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Calibration and data processing in gas chromatography combustion isotope ratio mass spectrometry

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Compound-specific isotope analysis (CSIA) by gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS) is a powerful technique for the sourcing of substances, such as determination of the geographic or chemical origin of drugs and food adulteration, and it is especially invaluable as a confirmatory tool for detection of the use of synthetic steroids in competitive sport. We review here principles and practices for data processing and calibration of GCC-IRMS data with consideration to anti-doping analyses, with a focus on carbon isotopic analysis ($^{13}C/^{12}C$). After a brief review of peak definition, the isotopologue signal reduction methods of summation, curve-fitting, and linear regression are described and reviewed. Principles for isotopic calibration are considered in the context of the $\Delta^{13}C = \delta^{13}C_M - \delta^{13}C_E$ difference measurements required for establishing adverse analytical findings for metabolites (M) relative to endogenous (E) reference compounds. Considerations for the anti-doping analyst are reviewed. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: gas chromatography combustion isotope ratio mass spectrometry; steroids; carbon isotope ratio; calibration; data processing

Introduction

Since the stable isotopic variation for carbon ($^{13}C/^{12}C$) in nature was first observed by Nier and Gulbransen[1] in 1939, high precision isotope ratio mass spectrometry (IRMS)[2–5] of light gases has been an important sourcing method in many research areas[6] such as paleoecology, geochemistry, archaeology, environmental chemistry, provenance studies, climate studies, food authentication, forensic sciences, and tracer studies in biomedical sciences. IRMS is used to measure the stable isotope ratios of light elements, such as $^{13}C/^{12}C$, $^{2}H/^{1}H$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$ in the form of pure gases CO_2, H_2, N_2, CO, and SO_2 respectively, and requires the conversion of organic molecule(s) to the appropriate gas.

Stable isotope measurements are a powerful means of determining if a synthetic version of an endogenous steroid, such as testosterone (T), has been used for performance enhancement in competitive sport. Stable isotopes are complementary to the more common approach by gas chromatography mass spectrometry (GC-MS), first introduced in 1983,[7] in which the testosterone to epitestosterone ratio (T/EpiT) is measured. Naturally occurring urinary T/EpiT in males is typically about 1.0 or lower; T/EpiT > 4 has been established as suspicious for T use. Molecular analyses using GC-MS can detect exogenous substances, such as stanozolol; however, it cannot differentiate endogenous and synthetic versions of T and EpiT because their chemical structures are identical. Becchi *et al.* proposed the use of gas chromatography combustion coupled to IRMS (GCC-IRMS) to confirm the use of external sources of T[8] which is difficult based on T concentration alone using GC-MS.[9,10] The GCC-IRMS approach[11,12] exploits the natural difference in $^{13}C/^{12}C$ between C3 plants (such as wheat, rice, yam, and soy) and C4 plants (such as corn and sugarcane), which fix carbon using different photosynthetic pathways.[6,13–16] Humans eat a diet derived from a varying mixture of C3 and C4 plants in which the ^{13}C content for endogenous steroids are higher than those of exogenously synthesized steroids, which are exclusively made with precursors from C3 plants (soy or yams). The $^{13}C/^{12}C$ difference is measured between an endogenous reference compound (ERC, e.g. pregnanediol), whose $^{13}C/^{12}C$ ratio is not affected by the administration of synthetic steroids, and a target compound (TC, e.g. T and its metabolites), whose $^{13}C/^{12}C$ ratio is affected. A threshold of a difference greater than 3‰ (units described later) to indicate synthetic steroids use was established by the World Anti-doping Agency (WADA) and is applied for a few combinations of ERCs and TCs.[17,18] In practice, some anti-doping laboratories use a higher threshold that incorporates the error associated with the measurement specific to the laboratory. Using this isotope ratio differential measurement, false adverse analytical findings are very rare, though false negatives may be common, considering the discovery of some synthetic T with isotope ratios similar to that of endogenous reference steroids.[19] It has been shown that between assay precisions can be as good as within assay standard deviations (< 0.3 ‰) over a few months,[20] but can be higher (0.6 to 1.7‰)[21] depending on the laboratory and analytical protocols. Between-assay standard deviations over a period of years have been shown to be as good as <0.5 ‰.[22]

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IRMS instrumentation

IRMS hardware and instrumentation has been previously reviewed in detail.[2–6] The IRMS is composed of a tight electron impact ion source, a magnetic sector, and Faraday cup detectors dedicated to the simultaneous measurements of two or more stable isotopes of a light gas. In contrast to molecular MS, compounds of interest must be converted to an appropriate light gas for isotopic analysis. For carbon, faraday cups are positioned to simultaneously measure the CO_2 isotopologues ion currents at *m/z* 44, 45, and 46 (Figure 1). This is done to maximize detection sensitivity, signal stability, and counting statistics in order to enable high precision measurements that distinguish small differences in isotope ratios.

The original principles of IRMS were established with the dual inlet system (also referred to as gas-IRMS or GIRMS) that allows alternating introduction and isotopic measurement of sample and standard gases from static volumes.[23,24] This approach requires the off-line conversion of the sample or compound(s) of interest to CO_2, and yields the highest precision results of all isotope techniques, SD(δ^{13}C) < 0.05 ‰. Continuous flow (CF) methodologies are now more common in many research areas due to the convenience of online compound separation and/or online chemical conversion before IRMS, eliminating laborious off-line sample preparation. Implementation of standard CF techniques are capable of average precisions of approximately SD(δ^{13}C) 0.10 to 0.50 ‰. The stable isotope analysis of solid or non-volatile liquid bulk samples[25–27] is achieved by elemental analyzer IRMS (EA-IRMS), which was first demonstrated for the bulk analysis of ^{15}N by Preston *et al.*[28] in 1983. If compound-specific isotope analysis (CSIA) is desired, EA-IRMS would still require off-line separation of analytes. The coupling of gas chromatography with on-line combustion (GCC) and isotope analysis was first introduced in 1978 by Matthews *et al.*,[29] allowing on-line CSIA. This was refined by Barrie *et al.*[30] in 1984 with the use of a dual collector MS creating the first GCC-IRMS system that resembles the commercial systems first available since 1990.[31] Subsequently, systems coupling GC to IRMS for the CSIA of ^{15}N/^{14}N,[32] ^{2}H/^{1}H,[33–35] and ^{18}O/^{16}O[36] have been introduced. In addition, customized systems capable of position specific isotope analysis (PSIA, or intramolecular isotope ratio measurements) have been reported[37–41] and can yield important insights into chemical processes but are not in routine use.

Recently, the integration of more advanced GC separation techniques coupled to IRMS has been demonstrated. These techniques enable more rapid analysis (Fast GCC-IRMS)[42,43] or improve separations of complex sample mixtures (comprehensive two dimensional GC-IRMS, GC × GCC-IRMS).[44,45] Fast GC and GC × GC both generate narrow peaks (<1 s wide), and coupling these techniques to IRMS requires numerous hardware modifications to conventional GCC-IRMS systems. In particular, components in the combustion interface must be optimized to minimize peak broadening, including reducing GC effluent pathway dimensions, dead volumes, and connections, with special considerations for the on-line reactor.

Schematically represented in Figure 1, GCC-IRMS for CSIA of C uses an online reactor held at approximately 950 °C positioned between the GC and IRMS to combust organic molecules to CO_2 and H_2O. This is accomplished using a fused silica or ceramic tube filled with CuO and NiO with a Pt catalyst or micro-reactors filled with CuO[42] or an oxidized Cu alloy.[43–46] A 4-port valve or back-flush system is used to divert organic solvent from the reactor, preventing the rapid depletion of the oxygen source; alternatively, a high-capacity injector that vents most evaporated solvent prior to introduction of analytes onto the column can be used to avoid dead volumes associated with additional connections. Thereafter, CO_2 is admitted to the IRMS through an open split for analysis. Prior to the open split, the H_2O is removed either by a Nafion tube[47] or a cryogenic trap [42,44,48] to avoid the protonation of CO_2 to HCO_2^+, which interferes with the *m/z* 45 ion current signal.

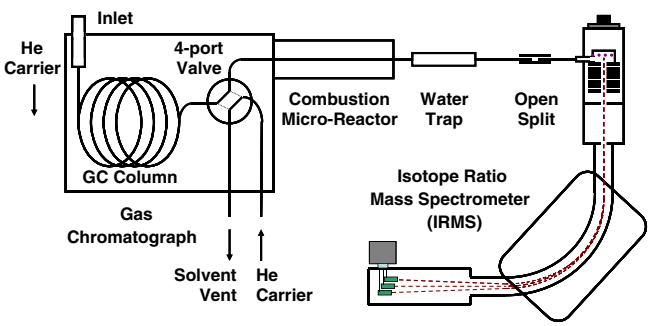

Figure 1. General schematic of a gas chromatograph combustion isotope ratio mass spectrometer (GCC-IRMS) system.

Delta notation

Standard notation for expressing high-precision IRMS measurements has very recently been proposed.[49] Stable isotope ratios are presented in delta (δ) notation, first formally defined by the Urey Group[23,50] based on a suggestion of Nier in 1946,[51] where the ion current ratios of a sample are measured relative to a standard traceable to an international standard, in units of permil (‰). Based on the International System of Units (SI) and recommendations by Coplen,[49] the carbon isotope ratio (CIR) difference, δ^{13}C, is defined as:

$$\delta^{13}C_{VPDB} = \frac{\frac{N(^{13}C)_P}{N(^{12}C)_P} - \frac{N(^{13}C)_{VPDB}}{N(^{12}C)_{VPDB}}}{\frac{N(^{13}C)_{VPDB}}{N(^{12}C)_{VPDB}}} = \frac{R\left(^{13}C/^{12}C\right)_P - R\left(^{13}C/^{12}C\right)_{VPDB}}{R\left(^{13}C/^{12}C\right)_P} \quad (1)$$

where $N(^{13}C)_X$ and $N(^{12}C)_X$ are the numbers of the ^{13}C and ^{12}C isotopes, $N(^{13}C)_X/N(^{12}C)_X$ and $R(^{13}C/^{12}C)_X$ are the carbon isotope ratio of sample P (x = P) and of the international standard Vienna PeeDee Belemnite (x = VPDB where $R(^{13}C/^{12}C)_{VPDB}$ = 0.011180).[52–55] VPDB is a carbonate with a high δ^{13}C relative to other natural materials, and typical δ^{13}C for other natural samples will carry a negative value. The precision associated with routine GCC-IRMS measurements is in the range of SD (δ^{13}C) = 0.1–0.5 ‰ depending on signal size, sample complexity, and other factors.

In anti-doping applications, the most relevant quantity is the difference in δ^{13}C between excreted steroid metabolite *M* and excreted steroid endogenous reference compound, ERC, or *E*. The isotope difference is often expressed as

$\Delta\delta^{13}C = \delta^{13}C_M - \delta^{13}C_E$. According to recent recommendations,[49] when the reference is VPDB, this difference should be expressed as:

$$\Delta^{13}C_{M/E} = \Delta^{13}C_{M/E,\ VPDB} = \Delta^{13/12}C_{M/E,\ VPDB} = \delta^{13/12}C_{M/VPDB} - \delta^{13/12}C_{E/VPDB}$$

where the various expressions are listed in order of specificity. Here we also use $\Delta^{13}C$ without subscript to refer to an isotope difference defined by context.

The ^{17}O correction

For carbon isotope analysis, the $^{13}C/^{12}C$ is not measured directly; rather it is calculated through the measurement of the CO_2 isotopologues. The IRMS measures ion currents at *m/z* 44 ($^{12}O^{16}O^{16}O^+$) and *m/z* 45 ($^{13}C^{16}O^{16}O^+$). The *m/z* 46 signal which originated mostly from $^{12}C^{16}O^{18}O^+$ (with minor contributions from $^{13}C^{17}O^{16}O^+$, and $^{12}C^{17}O^{17}O^+$) is also measured to correct for the isobaric contribution of $^{12}C^{17}O^{16}O^+$ in the *m/z* 45 signal; this procedure is referred to as the ^{17}O correction. The ^{17}O bearing ions form about 6.5% of the *m/z* 45 signal, consequently, accurate $^{13}C/^{12}C$ isotope ratios can only be calculated by correcting the raw isotope ratio of *m/z* 45/44 from an assumed relationship between the ^{17}O and ^{18}O isotopic abundances using one of various ^{17}O correction algorithms.

In 1957, Craig[56] was the first to apply an ^{17}O correction; followed by the development of alternate treatments by Allison *et al.*,[57] Santrock *et al.*,[58] Werner and Brand,[53,59] Miller *et al.*,[60] and Assonov *et al.*,[61,62] Craig,[56] and Santrock *et al.*[58] are the two most frequently used methods and are employed in commercial software. Generally, the corrections are based on the ^{18}O abundance determined from the ratio of *m/z* 46/44, as well as an assumed relationship between the ^{17}O and ^{18}O isotopic abundances. The various corrections lead to slightly different results over a range of less than 0.1‰. These differences are important for highly precise measurements, such as in static dual inlet analyses but are of little concern for steroid analyses by GCC-IRMS, which yields precisions of approximately SD($\delta^{13}C$) = 0.3‰, typical for replicate GCC-IRMS analyses of a single worked-up sample. For example, application of various ^{17}O correction methods to the GCC-IRMS analysis of our steroid standard CU/USADA 33–1, containing 5α-androstanol acetate (5α-androstanol-AC), androsterone acetate (A-AC), 11-ketoetiocholanolone acetate (11KE-AC), and 5α-cholestane (Cne) is shown in Table 1. The change in $\delta^{13}C$ values via application of the ^{17}O corrections is less than +0.03 ‰ and ranges from 0.021‰ to 0.030‰. The variation amount of these corrections is much less than can be practically detected with the precision of GCC-IRMS, and is only important for the highest precision dual inlet IRMS analyses. Also, the corrections are similar from steroid to steroid for a particular correction. Because the most relevant quantity is a difference measurement, $\Delta^{13}C_{M/E}$, differences tend to cancel and only errors that bias measurement of metabolite and ERC differently are important. Additionally, in GCC-IRMS of steroids, at least 90% of O in analyte CO_2 originates from the oxidation reactor, and only about 10% from the steroid(s) itself.[63] The $\delta^{18}O$ contributed from the combustion reactor reagent is consistent within experimental error over the course of a series of measurements, indicating that the $\delta^{17}O$ is uniform, and the resulting ^{17}O corrections will be similar and biases for $\Delta^{13}C_{M/E}$ negligible.

Calibration in GCC-IRMS

The raw data obtained in continuous flow IRMS measurement, such as GCC-IRMS, consists of a four component array as illustrated in Figure 2. The retention time and ion currents from three faraday cups detecting the three major isotopologues of CO_2 are acquired and recorded digitally at regular intervals. The ion currents are integrated over a specified time period and written

Time (s)	*m/z* 44	*m/z* 45	*m/z* 46
1033.2	29.7	33.1	53.2
1033.7	29.5	32.8	52.8
1034.1	29.8	33.1	53.0
1034.5	53.1	58.7	74.3
1034.9	388.2	433.4	406.7
1035.3	2156.0	2444.3	2321.2
1035.7	5493.9	6342.3	6468.3
1036.2	7007.9	8234.5	9164.6
1036.6	5510.6	6571.3	7941.5
1037.0	3321.7	3997.8	5133.5
1037.4	1803.3	2179.3	2891.8
1037.8	968.9	1171.6	1571.2
1038.2	545.1	657.4	879.9
1038.7	332.2	398.5	529.4
1039.1	223.0	265.3	350.0
1039.5	164.4	194.4	254.4
1039.9	130.9	153.7	200.6
1040.3	110.3	129.0	169.1
1040.7	96.6	112.7	148.6
1041.2	86.9	100.9	133.9
1041.6	79.5	91.9	123.0
1042.0	73.7	85.2	114.6
1042.4	69.2	79.7	108.4

Data Processing
Isotopic Calibration

$\delta^{13}C$

Figure 2. Illustration of the raw data stream obtained from a GCC-IRMS instrument during elution of a peak. The four array components are time, *m/z* 44, *m/z* 45, and *m/z* 46. High-precision isotope ratios are calculated through peak definition and integration, calibration of areas, and referencing to an isotopic standard. The process is automated in IRMS instrument software, where analysts have access to parameters.

Table 1. The average deviation of $\delta^{13}C$ values (n = 4) of steroids in the CU/USADA 33–1 steroid isotopic standard (SIS) mixture analyzed by GCC-IRMS, before and after ^{17}O corrections calculated using seven different methods.

	$\Delta^{13}C_{VPDB}$ Deviation after ^{17}O Correction (‰)						
CU/USADA 33-1	Craig[56]	Allison [57]	Santrock [58]	Werner&Brand (Backscaled to Craig) [53]	Werner&Brand (Original) [53]	Miller [60]	Assonov [61,62]
5α-androstanol-AC	0.021	0.021	0.025	0.026	0.026	0.027	0.027
A-AC	0.021	0.021	0.024	0.025	0.025	0.026	0.027
11-KE-AC	0.021	0.021	0.030	0.027	0.027	0.029	0.029
Cne	0.021	0.021	0.027	0.026	0.026	0.027	0.028

to disk for processing and conversion to isotope ratio. Sampling rates are usually modest, 4–10 Hz, since peaks are generally >5 s wide as a result of band broadening due to the interface, but narrow peaks like those generated in Fast GCC-IRMS or GC × GCC-IRMS demand faster sampling rates, typically about 25 Hz or greater. A requirement for IRMS that is unique among MS techniques is to convert these raw intensities into isotope ratios at high precision, typically with relative standard deviations of replicate injections in the range of 0.03% (SD($\delta^{13}C$) = 0.3 ‰) of the mean isotope ratio to enable the technique to reveal natural isotopic variability. This stringent criterion places strict demands on instrumentation, data processing approach, and calibration.

Isotopic calibration is fundamental to obtaining accurate isotope ratios in GCC-IRMS analyses. This is accomplished through the measurement of the ion current ratios relative to that of an international reference standard, such as VPDB for calculation of $\delta^{13}C_{VPDB}$ values. However, the international reference standard itself cannot be used on a daily basis due to its limited availability. Therefore, working standards are normally used that have isotope ratios traceable to an international reference standard. Ultimately, both precision and consistent accuracy influence the ability to confidently establish that a differential measurement is above or below a given threshold.

In GCC-IRMS analysis, there are many potential sources of isotopic fractionation in the system that can lead to inaccuracies. These sources include sample introduction into the GC inlet, gas chromatography of isotopologues, chromatographic peak distortion such as fronting or tailing peaks, the completeness of chemical conversion, specifically combustion in the case of $\delta^{13}C$ determination, the open split, and the ion source and analyzer of the IRMS. Ideally, comparison of samples to standards should be done under conditions as identical as possible, but this may not always be possible in a single GCC-IRMS run. Calibration can be approached in numerous ways and can be categorized as internal or external, referring to whether the standard is or is not part of the analysis mixture.

Internal calibration

To account for all sources of isotopic fractionation, it is preferable to perform an internal calibration. An isotopically calibrated standard is added to the sample mixture before injection into the system. The standard undergoes identical chemical reaction(s) and passes through the same pathway as the analytes. The standard(s) would ideally be similar in structure to the analytes and be completely separated from any components in the mixture. In addition, it is desirable to use standards that bracket the isotope range expected for the samples. In practice, however, the separation of a mixture of internal standards in a complex sample can be difficult to optimize, especially with the variations of constituents in real samples. An isotopically calibrated standard would be necessary for each matrix and an analytical protocol individually developed to enable it to elute in an unobstructed region of the chromatogram. Moreover, the amount of internal standard that can be analyzed at any point in a chromatogram is limited, thus restricting the signal available from the internal standard. For these reasons, internal standards are rarely used in routine GCC-IRMS applications.

External calibration

External calibration in GCC-IRMS refers to the use of a standard gas (such as CO_2 for $\delta^{13}C_{VPDB}$) that is traceable to an international reference standard. During the GC separation, when no interfering compounds elute, this standard gas can be introduced as pulses directly from an independent static volume such as a bellows from a dual inlet system or at the open split or a valve. This practice produces reliable results, but it cannot take into account the potential isotopic fractionation associated with all aspects of the GC system and online reactor(s) upstream of the standard gas introduction.

A more robust approach to external gas calibration is to analyze an isotopic standard compound mixture after every few samples to monitor and correct for any system drift. The advantage of this scenario is the ability to use standards that contain at least a few of the same compounds as the ones targeted in the sample and that go through the same analytical flow path as the samples. Werner and Brand refer to the 'identical treatment' principle, emphasizing the importance of using chemically similar sample and reference materials to mitigate the effects of isotopic fractionation.[53] Because the standards are run separately, no additional chromatographic methods development is necessary. Also, a large range of isotope ratios in standards can be used to bracket the expected range of isotope ratios in samples. Calibration methods using two or more certified reference standards should produce less error for $\delta^{13}C$ measurement than using one standard.[64] This may be the best approach for comparison of isotopic values generated in different labs, where a common standard mixture can be used as an external standard for inter-laboratory comparisons. Examples of this approach are given in the next section.

Calibration in anti-doping applications

Steroid isotopic standards were not available in the 1990s when anti-doping laboratories were developing and implementing the first GCC-IRMS analyses. Reliable measurements are certainly possible with externally calibrated analytes. A 2003 United States Anti-doping Agency (USADA) workshop on IRMS recommended the creation of steroid isotopic standards (SIS) for harmonization of the reported isotope ratios between the anti-doping laboratories.[65] In 2009, two sets ampoules of SIS were developed by Zhang *et al.*[66] and are the first comprehensively characterized steroid standards, enabling calibration traceable to VPDB. As shown in Figure 3, CU/USADA 33–1, a steroid acetate mixture, and CU/USADA 34–1, a native steroid mixture, were exhaustively characterized and calibrated against a natural gas reference material, NIST RM 8559, traceable to the international standard VPDB for $\delta^{13}C_{VPDB}$ values. The $\delta^{13}C$ values of CU/USADA 33–1 steroids range from -33.04 ‰ to −16.70 ‰, and CU/USADA 34–1 steroids range from −27.06 ‰ to −31.49 ‰, bracketing the isotopic range of interest in anti-doping tests. The within ampoule and between ampoule precisions averaged SD($\delta^{13}C$) = 0.13‰ and SD ($\delta^{13}C$) = 0.09‰, respectively.

A calibration procedure reportedly employed by the Cologne laboratory[67] and our laboratory was suggested for use because it incorporates the best features of internal and external calibration and is based on the concept of identical treatment.[53] In the procedure, an SIS is used to calculate an apparent isotope ratio of a CO_2 gas sampled from a static volume. Anti-doping samples are then calibrated against the apparent isotope ratio of the CO_2 gas. This forward and backward calibration minimizes any differential fractionation associated with the different flow paths while maintaining the flexibility of an external CO_2 gas standard and eliminating the need to develop internal standards

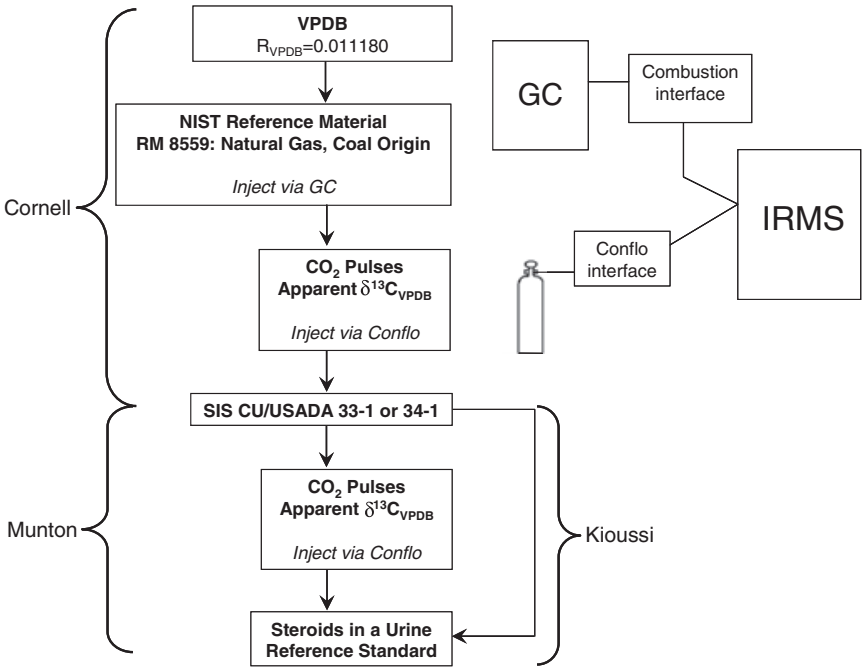

Figure 3. Isotopic calibration procedures for the Steroid Isotopic Standards (SIS) (CU/USADA 33–1 or CU/USADA 34–1), and the use of these SIS for the external calibrations of the steroids in a urine matrix in anti-doping tests, including work by Munton [68] and Kioussi.[63]

specific to the chromatography performed in any particular laboratory. In this procedure, the SIS is included within a sample queue during runs to monitor any system drift. An important feature of this approach is that the isotope ratio of the CO_2 gas standard is not relevant because it is calibrated through the steroid. Major practical advantages accrue with this method. Isotopic fractionation that might be induced during transfer of CO_2 from a calibrated sample bottle to the IRMS inlet static volume cancels out. The apparent isotope ratio may change when the method is changed, without affecting results. Finally, any convenient CO_2 can be used rather than costly calibrated gas.

Munton *et al.*[68] adapted a modified version of this external calibration procedure in order to calibrate and certify steroid components in a urine reference standard intended for use among the anti-doping laboratories. In preliminary work, the two CU/USADA SISs were used to calibrate the apparent isotope ratio of CO_2 gas pulses, and then the isotope ratios of the steroid standard in the urine matrix using the gas according to Figure 3. They use only the raw *m/z* 44 and 45 ion current areas to calculate $\delta^{13}C$ values, ignoring the *m/z* 46 signal and thereby the ^{17}O correction.[68] Certification of the urine reference material is in progress at this writing.

A calibration method that does not rely on CO_2 gas standards was recently introduced by Kioussi *et al.*,[63] in which $\delta^{13}C$ values were calibrated directly against external standards by a calibration curve. This calibration approach is outlined in Figure 3. Only the *m/z* 44 and 45 ion currents were used in the calibration, since the authors noted that at least 90% of the O in CO_2 originated from an isotopically uniform pool of O in the combustion reactor, and thus was constant within acceptable measurement error. CU/USADA 33–1 and 34–1 SIS were used as external calibrants bracketing the entire range of $\delta^{13}C_{VPDB}$ relevant to the analysis of TC and ERC steroids from urine samples for synthetic steroid detection. The certified $\delta^{13}C_{VPDB}$ of the calibration steroids were plotted against the $^{45}CO_2/^{44}CO_2$ raw peak area ratios to create a calibration curve, and the fitted linear regression was used to directly calculate the isotope ratios of the analytes. The external calibration corrects day-to-day systematic drift of GCC-IRMS. As part of the work, an inter-laboratory study between the Athens Greece and Sydney Australia WADA-accredited laboratories showed the feasibility and appropriateness of the procedure, for two TC and two ERC steroids in urine. The relative correspondence between the two laboratories was reported as $\Delta^{13}C = 0.12$ ‰, 0.58 ‰, -0.34 ‰, and −0.40 ‰ for androsterone, etiocholanolone, 5β-androstane-3α, 17β-diacetate, and pregnane-diacetate, respectively.

GCC-IRMS linearity

In static dual inlet analyses, it has long been known that there is a strong non-linear dependence of measured ion current ratios on the total gas pressure in the IRMS ion source[2] and is the reason why sample and standard pressures (i.e. *m/z* 44 ion signals for CO_2) are matched during analysis. Nevertheless, the ion source and ion optics can be tuned to reduce this non-linearity at the expense of some sensitivity.[3] On the other hand, in continuous-flow IRMS, He carrier gas entering through an open split acts as a buffer gas and minimizes this ion source effect. This is of particular importance to GCC-IRMS analyses, where analyte signal levels vary continuously as peaks rise and fall during a run. Other sources of non-linearity may be more important in GCC-IRMS. For example, sample-size-dependent isotopic fractionation can occur when GC or interface conditions are suboptimal,[69] especially GC inlet parameters.[70] These sources of non-linearity then effectively limit the useful dynamic range, and linearity checks are a required part of GCC-IRMS system validation. Normally, linearity is verified by admission of CO_2 to the ion source over a range of ion currents from a static volume or at a constant rate from a valve, and the isotope ratio assessed. However, this approach

will overlook analyte specific fractionation, i.e. due to non-quantitative transfer of an analyte from the injector to the column. An additional check can be made by injecting increasing amounts of a single analyte through the entire analytical path over the desired signal dynamic range to verify that the measured isotope ratio falls within acceptable limits. Trace component analysis of urinary steroids near the 10 ng lower limit for GCC-IRMS linearity has been successfully demonstrated.[22,71] However, in some cases where sample size is severely limited and non-linearity cannot be avoided, the effect can be characterized and corrected for [53,70] but this strategy is not recommended for routine anti-doping applications.

Data processing

Outside of anti-doping applications, the most common application of continuous flow IRMS is bulk sample analysis employing an EA-IRMS. The EA converts solid samples to CO_2 and, for N-containing samples, N_2, and they are admitted to the IRMS.[2] Calculation of isotope ratios from an EA-IRMS raw data stream is straightforward because the background is constant relative to GCC-IRMS, and the peak shape and size varies little from run to run, which facilitates peak integration and background definition. Data analysis in GCC-IRMS can be far more complicated, due to the potential for changing backgrounds, co-eluting interferences, and a wider dynamic range of analyte concentrations within an analysis. As a further complication, vendor provided software code is not available to users and thus is not amenable to detailed investigation. In our IRMS development lab, we rely on home-written LabVIEW-based software (SAXICAB) [72] which enables many of the features of the peak detection and summation algorithms used in manufacturers' software and enables customization and detailed investigation of the effects of data processing parameters on the final reported $\delta^{13}C$.

Peak detection

In all continuous flow IRMS methods, peaks must be detected, and peak starts and stops defined. Peak detection usually occurs as the first step, but in the case of the dynamic summation approach, peak detection occurs after a background correction step. Peaks can be detected and defined in several ways. The method described by Ricci *et al.*[73] yields satisfactory results, and is reportedly implemented in at least one manufacturer's GCC-IRMS software. In this approach, the slope at each point along one of the traces, usually the major isotope, is determined by linear regression, and a slope threshold is defined for the start and stop of a peak. Alternatively, peak starts and stops can be detected from changes in the minor/major ion current ratio.

A critical but often overlooked part of all high-precision GCC-IRMS data processing methods is that good results are achieved only if the same conditions are applied to all traces, for all analyses.[73] An important example is that the retention times associated with the peak start and peak stop for a given peak must be identical across all isotopologue traces if high precision is to be achieved. In practice, this means that starts, stops and background points are defined on one chromatographic trace, usually *m/z* 44 for $^{13}C/^{12}C$ studies, and the same retention times applied to the other traces, with subsequent minor adjustments made to account for the chromatographic time shift as described below. If the peak starts and stops are correlated across isotope traces, then changes in peak areas across traces will also be correlated and the calculated isotope ratio will change very little. If a peak start or stop changes for only one trace, a considerable change could be observed for the measured isotope ratio.

Approaches to calculating ion current ratios

There are three general approaches for calculating R for detected GCC-IRMS peaks: summation, curve fitting, and linear regression, depicted visually in Figure 4. Importantly, all of these approaches consider the entire peak, and not just the peak top. Using peak heights instead of peak areas, as discussed in at least one text on molecular GC-MS analysis of steroids [74] is not appropriate for GCC-IRMS due to the need to maximize the total number of counts

Summation

In the summation approach, the peak start and peak stop are defined, and the major and minor isotope traces are integrated. A background subtraction on the ion currents is performed either before or after peak detection and integration. The summation approach is the most intuitive approach for GCC-IRMS data reduction, and since it is used as part of the Thermo ISODAT software, it is almost certainly the most commonly utilized. In cases where peaks are well-resolved (<10% valley) and the baseline is roughly linear over the peak, it gives very satisfactory results.[75] The elements of the summation method were described in detail by Ricci *et al.*[73]

There are two approaches to the summation method, based on the order of the peak detection and background correction steps. One is 'individual background correction', in which peak detection precedes background definition. Starts and stops of peaks are detected on one trace, and these points are used to define peak windows across all channels. Background points are defined before and/or after the peak start and stop. Ricci *et al.* suggested using an average of points before or after the peak start or stop, or else the low point over an interval before or after the peak.[73] For each isotope trace, the resulting trapezoid is integrated, and this background subtracted from the total peak area to yield background-corrected peak areas from which isotope ratios can be calculated.

The other approach to the summation method is the 'dynamic background correction', in which the background correction step precedes peak detection. An array of background points is generated for one chromatographic trace by selecting points with the lowest ion current within regular 100-s intervals, then eliminating points that are valleys rather than true baseline points. Corresponding background point arrays, with identical retention times, are then constructed for the other isotope traces. The arrays of background points are converted into background chromatograms by interpolation, and subtracted from their corresponding chromatograms. Starts and stops of peaks are detected on one background-corrected trace, and the peak windows integrated to yield background-corrected peak areas from which isotope ratios can be calculated.

The individual summation method is our laboratory's method of choice. The individual method is better at consistently defining background points for well resolved peaks, while the dynamic method sometimes chooses background points far away from

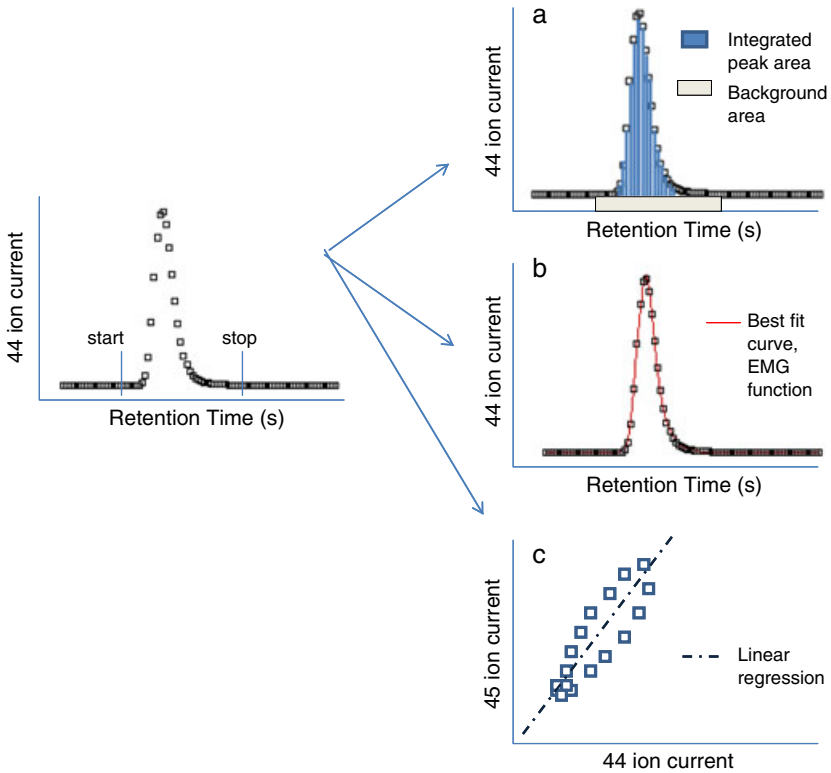

Figure 4. The three approaches to determining ion current ratios. A peak is detected on one of the ion current traces, and the start and stop defined. (a) Summation. The ion current is integrated for each ion trace. A trapezoidal background is subtracted by defining points on either side of the peak. (b) Curve-fit. The peak is parameterized as one of several functions for each ion trace, and the peak area extracted from the best fit parameters. (c) Linear regression. A plot of two ion currents is constructed. The slope of the least squares linear regression is taken as the ion current ratio.

the location of the peak. Because background correction is performed first, peak detection is somewhat improved with the dynamic correction when the background changes very rapidly, although in practice fast ramps are not usually employed in GCC-IRMS.[73] Also, the dynamic approach provides advantages by avoiding selection of background points that are located in 'valleys' between co-eluting peaks, somewhat improving results when 10–30% overlap occurs.[73] However, in most manufacturers' software, it is possible to manually define the background to ensure that valley points are not selected. For reasons described below, it is usually wiser to alter chromatography or sample clean-up in cases where co-elutions occurs, or else to rely on a data reduction approach such as curve-fitting that is less sensitive to co-elutions.

In practice, the summation method is not able to achieve the precision limit predicted by counting statistics, at least for small peak sizes. For example, a vendor of GCC-IRMS systems (Thermo Fisher) quotes a specification that 800 pmol C (= 800 pmol CO_2) is required to achieve SD($\delta^{13}C$) = 0.2‰ for the MAT 253 IRMS. This is equivalent to 160 pmol of C to the IRMS assuming a typical open split ratio of 5:1. However, the sample size necessary to achieve a precision of 0.2‰ based on counting statistics is 5 pmol C (our estimate). Similarly, Goodman and Brenna observed a severe loss of precision, SD($\delta^{13}C$) > 2‰, for sample sizes ≤300 pmol C on column (~60 pmol C to the IRMS) during analysis of FAMEs under normal chromatographic conditions.[76]

The disagreement between theoretical and practical limitations is likely explained by the increasing importance of uncorrelated noise in the background at small peak sizes. Usually, the noise in one GCC-IRMS trace is well correlated with the other isotopic traces, and this correlated noise will have a minimal effect on the precision of isotope ratio measurements.[77] However, with small peak areas, the small amount of uncorrelated noise will become increasingly important. Because the background area is defined by a small number of points, any errors made in defining the background points will be multiplied across the entire length of the peak.[78] Since the background area correction scales with peak width, precision will be worse for wider peaks as compared to narrower peaks.[42] The precision of defining the background can be improved by signal averaging multiple background points,[73] but the use of arbitrarily large intervals is rarely appropriate due to presence of other peaks or changing backgrounds.[77]

A portion of uncorrelated noise appears to result from shot noise associated with the background ion current.[77] We have also shown that uncorrelated noise and its effects on precision can be calculated exactly in cases where quantization error ('bit noise') is present.[78] However, a more general approach for calculating uncorrelated noise in GCC-IRMS, and an understanding of its causes, has not yet appeared.[42]

A time-shift correction is required in GCC-IRMS data processing, in which the integration window is slightly shifted for one or more traces to compensate for the chromatographic separation of the ^{13}C-containing isotopologues compared to the all ^{12}C-containing (monoisotopic) isotopologue.[73] In carbon isotope analyses, the $^{45}CO_2$ peak elutes 50–150 ms before the $^{44}CO_2$ peak under typical chromatographic conditions,[42] although this value is sensitive to both the analyte and the chromatographic conditions. In the case of a 50 ms peak shift, the peak integration window for the *m/z* 45 trace would be

shifted forward in time by 50 ms, or the *m/z* 44 trace shifted back by 50 ms.

The time shift is accounted for automatically by manufacturers' software, and the correction can be implemented in multiple ways. Ricci *et al*. performed the correction by first determining Δt, the difference in the *m/z* 44 and 45 ion peak tops, by modelling the apex of each trace as a parabola.[73] The integrated area of the major isotope trace (*m/z* 44) was then corrected by the term Δt ($^{44}F_{n+1}$ - $^{44}F_1$), where Δt is the time shift, and $^{44}F_{n+1}$ and $^{44}F_1$ correspond to points on the *m/z* 44 ion trace at the end and beginning of the peak window, respectively. In SAXICAB, we perform the time correction by shifting the integration window for the *m/z* 45 ion trace forward by Δt.[72] Since Δt is usually smaller than the data acquisition period, the signal is interpolated between surrounding points.

The original paper on the summation method implemented in IRMS assumed that the time shift would be less than the sampling interval, and thus the start and stop ion currents of the shifted peak could be interpolated from the two surrounding points.[73] With fast acquisition rates (>20 Hz), the sampling interval may be smaller than the chromatographic time shift, which would necessitate extrapolation to calculate ion currents, a potential source of error. When performing the time-shift correction with fast GCC-IRMS data, it is instead advisable to shift the sampling interval for the *m/z* 45 trace.[42] This is less of an issue with GC × GCC-IRMS data, since the time-shifts observed for individual slices are much smaller. However, all GC × GCC-IRMS slices must be corrected individually for their time shift. As a further complication, the time-shift correction only compensates for the time shift on GC2 peak slices; to date, there are no publications on correcting for the GC1 time shift, and therefore full integration of all GC2 slices is necessary to achieve good accuracy.[44]

In our experience, the time-shift correction is only partially effective when attempting to integrate a peak with a restricted window, i.e. if the peak stop is placed on the peak shoulder to avoid integrating a co-eluting peak. There are several possible explanations for this imperfect behaviour. One is that the *m/z* 45 ion trace is usually 1–3% broader than the *m/z* 44 ion trace, based on the full width at half maximum, presumably because of there are multiple ^{13}C-containing isotopolgues.[73] A second reason is the interpolation may be imperfect. In cases of co-elution, a third reason may be that the time shift for the two co-eluting peaks may not be identical. To our knowledge, this third explanation has not been well investigated. However, in most cases, the quality of the time shift correction is not an issue; for well resolved peaks with a flat baseline on the leading and trailing ends, integration windows can be made sufficiently large such that the integrated peak areas will not change meaningfully in response to a 50–150 ms shift in the peak window, and the time-shift errors will be irrelevant.

As a general rule, the summation method is inappropriate when even modest peak co-elution occurs, since the co-eluting peaks can differ in isotope ratio. In the case of δ^{13}C measurements, natural abundance typically spans 30‰.[54] A co-eluting interference integrated together with the target analyte constituting 1% of the analyte peak area, could potentially alter the measured δ^{13}C value by up to 0.3‰. More realistically, an analyst should limit the amount of interferences in their integrated peak to <5% if employing the summation method if they are trying to achieve accuracy within 0.5‰. The effects of co-elution on precision are discussed further below.

It is therefore critical for the analyst to evaluate the occurrence of co-elutions during methods development. For two co-eluting peaks of equal area, recognition of co-elutions is usually apparent from an unusual and/or asymmetric peak shape. Minor co-eluting interferences may not noticeably change the peak shape of the major peak. Multiple authors have suggested that co-eluting peaks can be detected by inspection of the minor/major isotope ratio trace for unexpected inflection points.[73,79] This approach for identifying co-elutions is more qualitative than quantitative, and in our experience may miss minor interferences, especially when the interference has a retention time within one standard deviation of the major peak.

A more robust strategy is to complement GCC-IRMS with molecular GC-MS and evaluate if any unexpected peaks are present.[37] As a point of caution, the response of compounds in GC-MS can vary by over an order of magnitude, so it may be necessary to run calibration standards by GC-MS to determine if a co-eluting peak will be quantitatively important. Finally, a strategy used widely for the validation of analytical methods is to run samples on two different column phases and confirm that similar results (i.e. δ values) are achieved on both columns.

When co-elution is modest and two peak tops are resolved, the analyst may be tempted to integrate the peaks separately to limit the contribution of a co-elution. The wisdom of this approach is questionable for even minor overlaps, not only because the peaks may have different isotope ratios, but also because the isotope ratio is not constant across a peak. The actual effect of co-elutions, and the maximum amount of allowable overlap, depends on the specific integration settings and other factors. Using two pairs of compounds with comparable isotope ratios ($\Delta^{13}C < 5‰$) Ricci *et al*.,[73] reported insignificant deviations for 10% peak overlap. A deviation of >0.5‰ was observed at 30% or greater peak overlap, with the early eluting enriched peak and the late eluting depleted peak. Goodman and Brenna observed far worse performance with co-eluting peaks, where a 10% peak overlap (valley = 10% of peak max height) resulted in a deviation of ~2 ‰ in accuracy.[75] Unexpectedly, the first peak appeared to be depleted in ^{13}C and the second peak enriched in ^{13}C, the opposite of the effect predicted from the isotopic separation. The different outcomes of these studies may be because the individual method was employed by Goodman,[75] while the dynamic method was employed by Ricci *et al*.[73] With the individual method, the end baseline point for the first peak would occur on the front shoulder of the second peak, a point which is enriched compared to the true baseline. Thus, when the baseline is subtracted, the remaining $^{13}C/^{12}C$ ratio of the first peak is too low. Defining the baseline points on either side of the doublet can be performed manually or automatically by the dynamic method and will yield better results. However, as mentioned earlier, it is preferable to improve chromatography, use preparative clean-up, or use curve-fitting, in cases where >10% valley is apparent.

Curve-fitting

In the curve-fitting approach, the major and minor isotope traces are parameterized by an appropriate form (e.g. skewed Gaussian). The areas of traces are extracted from the best-fit parameters, and isotope ratios calculated from the peak areas. One peak or two co-eluting peaks are modelled by a non-linear equation. In addition to the parameters necessary to define the peak shape, a background function is also incorporated into the curve fit,

typically as a straight line. A clear advantage to this approach is that it allows the analyst to deconvolve overlapping peaks without the issues described for the summation method. Various combinations of curve-fitting co-eluting peaks were investigated using exponentially modified Gaussian (EMG) and/or Haarhoff-Van der Linde (HVL) functions, which are designed to emulate real chromatographic peaks with the best precision achieved when modelling both peaks as EMG functions.[75] Impressively, curve-fitting was able to maintain high precision and accuracy even in cases of 30% peak overlap, while summation gave poor results with as little as 10% overlap. Ricci *et al*. reported that curve-fitting with EMG and related functions was not successful for unknown reasons, but had some success in modelling co-eluting peaks as triangles (parameters = height, width, and position).[73] A curve fit based on a polynomial has also been proposed.[80]

While the advantages of curve fitting in cases of co-elution are easily appreciated, curve-fitting also results in improved precision as compared to summation in cases of small peak size[75] and in the presence of quantization error.[78] The reason for the better performance of curve-fitting than summation is the susceptibility of the summation method to errors in background point definition, and any error in defining a background point in summation will be multiplied through the entire length of the integration window. In the curve-fitting approach, all points contribute to determining the background.

Linear regression

In the linear regression approach, a plot of the minor vs. major isotope ion current is constructed and the slope of the best-fit line is equal to the ion current ratio. This approach was utilized by Europa-PDZ as part of its Orchid Post-processing software. It is unique in that it does not rely on separate integration of the different traces, but rather determines the R directly. The approach begins with detection of peak starts and stops, after which a plot of the minor isotope vs. the major isotope, e.g. *m/z* 45 vs. *m/z* 44, is generated. Each point represents a time point across the peak, and the resulting plot has the shape of an ellipse, a result of the chromatographic separation of isotopologues. A linear least squares regression is then performed and the slope of the best-fit line is reported as the ion current ratio. Although not immediately obvious, the use of a linear regression assumes that the background is constant.

The robustness of the linear regression approach has not been well studied. Since all data points are used in definition of the background, it is not expected to suffer from uncorrelated background noise to the same extent as the summation method. However, the use of a linear fit assumes the background to be constant, so this approach is expected to perform poorly in cases where the background contribution is changing appreciably with respect to the peak area. Beyond this, linear least squares regression relies on the assumption that the Y-variables have constant variance (homoscedasticity), a condition that is clearly not satisfied in isotope ratio analyses since counting statistics predict that variance should scale with the instantaneous sample size. This may introduce bias or imprecision due to fluctuations at the low signal, though these factors have not been investigated to our knowledge.

Comparing the linear regression and summation algorithms is not straightforward, since individual vendors' software use one approach or the other. In our laboratory, we performed a comparison of the best-fit algorithm and the individual summation algorithm using the same raw GCC-IRMS data. A standard mixture of 14 fatty acid methyl esters (FAMEs) were analyzed by GCC-IRMS, and the raw $^{44}CO_2$, $^{45}CO_2$, and $^{46}CO_2$ data analyzed by the individual summation algorithm using ISODAT. The raw data was then exported as a text file, and converted by a home-written VB6 script into a PDZ Europa Orchid GC Graph Processor format and evaluated by the best-fit algorithm. This approach mimics an experiment in which the FAME mixture was analyzed on two different GCC-IRMS manufacturers' systems, but eliminates any variability derived from the instrumentation and focuses only on differences resulting from the data analysis software. Only baseline resolved peaks were used in the analysis. The $\delta^{13}C$ values for 14 FAMEs analyzed by the two methods are displayed as a scatter plot in Figure 5. Even though the methods selected similar peak starts and stops, and the average SD ($\delta^{13}C$) for both methods was 0.4‰; the best-fit line for the plot had an r^2 of 0.65 and the root-mean square error was 2.30‰. The reasons for the discrepancies between the methods are not clear, and we did not determine the $\delta^{13}C$ values of the individual components by another method (i.e. by EA-IRMS) and therefore we cannot evaluate which method was more accurate. Two important conclusions can be drawn from our observations. First, good precision does not ensure good accuracy. Second, $\delta^{13}C$ and $\Delta^{13}C$ values determined by different algorithms may not be comparable unless isotopic standards are available to validate results.

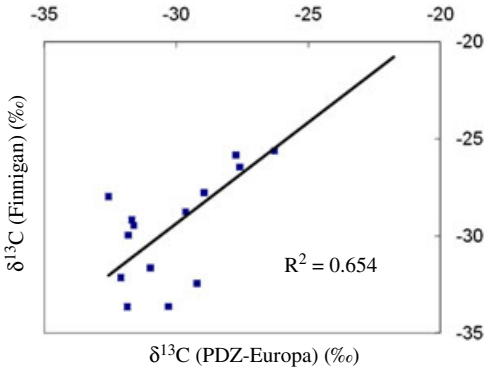

Figure 5. Comparison of $\delta^{13}C$ values calculated by ISODAT (Finnigan, now Thermo Scientific) and GC-Graph (PDZ-Europa) software algorithms. Identical raw data sets from a fatty acid methyl ester mixture were analyzed. Each data point represents a single component.

Conclusions

Anti-doping applications of steroid isotopic analysis rely on a difference measurement between a metabolite and an ERC. Calibration errors tend to cancel when analysis conditions and calculation parameters are identical, thus delivering robust $\Delta^{13}C$ as the final figure of merit for determining an adverse analytical finding. However, best practices for determination of the $\delta^{13}C$ for all steroids should further improve the consistency of results. The process of converting raw GCC-IRMS data to reliable $\delta^{13}C$ requires a combination of robust practices, well-defined isotopic standards, and properly implemented digital methods. For routine anti-doping applications within a single laboratory, calibration against working standards traceable to the international standard VPDB remains best practice. Most IRMS labs maintain standards specific to the sample types of interest to account for subtle, sample-dependent effects. Isotopic

calibration against steroid standards in a manner that takes into account possible sources of fractionation in the flow paths and chemical reactors is expected to yield results that are readily harmonized. Baseline resolution of analyte peaks avoids possible biases that depend on the choice of data processing parameters.

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