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Automated headspace gas chromatographic analysis of faecal short-chain fatty acids

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ABSTRACT

A method was developed and validated for analysis of faecal short-chain fatty acids using automated headspace gas chromatography. Quantification was by standard addition. Ghosting was minimized by lining the transfer tube from the headspace sampler to the gas chromatograph with deactivated fused silica and addition of formic acid to sample vials. Saturation of samples with lithium sulphate increased recoveries. The method was used to analyse small amounts of faecal matter collected from premature babies. Advantages of the technique are rapid, accurate, analysis of faecal specimens in batches, with minimum sample preparation.

INTRODUCTION

Short chain fatty acids (SFCAs, chain lengths C_1–C_6) are end products of bacterial fermentation of undigested carbohydrates or amino acids in the colon [1–3]. Normally, the sources are plant wall polysaccharides (dietary fibre), starch, sugar residues of mucus secreted into the colon and, in premature babies, undigested lactose [4]. Other simple sugars are substrates in patients with malabsorptive syndromes. SCFAs are absorbed readily into the body and provide energy. They also have important, beneficial, effects on the colonic mucosa [3]. After infancy, acetate, propionate and butyrate constitute 90% of intestinal SCFAs, in molar ratios of *ca.* 60:25:15, respectively. About 10–20% of SCFAs are excreted in faeces and probably reflect colonic production [5]. Measuring faecal SCFAs is important when the bowel microflora is altered in infancy by feeding with non-pathogenic bacteria ("probiotics") [6] and, perhaps, treatment with prolonged courses of antibiotics [7]. It is useful in older subjects with certain bowel disorders, and may find a place in selection of patients for treatment with SCFA supplements [3,8].

Analysis of SCFAs in faeces is problematic because of their volatility, reactivity and sample matrix effects [9]. The automated headspace method described was developed for batch analysis of small faecal samples collected from new-

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born babies. Accurate quantification by gas chromatography (GC) was achieved by minimizing peak ghosting with formic acid and use of standard addition [10].

EXPERIMENTAL

Patients and materials

Faecal samples were collected from premature babies, born before 34 weeks of gestation, nursed on the Neonatal Unit, Princess Anne Hospital, Southampton, UK. Samples were frozen immediately in air-tight containers and stored at −20°C. Aristar formic acid (acetate-free) and Analar lithium sulphate were from BDH (Poole, UK), and 2-ethylbutyric acid and Aristar acetic acid from Aldrich (Gillingham, UK). All other chemicals were of Analar grade and obtained from Sigma (Poole, UK). Water was distilled and deionized. All standard solutions were prepared weekly and stored at 4°C.

Instrumentation

The automated headspace sampler (HP19395A) and the gas chromatograph with a flame ionization detector (HP5890 series II) were from Hewlett Packard (Bracknell, UK). Data handling was performed by a Trio 2+ computing integrator (Trivector Systems International, Sandy, UK). The headspace analyser and the chromatograph were directly interfaced via a heated transfer line, maintained at 95°C to avoid condensation. In order to reduce ghosting of the acids on the hot metal surfaces, a length of narrow bore (0.25 mm), deactivated fused-silica tubing was inserted into the stainless steel transfer line and sealed in position with polyimide resin.

Sample preparation (definitive method)

A 1–10 g (wet weight) mass of faeces was homogenized using a laboratory-made hand blender in 10 ml of distilled water. A 2-ml aliquot was dried in a heated sand-bath and weighed. Two 4-ml aliquots were placed in headspace vials with 1 g of lithium sulphate and 0.5 ml of formic acid. A 1-ml volume of 2-ethylbutyric acid (15.62 m*M* in water) was added as the internal standard (I.S.) to one vial (A), and to the other (B), 1 ml of an aqueous standard mixture containing 15.62 m*M* of I.S. together with nine acids that may be present in faeces: acetic (35.72 m*M*), propionic (13.16 m*M*), *n*-butyric (10.87 m*M*), isobutyric (10.64 m*M*), *n*-valeric (9.26 m*M*), isovaleric (9.10 m*M*), hexanoic (7.81 m*M*), isocaproic (7.94 m*M*), and heptanoic (6.76 m*M*). For each pair of sample vials, a third vial ("formate") was prepared, containing only 5 ml of water, 0.5 ml of formic acid and 1 g of lithium sulphate. The vials were capped with a rubber septum and sealed. After incubation at 90°C for 30 min in the headspace analyser, they were pressurized with helium at 1 bar for 40 s, then vented for 4 s so that headspace vapour above the faecal mixture transferred to the sample loop of 1-ml capacity. The loop was then swept by a stream of helium for 1 min (flow-rate 100 ml/min) via the transfer line into the chromatograph. The sample loop was maintained at 95°C. The sequence of vials analysed was: formate; sample with I.S. (A); sample with I.S. plus mixed standards (B).

Gas chromatography

A megabore fused-silica column (25 m × 0.53 mm I.D.) coated with a 1.0-μm film of poly(ethylene glycol), BP-20 (SGE, Milton Keynes, UK) was used for the separation of the SCFAs. A FFAP capillary column (Alltech Associates, Carnforth, UK), tested in early studies, was unsatisfactory because of peak tailing and ghosting. Helium was the carrier gas, at a flow-rate of 3 ml/min, with a detector make-up gas flow-rate of 30 ml/min. The split injector (1:7 split ratio) and detector were maintained at 200°C. The oven temperature programme was adjusted to give a good separation of the SCFAs [initial temperature 50°C (2 min) then 40°C/min to 100°C (0.1 min), and 8°C/min to 215°C (1 min)]. The amounts of individual acids in the unspiked vial (A) were calculated from their integrated peak areas using the following formula:

$$\mu\text{mol of acid per vial} = \frac{A_1}{A_2 - A_1} \times S$$

where A_1 is the acid/I.S. peak-area ratio in un-

spiked sample (vial A), A_2 is the acid/I.S. peak-area ratio in spiked sample (vial B), and S is the amount (μmol) of standard added to vial B.

Results were expressed as μmol/g dry weight of faeces.

RESULTS

Retention times and response factors

The peak shapes for the nine standard SCFAs and I.S., were sharp, well resolved and showed little tailing (Fig. 1). Their absolute retention times were reproducible (Table I). The detector response was lowest for acetic acid and increased with acid chain length. When aqueous standard mixtures were injected directly into the chromatograph, there was no ghosting between samples.

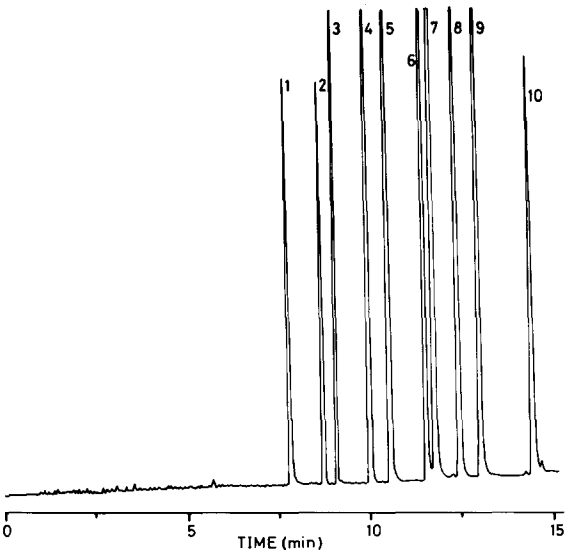

Fig. 1. GC profile of short-chain fatty acid standards. Headspace and chromatographic conditions as described in Experimental. Peaks (μmol): 1 = acetic (35.72); 2 = propionic (13.16); 3 = isobutyric (10.64); 4 = *n*-butyric (10.87); 5 = isovaleric (9.10); 6 = *n*-valeric (9.26); 7 = 2-ethylbutyric (I.S.) (15.62); 8 = isocaproic (7.94); 9 = hexanoic (7.81); 10 = heptanoic (6.76).

Headspace analysis

Incubation conditions: temperature, time, salting out. Using an incubation time of 30 min [11], the effects of incubating at 60, 70, 80 and 90°C were investigated for 2.5 μmol of hexanoic acid in aqueous solution. The concentrations of acid detected increased linearily as the temperature increased above 70°C, and 90°C was selected for maximum sensitivity. At higher temperatures, vials may explode. Using this temperature, the effect of increasing equilibration times in the range 30 to 90 min was investigated. The detected concentrations increased linearly from 30 to 60 min and then plateaued. A time of 30 min was selected for speed, because the sensitivity was ad-

TABLE I

RETENTION TIMES AND RELATIVE RESPONSE FACTORS OF SCFAs

Acid	Retention time			Relative response factor[a]		
	mean (n = 20) (min)	S.D. (min)	C.V. (%)	0.5 nmol	1.0 nmol	2.0 nmol[b]
Acetic	7.87	0.017	0.22	17	16	15
Propionic	8.82	0.013	0.15	41	43	37
Isobutyric	9.20	0.015	0.16	58	61	51
n-Butyric	10.08	0.015	0.15	61	61	53
Isovaleric	10.65	0.015	0.14	73	76	67
n-Valeric	11.63	0.017	0.15	76	79	73
I.S.	11.89	0.017	0.14	100	100	100
Isocaproic	12.54	0.020	0.16	114	109	125
Hexanoic	13.12	0.019	0.15	127	118	144
Heptanoic	14.50	0.020	0.14	236	184	300

[a] Response relative to 2-ethylbutyric acid (internal standard, I.S.).

[b] Amount injected (in water) onto the GC column.

TABLE II

EFFECTS OF GHOSTING ON MEASURED FAECAL SCFAs WHEN SAMPLES OF HIGH AND LOW CONCENTRATION ARE ANALYSED SEQUENTIALLY

Acid	Per cent increase in peak area (n = 5 pairs)[a]		Per cent increase in peak area relative to I.S. (n = 5)[b]	
	mean	range	mean	range
Acetic	+7.9	−8.5–+12.8	+1.0	0–+4.0
Propionic	+6.4	−9.0–+15.5	−0.8	0–−3.2
n-Butyric	+6.1	−10.3–+16.0	−1.0	− 6.3–+2.5
Isobutyric	+4.5	+0.3–+12.6	−1.4	− 3.3–+1.1
n-Valeric	+5.4	+1.0–+13.4	−0.5	−3.1–+3.0
Isovaleric	0	−8.9–+ 4.8	0	−2.3–+3.1
Hexanoic	+3.5	−1.7–+10.0	−2.5	−5.1–+3.0
Isocaproic	+4.9	+1.0–+12.4	−0.9	−2.3–+1.5
Heptanoic	+1.8	−4.2–+ 7.4	−4.4	−8.2–+3.0
I.S.	+5.8	+3.6–+10.9		

The increase in measured concentrations in successive *low* samples were calculated:

[a] Increase in absolute areas.

[b] Increase in areas relative to I.S..

equate. A range of salts was added in saturating amounts to aqueous standards, to increase the concentration of SCFAs in the headspace vapour: sodium chloride, calcium chloride, ammonium chloride, potassium phosphate, ammonium sulphate, sodium sulphate, lithium sulphate and magnesium sulphate. Of these, lithium sulphate was the most effective, increasing the amounts of acids detected by 145% (acetic acid), 183% (propionic acid), 200% (butyric acid and n-valeric acid) and 190% (hexanoic acid), compared with amounts detected without added salt.

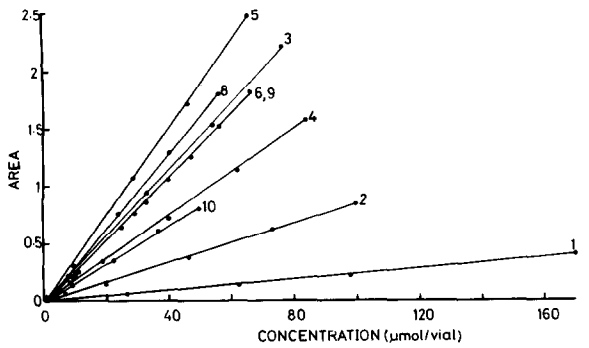

Fig. 2. Linearity of short-chain fatty acids analysed by the definitive headspace procedure. Peak areas were related to area of I.S. Peaks as in Fig. 1.

Ghosting. Initially, carry-over between samples due to ghosting was a major problem. However, after adding formic acid (*ca.* 11% v/v) to the sample, including a formate vial before analysis of each sample pair, and lining the transfer tube with fused silica, ghosting was reduced to acceptable levels. With ten successive analyses of 25 μmol of acetic acid per vial in aqueous solution, the mean increase in peak area due to ghosting was 4.5% (−6.0% to +11.2%), (areas not related to I.S.). In a more rigorous test, faeces spiked with nine SCFAs to low concentrations (0.7–21.9 μmol per vial) were analysed five times in alternation with faeces spiked to high concentration (13.5–71.4 μmol per vial). Table II shows that ghosting caused an increase in peak areas of eight acids and I.S., in the low-spike sample. When the peak area was related to the area of I.S., as it is in the definitive assay, the error decreased.

Linearity, recoveries, precision and detection limits. Standard acids at five concentrations were added to aliquots of a single sample of faeces and analysed. Unspiked faeces was analysed in parallel. The calibration curve was linear to concentrations above those normally found in faeces (Fig. 2). Linear regression correlation coefficients

TABLE III

RECOVERY OF SCFAs FROM FAECES AND WITHIN BATCH PRECI.S.ION

Acid	Concentration (μmol per vial)			Recovery (%)	C.V. (%)
	Expected	Mean recovered (n = 6)	S.D.		
Acetic	17.9	20.0	1.71	112	8.5
	35.7	39.9	4.85	112	12.0
	53.6	51.7	4.20	96	8.1
Propionic	6.6	6.8	0.48	104	7.1
	13.2	13.8	1.24	105	9.0
	19.7	19.6	1.31	99	6.7
n-Butyric	5.4	5.6	0.35	102	6.3
	10.9	11.2	0.95	103	8.5
	16.3	16.4	1.23	100	7.5
Isobutyric	5.3	6.1	0.16	114	2.6
	10.6	11.2	0.33	105	3.0
	16.0	16.3	0.51	100	3.1
n-Valeric	4.6	5.6	0.22	120	4.0
	9.3	9.8	0.33	106	3.4
	13.9	14.1	0.53	106	3.7
Isovaleric	4.6	5.2	0.15	115	2.8
	9.1	9.4	0.24	103	2.6
	13.7	14.1	0.48	103	3.4
Hexanoic	3.9	4.7	0.23	122	4.8
	7.8	8.0	0.26	103	3.2
	11.7	11.8	0.42	100	3.6
Isocaproic	4.0	4.6	0.18	116	3.9
	7.9	8.1	0.19	102	2.4
	11.9	11.9	0.40	99	3.3
Heptanoic	3.4	4.5	0.25	133	5.6
	6.8	7.0	0.30	102	4.3
	10.1	10.1	0.62	100	6.2

ranged from 0.9981 to 0.9999. The recoveries of acids from faeces were excellent at low, medium and high concentrations (Table III). Within- and between-batch precision was acceptable (Table IV). Detection limits for SCFAs in water were (μmol per vial): acetic, 17.9; propionic, 5.3; *n*-butyric, 2.2; isobutyric, 1.1; *n*-valeric, 0.9; isovaleric, 0.9; hexanoic, 0.8; isocaproic, 0.8; and heptanoic, 0.7. For an 8-g sample of faeces from a newborn baby (0.67 g dry weight per sample vial), detection limits for acetic, propionic and butyric acids were calculated as *ca.* 27, 8 and 3 μmol/g dry weight, respectively.

Clinical studies

Fig. 3 shows GC profiles for a faecal sample from a premature baby aged 8 days, with and without standard addition. Ethanol was detected as two peaks, and was a frequent finding in samples. Ninety specimens of faeces were collected from eight healthy, premature babies born before 34 weeks of gestation during the first 6 weeks of life. They were milk-fed and received normal clin-

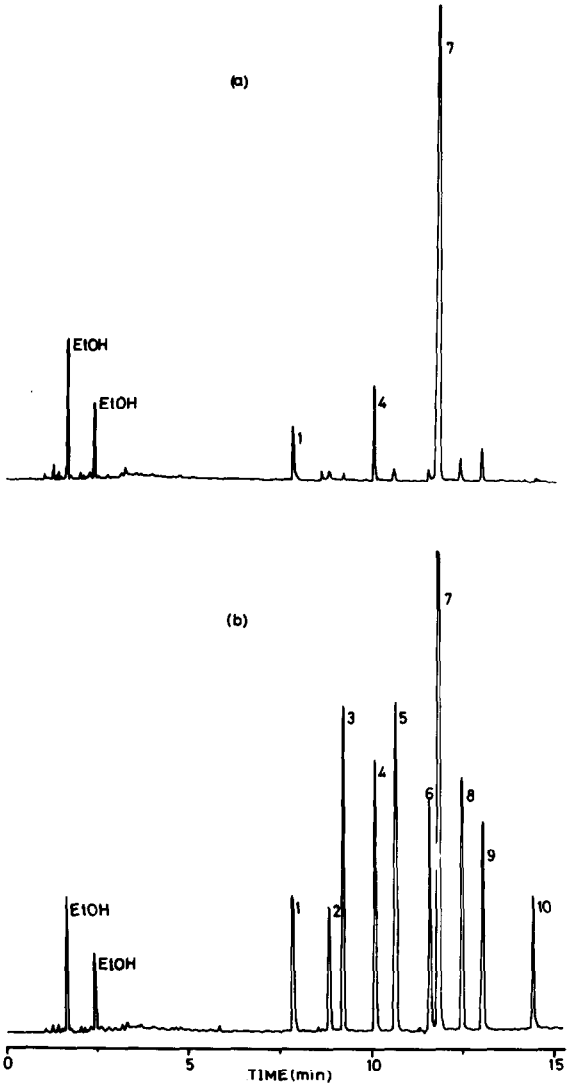

Fig. 3. Faecal short-chain fatty acid profiles of an 8-day-old premature baby analysed by the definitive procedure: (a) faeces with I.S. only; (b) faeces with standard additions. Peaks as in Fig. 1; EtOH = ethanol.

TABLE IV

BETWEEN BATCH PRECI.S.ION OF FAECAL SCFAs

Acid	Concentration (μmol/g dry weight)		C.V. (%)
	Mean (n = 5)	S.D.	
Acetic	290.0	34.80	12.0
Propionic	80.4	6.66	8.3
n-Butyric	72.0	2.20	3.0
Isobutyric	23.0	0.98	4.3
n-Valeric	22.6	1.00	4.5
Isovaleric	24.1	1.39	5.8
Hexanoic	17.8	0.97	5.4
Isocaproic	15.8	0.92	5.8
Heptanoic	17.9	1.35	7.6

ical care. Five were treated with the antibiotic cefotaxime in the first 1–5 days of life, but this did not alter faecal SCFAs significantly. Some faecal samples were very small (*ca.* 1 g wet weight). Ethanol was detected in 39% of samples, acetic acid in 57%, propionic acid in 29% and butyric acid in 20%. Concentrations of acetic, propionic and butyric acids in positive samples were (median (range): 185 (trace–963), 37 (11–229) and 37 (2–114) μmol/g dry weight, respectively. The branched-chain SCFAs, isobutyric, isovaleric and isocaproic acids were found in only five samples, at concentrations of 5–28 μmol/g. No other SCFAs were found. We noted that concentrations varied, sometimes, among samples collected from individual babies on the same day.

DI.S.CUSSION

There is a clinical need for reliable quantitative analyses of faecal SCFAs. Although they have been measured by high-performance liquid chromatography (HPLC) in faecal filtrates [12,13], most methods use an extraction procedure followed by GC for quantification. Both steps are problematic. Extraction with organic solvents has poor recoveries, is relatively insensitive and requires high purity solvents. Recoveries with steam distillation are also poor, and acetate concentrations may be increased by decomposition of other compounds during analysis. Vacuum distillation is an accurate, sensitive, method but is time consuming. The purge-and-trap method is sensitive but susceptible to many analytical variables [14,15].

With automated headspace analysis, little sample manipulation is required and losses of volatile compounds are therefore minimized. The principle is to allow volatile solutes to equilibrate between condensed (sample) and vapour phases in a heated, sealed, vial and to determine the total content of solutes in the system by analysing only the gaseous phase [10]. In an automated system, errors associated with gas sampling and injection into the GC are reduced, and batch analysis is possible. With our method, 14 samples may be analysed per day. Solute distribution between the phases is very sensitive to temperature and to the composition of the condensed phase. Partition into the vapour phase can be enhanced by the addition of salts [10]. Lithium sulphate was the most effective salt tested and, to our knowledge, has not been used before in headspace analysis. Because of matrix complexity and variability of faecal material, we used standard addition to quantify SCFAs. In effect, each sample is calibrated individually [10]. 2-Ethylbutyric acid was chosen as I.S. because it is not present in faeces, has been used for human faecal analysis [16] and gave a sharp peak on GC.

Dissociated SCFAs adhere readily to hot surfaces, causing tailing and ghosting, which leads to spurious increases in measured concentrations. Adhesion can be reduced by adding strong acids to the analytical system: for example, phosphoric or isophthalic acids to the column packing material [9]; oxalic [17] or formic acid to the headspace vials [9]; or formic acid to saturate the GC carrier gas [18]. Others have injected formic acid onto the column after each sample. Flame ionization detectors have a negligible response to this acid. Ghosting and tailing were not problems when we injected SCFAs directly onto the poly (ethylene glycol) GC column, but were with an FFAP column. The stainless-steel transfer line of the headspace sampler, however, introduced serious ghosting problems. We overcame these firstly

by lining the transfer tube with deactivated fused silica, secondly by adding formic acid (11% v/v) to the sample vials, and thirdly by preceding each pair of sample vials with a vial containing formic acid in water to clean the sample lines. It was not feasible to introduce formate vapour directly into the carrier gas with our instrumentation. We cannot readily explain why ethanol eluted as two peaks (Fig. 3). This was found only when using the automated headspace analyser, and not when ethanol was injected directly onto the poly(ethylene glycol) column. *n*-Propanol and *n*-butanol also produced double peaks. All three compounds had retention times of less than 4 min. Adsorption of the alcohols in the analyser may have accounted for the phenomenon.

We used the method to measure faecal SCFAs of premature newborn babies. There are few published data for comparison. The concentrations were of similar order to reported values for premature babies using a complex and laborious extraction procedure [19]. Some of the samples we analysed were probably too small to detect low concentrations of SCFAs. In addition, we noted variation in faecal SCFAs over 24 h. In future studies, it would be preferable to analyse aliquots of a pooled 24 h collection to obtain a more representative profile, and ensure adequate sample (8–10 g), and to reduce the concentrations of acids used for standard addition.

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