J. Thomas Brenna, Gavin L. Sacks, Ying Zhang, Margaret E. Brenna

Data Analysis in GCC-IRMS

Cornell University

Ithaca, New York, USA

Isotope ratio mass spectrometry (IRMS) is a relatively rare technique in drug analysis. It is much more common in fields of natural science that ask questions about the source of a material, rather than about its chemical composition or its concentration. Consequently, for decades the principles of IRMS evolved primarily within the field of geochemistry and environmental sciences, where it was used to trace carbon, nitrogen, and related elements as they move through the natural world. Ecologists have also recently adopted it for studies of migration patterns in whales, butterflies, and finches. We have reviewed IRMS and applications to natural variability recently [1].

High precision IRMS instruments produce data that is precise to 4-6 significant figures. The entire range of isotope ratios encountered in modern, terrestrial, natural samples is about 0.03% $^{13}C/^{12}C$. Thus, a notation is required that will emphasize the change in isotope ratios from sample to sample within this range.

The appropriate properties for a universal carbon standard were described by A. O. Nier in 1946 [2], who designed the first high precision mass spectrometers [3, 4] and also was the first to detect natural variation of $^{13}C/^{12}C$, in 1939 [5]: "…it would seem highly advisable to always include in the published results the excess of tracer isotope … above that found in some arbitrary standard. … If … ordinary chemical carbonate is used … the normal average biological material will probably contain less C13 than does the laboratory standard and it and the more dilute samples studied will have a negative excess of C13 over the standard. While this may be disconcerting to the reader, in any biological experiment what is really important is the difference in C13 concentrations in different compounds …" *[emphasis in original]*

The $\delta^{13}C$ is defined as a unit parts-per-thousand change in isotope ratio relative to a standard isotope ratio. The standard is a material with a relatively high $^{13}C/^{12}C$, known as Pee-Dee

Belemnite, from the Pee Dee River in South Carolina, USA. That original material was assigned a $^{13}C/^{12}C$ = 0.0112372, but this figure was probably not more accurate than 3 significant figures. It was replaced with Vienna-PDB (VPDB) scale, calibrated against NBS-19 calcite assigned a value of +1.95‰[6]. NBS-19 is still available, as are standards calibrated against it from the US National Institutes of Standards and Technology (NIST) and the International Atomic Energy Agency (IAEA).

The advantages of this system are most apparent when considering real data. Table 1 lists replicate analyses of a sample using $^{13}C/^{12}C$ and the $\delta^{13}C$ notation. Changes in $^{13}C/^{12}C$ are in the fifth decimal place, while changes in $\delta^{13}C$ are in the first decimal place. Note also that the SD associated with these replicate measurements is in the sixth decimal place, yielding % coefficients of variation = % relative standard deviations in the hundreds of parts per million.

One more example of comparative analyses is shown in Figure 1. Here, various plant-based foods were analyzed by IRMS. The foods made from exclusively C3 photosynthetic plants, which deplete $^{13}C/^{12}C$ of atmospheric CO_2 from its normal value around $\delta^{13}C$ = -7.8 ‰ to well below -25 ‰ are all around the same values. One breakfast cereal made exclusively from the C4 photosynthesizer corn, and including corn sweetener, is at around -12 ‰. Cereals made of mixes of corn and other grains are of intermediate $^{13}C/^{12}C$. It is notable that

Table 1. Sample IRMS Data comparing the $\delta^{13}C$ notation with traditional isotope ratio data. The isotope ratios are expressed as mole ratios (mol ^{13}C / mol ^{12}C)

	$\delta^{13}C$	$^{13}C/^{12}C$
	-30.01	0.010900
	-30.14	0.010899
	-30.74	0.010892
	-30.29	0.010897
Mean±SD	-30.30±0.32	0.010897±0.000004
%CV = %RSD	N/A	0.0328
CV(ppm)	N/A	328

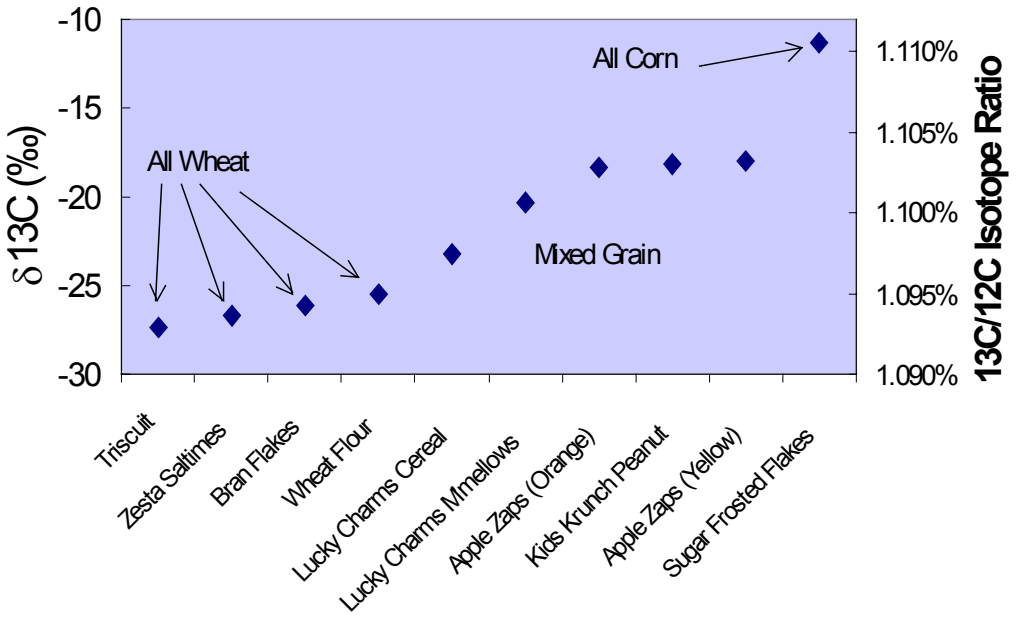

Figure 1. Isotopic analysis of plant-based foods, with emphasis on breakfast cereals. The $\delta^{13}C$ scale is on the left, and corresponding $^{13}C/^{12}C$ is on the right. Experimental error is within the markers.

the entire range of variability in this study was within $^{13}C/^{12}C$ = 1.09% to 1.11%. This study also illustrates the principle that isotope ratios of mixtures are linear combinations of the isotope ratios of their constituents.

To achieve this precision, sufficient numbers of ions must be detected to satisfy the sampling theorem. The sampling theorem says that for a given number of counts, the precision of repeated measures is the square root of the number of counts, or RSD = sqrt(cts)/cts, where "cts" is the number of counts. Using this result, we can estimate the minimum requirements for 1 ‰ precision.

First, it has long been known that the precision is limited to the number of counts detected for the minor beam (e.g. [7]). For carbon this is ^{13}C. To achieve a precision of 1e-4 (0.1‰), we require at least 1e8 ^{13}C. Detection of 1e8 ^{13}C requires 1e10 ^{12}C, which is roughly 1e10 C atoms. Detection efficiency is about 1e-3, so we require 1e13 CO_2 to be admitted to the ion source to yield 1e10 CO_2^+. 1e13/6e23 ~ 2e-11 or 0.02 nanomoles of CO_2. This is the statistical limit, and does not take into account noise from real power supplies, detectors, amplifiers, and other devices involved in the measurement, or splits that are necessary to maintain proper pressures in the instrument. It also does not take into account real chemical

noise such as is encountered in background due to column bleed. These factors account for the difference between this limit and the 50-fold greater the benchmark typically quoted in IRMS of about 1 nanomole.

The data stream from carbon analysis in gas chromatography-combustion-IRMS (GCC-IRMS) consists of three arrays of data, one from each of the detectors for the *m/z* 44, 45, and 46 cups. Each of these data streams resembles a chromatogram obtained from an FID detector. Some mathematical processing is then required to convert these data into isotope ratios while maintaining the precision of the measurement insofar as possible. To do so with a precision of hundreds of parts per million is non-trivial.

There are two major approaches to data analysis in chromatography, known as the *summation* and *curvefitting* methods. The summation method is by far the most prevalent but has certain drawbacks that become important GCC-IRMS. The two methods are illustrated in Figure 2 [8].

Summation. The key to the summation integration algorithm is to define the peak start and stop points and to define the background signal level. The peak start and stop points are typically detected by determining when the slope of the signal rises above a certain predetermined value. The stop is found after the peak, when the slope returns to a second value below a threshold. Because chromatography peaks are, strictly speaking, not symmetric under even ideal conditions, refinements are necessary to capture the entire peak including the tail. The more challenging issue is definition of the background level. The background may have a non-zero slope, and may not be linear, though it is almost always modeled as linear in GCC-IRMS. More challenging is the struggle with noise, and in particularly in defining the backgrounds. Reduction in precision and accuracy due to low signal to noise ratio first manifests with difficulties in defining the backgrounds for the summation method, which must be done for each of the three *m/z* traces.

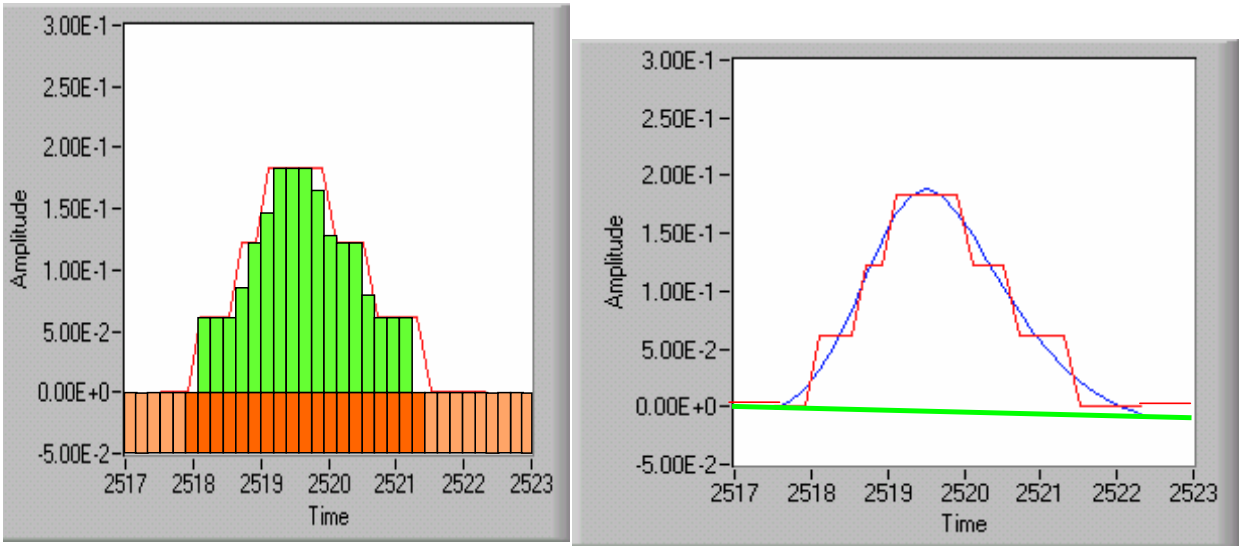

Figure 2. Schematic of the (left) summation method and (right) curvefitting methods. for peak integration. The signal granularity is exaggerated for illustrative purposes. The summation method defines peak start and stop, integrates between these limits, and subtracts a background. Curvefitting models the peak with a mathematical function, and then extracts the area as a fitted parameter.

Curvefitting. A straightforward case of curvefitting is the familiar straight line linear least squares fit (regression) to a series of points. A series of experimentally determined xy pairs are fit to an equation of a line of the form y=ax+b. The slope of the line can be read off from the "a" parameter. A curvefit to a peak is similar but uses an equation that is a sum of an equation describing the peak, and an equation – typically that of a straight line – that describes the background. The equation for the peak will be written to have fitted four terms, one each for peak position, width, area, and skew. The area can then be read off as the fitted parameter. The equation for the slope and intercept for a simple linear least squares fit can be written in terms of the xy pairs in closed form and thus computed uniquely. For more complicated line shapes, such as are necessary to describe a chromatographic peak, no such closed form can be written. Curves are fitted by using initial guesses, computing sum of squared errors, and then a parameter search algorithm such as the Marquardt-Levenberg algorithm, are employed to hone in on a minimal solution. In practice, this works best in GCC-IRMS for similar multiple analyses, such as for analysis of selected analytes from a common matrix, so that the initial guesses insure convergence to the global minima.

Summation is the more generally used method, however curvefitting has been shown to be more robust to peak overlaps [9] and to low signal levels [10]. It has not come into wide use however. We speculate that this is in part due difficulties in generalizing the algorithms to work with a wide array of analytes. However, for most specific applications where initial guesses are well characterized, we believe that curvefitting will outperform summation. To this end, we are currently developing software that can read all IRMS data formats and be customized for specific applications.

Standards for GCC-IRMS

Isotopic standards are particularly important for IRMS because of the subtle differences in isotope ratio that the technique typically must reliably report. Working standards, and round robin standards, with isotope ratios traceable to the international isotope standards, are essential to long-term reliance on the method.

Several characteristics are desirable for isotope standards, discussed previously in the context of a very early effort to create isotopic standards for IRMS [11].

- Chemical stability against heat, light, and oxidation;
- Conveniently available in high purity;
- Soluble in high-purity solvents;
- Nonvolatile (i.e., very low vapor pressure) at room temperature and atmospheric pressure;
- Environmentally rare, to lower the probability of contamination;
- Useful for volatile (e.g., GC) and nonvolatile (e.g., Elemental Analyzer) introduction techniques.
- For internal standards: elute from column at an unobstructed region of the chromatogram.

These criteria apply strictly to internal standards that would be added to samples prior to analysis and used for calibration purposes. With these issues in mind, it is possible to create standards with vial to vial isotope ratios that vary by $\delta^{13}C$ of about 0.1‰, which is better than the typical precision of GCC-IRMS for real samples.

Importantly, as pointed out explicitly by another speaker at this 2006 symposium (U. Flenker), isotope calibration in GCC-IRMS must focus on the actual analyte eluting from the GC column and deemphasize adherence to externally calibrated gas standards. That is to say, traditional IRMS calibration has focused on sample-standard comparisons, but in GCC-IRMS the analyte is processed through a specific set of steps to which standards may not be subject. Because all steps are suspect as sources of isotopic fractionation, the very best isotopic calibration is obtained with a calibrant that undergoes the very same processing as the analyte. Our ongoing efforts to create isotopic standards for GCC-IRMS of endogenous steroids of interest to the anti-doping community will take these important issues into account.

References

1. Asche, S., A.L. Michaud, and J.T. Brenna, *Sourcing Organic Compounds Based On Natural Isotopic Variations Measured By High Precision Isotope Ratio Mass Spectrometry.* . Curr Org Chem, 2003. **7**(15): p. 1527-1543.
2. Nier, A.O., *The mass spectrometer and its application to isotope abundance measurements in tracer isotope experiments*, in *Preparation and Measurement of Isotopic Tracers*, D.W. Wilson, A.O.C. Nier, and S.P. Reimann, Editors. 1946, J. W. Edwards: Ann Arbor, MI. p. 28.
3. Nier, A.O., *A mass spectrometer for routine isotope-abundance measurements.* Rev. Sci. Instrum., 1940. **11**: p. 212-16.
4. Nier, A.O., *Mass spectrometer for isotope and gas analysis.* Rev. Sci. Instrum., 1947. **18**: p. 398-411.
5. Nier, A.O. and E.A. Gulbransen, *Variations in the relative abundance of the carbon isotopes.* J. Am. Chem. Soc., 1939. **61**: p. 697-8.
6. Stichler, W., *Interlaboratory comparison of new materials for carbon and oxygen isotope ratio measurements*. 1995, IAEA: Vienna. p. 67-74.
7. Hayes, J.M., et al., *High precision stable isotope ratios from microgram samples*, in *Advances in Mass Spectrometry*, N.R. Daly, Editor. 1978, Heyden & Son: London. p. 475-480.
8. Wolyniak, C., G.L. Sacks, and J.T. Brenna. *Effect of quantization error on high precision continuous flow isotope ratio mass spectrometry*. in *51st ASMS Conference on Mass Spectrometry and Allied Topics*. 2003. Montreal, Ontario, Canada.
9. Goodman, K.J. and J.T. Brenna, *Curve-fitting for Recovery of Accuracy in High Precision Isotope Ratios in Gas Chromatography-Combustion Isotope Ra-tio Mass Spectrometry.* Anal. Chem., 1994. **66**(8): p. 1294-1301.
10. Goodman, K.J. and J.T. Brenna, *High Precision Gas Chromatography-Combustion Isotope Ratio Mass Spectrometry at Low Signal Levels, K. J. Goodman and J. T. Brenna, Journal of Chromatography A, 1995, 689, 63-68.* . J. Chromatogr. A, 1995. **689**: p. 63-68.
11. Caimi, R.J., L. Houghton, and J.T. Brenna, *Condensed Phase Carbon Isotope Standards for Compound-Specific Isotope Analysis (CSIA).* Anal. Chem., 1994. **66**(18): p. 2989-2991.