 1 Chemical structures of SCFAs (created in PubChem Sketcher V2.4)
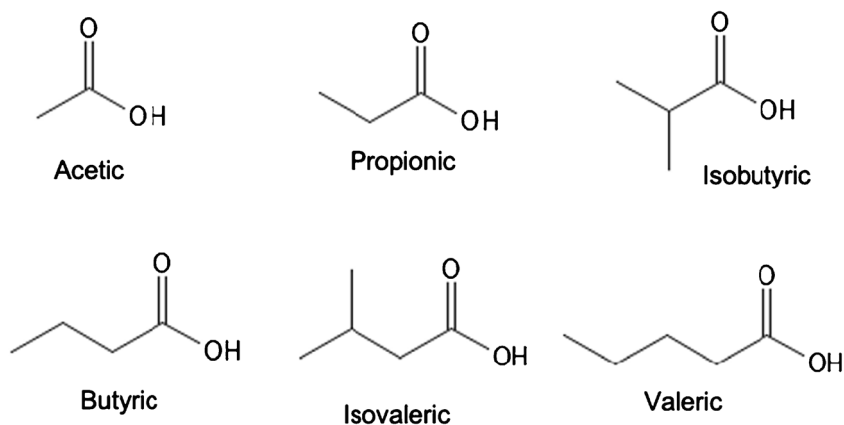
SCFAs are an emerging biomarker in metabolic diseases. A high concentration of SCFAs in fecal matter is associated with central obesity, hypertension, low-grade chronic inflammation, and dyslipidemia. The underlying mechanism begins with gut microbiota dysbiosis, which is caused by both an imbalance in SCFA production and gut permeability alteration [9]. The high SCFA concentration can act as an overnutrition signal to the host metabolism. This signal can be meaningful because SCFAs contributed up to 10% human energy requirement [10]. SCFA concentration in the gut varies from 20 to 140 mmol/L [11]. These levels of concentration are quite high but there was still a gap about how SCFAs produced in the gut can modulate or influence the tissue metabolism outside the gut. Moreover, there was an evidence that fecal SCFAs may not accurately reflect in vivo colonic fermentation since approximately 95% of colonic SCFA are absorbed and only the remaining 5% are excreted in feces [4]. To further understand the distribution and function of SCFAs in organs and tissues, it is vital to accurately measure blood levels.

Recent studies show that SCFA concentration in the blood, especially in the serum or plasma, varies according to type, ranging from 1.3 to 220 μmol/L in healthy human subjects [11]. The concentration SCFAs in the blood are in micromolar level that is considered much lower than their concentration in feces. Moreover, the turnover rate of SCFAs in the blood is rapid, resulting in low but dynamic concentrations [12]. Although serum SCFA concentration is at low levels, it is assumed to be associated with metabolic disease because serum SCFAs directly interact with target tissue and organs through their receptors. Gas chromatography–mass spectrometry (GCMS) is the most common analytical method to measure SCFA concentration in biological samples, including fecal and serum [13]. Therefore, the aim of present study is to develop a high-sensitive, simple, and fast GCMS method for serum SCFA quantification and investigate the potential features of serum SCFAs in central obesity as an emerging biomarker.

Methods

Chemicals, reagents, and solutions

Volatile free acid mix SCFA standard CRM49675 (Supelco, Darmstadt, Germany), 2,2-dimethylbutyric acid as an internal standard (Aldrich, Darmstadt, Germany), meta-phosphoric acid (Merck, Darmstadt, Germany), isopropanol liquid chromatography grade (Merck, Darmstadt, Germany), hydrochloric acid (Merck, Darmstadt, Germany), and ultrapure water obtained from PURELAB Ultra (ELGA, UK).

Working stock of ten levels of standard was prepared in isopropanol with concentration ranged from 0.7 to 3000 μmol/L. The internal standard working stock was prepared in ultrapure water with concentrations of 1000 and 120 μg/mL, the former of which was used to obtain a standard diluent, and the latter of which was used to obtain the sample precipitant. The standard diluent was prepared from isopropanol, hydrochloric acid (0.375 N), 2,2-dimethylbutyric acid (120 μg/mL), and ultrapure water. The sample precipitant was prepared from meta-phosphoric acid (0.5 g/L in water) and 2,2-dimethylbutyric acid (1000 μg/mL). The calibration standards were prepared from 50 μL each level of serial diluted working stock added with 250 μL standard diluent to obtain final concentration ranged 0.12–500 μmol/L. All working solutions were freshly prepared for each batch.

Sample preparation

Sample preparation consisted of mixing 20 μL of the precipitant with 180 μL of the serum to precipitate protein. The mixture was then homogenized with a vortex mixer for 2 min and centrifuged at 14000 RPM for 10 min; after which, 100 μL of the clear supernatant was then transferred to a new tube and 100 μL of isopropanol was added. The mixture was then homogenized and transferred to the GCMS sample vial (Agilent Technologies, Santa Clara, USA) with 1 μL injected for analysis.
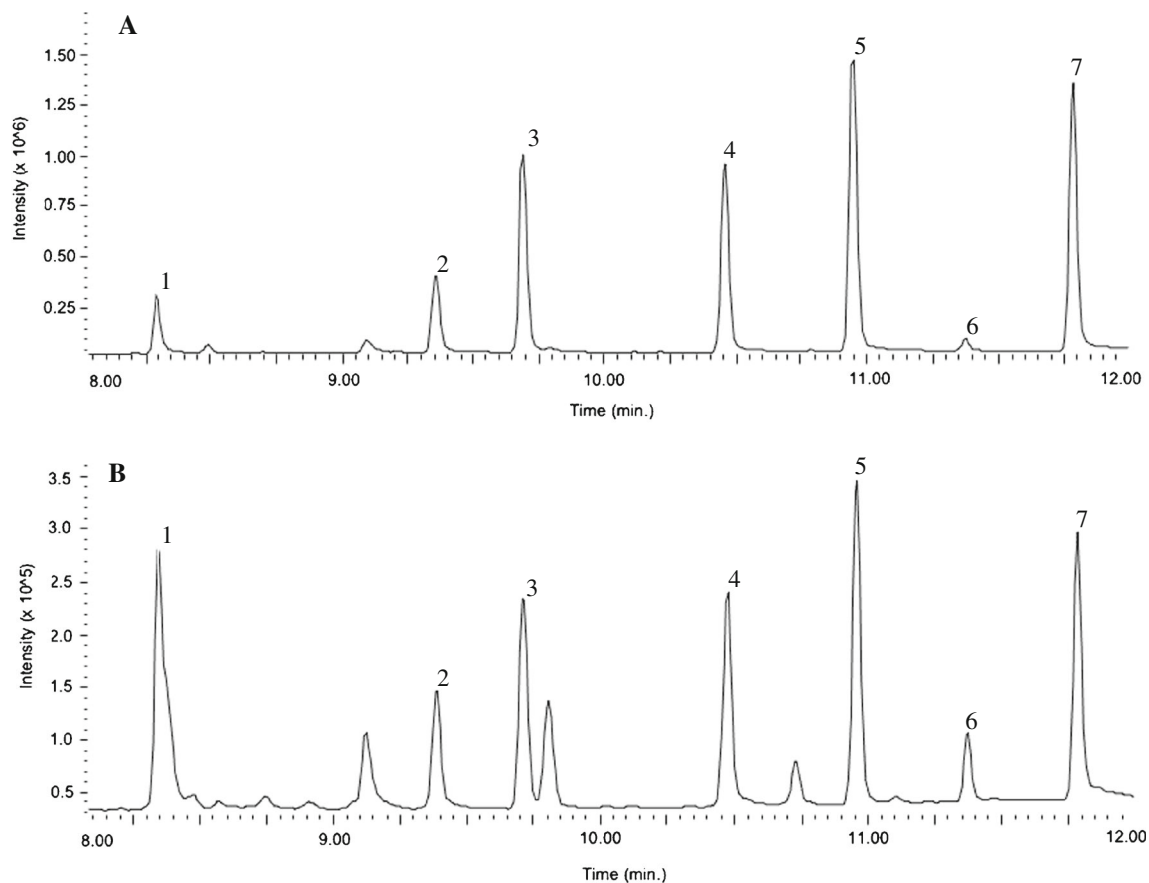
Fig. 2 **A** Typical chromatogram of identified short-chain fatty acids of standard material and **B** spiked serum sample with 31.2 μmol/L of the standard material ((1) acetic acid, (2) propionic acid, (3) isobutyric acid, (4) butyric acid, (5) isovaleric acid, (6) 2,2-dimethylbutyric acid, and (7) valeric acid))

Gas chromatography–mass spectrometry development and validation

This method was developed on a Shimadzu QP2030NX GCMS (Shimadzu Corporation, Japan) equipped with the LabSolutions Insight software for data processing. For the GC oven, the temperature was increased from 60 to 180 °C at a rate of 10 °C/min and hold for 12 min for cleaning process prior to next injection. The gas flow rate was set to 2.29 mL/min, pressure was set to 20 psi, and average velocity was read equal to 55.42 cm/s. GC was set to the constant-pressure mode. The MS detector was set to full-scan mode with a mass range of 40–150 m/z. Ion-source temperature and interface temperature were both set to 200 °C. Detector voltage was set to relative to the tuning result. The standards and samples were injected into a Nukol-fused silica capillary column (30 m long with a diameter of 0.25 mm; Supelco, Germany). The total run time was 25 min, but complete SCFA separation was achieved in less than 12 min. SCFA identification was achieved after injection using the National Institute of Standard and Technology (NIST) library search. All SCFAs were easily identified as a result of good separation.

Linearity was examined by injecting 10 levels of standard calibration in every batch. Standard curve linearity was automatically determined by the software. Curve fitting was selected to quadratic with the origin set to force through without curve weighting. Accuracy was evaluated using multipoint recovery testing from 125 to 7.8 μmol/L for acetic acid, 125 to 3.9 μmol/L for propionic acid, and 125 to 1 μmol/L for butyric, isobutyric, valeric, and isovaleric acids. In brief, 10 μL of standard that known in concentration was added to 170 μL serum and followed the sample preparation procedure. Recovery percentage was obtained from calculating the observed and expected concentrations. Precision was evaluated by sequential pooled serum injection twice a day from three different batches to obtained total six data. Serum from 32 centrally obese men and 17 lean men was injected into the GCMS after the method was developed and validated. Statistical analysis was conducted using SPSS version 24 for Windows.

Results

All of SCFAs in standard and also in sample was separated in less than 12 min and separation happen in gradient-temperature condition with the developed GCMS method. Good GC
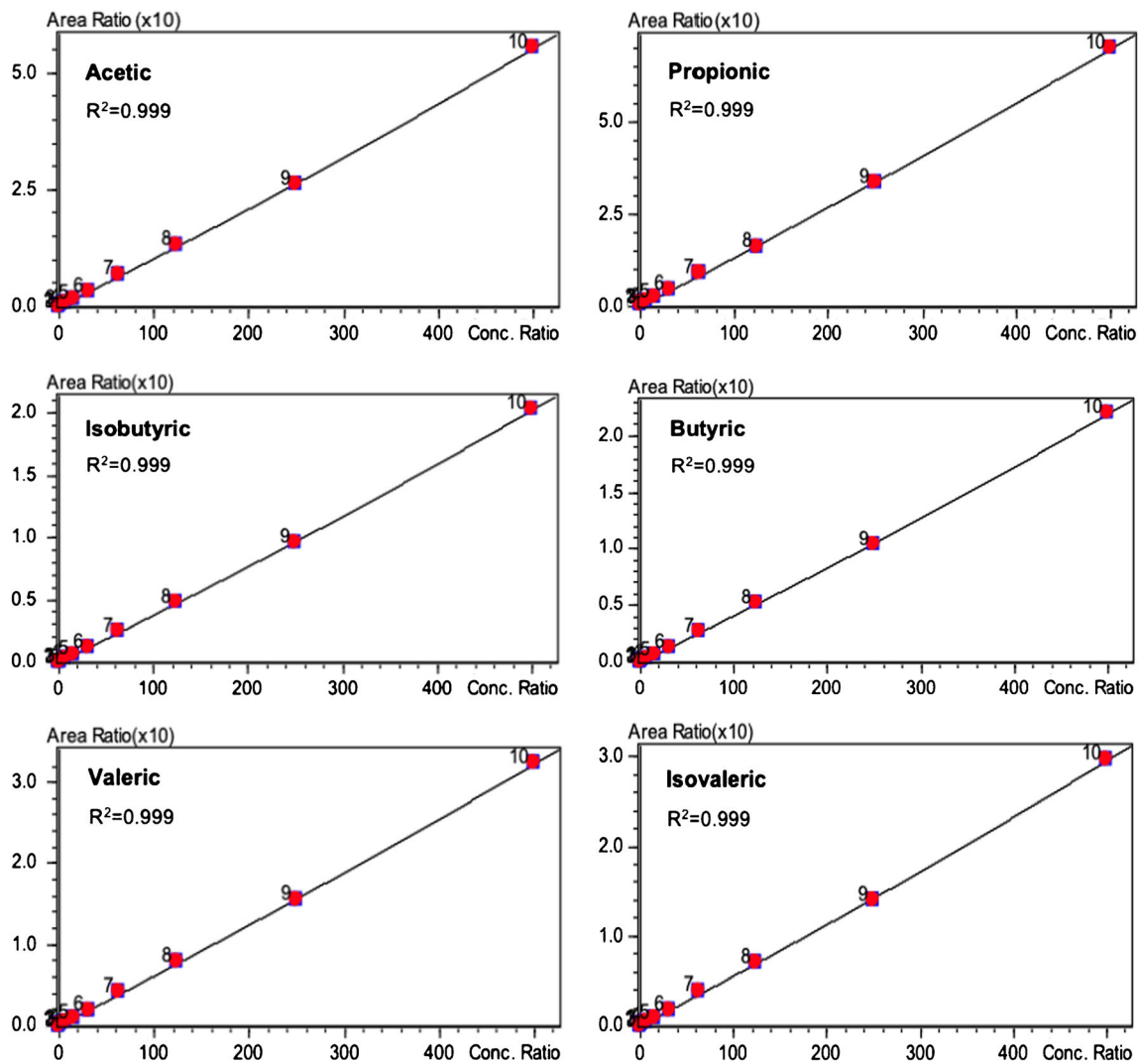
Fig. 3 Calibration curves of quantified short-chain fatty acids

separation is required for further method development and validation. Accordingly, SCFA identification was completed after GCMS separation using the NIST library search; the SCFA list was obtained based on their retention time and also in spiked serum sample (Fig. 2). The dominant ions of each SCFA were selected for quantifier ion. Two different ions of each SCFA were also automatically selected as qualifier ions to ensure that the identified SCFAs always show the consistent ion being both a quantifier and qualifier. The standard material consisted of 10 SCFA types, but we did not quantify isocaproic, caproic, and heptanoic acids, because they had extremely low concentrations in the serum samples. Sample preparation was conducted by adding meta-phosphoric acid to the serum sample to precipitate protein, which was then diluted with isopropanol. This sample preparation method was very simple, applicable in daily application, and also contributed in sensitivity because the total dilution was only 2.2 times.

Linearity was assessed for 10 levels of calibration. The calibration curves were constructed using post-run software and analyzed using the LabSolutions Insight software to obtain the regression coefficient. All analyzed SCFAs show good linearity for 10 levels of calibration, ranging from 500 to 0.12 μmol/L (Fig. 3). Moreover, the regression coefficient is larger than 0.995 for all SCFAs. Accuracy assessment was conducted through recovery testing. Multilevel recovery was conducted for every SCFA to monitor the accuracy of each representative concentration. Recovery testing was also conducted, which is useful for matrix effect monitoring due to sample extraction. In this method, SCFA recovery ranged from 80 to 117% (Table 1). Precision was evaluated from the double analysis of the pooled serum sample in three different batches. All SCFAs have an excellent coefficient of variation (CV; Table 2).

The data of serum SCFA concentration in 32 centrally obese men and 17 lean men were obtained after the GCMS method was developed and validated. Table 3 compares the SCFA concentration between lean and

Table 1 Recovery test results for all short-chain fatty acids

SCFAs	Concentration (μmol/L)		Recovery (%)
	Expected	Observed	
Acetic acid	7.8	7.3	93
	15.6	18.3	117
	31.3	33.5	107
	62.5	70.6	113
	125	141.5	113
Propionic acid	3.9	4.6	117
	7.8	8.8	112
	15.6	18.3	117
	31.3	36.2	116
	62.5	69.9	112
	125	139.7	112
Isobutyric acid	1	1.1	110
	3.9	4.4	112
	7.8	7.9	101
	15.6	15.2	98
	31.3	31.6	101
	62.5	63.8	102
	125	125.3	100
Butyric acid	1	0.9	97
	3.9	3.9	99
	7.8	7.9	101
	15.6	16.1	103
	31.3	32	102
	62.5	64.8	104
	125	130.7	105
Isovaleric acid	1	1	105
	3.9	4	101
	7.8	7.2	93
	15.6	14	90
	31.3	28.6	92
	62.5	57.5	92
	125	114.8	92
Valeric acid	1	0.8	80
	3.9	3.5	91
	7.8	7.1	90
	15.6	14.1	90
	31.3	29.2	94
	62.5	58.6	94
	125	118.5	95

Table 2 Precision results of all short-chain fatty acids

SCFAs	Mean (μmol/L)	Total CV (%)
Acetic acid	175.5	3.8
Propionic acid	11.6	11.9
Isobutyric acid	4.0	14.1
Butyric acid	2.7	8.4
Isovaleric acid	4.9	8.3
Valeric acid	1.3	12.1

Discussion

The GCMS method was selected based on the chemical properties of SCFAs: chemically polar and volatile compounds [14]. The basic sample requirements are thermal stability and appreciable vapor pressure at the column temperature, as this allows the sample components to vaporize and move with the gaseous mobile phase. GCMS is commonly used as a quantification method for volatile compounds as well as for those that can be derivatized into volatile compounds [15]. In this study, the serum SCFA sample did not undergo a derivatization process. This means that the proposed method is very simple and routine. Although derivatization can improve the selectivity and sensitivity, it also causes some problems, such as complex sample preparation and concentration uncertainty due to the addition of a derivatizing agent [16]. Several analytical methods have been developed for SCFA quantification in various matrices. Besides gas chromatography, high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and also capillary electrophoresis are typically SCFA quantification method [17]. In the last few years, HPLC coupled to tandem mass spectrometry (LC-MS/MS) was increasingly applied to quantified SCFAs. It reduced the analysis time of 45–60 min required from GCMS to as short as 15 min or less [18]. But compared to that of the LC-MS/MS method, our developed GCMS method offers the equal analysis time.

In this study, GCMS was developed using the temperature gradient approach. Theoretically, the temperature gradient for GC follows the same principle as gradient elution in liquid chromatography, where the elution composition changes over the time. The gradient temperature program of the GC oven increased the analytical speed and also improved the resolution of analytes in the column. In general, the chromatographer usually has two competing concerns with regard to GC separation: resolution and separation speed. Resolution is classically recognized as being a function of the selectivity of the column for the analytes in the sample and the efficiency of the column [19, 20]. Accordingly, SCFA separation was achieved within the temperature gradient in less than 15 min, and, as such, the separation was optimal and useful for

centrally obese men. In general, all SCFAs show a higher concentration in centrally obese men than in lean men. Correlation data were also obtained in this study, in which all of SCFAs show positive correlation with the waist circumference (Table 4).

compound identification and rapid for GCMS analysis. Because of the optimal separation, every single SCFA is separated from each other and also interfering compounds especially in complex sample such as serum. Indeed, the separated SCFAs ensured that mass spectrum identification was easy. Compound identification in GCMS is currently achieved by comparing a query mass spectrum with a reference mass spectrum in a library via spectrum matching. In this method, we used the NIST library, which is commonly used and already embedded with the GCMS software [21]. Briefly, after we obtained the chromatogram, we conducted a similarity search and identified compounds that were automatically annotated, including their retention time. After the identification process, three dominant ions from each SCFA mass spectrum were selected for quantifier and qualifier ions, which were used for quantification.

The optimized sample preparation in this study involved using protein precipitation. This is because, in preparing the serum sample, it is crucial to remove the protein. For this step, acids are commonly used to lowering the pH level of sample below the pKa of SCFA that result in their protonation, thereby promoting evaporation from the sample matrix and precipitating the protein [22]. The expectation is as follows: the lower the pH due to the addition of a stronger acid, the more pronounced the effect will be. Meta-phosphoric acid was used in this method, because it has a good protein precipitation efficiency and can be applied universally to various species. It can also remove plasma protein up to 90% in human samples [23]. This sample preparation procedure is simple and provides a clean sample prior to injection to the GCMS system. The developed GCMS method in this study also offers superior sensitivity that can achieve 0.12 μmol/L concentration. Meanwhile, some studies show that the lowest concentration that can be detected is 7.5 μmol/L [22]; others suggest it ranges from 1 to 20 μmol/L [24].

With some modifications, method validation criteria were adopted from the College of American Pathologist (CAP) chemistry checklist as well as from the Clinical & Laboratory Standards Institute (CLSI) C62A for Liquid Chromatography Tandem Mass Spectrometry and C50AE for Mass Spectrometry in the Clinical Laboratory [25–27]. Linearity was assessed for 10 levels of calibration to cover a wide range of SCFA concentrations. Every SCFA showed excellent linearity for the 10 levels of calibration with a correlation coefficient higher than 0.995, which is consistent with CLSI criteria. Furthermore, accuracy, matrix effect assessment, and sample extraction efficiency were examined by recovery testing. Based on CLSI and CAP requirements, recovery testing requires three levels of concentration: low, middle, and high. In this study, we used at least five levels of recovery concentration within the calibration range to ensure a high level of accuracy. Indeed, every level of recovery concentration is nearly 100%. According to CAP requirements, $100 \pm 25\%$ recovery is acceptable, and, therefore, SCFA concentration is accurate, and the proposed sample preparation is effective. The last parameter of method validation was precision assessment, and, based on CLSI requirements, a CV value of 15% in the dynamic range is acceptable. The results suggest that the highest CV is for isobutyric acid at 14.1%, whereas, for the other SCFAs, CV is below 10%. In light of this information, the proposed GCMS method for serum SCFA quantification is validated and meets the CAP and CLSI requirements.

SCFAs are the main product of the anaerobic fermentation of dietary fiber by gut microbiota in humans [4]. Since humans do not have an enzymatic system to digest complex fiber, these nondigestible carbohydrates pass through the upper gastrointestinal tract and are fermented in both the cecum and the large intestine by anaerobic cecal and colonic microbiota [28]. For gut microbiota, SCFAs are a necessary waste product, which are required to balance redox equivalent production in the anaerobic environment of the gut [29]. Depending on diet, the total concentration of SCFAs ranges from 70 to 140 mM in the proximal colon and from 20 to

Table 3 Mean differences of short-chain fatty acids in lean and centrally obese men

Variables	Lean (N=17) Mean±SD	Centrally obese (N=32) Mean±SD	p
Age (years)	39±7	39±6	0.875
Waist circumference (cm)	81±8	107±10	<0.01*
HbA1c (%)	5.2±0.3	6.5±2.1	<0.01*
Acetic acid (μmol/L)	152±24	210±30	<0.01*
Propionic acid (μmol/L)	6.9±1.0	11.5±4.8	<0.01*
Isobutyric acid (μmol/L)	16±2.3	19.8±4.2	<0.01*
Butyric acid (μmol/L)	1.6±0.7	2.1±1.1	0.01*
Isovaleric acid (μmol/L)	1.7±0.6	2.3±0.5	<0.01*
Valeric acid (μmol/L)	0.35±0.05	0.61±0.18	<0.01*
All SCFAs (μmol/L)	179±25	246±34	<0.01*

*Significance at the 95% confidence interval; Mann–Whitney U test

Table 4 Correlation between short-chain fatty acids and waist circumference

Correlation	Waist circumference	p
Acetic acid	$R=0.576$	<0.01*
Propionic acid	$R=0.514$	<0.01*
Isobutyric acid	$R=0.598$	<0.01*
Butyric acid	$R=0.396$	<0.01*
Isovaleric acid	$R=0.545$	<0.01*
Valeric acid	$R=0.560$	<0.01*
All SCFAs	$R=0.601$	<0.01*

*Significance at the 95% confidence interval; Spearman's rho test

70 mM in the distal colon [30]. When examining feces, the concentration range of SCFAs in the colon is at the millimolar level, which is too high to be detected by any method. Although fecal SCFAs are commonly used as an indicator of microbial fermentation, they may not accurately reflect in vivo colonic fermentation, since approximately 95% of colonic SCFAs are absorbed, and the remaining 5% are excreted in feces [31]. To further understand the distribution of SCFAs in the different biocompartments, it is necessary to accurately measure circulating levels of SCFA. Moreover, circulating concentrations of SCFA reflect high or low consumption of dietary fiber [32].

Throughout this paper, we developed a high-sensitive, simple, and fast method of measuring circulating SCFAs in the serum to assess the interaction between SCFAs and metabolic-related organs. SCFAs are the byproduct of gut microbiota, so they are transferred to the bloodstream before they reach target organs and affect metabolism. To date, SCFAs are known to directly regulate tissue and organ metabolism as well as influence their specific mechanisms. Accordingly, they are a metabolic regulator [5]. Due to the involvement of SCFAs in many metabolic processes, researchers believe that a cause–effect relationship exists between metabolic diseases, especially central obesity. A recent study showed that serum SCFAs [28], rather than fecal SCFAs, are correlated with metabolic markers such as glucagon-like peptide 1, fatty acid metabolism, and insulin sensitivity. This is consistent with the results of our study, which suggest a significant positive correlation between all SCFAs and waist circumference. This is also consistent with a study that demonstrated significantly different concentrations of fecal SCFAs for lean and obese subjects [33] and another study that showed a link between type 2 diabetes mellitus and SCFAs through the disturbance of pancreatic beta cell proliferation and function [34].

Conclusions

The validated GCMS method offers high-sensitive, fast, simple, and reliable SCFA quantitation in the serum and demonstrates the potential features of circulating SCFAs in central obesity.

Data availability The authors declared that the data supporting the findings of this study are available within the article.

Code availability Not applicable.

Declarations

Ethics approval All procedures performed in this study was approved by Health Research Ethics Committee of Faculty of Medicine Universitas Padjadjaran (No. 1039/UN6.C.10/PN/2017).

Source of biological material The biological material was human serum, which was obtained from a stored biological samples from a previous study in 2017.

Conflict of interest The authors declare no competing interests.

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