High-Precision Gas Isotope Ratio Mass Spectrometry: Recent Advances in Instrumentation and Biomedical Applications

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Introduction

Gas isotope ratio mass spectrometry (GIRMS, or simply IRMS) is probably the oldest branch of *analytical* mass spectrometry. GIRMS is now a standard tool in disciplines as diverse as biomedicine and geochemistry and continues to be vibrant area of instrumentation development. In this decade, GIRMS based on the widely employed dual inlet is being replaced by continuous flow methods, which produce comparable precision in a fraction of the time and enable interfacing of the GIRMS instrument to a wide variety of inlets. Applications to biomedical tracer studies have increased in recent years, partially because of the commercial introduction of gas chromatography (GC) interfaces for rapid and convenient "compound-specific isotope analysis" (CSIA) of $^{13}C/^{12}C$ ratios. This technology enables high-sensitivity stable-isotope-based tracer experiments which analytically are competitive with radiotracers without risks to subjects and disposal concerns.

GIRMS instrumentation developments of the past two years include capabilities for GC analysis of $^{15}N/^{14}N$ and extension to liquid chromatography (LC). In this Account, we discuss the GIRMS technique in its traditional form using the classical dual inlet and the increasing use of the continuous flow approach for rapid gas analysis or CSIA, with emphasis on selected biological applications driving instrumental and methodological developments.

Historical Background and Fundamentals

The roots of GIRMS are traceable to the first mass spectrograph of F. W. Aston in 1919, who showed definitively that *m/z* 22 observed by J. J. Thompson in 1912 was indeed a minor isotope of Ne.[1] Continuing developments evolved to the level of applications by the 1930s and contributed to dramatic changes of our understanding of physiology and of natural processes. A succession of instruments led to the 1940 Nier design[2] suitable for routine measurements, which included a 60° magnetic sector similar to those employed on modern GIRMS spectrometers. The core of high-precision measurements is the dual inlet, developed by Murphey[3] in 1947 for the study of diffusion of H isotopes. The original device, which permitted rapid sample/standard comparison, was the forerunner of the modern "changeover valve". The importance of sample/standard comparison was recognized quickly, as a refined version of this instrument was described in 1950 by the Urey group.[4] It is, in fact, the *difference* in isotope ratio which can be determined with great precision and accuracy, rather than the absolute isotope ratio. Modern, commercially available dual inlet GIRMS instruments employ the principles established in this latter paper and differ primarily in technological innovation.

High-precision IRMS is the standard technique for determination of the isotope ratios D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{17}O/^{16}O$, and ratios of the minor isotopes of S (^{33}S, ^{34}S, and ^{36}S) to the major isotope, ^{32}S. Pure gases, CO_2, H_2, N_2, O_2, and either SO_2 or SF_6, are employed for measurement of each element in order to optimize precision and accuracy per unit time. Each element must be converted from its current chemical form into the required gaseous species and purified prior to introduction to the ion source.

Notation. The absolute precision of IRMS measurement is routinely <1000 ppm relative standard deviation (RSD), and important differences in isotope ratio often occur over a very limited range. IRMS data is usually expressed in the $\delta^y X$ notation, given as

$$\delta^y X_{STD} = \left(\frac{R_{SPL} - R_{STD}}{R_{STD}}\right) \times 1000 = \left(\frac{R_{SPL}}{R_{STD}} - 1\right) \times 1000; \qquad R_X = \frac{[^yX]}{[^zX]} \quad (1)$$

where ^yX is the minor isotope and ^zX is the major isotope, "SPL" and "STD" refer to sample and standard, respectively, and the value of δ is referred to as "per mil", denoted "‰". The international standard for C is a sample of carbonate obtained from the Pee Dee formation in South Carolina (Pee Dee belemnite, "PDB"), relatively rich in ^{13}C, with an accepted value of $R_{PDB} = 0.011\,237\,2 \pm 0.000\,009\,0$.[5] For comparison, a compound of $\delta^{13}C_{PDB} = -1‰$ corresponds to a value of $R_{SPL} = 0.011\,226\,0$, and terrestrial plants have a ^{13}C content of $-40‰ < \delta^{13}C < -10‰$. The typical precision and accuracy associated with GIRMS measurements is $\delta^{13}C < 0.4‰$, so the $\delta^{13}C$ notation has the advantage of eliminating unchanging leading digits. A very useful brief discussion of standards for

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(1) Aston, F. W. *Mass Spectra and Isotopes*; Longmans, Green, and Co.: New York, 1942.
(2) Nier, A. O. *Rev. Sci. Instrum.* **1940**, *11*, 212–216.
(3) Murphey, B. F. *Phys. Rev.* **1947**, *72*, 834–837.
(4) McKinney, C. R.; McCrea, J. M.; Epstein, S.; Allen, H. A.; Urey, H. C. *Rev. Sci. Instrum.* **1950**, *21*, 724–730.
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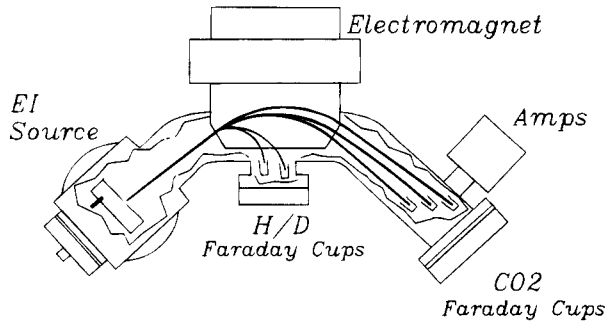

Figure 1. Diagram of a high-precision GIRMS instrument, the major features of which are a tight electron impact source, a single magnetic sector, and multiple Faraday cup detectors with dedicated amplifiers. The split flight tube is used for analysis of H isotopes.

all IRMS elements can be found elsewhere.[6] Isotope data are also expressed in atom % (AP), calculated directly from $\delta^{y}X$.

Instrumentation for Gas Analysis

We first discuss the mass spectrometer common to all GIRMS experiments, followed by a discussion of the classical dual variable-volume inlet and the various continuous flow inlets.

Mass Spectrometer. A diagram of a GIRMS instrument is shown in Figure 1. A tight electron impact (EI) ion source is employed to maximize sample residence time and thereby maximize ionization probability. This is in contrast to organic mass spectrometers where open EI sources minimize collisions which induce (i) ion−molecule reactions and (ii) an unacceptable accumulation of insulating organic material on ion optical elements. The GIRMS ion source does not require frequent cleaning because relatively unreactive gases are used exclusively. The ion source potential is typically 3−10 kV. Molecular ions emerge from the ion source and are separated by a magnetic sector set to a single field strength throughout the experiment. No energy filtering is used so as to optimize transmission near unity, giving a typical absolute sensitivity of 10^{-3} (about 1000 molecules enter the ion source per ion detected). Mass-filtered ions are focused onto dedicated Faraday cup (FC) detectors positioned specifically for the masses of interest. For example, GIRMS instruments equipped to determine CO_2 have three FCs for measurement of *m/z* 44, 45, and 46, positioned so that the ion beam of each mass falls simultaneously on the appropriate cup. Each FC has a dedicated amplifier mounted on the vacuum housing to minimize noise pickup, and dedicated counters/recorders for continuous and simultaneous recording of all relevant ion beams.

Inclusion of more than six FCs along the focal plane is difficult due to space constraints, and so the "split flight tube" arrangement is employed for analysis of H isotopes. Because of their small mass and relatively large mass difference, H isotopes can be focused and mass resolved around a tighter radius than the other isotopes, where FCs for H_2 and HD can be located in an uncluttered region of the instrument. It should be noted that just one gas can be analyzed at a time because the magnetic field is set at the characteristic value to direct particular ions into their respective detectors.

(6) Ehleringer, J. R.; Rundel, R. W. In *Stable Isotopes: History, Units, and Instrumentation*; Rundel, P. W., Ehleringer, J. R., Nagy, K. A., Eds.; Springer-Verlag: New York, 1988; pp 1−15.

While modern organic mass spectrometers (except FTMS) universally employ electron multipliers (EM), FCs are the detectors of choice for IRMS, due to two major considerations. Firstly, the absolute precision required for IRMS determinations is at leat 10^{-4}, which is attainable based on counting statistics with at least 10^8 particles detected. Ion currents that achieve these levels are well within the range detectable by FCs. For instance, the major ion beam current for atmospheric CO_2 analysis, *m/z* 44, will typically be around 10 nA, with the minor *m/z* 46 beam around 40 pA. Secondly, FCs are highly stable and rugged and rarely need replacement, compared to EMs, whose sensitivity degrades with use.

Dual Inlet. The dual adjustable-volume inlet system facilitates sample/standard comparison under the most nearly identical of circumstances. It is necessary for highest precision to compensate for (1) normal fluctuations in instrumental response and (2) ion source nonlinearity, which produces isotope ratios that depend on the source gas pressure. The latter phenomenon can be understood from consideration of the physical arrangement of an EI ion source. The source includes magnets that collimate the electron flow from the filament through the ion box. Once formed, ions are accelerated across magnetic field lines which induce minor but measurable mass selectivity. As the mean free path of ions depends on the relatively high pressure in the closed ion source, the mean position from which ions escape the ion box depends on pressure, as well as on mass. This translates into a subtle dependence of isotope ratio on pressure which becomes apparent at the precision of these measurements. The severity of this effect in any particular source defines the relative importance of sample/standard pressure matching. Continuous flow methods have now become routine because modern ion sources are designed for maximum linearity, and because the He carrier gas maintains the ion source pressure nearly constant and independent of the level of sample gas flowing into the source.

Modern inlets are designed for handling of minimal gas sample volumes to maintain viscous flow for small sample sizes. At lower pressures, molecular flow occurs and time-of-flight fractionation is observed. In order to maintain high pressure for small sample volumes, gas is transferred around the inlet via capillaries and low dead volume valves. The minimum sample size that can be analyzed by dual-inlet GIRMS is limited by the level at which viscous flow is maintained and is typically >100 μg of CO_2, or >2 μg of CO_2 for instruments equipped with a cryogenic concentrator and microvolume. Variable-volume welded metal bellows with an expanded-to-compressed volume ratio of about 20/1 are employed as analyte containers. After pressure equalization, the standard is admitted to the ion source while the sample is open to a flow-matched waste pump to maintain pressure balance between the two volumes. After a measurement time of typically 8−60 s, flows are switched and sample is measured. Usually the cycle is repeated six to eight times and the results are averaged to produce one determination. This method provides the most precise isotope ratio, with repeated measurements of a single gas aliquot routinely $\delta^{13}C < 0.1‰$ (SD).

Continuous Flow. A more recent approach to IRMS inlet systems is referred to as "continuous flow", where He carrier gas passes continuously into the ion

source and sweeps bands of analyte into the source for analysis.[7] This approach overcomes the sample size requirements for viscous flow because He carrier maintains viscous conditions independent of sample size. Although originally developed for online GC coupling, this approach is supplanting dual inlet for traditional dual-inlet applications.

Until recently, complex automated cryogenic purification systems were employed prior to the inlet for analysis of breath gases and headspace CO_2. The direct coupling of GC separation with IRMS is now offered commercially, and instrumentation dedicated to headspace gas analysis has been introduced. As little as 2 μL of headspace gas is injected onto a GC column which resolves N_2 and CO_2 and passes the effluent through a water trap and into the IRMS. Isotope ratios are calculated by integrating the peaks in each mass channel and standardizing on a calibrated standard, as discussed in more detail below for GCC–IRMS. Replicate determinations give a precision of $\delta^{13}C < 0.2‰$ with similar accuracy in an analysis time of about 1 min.[8] For comparison, conventional cryogenic systems can achieve similar performance in about 15 min and are of much greater complexity and expense to build and maintain.

Compound Specific Isotope Analysis (CSIA)

While studies of isotope ratios of bulk materials continue to be extremely useful, studies of C isotope ratio associated with individual compounds should be even more useful. There is a wealth of experience with specific compounds that are easily separated from their surrounding matrix, such as water, which, for instance, has yielded much of what we know about ancient climate conditions. Easily purified compounds are few, although many reports document chemical separation of individual chemical species from natural complex mixtures, with subsequent conversion to the required gas (e.g., CO_2) and determination of isotope ratio with high precision. The time-consuming nature of this work coupled with the relatively poor detection requirements (>2 μg) conspire to limit such studies to a small number of major components. True CSIA awaited the advent of online treatment, which made possible the direct coupling of separation techniques with high-precision IRMS.

GCC–IRMS. The first such device for coupling of GC and IRMS for C isotope determination was described by Matthews and Hayes in 1978,[9] based on earlier low-precision work of Sano et al.[10] A schematic diagram of a modern, commercially available instrument is shown in Figure 2. The GC column is connected to a combustion furnace loaded with CuO and Pt and held at about 850 °C. At this temperature, CuO exists in equilibrium with Cu and O_2, while Pt serves as a combustion catalyst, via

$$CuO(s) \xrightarrow{850\ °C} Cu(s) + O_2\uparrow \qquad (2)$$

Separated organic species are swept into the furnace by He carrier gas and quantitatively combusted to CO_2 and H_2O. As material is combusted, reaction 2 is driven to the right and so the CuO serves as a metered

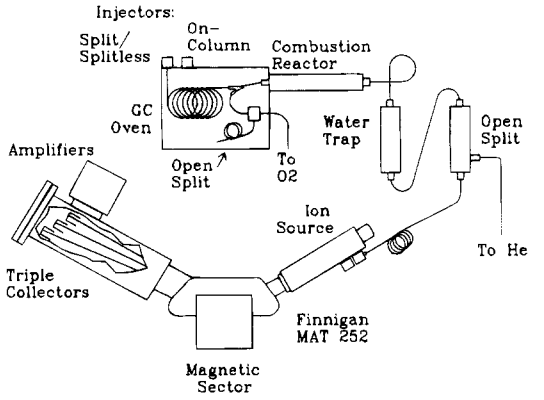

Figure 2. Schematic diagram of a gas chromatography–combustion–IRMS system. The GC effluent is combusted, dried, and admitted to the GIRMS instrument, where the signal from the C isotopes is continuously monitored.

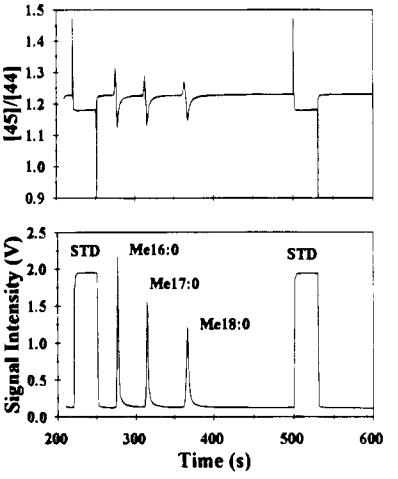

Figure 3. Illustrative GCC–IRMS chromatogram of a standard mixture of three fatty acid methyl esters. The lower trace is the *m/z* 44 signal; the upper trace is the ratio of the *m/z* 45 to *m/z* 44 signals. The square pulses are calibrated CO_2 gas admitted from an external bellows. The ratio trace responds whenever the CO_2 isotope ratio changes, due to analyte or standard, from the column bleed background.

source of O_2. These products are then passed through a water trap, which is often a length of Nafion sulfonated perfluorinated polymer which passes H_2O freely while retaining CO_2 nearly quantitatively. Cryogenic water traps have also been successfully implemented. The dried CO_2 passes through an open split to regulate pressure and flow and then directly into the ion source.

A chromatogram is shown in Figure 3, with the *m/z* = 44 trace shown on the bottom and the corresponding [45]/[44] ratio trace shown on the top. The chromatogram in each mass channel is nearly identical to that from a flame ionization detector (FID), which also generates a signal when CO_2 is produced by combustion. The ratio trace shows a level change at each peak corresponding to the shift of isotope ratio from background column bleed C to that of the eluting compound. The positive-going/negative-going peak shape is thought to be due to chromatographic separation of ^{13}C-substituted from all-^{12}C isotopomers. The peaks labeled "STD" are pulses of isotopically calibrated CO_2 from a gas bottle.

A peak-defining algorithm defines tops for one mass channel, adjusts the other mass channels for the time delay due to isotopic substitution, defines peak start/stop for a single mass channel, and applies those

(7) Guilluy, R.; Billion-Rey, F.; Pachiaudi, C.; Normand, S.; Roud, J. P.; Jumeau, E. J.; Brazier, J. L. *Anal. Chim. Acta* **1992**, *259*, 193–202.
(8) Hilkert, A. W.; Brand, W. A. *World Conf. Stable Isot. Nutr. Metab. Res., 2nd* **1994**, L14.
(9) Matthews, D. E.; Hayes, J. M. *Anal. Chem.* **1978**, *50*, 1465–1473.
(10) Sano, M.; Yotsui, Y.; Abe, H.; Sasaki, S. *Biomed. Mass Spectrom.* **1976**, *3*, 1–3.

values to the others to calculate areas. Area ratios are then calculated and calibrated either against the CO_2 pulses or against calibrated compounds within the chromatogram. Precision and accuracy are about $\delta^{13}C < 0.4‰$ for well-resolved peaks containing at least 10 ng of C. The highest quality results are obtained for peaks of intensity comparable to the standard, probably because of ion source nonlinearities. An attractive alternative method of calibration is to add an internal standard of calibrated isotope ratio in the chromatography mixture.[11] The advantage to this procedure is that the standard and the analytes are subjected to identical physical conditions. In our hands, this advantage is offset for standards eluting later in the chromatogram where peak broadening degrades subsequent peak definition and the resulting isotope ratios. CO_2 standard gas has the advantage that the pulse can easily be positioned anywhere in the chromatogram and it always has a sharp beginning and end for easy integration.

GCC–IRMS for $^{15}N/^{14}N$. Very recently, two groups have reported instruments for online determination of the isotopes of N for compounds eluting from the GC column,[12,13] with both applied initially to amino acid analysis. The principle analytical challenges associates with N, as compared to C, are that (1) N is found at substantially lower concentrations in organic molecules than C, so that sensitivity on a per-weight-sample basis is much lower, and (2) N is analyzed as N_2, which requires reduction chemistry, as opposed to C, which is analyzed as CO_2, which requires only oxidation chemistry.

The apparatus described by Preston and Slater[13] uses a conventional combustion interface which generates CO_2, CO, H_2O, and N_2 from combustion of volatile amino acid derivatives. Water, CO_2, and any residual higher *N*-oxides are removed by a cryogenic trap, with products directed to a second GC column for separation and removal of CO from N_2. The authors quote a typical analytical precision of ±0.0002 AP ($\delta^{15}N_{AIR} = 5‰$) near natural abundance for a sample size of 7 nmol of phenylalanine using an internal standard, and they indicate that this is largely limited by the MS sensitivity of their pilot system. The system reported by Merritt and Hayes[12] is similar in design as it also includes a combustion furnace reactor and cryogenic trap for removal of CO_2, but it is interfaced to a higher sensitivity IRMS instrument. In addition, these authors included a reduction reactor loaded with Cu wire, placed after the combustion furnace, for reduction of *N*-oxides and removal of O_2. For 2 nmol of amino acid on the column with 20% of the sample flowing into the mass spectrometer, this system provided a precision of $\delta^{15}N_{AIR} = 0.2‰$ with accuracy better than one-quarter that value.

LCC–IRMS. Recently, we described the first device capable of coupling a liquid chromatography to a high-precision IRMS instrument.[14] The interface is based on moving wire systems originally developed in the mid-1960s for interface of LC to an FID, devices which gained some commercial success but were superceded by other advancing detection technologies. The key to an effective LC interface is the reproducible desolvation of the analyte prior to combustion. The moving transport system accomplishes this efficiently and therefore was the approach of choice for initial efforts. Figure 4 is a diagram of the original system. The LC eluent flows to a coating block where the wire moves through the stream and carries away an estimated 1–5 μg/m, with the remainder flowing to waste. This region can be considered a "coating split" with a collection efficiency under our conditions calculated to be about 1%. After solvent drying at 200 °C, the analyte is combusted in an 800 °C combustion furnace loaded with CuO, picked up by a He carrier stream and directed to a capillary leading to the water trap. This latter "collection" split is estimated to accept 10–50% of the stream, depending on flow settings. After drying, the stream of carrier He and analyte-derived CO_2 passes directly to the IRMS. Routine precision and accuracy for the flow injection mode, without an LC column, are about $\delta^{13}C = ±0.5‰$ for injections of 50 μg of linoleic acid over a range of enrichments up to $\delta^{13}C = 90‰$. For a mixture of carbohydrates, precision and accuracy drop to about ±1–2‰, probably due to poorer performance of the peak detection algorithm for peaks broadened during time in the LC column.

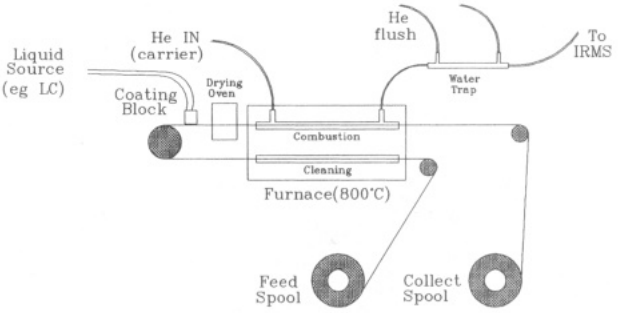

Figure 4. Schematic diagram of the first LCC–IRMS interface, based on the moving transport. The wire is coated with LC eluent, the solvent driven off at 200 °C, and the analyte combusted, dried, and admitted to the IRMS.

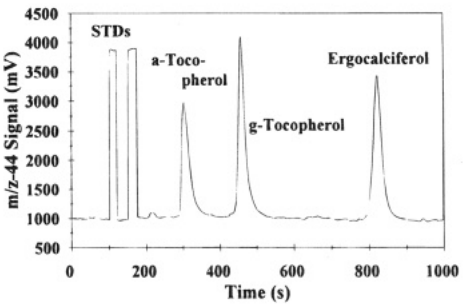

Figure 5. LCC–IRMS chromatogram of a mixture of α- and γ-tocopherol and ergocalciferol (45 μg injected per component). A 4.6 mm column with 2 mL/min flow rate, isocratic acetonitrile mobile phase was used.

Recent efforts to improve sensitivity in this system[15] have yielded signal enhancement by more than 2 orders of magnitude. Figure 5 shows an LCC–IRMS separation of a mixture of two tocopherol isomers and ergocalciferol. Considering the splits and the sample loading levels of 45 μg per component, we can estimate that the mass of CO_2 entering the ion source is between 10 and 400 ng to yield this chromatography. The intensity differences for these equimolar compounds are likely to be due to differences in coating properties of the analytes. Precision is not limited by counting statistics as signal levels of <10% yield

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(12) Merritt, D. A.; Hayes, J. M. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 387–397.
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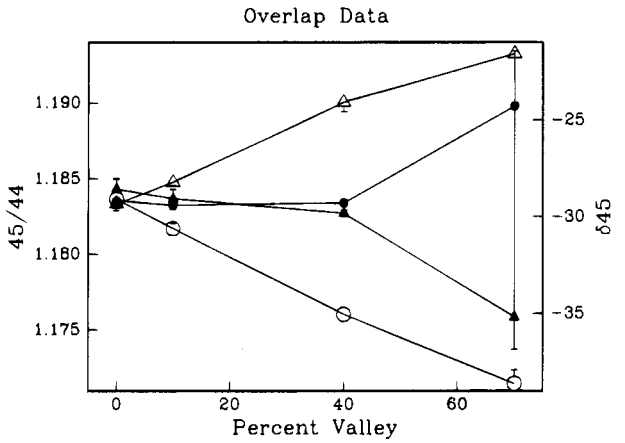

Figure 6. Effects of peak overlap on GCC–IRMS determined isotope ratio for two compounds, methyl tridecanoate (circles, first eluting) and butylated hydroxytoluene (triangles). Data was processed with either the conventional vertical drop method (open symbols) or fitting using the exponentially-modified Gaussian. Overlaps result in precise but biased isotope ratios, while fitting is nearly immune to overlaps to at least 40%. Error bars are 95% confidence limits.

similar results. It is probably limited by the LC peak width and by base-line instability, partly due to the chemical noise associated with this mixture, which makes peak definition less certain. We can therefore estimate that the minimum sample required to yield $\delta^{13}C = \pm 1-2‰$ precision is 10-fold lower than those used here. Redesign of the system to move toward unit collection and transfer efficiency by, for example, use of a belt rather than a wire can be expected to provide useful data for an injected sample of 1–40 ng, which is competitive with current GCC–IRMS systems.

Data Processing. One of the great strengths of the conventional organic mass spectrometers is the ability to provide an additional level of selectivity when analyzing compounds eluting from a separation device. Retention time in chromatography seldom depends directly on mass, and so it usually is possible to track individual compounds by monitoring the molecular ion or a suitable fragment ion in the selected ion monitoring mode (SIM). This ability enables the resolution of severely overlapping peaks and, with prior information about the mass spectra of each compound, completely overlapping peaks. The analogous situation does not exist in GCC–IRMS, since each compound is combusted to CO_2 and has an identical qualitative signature in the mass spectrometer. GCC–IRMS traces from each monitored channel are nearly identical to FID traces, since they arise from detection of C. It is known empirically that the precision for moderately overlapping peaks is not degraded significantly compared to the nonoverlapping case, implying that the chromatography and the summation peak definition algorithm are highly reproducible compared to other sources of noise in the system.

The accuracy of isotope ratios for overlapping peaks is a more subtle matter. Recently, we systematically evaluated the effect of graded degrees of overlap on the accuracy of isotope ratio determinations for two compounds differing in isotope ratio by $\delta^{13}C < 0.5‰$.[16] Results for compounds of equal abundance are shown in Figure 6. Even for 10% valley, the isotope ratios calculated using the vendor-supplied perpendicular drop algorithm diverge by ±2‰ compared to the completely resolved case, with the earlier eluting peak showing isotope depletion and the later eluting peak showing enrichment. These effects were corrected for overlaps up to 40% valley by using Levenberg–Marquardt nonlinear least squares fits for each peak and for the background, using combinations of exponentially-modified Gaussian and Haarhoff–Van der Linde[17] functions to approximate line shapes, with no appreciable loss of precision. This approach has the inherent advantage that an algorithm for definition of peak beginning and ending is not necessary, as the function are integrated over all space. Recent measurements in our lab indicate that curve-fitting improves precision for low-level compounds[18] and may be particularly useful for highly enriched compounds commonly encountered in tracer work.

Coupling to Organic MS. One of the principal strengths of the GCC–IRMS system is its ability to produce high-precision isotope ratio data for many components in a complex mixture. Inherent in any experiment involving complex mixtures is the problem of unequivocal identification, as the usual figure of merit, retention time, can only be considered indicative of chemical identity. A recent paper reports the interface of an rf ion trap with a GCC–IRMS system to facilitate splitting of the GC effluent to the combustion furnace with simultaneous admission to the ion source of an organic mass spectrometer.[19] Initial experiments with labeled amino acid metabolites indicate that doses 60× smaller than those used for GC/MS detection could be reliably quantified by GCC–IRMS.[20]

Stable Isotopes in Biomedical Tracer Experiments

High-precision GIRMS has had a formative impact on a wide variety of fields over the decades, and several volumes would be required for an adequate treatment of applications. This section will discuss selected biomedical applications, and the reader is referred to surveys of current research in literature devoted to applications in other areas such as ecology,[6] biogeochemistry,[21] geology,[22] and nutrition.[23]

An excellent historical reference is the 1947 "Symposium on the Use of Isotopes in Biology in Medicine",[24] which discusses applications familiar to modern biomedical IRMS practitioners, including protein biosynthetic rates and amino acid turnover, transmination/deamination, fatty acid biosynthesis and turnover, and gluconeogenesis. Isotopic biotracers research dates to the 1930s, when Urey's discovery of D gave biologists a tool for tracing organic molecules.[25]

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(25) Urey, H. C.; Brickwedde, F. G.; Murphy, G. M. *Phys. Rev.* **1932**, *39*, 164–165.

By 1936, Schoenheimer, Rittenberg, and others had used D for tracer applications, and by decade's end, they had transformed then-current thinking about metabolism by showing the dynamic character of mammalian lipids and proteins. Most of the early studies used either refractive index or density (i.e., falling drop method) measurements to assay for D content of combusted samples. By the 1940s, GIRMS was routinely employed for analysis of ^{13}C in doubly labeled acetate ($^{13}C/D$) and other species, in experiments where the analyte was isolated chemically and combusted to CO_2 prior to analysis.[26,27]

The vast majority of current biomedical IRMS experiments involve tracers and analysis by both dual-inlet and continuous flow methods. Reviews of conventional methods are available (e.g., ref 28). We briefly mention a couple of prominent examples, first requiring general CO_2 or H_2 gas analysis and, second, CSIA.

Analysis of Breath and Water. Breath tests have been developed to detect oxidation of a variety of compounds by analysis of labeled C of expired CO_2. Originally, ^{14}C radiotracers were used, but this has been almost completely overtaken for human clinical applications by ^{13}C labeling. Breath tests are increasingly employed in clinical situations for detection of, for example, protein and lipid absorption, hepatic detoxification capacity, digestive enzyme function (amylase; lipase), and gastric microbial colonization for ulcer diagnosis. This expanding role in clinical medicine has prompted an organized European effort to standardize protocols.[29]

Determinations of total body water dilution space using oral D_2O or $H_2{}^{18}O$ with sampling of urine, saliva, or serum are now routine research tools. In the 1940s, Nathan Lifson, working with Nier, discovered that the turnover of body H is predominantly by water loss, while the turnover of O is by water loss and by expired CO_2. On the basis of this insight, Lifson proposed that CO_2 production could be monitored by following the difference in washout rates between H and O in body water after administration of a dose of water labeled with D and ^{18}O, in a technique that is commonly referred to as the doubly labeled water method (DLW).[30,31] Since CO_2 production can be related to calorie production, this is a very convenient means to determine energy expenditure with minimum perturbation in free living animals. High-precision GIRMS is required to permit very small doses of the expensive isotope ^{18}O. In fact, the cost of ^{18}O limited applications to small animals until about 1980, when it was first applied in humans.[32] To date the total number of papers using this technique is approaching 1000.[33] A review of the method including technical aspects has recently been published.[34]

High-Sensitivity Tracers Using GCC–IRMS.

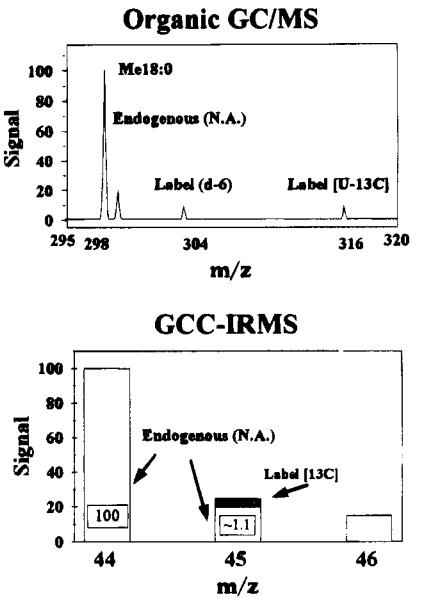

Figure 7. Diagrammatic comparison of organic GC/MS and GCC–IRMS for stable isotope tracer studies. Top: Conventionally, GC/MS SIM tracers are partially deuterated to shift the tracer mass beyond the natural abundance envelope. Use of perlabeled tracers results in further mass shift but no increase in sensitivity; the sensitivity is therefore independent of the number of labeled atoms on the molecule. Also the detection sensitivity is directly related to moles of *analyte* and inversely related to background noise and the number of monitored masses. Detection of a particular labeled compound depends on initially setting the MS to cycle through the selected peaks during the run. Bottom: In contrast, the detection sensitivity of GCC–IRMS for enriched tracer compounds is strictly related to precision, which defines the minimum enrichment level detectable compared to the background levels. Since each analyte C is analyzed, detection sensitivity is related to overall tracer enrichment; greater enrichment translates into greater sensitivity. Further, sensitivity is related to moles of *C in analyte*, and all peaks are detected with equal sensitivity since no peak switching or time sharing of detectors is necessary.

The postcolumn combustion system of Sano et al.[10] (1976), which predates the first GCC–IRMS system[9] (1978), was intended for detection of unknown drug metabolites. While Barrie et al.[35] discussed the sensitivity advantage for high-performance tracer studies as early as 1984, GCC–IRMS tracer applications have begun to appear only since the introduction of commercial instrumentation in 1990.

The xCC–IRMS (x = G or L) instrument is uniquely suited to take advantage of recent developments in the production of highly enriched tracers using microalgae.[36] We have introduced and evaluated the coupling of highly enriched, uniformly labeled tracers derived from microalgae grown in the presence of 99+% $^{13}CO_2$ with analysis by GCC–IRMS.[37] Because combustion permits the detection of each analyte C individually, the sensitivity of GCC–IRMS is proportional to the overall enrichment of the tracer molecule. This contrasts with organic GC/MS, in which higher order labeling shifts the isotope peak by additional mass units with no increase in sensitivity, as shown in the top panel in Figure 7. For GCC–IRMS, we have calculated that the minimum detectable label is

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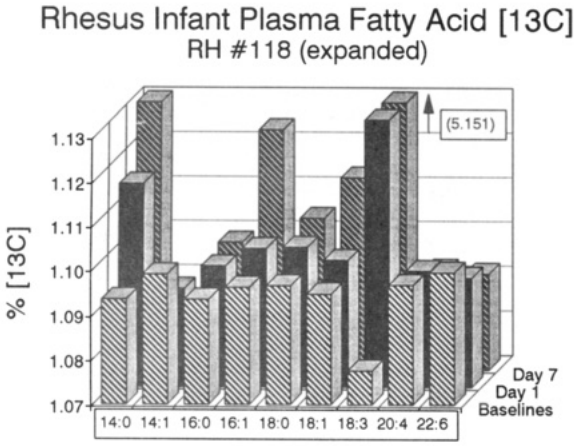

Figure 8. Atom % ^{13}C for fatty acids extracted from nursing infant rhesus monkey plasma. Samples obtained at days 1 and 7 show enrichment from a single dose of [U-^{13}C]-α-linolenic acid (20 mg) to the lactating female. Enrichment of the tracer at day 7 is >5%.

in the 10 fmol of C range under moderately well-behaved chromatographic conditions. Further, these detection limits are achieved for all compounds in the chromatographic mixture and are independent of the analyte molecular weight, as they depend solely on moles of C present in the analyte peak. The detection limits of organic GC/MS degrade on a per-mole-of-C basis with increasing molecular weight and with the number of monitored masses in the selected ion monitoring mode (SIM), as dwell time must be split. In this regard, GCC−IRMS with U-^{13}C-labeled tracers is similar to radiochromatography using U-^{14}C-labeled tracers, but is orders of magnitude more sensitive than possible with practical radiotracer levels. It is therefore ideally suited for detection of unknown metabolites and can be applied directly to humans.

We illustrate the sensitivity of this approach in a recent study of ω-3 fatty acid metabolism in lactating rhesus monkeys.[38] [U-^{13}C]-α-Linolenic acid (ALA*, 20 mg; <2 mg/kg of body weight) was administered to lactating rhesus monkeys by iv infusion. Plasma from the nursing infant was drawn at 1 and 7 days and analyzed for transfer products. Results for one subject show in Figure 8 that ALA* comprises >4% of the plasma ALA at 7 days. Conversion of label into saturated and mononunsaturated fatty acids, presumably via β-oxidation and reutilization in lipogenesis, is easily detected at both 1 and 7 days. These data demonstrate that tracer doses can be employed to detect a variety of minor products simultaneously.

GCC−IRMS has been investigated for determination of glucose kinetics and metabolism in adults using [U-^{13}C]glucose enriched by only 0.1% and derived from the C4 photosynthesizer maize.[39] These authors report sample size requirements at about 10 nmol of glucose and successful use of the very low enrichment precursor. Guo et al. have investigated the use of a 50-mg dose of [3,4-^{13}C]cholesterol in a human subject with analysis of plasma cholesterol over 57 days. Specifically labeled cholesterol was employed in these studies because there are no good sources of the highly enriched U-^{13}C-labeled species. Nevertheless, these workers conclude that detection limits for the partially labeled cholesterol are 15-fold improved compared to organic GC/MS and permitted enriched cholesterol to be detected in plasma for 40−50 days after the single dose. Demonstrations of low level tracer detection by GCC−IRMS for plasma lactate,[40] very low density lipoprotein triglycerides derived from ^{13} labeled ethanol,[41] and plasma amino acids[42] have appeared. Taken together, these early studies indicate that tracer detection by GCC−IRMS is straightforward and is substantially more sensitive than conventional methods.

Natural Variability. All C in biomolecules is ultimately derived from atmospheric CO_2 fixed by one of two photosynthetic processes denoted "C3" or "C4", which result in $\delta^{13}C$ of approximately −26‰ and −12‰, respectively.[6] The mix of C3 and C4 plants from which the C of any particular biomolecule is derived determines its ^{13}C content. Omnivorous animals consuming a varied diet will derive C from a mixture of C3 and C4 sources, and so $\delta^{13}C$ will fluctuate within this range. In contrast, the C derived from terrestrial sources can have $\delta^{13}C$ well outside this range,[43] suggesting that synthetic hormones may be distinguished from biosynthesized hormones on the basis of isotope ratio. Recently, GCC−IRMS has been applied to detection of misuse of synthetic testosterone to improve sports performance.[44] Comparison of $\delta^{13}C$ of testosterone and its metabolite androstanediol with the precursors cholesterol and dehydroepiandrosterone yielded consistently lower $\delta^{13}C$ for the former two compounds as expected for samples containing synthetic hormone. The authors, working with subjects drawn from European population, conclude that evidence of testosterone misuse is present for samples with $\delta^{13}C > -27‰$, but recognize that differences in dietary patterns may affect endogenous $\delta^{13}C$ values. This concern is well founded as an early study of baseline serum cholesterol of South African residents reported remarkably stable values of $\delta^{13}C = -17‰$, all of which might yield testosterone considered above the cutoff in the European population, and emphasizes the need to reference ^{13}C content to that of the internal precursor pool.

Conclusion

High-precision GIRMS is in the midst of a period of rapid evolution of continuous flow methods for analysis of gases and for compound-specific isotope analysis. These developments will make GIRMS an increasingly important tool in tracer methodology as well as other traditional applications of this enduring form of mass spectrometry. Challenges on the horizon for GIRMS instrumentation include the continuing improvement of specialized inlets for liquid chromatography and development of interfaces for high-precision GCC−IRMS for isotopes of H and O. Fundamental studies describing the natural variability of isotope ratios in complex molecular species, followed by theoretical advances to exploit this information, hold the key to insights available by no other means.

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