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Screening method for the determination of volatiles in biomedical samples by means of an off-line closed-loop trapping system and high-resolution gas chromatography–ion trap detection

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Abstract

A method is described for the analysis of volatile organic compounds in biological matrices (faeces and urine). The technique is based on off-line preconcentration by means of a closed-loop trapping system followed by high-resolution gas chromatography–ion trap detection (HRGC–ITD) for separation and identification of the compounds. The technique has been validated for pattern recognition in faecal and urine samples from healthy volunteers. It is considered a very promising tool in metabolic research.

1. Introduction

For many years, volatile substances are an important object of research in medicine. Organic volatiles are studied in different human matrices such as skin [1], saliva, tissue homogenates, cerebrospinal fluid, serum [2], breath [3,4], flatus [5,6], faeces [7–9] and urine [10]. In gastroenterology volatile organic compounds (VOCs) are related to health and diseases in the gastrointestinal tract [11–14].

In this study VOCs are especially related to fermentation products released by the bacterial degradation process in the human colon. Because the colon is difficultly accessible for *in vivo* studies, we used an *in vitro* faecal incubation system. These *in vitro* studies have been applied successfully for the study of colonic fermentation [15–18].

The first aim of this study was to develop a technique useful for determination of the volatile fermentation products in biomedical samples such as faeces and urine. The second aim was to demonstrate different patterns of fermentation products, formed when different substrates were added to an *in vitro* faecal incubation system. The substrates used were a carbohydrate, a protein and a lipid suspension.

2. Experimental

2.1. Closed-loop trapping apparatus

The device described in this paper is a slightly modified one, basically described by Grob and co-workers [19–21]. This technique has successfully been applied for different complex matrices in environmental studies [22–24], but at our

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knowledge has never been evaluated in biomedical samples.

The closed-loop trapping system is modified by adding a water-condensing system, a flow meter and a gas bulb as shown in Fig. 1 (numbers between parentheses refer to the figure).

Pure nitrogen (99.9995%) (Air Products, Vilvoorde, Belgium) (1) is entering the system by a 4-way valve (Whitey, Highland Heights, OH, USA) (2) with a stainless-steel inner housing. Switching the valve opens or closes the loop. When it is closed, the nitrogen gas is continuously flowing through the loop. When the valve is open, the system is continuously flushed by pure nitrogen. For a very clean background, a charcoal filter between the nitrogen gas bulb and the 4-way valve is recommended.

The gas enters the sample container (3) vertically and leaves through an elbow. This vessel is made of glass and is easily connectable with finger tight Cajon ultra-torr couplings (Macedonia, OH, USA). The sample vessel is dipped in a thermostated water bath (4) at 37°C.

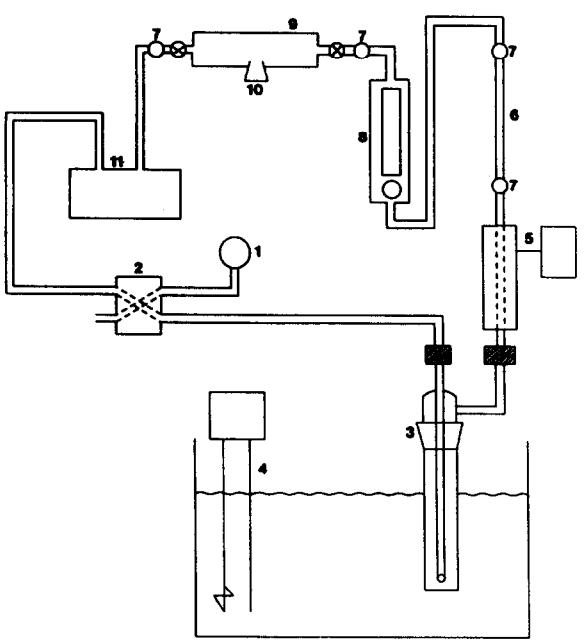

Fig. 1. The closed-loop trapping system: (1) nitrogen supply; (2) 4-way valve; (3) sample container; (4) thermostatic water bath; (5) water condensing system; (6) adsorption trap; (7) Rotulex joints; (8) flow meter; (9) gas bulb; (10) septum; (11) pump system.

Just before the adsorption trap, the glass tube is passed through a heating block (5) to minimize possible condensation of water from the gas leaving the sample container. The temperature of the heated block is kept at 60°C above the temperature of the sample container.

The adsorption trap (6) is filled with 160 mg Tenax GR (Chrompack, Middelburg, Netherlands) and is connected in the system by Rotulex knee-joints (7), providing an excellent tightness and a quick exchange. The traps are preconditioned overnight in an oven at 240°C with a gentle stream of nitrogen (99.9995%) (SoVirel, France). They are used the same day. An aluminium foil is kept around to prevent Tenax from degradation by light.

The gas bulb (Alltech, Laarne, Belgium) (9) has a volume of 125 ml and is modified with Rotulex knee-joints at the ends for quick exchange. Through the septum (10) of the flask it is possible to add internal standards or to withdraw samples.

A PTFE membrane pump (KNF Neuberger, Freiburg, Germany) (11) and a flow meter (GEO/National, Hasbrouck Heights, NJ, USA) (8) provide the flow through the system. The pump has a stainless-steel diaphragm coated with PTFE. This diaphragm is easily accessible for cleaning or exchange. The outlet of the pump is connected to the 4-way valve. The flow through the system is about 250 ml/min. All tubing is made of 1/8 in. (1 in. = 2.54 cm) stainless steel, except for the connection between the sample container and the adsorption trap, which is made of glass.

2.2. Desorption–separation–detection system

To release the VOCs from the adsorption trap, a modified thermodesorption cold trap (TCT) injector (Chrompack) is used [25,26]. Thermodesorption and cryofocussing were carried out in the following manner: holding the desorption trap at 200°C for 5 min with a helium (99.9999%) (Air Products) flow of 20 ml/min (sufficient for the desorption of the VOCs). Meanwhile the cryotrap, 0.32-mm uncoated deactivated fused silica, was held at − 120°C.

Breakthrough was non-existent under these conditions.

The VOCs concentrated in this manner in the cryotrap were released into the analytical column by heating the cryotrap to 200°C for another 20 min.

Separation and identification of the compounds were performed on a HRGC–ITD system. The gas chromatograph was a Vega 6000 instrument (Carlo Erba, Milan, Italy) equipped with a cryostat (cryo 620) with carbon dioxide, a split–splitless injector and a TCT injector. The analytical column was a 25 m × 0.32 mm CP-Sil 5 CB, film thickness 1.2 μm (Chrompack). Helium (99.9999%) at 70 kPa inlet pressure was the carrier gas. Oven temperature program: 30°C (5 min); 5°C/min; 200°C (6 min). The analytical column was coupled to the ion trap detector, model ITD 700 (Finnigan, San Jose, CA, USA) by means of an open split interface. The transfer line was held at 220°C.

Detection was carried out both in electron impact (EI) and chemical ionization (CI) mode. In the EI mode, multiple ion monitoring was used from *m/z* 34–39, *m/z* 41–43 and *m/z* 45–300. Automatic Gain Control was "on". Scans were averaged every second, as the result of 5 μscans. In the CI mode, isobutane 99.95% (Air Products) was used as reaction gas with a maximum ionization time of 800 μs and a maximum reaction time of 800 ms.

2.3. Reagents and standard solutions

Tenax GR was purchased from Chrompack. $HNa_2PO_4 \cdot 12H_2O$ and $H_2NaPO_4 \cdot 2H_2O$ were purchased from Fluka (Buchs, Switzerland). Pure water was purchased from the Central Bureau for Nuclear Measurements (CBNM) (Geel, Belgium). Sterile pyrogene-free water was obtained from Baxter (Lessines, Belgium). The internal standard mixture containing bromochloromethane, 1,4-dichlorobutane and 1-chloro-2-bromopropane, each 20 mg/ml methanol, was purchased from Supelco (Bellefonte, PA, USA). Hexane p.a. and acetone p.a. came from Merck (Darmstadt, Germany).

For closed-loop trapping performance control, a standard solution of acetone is made by diluting 100 ml acetone in 100 ml pure water. For urine and faecal analysis 5 μl internal standard mix is diluted in 20 ml pure water. An aliquot of 5 μl of this dilution is used for standardisation, so an amount of 25 ng for each standard on the analytical column is achieved. The standard was freshly made every day and kept at 4°C in the dark.

The buffer solution contained 2.9 g $Na_2HPO_4 \cdot 12H_2O$ and 0.7 g $NaH_2PO_4 \cdot 2H_2O$ per litre (pH 7). This solution was freshly made every day with pyrogene-free water.

The substrates added to the incubation medium were glucose, casein and Intralipid. Glucose was purchased from Cerestar (Vilvoorde, Belgium), casein from the Melkindustrie (Veghel, Netherlands) and Intralipid from Kabi Pharmacia (Stockholm, Sweden).

2.4. Sample preparation

Faecal samples

Total fresh faecal samples were diluted to obtain a faecal suspension of 1 part faeces per 124 parts of buffer solution (w/w). Dilutions were made by means of a Kenwood-mixer and kept anaerobic by pouring 30 ml of liquid nitrogen into the mixing beaker before sample addition. A 5-ml aliquot of the faecal suspension was transferred to the sample container, flushed with dry nitrogen and firmly closed by means of a clamp and two Swagelok fittings with PTFE seals at the small glass ends .

The sample container was placed in the incubator (Polylab, Antwerpen, Belgium) at 37°C for 24 h before it was plugged into the closed-loop trapping system.

For fermentation pattern recognition with different substrates 10 mg of each substrate was added to the diluted 5 ml faecal sample.

Urine samples

From a morning mid-stream urine, a 2-ml sample was transferred to the sample container and 3 ml of pyrogene-free water was added. No incubation of urine samples was performed prior to the closed-loop trapping procedure.

2.5. Methods

For determination of the reproducibility of the closed-loop trapping system, 5 ml acetone standard was added to the sample container. No adsorption trap was used in the closed loop. After 15 min of closed-loop running, the stopcocks on both ends of the gas bulb were closed simultaneously and 0.2 μl hexane was added to the flask through the septum as a calibration standard. After 15 min of equilibration, 100 μl gas was withdrawn from the flask and was injected into the splitless injector of the GC.

For determination of the faecal and the urine samples, an adsorption trap was added to the closed-loop system just before plugging in the sample container. The loop was closed by means of the 4-way valve, 5 μl of internal standard solution was added through the septum of the gas bulb and the sample container was immersed into the water bath at 37°C. After 15 min of closed-loop trapping, the adsorption trap was removed and closed on both ends with glass cocks and Rotulex clamps until analysis was carried out on the same day. Internal standard could not be added to faecal suspensions before incubation since some methanogenic bacteria may break down halogenated hydrocarbons [27]. Between each sample run a wash-out period of 30 min was performed with an empty adsorption trap and an open loop, to make sure that all trace organics left the system. The contribution of the VOCs from the buffer solution was determined by handling 5 ml buffer in the same way as the faecal samples. Blank procedure for handling urine samples was done by determination of VOCs in 3 ml water.

3. Results

3.1. Quality control of the system

To determine the purity of the whole system, an empty sample container is plugged into the closed-loop system. Internal standard is added and the procedure is run for 15 min at 37°C. A chromatogram of the system purity is given in Fig. 2. Some methylated benzene peaks are present due to the Tenax of the adsorption trap.

The reproducibility of the closed-loop system was determined by computing the ratios of the peak areas of the specific m/z from acetone and hexane (Table 1) on nine different tests. The mean of the ratios was 1.13 with an R.S.D. of 4%.

The reproducibility of the whole system (closed-loop system and HRGC–ITD) was determined by means of the three internal stan-

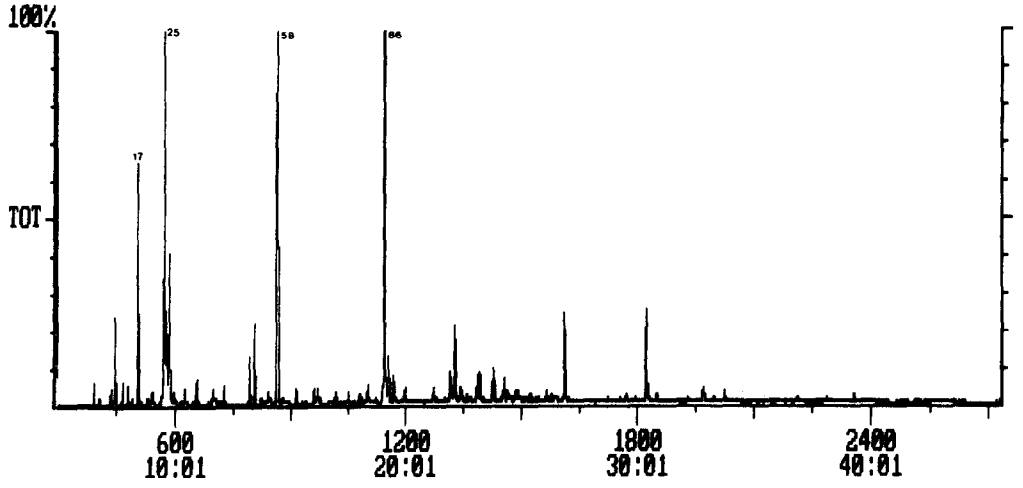

Fig. 2. Total ion chromatogram of the system purity. x-Axis: scan number and time (min:s). Peak numbers indicate compound No., listed in Table 2.

Table 1
Specific m/z values used for the calculation of the quality control of the closed-loop system

Product	Specific m/z used for calculation
Acetone	43
Hexane	57
Bromochloromethane	49
1,4-Dichlorobutane	41
1-Chloro-2-bromopropane	55

dards (I.S.) added to 5 ml pure water. The mean of the ratio of I.S. 3/I.S. 1 was 4.46 with an R.S.D. of 5.8%. The mean of the ratio of I.S. 3/I.S. 2 was 1.50 with an R.S.D. of 6.2%.

3.2. Volatile compounds in faeces and urine

For the determination of the volatile fermentation products, five healthy volunteers were studied. During a normal western European diet period, samples were collected and assayed as

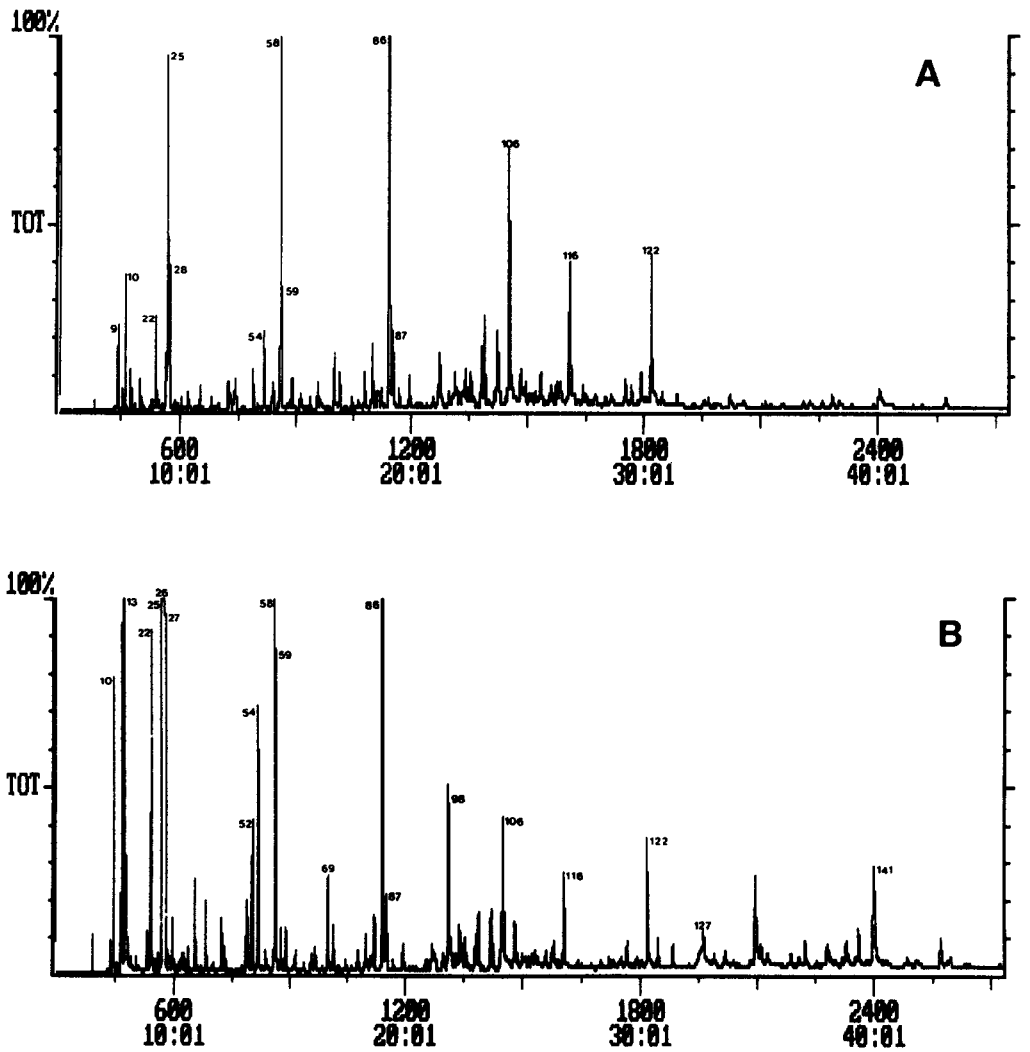

Fig. 3. Total ion chromatogram of the VOCs: (A) in the buffer solution; (B) of a typical faecal sample. Labelling of axes and peaks as in Fig. 2.

described above. Fig. 3A and B shows chromatograms of the background peaks resulting from both the system and the buffer solution and from a typical normal faecal sample.

Fig. 4A and B shows chromatograms of the background peaks from both the system and the pure water and from a typical normal urine sample.

Compounds were identified on the basis of purity and fitted matches between the EI mass spectrum generated by the ion trap detector and the EI spectrum in the computer-based NBS library (Environmental Protection Agency, National Institute of Health, Washington, DC, USA). CI was used to determine molecular masses of the different compounds. Peaks with a signal-to-noise ratio less than 10 in total ion content were not taken into account. Some products remained unknown due to difficulties in spectrum interpretation.

The peak area of the specific m/z value of all peaks was divided by the peak area of the specific m/z value of internal standard **2**, 1-chloro-2-bromopropane, to give a relative index (RI) for each compound. After subtraction of the RI of the background compounds from the

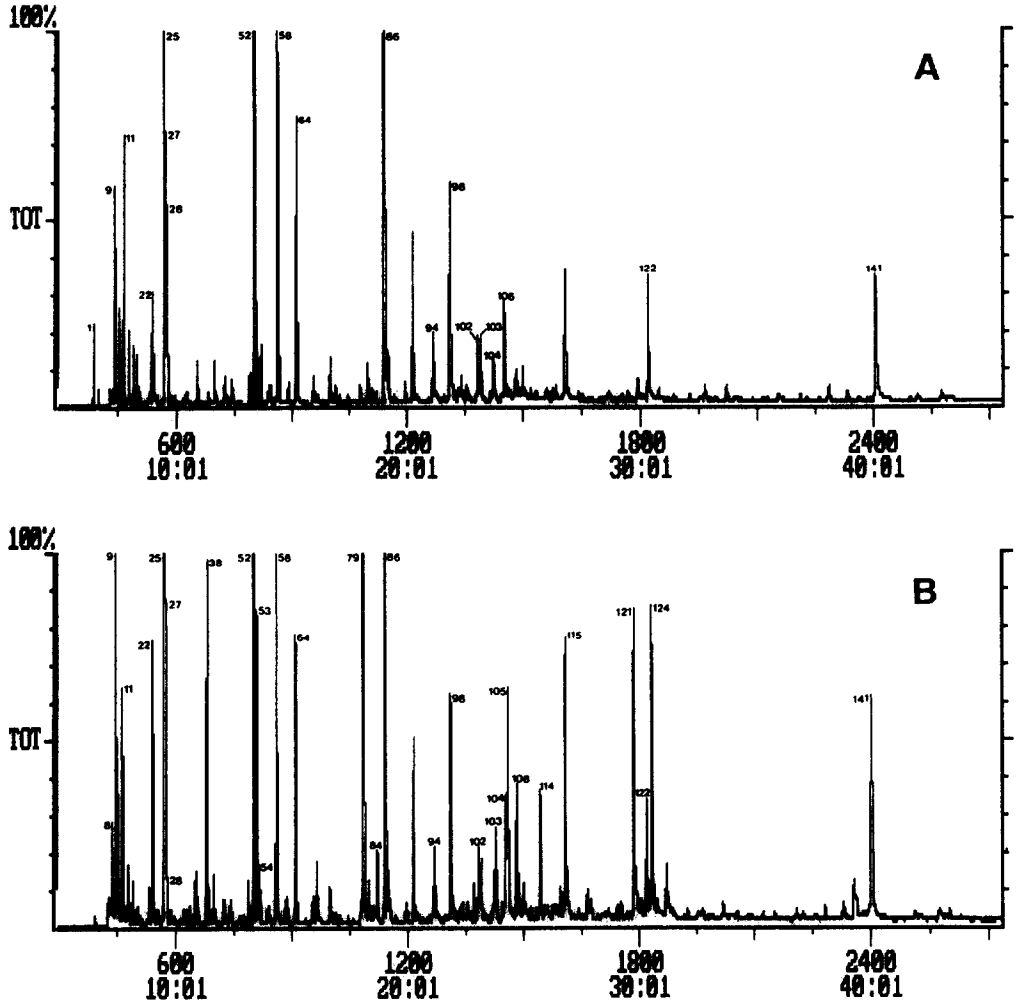

Fig. 4. Total ion chromatogram of the VOCs: (A) in the pure water; (B) of a typical normal urine sample. Labelling of axes and peaks as in Fig. 2.

Table 2
List of volatile compounds present in faeces and/or urine (F/U) and their *m/z* values, used for RI-computing

No.	Compound	F/U	*m/z*	No.	Compound	F/U	*m/z*
1. S-compounds				*3. Alcohols*			
1	Sulphur dioxide	FU	64	7	?4-Pentene-2-ol	FU	45
2	Hydrogen sulfide	FU	34	24	3-Methyl-2-butanol	FU	45
5	Methanethiol	FU	47	26	4-Methyl-2-pentanol	FU	45
6	Thioglycolic acid	FU	47				
11	Methylthiirane	FU	45	*4. Phenyl compounds*			
13	Methyl sulfide	FU	62	37	Benzene	FU	78
15	Carbondisulfide	FU	76	59	Toluene	FU	91
17	Dimethylsulphoxide	FU	79	77	Phenyl compound	FU	91
39	Methylthiocyanate	F	73	80	1,2-Dimethylbenzene	FU	91
40	2-Methyl-2-propane thiol	FU	41	87	Phenyl compound	FU	91
41	Thioacetic acid	FU	43	94	Benzaldehyde	FU	105
52	Dimethyl disulfide	FU	94	100	Phenol	FU	94
60	Methanesulphonylchloride	FU	79	103	Phenyl compound	FU	105
72	3-Pentanethiol	FU	43	105	3-Carene	FU	93
81	2-Phenyl-4-(phenylmethylene)-5(4H)-thiazolone	FU	77	108	Limonene	FU	67
				112	4-Methylphenol	FU	107
85	1,3-Propanedithiol	U	61	127	Indole	F	117
90	Methylpropyl disulfide	F	80	131	Skatole	F	130
91	2-Methyl-2-[(1-methylethyl)-thio]propane	FU	41	141	Phenol compound	F	205
				142	Diphenylamine	FU	169
98	Dimethyl trisulfide	FU	45				
110	4-Methylthiobutanenitrile	U	61	*5. Alkanes/alkenes*			
113	1,1-Methylenebis(thio)bis-ethane	U	75	8	?1-Propene	FU	41
				9	2-Methylpropane	FU	43
117	Methyl(methylthio)methyl-disulfide	F	61	12	1,3-Pentadiene	FU	67
				16	Pentane	FU	41
123	Dimethyl tetrasulfide	FU	45	29	Methylpropene	FU	41
				36	1-Butene	FU	41
2. Acids and esters				65	3-Octene	FU	41
30	Methylacetate	FU	43	97	Methyl-1,3-cyclopentadiene	FU	79
32	Acetic anhydride	FU	43				
35	2-(2-Propenyloxy)-ethanol	FU	41	*6. Ketones/aldehydes*			
43	Propanoic acid	FU	45	20	?4-Pentene-2-one	F	43
56	2-Methylpropanoic acid	FU	41	23	2-Ethylbutanal	U	43
63	Butanoic acid	FU	60	34	3-Methyl-2-butanone	FU	43
71	Pentanoic acid	FU	60	38	3,3-Dimethyl-2-hexanone	FU	43
73	2-Methylhexanoic acid	FU	74	51	2-Butenal	FU	41
74	Ester	F	57	54	3-Methyl-2-pentanone	FU	43
75	Ester	FU	43	62	2-Hexanone	FU	43
82	Hexanoic acid	FU	60	69	3-Methyl-3-pentene-2-one	FU	55
96	Ester	FU	41	79	4-Heptanone	FU	43
99	Heptanoic acid	FU	60	84	2-Heptanone	FU	43
111	Ester	FU	43	101	6-Methyl-5-heptene-2-one	FU	43
118	Ester	U	41	115	2-Nonanone	FU	43
119	Ester	F	43	129	Undecanone	FU	43
121	Ester	FU	73				
126	Ester	F	41	*7. Halogenated compounds*			
132	Ester	F	104	3	Acetylchloride	FU	43
134	Ester	F	104	28	Trichloromethane	FU	83
138	Ester	F	104	31	Fluoro compound	FU	51
139	Ester	F	104	44	Fluoro compound	FU	51

(*Continued on p. 340*)

Table 2 (*continued*)

No.	Compound	F/U	*m/z*	No.	Compound	F/U	*m/z*
50	Bromohexane	FU	43	78	2,3-Dihydropyrane	FU	41
55	Fluoro compound	FU	51	83	Unknown	FU	39
66	Fluoro compound	FU	51	88	Unknown	FU	41
67	2-Bromo-2-methylpentane	FU	41	92	Unknown	F	43
68	Tetrachloroethene	FU	35	93	Unknown	F	57
76	Fluoro compound	FU	51	95	β-Pinene	FU	93
89	Fluoro compound	FU	51	102	Unknown	FU	41
				104	Unknown	FU	281
8. Miscellaneous/unknown				106	Unknown	FU	57
4	Unknown	FU	43	107	Unknown	F	67
10	Diisopropyl ether	FU	45	109	Unknown	FU	41
14	Unknown	FU	59	114	Unknown	FU	93
18	1,2-Dimethylhydrazine	FU	42	116	Unknown	FU	41
19	Unknown	FU	43	120	Unknown	U	45
21	Unknown	FU	51	122	?Tetradecyloxirane	FU	41
22	Unknown	FU	43	124	Unknown	FU	41
27	Unknown	U	43	125	Unknown	U	39
33	Unknown	FU	41	128	Unknown	F	148
42	Unknown	U	43	130	Unknown	FU	43
45	2-Methyl-1-nitropropane	FU	41	133	Unknown	F	161
46	Hydroxylamino compound	FU	41	135	?O-Decylhydroxylamine	FU	41
47	Unknown	FU	39	136	Unknown	FU	43
48	Furanone compound	FU	41	137	?Caryophyllene	F	41
49	Pyridine	FU	52	140	Unknown	FU	43
53	1H-Pyrrole	FU	67				
57	Furanone compound	FU	41	*9. Internal standards*			
61	1,3-Epoxy-4-methylpentane	FU	43	25	Bromochloromethane (I.S. **1**)		49
64	Unknown	FU	41	58	1-Chloro-2-bromopropane (I.S. **2**)		41
70	Unknown	FU	207	86	1,4-Dichlorobutane (I.S. **3**)		55

Compounds were identified on the basis of purity and fitted matches between the electron impact mass spectrum generated by the ion trap detector and the electron impact spectrum in the computer-based NBS library. Additional information on molecular mass was given by the chemical ionization mode.

RI of the sample compounds, 142 compounds (internal standards included) remained. These compounds are listed in Table 2. An attempt is made to classify these compounds in nine categories following their chemical nature. Additional characterization is done by appearance of each compound in faeces and/or urine, and by specific *m/z* value used for RI computation. This table is the result of all compounds found in 5 healthy volunteers.

3.3. Quality control of the sample handling

The reproducibility of the analytical procedure was examined from homogenization of the faecal samples to detection and quantitation of the volatile compounds by mass spectrometry. For this purpose, three faecal samples from the same fresh faecal aliquot have been taken. Peak areas at specific *m/z* values (Table 2) were determined and R.S.D. values of each compound have been calculated. Out of 90 products found in this faecal sample, 58 had an R.S.D. $< 10\%$, 18 had an R.S.D. between 10 and 20% and 14 products had an R.S.D. $> 20\%$. Of the 14 compounds in the last series, 7 had a signal-to-noise ratio < 10 in specific *m/z* mode, 2 were overloaded and 5 showed bad chromatographic resolution.

The reproducibility of handling urine samples was examined in the same way. Three urine samples from the same urine stock sample were examined. Out of 81 products found in the urine

sample, 69 had an R.S.D. < 10%, 8 had an R.S.D. between 10 and 20%, and 4 had an R.S.D. > 20%. From the last series, 2 had a signal-to-noise ratio <10 in specific *m/z* mode and 2 were overloaded.

3.4. Fermentation patterns of different substrates

To determine the influence of substrates on the formation of volatile compounds by fermentation processes, different substrates were added to the faecal samples.

Figs. 5, 6 and 7 show a faecal sample incubated with glucose, casein and Intralipid, respectively.

Upon careful examination of chromatographic patterns of Figs. 5–7, different patterns in formation of VOCs are obtained, which are summarized in Table 3. Upon adding glucose to the incubation system, short-chain fatty acids (SCFA) are predominant. They are quantitatively the principal metabolites of carbohydrate fermentation. No primary alcohols could be detected due to limitations in the mass selection of the ITD. A faecal sample incubated with casein shows a different pattern, as mainly the sulphur compounds are present in relatively high concentrations. Indole and skatole could only be demonstrated in case Intralipid was added to the incubation medium.

4. Discussion

In this study, a closed-loop trapping system has been developed to study the formation of volatile compounds in faecal media and urine. Different methods of trapping and concentrating trace substances have been described. A short survey of preconcentration methods in capillary GC has been published by Roeraade [28], and trapping on solid adsorbents has been reviewed by Núñez *et al.* [29].

A closed-loop system for trapping VOCs, hitherto only used for environmental studies [22,23,24], has been applied to demonstrate VOCs in faecal and urine samples. At our knowledge, this is the first time that this system has been applied for the study of the formation of volatile bacterial fermentation products in the human colon. The rationale of this study is that in the last decade there is a growing interest in bacterial fermentation products as causing agents for colonic cell epithelium damage and systemic diseases. Indeed, the colon is an open system with nutrients continuously flowing into the

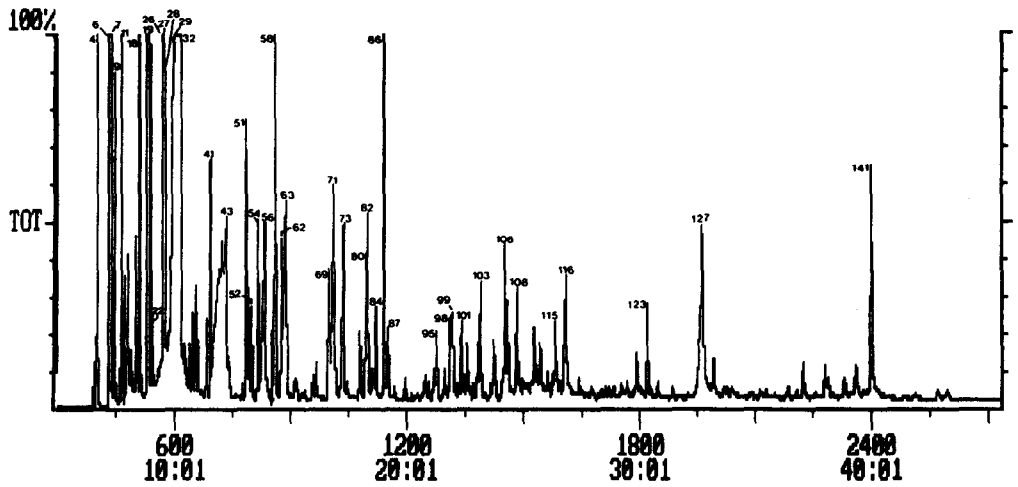

Fig. 5. Total ion chromatogram of the VOCs of a faecal sample incubated *in vitro* with glucose. Labelling of axes and peaks as in Fig. 2.

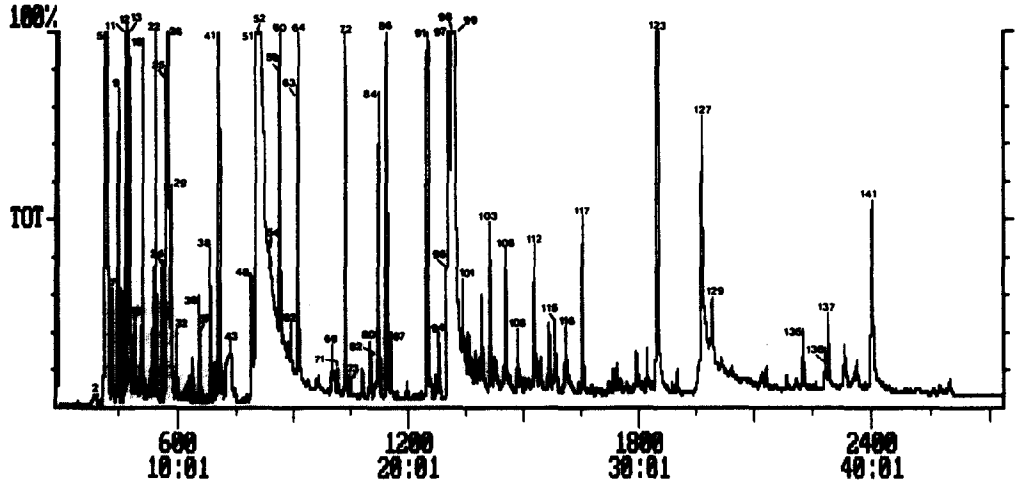

Fig. 6. Total ion chromatogram of the VOCs of a faecal sample incubated *in vitro* with casein. Labelling of axes and peaks as in Fig. 2.

caecum, and where the end products are excreted as faeces. This 1.5 meter anaerobic system contains about 90 g of total bacterial mass [9], representing more than 400 different bacterial species. They degrade the luminal content of the colon to a wide variety of metabolites. Some of them are beneficial for the host [30], others are known to be toxic [31,32] or even potentially carcinogenic [33,34]. The control of fermentation is thus of critical importance to man. An excellent overview of the degradative processes occurring in the colon is given by Macfarlane and Cummings [8,35].

To understand the profile of fermentation products in normal individuals, methods to analyse short-chain fatty acids [36] and bacterial fatty acids composition [37] have been described. The present study on analysis of VOCs is to be considered as an additional tool to gain insight in the fermentation processes.

The authors are well aware of the fact that these analyses show some fundamental short-

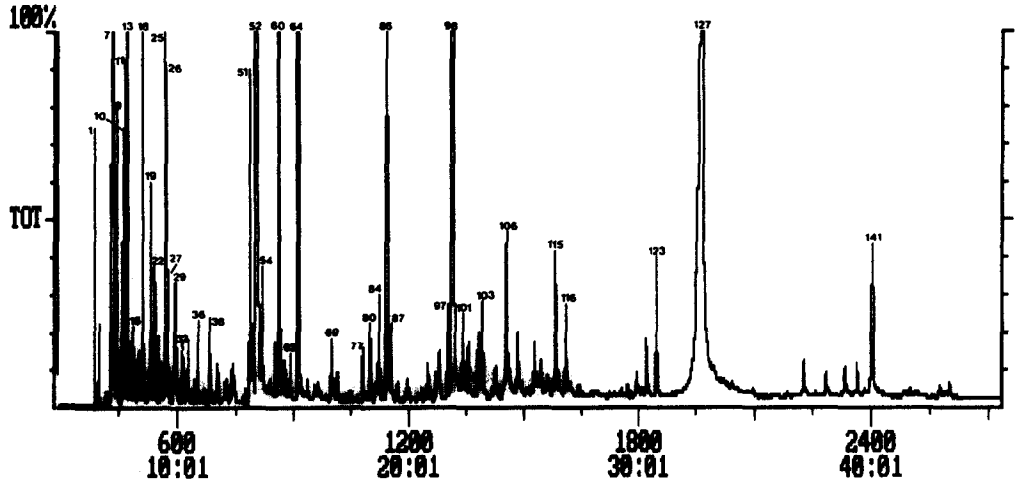

Fig. 7. Total ion chromatogram of the VOCs of a faecal sample incubated *in vitro* with Intralipid. Labelling of axes and peaks as in Fig. 2.

Table 3
The most important volatile compounds of the *in vitro* substrate study with their RI-values

No.	Blank	Glucose	Casein	Intralipid	Product
2	0	0	26	2	Hydrogen sulfide
5	0	0	15 198	8	Methanethiol
6	0	9801	0	0	Thioglycolic acid
7	22	4426	427	0	4-Pentene-2-ol
13	1210	54	8718	980	Dimethyl sulfide
26	394	2313	3505	304	4-Methyl-2-pentanol
32	0	8121	660	190	Acetic acid anhydride
35	0	102	4	27	2-(2-Propenyloxy)ethanol
41	0	614	9008	66	Thioacetic acid
43	0	1714	384	68	Propanoic acid
52	123	80	50938	1644	Dimethyldisulfide
56	0	524	0	0	2-Methylpropanoic acid
60	0	0	184	7	Methanesulphonylchloride
63	0	630	20	0	Butanoic acid
71	0	537	10	0	Pentanoic acid
72	0	0	760	0	3-Pentanethiol
73	0	333	3	0	2-Methylhexanoic acid
82	0	360	24	0	Hexanoic acid
91	6	36	1875	58	2-Methyl-2-[(1-methylethyl)thio]propane
98	83	98	4674	554	Dimethyl trisulfide
99	0	28	534	0	Heptanoic acid
100	8	8	50	27	Phenol
112	95	94	291	80	4-Methylphenol
117	0	0	555	6	Methyl(methylthio)methyldisulfide
123	4	5	4087	197	Dimethyl tetrasulfide
127	44	616	1136	2364	Indole
131	0	0	0	5	Skatole
142	2	3	11	7	Diphenylamine

comings because they have been done on faeces, which is an end product. But sampling "*in situ*" is not possible. In the past, this indirect approach, together with faecal incubation studies, has been proved to give reliable results on colonic fermentation processes [15–18]. Moreover, authors agree that inter-individual changes, due to different diets and different bowel habits, may influence analysis outcome.

Therefore, maximal precaution has been given to the described method to minimize errors in analytical data. As the described system, *i.e.* closed-loop trapping thermodesorption–gas chromatography–ion trap detection, is very sensitive for external contamination, each step has been controlled carefully. As an example, the contamination of water, originating from the Central Bureau of Nuclear Measurements, is given. Although this water has been chosen out of six other water samples and considered the purest, there are still volatile contaminants present. Additionally highest attention in interpreting mass spectra has been paid, and, although CI mass spectra have been used to complete EI data, it was not possible to identify all compounds. Due to the mass spectrometric overload at lower mass range, multiple ion monitoring could only be performed within specific mass ranges. By doing this, all primary alcohols (specific m/z 30 and 31) have been excluded from analysis. Compounds with signal-to-noise ratios less than 10 also have been excluded (in an arbitrary way) from data interpretation. This does certainly not mean that they would not exert any physiological significance, as trace quantities also may have great impact on colonic cell functions.

In this study, quantitative analysis of com-

pounds has not been done, due to the quasi impossible task to study response factors of each compound *versus* internal standards. These internal standards however are of great importance to control the stability of the system and to support the reliability of the qualitative data. Due to the sometimes poor chromatographic resolution in total ion mode, all compounds were examined with their specific m/z value. The calculation with relative indices gives only a first approximation on quantitative interpretation of data. There is a large difference between the contribution of the specific m/z values of each compound to the total mass of each compound. Attention should also be paid to the fact that equimolar quantities of different compounds give different responses in the detector.

Out of 90 compounds in faeces, 58 had an R.S.D. value of less than 10%; 14 products had an R.S.D. value of more than 20%. The latter was due, either to very low signal-to-noise ratios (≤ 10) or to product overload, which may cause misinterpretation of mass spectra. This study is the first attempt to gain insight in formation of volatile compounds upon bacterial fermentation in the colon. Some firm statements may already be made: formation of short-chain fatty acids upon carbohydrate presence. They are rapidly absorbed from the human large bowel, so their concentration in a blank faecal sample is relatively low [8]. Another statement is the formation of sulphur-containing compounds upon protein (casein) incubation. These compounds are intermediary or end products of the breakdown of cysteine and methionine, two amino acids present in casein. Phenol and *p*-cresol are end products of the fermentation of tyrosine, also present in casein [38]. The formation of acids has already been confirmed in other "*in vivo*" studies, whereas ongoing "*in vivo*" investigations on supplementary protein intake do confirm the faecal incubation studies, described in this study [39]. Addition of lipid (as Intralipid) shows only minor differences in the VOCs chromatogram. It seems reasonable to assume that the appearance of indole and skatole might be due to the protein content [40] of the Intralipid solution, rather than to the lipid content itself.

This study aims to contribute substantially to better knowledge of metabolite formation due to bacterial anaerobic metabolism. Together with other chemical compounds, VOCs may contribute in metabolic pattern recognition. This may lead to better understanding of pathophysiological processes occurring in the colon, and may also influence the etiology of diseases, related to the systemic system.

In conclusion the combination of an off-line preconcentration by means of a closed-loop trapping system followed by GC–ITD is a reliable method for screening volatiles in faecal and urine samples on a qualitative base.

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