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A sensitive method for the quantification of short-chain fatty acids by benzyl chloroformate derivatization combined with GC-MS†

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Short-chain fatty acids (SCFAs) were identified as critical markers in the diagnosis of chronic and metabolic diseases, but a sensitive and stable method to determine SCFAs in feces is a challenge for analysts due to the high volatility. Herein, a sensitive and accurate method to determine SCFAs adopting pre-column derivatization coupled with gas chromatography-mass spectrometry (GC-MS) has been developed. Benzyl chloroformate (BCF) was chosen as the reaction reagent and emulsified derivatization was applied to homogenize the reaction system. Higher sensitivity, wider application and satisfactory derivatization efficiency were obtained using the developed method. An excellent method validation showed a good linearity ranging from 0.9947 to 0.9998. At the same time, the intra-day and inter-day precision were achieved in the range of 0.56% to 13.07%. The lower limits of detection of all target analytes varied from 0.1 to 5 pg. The recovery ranged from 80.87% to 119.03%, and storage stability under three different conditions was also determined. This method was also successfully applied to the analysis of SCFAs in mice fecal samples to illustrate the significant differences between normal and type 2 diabetes mellitus mice.

Introduction

In recent years, much attention has been paid to short-chain fatty acids (SCFAs) in biological samples, which play an important role in pathological and physiological processes.[1] SCFAs, also referred to as "volatile fatty acids" (VFAs), are fatty acids with less than six carbon atoms.[2] Straight-chain SCFAs are mainly derived from the microbial fermentative activity of unabsorbed dietary fiber in the gut while branched-chain SCFAs are derived from the metabolism of branched-chain amino acids. Acetic, propionic and butyric acids are the three most common SCFAs, accounting for 90%–95% of the total.[3] SCFAs are important organic acids in the colon cavity and provide energy support for colon and small intestinal epithelial cells.[4,5] Most SCFAs are absorbed through the portal vein and only 5–10% of them can be found in feces, blood and urine.[6] In addition to acting as a crucial energy source of body, SCFAs also play a multi-effect role in human biology. Recent studies suggest that SCFAs are important signalling molecules regulating biological responses through the inhibition of histone deacetylases (HDACs) and G-protein-coupled receptors (GPCRs).[7] In addition, a direct link between the changes in SCFAs, enteroendocrine hormones and glucose homeostasis has also been observed in many studies.[8] SCFAs in biological samples, especially acetic, propionic and butyric acids, are associated with many diseases such as inflammatory bowel diseases (IBD), colorectal cancer (CRC),[9,10] hypertension, type 2 diabetes (T2D),[11] obesity and cardiovascular diseases.[12–14] These findings indicate the importance of quantification of SCFAs in biological samples for clinical diagnosis of various diseases.

A handful of analytical approaches have been developed to analyze SCFAs in a variety of biological matrices,[1,15] such as capillary electrophoresis (CE),[16] nuclear magnetic resonance (NMR),[17] gas chromatography-flame ionization detection (GC-FID) and high performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV).[18–20] However, these methods show poor resolution and sensitivity for SCFA determination in complex biological matrices. Chromatography-mass spectrometry has been widely used due to its high sensitivity and selectivity, and derivatization was

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performed to improve its chromatographic behavior.[15,21,22] Compared with liquid chromatography-tandem mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS) equipped with a complete database can provide easy access to metabolite identification.

Different chemical derivatization reagents such as pentafluorobenzyl bromide (PFBBr),[23] trimethylsilyl (TMS), chloroformate *etc*, have been recently used.[15,30] Nevertheless, PFBBr derivatization is time-consuming and may cause the evaporation of volatile derivatives.[24,25] Besides, TMS derivatization must be performed under anhydrous conditions, which is not convenient for the processing of biological samples. Propyl chloroformate or isobutyl chloroformate is more widely used for the derivatization of SCFAs due to its advantages of shorter reaction time and aqueous reaction system.[26,27] However, potential loss of SCFAs may also occur due to the strong volatility of derivatives. In addition, these methods are not able to quantify formic acid which overlaps with the solvent, and since acetic acid is near the injection peak, the peak is poorly resolved. The high toxicity of the derivatization reagent is also a drawback.

Herein a fast and accurate chemical derivatization-GC-MS method is presented which can reliably evaluate SCFAs in fecal samples. This method uses benzyl chloroformate (BCF) and benzyl alcohol for chemical derivatization under mild reaction conditions and quantitatively converts SCFAs to their benzyl ester derivatives. Since BCF and benzyl alcohol are insoluble in aqueous solution, DMSO was selected as the emulsifier to homogenize the reaction system. It suggests that this new derivatization method greatly improves the sensitivity and accuracy of quantification and can be used to quantify formic acid. Furthermore, this method uses low-toxic reagents for derivatization which can reduce the risk of occupational exposure. Also, this method was applied to quantify SCFAs in biological samples to demonstrate the utility of our procedure.

Materials and methods

Chemicals and reagents

Formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, 2-methylbutyric acid, valeric acid, isovaleric acid, 2-methylvaleric acid and 3-methylvaleric acid were purchased from Aladdin (Shanghai, China). Hexanoic acid, 2-methylhexanoic acid, heptanoic acid, benzyl alcohol (BnOH), pyridine (Py) and benzyl acetate were purchased from Sigma-Aldrich (St Louis, MO, USA). Benzyl chloroformate (BCF), benzyl butyrate, sodium hydroxide, acetone, dimethyl sulfoxide (DMSO), HPLC grade acetonitrile (ACN), cyclohexane and hexane were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ultrapure water was prepared by the Milli-Q system (Millipore, Billerica, MA).

Mice fecal sample

Four week male C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center (SPF II Certificate) and kept under controlled light and temperature conditions. The type 2 diabetes mellitus (T2DM) mice models were induced according to a previous study.[28] After adapting to the environment for one week, all the mice were randomly divided into three groups: a normal group (n = 8), a diabetic model group (n = 8) and a control group (n = 8). The normal group mice were fed a standard diet, and the diabetic model and control group mice were given a high fat diet (D12492). After being given the high-fat diet for four weeks, the diabetic model and control group mice were fasted for 12 h and then given intraperitoneal injections with STZ (35 mg kg^{-1}) for three successive days. Mice with fasting blood glucose levels higher than 16.7 mmol L^{-1} were chosen for experiments. The control mice were intragastrically administered with gefitinib (20 mg kg^{-1}) every other day. The fresh fecal samples were collected after the mice were fed for 16 weeks. In order to reduce the volatilization and degradation of SCFAs in the fecal samples, freshly excreted feces were collected immediately in sealed plastic tubes and stored at −80 °C until analyzed. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Fudan University and approved by the Animal Ethics Committee of School of Pharmacy, Fudan University.

Preparation of standard solutions, calibration and quality control (QC) samples

A mixture of standard stock solution containing formic acid (0.2 mg mL^{-1}), acetic acid (4 mg mL^{-1}), propionic acid (2 mg mL^{-1}), isobutyric acid (0.2 mg mL^{-1}), butyric acid (2 mg mL^{-1}), 2-methylbutyric acid (0.2 mg mL^{-1}), isovaleric acid (0.2 mg mL^{-1}), valeric acid (0.2 mg mL^{-1}), 2-methylvaleric acid (0.02 mg mL^{-1}), 3-methylvaleric acid (0.02 mg mL^{-1}), hexanoic acid (0.02 mg mL^{-1}) and heptanoic acid (0.06 mg mL^{-1}) was prepared by dissolving the above-mentioned analytical standards in 0.2 M aqueous NaOH. The mixed standard working solutions were prepared weekly by serial dilution with 0.2 M aqueous NaOH. A stock solution of 2-methylhexanoic acid (0.1 mg mL^{-1}) was also prepared with 0.2 M aqueous NaOH and used as an internal standard (IS). Quality control (QC) samples were prepared from biological samples spiked with 100 μL mixed standard solutions at high, medium, and low concentrations and a lower limit of quantitation. All the standard stock solutions were stored at −20 °C, while all the working solutions were stored at 4 °C.

Sample preparation and derivatization

A 200 μL aliquot of ice-cold IS stock solution was added to 50 mg fecal sample and the sample was homogenized at 70 Hz for 2 min. The sample was then centrifuged at 4000 rpm for 5 min and 100 μL of supernatant was transferred into a plastic tube. The extraction procedure was then repeated by adding 100 μL of ice-cold IS stock solution to the sample. Another 100 μL of supernatant was transferred into the tube with the first extraction. The resultant mixture was briefly vortexed and stored for derivatization. Then 200 μL of a BnOH–Py solvent mixture (3 : 2, v/v) and 100 μL of DMSO were subsequently

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added into the plastic tube and vortexed for 5 s. Then 100 μL BCF was added slowly and the cap was kept open for 1 min to release the generated gas. The cap was then closed and the mixture was vortexed for 3 min. After derivatization, 200 μL cyclohexane was added to the reaction mixture and the sample was vortexed for 1 min followed by centrifugation at 4000 rpm for 5 min. Subsequently, 100 μL derivative extract (upper cyclohexane layer) was transferred to a glass insert and 1 μL of this sample was injected into the GC-MS instrument for analysis.

GC-MS analysis

The derivatized samples were analyzed using an Agilent 7890B gas chromatography system coupled with an Agilent 5977A quadrupole mass spectrometric detector (Agilent Technologies, Santa Clara, CA). Derivatives were separated using a DB-5 MS UI capillary column (30 m × 0.25 mm id, 0.25 μm). One microliter of derivative was injected in split mode in the ratio of 20 : 1, and helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}. The temperatures of the front inlet, transfer line and ion source were set to 250, 280, and 230 °C, respectively. The initial column temperature was held at 70 °C for 3 min, ramped to 200 °C at a rate of 10 °C min^{-1} and was finally increased to 290 °C at a rate of 35 °C min^{-1} and held at this temperature for 7 min. A single run took 25.5 min and the solvent delay time was set to 6.7 min. The electron energy was −70 eV and the gain factor was set to 2.0. During qualitative analysis, the mass spectral data were collected under a full-scan mode (*m*/*z* 30–600), while selected ion monitor (SIM) mode was applied for quantitative analysis. The data were processed using Agilent MassHunter Qualitative Analysis (version B.06.00) and Agilent MassHunter Quantitative Analysis (version B.06.00) software.

Method validation

In order to ensure the reliability of the quantitative study, method validation was performed in terms of the linearity range, precision, recovery, derivatization reaction yield and stability. The linearity was determined from the calibration curves constructed from mixed standard working solutions at six concentration levels. Each concentration was prepared and determined in triplicate. The regression analysis of Aa/Ai (Aa: peak areas of derivatized analytes; Ai: the peak area of derivatized IS) was carried out to determine the linearity of the calibration curves. The LOD was determined as the lowest amount of analytes injected with an S/N ratio >3 and the LLOQ was determined as the amount which yielded at least 10 times the S/N ratio. To determine whether the fecal sample matrices would interfere with the SCFA quantitation, the matrix effect (ME) was evaluated as described below: ME = $[(A - B)/C] \times 100\%$, where A is the area of the analytes in the post extraction spiked sample, B is the area of the analytes in the blank matrix sample, and C is the area of the analytes in a 0.2 M NaOH solution.

The intra-day precision was determined by analyzing the four concentration levels of the QC samples within a day and each concentration was determined five times. The inter-day precision was confirmed by analyzing the QC samples for three days. The recoveries were assessed for the QC samples and calculated according to the concentration ratio of the measured to the spiked analytes. Recovery (%) = $[(A - B)/C] \times 100\%$, where A is the measured concentration of the analytes in the spiked samples, B is the measured concentration of the analytes in the unspiked samples, and C is the theoretical concentrations of the analytes in the spiked samples.

Stability studies for the standard working solutions at two different concentrations were performed under three different storage conditions, *i.e.*, freeze/thaw cycles three times, 4 °C for 24 h, and −80 °C for a week.

Herein, formic acid, acetic acid, propionic acid, butyric acid, hexanoic acid, isobutyric acid and isovaleric acid were used as the representative analytes, and the derivatization reaction yield was assessed according to the published method.[26] Briefly, the standard working solutions at high and low concentrations were processed using the developed method, while the blank water sample was processed in parallel until the derivative extraction step. Instead of pure cyclohexane, cyclohexane dissolved with esters was used for the blank sample. Moles of benzyl esters and analytes in the standard mixtures were equal, and the peak areas of the added benzyl esters could be used as a benchmark.

Results and discussion

Benzyl chloroformate derivatization

The SCFAs containing easily-derivatized carboxylic acid functional groups can be converted into benzyl ester using BCF. The carboxylic group first attacks BCF and the SCFA-BCF intermediate (**I**) is subsequently attacked by BnOH, generating a second intermediate (**II**), which undergoes further rearrangements to form the corresponding benzyl ester (Fig. 1). Compared to methyl-, propyl-, and isobutyl-chloroformate,[26,27,29] the use of BCF results in less volatile derivatives while introducing a structurally stable benzyl group into the derivative, which greatly improves the accuracy and sensitivity of the developed method. In addition, the derivatives of formic and acetic acids were successfully separated from the solvent peaks by introducing a benzyl group. However, the low solubility of BCF in water can lead to phase

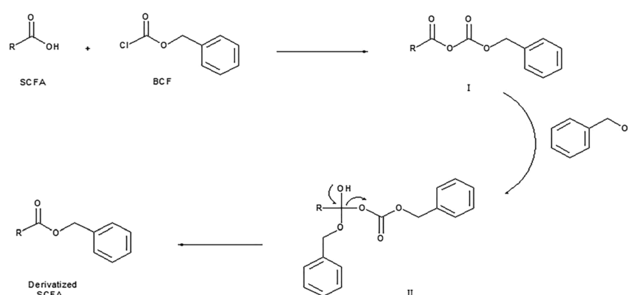

Fig. 1 Chemical derivatization reactions of SCFAs.

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separation and the failure of derivatization reaction. In order to deal with this problem, DMSO was chosen as the emulsifier to homogenize the reaction system so that the derivatization can be completed smoothly.

Then the derivatized products were first analyzed in full scan mode by GC-MS, and MassHunter Qualitative Analysis software (version B.06.00) was used to perform product identification of raw data. The derivatized products are all separable and the mass spectrum of each compound is consistent with the expected product (ESI, Fig. 1†). Fig. 2A shows a typical SIM chromatogram of the derivatized products, and the selected ions monitored are listed in Table 1.

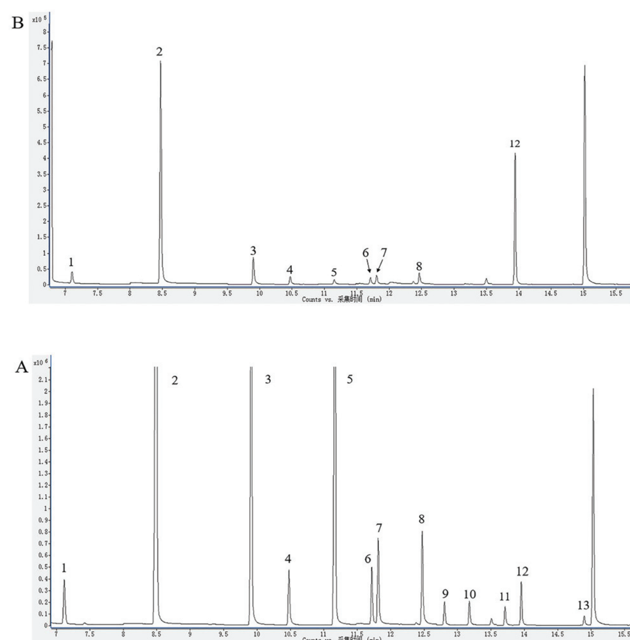

Fig. 2 Typical GC-MS SIM chromatograms of (A) a standard mixture and (B) a mice fecal sample. (1) Formic acid, (2) acetic acid, (3) propionic acid, (4) isobutyric acid, (5) butyric acid, (6) 2-methylbutyric acid, (7) isovaleric acid, (8) valeric acid, (9) 2-methylvaleric acid, (10) 3-methylvaleric acid, (11) hexanoic acid, (12) 2-methylhexanoic acid (IS), and (13) heptanoic acid.

Table 1 Selected ions for the derivatized compounds

Analytes	RT (min)	m/z*
Formic acid	7.09	91, 136[a]
Acetic acid	8.48	108, 150[a]
Propionic acid	9.90	91, 164[a]
Isobutyric acid	10.47	91[a], 178
Butyric acid	11.15	108, 178[a]
2-Methylbutyric acid	11.71	91, 108, 192[a]
Isovaleric acid	11.80	91[a], 108, 192
Valeric acid	12.46	91, 108[a], 192
2-Methylvaleric acid	12.80	91, 164[a]
3-Methylvaleric acid	13.17	65, 91[a], 115
Hexanoic acid	13.71	99, 108[a], 206
2-Methylhexanoic acid	14.00	91[a], 113
Heptanoic acid	14.88	91[a], 113

*Qualitative ions. [a]Quantitative ions.

Optimization of derivatization and extraction

In order to obtain an ideal method based on the reaction of BCF derivatization, the optimization of derivatization and extraction conditions was performed using acetic acid as the representative compound in four different respects: (1) the concentration of aqueous NaOH, (2) the type and volume of emulsifiers, (3) the procedures of derivatization and (4) the type of extraction solvent. The reproducibility was ensured by triplicate measurements under each condition.

The efficiency of derivatization would be affected by the pH of the system according to the previous studies.[30] Fig. 3A indicates that the best derivatization efficiency can be obtained with 0.2 M aqueous NaOH. The volume and ratio of BnOH and Py were also tested, and 200 μL BnOH–Py solvent mixture (3 : 2, v/v) was selected for derivatization. Subsequently, the types and volumes of emulsifiers were compared (Fig. 3B). As indicated by the plots, DMSO provides the best derivatization efficiency compared with acetone and acetonitrile, and 100 μL DMSO is considered to be the optimal condition. Based on the above results, an investigation of the reaction conditions and reaction time was performed. As shown in Fig. 3C, higher derivatization efficiency would be obtained by vortex compared to ultrasound, and the peak area of benzyl acetate plateaued when vortex time ranged from 3 to 10 min. For operational convenience, vortex for 3 min was considered to be the optimal reaction time. The type of extraction solvent was also optimized and cyclohexane was selected. In comparison with hexane used in other reports,[26] cyclohexane shows a higher extraction efficiency, providing a lower volatility and ensuring accuracy and precision of the analytical method.

Method validation

Linearity, limit of detection and lower limit of quantitation. Table 2 lists the calibration curves for all the SCFAs, based on the measurement of the standard working solutions. As is shown, the linear regression (R^2) indicates a linear relationship between the MS response and the concentration of standards, ranging from 0.9947 to 0.9998. The LODs of each of the compounds measured in standard working solutions are also included in Table 2. As is shown, the LODs of these targeted SCFAs vary from 0.1 to 5 pg and the LLOQs from 0.1 to 10 pg. The sensitivity of the method reported herein is higher than those in previous reports using GC/MS.[26,31,32] The ME values calculated with peak areas are in the range of 93–105%, indicating that the quantitative determination of SCFAs in the fecal samples is not affected by the sample matrix. The ME results of compounds are listed in Table 3.

Precision and accuracy. The recovery and precision of this method were determined from the extraction of the spiked fecal samples and the corresponding percentage mean values are given in Table 3. Recoveries ranged from 80.88% to 119.03%, which are higher than those in the previous report using GC-MS with chloroformate derivatization.[27] The intra- and inter-day precision (RSDs) also showed satisfactory results, ranging from 0.56% to 13.07%.

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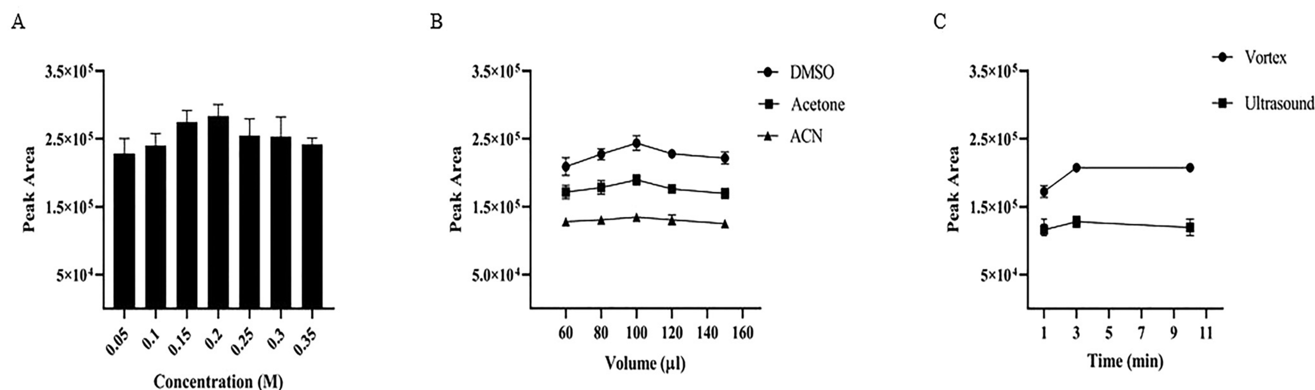

Fig. 3 Optimization of derivatization and extraction conditions using acetic acid as a representative. (A) Concentration of aqueous NaOH. (B) Type and volume of an emulsifier. (C) Procedures of derivatization.

Table 2 Linearity data and LODs of 12 analytes determined with GC-MS (n = 3)

				LOD[b]	
Analytes	Calibration equation[a]	Linear range (μg mL^{-1})	R^2	pg on column	Signal/noise
Formic acid	$y = 0.5685x + 0.0003$	1–100	0.9947	—	—
Acetic acid	$y = 0.2860x - 0.0156$	20–2000	0.9990	—	—
Propionic acid	$y = 0.2780x - 0.0095$	10–1000	0.9990	—	—
Isobutyric acid	$y = 1.6416x - 0.0001$	0.5–100	0.9996	1	10
Butyric acid	$y = 0.2334x - 0.0056$	10–1000	0.9991	—	—
2-Methylbutyric acid	$y = 0.1419x - 0.0001$	0.3–100	0.9993	1	4
Isovaleric acid	$y = 1.4281x - 0.0011$	0.5–100	0.9990	1	18
Valeric acid	$y = 1.0666x - 0.0034$	1–100	0.9991	1	6
2-Methylvaleric acid	$y = 0.0764x - 0.0001$	0.1–10	0.9998	2.5	6
3-Methylvaleric acid	$y = 1.1434x - 0.0005$	0.1–10	0.9991	0.1	10
Hexanoic acid	$y = 1.0648x - 0.0002$	0.05–10	0.9991	2.5	5
Heptanoic acid	$y = 2.2122x - 0.0067$	0.3–30	0.9992	5	7

[a] x: Concentration (μg mL^{-1}); y: peak area ratio (area of each analyte/area of the internal standard). [b] LOD: The LODs of formic acid, acetic acid, propionic acid and butyric acid were not calculated as the background signals of these four analytes could be detected in blank water samples.

Stability. The stability results are listed in Table 4. The standard solutions showed good stability under three different storage conditions, with recoveries in the range of 88.46%–115.56%, 89.75%–109.12% and 88.10%–113.24%, respectively.

Derivatization reaction yield. The derivatization efficiency was assessed under optimized conditions using seven SCFAs as the representative analytes, which was calculated as the peak area of derivatized standard mixtures/the peak area of the blank sample spiked with benzyl esters × 100%. As shown in Table 5, the derivatization reaction yields showed satisfactory results for target SCFAs, ranging from 80.04% to 108.00%.

Method application

Feces is a valuable biological sample for the study of diabetes mellitus. However, most of the existing studies focus only on common SCFAs such as acetic, propionic, and butyric acid, while the changes in other SCFAs in mice remain unknown. Herein, the developed method was successfully applied to quantify SCFAs in mice feces, including formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, 2-methylbutyric acid, valeric acid and isovaleric acid. A typical SIM chromatogram of derivatized mice feces is shown in Fig. 2B. The data were statistically processed using GraphPad Prism 8.0.1 software.

As shown in Fig. 4, the total content of SCFAs in the feces of diabetic mice induced by a high-fat diet was significantly lower than that of normal mice, indicating that the deficiency of SCFAs is associated with T2DM.[33] Similarly, most individual SCFAs in diabetic mice also showed a significant decrease. Acetic, propionic and butyric acid significantly decreased in diabetic mice, consistent with the previous study which demonstrated that acetic, propionic and butyric acid have beneficial effects on β-cell function and insulin secretion.[11,34] In addition, changes in formic and valeric acids were also observed in our model mice, which were less studied before due to their low levels in biological samples. In particular, formic acid in biological samples has rarely been reported due to the lack of detection methods. The developed method can benefit the research of formic acid *in vivo* in the future.

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Table 3 ME, accuracy and precision of 12 analytes determined by GC-MS (n = 5)

Analytes	Spiked concentrations (μg mL^{-1})	Measured concentrations (μg mL^{-1})	Recovery (%)	Intra-day precision (RSD%)	Inter-day precision (RSD%)	ME (%)
Formic acid	0.00	5.69	—	6.39	6.13	—
	1.00	6.59	90.82	6.54	7.12	—
	2.00	7.67	99.21	10.54	8.16	102.32
	35.00	41.36	101.91	5.45	12.67	—
	80.00	90.46	105.97	8.15	11.91	104.15
Acetic acid	0.00	172.41	—	4.37	6.61	—
	20.00	188.59	80.88	6.50	5.99	—
	40.00	209.97	93.89	8.18	6.01	97.91
	700.00	909.11	105.24	3.61	6.78	—
	1600.00	1834.77	103.90	5.96	7.13	104.86
Propionic acid	0.00	14.97	—	2.12	5.08	—
	10.00	23.07	80.92	5.06	4.89	—
	20.00	32.25	86.40	3.14	4.51	102.24
	350.00	368.69	101.06	2.43	4.93	—
	800.00	842.91	103.49	3.22	4.82	104.22
Isobutyric acid	0.00	0.72	—	2.96	6.77	—
	1.00	1.57	84.23	4.97	4.86	—
	2.00	2.57	92.11	7.44	4.46	93.14
	35.00	36.28	101.60	2.86	2.98	—
	80.00	81.24	100.65	1.85	2.60	104.96
Butyric acid	0.00	11.03	—	3.08	7.21	—
	10.00	19.37	83.38	6.09	5.50	—
	20.00	28.58	87.75	5.36	4.06	97.72
	350.00	371.13	102.89	2.40	4.45	—
	800.00	850.77	104.97	3.04	3.77	104.82
2-Methylbutyric acid	0.00	0.39	—	3.17	7.29	—
	1.00	1.23	83.35	1.66	4.88	—
	2.00	2.16	88.59	4.41	4.75	95.14
	35.00	36.17	102.23	0.56	2.10	—
	80.00	84.64	105.31	2.41	3.00	103.79
Isovaleric acid	0.00	0.60	—	4.22	7.13	—
	1.00	1.43	83.10	3.86	3.52	—
	2.00	2.33	86.37	3.44	2.82	99.46
	35.00	36.44	102.38	1.10	2.41	—
	80.00	86.75	107.69	1.47	1.81	104.09
Valeric acid	0.00	1.18	—	3.79	7.65	—
	1.00	2.03	85.56	6.59	8.80	—
	2.00	2.95	88.76	4.05	4.70	95.16
	35.00	37.08	102.59	2.95	5.60	—
	80.00	84.25	103.84	3.21	4.41	101.84
2-Methylvaleric acid	0.00	0.00	—	—	—	—
	0.10	0.09	91.32	4.14	8.63	—
	0.20	0.18	89.56	2.78	4.92	101.13
	3.50	3.41	97.41	2.36	3.18	—
	8.00	8.08	101.02	4.63	4.04	98.35
3-Methylvaleric acid	0.00	0.00	—	—	—	—
	0.10	0.10	104.28	7.50	6.63	—
	0.20	0.22	107.95	6.68	5.20	95.98
	3.50	3.62	103.44	1.26	2.88	—
	8.00	8.45	105.58	0.82	2.74	101.21
Hexanoic acid	0.00	0.06	—	12.85	13.07	—
	0.10	0.15	89.52	6.02	6.69	—
	0.20	0.23	82.52	4.92	6.56	98.74
	3.50	3.42	95.88	7.72	5.89	—
	8.00	8.05	99.81	2.73	4.77	101.60
Heptanoic acid	0.00	0.00	—	—	—	—
	0.30	0.36	119.03	2.55	5.18	—
	0.60	0.63	104.25	4.97	4.21	102.12
	10.50	10.50	100.01	2.33	5.05	—
	24.00	24.77	103.20	2.18	3.35	102.86

As an epidermal growth factor receptor inhibitor, gefitinib can significantly improve insulin sensitivity in type 2 diabetic rats.[35,36] The results of our study reveal that gefitinib significantly increases SCFA levels in the feces of mice, especially acetic acid and butyric acid. SCFAs have beneficial effects on β-cell function and insulin secretion and they can prevent insulin resistance.[11,34] Therefore, the effect of gefitinib on SCFA levels may contribute to the improvement of insulin sensitivity, suggesting that SCFAs may serve as biomarkers for screening diabetes mellitus drugs.

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Table 4 Stability of 12 analytes determined by GC-MS (n = 5)

	Freeze and thaw (3 cycles)		4 °C (24 h)		−80 °C (a week)	
Analytes	Low-con	High-con	Low-con	High-con	Low-con	High-con
Formic acid	95.80	100.33	98.23	103.77	96.45	104.14
Acetic acid	90.99	106.33	91.65	106.73	88.10	107.54
Propionic acid	90.58	107.06	91.90	104.54	90.83	108.58
Isobutyric acid	102.28	108.83	103.92	108.51	98.31	108.95
Butyric acid	88.46	107.00	90.67	104.67	89.90	109.30
2-Methylbutyric acid	90.14	101.82	89.75	106.05	92.51	110.76
Isovaleric acid	89.74	106.05	92.19	105.75	91.27	107.91
Valeric acid	88.71	102.32	93.99	103.51	96.40	107.41
2-Methylvaleric acid	92.50	100.39	90.51	101.72	89.59	107.99
3-Methylvaleric acid	115.56	108.14	99.79	108.76	99.23	108.17
Hexanoic acid	91.71	103.32	94.69	109.12	90.49	106.23
Heptanoic acid	93.61	102.93	97.48	108.15	91.46	113.24

Low-con: Recovery of standard solutions at low concentration. High-con: Recovery of standard solutions at high concentration.

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Table 5 Derivatization yields of 7 analytes determined by GC-MS (n = 5)

Analytes	Low-con (%)	High-con (%)
Formic acid	92.73	104.15
Acetic acid	93.03	108.00
Propionic acid	87.98	80.04
Butyric acid	96.63	89.44
Hexanoic acid	80.65	90.48
Isobutyric acid	92.48	81.34
Isovaleric acid	94.95	97.32

Low-con: Derivatization yields of standard solutions at low concentration. High-con: Derivatization yields of standard solutions at high concentration.

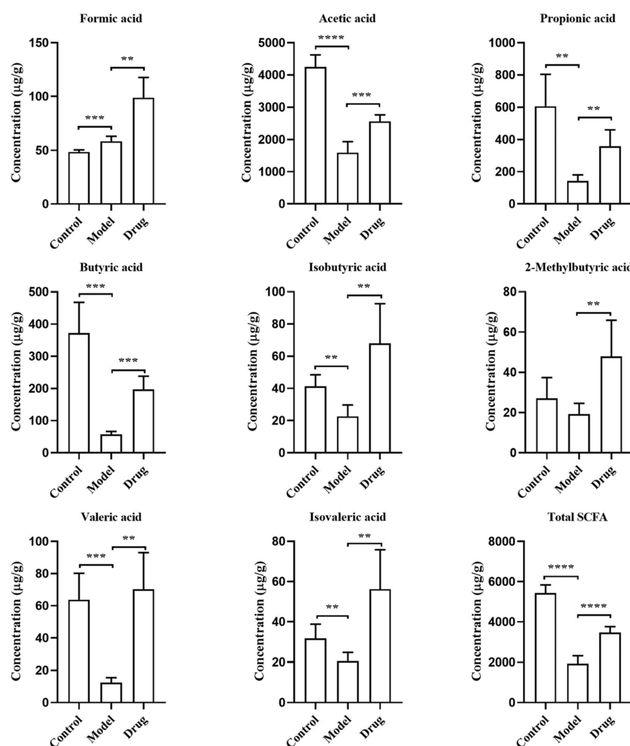

Fig. 4 Column graphs of SCFAs detected in mice fecal samples. An unpaired two-tailed t-test was applied for statistical processing; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Conclusions

In this study, a rapid and sensitive method for the quantification of SCFAs in biological samples was developed, which used BCF derivatization and GC-MS analysis. The analytical protocol that we described overcomes the shortcomings of previous methods, and the validation shows satisfactory results for linearity, LOD, recovery, repeatability and stability. This method was successfully applied to analyze fecal samples. The established method is an efficient tool and will benefit the disease research involving SCFAs. In addition, this method can also be applied for the analysis of amino acids, which will be studied soon.

Conflicts of interest

There are no conflicts of interest to declare.

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