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Note

Rapid, single sample analysis of H_2, O_2, N_2, NO, CO, N_2O and CO_2 by isothermal gas chromatography: applications to the study of bacterial denitrification

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Microbial denitrification is a fundamental reaction sequence within the global cycle of nitrogen that returns fixed nitrogen to its elementary state. In denitrification studies there is a need for an efficient gas analysis system that should meet the following requirements: (i) NO, N_2O, N_2 and O_2 as one group of gases should be quantified in a single sample; (ii) quantitation should cover a wide range of gas concentrations; (iii) short intervals between successive determinations are required for kinetic measurements; and (iv) the sample volume should be small to keep changes in the reaction system negligible. Use of a sample loop is not possible because of this latter limitation.

Shortly after the introduction of porous polymer beads by Hollis[1], this material was adapted for gas determinations in denitrification studies[2]. Although this meant a substantial improvement over manometric methods, several limitations still had to be accepted: simultaneous analysis of the pertinent components for denitrification cannot be achieved within a sufficiently short time span, O_2 and N_2 are not separable and water causes straggling peaks on all types of porous polymers[3]. Molecular sieve, activated alumina or silica gel inorganic adsorbents show similar limitations when a simultaneous and rapid analysis is needed. Temperature programmes, including recooling cycles and dual detector systems, were developed but do not meet the above requirements for gas separation[4–6].

An isothermal multi-column system is the only alternative for taking advantage of different adsorbents and circumventing their undesirable features. Arrangements of multi-column systems for the analysis of gas mixtures as produced by denitrification have been tested previously. The time requirement for one determination[7], however, hampered fast kinetic studies, and straggling peaks of another column system[8] would interact with consecutive measurements.

This paper describes the design and performance of an isothermally operating dual-column system that rapidly separates H_2, O_2, N_2, NO, CO, N_2O and CO_2 and allows subsequent determinations within 3-min intervals.

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EXPERIMENTAL

Column arrangement

The separation columns were installed in an isothermally (40°C) operating Shimadzu 3BT gas chromatograph. The electric four-port valve and the restrictor column were placed outside the oven (Fig. 1). Gas samples (100 μl) from the headspaces of reaction mixtures were applied through inject port 1 with a gas-tight syringe.

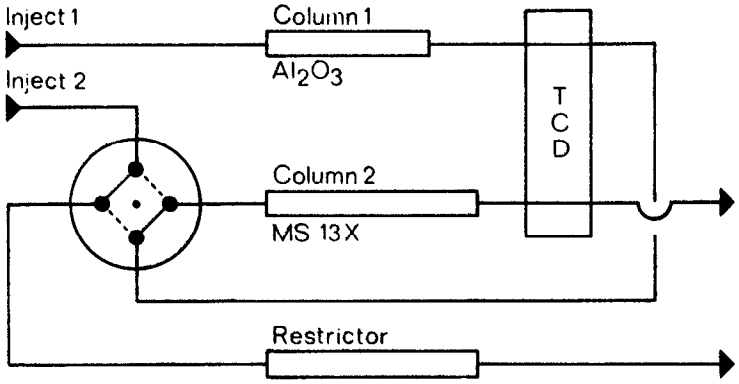

Fig. 1. Column arrangement and flow diagram of the chromatographic system. The SAD arrangement of columns 1 and 2 is indicated by the continuous lines inside the valve. The dashed lines show the parallel arrangement of the two columns, while the restrictor column was connected in series with column 1. Samples are injected through port 1.

The light gases were eluted as a composite peak from column 1. After they had passed the detector and were flushed on to column 2, the valve was activated by a Spectra-Physics SP 4100 integrator, whereby columns 1 and 2 were switched from the in-series across-detector arrangement to the parallel position. The light gases were first separated on column 2 and detected before N_2O or CO_2 were released from column 1. Serial measurements were made at intervals of 150 sec without peak interferences from preceding determinations.

Helium was used as the carrier gas at a flow-rate of 210 ml/min through channel 1 (column 1 and 2 or restrictor) and 195 ml/min through channel 2 (column 2 or restrictor). The flow-rates were controlled by the inlet pressure. In column 2 and in the restrictor the back-pressure had to be closely balanced to avoid flow changes in both channels after valve switching. Baseline restabilization after changing the flow lines was completed within 5 sec. The detector range was set to 1 mV full-scale.

Packing materials

The lighter gases were separated from heavier gases by an activated alumina column (2 m × 5 mm I.D.; WGA, Düsseldorf, F.R.G.). Water and CO_2 were adsorbed nearly irreversibly at the operating temperature of 40°C. When the retention time of N_2O had decreased (caused by deactivation of the alumina column by H_2O) the column was reactivated at intervals of *ca.* 6 months by heating it at 120°C for 30 min under the usual carrier gas flow-rate. On cooling to the operating temperature

water was injected in several portions, totalling *ca.* 50 μl, to obtain an activation state of alumina that gave a retention time for N_2O of about 2 min. When analysis of CO_2, in addition to the other gases was required, the alumina column was replaced with a Porapak Q column (80–100 mesh; 2.50 m × 3 mm I.D.), preceded by a short tube of silica gel (45–60 mesh; 0.2 m × 3 mm I.D.; WGA) to adsorb water vapour. It was important to use only a short silica gel column, as the order of elution of N_2O and CO_2 is reversed on the two adsorbents.

H_2, O_2, N_2, NO and CO were separated from each other on a molecular sieve 13X column (45–60 mesh; 2.0 m × 3 mm I.D.; Serva, Heidelberg, F.R.G.). The commercial material had to be activated for gas separation to eliminate tailing of NO. The molecular sieve grains were washed five times with distilled water[9], which removed about 10% of the original material. It was then dried under air to constant weight and activated for optimal performance by further heating for 12 h at 195°C.

Detection

Gases were detected with a thermal conductivity detector. Surface corrosion of the rhenium–tungsten filaments of the Wheatstone bridge became a problem, as they were frequently exposed to O_2 and/or NO from the samples. When the bridge could not be balanced by the built-in resistors because of an unsymmetrical increase in the filament resistance, trimmer potentiometers were inserted in parallel with each filament. The useful life of the detector could be prolonged at least three-fold by this method. The corresponding decrease in sensitivity was negligible.

Calibration

Enzymatic tests were carried out in stoppered 12-ml vials containing 3 ml of liquid phase under helium. The pressure within the vials was kept slightly above atmospheric pressure to minimize air contamination during sampling. The resulting small loss of gas sample from the syringe was accounted for by external standard calibration. The accuracy of the system was better than ± 1% for each component, except H_2 (Table I).

TABLE I

DETECTION LIMITS AND RELATIVE STANDARD DEVIATIONS FOR VARIOUS GAS COMPONENTS

Gas species	*Detection limit** (*vpm*)	*R.S.D.*** (*%*)
Hydrogen	1000	3.5
Oxygen	100	0.8
Nitrogen	100	0.6
Nitric oxide	200	0.9
Nitrous oxide	200	0.8
Carbon monoxide	150	***
Carbon dioxide	200	0.7

* The detection limit (twice the noise level) was determined for a sample of 100 μl and a bridge current of 150 mA.

** The relative standard deviation was determined with a mixture containing *ca.* 1% of each component in He.

*** Not determined.

RESULTS AND DISCUSSION

Rapid separation of NO and N_2O within a mixture of other gases on a dual-column system requires the elimination of tailing of NO on molecular sieve 13X. As the adsorption of NO is dependent on the activation state of the molecular sieve, three different activation states were compared with respect to the separation of NO from the preceding components: variation of its retention time, peak height and area with sample size. Molecular sieve 13X, dried to constant weight under air at 120°C, showed insufficient separation properties for the present purpose and was not studied further. On the other hand, on completely dry and fully activated stationary phase, the retention time of NO increased non-linearly with decreasing amount of NO and the peak height was also non-linearly related to NO at low concentrations (Fig. 2). The peak shapes with small amounts of NO also differed from run to run because of the adsorption of NO. Similar behaviour had been reported for molecular sieve 5A[5].

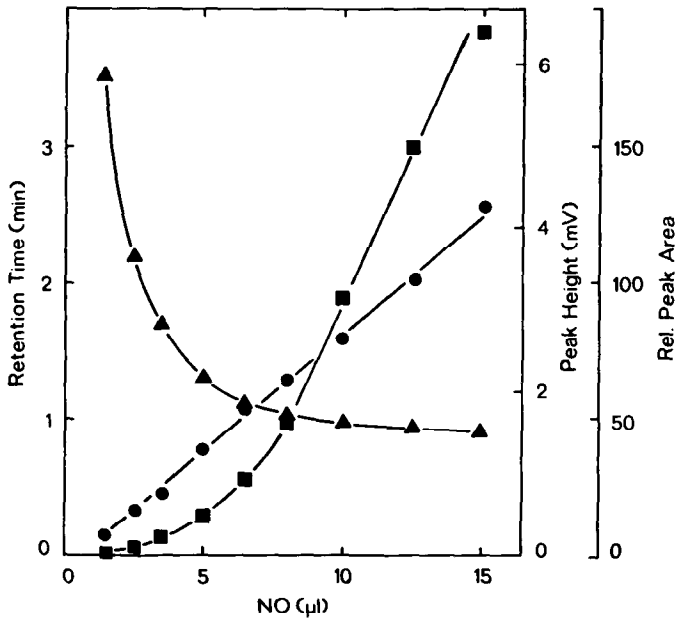

Fig. 2. Correlation between sample size and peak area, peak height and retention time on fully activated molecular sieve 13X. Activation was effected on-column at 300°C under a helium flow (50 ml/min) for 8 h. Operating conditions: steel column, 0.8 m × 3 mm I.D.; oven temperature, 40°C; carrier gas (He) flow-rate, 60 ml/min. NO was injected as a 20% mixture with He. Symbols: ▲, retention time; ■, peak height; ●, peak area.

These observations indicate that the elution of NO from the molecular sieve represents a superposition of different partitioning processes. The aim of conditioning the adsorbent is therefore either the elimination or the permanent saturation of secondary strong binding sites for NO. On molecular sieve 5A secondary adsorption

was eliminated by oxidizing NO *in situ* to NO_2, which was not removed under normal operating conditions[6]. On highly activated molecular sieve 13X these sites are transiently covered only above 8 μl of NO; smaller samples are difficult to quantitate accurately (Fig. 2).

Reproducible performance of molecular sieve 13X over the range 1.5–15 μl of NO was achieved with material heated for 12 h at 195°C (see Experimental). Fig. 3 compares the retention times of this material for O_2, N_2 and NO with that of fully activated molecular sieve. In contrast to previous findings[10,11], the retention time for NO on molecular sieve 13X in this intermediate activation state was now independent of the sample volume (Fig. 3A) and tailing was minimal. The activation state chosen was just sufficient for baseline separation within a minimal time span, but still below an activation level that would cause a non-linear response (Figs. 2 and 3B).

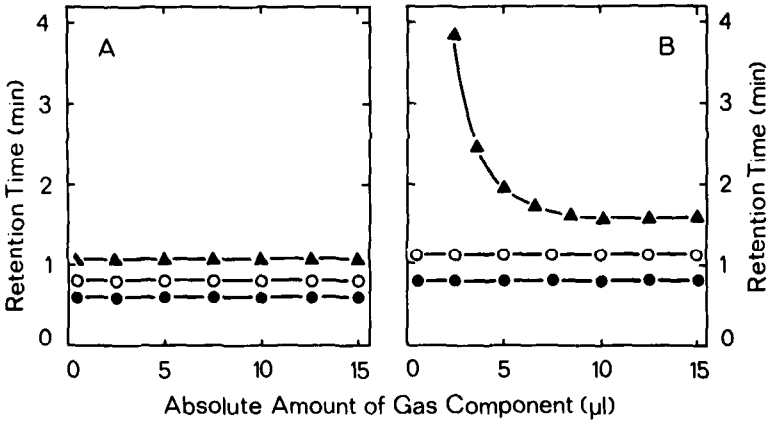

Fig. 3. Retention characteristics of two different activation states of molecular sieve 13X for O_2, N_2 and NO. (A) Activation under air at 195°C for 12 h; (B) activation on column as in Fig. 2. Running conditions: oven temperature, 40°C; carrier gas (He) flow-rate, 100 ml/min; steel column, 2.5 m × 3 mm I.D., molecular sieve 13X (45 60 mesh). NO was injected as a 20% mixture with He; O_2 and N_2 were injected as air. Symbols: ●, O_2; ○, N_2; ▲, NO.

Representative gas chromatograms of composite gas mixtures are shown in Fig. 4. The simultaneous analysis and complete separation of H_2, O_2, NO, CO, CO_2 and N_2O was achieved within 2.5 min and subsequently measurements can be carried out in this time interval. By use of the molecular sieve column only, the group of light gases was separated within 45 sec at an accelerated carrier gas flow-rate through channel 2 (Fig. 5). For this purpose columns 1 and 2 were switched into a parallel arrangement and the sample was injected through port 2.

The detection limits for the different gases were approximately one order of magnitude higher than those of other separation systems[6,11], using a sample volume of only 100 μl. The relative standard deviations, however, are comparable to those of other methods (Table I).

Application of the dual-column system is exemplified by following the evolution of gas from nitrite respiration by *Pseudomonas stutzeri*. Cultures grown with O_2

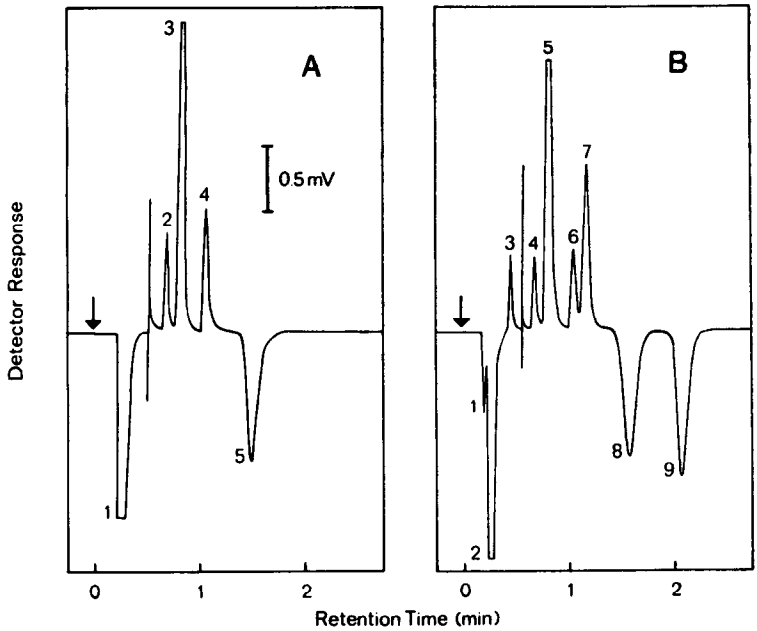

Fig. 4. Gas chromatograms of representative separations. Running conditions: oven temperature, 40°C; carrier gas (He) flow-rates, 190 ml/min through column 1 and 205 ml/min through column 2. The gas mixtures were prepared immediately before injection because of the reactivity of O_2 with NO. (A) Activated alumina as stationary phase in column 1. Elution sequence: 1, composite peak (O_2, N_2, NO); 2, O_2; 3, N_2; 4, NO; 5, N_2O. (B) Porapak Q and silica gel as stationary phases in channel 1. Elution sequence: 1, H_2; 2, composite peak (O_2, N_2, NO, CO); 3, H_2; 4, O_2; 5, N_2; 6, NO; 7, CO; 8, CO_2; 9, N_2O.

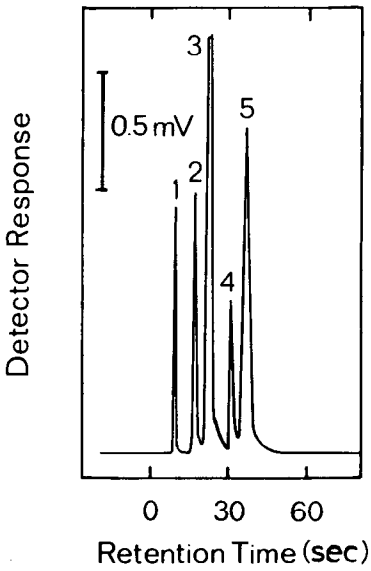

Fig. 5. Gas chromatogram of rapid separation of light gases. Conditions: molecular sieve 13X (column 2 only); oven temeprature, 40°C; carrier gas (He) flow-rate, 295 ml/min. Elution sequence: 1, H_2, 2, O_2; 3, N_2; 4, NO; 5, CO.

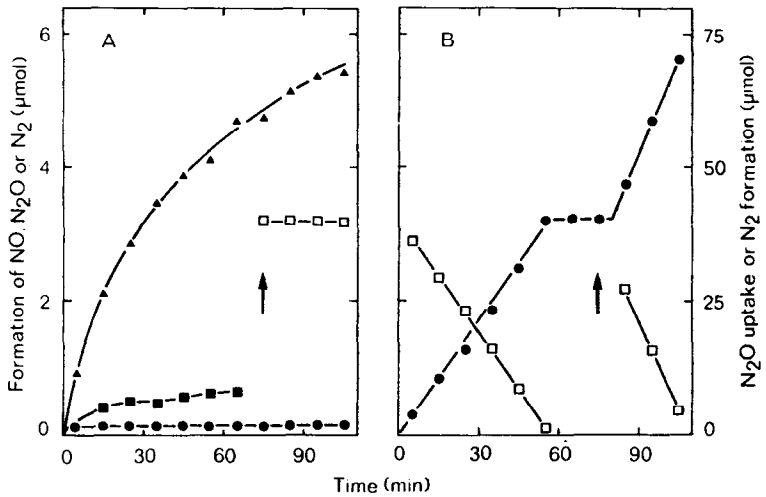

Fig. 6. Products of nitrite respiration by *Pseudomonas stutzeri* grown under O_2 limitation in the presence of nitrate. Two reaction vials containing in a 3-ml total volume whole cells (4.1 mg protein), 100 μmol of phosphate buffer (pH 7.0) and 150 μmol of Na lactate were incubated under helium at 30°C in a shaking water-bath. The reactions were started by injection of 50 μmol of $NaNO_2$ (A) or 40 μmol of N_2O (B). After 75 min (arrow), 40 μmol of N_2O were added to each assay mixture. The scale for N_2O uptake in A is identical with that in B. Symbols: ▲, formation of NO; ●, N_2; or ■, N_2O; □, N_2O uptake.

limitation in a nitrate-containing medium formed NO and N_2O as the only products (Fig. 6A). N_2 was not produced from nitrite, even though the cells exhibited strong N_2O reductase activity (Fig. 6B). The inhibition of N_2O reductase by NO[12] and the poor expression of NO reductase in cells exposed to low O_2 tension may explain this truncated denitrification (unpublished results). The simultaneous analysis of NO and O_2 by this chromatographic system also makes it a useful tool for the detection of air contamination in anaerobic reaction compartments.

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