

Ying Zhang¹, Gavin L. Sacks², J. Thomas Brenna¹

Fast Gas Chromatography Combustion Isotope Ratio Mass Spectrometry

¹Cornell University, Division of Nutritional Sciences, Savage Hall, Ithaca, New York 14853

²Dept of Food Science & Technology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456

Introduction

Compound specific $^{13}\text{C}/^{12}\text{C}$ analysis by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) is widely employed to establish the provenance or history of samples. Applications include reconstructing ancient habitats in biogeochemistry, [1] authenticating foods and flavors and determining food webs or migratory patterns.[2] GC-C-IRMS is now routine for distinguishing exogenous and endogenous steroids in athletic doping detection [3,4]. In this application, the time required for instrumental analysis is limiting, and the GC-C-IRMS is used primarily as a confirmatory test after a GC/MS screen detects an anomaly. Fast GC-C-IRMS holds the potential to enable analysis of all samples, and thus detect doping in samples for which the molecular marker concentrations have returned to normal. Narrow-bore fast GC columns (0.10-0.20 mm i.d. columns) can achieve up to 10-fold faster run times than conventional GC columns (0.25-0.32 mm i.d.), but this speed can only be achieved with fast detectors (e.g. flame ionization detector (FID) or time-of-flight mass spectrometry (TOF-MS)).[5] An added advantage of fast GC is improved detection limits due to diminished band broadening of the eluting peak.

Despite the obvious advantages, the successful coupling of fast GC to IRMS has not been reported. Sessions has correctly noted that fast GC-C-IRMS may not be possible with commercial systems because fast GC demands a fast detector response that is incompatible with the high impedance feedback resistors.[6] At least as importantly, the post-column combustion interface is known to broaden eluting peaks considerably.

In this work, we address and comprehensively evaluate these issues and develop a fast GC-C-IRMS system. We demonstrate fast GC-C-IRMS on ten underivatized steroids for

eventual application to urinary steroid analysis. A more comprehensive report has appeared elsewhere.[5]

Design considerations

Fast GC detectors and IRMS. The chemical and physical nature of a fast GC detector must be responsive to peaks of width <500 ms without introducing peak broadening or distortion, and data acquisition must be sufficiently rapid to adequately define the peak. The IRMS should be conducive to fast applications, as the ionization and extraction process is very fast, and the faraday cups have comparable speed to FID; however, response time of the IRMS can be limited by the R-C time constant of the head amplifiers. Tobias and Brenna previously reported peak broadening during D/H analyses on the $m/z = 2$ cup caused by the high impedance and resulting high RC time constant in 1996.[7] In our CO₂ analysis, the amplifier circuit capacitance is 1 pF, and the RC time constants for the 44 (feedback resistor = 300 MΩ), 45 (30 GΩ), and 46 (100 GΩ) cups are 0.3, 30, and 100 ms. Noticeable broadening for 200-1000ms fast GC peaks would be a problem only for the 46 cup based on Gaussian summation of peak widths. In order to fix this problem, the 46 cup feedback resistor was replaced with a 30 GΩ resistor.

Minimizing Peak Broadening Effects Prior to Ionization. Of greater concern is that the post-column interface and dead volume in the gas path leading to the ion box will introduce intolerable extra-column broadening. In GC-C-IRMS, the entire post column interface (combustion, water trap, transfer lines, open split, IRMS) can be viewed as the detector. While the combustion step is expected to be rapid (1-2 ms timescale), the volumes associated with combustion interface can introduce considerable broadening. The combustion interface components are (1) solvent diversion, (2) combustion reactor, (3) transfer lines, (4) water trap, and (5) open split. Goodman compared his continuous capillary combustion interface design that used a rotary valve to a conventional combustion interface with a backflush system to divert the solvent. To our knowledge, there is no other literature on the post-column combustion interface on peak broadening. We therefore undertook an analysis of each component.

Solvent Diversion. Typical GC-C-IRMS requires 1 μL injection size for the solvent (50 μmol C). If the solvent is allowed to enter the combustion reactor, the oxygen in the reactor will be exhausted. Two systems, a backflush system [13] with T-connectors before and after the combustion reactor, or a four-way rotary valve which directs the column effluent to either a vent or to the combustion interface and IRMS. [8] The latter has been routinely used

in our lab for many years, although the internal volume of the rotor channels may cause peak broadening. We performed injections of CO₂ into the IRMS via a transfer line or a rotary valve. Peak widths (FWHM) of 242 ms were attainable without the rotary valve, compared to 296 ms with the rotary valve. Thus, 54 ms improvement of peak width can be achieved with the transfer line no interruption of rotary valve.

Flenker has recently proposed an alternative solvent diversion system which demands no extra connections.[8] The sample is injected onto a cold programmable temperature vaporization (PTV) injector with split on to purge the solvent. The split is turned off and the PTV temperature ramped to transport the sample to the column. Thus, the solvent is vented out of the septum purge. This approach is advantageous because it adds no extra components, although it would not be compatible with similar boiling points to the solvent

Combustion Reactor. Conventional combustion reactors employ a 30cm × 0.5mm i.d. alumina combustion tube hand-loaded with a twisted strand of 20cm × 0.1mm Cu, Pt, and Ni wires, which are connected in series to the column effluent. The continuous capillary design replaces the ceramic tube with a continuous 30cm × 0.25mm i.d. FS capillary loaded with a single strand of 20cm × 0.1mm Cu wire for the combustion zone. One caveat for the narrow bore capillary is that the capillary is too fragile to be used in a long run at high temperature. Our recent experience is that thick wall capillary with the same i.d. of 0.25 mm is much more robust than thin wall capillary. We also used a tee fitting on the outlet side of the reactor to reveal when the capillary is cracked. The furnace temperature was set at 950°C, which is normally regarded as the optimum temperature for quantitative combustion of CH₄. To prevent depletion of the oxygen source, the furnace was recharged every 24 hours.

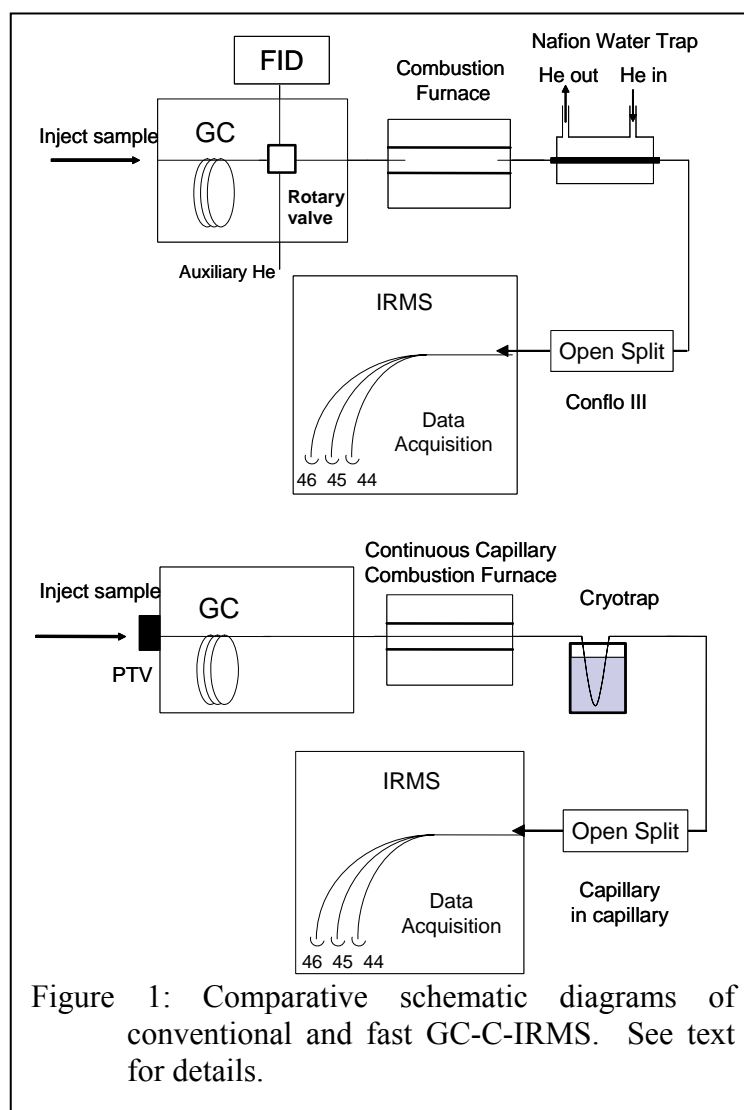
Experimental Details

Chemicals and Standard Mixtures. A mixture of steroids (99% purity) with a wide range of isotope ratios and eluting over a range of retention times and was prepared: 3β-hydroxy-5α-androstane, OH-Andro; 19-noretiocholanolone, 19-NA; Etiocholandioliol, Etio; Dehydroepiandrosterone, DHEA; Androsterone, Andro; 11-ketoetiocholanolone, 11-keto; Pregnenediol, 5βP; Cholestane, Chln; Pregnanetriol, Preg; Cholesterol, Ch-OH. All steroids were purchased from Steraloids (Newport, RI) with the exception of 5βP purchased from Acros Organics USA (Morris Plains, NJ). One hundred ng/μL of each component was dissolved in isopropanol (Mallinckrodt Baker).

Conventional GC-C-IRMS System. An HP 5890 GC equipped with split/splitless injector and a Varian 8200 autoinjector was interfaced to a home-built combustion

reactor/water trap/open split and the effluent sent to a Finnigan MAT 252 (Bremen, Germany) IRMS run in high linearity mode, as shown in figure 1a. A four-way rotary valve within the GC enabled venting of solvent prior to the combustion reactor. The GC column was connected via Valco (Houston, TX) stainless steel ZDV fittings to a 30cm × 0.5mm i.d. alumina combustion tube hand-packed with a twisted strand of 20cm × 0.1mm Cu, Pt, and Ni wires. The combustion tube was maintained at 950°C during operation with a 30 cm Thermcraft tube furnace (Winston Salem, NC). Combustion products were dried in a Nafion® water trap (dimensions = 10cm × 0.8mm i.d.), and sent to the IRMS via a 2m × 0.10mm open split directly inserted into the post-water trap transfer line. The post GC transfer lines were 0.32 mm i.d. fused silica (FS) tubing and had a total length of ~2 m. In high linearity mode, the IRMS operated at a source pressure of 1×10^{-6} Torr with an absolute sensitivity of ~3000 molecules/ion as estimated from CO₂ injections. All connections were made with Press-Tight® connectors (Restek; Belafonte, PA).[5]

Fast GC-C-IRMS System. The fast GC-C-IRMS system used the FMAT 252 IRMS and followed the same stages as the conventional system but was modified to minimize broadening and dead volumes, as shown in figure 1b. 1) A Varian 3800 GC with programmable temperature vaporization (PTV) injector was used. The innovative use of the PTV was based on data shown previously at this workshop [8], and enables 2) elimination of the post-column rotary valve from the system. 3) The combustion tube was 40cm x 0.25mm i.d. FS tubing loaded with a single strand of 20cm × 0.1mm i.d. Cu wire,



positioned in the center of the combustion tubing. A postcolumn reaction tee fitting on the one end of combustion tubing was used to diagnose if the capillary was cracked at high temperature. The combustion tubing was inserted into a 30cm x 0.5mm i.d. ceramic tube and connected on either side for mechanical stabilization, as described by Goodman.[9] 4) The post furnace transfer line was 2m x 0.15mm FS capillary. 5) The Nafion water trap was replaced with a cold trap (dry ice in acetone) with the cooled zone of the transfer line ~30cm in length .

Data Collection and Processing. Data were collected from the FMAT 252 by attaching three 24-bit National Instruments (Austin, TX) NI4351 digitizers directly to the output of the $m/z = 44, 45,$ and 46 cup head amplifiers. Data were acquired and processed by SAXICAB,[10] a home-built Lab VIEW-based [11] data acquisition system. The data was collected at 50 Hz, and consecutive points were averaged to reduce the size of the files by half and the effective data acquisition rate to 25Hz.

Two modifications were made to record the data from the fast GC: 1) The 46 cup with 100G Ω feedback resistor was replaced with a 30G Ω resistor to avoid the problem of noticeable broadening for 200-1000 ms fast GC peaks. This was necessary only for the high resistance m/z 46 cup, which had a response time <100 ms. 2) The second is that the algorithm used to integrate and calculate background windows on the 45 trace were modified to shift properly account for the time shift, Δt_R , of greater than one bin between the $^{44}\text{CO}_2$ and $^{45}\text{CO}_2$ peaks. High precision isotope ratios of the CO_2 expressed as $\delta^{13}\text{C}$ (‰), were calculated using pulses of CO_2 gas that had been isotopically calibrated to the VPDB reference via isotopically calibrated condensed phase FAME standards.[12]

Comparison of Underivatized Steroid Analyses by Conventional and Fast GCC-IRMS. A steroid standard mixture was prepared with 100 ng/ μL of each steroid in 75% isopropanol / 25% cyclohexane. One μL of the mixture was injected on column for both conventional and fast analyses.

For conventional GCC-IRMS, a Varian 25m x 0.25mm x 0.25 μm VF-5ms (5% phenyl) column was used. The oven program was 80°C (initial, no hold) to 260°C (70 °C/min no hold) to 330°C (2 °C/min). The injector was held at 300°C. Injections were performed splitless, and the purge was turned at 0.8min. The GC was operated in constant pressure mode, and the head pressure was adjusted such that the flow rate at 80°C was 1 mL/min.

For fast GCC-IRMS of native (underivatized) steroids, a 20m x 0.15mm x 0.60 μm Varian VF-5ms (5% phenyl) column was used. The oven parameters were: 80°C (initial, hold 3 min) to 320°C (90°C/min, no hold) to 360°C (5°C/min, hold 2 min). The PTV injector

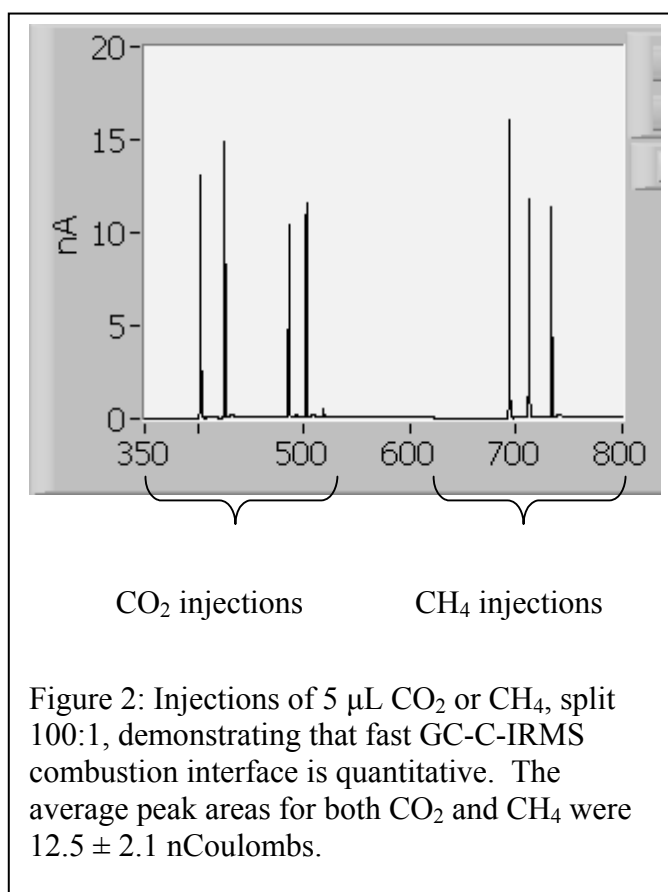
parameters were 80°C (initial, hold 0.5 min) to 320°C (150°C/min). The sample was injected on the cool PTV with the split at 1000:1 to purge the solvent. The split was turned off at 0.4 min while the analytes were vaporized, and returned to 300:1 at 3 min to purge the injector during the column temperature program. The GC was operated in constant flow mode (1 mL/min).

Results and Discussion:

To evaluate the efficiency of the continuous capillary (fast) furnace, we performed 5 uL injections of each CH₄ and CO₂ at 100:1 split ratio on the fast GC-C-IRMS system. The results in Figure 2 show that furnace efficiency is near 100% because the average peak area of three injections of CH₄ is the same as those of four injections of CO₂.

Transfer Lines and Connectors. For conventional GC-C-IRMS system, 1-3 m of 0.32 mm i.d. FS is used for transfer lines between the reactor and the water trap and open split. We used two meters of 0.32 mm i.d. FS capillary resulted in minimal achievable peak widths of 120 ms. We also observed no noticeable broadening from FS Press-Tight connectors, consistent with Matisova et al.'s report. [13]

Water trap. Water must be removed prior to admission to the ion source because it protonates CO₂ to produce HCO₂⁺, which interferes with analysis at *m/z* 45. Either a Nafion™ tube or a cryogenic trap is employed to remove the water of combustion. Our conventional system successfully removes water with a 20cm × 0.8mm Nafion tube. This trap offers the advantage that it operates continuously at room temperature without attention. Its principle disadvantage is that it introduces two connections, which offer the possibility of leaks, and has a considerably larger ID than the transfer lines which compromise chromatographic resolution due to diffusion. The



injection of CO₂ showed that a Nafion trap results in a minimal peak width of >1600 ms. The fast GC trap, consisting of narrow-bore capillary inserted to a cryogenic trap without any extra connections or diameter changes achieved a peak width of 242 ms, and thus retained chromatographic quality.

Open Split Design. The conventional open split design [9,14] employs a narrow diameter piece of FS capillary (e.g. 2m x 0.10 mm i.d.), that provides an intentional leak into the IRMS. A commercial ConFlo III system is used as a diluter to direct the standard CO₂ and He carrier gas mix into a closed glass tube. The mixture is then sampled into the IRMS via the capillary leak. Our measurements show that the ConFlo design would allow for a minimal peak width of ~800 ms.

We normally employ a simplified open split, with a small i.d. capillary leak threaded inside a large i.d. capillary coming from the water trap. The o.d. of the capillary leak must be small enough to allow its insertion, and the junction is jacketed with dry He gas. A typical target leak rate for IRMS is 0.2 mL/min. Our measurements indicate that minimal attainable peak widths for two open split capillaries (12.5 cm x 0.05 mm and 2 m x 0.10 mm) yield similar leak rates, determined to be 127 ms for the 0.05 mm i.d. and 183 ms for the 0.10 mm i.d. In practice, the 0.05 mm i.d. capillary is too short to be used because the inlet flow rate will be changed significantly with minor changes in the capillary length. A 70 cm x 0.075 mm open split was eventually chosen as a good compromise.

Most narrow GCC-IRMS Peak achievable with Minimal Broadening interface.

Figure 3 shows results with all the above modifications to the interface, and a 1m x 0.1mm ID FS capillary used as a column. Peak widths of about 250 ms for both CO₂ and CH₄ injections were achieved. Given that fast GC yields peaks of less than 1 s width, the interface can be expected to perform suitably for many fast GC applications.

Underivatized Steroid Analyses by conventional and fast GC-C-IRMS. We analyzed a synthetic mixture of 10 steroids (100 ng each OH-Andro, 19-NA, Etio, DHEA, Andro, 11-keto, 5βP, Ch, Preg, and Ch-OH) that are widely used in antidoping analysis via 1 uL splitless injection by conventional and fast GC-C-IRMS.

Figure 4 shows that

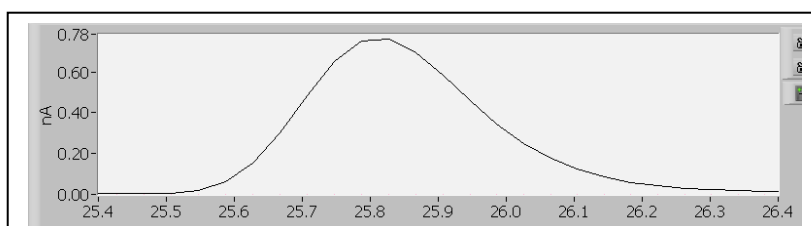


Figure 3: ⁴⁴CO₂ traces of CH₄ peak in the optimized fast GC-C-IRMS system, showing 250 ms wide peaks (FWHM). Data acquisition was at 25 Hz (40 ms per data point).

compared to conventional GC-C-IRMS, fast GC-C-IRMS using smaller i.d. and thicker film column can achieve near baseline separation of the 10 steroids in 620 s, about 50% of the run length of conventional GC. Also, peak widths of fast GC-C-IRMS are less than 1s. Two of the steroids (DHEA and Andro) that

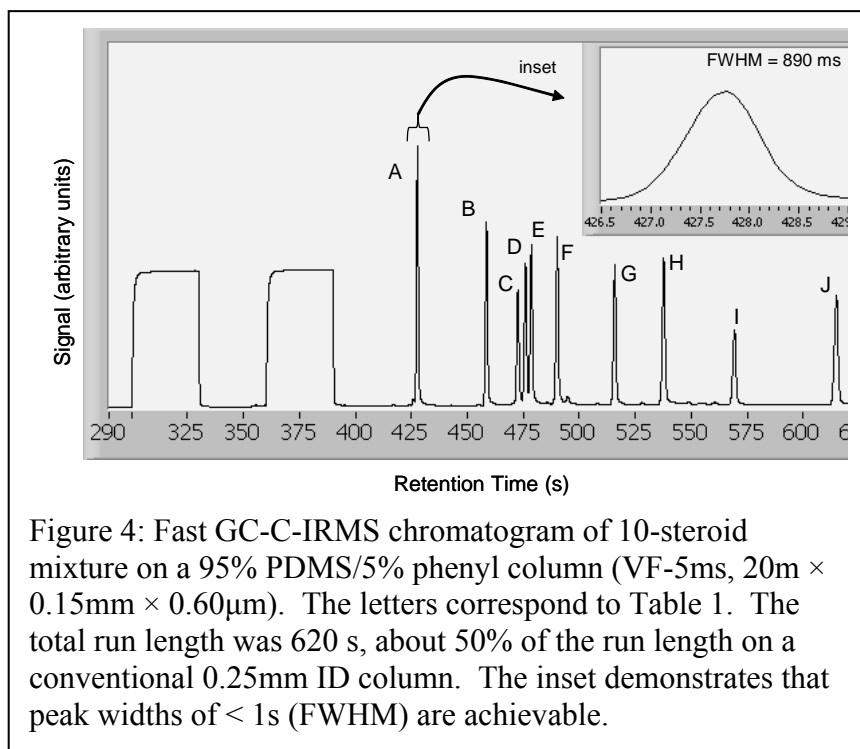


Figure 4: Fast GC-C-IRMS chromatogram of 10-steroid mixture on a 95% PDMS/5% phenyl column (VF-5ms, 20m \times 0.15mm \times 0.60 μ m). The letters correspond to Table 1. The total run length was 620 s, about 50% of the run length on a conventional 0.25mm ID column. The inset demonstrates that peak widths of < 1s (FWHM) are achievable.

could not be resolved by conventional GC can be resolved by fast GC. The precision of steroids on both systems are comparable ($SD(\delta^{13}C) = \pm 0.2\%$). A comparison of $\delta^{13}C$ values between the two systems in Table 1 shows that differences are $\leq 2\%$ for all steroids and $\leq 1\%$ for six of the eight steroids. In all, fast GC-C-IRMS for steroid analysis can provide shorter run time and better resolution than conventional GC with similar precision and accuracy.

Conclusion:

Compared to conventional GC-C-IRMS, fast GC-C-IRMS achieves shorter run times and improved precision and accuracy at low signal levels. The optimized interface can produce 250 ms wide chromatographic peaks (FWHM), one fifth the width of previously reported GC-C-IRMS peaks. To demonstrate the applicability of fast GC-C-IRMS, we have tested underivatized steroids on our optimized fast GC-C-IRMS system. The results show the fast GC-C-IRMS system can achieve equivalent or better precision ($SD(\delta^{13}C) = \pm 0.2-0.5\%$) and resolution than conventional GC-C-IRMS with about 2 to 3 fold decreased run time. Differences of $\delta^{13}C$ values between fast and conventional systems are $\leq 1\%$ for six of the eight steroids. Although the narrow bore columns provide lower sample capacity, the improved precision at low sample sizes raises the possibility of injecting samples in split mode without compromising performance.

Future modifications should permit even narrower GC-C-IRMS peaks. In particular, replacing the current 40 cm \times 0.25 mm i.d. FS combustion reactor with a narrower capillary

(i.e. 0.10 mm) would reduce broadening. Further work will investigate ways to effectively include an oxygen source (CuO, ZnO) in such a narrow bore design. Another necessary modification will be the IRMS inlet, as our data show that the inlet limits peaks to at least 124 ms. The internal volume of the IRMS ion source may contribute to this broadening.

Acknowledgements: This work was supported by the US Antidoping Agency.

Table 1: Comparison of $\delta^{13}\text{C}$ values for a steroid mixture between conventional and fast GC-C-IRMS, corresponding to the chromatograms in Figure 4. Mean precision for both systems was SD ($\delta^{13}\text{C}$) = 0.2‰ and the difference in $\delta^{13}\text{C}$ values between the systems was for all eight steroids, values were not determined for DHEA and Etio. Abbreviations are defined in the Experimental section.

Peak	Steroid	$\delta^{13}\text{C}$ (‰, mean \pm SD)	
		Conventional	Fast
A	OH-Andro	-30.3 \pm 0.1	-30.2 \pm 0.1
B	19-NA	-29.7 \pm 0.3	-30.2 \pm 0.1
C	Etio	-33.1 \pm 0.1	-32.1 \pm 0.1
D	DHEA	n.d.	-26.0 \pm 0.4
E	Andro	n.d.	-26.6 \pm 0.4
F	11-keto	-13.7 \pm 0.3	-14.7 \pm 0.2
G	5 β P	-30.3 \pm 0.1	-30.8 \pm 0.2
H	Chln	-23.8 \pm 0.2	-25.8 \pm 0.1
I	Preg	-31.6 \pm 0.2	-31.4 \pm 0.2
J	Ch-OH	-20.7 \pm 0.2	-22.3 \pm 0.4

References

1. Freeman, K.H., Hayes J.M., Trendel J.M., Albrecht P. (1990) Evidence from carbon isotope measurements for diverse origins of sedimentary hydrocarbons. *Nature* **343**: 254-256.
2. Woodbury, C.P., Jr., Fitzloff J.F., Vincent S.S. (1995) Sample multiplexing for greater throughput in HPLC and related methods. *Anal Chem* **67**: 885-890.
3. Aguilera, R., Chapman T.E., Catlin D.H. (2000) A rapid screening assay for measuring urinary androsterone and etiocholanolone delta(13)C (per thousand) values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom* **14**: 2294-2299.
4. Bowers, L.D. (1997) Analytical advances in detection of performance-enhancing compounds. *Clin Chem* **43**: 1299-1304.
5. Sacks, G.L., Zhang Y., Brenna J.T. (2007) Fast gas chromatography combustion isotope ratio mass spectrometry. *Anal Chem* **79**: 6348-6358.
6. Sessions, A.L. (2006) Isotope-ratio detection for gas chromatography. *Journal of Separation Science* **29**: 1946-1961.
7. Tobias, H.J., Brenna J.T. (1996) High-precision D/H measurement from organic mixtures by gas chromatography continuous-flow isotope ratio mass spectrometry using a palladium filter. *Analytical Chemistry* **68**: 3002-3007.
8. Flenker, U., Hebestreit M., Pipert T., Hulsemann F., Schanzer W. (2007) A new robust method for gas chromatography-combustion isotope ratio mass spectrometry. *Donike Workshop submitted*.
9. Goodman, K.J. (1998) Hardware modifications to an isotope ratio mass spectrometer continuous-flow interface yielding improved signal, resolution, and maintenance. *Analytical Chemistry* **70**: 833-837.
10. Sacks, G.L., Brenna J.T., Sepp J.T. SAXICAB: Software for automated high-precision continuous-flow isotope ratio mass spectrometry; 2001; Chicago, IL.
11. (2000) Labview 6i. Austin, TX: National Instruments.
12. Caimi, R.J., Houghton L.A., Brenna J.T. (1994) Condensed-Phase Carbon Isotopic Standards for Compound-Specific Isotope Analysis. *Analytical Chemistry* **66**: 2989-2991.
13. Mastovska, K., Lehotay S.J. (2003) Practical approaches to fast gas chromatography-mass spectrometry. *Journal of Chromatography A* **1000**: 153-180.
14. Merritt, D.A., Freeman K.H., Ricci M.P., Studley S.A., Hayes J.M. (1995) Performance and Optimization of a Combustion Interface for Isotope Ratio Monitoring Gas-Chromatography Mass-Spectrometry. *Analytical Chemistry* **67**: 2461-2473.