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Note

Gas chromatographic technique to simultaneously quantitate the gases produced by intestinal microorganisms from fermentation mixtures★

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When carbohydrates are metabolized by intestinal microflora of human systems, carbon dioxide, methane, and hydrogen are produced [1]. Usually the total gas evolved is measured manometrically [2, 3] or with a pressure transducer [4]. Specific gases are then determined by various techniques. Hydrogen is measured electrochemically [5]. Carbon dioxide is determined by absorption and titration [6]. Gas chromatographic (GC) techniques have been applied, but they only quantitate one or two of the gases [4, 6–9] at the same time. Methods which could separate two or more of the gases require low temperatures [10] or multiple columns, and/or temperature programming [7, 11–13]. The sensitivity of the thermal conductivity detector, the detector of choice for gases, precludes using temperature programming. A single method for all three gases would facilitate the investigation of these gases evolved from fermentation mixtures. In this paper an isothermal method that allows for the rapid determination of all three gases with one sampling in less than 12 min is described.

EXPERIMENTAL

Materials

Gas mixtures used as standards were obtained from Linde Specialty Gas (Union Carbide, Danberry, CT, U.S.A.) and Scott Specialty Gases (Scott Environmental Technology, Houston, TX, U.S.A.). A series of individual standards ranging from 0.01 vol.% (100 ppm) to 30 vol.% was used to check linearity and sensitivity

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of the method. Standard gas mixtures (hydrogen, methane, and carbon dioxide) in argon were run daily to check instrument variability and to quantitate the samples. The nutrient broth (dehydrated form) tryptone and yeast were obtained from Difco Labs. (Detroit, MI, U.S.A.), the sugars from J.T. Baker (Phillipsburg, NJ, U.S.A.) or Mallinckrodt (St. Louis, MO, U.S.A.). The innoculation system (Prompt) was obtained from 3M (St. Paul, MN, U.S.A.). The gas samples were stored in evacuated tubes (Vacutainer, Becton-Dickinson, Rutherford, NJ, U.S.A.). The syringe and needle used to take the samples were obtained from Becton-Dickinson. A Pressure Lok (Series A) gas syringe (Dynatech Precision Sampling, Baton Rouge, LA, U.S.A.) was used to inject the sample onto the column.

Apparatus

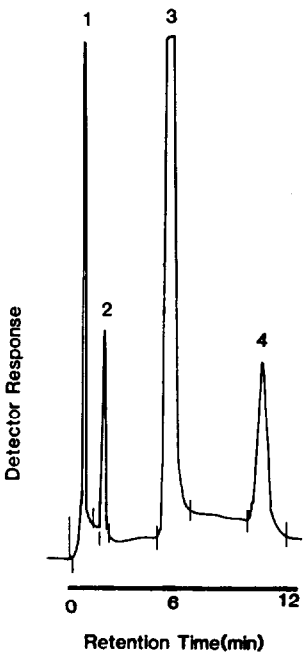
A Packard Model 7300 gas chromatograph equipped with a Model 886 thermal conductivity detector (United Technologies, Downers Grove, IL, U.S.A.) was used with a Hewlett-Packard Model 3380A integrator (Avondale, PA, U.S.A.). The stainless-steel column (210 cm×3.2 mm) was packed with a special carbon packing, 100/120 Carbosieve S (Supelco, Bellefonte, PA, U.S.A.).

Gas chromatographic conditions

Argon was used as the carrier gas at a flow-rate of 35 ml/min. The column was heated and maintained at 95°C. The inlet temperature was 110°C. The thermal conductivity detector was heated to 150°C. Bridge current on the thermal conductivity detector was set at 135 mA and the sensitivity at 1 mV. Attenuation on the integrator was set at 2 and the slope sensitivity at 0.3 mV/min.

Bacterial samples

The three intestinal microorganisms used for this study, obtained from the American Type Culture Collections (Rockville, MD, U.S.A.), were two gram-negative rods, *Escherichia coli* (ATCC No. E11775) and *Klebseilla pneumoniae* (ATCC No. E13883), and a gram-positive cocci, *Streptococcus faecalis* (ATCC No. E119433). The microorganisms were incubated overnight on tryptone glucose yeast agar [14] at 37°C. An inoculation of 10^8 microorganisms diluted with 1 ml of 0.85% sodium chloride solution was prepared and added to 20 ml of 1% carbohydrate in nutrient broth in a closed vessel. The four sugars used in the study were glucose, fructose, lactose, and sucrose. A 10-ml volume of head space was taken at time of inoculation and after incubation for 24 h at 37°C. Samples were collected with a plastic Luer Lok syringe fitted with an 18-gauge needle and stored under positive pressure in a 5-ml evacuated tube at 0°C [15, 16]. Aliquots (1 ml) of the samples were injected onto the column with a Pressure Lok syringe. The difference between the initial and 24-h samples indicates the concentration of gas evolved from the carbohydrate metabolism by the intestinal microorganism in 24 h. The fermentation studies with the three enteric microorganisms were done in triplicate.



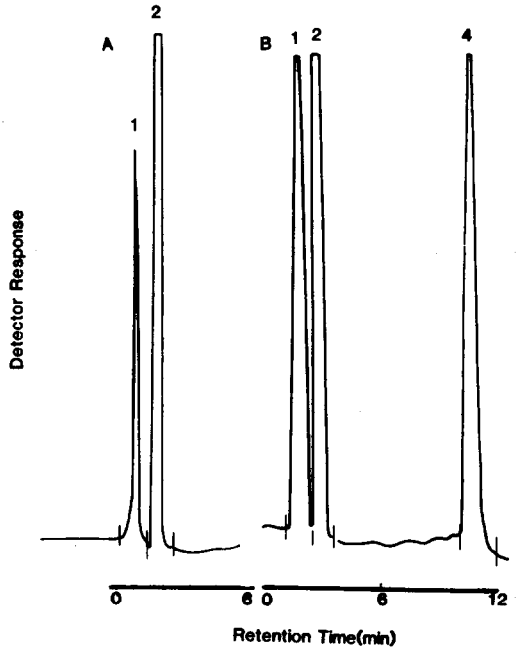


Fig. 1. Gas chromatogram of standard gas mixture (1 vol.%). Conditions: column, 2.1 m×3.2 mm stainless-steel column packed with 100/120 Carbosieve S; column temperature, 95°C, inlet temperature, 110°C; carrier gas, argon; flow-rate, 35 ml/min; thermal conductivity detector: bridge current, 135 mA; sensitivity, 1 mV; temperature, 150°C; attenuation, 2; slope sensitivity, 0.3 mV/min. Elution sequence: 1, hydrogen; 2, air; 3, methane; 4, carbon dioxide.

Fig. 2. Chromatogram of *Klebseilla pneumoniae* in 1% fructose nutrient broth at 0 h (A) and 24 h (B). Conditions as in Fig. 1.

RESULTS AND DISCUSSION

A chromatogram of the working standard is given in Fig. 1. The order of elution is hydrogen, methane, and carbon dioxide at approximately 0.9, 6.0, and 11.0 min, respectively. At approximately 2 min, there is an air peak (nitrogen and oxygen). (N.B.: manual activation of the integrator caused a slight variation in retention time.) The concentration of each gas in a sample was calculated by comparing peak areas of the sample to those of the standards. The sensitivity for hydrogen is 0.01 vol.%, methane 0.02 vol.%, and carbon dioxide 0.05 vol.%. The response factors (i.e. vol.%/peak area) are 1.37, 4.90, and $16.24 \cdot 10^{-5}$ for hydrogen, methane, and carbon dioxide, respectively. Least-squares analysis was used to determine linearity. A linear relationship up to 30 vol.% for hydrogen and carbon dioxide and up to 25 vol.% for methane was observed when different concentrations of the gases were plotted against areas under the peaks (Table I). The correlation coefficient for each of the three was greater than 0.99. The precision of the method was assayed by repeated analysis of standards of the three gases. Within-day variation (sample numbers 9–11) ranged from 4.7 to 8.0% while day-to-day variation (sample numbers 16–19) ranged from 2.9 to 9.0% (Table II). Subsequent

TABLE I

LINEAR REGRESSION ANALYSIS

Gas	Slope (mean ± S.D.)	y-Intercept (mean ± S.D.)	Correlation coefficient
Hydrogen	0.000018 ± 0.0000009	−0.148 ± 0.139	0.994
Carbon dioxide	0.000227 ± 0.0000110	−1.706 ± 0.807	0.996
Methane	0.000064 ± 0.0000035	−0.474 ± 0.644	0.994

to this study a 1.8 mm × 3.2 mm, 120/140 Carbosieve B column was used in place of the 100/120 Carbosieve S column. The Carbosieve B column gave the same sensitivity, linearity, and reproducibility but decreased the run time by two thirds. The retention times for hydrogen, air, methane, and carbon dioxide were 0.34, 0.64, 1.58, and 3.66 min, respectively.

The chromatographs of the 0- and 24-h samples taken for *Klebseilla pneumoniae* in 1% fructose are shown in Fig. 2. The difference in the concentration in the two samples is used to determine the concentration of gas produced per 24 h. The concentrations of hydrogen and carbon dioxide evolved from each of the four sugars by *Escherichia coli, Klebseilla pneumoniae,* and *Streptococcus faecalis* are shown in Figs. 3 and 4. The three microorganisms used in this study are facultative aerobes which do not normally produce methane. Some of the intestinal anaerobic microorganisms do produce methane; therefore, a method was needed which could measure the methane produced when these microorganisms are present in a mixture of intestinal microorganisms. There is a difference in the concentration of gas evolved by *Escherichia coli* from sucrose as compared to the other three sugars. Some strains of *Escherichia coli* are known to metabolize sucrose differently or not at all [17]. Also there was no hydrogen and only a small amount of carbon dioxide evolved from all four sugars by *Streptococcus faecalis.* This microorganism is known to fix the hydrogen produced by forming water [18].

The method in a time span of 12 min allows the simultaneous measurement of hydrogen, methane, and carbon dioxide from one sampling using one column and one detector. With the Carbosieve B column the sampling time is further reduced

TABLE II

PRECISION STUDY

C.V. = coefficient of variation.

Gas	Concentration (vol.%)	Within-day precision		Day-to-day precision	
		Peak area (mean ± S.D.)	C.V. (%)	Peak area (mean ± S.D.)	C.V. (%)
Hydrogen	1	71934 ± 3402	4.7	72979 ± 2404	3.2
Methane	5	97318 ± 5972	6.1	101859 ± 3029	2.9
Carbon dioxide	5	27566 ± 2770	8.0	30784 ± 2770	9.0

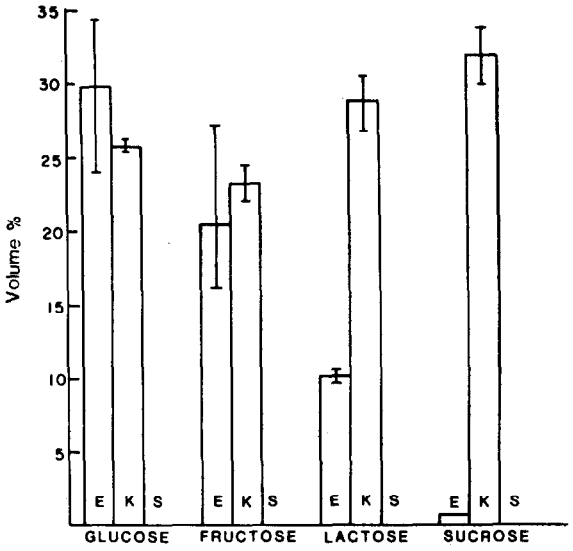


Fig. 3. Hydrogen evolved by 10^8 microorganisms in 24 h from 1% solutions of glucose, fructose, lactose, and sucrose in nutrient broth. Bar gives mean and line indicates range of triplicates. E = *Escherichia coli*; K = *Klebseilla pneumoniae*; S = *Streptococcus faecalis*.

to 4 min. Thus the sampling time is considerably less than found with other commonly used packing material, such as silica gel [10]. Using argon as the carrier gas optimizes the method for hydrogen but still allows the detection and quantitation of carbon dioxide and methane at 0.05 and 0.02 vol.%, respectively, which is approximately four to ten times lower than other systems [7]. The response is linear for the range of expected concentrations. This method can be used to assess

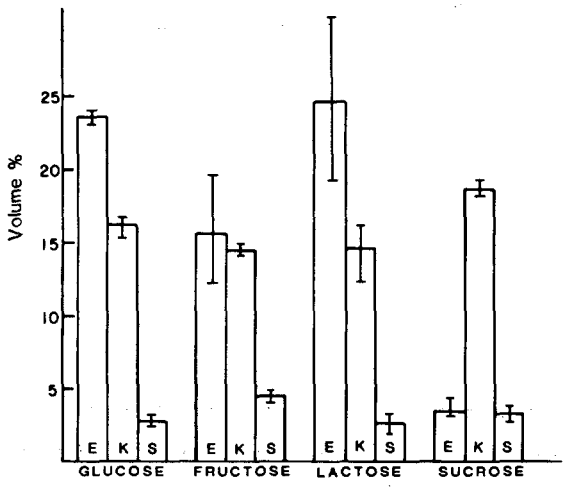


Fig. 4. Carbon dioxide evolved by 10^8 microorganism in 24 h from 1% solutions of glucose, fructose, lactose, and sucrose in nutrient broth. Bar gives mean and line indicates range of triplicate. E = *Escherichia coli*; K = *Klebseilla pneumoniae*; S = *Streptococcus faecalis*.

the gases evolved by intestinal microflora from fermentation mixtures. It has been applied to a study of carbohydrate fermentation by the intestinal microflora from infants on different feeding regimens.

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