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Determination of short-chain fatty acids in serum by hollow fiber supported liquid membrane extraction coupled with gas chromatography

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Abstract

A method based on hollow fiber supported liquid membrane extraction coupled with a gas chromatograph equipped with flame ionization detector (GC-FID) was developed for the determination of six short-chain fatty acids including acetic acid, propionic acid, *i*-butyric acid, *n*-butyric acid, *i*-valeric acid and *n*-valeric acid in serum. Hollow fiber supported liquid membrane extraction was employed for preconcentration and clean-up of the samples. The fatty acids were extracted from the acidic donor (diluted serum) into a liquid membrane formed in the wall of the hollow fiber with 10% tri-*n*-octylphoshphine oxide (TOPO) in di-*n*-hexyl ether, and then extracted back into a basic acceptor solution filled in the lumen of the hollow fiber. After being acidified with HCl, the acceptor was directly analyzed by GC-FID. The acceptor concentration, donor pH, membrane liquid and extracting time were optimized giving an enrichment factor up to 155 times. The good linearity ($r^2 > 0.980$), reasonable recovery (87.2–121%), and satisfactory intra-assay (8.2–11.5%) and inter-assay (6.1–11.6%) precision illustrated the good performance of the present method. Limits of detection (LOD) ranged from 0.04 to 0.24 μM and limits of quantification (LOQ) varied from 0.13 to 0.80 μM.

Keywords: Hollow fiber supported liquid membrane extraction; Short-chain fatty acid (SCFA); Serum; Gas chromatography; Determination

1. Introduction

Short-chain fatty acids (SCFAs) are mainly produced from indigestible carbohydrates in the human colon by bacterial fermentation [1,2]. The growing interest in the formation of SCFAs in the recent years is related to increasing evidence of positive physiological effects of some of these acids [3]. The main SCFAs of the large bowel are acetic acid, propionic acid and butyric acid. BUT is the preferred energy substrate for the mucosal cells and it has been suggested that this acid plays a role in the prevention and treatment of colonic diseases [4]. SCFAs that escape metabolism in the colonocytes enter the hepatic portal blood. Acetic acid acts as a precursor for lipogenesis but also stimulates gluconeogenesis, while propionic acid has been shown to inhibit gluconeogenesis and hepatic synthesis of cholesterol from acetic acid [5,6]. It is generally believed that most of the butyric acid is efficiently metabolized by the colonic epithelium, but recent studies on catheterised pigs have shown that the concentration of butyric acid in the portal and jugular vein increased with the production rate in the colon [7]. In this connection, it should be noted that butyric acid has also been shown to influence the lipid-metabolism in caco-2 cells [8,9]. Since the level and pattern of SCFAs formed are dependent on the type of carbohydrate reaching the colon it may be possible to regulate these parameters by the diet. Thus, determination of SCFAs in blood plays an important role in screening, diagnosis and monitoring effects of dietary fiber and other dietary carbohydrates reaching colon when designing foods with specific health effects. However, compared with feces, whole blood, plasma or serum contains small concentrations of SCFAs and highly sensitive techniques are required for this measurement.

The usual methods for SCFA determination include capillary gas chromatography (GC) with or without mass spectrometry after solvent extraction, ion exchange, vacuum distillation, solid phase microextraction or derivatization [10–17].

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Other routine techniques for analyzing SCFAs in blood include high-performance liquid chromatography (HPLC) [18], stable isotope tracer technique [19–20] and capillary electrophoresis [21].

Although SCFAs including acetic acid (ACE), propionic acid (PRO), *i*-butyric acid (IBUT), *n*-butyric acid (BUT), *i*-valeric acid (IVAL) and *n*-valeric acid (VAL) were found in blood [12,13,15], only ACE, PRO and BUT can be identified and quantified by most of the above-mentioned analytical methods, while IBUT, IVAL and VAL cannot be quantified as they are almost always present in trace amounts in various blood samples. Further, these methods always require at least 400 μl of blood sample that could sometimes be a limiting experimental factor, particularly in case of studies on small animal models, such as rats [22]. Therefore, the development of a method for preconcentration and clean-up of these analytes from small amounts of samples is of great importance.

Supported liquid membrane extraction has been utilized as a selective cleanup and pre-enrichment step for various analytes in biological fluids [23,24] including small amounts of carboxylic acids in various matrices [25–27]. In this work, a hollow fiber supported liquid membrane approach has been developed for extraction/pre-enrichment of SCFAs from serum before GC analysis. The present method was aimed to provide high enrichment enough to quantify all of the above-mentioned SCFAs in a very restricted volume (100 μl) of serum sample.

2. Experimental

2.1. Apparatus

Chromatographic analysis was carried out using an Agilent 6890 N GC system equipped with a flame ionization detector (FID) and an N10149 automatic liquid sampler (Agilent, USA). A fused-silica capillary column with Free Fatty Acid Phase (DB-FFAP 125–3237, J&W Scientific, Agilent Technologies Inc., USA) of 30 m × 0.53 mm I.D. coated with 0.50 μm film thickness was used. Data handling was carried out with a HP ChemStation Plus software (A.09.xx, Agilent).

The polypropylene hollow fiber tube (50 μm wall thickness, 280 μm inner diameter, 0.1 μm pore size, model 50/280 Accurel PP) was obtained from Membrana GmbH (Wuppertal Germany). BD Micro-Fine Syringes (with a needle of 0.30 mm outer diameter, 8 cm length, 0.5 ml holding volume and prepared for U-100 insulin injection) were obtained from BD Consumer Healthcare (via a local pharmacy), and used to fill the acceptor into the lumen of the hollow fiber for extraction and to flush out the acceptor from the lumen of the hollow fiber into a 100 μl pulled point conical glass vial (Agilent, USA).

2.2. Materials

High purity SCFAs were used to prepare the standard solutions. ACE (100%) and VAL (99%) were obtained from Merck (Darmstadt, Germany). PRO (100%) was purchased from Janssen Chimica (Belgium), while IBUT (99%), BUT (99%) and IVAL (99%) were purchased from Sigma (St. Louis, MO, USA). 2-Ethylbutyric acid, used as internal standard, was obtained from Sigma–Aldrich (Chemie GmbH, Steinheim, Germany).

An aqueous stock standard solution was prepared for each acid with a concentration of 25 mM for ACE, 1 mM for PRO, BUT and IBUT, 3 mM for VAL and 0.5 mM for IVAL. A working standard solution containing the six target analytes was prepared by mixing appropriate volumes of the individual standard stock solution and diluting it with water. An internal standard stock solution containing 2-ethylbutyric acid with 12% formic acid was also prepared. All the stock standard and working solutions were stored at −20 °C until used.

Tri-*n*-octylphosphine oxide (TOPO) and dihexyl-ether (DHE) were obtained from Sigma (St. Louis, MO, USA). The organic membrane liquid was prepared by dissolving appropriate amounts of TOPO in DHE. Analytical-grade HCl (30%) and formic acid (98–100%) were used. The donor was acidified using 12 M HCl and 12% (v/v) formic acid was used to clean the GC column. Analytical-grade NaOH from Merck (Darmstadt, Germany) and its water solution (0.1–0.3 M) was used as basic acceptor. All water used was purified using a Milli-Q® reagent water system (Millipore, Molsheim, France).

2.3. Sample preparation

Frozen human serum supplied by Lund University Hospital (Sweden) was used for method development and validation. For method application, human serum from four healthy subjects and portal serum samples from nine Sprague–Dawley rats were included. The Ethics Committee for Human and Animal Studies at Lund University approved the experiments. Among the rat samples, two portal blood samples were taken from rats fed with a diet containing 8% inulin (w/w calculated on dry weight basis, dwb) for 13 days, and the other seven samples were taken from rats fed a diet containing 12% blueberry husk (w/w, dwb) and 1 ml probiotics/day (L. *gasseri*, L. *crispatus*, L. *plantarum*, B. *infantis*) for 13 days. The portal blood from the rats was transferred to serum tubes and centrifuged for 15 min (2500 × g, 19 °C) and stored at −20 °C before sample preparation and analysis.

To prepare the acidic donor, 100 μl serum sample was diluted with 1350 μl water. The pH of the diluted serum solution was then adjusted to 2 with 2 M HCl (about 11 μl needed) and the volume of the diluted serum solution was made up to 1500 μl by adding water.

2.4. Extraction procedure

The extraction procedure was carried out as described by Liu et al. [28] with some modifications. Briefly, the lumen of a piece of hollow fiber (∼15 cm length) was flushed and filled with acceptor solution by using a BD Micro-Fine Syringe. Then the fiber was dipped into the membrane liquid for a few seconds to impregnate the pores of the hollow fiber wall, thus forming the organic liquid membrane. After that, the lumen of the fiber was slowly flushed and completely filled with the acceptor solution. To seal the fiber, the two ends of the fiber were folded and enveloped with a piece of aluminum foil. The obtained extrac-

tion device was immersed into water and shaken for about 1 min to wash out surplus membrane liquid on the fiber surface. For extraction, the finished extraction device was immersed into the 1.5 ml donor solution held in a 2 ml capped vial. It is important to immerse the whole fiber fully in the donor solution and obtain an even distribution of the fiber in the vial. After the hollow fiber has been installed in the vial, the vial was capped and shaken on a shaker (VXR, IKA®-Labortechnik, Sweden) for 16 h at 350 r/min to perform the extraction and enrichment. After extraction, the hollow fiber extraction device was harvested and one of the sealed ends was cut and the fiber was connected to the needle of a BD Micro-Fine Syringe filled with air, then the other sealed end was cut and the acceptor containing the extracted analytes was flushed from the fiber lumen into a clean and dry 100 μl pulled point conical glass vial (Agilent, USA). Normally 8–10 μl of acceptor could be harvested, of which 8 μl was transferred into another clean and dry 100 μl small pulled point conical glass vial (Agilent, USA) and 10 μl 1 M HCl containing 1 mM internal standard was added. Then the vial could be directly placed on the auto-sampler for GC analysis.

2.5. Gas chromatographic analysis

Helium was supplied as the carrier gas at a flow rate of 14.4 ml/min. The initial oven temperature was 100 °C and was kept there for 0.5 min, and then raised to 180 °C by 8 °C/min and held there for 1.0 min, then further increased to 200 °C by 20 °C/min, and finally held at 200 °C for 5 min. Glass wool (Supelco) was inserted in the glass liner of the splitless injection port. The temperatures of the FID and the injection port were 240 and 200 °C, respectively. The flow rates of hydrogen, air and nitrogen as makeup gas were 30, 300 and 20 ml/min, respectively. The injected sample volume for GC analysis was 1 μl, and the running time for each analysis was 17.5 min. In order to decrease the small ghosting peaks of SCFAs, the GC column was cleaned with an injection of 1 μl 12% (v/v) formic acid once every five sample injections.

3. Results and discussion

3.1. Evaluation of the supported liquid membrane extraction conditions

Fig. 1 shows the principle of the extraction procedure [24]. When the donor was adjusted to pH 3–2, the SCFAs in the donor were completely protonated because of the comparatively high p*K*a values that varied from 4.5 to 4.9 [29] (Table 1). During extraction, undissociated SCFAs diffused from the bulk donor solution to the surface of the hollow fiber and then they partitioned into the membrane liquid. At the lumen surface of the hollow fiber, these undissociated SCFAs were deprotonated and extracted into the acceptor, where they were ionized and consequently could not be extracted back into the membrane liquid, i.e. they were trapped in the acceptor. These two processes occurred simultaneously with highly efficient overall extraction. Concentrations of the disturbing neutral compounds remained unchanged in donor and acceptor, which implied no enrichment.

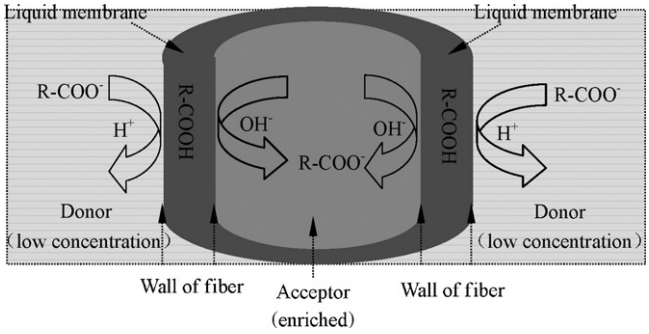

Fig. 1. Sketch map of extraction SCFAs with hollow fiber supported liquid membrane.

Table 1
Names, abbreviations, pK_a and log P values of the six SCFAs

Full name	Abbreviation	pK_a	log P[a]
Acetic acid	ACE	4.76	−0.29
Propionic acid	PRO	4.87	0.25
i-Butyric acid	IBUT	4.86	0.59
n-Butyric acid	BUT	4.83	0.78
i-Valeric acid	IVAL	4.58	1.12
n-Valeric acid	VAL	4.83	1.31

[a] Calculated by the computer program ACD/Log *P* 3.00 (Advanced Chemical Development Inc., Toronto, Canada).

Further, basic compounds in the donor were in their protonated form and could not be extracted. Therefore, the hollow fiber supported liquid membrane extraction in this study provided both high enrichment and high selectivity for the acidic compounds. It was proved to be an effective sampling method for analysis of small amounts of SCFAs such as in blood samples. The enrichment factor in this study is defined as the analyte concentration in the acceptor after extraction divided by the original analyte concentration in the donor.

3.1.1. Influence of acceptor concentration

Experiments showed that variation in concentration of the NaOH acceptor in the range of 0.1–0.3 M had little effect on the enrichment of the SCFAs (Fig. 2), which was explained by the high pH of the acceptor (about 12) after extraction at the lowest acceptor concentration (0.1 M). This is in agreement with

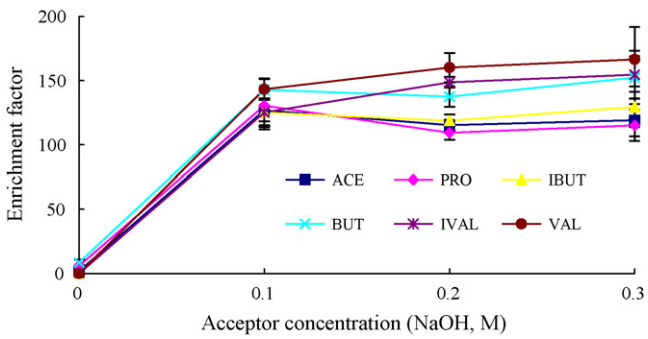

Fig. 2. Enrichment factor as a function of acceptor concentration [ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid)].

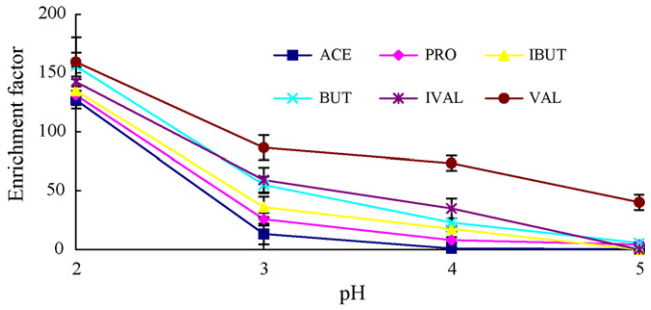

Fig. 3. Enrichment factor as a function of the pH of the donor [ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid)].

studies reported by Kou et al. [30]. It was also found that the pH of the acceptor might drop if a donor with high concentration of SCFAs was subjected to extraction. To increase the reservoir capacity an acceptor with high NaOH concentration is preferable during such conditions. As previously reported, to ensure that the fraction of SCFAs in the undissociated form is below 0.0005, the pH of the acceptor must be at least 3.3 pH units higher than the highest pKa value of the SCFAs [31], which is well met at the actual conditions. However, the higher the concentration of NaOH, the more solid residues (NaCl) will be produced at the GC injection, which may result in contamination or block in the inject port liner and GC column.

3.1.2. *Influence of donor pH*

HCl (2 M) or NaOH (2 M) was added to the water sample to adjust its pH to a certain level. As shown in Fig. 3, the enrichment factor was not changed much when the pH of the donor decreased from 5 to 3. However, a sharp increase in enrichment was observed when the pH of the donor decreased from 3 to 2. Thus, the higher the fractions of SCFAs in the undissociated form in the donor, the higher enrichment factor could be achieved. For extraction of weak acids, the donor pH must be at least 2 pH units lower than the pKa of the acid, to assure that the fraction of SCFAs in the undissociated form in the donor is beyond 0.99 [31].

3.1.3. *Influence of the type of membrane liquid*

The effect of the membrane liquid on the enrichment factor is shown in Table 2. A certain amount of SCFAs could also be transferred to the acceptor without any membrane liquid, which is due to some type of membrane distillation of the SCFAs [32], transferring the acids in gas phase over the membrane. In this type of extraction, the enrichment factor was decided by the volatility of the SCFAs, and increased in the order; ACE < PRO < IBUT ≈ BUT < IVAL ≈ VAL. When pure DHE was used as membrane liquid, the enrichment factor increased with the hydrophobicity or Log *P* of the SCFA. The presence of TOPO in DHE significantly changed the behavior of the membrane liquid due to the hydrogen bonding between TOPO and the SCFAs [33]. With increased concentration of TOPO in the membrane liquid from 5 to 10%, the enrichment factor was rapidly elevated. A further increment of TOPO in the membrane liquid from 15 to 20%, however, deteriorated the enrichment factor. This was partly explained by the fact that TOPO is an effective hydrogen-bonding reagent and the higher the concentration of TOPO in the membrane liquid the higher NaOH concentration is required in the acceptor [30]. Therefore, 10% was chosen to be the optimum TOPO concentration in the membrane liquid. Another interesting phenomenon was that with 15 or 20% TOPO in the membrane liquid, the enrichment factors for all SCFAs at 16 h extraction were lower than that of 8 h extraction. This was also in contrast to the results obtained with pure DHE, 5 and 10% TOPO as membrane liquid or when extraction was performed without any membrane liquid. These behaviors of the membrane liquid with TOPO are not well understood. It was also found that the enrichment factor with 10% TOPO in DHE as membrane liquid was comparatively higher for SCFAs with long hydrophobic chains than for SCFAs with short hydrophobic chains. Similar results were obtained by Shen et al. [26]. Other membrane liquids with TOPO such as in 6-undecanone

Table 2
Effect of different membrane liquids on the enrichment factor of SCFAs

Membrane liquid	Extracting time	Enrichment factor					
		ACE[a]	PRO	IBUT	BUT	IVAL	VAL
No liquid	8 h	17.8 ± 1.6	24.2 ± 1.2	29.3 ± 1.8	29.5 ± 2.3	30.7 ± 1.2	32.7 ± 0.7
	16 h	25.8 ± 5.0	34.6 ± 3.6	39.7 ± 2.1	45.9 ± 4.2	41.3 ± 5.0	45.0 ± 11.9
DHE	8 h	22.2 ± 0.8	61.6 ± 6.9	73.7 ± 5.5	82.0 ± 5.5	80.2 ± 6.2	115.1 ± 6.4
	16 h	35.3 ± 3.8	49.0 ± 6.7	48.0 ± 3.0	69.0 ± 7.5	66.3 ± 4.1	123.6 ± 7.8
20% TOPO	8 h	6.59 ± 0.03	8.43 ± 1.37	4.65 ± 1.83	9.75 ± 3.44	5.38 ± 2.45	11.76 ± 6.75
	16 h	5.91 ± 0.86	8.13 ± 1.11	2.61 ± 0.77	8.88 ± 2.35	2.76 ± 0.72	5.57 ± 1.77
15% TOPO	8 h	44.5 ± 1.2	36.7 ± 0.3	28.9 ± 3.8	37.3 ± 4.2	29.7 ± 3.3	48.6 ± 6.2
	16 h	5.09 ± 0.33	7.72 ± 0.75	2.28 ± 0.27	6.88 ± 0.36	1.93 ± 0.16	3.95 ± 0.72
10% TOPO	8 h	126.2 ± 5.2	129.6 ± 6.8	125.1 ± 8.8	142.4 ± 9.6	125.6 ± 10.4	163.2 ± 13.6
	16 h	143.6 ± 5.2	146.6 ± 6.2	150.8 ± 6.4	157.3 ± 11.6	153.3 ± 8.0	156.3 ± 12.5
5% TOPO	8 h	58.1 ± 2.8	49.6 ± 2.2	33.2 ± 2.3	55.0 ± 3.7	47.8 ± 2.3	56.9 ± 4.5
	16 h	77.8 ± 2.3	78.4 ± 3.1	55.6 ± 2.9	84.8 ± 3.9	60.6 ± 0.1	81.9 ± 2.2

[a] ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid).

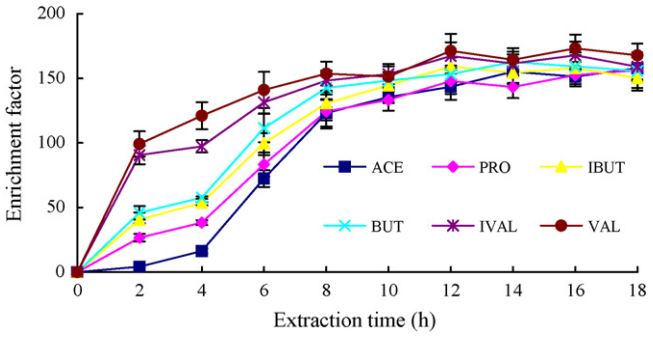

Fig. 4. Enrichment factor as a function of extracting time [ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid)].

or xylene could also be used for organic acid extraction, but these may have other characteristics [27].

3.1.4. Influence of extraction time

As shown in Fig. 4, the enrichment factor successively increased in the first 12 h of the extraction. After that, the enrichment factor kept stable for the following 4 h investigated (i.e. to 16 h). Considering the actual application of the method, 16 h was selected as the time of extracting of practical reasons, as the extraction then can be started at four o'clock in the afternoon and be finished at eight o'clock the next day. Under these extraction conditions, enrichment factors around 155 times were obtained for all of the SCFAs. This corresponds nicely to the expected enrichment factor calculated from the ratio of the donor and acceptor volumes and shows that the extraction is essentially complete.

3.1.5. Other conditions

Other conditions including environment temperature, shaking rate, sample volume, concentration of analytes and viscosity of the donor may also have an effect on the extraction. In this study, the extraction was performed at an environment temperature of about 25 °C and the shaking rate was set to 350 r/min on a shaker. It was also found that the lower the temperature and shaking rate the lower was the enrichment factor. This is due to the fact that the diffusion coefficient of the SCFAs in the donor increases with temperature and shaking rate. However, a comparatively high shaking rate resulted in a decreased enrichment factor, which may be due to emulsification and leakage of organic membrane liquid into the donor [34]. When the sample volume and the concentration of SCFAs increased, the enrichment factor first elevated and reached a maximum value and then decreased. This could be due to a decrease in pH when the amount of analytes in acceptor increase. To keep the amount of serum within a very restricted volume, 100 μl serum was diluted up to 15 times to obtain a 1.5 ml sample volume, even though the enrichment factor was not at maximum during these circumstances. As for the viscosity, it was found that serum must be diluted up to more than 10 times to ensure a good performance of the extraction, since the solubility of the protein in serum decreases when the donor is acidified. With whole blood samples, the protein and the foam formed in preparing the acidic donor made the extraction unrealizable. When plasma was deproteinized, it was possible to extract the SCFAs but the recovery was unsatisfactory which probably is due to that the denatured proteins can absorb the SCFAs.

3.2. Evaluation of method performance

The chromatograms of direct injection of a standard solution, extracted standard solution and extracted human serum are shown in Fig. 5. As can be seen, all of the peaks were very well separated by GC. Chromatograms of extracted serum reflected the high enrichment and high selectivity for the SCFAs. It should be pointed out that a peak around 5.7 min always appeared in the chromatograms of extracted standard solutions and serum samples with consistent peak area counts. This peak may be a result

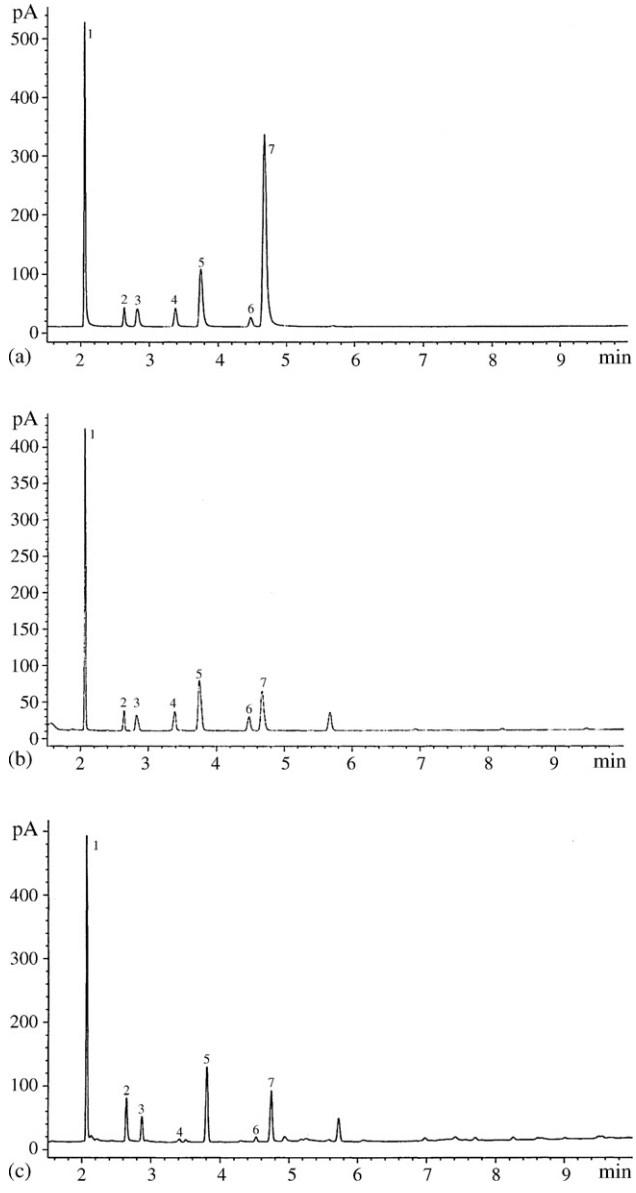

Fig. 5. Gas chromatograms of standard mixture (a), extracted standard solution (b), and extracted human serum (c). Peak identification: 1, acetic acid; 2, propionic acid; 3, *i*-butyric acid; 4, *n*-butyric acid; 5, *i*-valeric acid; 6, *n*-valeric acid; 7, 2-ethylbutyric acid.

Table 3
Parameters for the regression equations ($y = ax + b$) of the SCFAs, where y is the donor concentration (mM) and x is the peak area counts[a]

SCFA	Slope a	Intercept b	R^2[b]	Linearity range (μM)
ACE[c]	0.00007	−0.0015	0.9904	3.72–120.43
PRO	0.00003	−0.0003	0.9840	0.10–2.50
IBUT	0.00002	−0.00005	0.9879	0.10–2.50
BUT	0.00002	−0.0002	0.9876	0.10–2.50
IVAL	0.00002	0.00003	0.9913	0.43–8.40
VAL	0.00002	−0.0002	0.9809	0.07–0.10

[a] The peak area counts refer to the area counts of the solution by mixing 8 μl acceptor and 10 μl 1 M HCl containing 1 mM internal standard.
[b] Linearity was expressed as the square of correlation coefficient between donor concentration and peak area counts.
[c] ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid).

Table 4
Recovery values of SCFAs at three spiking levels in human serum[a]

	ACE[b]	PRO	IBUT	BUT	IVAL	VAL
Serum (μM)	181.0	8.20	11.99	11.64	66.38	4.87
Spiked (μM)	123.6	5.00	5.00	5.08	14.60	2.53
Recovery (%)	104.9	87.2	96.4	90.1	90.1	107.7
Spiked (μM)	247.7	10.0	10.0	10.2	29.2	5.01
Recovery (%)	118.4	93.3	112.7	100.8	97.8	113.9
Spiked (μM)	371.6	15.0	15.0	15.2	43.8	7.63
Recovery (%)	107.3	90.7	97.3	92.6	106.0	121.1

[a] To determine the recovery of SCFAs, the extraction parameters were set as followed; donor pH 2, fiber length 15 cm, acceptor 0.1 M NaOH, extracting time 16 h, and membrane liquid 10% TOPO in DHE.
[b] ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid).

of an impurity in the DHE solution, because it also appeared when water was extracted with pure DHE. Apart from the peaks corresponding to the identified SCFAs and the internal standard, two unknown peaks located at 3.45 and 4.97 min were found in the chromatogram of the extracted serum.

As shown in Table 3, the linearity of the present method was excellent for all SCFAs, with square of correlation coefficients higher than 0.980. For the linearity range, it is enough to cover the possible concentration in various serum samples and the present conditions allow a dilution of the serum up to ten times when preparing the donor.

A mixture of the six studied acids was added to a human serum at three levels and the recovery of the acids (concentration found/concentration spiked) was calculated. The results are presented in Table 4, showing that these recoveries varied from 87.2 to 121%. Due to the significant difference among the concentrations of the SCFAs in serum, the relatively wide range of recoveries is acceptable.

As shown in Table 5, the intra-assay relative standard deviation (RSD) measured at 10 runs a day of a serum sample was relatively low (8.2–11.5%), and the inter-assay precision evaluated on different days with a total of 15 runs provided RSD

Table 5
The intra-assay and inter-assay reproducibility of the method and instrument on serum sample

	ACE[a]	PRO[a]	IBUT[a]	BUT[a]	IVAL[a]	VAL[a]
Intra-assay						
Concentration (μM) ($n = 10$)	215.8	11.16	11.89	11.87	60.73	5.33
RSD (%)	8.2	11.5	9.8	10.4	9.0	8.4
Inter-assay						
Concentration (μM) ($n = 15$)	190.2	11.28	10.92	10.83	58.93	6.11
RSD (%)	6.4	10.8	6.1	11.6	6.9	7.9

[a] ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid).

Table 6
The concentration of SCFAs of actual samples in μM

Sample[a]	ACE[b]	PRO	IBUT	BUT	IVAL	VAL
H1	162.4 ± 6.9	14.4 ± 3.4	15.1 ± 0.6	9.04 ± 0.04	88.7 ± 0.9	1.71 ± 0.25
H2	171.6 ± 6.7	11.0 ± 0.8	9.02 ± 0.05	14.5 ± 0.3	38.2 ± 0.6	1.48 ± 0.03
H3	134.2 ± 1.5	13.6 ± 1.2	12.9 ± 0.7	14.5 ± 0.3	69.9 ± 1.3	2.05 ± 0.17
H4	227.2 ± 9.7	13.4 ± 0.7	5.93 ± 0.21	16.5 ± 0.8	42.8 ± 2.4	1.66 ± 0.29
R1	1449.4 ± 139.3	96.0 ± 7.0	6.42 ± 0.19	17.4 ± 1.0	15.2 ± 0.6	11.4 ± 0.6
R2	1488.0 ± 19.9	82.0 ± 1.7	9.84 ± 0.73	15.3 ± 0.6	20.6 ± 1.4	8.29 ± 1.78
R3	553.7 ± 23.0	7.15 ± 0.95	5.56 ± 0.19	19.0 ± 0.9	17.5 ± 0.1	1.10 ± 0.14
R4	667.9 ± 37.4	6.36 ± 1.07	2.54 ± 0.26	10.9 ± 0.6	8.29 ± 0.64	1.45 ± 0.23
R5	589.1 ± 27.4	11.9 ± 0.9	2.64 ± 0.37	17.3 ± 0.3	10.2 ± 0.4	1.39 ± 0.24
R6	627.1 ± 78.1	7.01 ± 1.09	2.49 ± 0.00	11.5 ± 1.0	10.2 ± 0.5	0.81 ± 0.20
R7	669.9 ± 71.2	10.8 ± 1.1	4.53 ± 0.72	18.8 ± 3.1	15.0 ± 1.1	0.53 ± 0.34
R8	872.9 ± 52.5	20.7 ± 0.8	4.47 ± 0.22	32.5 ± 2.8	12.8 ± 0.6	2.71 ± 0.18
R9	773.0 ± 22.9	13.8 ± 0.5	2.87 ± 0.31	19.7 ± 2.7	10.4 ± 1.1	1.55 ± 0.02

[a] H1–H4 human serum from four apparently healthy persons; R1–R2 rat serum from Sprague–Dawley rats fed diet containing 12% blueberry husk and a probiotic mixture (L. *gasseri*, L. *crispatus*, L. *plantarum*, B. *infantis*) for 13 days; R3–R9 rat serum from Sprague–Dawley rats fed inulin 8% (w/w) for 13 days.
[b] ACE (acetic acid), PRO (propionic acid), IBUT (*i*-butyric acid), BUT (*n*-butyric acid), IVAL (*i*-valeric acid) and VAL (*n*-valeric acid).

values in the range 6.1–11.6%. Consequently, the method had a very good repeatability and comparatively high reproducibility.

When calculated from the standard calibration curves by considering the peak area corresponding to three and ten times of the signal-to-noise ratio [35], limits of detection (LOD) and quantification (LOQ) varied in the range 0.04–0.24 μM and 0.13–0.80 μM, respectively. This implies a high sensitivity of the present method.

3.3. Application to serum samples

To demonstrate the applicability of the described technique, four human serum and nine rat serum samples were analyzed (Table 6). In the human serum from healthy subjects, the concentrations of ACE, PRO and BUT were in the ranges 130–230 μM, 11–15 μM and 9–17 μM, respectively. This is significantly higher than the data reported by Tollinger et al. [10], Schatowitz et al. [13] and Vogt et al. [17]. Serum from rats was from the portal vein, while that from humans was venous blood. This fact most probably explains the higher concentration of ACE in serum of rats than in serum from human beings.

4. Conclusions

Hollow fiber supported membrane liquid extraction followed by GC-FID analysis was developed as a technique for the quantification of six SCFAs in serum. It is a simple and selective method. Further, no derivatization is required and only 100 μl serum sample is needed. The hollow fiber supported membrane liquid device is inexpensive, easy to make, and only a few microliters of organic membrane liquid per sample is needed. Finally, this method showed excellent precision and stability in the actual application.

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