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## A Gas Chromatographic Analysis of Fecal Short-Chain Fatty Acids, Using the Direct Injection Method

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A simple, reproducible, and rapid gas chromatographic method for short-chain fatty acid determination in human feces was developed. It involves direct injection of fecal supernatants into the gas chromatograph, without any pretreatment. Contamination of the gas chromatographic column with nonvolatile fecal material was prevented by the use of a glass liner in the injector. This liner, which acted as a precolumn, was stoppered with a glass wool plug at the lower end of the liner. Injection was performed against the glass wall of the liner, ensuring an immediate contact of the injected sample with the hot glass wall. More than 100 injections of fecal supernatants could be carried out before the liner had to be replaced by a new one. Peak tailing and ghosting was prevented by the use of formic acid in the fecal samples. The method gave sharp peaks with baseline separation for all the fatty acids.

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Short-chain fatty acids (SCFA)<sup>2</sup> are produced largely as a result of the breakdown of dietary carbohydrate in the gut by anaerobic bacterial fermentation (1). Acetic, propionic, and *n*-butyric acid are quantitatively the most important ones. The SCFA present in minor amounts in the human colon (*i*-butyric, *n*-valeric, *i*-valeric, and *n*-caproic acid (2)) primarily originate from protein catabolism and in particular from degradation of certain amino acids (3).

About 80-90% of the SCFA are absorbed; the rest are excreted in the feces (4, 5). Despite their presence in the colon in high millimolar concentrations, little information exists regarding the role of SCFA in health and disease (6-8). This is partly due to the fact that

techniques to measure fecal SCFA concentrations are rather cumbersome and time-consuming. Most procedures involve some kind of pretreatment of the fecal samples followed by gas chromatography (GC). Feces have been pretreated by extraction in organic solvents (9-11), ultrafiltration (12, 13), and derivatization (14, 15), steam distillation (16), and vacuum distillation (3, 17-19). At present, the latter method of vacuum distillation followed by GC of the aqueous SCFA solutions is used most often. Although GC on capillary columns has been used more and more, GC on packed columns is still the method of choice for the separation of fecal SCFA. In addition to GC, high-performance liquid chromatography has also been applied to the analysis of fecal SCFA (13, 20). A major drawback of the above-mentioned methods is the time-consuming pretreatment procedures, e.g., for the vacuum distillation technique about 45 min are required for one complete analysis. Moreover, losses of the more volatile acids may occur during pretreatment. No methods were found whereby direct injection of human fecal supernatants into the gas chromatograph was applied, without using any pretreatment.

In the present study we describe a very simple, reproducible, and rapid method for fecal SCFA determination, involving direct injection of fecal supernatants into the gas chromatograph, without any pretreatment.

### MATERIALS AND METHODS

#### Reagents

Formic acid, acetic acid (C<sub>2</sub>), propionic acid (C<sub>3</sub>), isobutyric acid (*i*-C<sub>4</sub>), *n*-butyric acid (C<sub>4</sub>), isovaleric acid (*i*-C<sub>5</sub>), *n*-valeric acid (C<sub>5</sub>), *n*-caproic acid (C<sub>6</sub>), and 2-ethylbutyric acid (internal standard, IS), all analytical grade (> 97% purity) were obtained from Merck (Darmstadt, Germany). The column packing material, 10% SP-1200/1% H<sub>3</sub>PO<sub>4</sub> on 80/100 Chromosorb W AW, came from Supelco (Bellefonte, PA). The glass wool (dimethylchlorosilane treated) was from Chrompack

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<sup>2</sup>Abbreviations used: SCFA, short-chain fatty acid; GC, gas chromatography; IS, internal standard.

(Middelburg, The Netherlands) and the small glass balls with a diameter of 1 mm were from Tamson (Zoetermeer, The Netherlands).

#### *Preparation of Fecal Homogenate*

Fresh fecal material was frozen on dry ice immediately after collection and stored at  $-20^{\circ}\text{C}$  until processing. Homogenates were prepared by suspending 10 g of fecal material in 50 ml of distilled water, giving a 16% (w/v) fecal suspension. One milliliter of the homogeneous suspension was transferred into a conical polypropylene micro sample tube (Eppendorf, 1 ml) and centrifuged for 1 min at 10,000g in an Eppendorf centrifuge. Ten microliters of a solution of 150 mmol/liter of 2-ethylbutyric acid (the gas chromatographic internal standard) in 100% formic acid was added to 100  $\mu\text{l}$  of the supernatant in an Eppendorf tube (1 ml), resulting in a 9% formic acid suspension. This latter suspension was centrifuged for 1 min at 10,000g and 0.6–1.0  $\mu\text{l}$  of the clear brownish supernatant was injected in the gas chromatograph for analysis. The final concentration of SCFA in the supernatant of the fecal homogenate (mmol/liter) must be multiplied by  $(5 + x)$  to obtain the concentration in the original feces in millimoles per kilogram wet weight and by  $(5 + x)/(1 - x)$  to calculate the SCFA concentration in millimoles per kilogram dry weight. In this equation,  $x$  represents the wet weight fraction of the feces. The wet weight fraction  $x$  was determined by freeze-drying 5 g of the fecal samples. Using this method for 40 fecal samples, we obtained an  $x$  of  $0.76 \pm 0.05$  (mean  $\pm$  SD; range, 0.65–0.85).

#### *Preparation of Fecal Supernatant*

Fecal samples were homogenized with a blender and ultracentrifuged for 2 h at  $4^{\circ}\text{C}$  and 30,000g. No fluid was added to the fecal sample for this homogenization. The supernatant was carefully removed. This fecal supernatant fraction was stored at  $-20^{\circ}\text{C}$  until analysis. For gas chromatographic analysis, 100  $\mu\text{l}$  of fecal supernatant was processed in the same manner as described above for fecal homogenate resulting in a clear dark-brown supernatant of which 0.6–1.0  $\mu\text{l}$  was injected. The final concentration in the fecal supernatant (mmol/liter) must be multiplied by the wet weight fraction  $x$  (about 0.75, see above) to obtain the concentration in the original feces in millimoles per kilogram wet weight and by  $(x/(1-x))$  to calculate the concentration in the original feces in millimoles per kilogram dry weight.

#### *Gas Chromatography*

The gas chromatograph used was a Chrompack Model CP 9001, equipped with a flame ionization detector and a CP-9010 automatic liquid sampler (Chrompack, Middelburg, The Netherlands). The sample injection rate of

the auto injector was 50  $\mu\text{l/s}$ . Data handling was carried out with the Maestro chromatography data system (Chrompack). The injection port of the chromatograph was installed with a hand-made glass liner (length: 8 cm; o.d. 6 mm; i.d. 3 mm) (Fig. 1A). This liner, which acted as a precolumn to prevent contamination of the chromatographic column with brown nonvolatile fecal material, was stoppered with a dimethylchlorosilane-treated glass wool plug. Injection was performed against the glass wall of the liner above the glass wool plug, by means of a 10- $\mu\text{l}$  Hamilton syringe with a slightly bent syringe needle, ensuring an immediate contact of the injected sample with the hot glass wall. The results in this study were all obtained using the above-mentioned injection technique. During preparation of this article, a slight modification in injection technique was developed. For ensuring an immediate contact of the injected sample with hot glass, the liner was partly filled with small glass balls with a diameter of 1 mm. Injection was performed by means of a 10- $\mu\text{l}$  Hamilton syringe inside the liner between the glass balls, penetrating the glass balls by about 1 cm (Fig. 1B).

The conditions were as follows: Column, 2 m  $\times$  2 mm i.d., glass, packed with 10% SP 1200/1%  $\text{H}_3\text{PO}_4$  on 80/100 Chromosorb W AW; column temperature,  $145^{\circ}\text{C}$ ; injection port temperature,  $200^{\circ}\text{C}$ ; detector temperature,  $180^{\circ}\text{C}$ . The carrier gas was  $\text{N}_2$ , 20 ml/min;  $\text{H}_2$ , 30 ml/min; air, 300 ml/min. Freshly packed columns were conditioned overnight at  $190^{\circ}\text{C}$ . A few 1- $\mu\text{l}$  injections of 10% formic acid were made to clear the column of unknown impurities. There was no need to regularly prime the column with formic acid or to add formic acid to the carrier gas.

#### *Calibration and Recovery Studies*

An aqueous stock standard (solution A) was prepared with a concentration of 250 mmol/liter for  $\text{C}_2$ , 100 mmol/liter for  $\text{C}_3$ , and 50 mmol/liter for  $\text{C}_4$ ,  $i\text{-C}_4$ ,  $\text{C}_5$ ,  $i\text{-C}_5$ , and  $\text{C}_6$ . This stock solution was diluted 2.5-, 5-, 10-, and 20-fold to obtain standard solutions B, C, D, and E, with concentrations ranging from 12.5 to 100 mmol/liter for  $\text{C}_2$ , 5 to 40 mmol/liter for  $\text{C}_3$ , and 2.5 to 20 mmol/liter for  $\text{C}_4\text{--C}_6$ . To 100  $\mu\text{l}$  of each standard solution 10  $\mu\text{l}$  of a solution of 150 mmol/liter of 2-ethylbutyric acid (IS) in 100% formic acid was added. These standards were used for daily calibration. A linear relationship was found between the peak area ratio SCFA/IS and concentration for each individual SCFA. The peak area responses in FID, although linear, differ for the different fatty acids. The peak areas relative to  $\text{C}_4$  for equimolar amounts of the various SCFA amounted to  $0.19 \pm 0.01$  for  $\text{C}_2$ ,  $0.49 \pm 0.01$  for  $\text{C}_3$ ,  $1.00 \pm 0.02$  for  $i\text{-C}_4$ ,  $0.94 \pm 0.03$  for both  $i\text{-C}_5$  and  $\text{C}_5$ , and  $1.01 \pm 0.05$  for  $\text{C}_6$  (mean values  $\pm$  SD,  $n = 8$ ).

Recovery studies were performed from fecal superna-

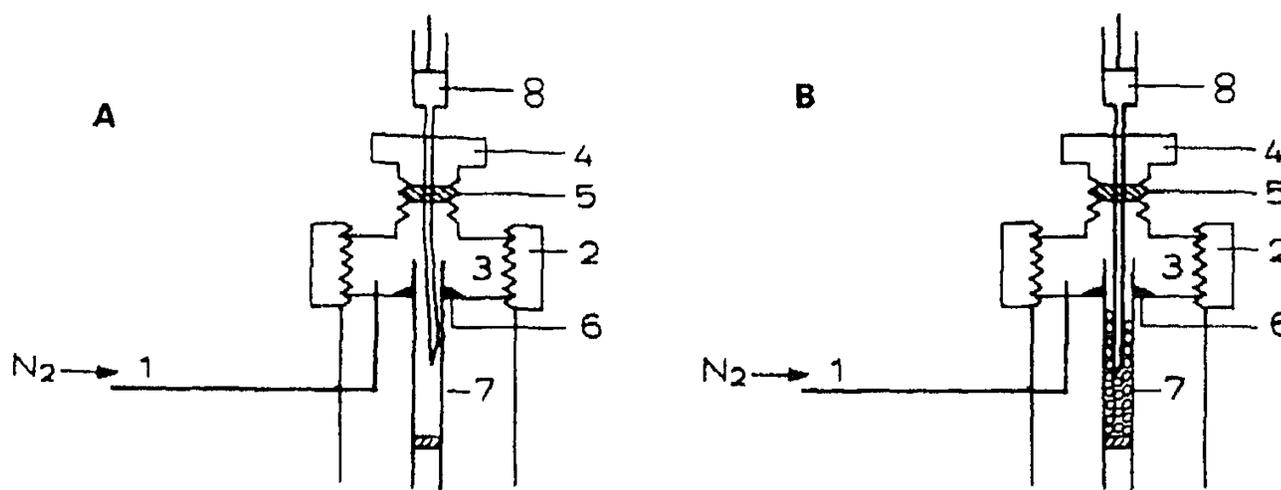


FIG. 1. GC injection port. 1, Carrier gas flow; 2, injection port head retainer; 3, injection port head; 4, septum retaining cap; 5, septum; 6, O-ring; 7, (A) empty glass liner, provided with a glass wool plug at the end; (B) glass liner, stoppered with a glass wool plug and partly filled with small glass balls; 8, (A) 10- $\mu$ l Hamilton injection syringe, provided with a slightly bent needle; (B) 10- $\mu$ l Hamilton injection syringe, penetrating the glass balls inside the liner by about 1 cm, during injection.

tant and from feces. Feces was processed as described above for fecal homogenate. Known amounts of SCFA were added to one fecal supernatant and to one fecal sample. After vortexing, the mixtures were kept at room temperature for 15 min and the clear fecal supernatants were analyzed by direct injection.

The intraassay reproducibility was determined for a standard SCFA solution and for a fecal supernatant by analyzing each sample eight times on the same day. For each gas chromatographic analysis, 100  $\mu$ l of the sample was processed as described under preparation of fecal homogenate. The interassay reproducibility was determined by analyzing the same samples on 5 different days, during a 3-month period. In between, the samples were stored at  $-20^{\circ}\text{C}$ .

#### Vacuum Distillation of Fecal Samples

The recoveries obtained with the direct injection method (see above) were compared with those obtained after vacuum distillation. For vacuum distillation of fecal samples, we adapted the procedure as described earlier for serum (21). In short, to 3.0 ml of fecal supernatant or fecal homogenate 0.3 ml of the IS solution (150 mmol/liter of 2-ethylbutyric acid in 100% formic acid) was added, resulting in a 9% formic acid solution (pH 2–2.5). After addition of one drop of 20%  $\text{H}_3\text{PO}_4$ , the mixture was vacuum distilled in an all-glass equipment by means of a water suction pump. During this procedure, the sample was heated from room temperature to  $70^{\circ}\text{C}$  while the receiver tube was cooled in liquid nitrogen. Distillation was complete within 30 min. The clear colorless distillate was thawed and 0.6–1.0  $\mu$ l was injected in the gas chromatograph for analysis.

#### Freeze-Drying of Fecal Samples

Five grams of feces was transferred into a glass vial of 15 ml. The feces was frozen by liquid nitrogen and

freeze-dried for 2 days, using a Hetosicc freeze-dryer (type CD 52, Heto InterMed, Birkerød, Denmark). The dry weight fraction was determined by weighing of the freeze-dried samples. The freeze-dried feces was made up with isotonic saline to the original weight of the feces sample, fecal supernatant was prepared, and the SCFA concentrations in the fecal supernatant of four freeze-dried fecal samples were compared with those before freeze-drying.

## RESULTS

### Gas Chromatographic Separation

Figure 2A shows a gas chromatogram of a standard mixture of the SCFA  $\text{C}_2$ – $\text{C}_6$ . A typical chromatogram of a fecal sample is shown in Fig. 3A.  $\text{C}_2$ ,  $\text{C}_3$ , and  $\text{C}_4$  are the main components in feces, whereas *i*- $\text{C}_4$ , *i*- $\text{C}_5$ ,  $\text{C}_5$ , and  $\text{C}_6$  are present in minor concentrations. Baseline separation was obtained for all the SCFA within 4 min. The peaks are symmetrical without any tailing. Ghosting of peaks as described by van Eenaeme *et al.* (22) was minimized by the use of formic acid in the sample. As shown in Fig. 2B, ghosting was very small. Ghosting amounted to only 3–10% for  $\text{C}_2$  and 1–5% for  $\text{C}_3$ – $\text{C}_6$ , depending on the state of the liner. A new liner gave somewhat less ghosting than a liner contaminated with fecal material after some 50–100 injections. However, in all cases it was reduced to less than 1% after a second injection of 1  $\mu$ l of 10% formic acid blank solution. Therefore, ghosting can be prevented by injection of 1  $\mu$ l of 10% formic acid blank solution in between two samples. When using automated injection, each fecal sample was followed by a blank solution. Both samples and blanks were injected twice, completely preventing ghosting and contamination of one sample with a previously injected one.

When performing injection into an empty liner, one should use a syringe with a slightly bent needle to ensure

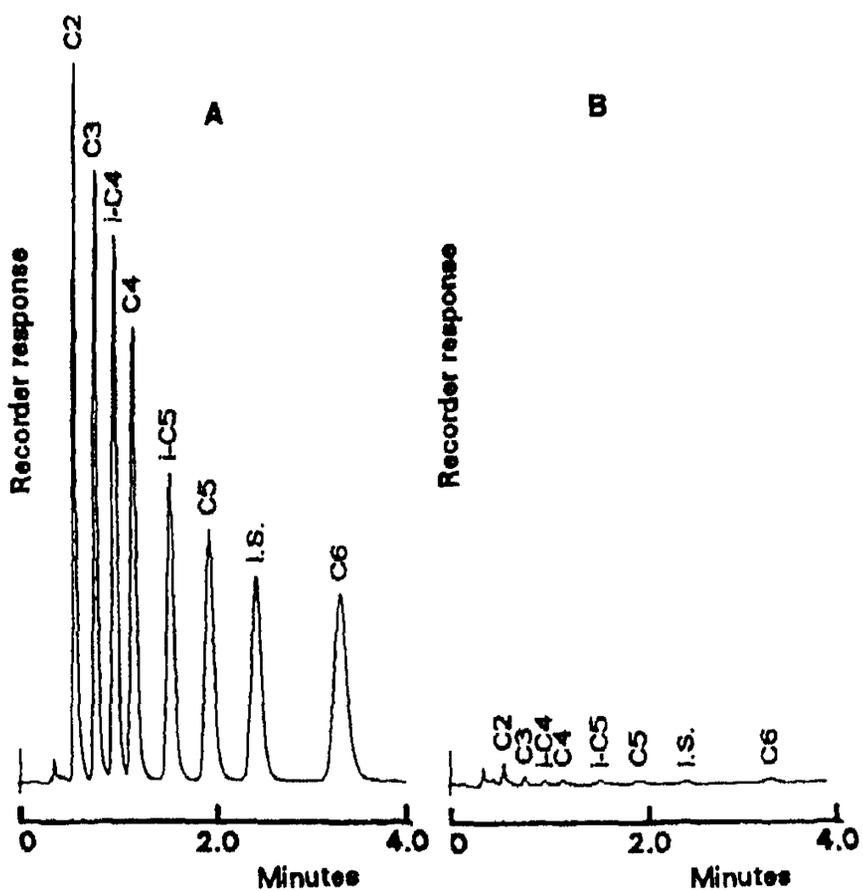


FIG. 2. (A) Gas chromatogram of a standard mixture of SCFA (standard solution B). Column temperature 145°C. Injection volume 0.8  $\mu$ l. (B) Gas chromatogram of a sample blank (0.8  $\mu$ l of a 9% formic acid solution), injected immediately after the standard sample shown in A (ghosting). Same detector attenuation as under A.

that the sample comes in immediate contact with the hot glass wall of the liner (Fig. 1A). If not, broad unresolved peaks were obtained (Fig. 3B), such as in the case of a slowly performed injection where immediately after injection the sample sticks at the end of the injection needle or after a normal injection with a straight needle where the sample was injected in the gas phase of the liner and not against the glass wall. This phenomenon was observed both for fecal samples and for the clear standard solutions. During injection of fecal samples, brown non-volatile fecal material adheres onto the glass wall inside the liner in the injection area. This disturbs the gas chromatographic separation after more than about 100 injections by hand. This is first seen for the peak of C<sub>2</sub> which then broadens and begins to tail. If so, one must replace the glass liner by a new one, which can be done within 1 min. Using automated injection, tailing of the C<sub>2</sub> peak was already observed after about 40 injections. During automated injection the sample was always injected in exactly the same way, thereby contaminating the wall of the glass liner at only one spot. The injection area became therefore sooner contaminated than when using injection by hand, the latter resulting in a more evenly distributed contamination.

An alternative injection technique is the use of a liner, partly filled with small glass balls with a diameter of 1 mm (Fig. 1B). Injection was performed inside the liner

between the glass balls with a 10- $\mu$ l Hamilton syringe, with the needle penetrating the glass balls by about 1 cm. This injection technique has two advantages. First, no differences were observed between automated and hand injection. The glass balls largely increased the glass surface in the injection area and gave an evenly distributed contamination of the glass balls and the liner's glass wall in the injection area, also during automated injection. Routinely, the liner was replaced after 100 injections of fecal samples. Second, this latter technique always ensures an immediate contact between sample and hot glass and always gave sharp resolved peaks, also in the case of a slowly performed injection as in Fig. 3B. The data presented here were all obtained using the first injection technique, viz. injection by hand against the glass wall of an empty liner. However, the injection technique between glass balls gave exactly the same results. When comparing the concentrations of the various fatty acids obtained using injection between glass balls with those obtained with the first injection technique, excellent correlations were seen ( $r = 0.9996-1.0000$ ,  $n = 10$ , 5 standards and 5 fecal samples).

#### Recovery and Precision

Recoveries of the individual SCFA from spiked samples of fecal supernatant ranged between 92 and 102% and of feces between 95 and 102% (Table 1).

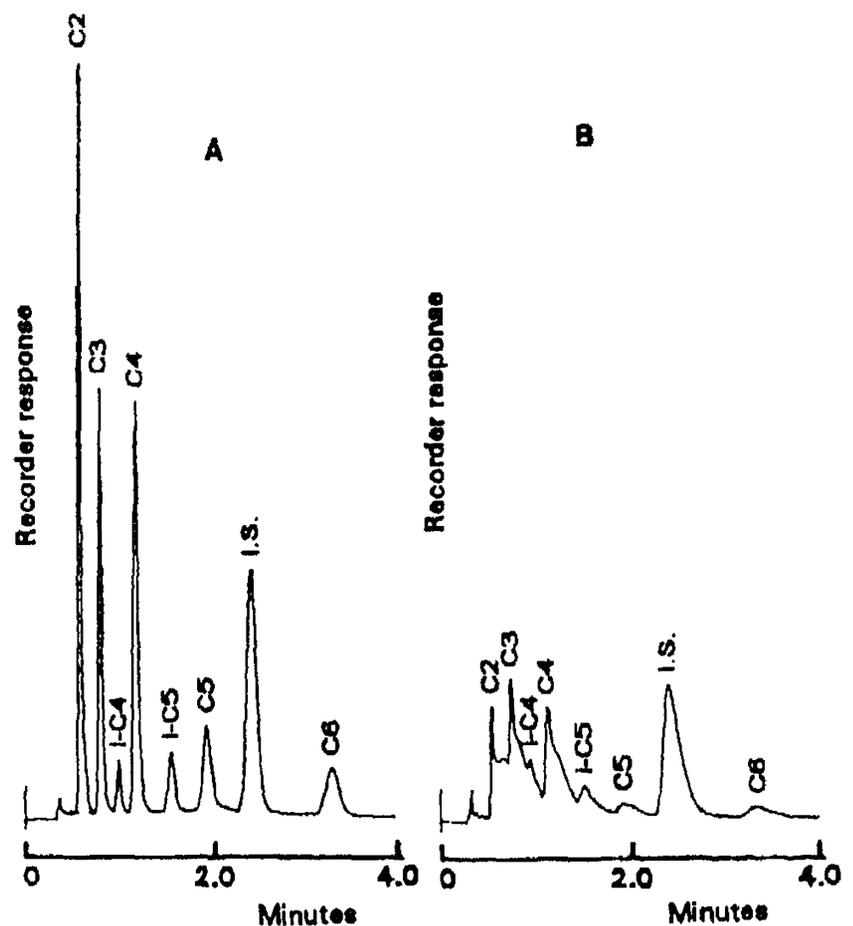


FIG. 3. (A) Gas chromatogram of a fecal supernatant. Column temperature 145°C. Injection volume 0.8  $\mu$ l. (B) Gas chromatogram of the same fecal supernatant as in A, injected more slowly than under A with a rate of 1  $\mu$ l/s, and using injection in the gas phase of an empty liner.

TABLE 1

Recovery of SCFA from One Fecal Supernatant and from One Fecal Sample, Spiked with Different Amounts of SCFA

	Acetic acid	Propionic acid	<i>i</i> -Butyric acid	<i>n</i> -Butyric acid	<i>i</i> -Valeric acid	<i>n</i> -Valeric acid	<i>n</i> -Caproic acid
Fecal supernatant (mmol/liter)							
Original amount	22.8	3.6	0.5	1.5	1.3	0.5	1.0
Amount added	25.0	10.0	5.0	5.0	5.0	5.0	5.0
Amount recovered	44.5	13.1	5.6	6.3	6.0	5.5	5.7
Recovery (%)	93	96	102	97	95	100	95
Amount added	50.0	20.0	10.0	10.0	10.0	10.0	10.0
Amount recovered	71.5	23.3	10.3	11.2	11.2	10.1	10.6
Recovery (%)	98	99	98	97	99	96	96
Amount added	75.0	30.0	15.0	15.0	15.0	15.0	15.0
Amount recovered	90.3	33.0	15.1	15.8	15.5	14.5	16.3
Recovery (%)	92	98	97	96	95	94	102
Feces (mmol/kg wet weight)							
Original amount	37.5	8.6	0.9	7.6	1.9	1.6	1.9
Amount added	125.0	50.0	25.0	25.0	25.0	25.0	25.0
Amount recovered	161.8	59.8	26.2	27.0	26.4	25.8	25.6
Recovery (%)	100	102	101	98	98	97	95

The intraassay and interassay reproducibility was excellent (Table 2), with very low variation coefficients. The interassay reproducibility was determined by analyzing the same samples on 5 different days, during a 3-month period. In between, the samples were stored at  $-20^{\circ}\text{C}$ . The low interassay variation shows that samples can be stored at  $-20^{\circ}\text{C}$  without any change in SCFA concentrations.

Once the samples have been prepared for GC injection, viz. after addition of the internal standard in formic acid, they are quite stable. Storage for 6 months at 4 or  $-20^{\circ}\text{C}$  and for 1 week at room temperature showed no change in SCFA concentrations.

The detection limit amounted to 0.1 mmol/liter for  $\text{C}_2$  and to 0.02–0.05 for  $\text{C}_3$ – $\text{C}_6$ , which is suitable for the analysis of fecal samples.

TABLE 2

The Intraassay and Interassay Reproducibility, as Measured for One Fecal Supernatant (Sample A) and for One SCFA Standard Solution (Sample B)

	Acetic acid	Propionic acid	<i>i</i> -Butyric acid	<i>n</i> -Butyric acid	<i>i</i> -Valeric acid	<i>n</i> -Valeric acid	<i>n</i> -Caproic acid
Intraassay for sample A							
Concentration (mmol/liter), mean $\pm$ SD ( $n = 8$ )	80.6 $\pm$ 2.2	23.8 $\pm$ 0.5	2.2 $\pm$ 0.2	13.6 $\pm$ 0.3	3.7 $\pm$ 0.2	3.0 $\pm$ 0.1	1.0 $\pm$ 0.1
CV <sup>a</sup> (%)	2.7	2.1	9.1	2.2	5.4	3.3	10.0
Intraassay for sample B							
Concentration (mmol/liter), mean $\pm$ SD ( $n = 8$ )	50.9 $\pm$ 1.3	22.5 $\pm$ 0.5	11.2 $\pm$ 0.2	11.2 $\pm$ 0.3	10.1 $\pm$ 0.2	11.4 $\pm$ 0.2	10.8 $\pm$ 0.2
CV (%)	2.6	2.2	1.8	2.7	2.0	1.8	1.9
Interassay for sample A							
Concentration (mmol/liter), mean $\pm$ SD ( $n = 8$ )	78.6 $\pm$ 3.3	24.3 $\pm$ 0.4	2.4 $\pm$ 0.1	14.1 $\pm$ 0.2	3.9 $\pm$ 0.2	3.1 $\pm$ 0.1	1.1 $\pm$ 0.1
CV (%)	4.2	1.6	4.2	1.4	5.1	3.2	9.1
Interassay for sample B							
Concentration (mmol/liter), mean $\pm$ SD ( $n = 8$ )	51.1 $\pm$ 1.5	22.7 $\pm$ 0.5	11.3 $\pm$ 0.2	11.3 $\pm$ 0.2	10.1 $\pm$ 0.2	11.2 $\pm$ 0.2	10.7 $\pm$ 0.4
CV (%)	2.9	2.2	1.8	1.8	2.0	1.8	3.7

<sup>a</sup> Coefficient of variation.

TABLE 3  
The Concentration Characteristics of SCFA in Human Fecal Supernatant

	Acetic acid	Propionic acid	<i>i</i> -Butyric acid	<i>n</i> -Butyric acid	<i>i</i> -Valeric acid	<i>n</i> -Valeric acid	<i>n</i> -Caproic acid	Total SCFA
Concentration (mmol/liter), mean $\pm$ SD ( $n = 24$ )	74.1 $\pm$ 27.6	22.4 $\pm$ 10.8	1.9 $\pm$ 1.0	13.6 $\pm$ 6.2	3.4 $\pm$ 2.1	2.8 $\pm$ 1.1	1.3 $\pm$ 1.0	119.5 $\pm$ 44.9
Range (mmol/liter)	25.0–121.5	6.2–47.6	0.6–5.0	3.2–28.5	1.0–10.2	0.8–5.1	0.0–3.4	37.7–202.7
Percentage of total, mean $\pm$ SD ( $n = 24$ )	62.1 $\pm$ 4.7	18.4 $\pm$ 3.5	1.7 $\pm$ 0.7	11.2 $\pm$ 2.1	3.2 $\pm$ 1.7	2.4 $\pm$ 0.5	1.0 $\pm$ 0.6	
Range (%)	50.2–70.4	13.3–26.5	0.5–2.8	8.3–15.3	0.6–6.1	1.3–3.2	0.0–2.5	

#### Concentration of SCFA in Fecal Supernatant

The characteristics of the SCFA concentrations in human fecal supernatant are depicted in Table 3. Acetic acid, propionic acid, and *n*-butyric acid are quantitatively the most important ones and constitute about 90% of intestinal SCFA in molar ratios of ca. 68:20:12.

#### Freeze-Dried Fecal Samples

Concentrations of SCFA after freeze-drying were compared with those before freeze-drying. Recoveries after freeze-drying were almost quantitative (mean  $\pm$  SD,  $n = 4$ : C<sub>2</sub>, 101  $\pm$  10; C<sub>3</sub>, 93  $\pm$  7; *i*-C<sub>4</sub>, 90  $\pm$  3; C<sub>4</sub>, 91  $\pm$  7; *i*-C<sub>5</sub>, 91  $\pm$  7; C<sub>5</sub>, 93  $\pm$  9; C<sub>6</sub>, 101  $\pm$  3). Almost no losses of SCFA were observed during freeze-drying of fecal samples. The pH as measured in 40 fecal supernatants amounted to 6.50  $\pm$  0.24 (range 6.1 to 6.9). The  $pK_a$  of the SCFA (4) is approximately 4.8. Thus, at fecal pH the SCFA are in ionized nonvolatile form for more than 95%, explaining the almost quantitative recovery after freeze-drying. One might also make the samples alkaline before freeze-drying to prevent any loss of SCFA during lyophilization (15).

#### Comparison of the Direct Injection Method with the Vacuum Distillation Method

The direct injection technique as described in this paper was compared with the most often used technique of vacuum distillation, for three samples, one fecal supernatant, a fecal homogenate 1, and a fecal homogenate 2, the latter spiked with stock solution A (2 vol of fecal homogenate and 1 vol of solution A). The absolute peak areas before and after vacuum distillation were compared, to assess potential losses of SCFA during vacuum distillation. The thus obtained absolute recoveries are shown in Table 4. The most volatile SCFA C<sub>2</sub> and C<sub>3</sub> showed a loss of, respectively, 20–30 and 15–25% during vacuum distillation, whereas the loss of the higher SCFA C<sub>4</sub>–C<sub>6</sub> amounted to only 0–

10%. Of course, these losses are accounted for when vacuum-distilled standards are used.

#### DISCUSSION

Many pitfalls have been found in the gas chromatographic analysis of SCFA (23, 24) such as peak tailing due to adsorption, ghost peaks after repeated injections, double peak formation, azeotrope formation, or dimerization and loss by evaporation during prechromatographic manipulations. Van Eenaeme *et al.* (22) stated that the injection area which includes the injector, the column plugs, and the column top might be the principle cause of ghosting. The results in the present paper show that this also holds for the phenomena of peak tailing, peak broadening, and double peak formation. All these disturbances can be prevented by using formic acid in the injected sample and by using the technique of injection against the hot glass wall of an empty liner or by using injection between glass balls in a glass liner, the liner acting as a precolumn. Injection of the sample against hot glass appears to be very important for obtaining sharp peaks. Broad unresolved peaks were seen after injection in the gas phase of an empty liner. This might be explained by a temperature difference. The glass wall of the liner and the glass balls have about the same temperature as the injector (200°C), whereas the temperature in the gas phase of the liner is surely lower, mainly due to cooling by the N<sub>2</sub> carrier gas stream through the liner. Injection in the gas phase might therefore result in a slower evaporation of the sample and as a consequence of that in peak broadening.

With the technique described in the present study, fecal samples can be analyzed by GC without any pretreatment. The use of a precolumn protects the GC column against serious contamination with nonvolatile fecal material. The same gas chromatographic column is now in use for about 3 years for fecal SCFA. Despite a brown coloring of the first part of the column after some 10,000 injections of fecal samples, the retention

TABLE 4

Absolute Recovery of SCFA after Vacuum Distillation of a Fecal Supernatant, of a Fecal Homogenate 1 and of a Fecal Homogenate 2 Spiked with SCFA

	Acetic acid	Propionic acid	<i>i</i> -Butyric acid	<i>n</i> -Butyric acid	<i>i</i> -Valeric acid	<i>n</i> -Valeric acid	<i>n</i> -Caproic acid
Fecal supernatant (mmol/liter)	64.2	14.4	0.6	27.9	0.6	1.4	3.5
Absolute recovery (%)	82	87	90	90	95	94	103
Fecal homogenate 1 (mmol/liter)	22.7	4.0	0.3	4.3	0.2	0.6	0.7
Absolute recovery (%)	71	75	90	90	100	90	100
Fecal homogenate 2 (mmol/liter)	111.5	41.7	19.2	22.8	17.1	19.2	16.8
Absolute recovery (%)	75	85	92	90	94	93	103

times of the SCFA remained stable and no deterioration of the column was observed, again stressing the fact that the injection technique is more important for a good separation than the condition of the column.

The use of about 10% formic acid in the injection sample is completely safe as was also shown by Cochran (23). No corrosion of the metal parts of the gas chromatograph was observed, not even after 10 years.

In the previous literature about GC analysis of SCFA in aqueous solution, it was very common to add phosphoric acid to the glass wool and/or to the sample (21, 23–25). In the present study, we used dichlorodimethylsilane-treated glass wool in the liner. However, different forms of glass wool (untreated, phosphoric acid-treated, and silanized) all gave the same results when injecting 9% formic acid test samples (data not shown), eliminating any influence of the glass wool on the SCFA separation in our setting. As previously reported (21–23), the use of formic acid as injection solvent is essential to prevent the adsorption of SCFA in the column, thereby eliminating peak tailing and ghosting. Another advantage of the use of formic acid in the sample is the high stability of the SCFA-end solutions, this in contrast to, e.g., phosphoric acid-end solutions, the latter resulting in a substantial reduction of SCFA within 30 min at room temperature, due probably to micellar separation (21). Compared with the volatile formic acid, use of the nonvolatile phosphoric acid will also result in a faster contamination of the injection area, resulting in peak broadening.

In the past, direct injection of urine onto the GC column was used to measure the SCFA in urine (26). No recovery and precision were determined in that study. The life span of the column was very shortened, due to contamination of nonvolatile compounds onto the column. The present method of direct injection in a glass precolumn may also be used for measuring SCFA in other biological fluids, e.g. in urine.

The fecal SCFA concentrations in normal healthy subjects found in this study were similar to those found by other groups (6, 13, 18, 20, 27–29) but were about twice as high as those found by Scheppach *et al.* (19).

We have no obvious explanation for this discrepancy. Acetic, propionic, and *n*-butyric acid are quantitatively the most important ones and constitute about 90% of intestinal SCFA in a molar ratio of 68:20:12.

In conclusion, the direct injection method as presented in this paper is a rapid, sensitive, and reliable procedure for measuring fecal SCFA. When analyzing fecal homogenates, one SCFA analysis requires only 10 min, including sample preparation time. This opens the possibility of analyzing many samples in a short time, facilitating research in this field.

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