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High-precision gas chromatography–combustion isotope ratio mass spectrometry at low signal levels

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Abstract

Precision and accuracy of gas chromatography–combustion isotope ratio mass spectrometry are investigated for sample levels down to about 5 pmol C in fatty acid methyl ester mixtures spanning 1000-fold in concentration. Precision and accuracy of isotope ratios diverge rapidly for conventional summation methods, and become unusable below 30 pmol material on column. At lower levels, mean isotope ratios were statistically different from reference values indicating bias as well as poor precision. In contrast, curve fitting, using the exponentially modified Gaussian line shape, gives improved precision for most peaks and useful results down to 3 pmol. The curve-fitting algorithm was also less sensitive to signal integration time than the summation method. These data indicate that curve fitting may be the method of choice for integration of noisy data when high-precision isotope ratios are desired.

1. Introduction

High-precision gas [1,2] (or liquid [3,4]) chromatography–combustion isotope ratio mass spectrometry (xC–C-IR-MS) for analysis of C or N isotopes [5,6] is gaining importance across many areas of natural science [7]. These techniques facilitate the analysis of individual compounds at very small sample sizes, with precision levels approaching that of conventional dual inlet [8]. Most applications take advantage of these improved parameters, as well as the capability to rapidly obtain high-precision compound-specific results without cumbersome chemical separation prior to analysis (e.g. Refs. [9–11]). However, a wide variety of applications require the analysis of complex mixtures, inevitably involving overlapping peaks. We previously reported that the conventional summation integration algorithm produces systematic bias at degrees of overlap as low as 10% valley, even for compounds of well matched isotope ratio (e.g. $\Delta\delta^{13}C < 0.5$) [12]. Most notably, this bias is observed while high precision is maintained. Further, isotope ratios for minor components (< 10 ng) are seldom calculated because of overlaps and the well known difficulties in defining peak start/stop in low signal-to-noise ratio (S/N) cases. This is true even though high-precision results ($\delta^{13}C < 0.5$) can be achieved for sub-ng sample sizes under ideal circumstances [8].

We have recently shown that curve fitting can be effective in recovering accurate isotope ratios from severely overlapping peaks while preserving high precision [12]. Curve-fitting methods are known to be more resilient to low S/N than

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summation algorithms, where peak definition is most difficult [13–15]. Analysis of low levels by GC–C-IR-MS is of particular interest for tracer studies where precision as poor as $\delta^{13}C < 100$ is useful for detecting enriched metabolites [2]. We report here a systematic study comparing curve fitting to conventional summation for well-resolved small sample injections over four orders of magnitude in single GC–C-IR-MS chromatograms.

2. Experimental

2.1. Instrumentation

A Varian 3400 GC system interfaced to a Finnigan MAT 252 high-sensitivity gas isotope ratio mass spectrometer via a combustion interface was used for this study. The instrument was operated at an accelerating potential of 8 kV and a source pressure of $2 \cdot 10^{-6}$ Torr (1 Torr = 133.322 Pa). Briefly, the effluent of the capillary column is directed to a combustion furnace held at 850°C and loaded with CuO as a source of O_2. Combustion products are dried and pass through an open split prior to entrance to the IR-MS ion source. CO_2 is monitored continuously at m/z 44, 45 and 46 with three dedicated faraday cups and associated electronics. Asymmetric peak shapes are commonly observed because of the numerous connections and changes in capillary diameter between the GC column and the mass spectrometer. Details of the system can be found elsewhere [2].

A fatty acid methyl ester (FAME) standard mixture was separated on a J & W (Folsom, CA, USA) DB-23 capillary column, (30 m × 0.32 mm I.D., 0.25 μm film). Carrier flow through the GC column was 45 cm/s He (99.999 + %), with in-line O_2 and H_2O traps. Fatty acid standards were obtained from Sigma (St. Louis, MO, USA) and were 99 + % pure by GC analysis. Hexane (Fisher Optima grade) was used whenever solvent was necessary, and samples were stored in glass vials with Teflon-lined caps. The test mixture was composed of methyl pentadecanoate (Me15:0), methyl heptadecanoate (Me17:0), methyl octadecanoate (Me18:0) and methyl heneicosanoate (Me21:0) at concentrations of about 0.16, 1.6, 16 and 160 ng/μl, respectively. Injections of 0.5 μl of this mixture were made in splitless injection mode. A second, equimolar mixture of these compounds (0.2 μg/μl each) was prepared and analyzed separately to establish reference isotope values for each compound.

The vendor-supplied data acquisition system collects a signal from each mass channel simultaneously for adjustable integration times. At the conclusion of an integration period, a counter is queried, zeroed, and restarted, giving a >99% duty cycle. Any particular integration time is a tradeoff between noise per data point and faithful reproduction of lineshape. Longer integration times improve S/N for each data point, but distort lineshape. This distortion can be important for accurate curve fits, as well as for precise definition of peak start/stop for summation. The test mixture was analyzed using integration times of 0.25, 0.125 or 0.0625 s to investigate the effect of this parameter on summation and on curve-fitting peak integration approaches. Four or five replicates were made at each integration time.

2.2. Data processing

The vendor-provided software "ISODAT" (Finnigan MAT, Bremen, Germany) was used to process the data by the summation method. A review of this software has been published recently [16]. Slope sensitivity (SS) for peak start/stop definition is set by the user. A rolling five-point linear least squares fit is applied to the chromatogram, with peak start defined when the slope exceeds the SS; stops are defined as the point beyond the peak where the slope falls below the absolute value of the SS. SS settings chosen to detect strong peaks and reject spurious noise-related peaks do not satisfactorily detect very small peaks, while settings used to detect small peaks are overly sensitive to noise. Therefore, two separate settings for slope sensitivity were compared, SS = 0.8 mV/s for higher level detection which has performed satisfactorily in

routine applications in our laboratory over the last three years, and SS = 0.1 mV/s for low detection. Other workers report SS = 1 mV/s as satisfactory for routine analyses [8].

The commercial program Peakfit (Jandel Scientific) was used for all curve fitting. Previously, we evaluated several functions for use with GC–C-IR-MS peaks [12]. The well-known exponentially modified Gaussian (EMG) function, constructed as the mathematical convolution of the Gaussian and exponential functions [15], accurately reproduces the line shapes as long as the column is not overloaded and appears to work particularly well for low signal levels. It was used exclusively in this work. Each set of four or five chromatograms for each integration time were processed with the SS = 0.8, SS = 0.1, and curve-fit methods.

As in previous work, we calculate high-precision isotope ratios according to the following:

$$\delta 45 = \left(\frac{R_{SPL} - R_{PDB}}{R_{PDB}}\right) \times 1000 \quad (1)$$

where R_X is the ratio of integrated areas for the m/z 45 to 44 channels, SPL and PDB refer to the sample and the international standard PeeDee Belemnite, respectively, international standard with $R_{PDB} = 0.0112372$. This expression does not take into account the contribution of $^{12}C^{16}O^{17}O$ to the m/z 45 signal, which is small and in the case of GC–C-IR-MS is nearly constant. Areas are obtained by either fitting the m/z 44 and 45 channels or using the summation methods. No outliers were excluded from this data set.

Tests of statistical differences of means were performed using the t-distribution at the 95% confidence level [17].

3. Results and discussion

A representative chromatogram is presented in Fig. 1, and plotted in semilog format so that the four orders of magnitude peak intensities and background noise levels are discernable. Peak intensities above baseline range from about 2 mV for the smallest, earliest eluting peak to a high of

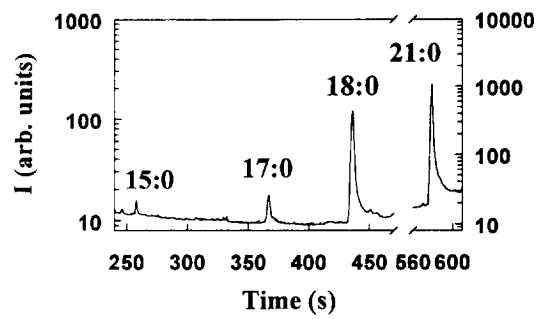

Fig. 1. GC–C-IR-MS chromatogram of the FAME test mixture plotted in semilog format.

500 mV for the most abundant but broader, latest eluting peak. There is a significant amount of chemical noise near the intensity of the smallest peak, which corresponds to 0.1% of the largest peak, Me21:0. As all FAMEs in the mixture are 99 + % pure, contaminants at this level entering the test mixture with Me21:0 are expected. Signal outputted in volts by the data system was converted to current, and peak area in nA s was calculated and used for comparison.

Deviations from reference values in $\delta 45$ units (‰) are presented in Figs. 2–4 for the individual determinations of FAMEs at the three integration times investigated. The abscissa represents signal area and is approximately logarithmic, although points have been offset horizontally for illustrative purposes. Both summation methods diverge more rapidly than the fitted data as sample size decreases for all integration times. Performance of the summation methods degrades significantly at 63 ms integration time compared with the 125 and 250 ms case. In contrast, the curve-fit performance did not degrade significantly at the lower integration time. At 200 and 2000 nA s, precisions were best at 63 ms integration time, while the lower signal levels gave slightly better results at 250 ms integration time.

Precisions for the higher SS are superior to those for the lower SS at all levels. At the lowest signal level investigated, the SS = 0.8 summation method failed to detect peaks reliably while the SS = 0.1 method produced highly inaccurate and imprecise ratios. The summation results are also seen to deviate substantially from the reference

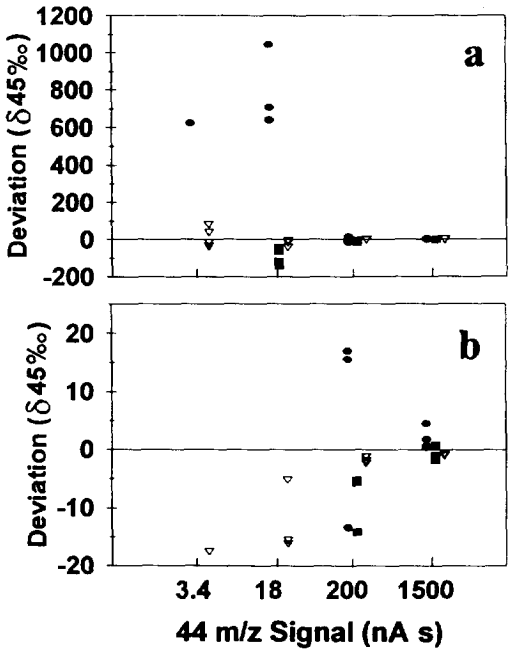

Fig. 2. δ45 deviations from reference values for 63 ms integration rate using the three different integration methods: ■ = SS 0.8 mV/s; ● = SS 0.1 mV/s; ▽ = curve fit. Points for SS = 0.8 mV/s and fitted data are offset their actual signal level for illustrative purposes. Data of a is plotted in b with expanded ordinate.

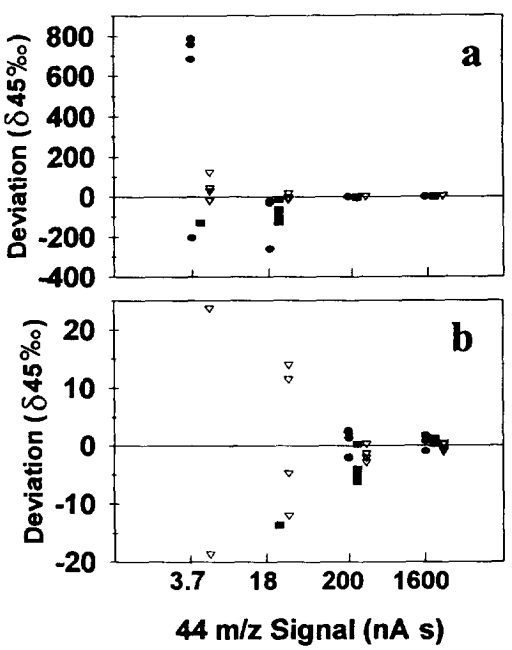

Fig. 3. δ45 deviations from reference values for 125 ms integration rate. Symbols as in Fig. 2.

levels. This observation is consistent with our previous report, and may also explain inaccuracy observed for highly enriched material at low sample levels [18]. In fact, S.D.s improve with shorter integration time for the strongest signal level.

Summary data of δ45 values are presented in Table 1, tabulated using average signal levels (in nA s), along with reference values from the equimolar mixture. Precisions (S.D.) for the reference values were all $\delta^{13}C < 0.4$ which are consistent with our routine measurements under nearly ideal GC–C-IR-MS conditions. The SS = 0.8 method detected only one peak at the lowest level across all integration times. Precisions and accuracy for the curve-fit data are superior for all but one cell.

Accuracy was assessed by statistically testing mean deviations for equivalence with zero. All of the 20 nA s deviations for the SS = 0.8 method were significantly different from zero, indicating systematic shifts in measured isotope ratio

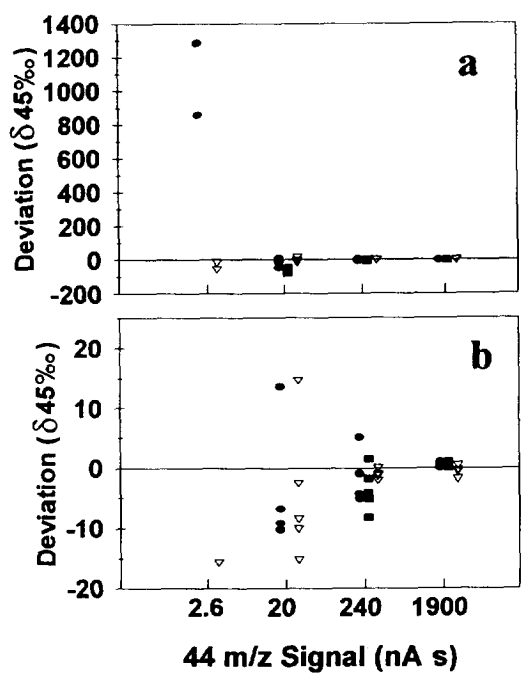

Fig. 4. δ45 deviations from reference values for 250 ms integration rate. Symbols as in Fig. 2.

Table 1
δ45 (Mean ± standard deviation)

	Average area (nA s)	δ45			
		63 ms	125 ms	250 ms	Reference
SS = 0.8 mV/s	3	(No peaks)	−156.65 ± (n = 1)	(No peaks)	−26.98 ± 0.36
	20	−126.7 ± 45.3[a]	−119.7 ± 46.2[a]	−102.1 ± 15.9[a]	−34.17 ± 0.17
	200	−27.59 ± 4.34[a]	−23.91 ± 2.47	−23.43 ± 3.64	−19.95 ± 0.27
	2000	−30.82 ± 1.16	−29.18 ± 0.42	−29.39 ± 0.46	−29.88 ± 0.23
SS = 0.1 mV/s	3	598.3 ± (n = 1)	534.3 ± 430.0[a]	1046 ± 301	−26.98 ± 0.36
	20	763.3 ± 216.2[a]	−107.9 ± 105.0	−45.69 ± 21.3	−34.17 ± 0.17
	200	−13.51 ± 17.2	−19.51 ± 2.30	−21.15 ± 4.01	19.95 ± 0.27
	2000	−28.05 ± 1.88	−64.91 ± 80.14 (−29.08 ± 1.27)[b]	−29.31 ± 0.40	−29.88 ± 0.23
Curve fit	3	−10.19 ± 54.03	0.93 ± 57.69	−63.18 ± 29.03	−26.98 ± 0.36
	20	−53.43 ± 14.85	−36.50 ± 14.72	−38.50 ± 11.52	−34.17 ± 0.17
	200	−21.65 ± 0.56[a]	−21.57 ± 1.18	−21.05 ± 0.75	−19.95 ± 0.27
	2000	−30.82 ± 0.14	−30.24 ± 0.59	−30.40 ± 0.82	−29.88 ± 0.23

[a] Statistically different from reference value ($p < 0.05$).
[b] Mean and error for SS = 0.1 mV/s (2000 nA s) excluding one aberrant determination.

for signal levels below 200 nA s using this method. The SS = 0.1 method showed significant differences for only the 63 ms integration time, but gave very large errors for all but the 250 ms integration time. At the 3 nA s level, where the higher SS failed to detect peaks, the SS = 0.1 method produced large errors ($\delta^{13}C > 300$) and statistically different mean isotope ratios. In contrast, only one of twelve fitted means is different from the reference values (63 ms, 200 nA s) at 95% confidence.

The relatively high quality of the curve-fitted data at low levels prompted investigation of the preservation of peak area with decreasing sample size, for quantitative analysis. Graded solutions of pure Me17:0 in hexane were injected at levels from about 10 pmol to 500 nmol C. Areas for the *m/z* 44 signal were obtained by each of the calibration methods and summary data are plotted in Fig. 5, in log–log format. Peak areas below about 1 nmol are not accessible in our version of the vendor software and so are not presented. The SS = 0.8 method parallels the curve-fit method to 1 nmol, while the SS = 0.1 method diverges, with slopes and S.D.s for the two methods of $6 \cdot 10^{-3} \pm 6 \cdot 10^{-6}$ ($r^2 > 0.999$) and $5 \cdot 10^{-3} \pm 20 \cdot 10^{-6}$ ($r^2 < 0.99$), respectively. The curve-fit method provides good linearity down to 10 pmol, with slope and S.D. of $5 \cdot 10^{-3} \pm 6 \cdot 10^{-6}$ ($r^2 > 0.999$) over this range.

The Finnigan MAT 252 instrument is specified to operate at an absolute sensitivity of 1000 CO_2 molecules/ion detected, and typically attains this

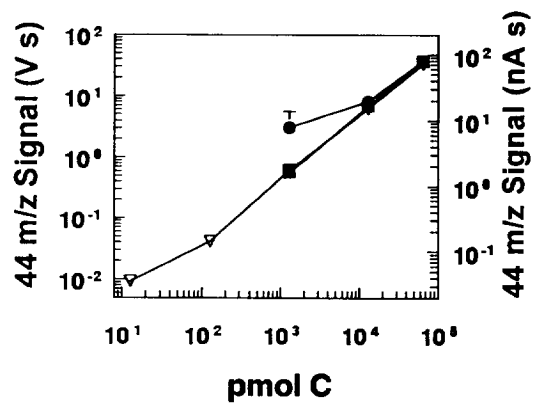

Fig. 5. Calibration curve showing the relationship of integrated area to mass of Me17:0 injected on-column for all three integration methods. ■ = SS 0.8 mV/s; ● = SS 0.1 mV/s; ▽ = curve fit.

level. For such an instrument, the 2000 nA s area would be produced with about 5 nmol C entering the combustion furnace, while the 3 nA s level would be attained with 5 pmol C. It should be emphasized that these detection limits are based on molar C content, and so are largely independent of the analyte molecular mass for most organic molecules of biomedical interest. These data indicate that curve fitting produces useful isotope ratios at this level of total analyte.

4. Conclusions

For tracer applications, a cutoff for "high precision" can be defined as an error of $\delta^{13}C = 100$ (S.D.), which corresponds to 0.1% in absolute terms and to the practical limit of organic mass spectrometers operated in selected ion mode (SIM) [2]. The SS = 0.1 mV/s detected peaks at levels below the SS = 0.8 mV/s method, but produced S.D.s greater than $\delta^{13}C = 100‰$ in most cases at the lower levels. SS = 0.1 did perform better for the largest integration time and lowest signal, further suggesting that the summation methods require a minimum S/N for adequate performance. For this reason, it appears that the higher value for SS is appropriate when using the summation method, as it detects peaks down to levels at which high precision is obtained. In cases where very small peaks must be detected by summation, consecutive data points can be summed to improve S/N per data point, with a lower SS then applied to the resulting chromatogram.

At all levels, curve-fitting produced satisfactory precision and accuracy, and was nearly immune to the choice of integration time. It was the only method that produced useful isotope ratios at the lowest levels. It also gives linear response over the entire usable dynamic range and so is also suitable for quantitative analysis. Our previous results showed that curve fitting satisfactorily recovers aberrant isotope ratios derived from overlapping peaks [12]. Together, these studies indicate that curve fitting may be the method of choice for integrating peaks in high-precision chromatography applications where complex mixtures are commonly encountered.

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