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Headspace solid-phase microextraction with 1-pyrenyldiazomethane in-fibre derivatisation for analysis of faecal short-chain fatty acids

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Abstract

Short chain fatty acids (SFCAs) are nutritionally important products of colonic bacteria. Their analysis in faeces is problematic. We report a headspace solid-phase microextraction procedure in which faecal SCFAs are derivatised on the fibre in-situ with 1-pyrenyldiazomethane. With this method sharp, well-resolved chromatographic peaks were obtained with no interferences. Inclusion of deuterated analogues enabled accurate quantification. Good linearity, recoveries and precision were achieved. Differences observed between the SCFA profiles of normal subjects and patients with cystic fibrosis indicate the potential of this new technique for clinical studies. 2-Methylbutyric acid was found in all faecal samples. Few have reported this before.

Keywords: Derivatisation, GC; 1-Pyrenyldiazomethane; Short-chain fatty acids

1. Introduction

The human colon normally harbours at least 175 g wet mass of bacteria [1]. They obtain energy and nitrogen for growth by fermenting carbohydrates and proteins in the colonic lumen, mainly polysaccharide of plant cell walls (dietary ''fibre'') some starch and secreted glycoproteins. The end-products include around 200 to 400 mmol/day of C_2–C_6 short-chain fatty acids (SCFAs). Over 90% are absorbed and used by the host, contributing 2 to 10% of daily energy requirements [1,2]. This is particularly important in premature babies with lactose malabsorption [3].

Extensive disease of the bowel, surgical bypass, prolonged antibiotic therapy, total parenteral nutrition, malabsorption, immaturity [4,5] and wide variation in dietary fibre intake may all disturb this important host/bacterial symbiosis [1,2]. Since faecal SCFAs reflect colonic fermentation [6] they are measured in studies to investigate and manipulate such disturbances.

Analysis usually requires isolation of SCFAs from faeces followed by gas chromatography (GC) with or without mass spectrometry (MS). Methods have included solvent extraction, partition chromatography, vacuum distillation and steam distillation, sometimes with a trapping procedure, or analysis of headspace (HS) vapour [1,6–8]. However, because SCFAs are volatile and adsorb readily to hot metal and glass surfaces, recoveries are often poor. In-

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corporation of formic acid vapour in the HS or GC carrier gas overcomes quantification problems arising from irregular peak shapes, peak tailing and ghosting [8–10] but with the risk of corrosion. Esterification of SCFAs prior to GC may lead to poor recoveries [10].

Solid-phase microextraction (SPME) is a recently introduced technique for extracting volatile and semi-volatile compounds from a variety of matrices [11]. The method uses a modified syringe assembly which houses a short fused-silica fibre externally coated with a GC stationary phase [12]. A range of phases [e.g. polydimethylsiloxane (PDMS), polyacrylate (PA), Carbowax–divinylbenzene] is now available. The technique involves partitioning analytes between the stationary phase and sample, followed by desorption of the analytes into the analytical instrument [12–14]. SPME can be operated in two modes, either HS sampling or with immersion directly into the sample [12]. The majority of analyses to date have been of underivatised compounds [15–17], including one report for the analysis of SCFAs in cheese [18]. However, in a novel application, C_2–C_{10} fatty acids in HS vapour were trapped on a fibre coated with a relatively polar PA phase which had been impregnated with a derivatising agent, 1-pyrenyldiazomethane (PDAM) [19]. This reagent requires mild derivatising conditions, has low volatility, is relatively stable and forms stable reaction products. The adsorbed acids were derivatised in-situ to pyrenylmethyl esters and then desorbed in a hot GC injection port. By combining extraction and derivatisation, there was reduced risk of sample loss. Chromatographic resolution was good and inclusion of isotopically labelled analogues enabled accurate quantification. The method has been applied to analysis of SCFAs in sewage sludge [19] and milk [20]. We report a GC–MS SPME method using PDAM in-fibre derivatisation for SCFAs in faeces that incorporates deuterated internal standards for accurate quantification.

2. Experimental

2.1. Faecal samples and materials

All faecal samples for method development were from one healthy adult on a normal diet, collected onto foil and transferred immediately to screw-capped glass containers. These were completely filled to minimise HS losses. Samples were analysed fresh, or frozen immediately and stored at −20°C. Fluid draining via an ileostomy from a four-month-old milk-fed baby who had undergone bowel resection was stored in a container at −20°C. The Institute of Nutrition, University of Southampton, donated samples from one adult and two patients with cystic fibrosis taking part in an ethically approved study. Samples collected onto polythene were frozen immediately on solid CO_2 and stored at −20°C. After partial thawing, the samples were homogenised in water (1.5:1 water-to-faeces), aliquoted into 20-ml silanised glass bottles and frozen at −20°C until analysis.

Polyacrylate (85 μm film thickness) SPME fibres and SPME fibre syringe holders were from Supelco (Poole, UK). Headspace vials (22 ml) with soft silicone rubber seals (20 mm) and aluminium caps were from Alltech Associates (Carnforth, UK). Straight- and branched-chain fatty acid (C_1–C_8) standards and deuterated D_1 formic, D_3 acetic and D_2 propionic acid standards were from Sigma–Aldrich (Poole, UK). Analytical grade chemicals and other reagents were from Sigma–Aldrich or Fisons (Loughborough, UK). PDAM derivatising reagent was from Molecular Probes (Leiden, The Netherlands) and was used without further purification. PDAM should be considered hazardous and handled with care. Water was deionised by reverse osmosis.

Aqueous deuterated fatty acids standards were prepared as concentrated stock solutions (formic 1063, acetic 873, propionic 657 mmol/l) and aliquots stored in sealed glass ampoules at −20°C.

Glassware, headspace vials and magnetic stirrers were silanised for 1 h in a solution of dichlorodimethylsilane (approximately 10%, v/v, in cyclohexane), thoroughly washed with methanol and dried prior to use.

2.2. Instrumentation

A GC system (Model 5890 series 2) linked to a bench-top quadrupole MS instrument (5971A) (Hewlett-Packard, Bracknell, UK) fitted with a narrow bore (0.75 mm I.D.) SPME injection liner (Supelco) was used for analyses. The GC column was a BPX-5

fused-silica capillary column (30 m×0.22 mm I.D., film thickness 0.25 μm) from Scientific Glass Engineering (Milton Keynes, UK). Helium was used as the carrier gas at a flow-rate of 1 ml/min.

2.3. Sample preparation (definitive procedure)

For most of the development work, approximately 1 g of thawed faeces was analysed. For the recovery experiments and clinical studies a small scoop of faecal homogenate or ileostomy fluid (approximately 2 ml) was used. Working on ice, fresh or freshly thawed sample was placed in a pre-weighed silanised 22 ml HS vial and weighed quickly. Three additional aliquots were dried overnight in a muffle furnace to establish faecal dry mass. To each HS vial were added quickly a magnetic stirrer, approximately 3.0 g of sodium chloride, 8 ml of water and 500 μl of working deuterated I.S. mixture (D_1 formic acid 53.16 μmol, D_3 acetic acid 21.83 μmol, D_2 propionic acid 8.22 μmol per vial). Vials were capped with a septum, crimped, vortex mixed vigorously and kept on ice until analysis (same day).

2.4. SPME derivatisation and extraction procedure

The procedure was based on Pan et al. [19]. PA fibres were pre-conditioned before use by inserting them into the GC injector according to the manufacturer's instructions. Fibres were loaded with derivatising reagent by placing them into an approximately 5 mg/ml PDAM–*n*-hexane solution for 15 min. Excess *n*-hexane was allowed to evaporate at room temperature before beginning sample extraction. Because it is light-sensitive, all vials containing PDAM were kept in the dark. The solution was stored at −20°C and could be used for several weeks.

Vials were mounted on a magnetic stirrer submerged in a water bath maintained at 50°C and the contents stirred continuously so as to release SCFAs into the HS. The septum of the sample vial was pierced with the SPME needle guide and the loaded SPME fibre exposed to the HS vapour for 30 min. The extracted, derivatised compounds were then desorbed (4 min) from the fibre in the GC–MS injector port, split valve closed for 4 min. The GC–MS system was operated under the following conditions: solvent delay 16 min; injector 260°C; interface transfer line 280°C; dual ramp oven temperature programme 100°C (2 min) then 20°C/min to 280°C (1 min) then 2°C/min to 310°C (10 min). The MS system was operated in the single ion monitoring mode for the specific fatty acid ions [*m*/*z*: formic, 260; D_1 formic, 261; acetic, 274; D_3 acetic, 277; propionic, 288; D_2 propionic, 290; isobutyric and *n*-butyric, 302; 2-methylbutyric, isovaleric and *n*-valeric, 316; *n*-hexanoic and isocaproic, 330; heptanoic, 344; 2-propylpentanoic (valproic), 2-ethylhexanoic and octanoic, 358]. The detector signals were collected, integrated and recorded using a HP Chemstation (Hewlett-Packard). With these conditions no carry over of acids was found between runs.

The amount of formic, acetic and propionic acid per vial was calculated from their peak areas related to their deuterated I.S. analogues. C_4–C_6 acids were quantified using D_2 propionic as I.S. with appropriate relative response factors (RRFs). RRFs were obtained by analysis of aqueous standard mixtures of different concentrations. For SCFAs found in large amounts in faeces the selected RRFs were *n*-butyric, 2.72; isobutyric, 3.87 (see Results). For other acids, RRFs were: *n*-valeric, 5.36; isovaleric and 2-methylbutyric, 6.43; *n*-hexanoic, 14.38; isocaproic, 13.14. Concentrations were calculated as μmol/g faecal dry mass.

3. Results

3.1. Retention times, response factors and mass spectra

With the definitive procedure, the peak shapes for the SCFA pyrenylmethyl esters were sharp and the derivatives of straight- and branched-chain acids were well resolved (Fig. 1). Of note, formic acid was extracted from aqueous solution and derivatised successfully. The branched-chain fatty acids eluted earlier than their straight-chain analogues. The unsaturated fatty acids, butenoic, butynoic, 2-*trans*-hexenoic and octenoic acid could also be analysed by this procedure and eluted much later than their respective saturated acids (data not shown). 2-Ethylhexanoic acid, an analytical contaminant from the plasticiser di-(2-ethylhexyl)-phthalate (DEHP) [21] produced a peak which co-eluted with valproic acid (Fig. 1). No SCFA peaks were found with a

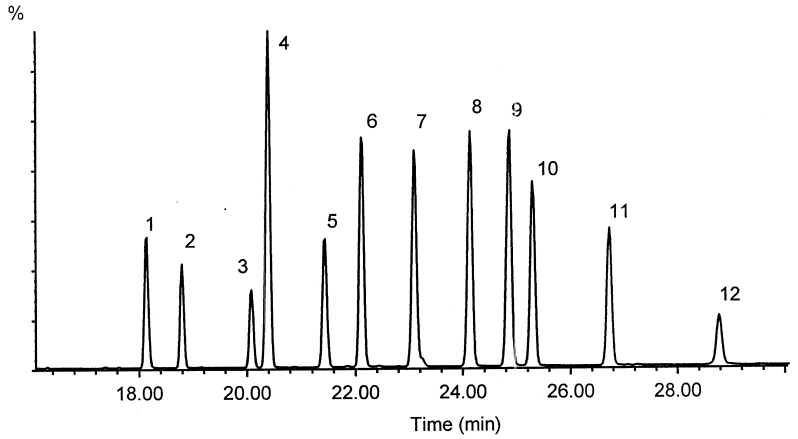

Fig. 1. GC–MS single ion monitoring profile of SCFAs using simultaneous derivatisation and extraction with PDAM. An 85-μm PA SPME fibre was loaded with derivatising reagent for 15 min at room temperature. The loaded fibre was exposed to the HS vapour of an aqueous solution of C_1–C_8 SCFA standards (concentrations/vial ranging from 4 μmol for *n*-octanoic acid to 53 μmol for formic acid). The vial was heated to 50°C and the HS vapour sampled for 30 min and desorbed for 4 min. Key to PDAM derivatised acids: (1) formic, (2) acetic, (3) propionic, (4) isobutyric, (5) *n*-butyric, (6) isovaleric, (7) *n*-valeric, (8) isocaproic, (9) *n*-hexanoic, (10) valproic or 2-propylpentanoic, (11) *n*-heptanoic, (12) *n*-octanoic.

procedural blank. Response factors increased with increasing chain-length and, except for C_6 acids, were higher for branched-chain than straight-chain isomers. For an aqueous standard mixture of SCFAs ranging in amounts from 3.97 μmol of isocaproic acid to 53.01 μmol of formic acid per vial, response factors relative to 8.2 μmol of deuterated propionic acid were: formic, 0.12; acetic, 0.42; *n*-butyric, 2.72; isobutyric, 3.87; *n*-valeric, 6.55; isovaleric, 7.62; *n*-hexanoic, 14.26; isocaproic, 12.79.

Electron impact mass spectra of the PDAM esters were simple showing intense molecular ions for all of the derivatised acids tested which were suitable for selected ion monitoring in the stable isotope dilution assay. The ion fragment at *m*/*z* 215 (Fig. 2) was thought to arise from cleavage of the bond

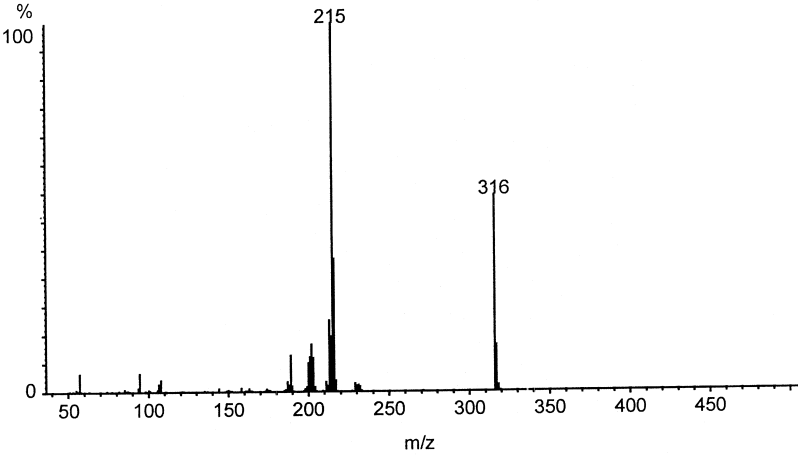

Fig. 2. Electron impact (70 eV) mass spectrum of the PDAM derivative of 2-methylbutyric acid.

between the pyrenylmethyl group and the fatty acid methyl mono-ester [19,22].

3.2. Incubation temperature, sampling time, salting out, carry over

Using an incubation time of 30 min [8], the effects of incubation at 30, 40, 50 and 60°C were investigated for 275 to 383 (mean 318) mg dry mass of faeces. Raising the temperature from 30 to 40°C resulted in a 2.1 to 6.5 (median 4.9)-fold increase in peak area/mg of the eight SCFAs identified in the samples. The increment increased with chain length. The peak areas of C_1–C_3 acids plateaued at 40°C but those of C_4 and C_5 acids continued to increase to 60°C. A temperature of 50°C was selected since good peak areas were obtained and there is risk of loss of PDAM from the fibre at high temperatures under humid conditions [19].

The effect of incubation time from 10, 15, 30, 45, 60 min at 50°C was investigated for 336 to 408 (mean 360) mg dry mass of faeces (Fig. 3). Peak areas for all C_2–C_5 SCFAs identified increased with time, with no evidence of plateauing at 60 min. Pan et al. [19] made similar observations for propionic and butyric acids. The formic acid peak area was low throughout, plateaued from 15 to 45 min and decreased at 60 min. Thirty min was selected for acceptable throughput of samples, since sensitivity was adequate.

In a previous study [8] we found that adding inorganic salts to faeces in saturating amounts increased the concentration of SCFAs in a standard HS procedure. Lithium sulphate was most effective [8]. However, this proved unsatisfactory with the SPME PDAM analysis since it caused rapid disintegration of the fibre coating. We therefore selected sodium chloride [19]. Pan et al. [19] observed increased SCFA recoveries with acidification to pH 1.5, but loss of fibre coating at pH 1.0. We found that acidification of aqueous SCFA mixtures to pH 2.0 with HCl caused fibre stripping. Because fine adjustment of the pH of faecal samples is difficult, with risk of evaporation of the acids, we opted not to acidify the samples. Using our conditions the fibre

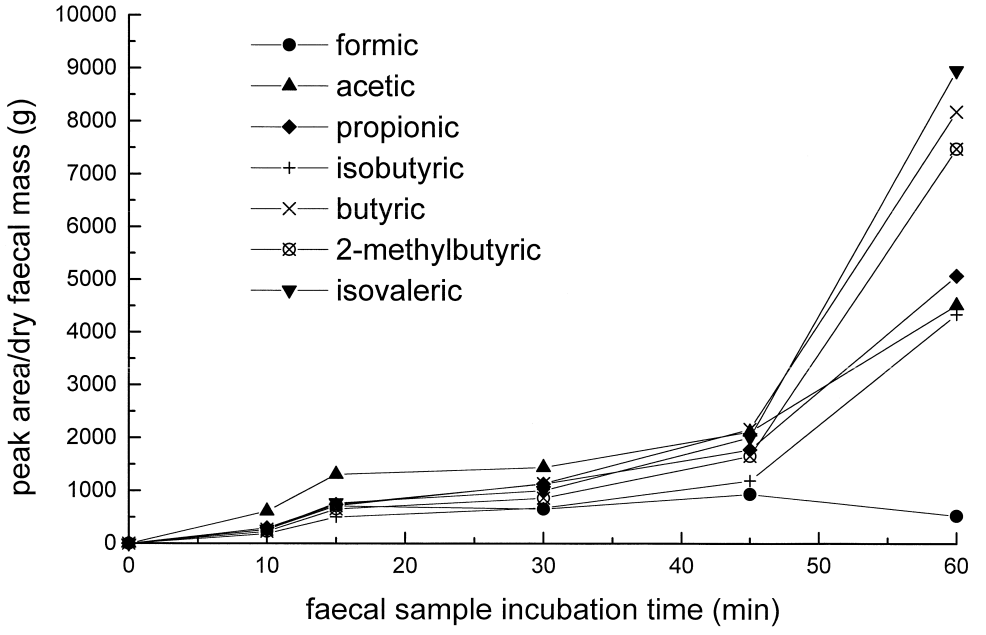

Fig. 3. Effect of incubation time at 50°C on the amounts of fatty acid released from faeces. 0.336–0.408 g dry mass of faeces was analysed using the conditions described in Fig. 1, but varying incubation times from 10 to 60 min.

Table 1
Linearity of SCFAs in aqueous solution ($n=5$)

Acid	Range (nmol/vial)	Regression equation	R^a	SD[b]
Formic	106.0–53009	$y=0.00002x+0.03375$	0.9983	0.0247
Acetic	35.0–17338	$y=0.00004x+0.01340$	0.9997	0.0096
Propionic	13.0–6702	$y=0.00013x+0.00294$	0.9999	0.0049
Isobutyric	11.0–5391	$y=0.00047x-0.00527$	1.0000	0.0037
n-Butyric	11.0–5471	$y=0.00033x-0.00469$	1.0000	0.0034
Isovaleric	9.0–4588	$y=0.00093x+0.00200$	1.0000	0.0191
n-Valeric	9.0–4597	$y=0.00080x+0.00430$	0.9999	0.0218
Isocaproic	8.0–3973	$y=0.00155x+0.04416$	0.9996	0.0866
n-Hexanoic	8.0–3990	$y=0.00173x+0.04657$	0.9996	0.0950

[a] R=Regression coefficient.
[b] SD=Standard deviation.

still performed well after analysis of 25 faecal samples.

We investigated the effect of using silanised incubation vials and magnetic stirrers. The peak areas of eight SCFAs in normal faeces were 1.5 to 3.4 (median 2.5)-times higher with silanised than unsilanised vials. Silanised apparatus was therefore used for the procedure. SCFA derivatives were desorbed completely from the fibre within 4 min [19] with no carry over between samples.

3.3. Linearity, detection limits, imprecision, recoveries

The procedure was linear for SCFAs in aqueous solution over a wide concentration range (Table 1). Formic acid was still detectable at 11 nmol per vial, acetic at 4 nmol, propionic at 2 nmol and C_4–C_6 acids at 1–2 nmol.

Increasing amounts of a normal faecal sample were analysed for SCFAs (Table 2). For C_2–C_6 acids, good linearity was observed over a ten-fold increase in faecal dry mass from 0.0488 to 0.4825 g. Only small amounts of isocaproic and *n*-hexanoic acids were detected in this sample. The response for formic acid was linear from 0.1051 to 0.4825 g dry mass.

Imprecision was assessed by analysis of eight aliquots of normal, homogenised, faeces (Table 3). Precision was good for the three major SCFAs, acetic, propionic and *n*-butyric acids (RSD 2.4–5.0%) and acceptable for acids present in lower concentrations. Precision was worst for formic acid.

Two experiments were undertaken to investigate

Table 2
Linearity of faecal SCFAs

Acid[a]	Range (μmol acid per vial)	Regression equation	R^b	SD[c]
Formic	2.180–7.180	$y=11.85812x+1.29181$	0.9390	0.6969
Acetic	1.880–32.420	$y=73.99210x-3.70182$	0.9867	1.9891
Propionic	0.840–14.890	$y=34.02105x-1.68449$	0.9876	0.8844
Isobutyric	0.138–2.428	$y=5.57246x-0.25210$	0.9878	0.1439
n-Butyric	0.418–7.714	$y=17.64425x-0.82055$	0.9894	0.4230
2-Methylbutyric	0.133–2.235	$y=5.09155x-0.19573$	0.9892	0.1236
Isovaleric	0.174–2.849	$y=6.46304x-0.25569$	0.9896	0.1534
n-Valeric	0.195–3.572	$y=8.10090x-0.30681$	0.9920	0.1688
Isocaproic	0.0003–0.0041	$y=0.00899x-0.00021$	0.9931	0.0002
n-Hexanoic	0.0044–0.0692	$y=0.15541x-0.15541$	0.9910	0.0034

[a] C_2–C_6 acids $n=7$, faecal dry masses 0.0488–0.4825 g; formic acid $n=6$, faecal dry masses 0.1051–0.4825 g.
[b] R=Regression coefficient.
[c] SD=Standard deviation.

Table 3
Within-batch imprecision of faecal SCFAs

Acid	Concentration (μmol/g dry mass)		RSD[b] (%)
	Mean (n=8)	SD[a]	
Formic	988.24	151.51	15.30
Acetic	149.54	5.02	3.30
Propionic	41.59	0.99	2.40
Isobutyric	9.40	0.96	10.20
n-Butyric	54.94	2.73	5.00
2-Methylbutyric	8.30	0.92	11.10
Isovaleric	9.22	0.90	9.80
n-Valeric	9.76	1.24	12.70

[a] SD=Standard deviation.
[b] RSD=Relative standard deviation.

analytical recovery by adding aqueous standard mixtures as spikes to faeces (dry masses 289–360 mg and 127–146 mg in experiments 1 and 2, respectively; Table 4). Recoveries of formic, acetic and propionic acids were calculated from deuterated internal standards. Those of C_4–C_6 acids were estimated using deuterated propionic acid as I.S. and appropriate response factors obtained from the analysis of aqueous standard mixtures. In both experiments, recoveries for acetic acid (all >82.3%) and propionic acid (all >93.3%) were good. Good recoveries for *n*-butyric acid (>82.2%) and isobutyric acid (>73.1%) were observed in both, and for *n*-valeric acid (>95.5%), isovaleric acid (>77.1%) and *n*-hexanoic acid (>85.6%) in experiment 1. Results for C_5 and C_6 acids from experiment 2 were not reported since an unsuspected injector blockage led to drift in their response factors. Recovery of formic acid was unacceptable. In experiment 1 the mean recovery was only 18.8%. In experiment 2, peaks of both deuterated I.S. and unlabelled acid were undetectable in two of the three unspiked vials and two of the three with a low spike.

3.4. Clinical studies

In a preliminary experiment to find out whether the analysis would be useful in a clinical setting, results for normal faeces analysed during method development were compared with those for two patients with cystic fibrosis and for small intestinal fluid from a four-month-old baby (Table 5). Well-resolved sharp peaks of the derivatised SCFAs with no interference from other compounds were observed for all of the samples (Figs. 4 and 5). Satisfactory analyses were achieved with as little as 109 mg faecal dry mass (CF2). In all faecal samples an unknown peak with m/z 316 eluted at 21.8 min between *n*-butyric and isovaleric acids. This was likely to be a C_5 acid. By comparison with authentic standards it was identified as 2-methylbutyric acid. Standard pivalic acid (trimethylacetic acid), a C_5 acid identified in sewage sludge [19], eluted earlier, with a retention time of 20.4 min. This acid was not found in faecal samples.

There were clear differences in the SCFA profiles (Table 5). In normal faeces (Fig. 4) the largest peaks by GC–MS were for acetic, propionic and *n*-butyric acids although formic acid, which has a low response factor, was the most abundant acid on quantification.

Table 4
Recoveries of SCFAs from faeces

Acid	Experiment 1 (n=3)			Experiment 2					
	Mean faecal SCFA (μmol)	SCFA added (μmol)	% Recovery range (median)	High spike (n=2)			Low spike (n=3)		
				Mean faecal SCFA (μmol)	SCFA added (μmol)	% Recovery	Mean faecal SCFA (μmol)	SCFA added (μmol)	% recovery range (median)
Formic	0.73	26.51	16.7–23.0 (18.8)	2.49	53.01	63.4; 80.0	2.52	13.25	64.0[a]
Acetic	38.40	8.67	82.3–97.8 (90.8)	8.54	17.34	92.6; 94.3	8.02	4.34	87.3–91.7 (88.1)
Propionic	12.31	3.35	94.7–102.4 (96.5)	3.85	6.70	100.3; 101.2	3.61	1.68	93.3–95.3 (93.8)
Isobutyric	2.61	2.70	78.2–88.6 (82.9)	0.26	5.39	73.1; 77.1	0.37	1.35	82.6–89.8 (84.4)
n-Butyric	16.97	2.74	89.8–95.2 (93.2)	2.96	5.47	82.2; 85.8	2.78	1.37	87.6–107.9 (103.0)
Isovaleric	2.04	2.29	77.1–92.9 (86.6)						
n-Valeric	2.48	2.30	95.5–107.2 (97.5)						
n-Hexanoic	1.79	2.00	85.6–100.3 (93.6)						

[a] n=1: deuterated and unlabelled formic acid not detectable in two vials.

Table 5
SCFA concentrations of normal faeces, faeces from two patients with cystic fibrosis (CF) and of ileostomy fluid

SCFA	Acid concentration (μmol/g dry mass)			
	CF1[a]	CF2[a]	Ileostomy[b]	Normal[c]
Formic	30.66	137.42	20.93	379.46
Acetic	772.00	517.99	2013.83	48.96
Propionic	74.72	51.93	125.35	17.71
Isobutyric	4.49	1.94	0.08	3.10
n-Butyric	66.40	5.05	2.47	8.00
2-Methylbutyric	3.73	1.26	0.06	2.77
Isovaleric	0.62	1.38	0.16	3.28
n-Valeric	0.75	0.11	Trace	3.70
Isocaproic	0.14	0.14	0.02	ND[d]
n-Hexanoic	0.10	0.26	Trace	0.07
Dry mass (mg)	270	109	455	171–322 (mean 227)

[a] CF1 and CF2 were faeces from two patients with cystic fibrosis.
[b] The ileostomy fluid was from a milk-fed baby aged four months who had intestinal resection.
[c] The normal data was from the imprecision experiment.
[d] ND=Not detected.

There were large peaks of three branched-chain acids. Compared with normal, both cystic fibrosis samples had large amounts of acetic and propionic acids and sample CF1 had a very large *n*-butyric acid excretion. The profile of the ileostomy fluid (Fig. 5) was different, being dominated by acetic acid, with a large amount of propionic acid, but low concentrations of the C_4–C_6 acids.

4. Discussion

Measurement of faecal SCFAs can make an important contribution to clinical studies. However, analyses have been problematic. SPME with in-situ derivatisation is a new approach with considerable potential.

It is not an equilibrium process since the extracted

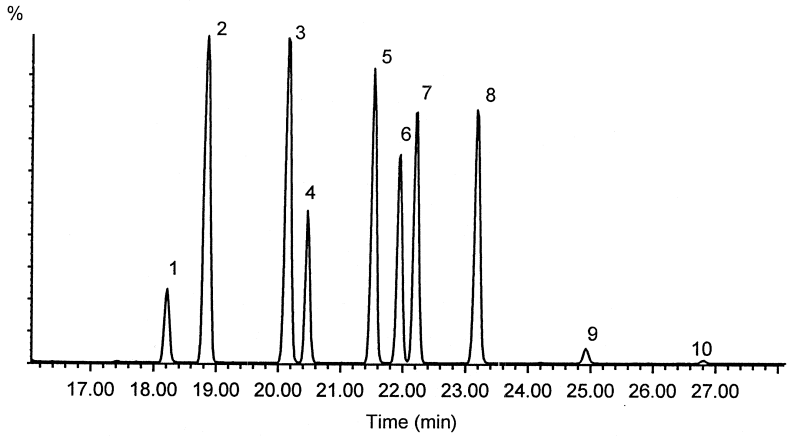

Fig. 4. Faecal SCFA profile of a normal adult on a normal diet. 0.193 g of dry mass of faeces was analysed using conditions as described in Fig. 1. Key to PDAM derivatised acids: (1) formic, (2) acetic, (3) propionic, (4) isobutyric, (5) *n*-butyric, (6) 2-methylbutyric, (7) isovaleric, (8) *n*-valeric, (9) isocaproic, (10) *n*-hexanoic.

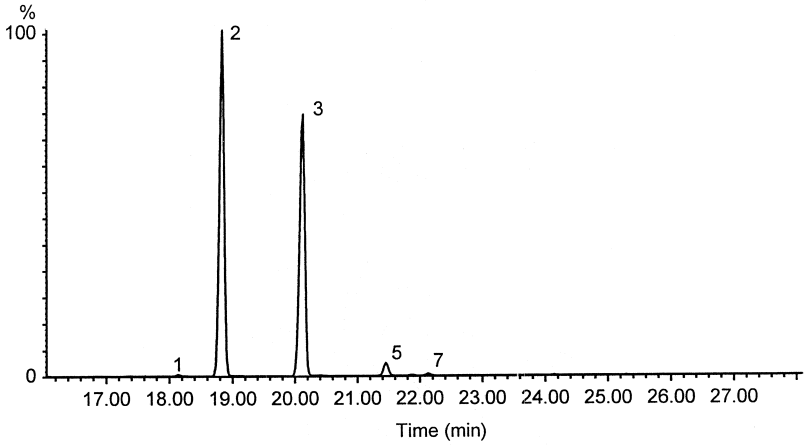

Fig. 5. SCFA profile of ileostomy fluid collected via a drain from a milk-fed baby aged four months, receiving no antibiotics. Approximately 0.455 g dry mass of fluid was analysed using conditions as described in Fig. 1. Peak identification as for Fig. 3.

analytes are converted to derivatives with a high affinity for the fibre coating. Pan et al. [19] investigated the procedure carefully and we followed their method in principle. A polar fibre is required. Sensitivity is poor with PDMS PDAM-coated fibres, probably because SCFAs do not penetrate the non-polar phase well. As is recognised in other HS analyses [23], saturation of the liquid phase with sodium chloride increased sensitivity. Lithium sulphate, which we have previously found effective for faecal HS procedures [8] was unsatisfactory since it caused damage to the fibre coating. This was also a problem with acidification of the matrix to less than pH 2.0 and we opted not to adjust the pH as in the reported method [19]. Raising the temperature during sampling increased the yield of derivatised acids, probably through increased mass transfer into the vapour phase. Sensitivity was also enhanced by silanisation of vials and stirrers, by removing free hydroxyl groups and hence reducing adsorption of polar SCFAs [10,19].

The partition of solute in a gas–liquid system depends upon the activity coefficient. Because of the complexity and variability of the faecal matrix, the only possible choice for calibration is by standard additions to the sample [23,24]. With MS, accurate quantification is possible with isotopically labelled analogues as I.S. [19]. Due to cost constraints, we used only D_1 formic, D_3 acetic and D_2 propionic acids in this exploratory investigation. For clinical studies, a longer chain deuterated acid (e.g. D_{11} hexanoic acid, Sigma–Aldrich) should be substituted for D_1 formic acid (see below). ^{13}C labelled SCFA analogues are an alternative [19].

The method performed well with faeces, with good linearity and precision, good recoveries for acetic and propionic acids quantified with their own labelled analogues, and acceptable recoveries for C_4–C_6 acids calibrated against D_2 propionic acid with allowance for relative response factors. Better results for these acids should be attainable with a more appropriate I.S.

Formic acid is not detectable with flame ionisation detection and hence by most GC procedures [8,9]. It was extracted and derivatised successfully from aqueous solution using the SPME-PDAM procedure, producing a sharp peak. Formic acid was detected in many of the faecal samples. However quantification was unsatisfactory. Peaks of both formic acid and D_1 formic acid added as I.S. were frequently very small or undetectable. Recoveries were poor and variable. Unlike other SCFAs, formic acid is an intermediate, and not an end-product of bacterial fermentation, and is converted readily to CO_2 and water [2,25]. Formic acid was probably metabolised by bacterial enzymes during incubation at 50°C. In addition, it is extremely volatile and losses are inevitable during sample preparation. For these reasons measurement of formic acid in faeces cannot be recommended. However, the procedure should be applicable to the

analysis of formic acid in fluids lacking faecal-type bacteria.

We found 2-methylbutyric acid in all faecal samples. This was first reported in normal faeces in 1986 [26], when it was identified by GC–MS. We are unaware of other reports and it is probable that the free acid is not well resolved with most GC columns [26]. It is produced from isoleucine by comparable reactions to isobutyric and isovaleric acids from valine and leucine, respectively [27]. Both these acids are well recognised faecal SCFAs and it would be surprising if 2-methylbutyric acid were not excreted as well. With SPME-PDAM, Pan et al. [19] identified a C_5 acid in sewage sludge as pivalic acid. However, this acid had a shorter retention time than 2-methylbutyric acid in our system and is not a known mammalian or bacterial metabolite. It is likely that they, too, were finding 2-methylbutyric acid.

Our preliminary quantitative data for normal faecal acids are comparable to our earlier study using HS analysis [3] and to published ranges for normal adults [6]. The profiles of the two cystic fibrosis patients differed from normal and from each other, presumably reflecting diet, malabsorption and/or antibiotic treatment. The profile for the ileostomy fluid was strikingly different, and lacked the protein-derived branched-chain acids. These observations support our view that SPME-PDAM will be valuable in a range of clinical studies. We have not addressed pre-analytical problems here. Clearly, careful storage and manipulation of samples is mandatory to minimise losses of these very volatile acids. We were careful to avoid contact with plastic containers, since the C_8 acid, 2-ethylhexanoic acid from the plasticiser DEHP [21], often produced a large contaminant peak in our parallel studies of urine collected into polystyrene containers.

In summary, HS-SPME is an exciting new approach to SCFA analysis in faeces, urine and other fluids. It is easy to perform, sensitive and, with stable isotope dilution, capable of accurate quantification of a group of compounds notoriously difficult to analyse well.

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