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Rapid ^{18}O Analysis of CO_2 Samples by Continuous-flow Isotope Ratio Mass Spectrometry

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Continuous-flow isotope ratio mass spectrometry (CF-IRMS) has been shown to minimize sample preparation and reduce the sample size requirements and time for the determination of carbon and nitrogen. An inexpensive CF inlet was developed for the determination of the ^{18}O isotopic abundance in CO_2. Initial efforts were hampered by significant exchangeable oxygen within the inlet system, which was minimized by design changes and post-analysis mathematical correction. The system was tested for accuracy in the measurement of ^{18}O abundance in standards and was accurate to 0.04‰ with a precision of 0.03‰. Additional tests were performed using urinary water for the measurement of energy expenditure by the doubly labeled water method and the results agreed to within 1% with those from traditional off-line, dual-inlet IRMS. The CF-IRMS reduced the analysis time to 2 min per sample compared with 15 min for traditional duel-inlet IRMS. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Continuous-flow isotope ratio mass spectrometry (CF-IRMS) was originally applied to the determination of ^{13}C.[1] It has provided excellent accuracy and precision and reduced carbon sample requirements to the nanomolar level, while decreasing the time required for sample preparation and isotopic analysis.[2,3] More recently, the development of new interfaces has expanded the utility of CF-IRMS to other light elements, i.e. hydrogen, nitrogen and oxygen.[4–10] The general advantages of CF-IRMS, including speed, small sample size and precision, however, were not fully realized for oxygen. The determination of ^{18}O in the carbon dioxide in air has been reported to have a precision of 0.2‰ for 10 μmol of carbon dioxide, but only 1‰ for 0.2–1 μmol samples.[4] The speed and ease of CF-IRMS for the determination of ^{18}O would be advantageous in the analysis of total body water and energy expenditure by stable isotope dilution (doubly labeled water), but because of the cost of ^{18}O, a precision of 0.1‰ is needed to minimize dose requirements.[11]

The aim of this investigation was to refine CF-IRMS further for ^{18}O determination in biological fluids to meet the demands for precision and accuracy necessary for the measurement of energy expenditure by doubly labeled water. To do so required that exchange between the analyte and exchangeable oxygen in the CF-IRMS inlet system be minimized.

EXPERIMENTAL

Instrumentation

A Finnigan MAT (San Jose, CA, USA) Delta S isotope ratio mass spectrometer with a combustion interface was modified by the addition of an injection port that by-passed the combustion oven (Fig. 1). Specifically, a septum-sealed injector was added to the He make-up gas like of the open-split interface. A 6 cm length of Nafion tubing (Perma Pure, Toms River, NJ, USA) was placed directly after the injector to remove water using an external counter-current He flow. A stainless-steel packed column containing porous polymer beads (Porapak; Millipore, Bedford, MA, USA) facilitated the separation of carbon dioxide gases.

The IRMS employed the standard electron impact ion source and triple collector using standard 3×10^8, 3×10^{10} and 1×10^{11} feedback resistors for the m/z 44, 45 and 46 ion beams, respectively. Ultrapure He (Linde Gas, East Chicago, IN, USA) was used for all analyses. Flow rates were 5–12 ml min^{-1} for the inlet and the water scrubber.

Methods

Sample preparation. Fluid samples, i.e. water standards or urine, were equilibrated with CO_2. An 0.08 mol (1.5 ml) aliquot of fluid was injected into a 3 ml red-topped (no additive) Venoject tube (Terumo Medical, Elkton, MD, USA) along with 45 μmol (1 ml) of CO_2. These were

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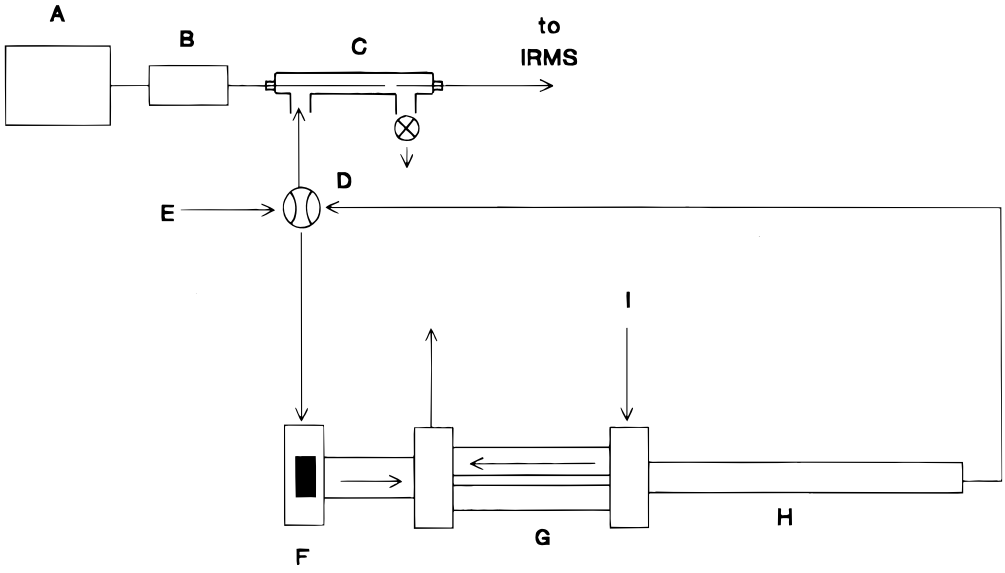

Figure 1. Schematic representation of the continuous-flow inlet for ^{18}O determination in CO_2. A, Gas chromatograph; B, combustion furnace; C, open split; D, four-port valve (Valco Instruments, Houston, TX, USA); E, make-up gas (He) line; F, septum; G, Nafion water scrubber; H, chromatographic column; I, gas line (He) for water scrubber.

equilibrated in an air bath at 30 ± 0.3 °C for at least 24 h. CO_2 samples (1–100 μl) were withdrawn through the septum with a gas-tight syringe (Scientific Glass and Engineering, Austin, TX, USA) or a 0.3 ml insulin syringe (Becton-Dickinson, Franklin Lakes, NJ, USA).

Off-line analysis. Comparisons with traditional off-line sample preparation and dual-inlet IRMS analysis were performed as described previously.[12] Samples were equilibrated as described above, except that the entire CO_2 sample was cryogenically purified under vacuum and introduced into the dual inlet of a Nuclide-360 IRMS system (State College, PA, USA).

Fluid samples. International water standards were obtained from the International Atomic Energy Agency (Vienna, Austria). Secondary water standards were prepared from tap water and tap water spiked with 10 atom% $H_2{}^{18}O$ (Cambridge Isotopes, Worchester, MA, USA). Urine samples were collected as spot urines after the oral administration of 2.5 g of 10 atom% $H_2{}^{18}O$ kg^{-1} total body water. Urine samples were decolorized with 200 mg of dry carbon black prior to equilibration with CO_2. All subjects gave informed written consent and the protocol was approved by the Institutional Review Board of the University of Chicago.

Calculation of oxygen exchange. Isotope exchange was calculated using a modification of the method described by Hayes.[13] The original model was developed for the purpose of detecting and measuring the isotopic contribution from an analytical blank that by itself was too small for isotopic analysis. The isotopic abundance (δ) of the mixture of N_t mol of CO_2 reaching the IRMS system represents the sum of the sample (N_s) plus material substituted by exchange (N_e):

$$\delta_t = (N_s/N_t)\delta_s + (N_e/N_t)\delta_e \quad (1)$$

Substituting $N_s = N_t - N_e$ and rearranging terms yields

$$\delta_t = \delta_s - N_e(\delta_s - \delta_e)/N_t \quad (2)$$

Hence a plot of δ_t *vs.* $1/N_t$ yields a line with an intercept equal to δ_s and a slope of $N_e(\delta_s - \delta_e)$. When this is performed with two specimens of different isotopic abundance, then N_e and δ_e can be calculated from the slope.

The original model of Hayes[13] was used to determine the size and isotopic enrichment of an analytical blank and considered the analyte reaching the IRMS system as a mixture of the sample (s) and the blank (b) such that the moles of total material (N_t) was the sum of the amount of sample input to the system plus the moles of blank, i.e. $N_t = N_s + N_b$. Under such conditions, both the moles of sample input to the system and the total moles derived from the system are measured and used in the calculations. In the presence of isotopic exchange, however, only moles of sample input to the system can be measured directly because for each molecule of material derived from the exchangeable pool during transit of the sample through the system, a molecule of sample is lost. Thus, an approximation that N_t equals the amount of sample input to the system is used in Eqn (2). This results in a non-linearity, but it will be negligible as long as the fraction of the sample subject to exchange is less than 5–10% of the total sample. The use of δ values in the mass balance equation is another potential source of non-linearity because it is derived from the $^{18}O/^{16}O$ ratio rather than an atom fraction. As such, δ values increase in a non-linear manner because the denominator decreases as the enrichment increases. The non-linearity, however, is negligible (<1%) as long as the enrichment is less than 5000‰, which roughly corresponds to an ^{18}O atom fraction of 0.01.

Exchange correction. All isotopic abundances of standards and physiological specimens were corrected for the contribution from isotopic exchange. An enriched and a natural abundance sample were analyzed daily to determine the abundance and amount of exchange. Equation (1) was then rearranged to solve for the isotopic abundance of the sample and used to correct each

apparent isotopic abundance for the contribution from exchange.

RESULTS AND DISCUSSION

A continuous flow inlet for analysis of ^{18}O isotopic abundances in CO_2 was constructed for under $300. The inlet was installed on the standard vendor's combustion interface as part of the make-up gas system for the open-split interface to the IRMS system so that it would not interfere with the operation of the standard interface by altering the dead volume. At the same time, it by-passed the copper(II) oxide reactor which would scramble the ^{18}O in the CO_2.

The major concern in the design of the interface was to minimize exchangeable oxygen so that samples as small as 40 nmol could be analyzed. This is no small concern. For example, if there were as little as a monolayer of water on the surface of the 0.8 mm i.d. capillary, we calculate that there would 0.2 nmol cm^{-1} of exchangeable oxygen. Even more daunting is the realization that room air at 50% relative humidity contains 650 nmol ml^{-1} of exchangeable oxygen in the form of water vapor. Based on surface area alone, however, our primary concern was the chromatography packing.

Initial attempts to determine the ^{18}O abundance were made without chromatographic separation of CO_2 from other gases in order to avoid potential exchange on the large surface area of the column packing. The isotopic accuracy was excellent when pure CO_2 was injected, but the accuracy was severely degraded when the CO_2 was mixed with air (Table 1). Loss of isotopic integrity was also observed when the CO_2 was mixed with N_2 or O_2, although the effect was worse for N_2. Because isotopic integrity was preserved when the CO_2 was separated from the air bolus by cryogenic isolation of CO_2 in a short length of transfer tubing prior to introduction into the IRMS system ($\delta^{18}O = -8.51‰$), it was concluded that the interference occurred in the ion source. This interference was eliminated with the use of a short gas–solid chromatographic column, and we therefore did not attempt to identify the spectroscopic source of the isotopic interference. We speculate, however, that the interference was due to the formation of nitrogen oxides, particularly N_2O_2, which has a molecular ion at m/z 46 that dramatically increases the apparent ^{18}O abundance.

Table 1. Effect of CO_2 purity on isotopic integrity by continuous-flow IRMS

Sample	$\delta^{18}O$ (‰)	SD (‰)
Pure CO_2 ($n = 12$)	−8.60[a]	0.11
CO_2 and air ($n = 8$)	52.51	6.89
CO_2 and N_2 ($n = 8$)	36.83	12.60
CO_2 and O_2 ($n = 8$)	23.84	5.42

[a] Off-line analysis of CO_2: $\delta^{18}O = -8.51‰$ *vs.* SMOW.

Separation of the CO_2 from the nitrogen and oxygen was most readily accomplished using a short (15–30 cm) gas–solid chromatographic column. We first tested Porapak-T because it offered the largest separation for CO_2. Porapak-T is a polar packing synthesized from ethylene glycol dimethacrylate. Porapak-T, however, also has a high affinity for water. We noted a significant column background at m/z 18 that could be reduced, but not eliminated, by preheating to 80–100 °C, and that the packing provided a major source of exchangeable oxygen (Table 2). We therefore tested Porapak-Q, a much less polar porous polymer synthesized from divinylbenzene. Although Porapak-Q has a lower separation power for CO_2 (retention relative to nitrogen of 2.7) it also has a lower affinity for water. The use of Porapak-Q eliminated the m/z 18 column background and reduced the isotopic exchange during CO_2 chromatography (Table 2). Using similar methodology, it was also found that a fused-quartz capillary was a lesser source of exchangeable oxygen than a nickel capillary (Table 2). We therefore used fused quartz for connecting the open slit interface to the IRMS system. Capillaries were used as obtained from the vendor without any attempt to pacify active sites.

To test for memory effects, samples of isotopically light and heavy CO_2 were injected in series (Fig. 2). No memory effect was detected, except in the injection syringe. Using either gas-tight syringes or less expensive, disposable 0.3 ml syringes could lead to up to a 10% memory for 100–400 nmol samples unless the syringe was flushed with air or sample between subsequent samples of different isotopic abundance.

The absence of memory was surprising because there was clearly a source of exchangeable oxygen in the system even after efforts to minimize exchange. This suggests that the exchange involves a large reservoir of exchangeable oxygen such that only a small percentage

Table 2. Amount of isotopic exchange associated with inlet components and its estimated isotopic abundance

Component	Exchangeable oxygen (nmol)	Isotopic abundance ($\delta^{18}O$, ‰)[a]
Chromatographic column:		
Porapak-T, 10 cm × 3.2 mm o.d.	1.4	38
Porapak-T, 20 cm × 1.6 mm o.d.	0.3	36
Porapak-Q, 10 cm × 3.2 mm o.d.	0.1	24
Transfer capillary between open split and IRMS system:		
Nickel, 0.3 mm i.d.		
	1.8	−3
Fused quartz, 0.3 mm i.d.	0.2	32

[a] $\delta^{18}O$ is expressed relative to SMOW.

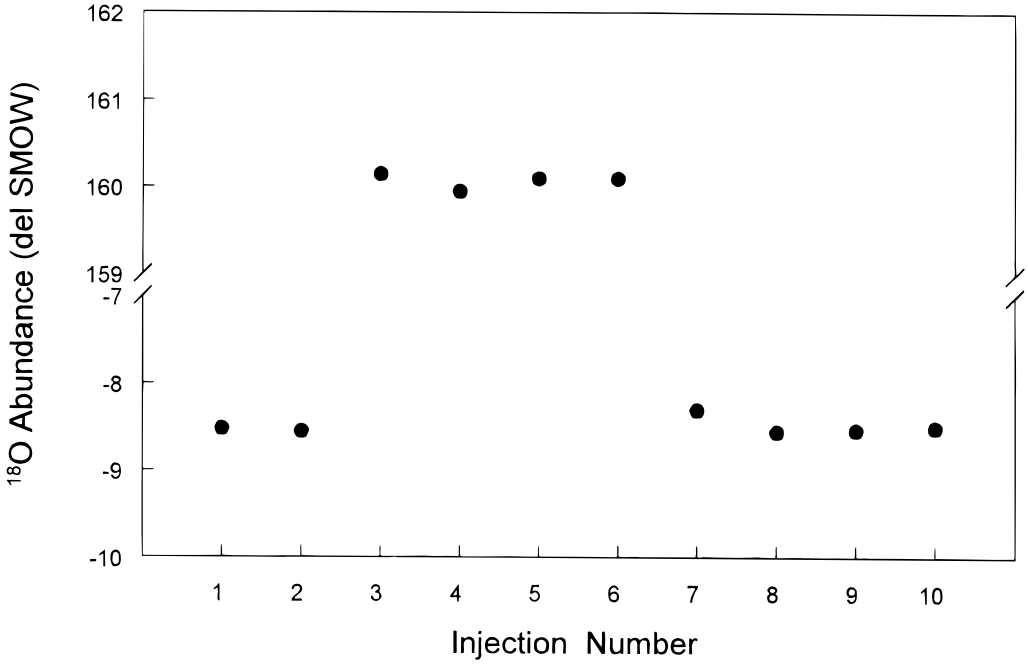

Figure 2. Effect of exchange on the isotopic abundance of a baseline (natural abundance) urine sample (injections 1, 2 and 7–10) and one obtained after a loading dose of ^{18}O (injections 3–6). Each point represents a single, consecutive injection of CO_2. A 10 cm × 1.6 mm o.d. Porapak-T fused-quartz transfer capillary was used during this analysis. Memory was calculated at less than 1% for these 100 nmol injections.

of the material exchanges or else there is a re-exchange with another source, such as trace water in the carrier gas, between injections. The latter was ruled out by passing the incoming He through a liquid nitrogen trap without any detectable change in performance.

The effect of sample size on ^{18}O abundance in an enriched water standard is illustrated in Fig. 3. With samples ranging in size from approximately 20 to 125 nmol on-column, the problem of oxygen exchange is most striking in the smaller samples, decreasing as samples increase in size, i.e. as the exchange constitutes a smaller proportion of the sample. After applying the oxygen exchange correction algorithm, sample size is no longer a concern. The accuracy of the system was tested using 100 nmol aliquots of CO_2 equilibrated with water standards (Table 3). The accuracy was 0.04‰, with a precision of 0.03‰.

The utility of the system for biomedical applications was tested using urine specimens obtained from two subjects who been given oral loading doses of 0.12 g kg

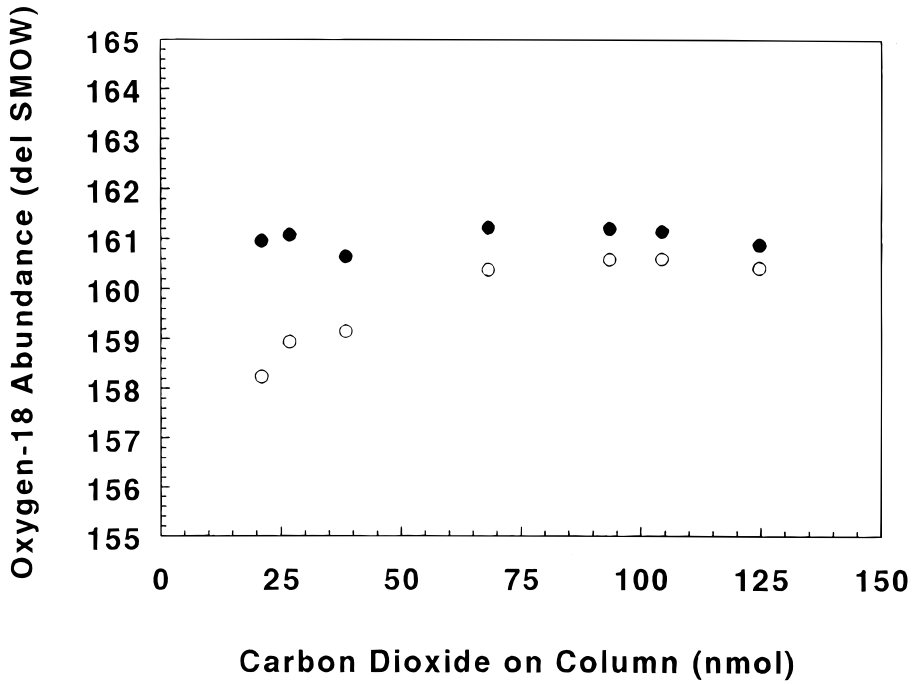

Figure 3. Effect of on-column sample size on ^{18}O abundance of an enriched water standard (○) before and (●) after correction for oxygen exchange as described in the Methods section. Mean (±SD) ^{18}O abundance after correction for exchangeable oxygen = 161.01 ± 0.12‰.

Table 3. Accuracy and precision of oxygen isotope analysis

	CF-IRMS			Off-line			
Sample	$\delta^{18}O$[a]	SD	*n*	$\delta^{18}O$[a]	SD	*n*	Difference
SLAP	−55.49	0.08	9	−55.50	0.02	4	−0.01
Tap water	−6.29	0.05	9	−6.37	0.02	4	0.08
Spiked standard	142.51	0.05	9	142.69	0.05	4	−0.18
Urine 1 before loading	−3.86	0.12	3	−3.92	0.11	2	0.06
Urine 1 after 6 h	180.69	0.06	3	180.91	0.10	2	−0.22
Urine 1 after 8 d	57.84	0.20	3	58.03	0.08	2	−0.19
Urine 2 before loading	−4.28	0.10	3	−4.18	0.08	2	−0.10
Urine 2 after 6 h	116.0	0.07	3	115.89	0.01	2	0.21
Urine 2 after 8 d	39.50	0.16	3	39.30	0.02	2	0.20

[a] $\delta^{18}O$ is expressed relative to SMOW.

$H_2{}^{18}O$ for measurement of energy expenditure by doubly labeled water.[14] Urine was collected before the loading dose and 6 h and 8 d after the loading dose. The biological elimination rates for the two subjects were calculated ($k_O = 0.1370$ and $0.1264\ d^{-1}$) and compared with that determined by tradition off-line, dual-inlet IRMS ($k_O = 0.1366$ and $0.1269\ d^{-1}$). The elimination rates determined by the two methods agreed to within 1% therefore the systems had adequate accuracy and precision for application to the doubly labeled water method for the measurement of energy expenditure.[12]

The use of continuous-flow IRMS for the determination of ^{18}O in CO_2 was tested. A significant potential for exchangeable oxygen in the system was observed and minimized by design changes and corrected for by post-analysis calculations. The system was shown to be accurate for ^{18}O abundances in urinary water for measurement of energy expenditure by the doubly labeled water method.[11] The major advantage of this system was that it reduced the analysis time to 2 min per analysis compared with about 15 min using the traditional off-line, dual-inlet IRMS.

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