HIGH PRECISION $\delta^{13}C$ ANALYSIS OF STEROIDS BY COMPREHENSIVE TWO DIMENSIONAL GAS CHROMATOGRAPHY COMBUSTION ISOTOPE RATIO MASS SPECTROMETRY

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HIGH PRECISION $\delta^{13}C$ ANALYSIS OF STEROIDS BY COMPREHENSIVE TWO DIMENSIONAL GAS CHROMATOGRAPHY COMBUSTION ISOTOPE RATIO MASS SPECTROMETRY

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Carbon Isotope Ratio analysis of urinary steroids using GCC-IRMS is a recognized test to detect illicit doping with synthetic testosterone. However, there are currently no universally used steroid isotopic standards (SIS) for calibration of GCC-IRMS data between antidoping labs. One dimensional GC (1DGC) has limitations with the separation of closely eluting components. This thesis focuses on challenges which have limited the scope of antidoping tests with existing instrumentation.

Two steroid mixtures were prepared, one with four derivatized steroids and one with three underivatized steroids. Steroids were calibrated using a Thermo Finnigan 253 IRMS and calibrated against NIST RM 8559, traceable to the international standard VPDB. Absolute $\delta^{13}C_{VPDB}$ and $\Delta\delta^{13}C_{VPDB}$ values from randomly selected SIS ampoules indicate uniformity of steroid isotopic composition within measurement reproducibility, SD ($\delta^{13}C$) < 0.2 ‰.

Recently, two dimensional GC (GC × GC) has developed into a commercial technique and has been applied to natural mixtures because it has ten times the peak capacity and sensitivity compared to 1D GC. Here the very first coupling of fast GC to IRMS with peak widths one-fifth of any previously reported GCC-IRMS system is shown. Fast GC was accomplished by systematically removing sources of peak
broadening. The first coupling of comprehensive GC × GC to on-line combustion IRMS was accomplished by interfacing to an optimized low dead volume combustion interface to preserve < 300 ms wide GC2 peaks, modifying the IRMS detector to enable fast detection, and advancing software to handle isotopic time shifts of less than one bin (40 ms), and to integrate peak slices to recover isotope ratios.

To avoid the addition of external carbons, analysis of underivatized steroids is preferred for IRMS analysis. Several column sets were evaluated for best dispersion of steroids in 2D analysis space. A thick film polar GC1 column and a nonpolar GC2 column were used for the detection of underivatized exogenous steroids and other drugs in a complex urine matrix using GC × GC-TOF-MS. The use of GC × GC-TOF-MS allows for full mass spectrum scanning, enabling the detection and identification of unknowns or designer drugs in the urine matrix.
Ying Zhang received the B.S. degree in Chemistry Department from State University of New York-College at Cortland in 2004, M.S. degree in Analytical Chemistry in 2006, and Ph.D degree in 2009 from Cornell University. Her research interests include development of advanced chromatography methods and application of isotope ratio mass spectrometry for analysis and characterization of biological molecules.
To

My parents Yunkun Xu, Tongyan Zhang

and

My husband

Hang
I would like to thank Dr. J. Thomas Brenna for kindly advising me through my Ph.D. program. Not only he taught me the depth and broadness in knowledge, but his exceptional enthusiasm, curiosity, and ever encouraging positive spirit have always been and will continue to be an inspiration to me.

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LIST OF ABBREVIATIONS

11k-AC: 5β-Androstan-3α-ol-11, 17-Dione Acetate
11-keto: 11-ketoetiocholanolone
11-OHA: 5α-androstan-3α, 11β-diol-17-one
19-NA: 19-noretiochonanolone
2DGC: Two-Dimensional Gas Chromatography
5αA: 5α-androstan-3α, 17β-diol
5αA-AC : 5α-Androstan-3β-ol Acetate
5βP: 5β-Pregnane-3α, 20α-diol
5βA: 5β-androstan-3α, 17β-diol
A: 5α-Androstan-3α-ol-17-one
A-AC: 5α-Androstan-3α-ol-17-one Acetate
AAS: Anabolic Androgenic Steroid
AP: Atom Percent
APE: Atom Percent Excess
BSTFA: N, O-bis (trimethylsilyl) Trifluoroacetamide
CAM: Crassulacean Acid Metabolism
CDT: Canyon Diablo Meteorite
CF-IRMS: Continuous Flow-Isotope Ratio Mass Spectrometry
Chln: 5α- Cholestane
Ch-OH: Cholesterol
CI: Chemical Ionization
CIR: Carbon Isotope Ratio
Cne : 5α-cholestane
COIL: Cornell University Stable Isotope Laboratory
(C)-PSIA: Carbon Position-Specific Isotope Analysis
CRI: Chemical Reaction Interface
CSIA: Compound-Specific Isotope Analysis
D/H: Deuterium/Hydrogen
D-D: Deuterium
DHEA: Dehydroepiandrosterone
(DI)-IRMS: Dual Inlet Isotope Ratio Mass Spectrometry
E: 5β-Androstan-3α-ol-17-one
EA-IRMS: Elemental Analyzer Isotope Ratio Mass Spectrometry
EI: Electron Impact
EM: Electron Multipliers
EpiT: Epitestosterone
ERC: Endogenous Reference Compound
F: Fraction
FAME: Fatty Acid Methyl Ester
FC: Faraday Cup
FeS: Troillite
FID: Flame Ionization Detector
FWHM: Full Width Half Maximum
GC × GCC-IRMS: Comprehensive Two-Dimensional Gas Chromatography Isotope Ratio Mass Spectrometry
GC × GC-TOF-MS: Comprehensive Two-Dimensional Gas Chromatography Time of Flight Mass Spectrometry
GC: Gas Chromatography
GC1: The First Gas Chromatography Column
GC2: The Second Gas Chromatography Column
GCC-IRMS: Gas Chromatography Combustion Isotope Ratio Mass Spectrometry
GC-MS: Gas Chromatography Mass Spectrometry
(GC)-Py-GCC-IRMS: (Gas Chromatography)-Pyrolysis-Gas Chromatography
Combustion Isotope Ratio Mass Spectrometry
H-H: Hydrogen
HP: Hewlett Packard
HPLC: High Pressure Liquid Chromatography
IAEA: International Atomic Energy Agency
IOC: International Olympic Committee
IRMS: Isotope Ratio Mass Spectrometry
KIE: Kinetic Isotope Effect
LMCS: Longitudinal Modulated Cryogenic System
MDGC: Multidimensional Gas Chromatography
MS: Mass Spectrometry
MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide
NIST: National Institute of Standards and Technology in the United States
NP-P: Nonpolar-Polar Column Set
OH-Andro: 3β-hydroxy-5α-androstane
PDB: PeeDee Belemnite
PEP: Phosphoenolpyruvate
P-midP: Polar-Medium Polar Column Set
P-NP: Polar-Nonpolar Column Set
Preg: 5β-pregnane-3α, 17, 20α-triol
PSIA: Position - Specific Isotope Analysis
PTV: Programmable Temperature Vaporization
RM-8559: Reference Material 8559
RuBP: Ribulosebiphosphate
S/N: Signal to Noise Ratio
SD: Standard Deviation
SIM: Single Ion Monitoring
SIS: Steroid Isotopic Standard
SLAP: Standard Light Antarctic Precipitation
SM1: Steroid Mixture 1
SM2: Steroid Mixture 2
SM9: Nine-Steroid Mixture
SM9-AC: Nine-Steroid-Acetate Mixture
SMOW: Standard Mean Ocean Water
SMRTL: Sports Medicine Research & Testing Laboratory
SMRTL70: A Standard Containing a Set of 70 Native Anabolic Steroids and Drugs
T/E: Testosterone/Epitestosterone
T: Testosterone
TBME: Tert-Butylmethylether
TIE: Thermodynamic Isotope Effect
TMS: Trimethylsilyl
TOF-MS: Time of Flight Mass Spectrometry
UHP: Ultra High Purity
USADA: United States Antidoping Agency
VPDB: Vienna PeeDee Belemnite
VSMOW: Vienna Standard Mean Ocean Water
WS: Working Standard
ZPE: Zero-Point Energy
CHAPTER ONE

Introduction

1.1. Stable Isotopes at natural abundance levels

The stable isotopes of light elements are composed of one overwhelmingly abundant isotope, and one or two isotopes of relatively minor abundance. The low abundance of these isotopes provides opportunities to use enriched sources of the isotopes as tracers in biological and biochemical studies.

Isotope variation due to natural processes, which include source effects, formation processes, enzyme selectivity, reaction rates and equilibrium constants, and environmental conditions, has provided important data and new insights into physiological studies among/between organisms. It was first recognized that small variations in the natural abundance of light stable isotopes such as $^2$H, $^{13}$C, $^{15}$N, $^{18}$O, and $^{34}$S in a variety of samples can be measured with high precision (4-6 significant figures) isotope ratio mass spectrometry (IRMS) in the late 1940s. The past 30 years have seen rapid growth in the development of better gas-phase stable isotope instruments, interfaces and measurements. Automated on-line sample preparation devices, and continuous-flow analysis, provide for a growing number of applications in the fields of agriculture, natural and atmospheric sciences, forensic science, biomedical research, and anti-doping tests.

1.2. Isotopic Fractionation

Isotopic fractionation refers to any process that changes the relative abundances of stable isotopes of an element. Isotopes of an element have the same electron configurations and have the same chemical properties. Isotopes differ in
mass and nuclear spin; consequently, two types of fractionation occur, known as kinetic and thermodynamic isotope effects.

1.2.1. Kinetic Isotope Effect

Kinetic isotope effects (KIE) are a result of differences in bond strength (e.g. vibration energy levels of bonds) between heavier isotopes and lighter isotopes. When different isotopes of the same element are involved in a reaction/process, this difference in bond strength can result in different reaction rates for the bond. KIE represent changes in bonding between the ground state and the transition state of a reaction. Statistical models predict that the lighter (lower atomic mass) of two isotopes of an element will form the weaker bond during kinetic isotope processes.

Figure 1.1 illustrates the zero-point energy (ZPE) level for D-D stretching vibration (105.3 kcal/mole) is higher than the zero-point energy of the same stretch for a H-H bond (103.2 kcal/mole). Thus, greater activation energy is required for cleavage of D-D bond. In a fast, incomplete, and unidirectional reaction in which the H-H (D-D) bond breaks, there will be a kinetic isotope effect. KIE can be expressed as the following equation:

\[ KIE = \frac{K_H}{K_D} \]  

(1)

Where \( k_H \) and \( k_D \) are both reaction rate constants for hydrogen and deuterium. Since the H-H bond starts out at a higher energy than the first activation energy of D-D, then \( K_H/K_D \) will be greater than 1.

1.2.2. Thermodynamic Isotope Effect

The thermodynamic isotope effect (TIE) is the second most common isotope
Figure 1.1. Lennard-Jones potential diagram for H\textsubscript{2}. Higher zero-point energies (ZPE) or lower numerical values, result in a particular molecule being less stable and therefore its chemical bonds being more easily broken.*

effect, and relates to the free energy change brought about when one atom in a compound is replaced by its isotope. To set it apart from the KIE, this effect manifests itself in processes where chemical bonds are neither broken nor formed [1]. As the phase changes proceed over time, the phase I will become enriched in the heavy isotope because the lighter isotope reacts more quickly, and the phase II becomes increasingly depleted. Eventually, as the heavy:light ratio increased in the phase I, transfer of the heavy isotope will predominate. Typical examples for the results of TIE can be observed as distillation and any kind of two-phase partitioning (e.g., liquid-liquid extraction) [2].

1.3. Delta Notations & Units of Measurement

IRMS data is usually expressed in conventional delta notation first formally defined in 1950 by Mckinney et. al. [3,4] based on a suggestion of Nier in 1946[5], and reported in permil (per thousand) units deviation from an internationally accepted standard, abbreviated as ‰. The general form of the equation for the case of carbon is expressed as:

\[
\delta^{13}C_{VPDB} = \left[ \frac{R_{SPL} - R_{VPDB}}{R_{VPDB}} \right] \times 1000
\]

(2)

with

\[
R_X = \left[ \begin{array}{c} ^{13}C \\ ^{12}C \end{array} \right]
\]

(3)

Where \(^{13}\text{C}\) and \(^{12}\text{C}\) are the abundances of the respective isotopes in the sample or Vienna PeeDee Belemnite (VPDB), \(R_{VPDB} = 0.0112372 \pm 0.0000090\) [6], and where the latter form \(\delta^{13}C_{VPDB}\) is more convenient for calculations. A positive \(\delta\) value indicates that the sample is higher than the standard and has more of the heavier
isotope compared to the standard, while a negative value indicates that the sample has a lower isotope ratio than the standard. For example, we might find that an unknown sample has a value of -20 ‰, which means that it has a $^{13}\text{C}/^{12}\text{C}$ isotope ratio 2 % lower than the international standard (i.e. VPDB). The precision associated with IRMS measurements is often expressed as SD ($\delta^{13}\text{C}$), the standard deviation (root mean square deviation) expressed in $\delta^{13}\text{C}$ units.

Besides the $\delta$ notations, fraction (F), abundance in units of atom percent (AP), and atom percent excess (APE) are also used as the units of measurement for stable isotopes in tracer studies. A detailed discussion of units of stable isotopes is available [7,8].

1.4. Isotopic Standards

A list of international standards for the lighter isotopes in the biosphere is shown in Table 1.1. For the five principal lighter elements of biological interest, there are four accepted isotopic standards. These are standard mean ocean water (VSMOW) for hydrogen and oxygen, VPDB for carbon and oxygen, atmospheric air for nitrogen, and the Troillite (FeS) from the Canyon Diablo meteorite (CDT) for sulfur. Since the original standards SMOW and PDB are exhausted and no longer available, Vienna SMOW (VSMOW) and Vienna PDB (VPDB) are available from the international Atomic Energy Agency (IAEA) in Vienna, which have isotopic compositions nearly identical to those of the original SMOW and PDB. This table also includes information about the abundance ratio measured for each standard. The $\delta$ value of all international standards is by definition 0 ‰. These standards can be obtained from either the IAEA or the National Institute of Standards and Technology (NIST) in the United States.

A single international standard is normally adequate for isotope ratio
Table 1.1. The isotope abundance ratios measured and their internationally accepted reference standards.

<table>
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<tr>
<th>Isotope</th>
<th>Ratio measured</th>
<th>Standard</th>
<th>Abundance ratio of reference standard</th>
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<tr>
<td>$^2\text{H}$</td>
<td>$^2\text{H}/^3\text{H}$ (D/H)</td>
<td>V-SMOW$^2$</td>
<td>$1.5575 \times 10^4$</td>
</tr>
<tr>
<td>$^{12}\text{C}$</td>
<td>$^{12}\text{C}/^{13}\text{C}$</td>
<td>V-PDB$^3$</td>
<td>$1.1237 \times 10^2$</td>
</tr>
<tr>
<td>$^{15}\text{N}$</td>
<td>$^{15}\text{N}/^{14}\text{N}$</td>
<td>N$_2$-atm.$^4$</td>
<td>$3.677 \times 10^3$</td>
</tr>
<tr>
<td>$^{18}\text{O}$</td>
<td>$^{18}\text{O}/^{16}\text{O}$</td>
<td>V-SMOW</td>
<td>$2.0052 \times 10^3$</td>
</tr>
<tr>
<td>$^{34}\text{S}$</td>
<td>$^{34}\text{S}/^{32}\text{S}$</td>
<td>CDT$^5$</td>
<td>$4.5005 \times 10^2$</td>
</tr>
</tbody>
</table>

the hydrogen stable isotope with mass two ($^2\text{H}$); $^2$ the original standard, ‘standard Mean Ocean Water’ (SMOW), is no longer available, but ‘Vienna’- SMOW’ is available from the IAEA; $^3$ the original standard, Belemnite from the PeeDee formation (PDB), is not available, but ‘Vienna’-PDB’ is available from the IAEA; $^4$ atm. = atmospheric gas; $^5$ the standard still used is Troillite (FeS) from the ‘Canyon Diablo’ meteorite (CDT).

*Taken from T. E. Dawson and P. D. Brooks, Stable isotope techniques in the study of Biological processes and functioning of ecosystems, 2001, 1-18.
calibration of naturally occurring C, N, O, S samples. However, hydrogen is unique in its requirement for standards, since the range of naturally occurring D/H concentrations is so large that calibration with a single standard can cause significant errors for samples with isotope ratios being very different from the single standard. Gonfiantini (1978) suggested a normalization of hydrogen and oxygen isotopic data by creating VSMOW-Standard Light Antarctic Precipitation (SLAP) scale [9].

Values are expressed against these international standards when isotope ratios are determined using an IRMS. However, since IAEA or NIST standards are rare and thus not used on a daily basis, all stable isotope facilities routinely use their own internal working standards (WS). A WS is most often unique to that particular analytical laboratory and matched to the particular types of analyses being performed. A WS must always be calibrated against an international standard. In practice, for carbon isotopic analysis, abundances from samples and WS calibrated against VPDB are used to calculate δWS, and the following formula is used to convert δWS to δ13CVPDB:

$$\delta_{\text{VPDB}}^{\text{SPL}} = \delta_{\text{VPDB}}^{\text{WS}} + \delta_{\text{VPDB}}^{\text{SPL}} \frac{\delta_{\text{VPDB}}^{\text{WS}}}{1000}$$

(4)

where superscripts refer to the reference ratio. Ehleringer et. al. presented a very useful brief discussion of international standards in their paper [10].

1.5. High Precision Isotope Ratio Mass Spectrometry

Isotope ratio mass spectrometry (IRMS) measures isotope abundances at high precision, which is defined as a standard deviation in the range of 4-6 significant figures. For isotope ratio measurement, the analyte must be converted into a simple gas, isotopically representative of the original sample, before entering the ion source of an IRMS. Continuous flow isotope ratio measurements of 13C/12C, 2H/1H, 15N/14N,
Figure 1.2. Diagram of a generic isotope ratio mass spectrometer, not including inlets.*

NO, O₂, CO, SO₂ or SF₆. Figure 1.2 shows the major components of IRMS: ion 
\(^{18}\text{O}/^{16}\text{O}\), and \(^{34}\text{S}/^{32}\text{S}\) are performed by analysis of the pure gases CO₂, H₂, N₂, N₂O, 
source, single magnetic sector and multiple collectors.

1.5.1. Ion Source

A “tight” electron impact (EI) ion source, which means to contain analyte 
molecules until they achieve maximum ionization probability, is employed in IRMS. The IRMS EI source is more like an organic chemical ionization (CI) source, which largely depends on collisions to facilitate proton exchange [11]. A 70-eV electron beam, produced by ion source and collimated by small source magnets, travels a tight helical path to increase the EI ionization efficiency of gas molecules. As a result, about 1 ion per \(10^3\) molecules that enter the source are produced by the tight source, which is a lot more efficient than that of about 1 ion per \(10^6\) molecules for the conventional open organic ion sources used for structural chemical identification.

The ion source is usually held at high potential (typically 70-100V) to accelerate the emitted electrons from the filament through the ion source. 20-50% of the electrons are collected by a trap electrode [11].

1.5.2. Analyzer

The extracted and accelerated ions from the ion source are collimated into a beam by electrostatic lenses, and deflected electrostatically in a circular path in the magnet field (about 0.75 tesla). An entrance slit of a fixed width prior to the magnetic sector results in a working resolution of about \(m/\Delta m=100\), which is sufficient for the mass separation of interest [7]. A single magnetic sector or sometimes a permanent magnet with requirement of mass adjustment to be made by adjusting the accelerating voltage is used in IRMS since it can produce flat-topped
peaks. The two most important features of IRMS mass analysis are stability and transmission, which are optimized by sacrificing mass resolution. To avoid the collision between ions and background gas molecules, which cause the ions be knocked off its path and ended up in the wrong collector, the analyzer pressure is always kept low by a high-vacuum system.

1.5.3. Detection and Data Acquisition

IRMS employ faraday cups (FC) for ion detection, while the majority of organic MS instruments employ electron multipliers (EM) to achieve the same purpose [7], except for the trap-type instruments such as Ion Cyclotron Resonance and RF ion traps [14]. Even though FCs are generally less sensitive than EMs, they are superior for IRMS for several reasons: (1) the high count rates required for high-precision place IRMS signals in the normal detection range for FCs, but would rapidly damage EMs; (2) FCs are very stable; (3) FCs linearly detect very large ion currents without gain adjustments; (4) They have very long lifetime, especially for the low atomic weight elements used in IRMS. Each individual cup, for each ion beam of interest, is used to maximize dwell times and eliminate the need for peak-jumping. To dramatically improve the precision, simultaneous measurement of the ion beam of different masses is used to eliminate variability in analytical conditions that cause correlated changes in signal intensity such as filament brightness. The cups are deep and narrow metal tubes, equipped with secondary electron suppressers positioned at the entrance of each collector, which prevent the analyte ions and secondary electrons emitted upon impact of the incoming high-energy ions from escaping out of the metal surface. Dedicated amplification of electronics by mounting circuits on a vacuum flange in close physical proximity to the detector is required for each mass channel. The feedback amplifiers are usually highly shielded, held under vacuum, and are made
with high performance resistors to produce a voltage high enough to be measured conveniently. For example, the feedback resistors for C, m/z 44, 45, and 46 detectors are in the normal ratio of 3:300:1000. The amplified ion beams are eventually digitized using high-linearity voltage to frequency converters. Carbon isotope data from a single GC peak are shown in Figure 1.3. Three traces, shown in Figure 1.3(B), representing masses 44, 45, and 46, are recorded as the analyte CO₂ passes through the ion source. The ratio of m/z 45 to 44 is plotted and shown in Figure 1.3(A) to illustrate the commonly observed phenomenon that the 45 peak is observed to elute prior to the 44 peak, yielding a “positive-negative going” ratio trace. This reversal is thought to be due to the heavier isotopic species of a compound eluting more rapidly from the high-resolution capillary column than the light species, which is known as the “inverse” isotope effect [13-15]. These traces are used to generate integrated areas by summation or curve-fitting algorithms.

1.6. Continuous-Flow Inlets

Continuous-flow MS was first demonstrated the feasibility for isotopic analysis in 1976 by Sano et al. and coworkers [16]. The principle of his design is to transport the separated analytes by a carrier gas into an online microchemistry stage to convert it to an admissible gas to the MS. This was achieved by interfacing a GC to an organic MS via a combustion furnace to convert the $^{13}$C-labeled analytes to CO₂ while setting the MS for peak switching between masses 44 and 45, thus measuring the carbon isotope ratios. In 1978, Matthews and Hayes reported a GC-combustion-MS system based on a single collector MS [13]. The combustion products were dried up with a semi permeable membrane, Nafion™, which remove water and send CO₂ to the MS. The system achieved a good precision that was enough to detect some samples of natural variability. In 1982, Markey and Abramson set up a
Figure 1.3. (A) Ratio trace of m/z 45 to 44 are analyzed in the IRMS. (B) Traces of the m/z 44, 45, 46 mass channels are analyzed as a GC peak in the IRMS. The signals are of comparable size, because the amplifications are set to match natural abundance. Peaks are integrated and areas are used to calculate isotope ratios.
chemical reaction interface (CRI) MS similar to on-line microchemistry approach [15]. This approach is based on the processing of a GC effluent in a microwave cavity to yield small molecules for isotopic analysis at low precision. In general, a carrier gas to transport the analyte through a stage of on-line chemistry for conversion to an acceptable form by the MS is required for all these systems, which is one of the fundamental principles of the CF-IRMS.

1.6.1. Bulk Samples: Elemental Analyzer

A great demand exists for the rapid analysis of large numbers of solid samples as a result of the wide use of stable isotopes for bulk analysis in different field [7]. Preston and Owens reported the first CF interface to a multi-(dual)-collector IRMS for the bulk analysis of \( \text{N}_2 \) from solid samples in 1983 [17]. This system was the first high-precision multicollector CF-IRMS. Elemental analyzers provide an automated form for on-line isotope ratio measurement of solid and nonvolatile liquid samples. Samples are placed in a tin or silver capsule, and loaded into a carousel, then combusted by a heated reactor that contains an oxidant, such as CuO to produce excess \( \text{O}_2 \) for C and S analysis. Combusted products are transported by He gas through a reduction furnace for removal of excess oxygen and conversion of nitrous oxides into \( \text{N}_2 \). During this process, any excess water produced by combustion step is removed by a drying tube. A GC is employed to separate the gas-phase products, which are detected by thermal conductivity before introduction to the IRMS. The sample sizes in the mg range are often analyzed with the elemental analyzer for all the elements.

In recent instrument work, low sample size analysis has been an important issue. In 1996, Fry et. al. introduced an elemental analyzer-IRMS system, which is based on conventional cryogenic techniques for collecting samples rather than
chromatographic separation, for measuring C, N and S isotopic compositions of gas samples in the 10-1000 nmol range [18]. In this system shown in Figure 1.5, samples are injected or dropped into a quartz tube that is partially filled with reduced copper and held at a temperature of 600-1000°C with a continuous He flow. If samples need to be combusted, O₂ can be added directly. The emerged gas samples after reaction are transported by high flow-rates of He through a series of traps for purification and collection of the target analyte, and then released into the IRMS after the flow rate is lowered and the trap is heated with a hot water bath. The actual isotopic measurements are made relative to a tank of gas standards introduced into the IRMS from tanks connected to the sample processing line.

1.6.2. Compound Specific Isotope Analysis

Fundamentals of the modern GCC-IRMS for high-precision compound specific isotope analysis (CSIA) was introduced in 1978 by Matthews and Hayes [13] and in 1984 by Barrie et al. [14], and commercial instruments has been available since about 1990. A schematic diagram of the instrument for carbon analysis is shown in Figure 1.6. The GC is a standard capillary system with split/splitless or on-column injectors, which is interfaced to a switchable valve that directs the column flow to waste while the solvent peaks elute, or to the combustion furnace when analyte elutes. It is necessary to divert solvent peaks away from the furnace, because solvent would deplete O₂ in the furnace. In the waste mode, He with a small concentration of O₂ for reagent recharge flows through the furnace. The combustion reactor loaded with Cu, Ni and Pt wires, charged with O₂, and held at about 950°C, converts organics to CO₂ and H₂O. CuO is a source of O₂ via

\[
2\text{CuO (s)} \xrightarrow{950°C} 2\text{Cu (s)} + \text{O}_2 (g)
\] (5)
Figure 1.4. Diagram of the Cryoflow system for cryofocusing of analyte gases derived from an elemental analyzer. Furnace temperature, He carrier flows, and trap conditions are adjusted for optimal analysis of CO$_2$, N$_2$, or SO$_2$. *

Figure 1.5. Schematic diagram of a gas chromatography combustion-IRMS system. The GC effluent is combusted, dried, and admitted to the IRMS instrument, where the signal from the C isotopes is continuously monitored.
The water must be removed by a Nafion™ tube or a cryogenic trap, prior to admission to the ion source because it protonates CO₂ to produce HCO₂⁺, which interferes with analysis at m/z 45. A Nafion-type trap consists of a length of the fluorinated polymer tube attached to a capillary at its entrance and exit. The analyte stream passes into the tube, where water passes through the walls and is swept away with counterflowing He, while CO₂ is quantitatively retained. This type of water trap is often used in our GCC-IRMS system because it offers the advantage of operating continuously at room temperature without attention. The capillary after the water trap is finally directed to an open split for admission of purified CO₂ to the IRMS.

One of the commercial data analysis algorithms in routine use for CSIA/CF data of carbon has been reported by Ricci et al. in 1994 [19], which is known as the “summation” method. This procedure works well near natural abundance, but is less effective at very high enrichments. At the same year, Goodman and Brenna investigated the effects of incomplete chromatographic resolution on the calculation of isotope ratios by the summation method and by curve-fitting, using several functions designed for chromatographic applications [20]. The results showed that curve-fitting with a common chromatographic function, the exponentially modified Gaussian, recovers the accurate isotope ratio without sacrificing precision, up to a 40% overlap. Further work on curve-fitting [21] showed that precision could be preserved at very low signal levels with curve-fitting for well-resolved peaks, as illustrated in Figure 1.7. Curve-fitting produces considerable better accuracy and precision than conventional summation. In 2001, Sacks et al. in our lab reported a new software, SAXICAB [22], which incorporates curve-fitting method into IRMS data reduction process. We have been using this software for isotope ratio data analysis since that time.
Figure 1.6. Results of a study of curve-fitting on well-resolved, low signal-to-noise peaks. Curve-fitting (open symbols) produces considerable better accuracy and precision than conventional summation (closed symbols). *

1.6.3. Position-Specific Isotope Analysis

Measurement of intramolecular isotope ratios for detection of natural variability is referred to as position-specific isotope analysis (PSIA). Unlike CSIA, PSIA requires the off-line or on-line fragmentation of the parent molecule, followed by IRMS analysis for isotopic characterization for the fragments. Few publications exist in the PSIA field because of the cumbersome chemical degradation and separation steps that are required prior to IRMS analysis. Off-line carbon (C-)PSIA studies relied upon chemical degradation, isolation, and combustion of the fragments to CO₂, followed by isotopic analysis using IRMS [23,24]. Although these reports yielded important insights into biological processes, these methods are tedious and require large sample sizes. Furthermore, collecting volatile fragments is cumbersome, making off-line PSIA of small molecules difficult.

Until 1997, Corso and Brenna [25] introduced a strategy for on-line PSIA, based on (1) pyrolytic fragmentation of analyte molecules, (2) measurement of fragment isotope ratios, (3) calculation of isotope ratios for specific positions. They showed that isotope ratio of a position or moiety in the parent compound can be calculated from the measurements of the appropriate fragments. Precision of measurements reported in this case was below 1 ‰. The on-line PSIA system is designed of coupling of an on-line pyrolysis (Py) furnace to a GCC-IRMS system following an initial GC separation step, as shown in Figure 1.8. Pyrolytic fragmentation can usually be generated throughout the molecule, including along the carbon backbone, which increases the number of sites accessible to PSIA, while online collection and separation of fragments improves throughput and reduces the possibility of contamination. C-PSIA by (GC)-Py-GCC-IRMS systems of fatty acid methyl esters [25], alkanes [26], fatty alcohols [27], and short chain organic acids [30, 31] have been reported by Brenna group and others. Since pyrolysis is non-
Figure 1.7. Diagram of a PSIA instrument that permits injection of a complex mixture into GC I, selection of a single peak into the pyrolysis furnace, separation of fragments in GC II, and analysis either of isotope ratio via combustion IRMS or structural analysis to identify fragments in an ion trap. *


identical standard [30].
quantitative, the fragmentation step is a potential source of isotopic fractionation. In 2003, Gavin and Brenna demonstrated that pyrolysis-induced fractionation can be taken into account reporting Intramolecular isotope ratios relative to a chemically identical standard [32].

1.7. Anabolic Androgenic Steroids

Steroids are lipid compounds with a perhydro-1,2 cyclopentanophenanthrene ring system, which can be classified into six groups according to the number of C-atoms. C_{17} gonanes; C_{18} oestranes, as estradiol and estrone; C_{19} androstanes, as testosterone and androstenedione; C_{21} pregnanaes, as progesterone and cortisol; C_{24} cholane, as the bile acids cholic acid and desoxycholic acid; and C_{27} cholestanes, as cholesterol. According to their function, steroid hormones can be divided into estrogens, androgens, glucocorticoids and mineralocorticoids [31].

Testosterone (T) is the principal anabolic androgenic steroid (AAS) and is produced in males mainly in the testis. In females, smaller amounts of T are produced by the ovary and the adrenal gland. T was first discovered in 1935 by David et. al. [34], who isolated it from the testis of bulls but did not identify its structure. Structures elucidation by synthesis was performed in the same year independently by Butenandt and Hanisch [33] and Ruzicka and Wettstein [34]. For this work, Butenandt and Ruzicka were awarded the Nobel Prize in 1939. Interest in T, which possesses anabolic and androgenic properties, is based on its ability to stimulate anabolic activities. In medical treatment the use of T improves recovery from catabolic states [34].

The first synthetic anabolic steroids were methyltestosterone, mestanolone, and methandriol, all synthesized by Ruzicka et. al. in 1935 [35]. The importance of the therapeutic use of AAS in treatment of catabolic conditions was recognized in the
1950s, after which an enormous number of steroids were synthesized and tested for potency. For example, metandienone [36,37] and stanozolol [38,39], two of the most frequently misused AAS, were synthesized in 1955 and 1950, respectively.

The metabolism of T can be discussed as a basic metabolic pathway for all synthetic AAS since the enzymes that convert T to its metabolites are also active towards AAS when similar groups and configurations are present. Figure 1.8 shows the metabolic pathway of cholesterol. Cholesterol is the precursor for the steroids that are monitored in athletic drug testing and is a freely available compound in the United States on the internet or in nutritional supplements with other pro-hormones. The main excreted testosterone metabolites androsterone, $5\alpha$A, etiocholanolone, and $5\beta$A are detected in routine urine samples in doping tests. Doping tests rely exclusively on urine samples, which can be collected non-invasively in sufficient amounts and provide longer detectability of many substances than blood. Also, drug and metabolite levels are much higher in urine than in blood. However, urinary T measurement is not trivial. Multistep procedures are necessary to achieve a high degree of sensitivity and specificity in the detection. Nowadays, most of the laboratories involved in athletic drug testing programs are using procedures based upon solid-phase extraction of the urine sample, enzymatic hydrolysis of glucuronides, followed by the liquid-liquid extraction at basic pH and trimethylsilyl or acetylation derivatization prior to the GC-MS or GCC-IRMS analysis [40,41].

1.8. Drug testing in Sports

Testing for drugs used to enhance performance were first carried out at the Olympic Games held in Mexico in 1968, when Australia’s Ron Clarke became the first athletes to be tested. Doping at the Olympic Games is banned for two very good reasons: (1) the use of drugs is cheating; (2) drugs have adverse effects on the health
Figure 1.8. Simplified metabolic pathways of pregnenolone. *

of athletes. The International Olympic Committee (IOC) has maintained a list of proscribed substances and methods for their detection since 1967, and this list is updated each year to form the basis for determining banned substances in all sports.

In 1974, The IOC banned the use of synthetic AAS by athletes. This prohibition was adopted by all international sports federations, and also encouraged drug testing laboratories to develop methods for the detection of misused anabolic steroids. The first methods, based on radioimmunoassay techniques [42,43], failed to take into account the high extent of metabolism of AAS. For detecting and identifying AAS metabolites, GC-MS is the current method of choice[40,44,45]. Several MS techniques have been used in doping test: quadruple low resolution full-scan and selected ion monitoring (SIM), ion-trap, magnetic high-resolution and tandem MS with electron impact (EI) and chemical ionization (CI). To normalize the varying T concentrations in subjects, Donike et. al. [46] in 1983 developed a method which used the ratio of urinary excreted T to EpiT as an indicator for T administration. In 1984, the use of T was also banned by the IOC, which defined 6/1 ratio as upper limit of T/E ratio and recently lowered to 4/1. However, pharmaceutical or exogenous T presents special challenges as it is not possible to distinguish it from natural endogenous testosterone by GC-MS.

In 1994, the use of IRMS after GC separation was proposed by Becchi et. al. [49] to distinguish external sources of T from endogenously increased concentrations or T/E ratios by the $^{13}\text{C}/^{12}\text{C}$ ratio. This GCC-IRMS technique overcomes problems that emerge due to physiological variations in the $\delta^{13}\text{C}$ of endogenous steroids. The $^{13}\text{C}/^{12}\text{C}$ of natural human T is a function of the aggregate sum of the $^{13}\text{C}/^{12}\text{C}$ of the plants and animals humans consume, and any additional effects of biological processing. Synthetic T is prepared from one plant source with an unusually low $^{13}\text{C}/^{12}\text{C}$. These slight differences can be detected through IRMS [47-53].
Figure 1.9. Some typical examples of natural $\delta^{13}C$ values grouped according to origin along the scale of $^{13}C$ natural abundance. *

It has long been known that isotope fractionation of carbon formed by photosynthesizing plants varies radically depending on the pathway of photosynthesis in which the species is engaging [54]. The principle reason for this is that carbon fixed by photosynthesis of CO₂, assimilating enzymes ribulosebiphosphate (RuBP) carboxylase (C₃ plants) and phosphoenolpyruvate (PEP) carboxylase (C₄ and Crassulacean Acid Metabolism (CAM) plants) via different pathways, which could cause varying degree of mass discrimination [55-60]. All living matter is created through photosynthesizing CO₂ in atmosphere (~ 7 ‰) or in marine environment (~0 ‰) as shown in Figure 1.9. In the United States, maize (corn) is extensively used in human diet, which CO₂ fixation results in the formation of a C₄ body. The average δ¹³C/¹²C values obtained from C₄ plant (e.g. wheat, barley, oat, grape or sugar beet) material are between -16 to -10 ‰, which is much higher than C₃ plant (e.g. yams, soy, corn, sorghum, millet or sugar cane), from -32 to -24 ‰ [60]. The synthetic T is made from phytosterol precursors derived from yams and soy, which are both warm-climate C₃ plants, thus leading to the different isotope ratios from C₄ plants. Consequently a comparison of the δ¹³C of T metabolites and steroids from an independent pathway can indicate the administration of synthetic steroids. A difference of 3 ‰ on the delta scale has been written down as a threshold level in sports legislation [61]. Androgens, corresponding pro-hormones or metabolites thereby represent the targets. Any endogenous steroid from an independent metabolic pathway may serve as a reference in contrast.

1.9. Summary

Compound-specific isotope analysis by IRMS following on-line combustion of compounds separated by GC is a relatively young analytical method. Due to its ability to measure isotope distribution at natural abundance level with great accuracy
and high precision, GCC-IRMS has increasingly become the method of choice in authenticity control of food, biomedicine and nutrition, and determination of origin in archaeology, geochemistry, and environmental chemistry. In the recent years, the fight against sports doping has been intensified. Therefore, GCC-IRMS is becoming a unique and powerful tool for detection of doping. This thesis offers an overview of the instrument calibration method using newly created steroid isotopic standards for GCC-IRMS in the field of anti-doping test, in chapter two. Thereafter, it presents the instrumental development of the fast GCC-IRMS and the comprehensive two dimensional GC (GC × GC) C-IRMS for application to natural mixtures such as urine, enabling efficient separation of complex mixtures in Chapter 3 and 4. A native steroid GC × GC chromatography with TOF-MS was explored in Chapter 5 as a prelude to IRMS for detection and identification of unknowns or designer drugs in a complex urine matrix. Finally, conclusions and future works for carbon isotope ratio analysis of steroids are presented in its last Chapter.
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CHAPTER TWO

Steroid Isotopic Standards for Gas Chromatography-Combustion Isotope Ratio Mass Spectrometry (GCC-IRMS)

2.1. INTRODUCTION

Anabolic androgenic steroid (AAS) use was prohibited by the International Olympic Committee (IOC) in 1976, and since various approaches to monitor compliance were developed [1]. Urinary testosterone (T) concentration is an unsuitable metric because of the large concentration range of inter-individual urinary steroid excretion, eliminating the possibility that T doping could be detected on the basis of concentration [2]. In 1983, Donike et al [3] proposed the testosterone/epitestosterone (T/E) excretion ratio measured by benchtop gas chromatography-mass spectrometry (GC-MS) for this purpose. This test was first employed in Olympic doping control during the 1984 Los Angeles Olympic Games [4]. Currently, a T/E ratio greater than 4:1 is used as a screen for exogenous T administration. There are several issues with the use of T/E alone to confirm T use. Normal inter-individual variations related to urinary steroid excretion gives a T/E ratio > 4:1 in some cases. Tests of endocrine function are necessary to rule out high T/E ratios due to T secreting tumors or other hormonal disorders [2]. Also, simultaneous T doping with exogenous E as a masking agent can prevent a high urinary T/E ratio [5]. Finally, a major caveat of GC-MS is that it does not establish whether urinary T in any particular sample is of exogenous origin.

High precision gas isotope ratio mass spectrometry (IRMS) is the classical technique for determination of isotope fractionation due to natural processes for C, H,

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1 Based on the reference of Steroids 2009, 74, 369-378.
High precision compound specific isotope analysis (CSIA) was introduced in 1978 by Matthews and Hayes [8] using previously established instrumental principles [9], and instrumentation has been available commercially since about 1990. Briefly, the effluent of a gas chromatography (GC) column is directed to a combustion (C) interface, and the CO$_2$ thus from analyte combustion is in turn directed to an IRMS for continuous isotope ratio monitoring. Modern GCC-IRMS instruments calibrate isotopic signals using isotopically calibrated CO$_2$ admitted to the IRMS from a separate, external volume.

Becchi et al [10] first proposed synthetic testosterone detection by CSIA using high precision GCC-IRMS in 1994, now known as the Carbon Isotope Ratio (CIR) test. The method exploits the natural difference in $^{13}$C/$^{12}$C between C3 and C4 plants the mix of which determines the $^{13}$C/$^{12}$C of biomolecules excreted in urine. The $^{13}$C/$^{12}$C of a steroid on a parallel metabolic pathway but unaffected T or E doping, known as an endogenous reference compound (ERC), is compared with the $^{13}$C/$^{12}$C of T or one of its metabolites [11-13]. Synthetic T produced commercially entirely from the C3 plant soy has a significantly lower $^{13}$C/$^{12}$C than the ERC which arises metabolically from mixed C3 and C4 precursors.

The $^{13}$C/$^{12}$C values detected by IRMS are expressed in terms of relative parts per thousand compared to an international standard reference material (VPDB), $\delta^{13}C_{VPDB} = \{(R_S-R_{VPDB})/R_S\} \times 1000$, where $R_S$ is the $^{13}$C/$^{12}$C of a steroid, and $R_{VPDB} = 0.0112372 \pm 0.0000090$. An isotope ratio difference of 3 ‰ has been used as a threshold level in sports legislation [14]. Although CIR analysis of urinary steroids using GCC-IRMS is in wide use worldwide for detection of T doping [2], GCC-IRMS continues to be a specialty technique that requires careful procedures for standardization. In 2003, a United States Antidoping Agency (USADA) research symposium recommended development of steroid isotopic internal standards to
harmonize reported values and achieve more uniform results [15]. Ideally, these would be isotopically calibrated steroids that could be analyzed under GC conditions identical to sample steroids, and their GC retention time and isotope ratios would bracket the range of interest.

Here we report a protocol for creation of isotopically uniform steroid standards for calibration of GCC-IRMS data between the antidoping labs. This procedure adapted from Caimi et. al. [16] ensures uniform stable isotopic composition among a prepared set of containers. The steroids were selected based on their isotopic composition, metabolic role, and GC retention times. Isotopic values are calibrated against a natural gas reference mixture to insure that the standard traversed the same analytical path as the steroids to be calibrated. We introduce a procedure for isotopic calibration of GC peaks that uses an external CO$_2$ tank but is immune to differential fractionation that may occur in the flow path between the volume containing the standard CO$_2$ gas and the IRMS, compared to the flow path between the GC and the IRMS.

2.2. EXPERIMENTAL

Chemicals and Standards. High purity He, N$_2$, O$_2$, and CO$_2$ were purchased from Airgas East (Salem, NH). The steroid acetate isotopic standard coded CU/USADA 33-1 was made with the following components: 5α-androstan-3β-ol acetate (5αA-AC), 5α-androstan-3α-ol-17-one acetate (androsterone acetate, A-AC), 5β-androstan-3α-ol-11, 17-dione acetate (11-ketoetiocholanolone acetate, 11k-AC) and 5α-cholestane (Cne). A second isotopic standard coded CU/USADA 34-1 was made with native (underivatized) steroids with the following components: 5β-androstan-3α-ol-17-one (etiocholanolone, E), 5α-androstan-3α-ol-17-one (androsterone, A), and 5β-pregnane-3α, 20α-diol (5βP). All steroids were 99%
purity, and were purchased from Steraloids (Newport, RI) with the exception of 5βP which was purchased from Acros Organics USA (Morris Plains, NJ). The steroid isotopic standards (SIS) were created and prepared as described in the sections below. The natural gas reference material NIST RM 8559 (coal origin, >80% methane) was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD).

Creation of Steroid Isotopic Standards (SIS). All glass containers were used to prevent any possible contamination due to solvent extraction of polymeric materials associated with plastic containers. The glass containers were thoroughly solvent washed and dried before use. For CU/USADA 33-1, 60 mg of each of the four steroids were dissolved into a single volume of 300 mL 2-propanol. For CU/USADA 34-1, 100 mg of each of the three steroids was dissolved into separate volumes of 20 mL 2-propanol. All steroids were allowed to dissolve completely overnight at room temperature in capped containers. For CU/USADA 34-1, a 1.5 mL aliquot of each pure steroid solution (~7.5 mg) was taken for elemental analyzer IRMS (EA-IRMS), while the remainder of the solutions was pooled to create a master solution containing all the steroids for the respective standards, and then diluted into 2-propanol to a total volume of 500 mL. For both CU/USADA 33-1 and CU/USADA 34-1, 2 mL aliquots of the master solution were dispensed into 2 mL amber glass ampoules (Fisher Scientific Inc.). High purity N₂ was used to evaporate solvent at 80°C, leaving crystallized standard in the container. The ampoules were then N₂-flushed and flame-sealed for safe and convenient storage and shipment. For each standard, approximately 100 ampoules were made.
Steroid Isotopic Standard (SIS) Preparation for GCC-IRMS Analysis. Randomly selected ampoules for each SIS were prepared for GCC-IRMS analysis. Each amber glass ampoule was carefully cracked open at the score at the neck, filled with 2 mL of 2-propanol and allowed to sit over 3 hours to fully dissolve steroid components. After full dissolution, the SIS solution was then removed from the ampoule into a clean working glass container. Before analysis with GCC-IRMS, the SIS was diluted to approximately 100 ng/µL in the case of CU/USADA 33-1 and 94 ng/µL in the case of CU/USADA 34-1. A 1 µL sample was analyzed in each GCC-IRMS run.

GCC-IRMS Instrumental Setup. An HP 5890 or 6890 GC with a split/splitless inlet (Agilent Technologies, Palo Alto, CA) and autoinjector were coupled to a Thermo MAT 253 IRMS (Bremen, Germany) via a home-built combustion interface. The IRMS was tuned for high linearity. The IRMS was operated at a source pressure of $2.3 \times 10^{-6}$ Torr, at 9.5 kV with a measured sensitivity of 850 molecules/ion. Data were collected from the IRMS and analyzed using ISODAT 2.5. The GC column was connected to an online micro-combustion reactor via a four-way rotary valve which permitted solvent diversion. The micro-combustion reactor was constructed with a 30 cm $\times$ 0.5 mm i.d. alumina tube hand-packed with three 20 cm $\times$ 0.1 mm wires (i.e. 1 Cu, 1 Pt, and 1 Ni wire). The tube was maintained at 950°C using a 30 cm Thermcraft tube furnace (Winston Salem, NC). Water generated due to combustion was removed from the system using a Nafion® water trap (dimensions = 10 cm $\times$ 0.8 mm i.d.) upstream of the IRMS. An open-split consisted of a 1 m $\times$ 0.075 mm IRMS inlet sampling capillary that was directly inserted into the post-water trap transfer line. The same combustion interface design was used for all measurements.
For CU/USADA 33-1 analysis, a 30 m × 0.25 mm × 0.25 μm Varian VF-5ms column (5% phenyl, 95% dimethyl polysiloxane) was used. The GC conditions were: head pressure at 21 psi in constant pressure mode, inlet at 280 °C, splitless injection, total flow at 25.4 mL/min, and purge flow at 10 mL/min. The oven parameters were: 80 °C (initial, hold 1 min) ramped to 270 °C (30 °C/min, hold 12 min), and ramped to 300 °C (10 °C/min hold 4.67 min).

For CU/USADA 34-1 analysis, a 30 m × 0.25 mm × 0.25 μm J & W Scientific DB-17ms (50% phenyl, 50% dimethyl polysiloxane) was used. The GC conditions were same as those for CU/USADA 33-1 analysis, except that the head pressure was 20 psi in constant pressure mode. The oven parameters were: 70 °C (initial, hold 1 min) ramped to 270 °C (30 °C/min, hold 11 min), and ramped to 300 °C (10 °C/min hold 3 min).

**GCC-IRMS Calibration Apparatus and Procedure.** A system for sampling the natural gas reference material, NIST RM8559 (natural gas, coal origin, >80% methane), without fractionation was constructed. A schematic is shown in Figure 1, where circles labeled as A, B, and C were Nupro(R) stainless steel shut off valves (Swagelok Company, Solon, OH) and circles labeled D and E were 9500 Series brass shut off valves (Circle Seal Controls Inc, Corona, CA). The RM 8559 was procured from NIST as a 50 ml stainless steel cylinder with valve A attached, containing 800 psig gas. This was secured to the system using Swagelok fittings in the position as shown in Figure 2.1. A septum sealed the end of the 25 ml sampling volume by an HP split/splitless inlet cap. All fittings and volumes in the system were stainless steel or brass. The system was thoroughly leak checked.

Operation was as follows. All volumes other than the 50 ml NIST RM 8559 cylinder were evacuated using a 1.3 ft³/min roughing pump (Edwards 1.5,
Figure 2.1. Schematic of apparatus for sampling of RM 8559 from a pressurized cylinder into a syringe for injection into a GC. A series of valves connecting fixed volumes enables expansion of gas from high to low pressure while minimizing isotopic fractionation. See text for operational details.
Wilmington, MA) by opening valves B, C, D, and E with valve A closed. After the vacuum stabilized below 20 mtorr for 15 min, all valves were closed. An aliquot of RM 8559 was expanded into a 7.5 ml volume by opening valve A. After equilibration over 10 min to minimize any isotopic fractionation, valve A was closed. Next, the aliquot of RM 8559 was further expanded into a 150 ml volume by opening valve C and equilibrated over 10 min, after which valve C was closed. Finally, RM 8559 was expanded into a 25 ml sampling volume by opening valve D and equilibrated over 10 min, after which valve D was closed. At this point, the 25 ml sampling volume was around 2 atm.

Approximately 2 μL, 30 μL, and 60 μL volumes of RM8559 gas from were sampled from the septum sampling port through the split/splitless inlet cap and septum. The needle of a gas tight syringe was inserted into the 25 ml sampling volume, the plunger drawn back, and the gas equilibrated for 30 seconds. The syringe was removed and the gas was injected into the GCC-IRMS system. A fresh aliquot of RM 8559 gas was expanded from the 150 ml volume into the 25 ml sampling volume each day the RM 8559 analysis was run or when the septum was changed.

Calibration of isotope ratios requires that isotopic fractionation during measurements be minimized, and particularly that all possible sources of fractionation be equivalent between samples and standards. Since fractionation can be subtle and difficult to characterize, this is best accomplished by using equivalent steps, including flow paths for sample and standard, as is embodied in the classic dual inlet [6, 17]. However, in GCC-IRMS the use of standards contained within a sample mixture is unreliable because of the possibility of unsuspected co-eluting materials and the need for a highly stable baseline. All commercial GCC-IRMS instruments perform calibration with CO₂ gas from an external volume [18]. Figure 2.2 illustrates the calibration traceability of our measurements for CU/USADA 33-1 and CU/USADA
Figure 2.2. Isotopic calibration procedure. NIST RM 8559 is injected directly into the GC. Methane and ethane are separated, combusted, and isotope signals determined and set to the reference values. In the same analysis, CO$_2$ from a pressurized tank is admitted as gas pulses through the Conflo III interface to the IRMS before and after the methane and ethane. Software is set to calibrate the CO$_2$ isotope ratio against the reference values for methane and ethane, thereby isotopically calibrating an apparent isotope ratio for the CO$_2$. Analytes, steroids in this case, are RM8559, methane and ethane, have values of $\delta^{13}$C$_{VPDB} = -29.11\%$ and $\delta^{13}$C$_{VPDB} = -25.9\%$[19], respectively, and were used to calibrate the CO$_2$ gas pulses admitted into calibrated against the apparent isotope ratio of the tank CO$_2$. The back calibration takes into account any differential fractionation associated with the alternative flow path. Steroid standard isotope ratios are thus traceable back to VPDB.
Two of the major components in the RM8559, methane and ethane, have values of $\delta^{13}C_{VPDB} = -29.11\%$ and $\delta^{13}C_{VPDB} = -25.9\%$ [19], respectively, and were used to calibrate the CO$_2$ gas pulses admitted into the IRMS from a high volume pressurized CO$_2$ tank. Three CO$_2$ gas pulses are admitted at the beginning and three pulses are admitted towards the end of each GCC-IRMS run for the SIS and the RM 8559. The CO$_2$ gas was admitted from a pressurized tank via a Conflo III interface (Thermo Finnigan). The value measured for the CO$_2$ is referred to as an “apparent” $\delta^{13}C_{VPDB}$ since it does not transverse the same flow path as the GC analytes. The apparent $\delta^{13}C_{VPDB}$ assigned to the CO$_2$ is then used to subsequently calibrate CU/USADA 33-1 and CU/USADA 34-1 peaks. With this scheme, any differential fractionation attributable to the different flow paths cancels out. Steroid calibration is traceable to the international standard Vienna Pee Dee Belemnite (VPDB, $R_{VPDB} = 0.0112372 \pm 0.0000090$). All $\delta^{13}C$ values reported here are with respect to VPDB ($\delta^{13}C_{VPDB}$ or $\delta_{VPDB}^{13}C$); for economy of space we use “$\delta^{13}C$”.

**CO$_2$ Isotopic Calibration Using NIST RM 8559.** A 30 m × 0.32 mm GS-GasPro column (Agilent Technologies, Palo Alto, CA) was used. The GC conditions were: head pressure at 12 psi in constant pressure mode, inlet at 200°C, split injection at 50:1, and purge flow at 10 mL/min. The oven parameters were: 25°C (initial, hold 4 min) and ramped to 80°C (10°C/min, hold 1 min). A 100 μL gas tight syringe (Hamilton) was used for manual injections of gas on each GCC-IRMS setup used for CU/USADA 33-1 and CU/USADA 34-1 analysis.

**EA-IRMS analysis.** Four replicates of each steroid (1.25 mg each replicate) was analyzed using a Carlo Erba NC 2500 elemental analyzer (Thermo Finnigan, Bremen, Germany) interfaced to a Delta Plus IRMS (Thermo Finnigan, Bremen,
Germany). Data were collected from the IRMS and analyzed using ISODAT 2.0. Analysis was performed in the Cornell University Stable Isotope Laboratory (COIL). A COIL in-house rice standard biannually calibrated against calcite reference material IAEA CO-1 and carbonate reference material IAEA CO-8 was used for calibration, and a chemical standard, methionine, was used to verify instrument linearity.

2.3. RESULTS AND DISCUSSION

Carbon Isotope Ratio Analyses. The GCC-IRMS chromatograms of SIS CU/USADA 33-1 and 34-1 are shown in Figure 2.3. They demonstrate baseline resolution of the components in each mixture. The contents of eight randomly selected ampoules containing the SIS mixture CU/USADA 33-1 were analyzed by GCC-IRMS, with four replicate injections analyzed for each ampoule. Two sets of data were acquired identically on two separate days. The absolute $\delta^{13}C$ data from all the measurements are presented in Table 2.1, with no outliers eliminated. The absolute $\delta^{13}C$ measured for the four steroids ranged from -33.05‰ to -16.65‰ with an average standard deviation SD ($\delta^{13}C$) = 0.11‰ within ampoules and 0.08‰ between ampoules. The $\Delta\delta^{13}C$ values between 11-keto-AC and the other three steroids, as shown in Table 2.2, ranged from 8.03‰ to 16.37‰ with an average SD($\delta^{13}C$) = 0.15‰ within ampoules and 0.09‰ between ampoules. Typical SD ($\delta^{13}C$) for well resolved peaks is less than 0.20‰, thus the magnitude of these SD($\delta^{13}C$) indicates uniformity of steroid isotopic composition within measurement reproducibility among the ampoule set.

The contents of eight randomly selected ampoules containing the SIS mixture CU/USADA 34-1 were analyzed by GCC-IRMS, with four replicate injections analyzed for each ampoule. Full sets of data were acquired identically on two separate days. For this SIS, 16 replicates of the master solution were also analyzed.
Figure 2.3. GCC-IRMS chromatograms of (A) the steroid isotopic standard CU/USADA 33-1, and (B) the steroid isotopic standard CU/USADA 34-1.
Table 2.1. CU/USADA 33-1 results.

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Table 2.2. CU/USADA 33-1 results reported as $\Delta^{13}C$, calculated as the difference between the $^{13}C$ of 11k-AC and 5α-A-AC, A-AC, and Cne. See caption to Table 1 for details.

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using GCC-IRMS on a separate day. The absolute $\delta^{13}C$ data from all the measurements are presented in Table 2.3, with no outliers eliminated. The absolute $\delta^{13}C$ measured for the three steroids ranged from -26.92‰ to -31.53‰ with an average SD($\delta^{13}C$) = 0.10‰ within ampoules and master solution, and 0.07‰ between ampoules and master solution. The $\Delta\delta^{13}C$ values between 5βP and the other two steroids, as shown in Table 2.4, ranged from 2.47‰ to 4.64‰ with an average SD($\delta^{13}C$) = 0.12‰ within ampoules and master solution, and 0.09‰ between ampoules and master solution. This indicates uniformity of steroid isotopic composition within measurement reproducibility among the master solution and ampoule set.

The $\delta^{13}C$ and $\Delta\delta^{13}C$ values for CU/USADA 34-1 components determined by EA-IRMS for the individual steroid master solutions are compared to that determined by GCC-IRMS for the master solution and eight randomly selected ampoules contents, and are presented in Table 2.5. The results show that the absolute $\delta^{13}C$ and $\Delta\delta^{13}C$ measured by EA-IRMS for the three steroids deviate by <0.2‰ on average from those measured with GCC-IRMS analysis. The results also confirm that there is no significant isotopic fractionation between the master solution and the ampoule contents.

**Effects on Carbon Isotope Ratio Measurements for CU/USADA 33-1 and CU/USADA 34-1.** JMP 7.0 (SAS Institute, Cary, NC), software for statistical analysis, was used to determine if there were any systematic effects on the carbon isotope ratio measurements by GCC-IRMS. Using a least squares fit categories for ampoule and day, an analysis of variance confirmed that there was a minor but statistically significant (p<0.05) ampoule effect on the absolute $\delta^{13}C$ for three of the seven total steroids in the two standards, as shown in Table 2.6. This effect
Eight randomly selected ampoules and the master solution were analyzed. The data sets were acquired identically on three separate days.

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Table 2.4. CU/USADA 34-1 results reported as $\Delta^{13}C$, calculated as the difference between the $^{13}C$ of 5$\beta$P and E and A. See caption to Table 3 for details.

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<td>4.45 0.09 0.09</td>
<td>4.45 0.09 0.09</td>
</tr>
<tr>
<td>5</td>
<td>4.42 0.19 0.19</td>
<td>4.42 0.19 0.19</td>
</tr>
<tr>
<td>6</td>
<td>4.40 0.04 0.04</td>
<td>4.40 0.04 0.04</td>
</tr>
<tr>
<td>7</td>
<td>4.45 0.09 0.09</td>
<td>4.45 0.09 0.09</td>
</tr>
<tr>
<td>8</td>
<td>4.42 0.19 0.19</td>
<td>4.42 0.19 0.19</td>
</tr>
<tr>
<td>1-8 8</td>
<td>4.34 0.09 0.06</td>
<td>4.34 0.09 0.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ampoule Data Set 2 (94 ng each on column)</th>
<th>$\delta^{13}C(E) - \delta^{13}C(5\beta P)$</th>
<th>$\delta^{13}C(A) - \delta^{13}C(5\beta P)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampoule n</td>
<td>$\delta^{13}C$ SD 95% CL</td>
<td>$\delta^{13}C$ SD 95% CL</td>
</tr>
<tr>
<td>1</td>
<td>4.57 0.11 0.10</td>
<td>4.57 0.11 0.10</td>
</tr>
<tr>
<td>2</td>
<td>4.45 0.11 0.10</td>
<td>4.45 0.11 0.10</td>
</tr>
<tr>
<td>3</td>
<td>4.48 0.13 0.13</td>
<td>4.48 0.13 0.13</td>
</tr>
<tr>
<td>4</td>
<td>4.71 0.21 0.21</td>
<td>4.71 0.21 0.21</td>
</tr>
<tr>
<td>5</td>
<td>4.44 0.07 0.07</td>
<td>4.44 0.07 0.07</td>
</tr>
<tr>
<td>6</td>
<td>4.45 0.07 0.07</td>
<td>4.45 0.07 0.07</td>
</tr>
<tr>
<td>7</td>
<td>4.51 0.13 0.13</td>
<td>4.51 0.13 0.13</td>
</tr>
<tr>
<td>8</td>
<td>4.53 0.06 0.06</td>
<td>4.53 0.06 0.06</td>
</tr>
<tr>
<td>1-8 8</td>
<td>4.52 0.09 0.06</td>
<td>4.52 0.09 0.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Master Solution Data Set (94 ng each on column)</th>
<th>$\delta^{13}C$ SD 95% CL</th>
<th>$\delta^{13}C$ SD 95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master Sol'n 16</td>
<td>4.64 0.13 0.06</td>
<td>4.64 0.13 0.06</td>
</tr>
</tbody>
</table>

48
Table 2.5. Comparison of the measured δ^{13}C values, and their respective standard deviations (SD) and 95% confidence limits (95% CL) for the steroids in CU/USADA 34-1 by GCC-IRMS and EA-IRMS.

<table>
<thead>
<tr>
<th>Technique</th>
<th>n</th>
<th>δ^{13}C</th>
<th>SD</th>
<th>95% CL</th>
<th>δ^{13}C</th>
<th>SD</th>
<th>95% CL</th>
<th>δ^{13}C</th>
<th>SD</th>
<th>95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA</td>
<td>4</td>
<td>-28.91</td>
<td>0.02</td>
<td>0.02</td>
<td>-27.06</td>
<td>0.04</td>
<td>0.04</td>
<td>-31.42</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>GCC-IRMS (master sol’n)</td>
<td>16</td>
<td>-28.82</td>
<td>0.13</td>
<td>0.06</td>
<td>-26.98</td>
<td>0.12</td>
<td>0.06</td>
<td>-31.62</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>GCC-IRMS (ampoules)</td>
<td>2’</td>
<td>-28.90</td>
<td>0.09</td>
<td>0.05</td>
<td>-27.06</td>
<td>0.17</td>
<td>0.08</td>
<td>-31.48</td>
<td>0.08</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Average of measurements made on two separate days (8 ampoules per day, n=4 per ampoule).
disappeared for all but one steroid (11k-AC – A-AC) when $\Delta \delta^{13}C$ were calculated, and in this case the statistical significance dropped from $p = 0.0079$ to $p = 0.02$, suggesting that instrument drift was the cause. Even so, the magnitude of the deviations is well within the acceptable SD of GCC-IRMS, $\text{SD}(\delta^{13}C) < 0.2\%$, and the statistical significance of the results is in part driven by the excellent precision and large data set. Overall, the results confirm the uniformity of the steroid isotope ratios from ampoule to ampoule, as well as the robustness of our steroid isotopic standards creation protocol.

Table 2.6 also shows that the analysis of variance identified a statistically significant day effect on the $\delta^{13}C$ for the steroids, except for A-AC in CU/USADA33-1, and statistical significance did not disappear for the $\Delta \delta^{13}C_{VPDB}$. The origin of the effect is unclear but may also be traceable to day-to-day drift. In our calibration procedure, the reference CO$_2$ gas isotope ratio measurement does not include a combustion step, and therefore it does not account for short term changes in combustion reactor and water trap efficiencies, and other issues elsewhere in the GC system and combustion interface that may lead to isotopic fractionation. Thus the day effect may ultimately be traceable to one or more of these components.

However, even though a day effect is statistically observed in some of the data from this study, the magnitude of that effect is still small when considering the measurement variability. The average deviation of absolute isotope ratios of the four steroids in CU/USADA 33-1 was $< 0.1\%$ between the two different days, and the average deviation of absolute isotope ratios of the three steroids in CU/USADA 34-1 was $< 0.17\%$ among the three different days, as shown in Table 2.7. In addition, the $\Delta \delta^{13}C$ values of the steroids in CU/USADA 33-1 was $< 0.05\%$ between the two different days; while, the $\Delta \delta^{13}C$ values of the three steroids in CU/USADA 34-1 was $< 0.18\%$ on three different days, also shown in Table 2.7. We conclude that the day
Table 2.6. Analysis of variance of the $\delta^{13}$C values measured for the components in CU/USADA 33-1 and CU/USADA 34-1 based on effect from ampoule and day.

<table>
<thead>
<tr>
<th></th>
<th>CU/USADA 33-1</th>
<th>CU/USADA 34-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$p_{\text{Ampoule}}$</td>
<td></td>
</tr>
<tr>
<td>$\delta^{13}$C</td>
<td>5$\alpha$A-AC</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>A-AC</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>11k-AC</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Cne</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>5$\beta$P</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$\Delta \delta^{13}$C (vs 11k-AC or 5$\beta$P)</td>
<td>0.26</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>$p_{\text{Day}}$</td>
<td></td>
</tr>
<tr>
<td>$\delta^{13}$C</td>
<td>5$\alpha$A-AC</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>A-AC</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>11k-AC</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>Cne</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$\Delta \delta^{13}$C (vs 11k-AC or 5$\beta$P)</td>
<td>0.0032</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Table 2.7. Deviation of $\delta^{13}C$ and $\Delta\delta^{13}C$ measured for the components in CU/USADA 33-1 on two different days for two ampoule data sets and CU/USADA 34-1 on three different days for two ampoule data sets and master solution from the average.

<table>
<thead>
<tr>
<th>SIS</th>
<th>$\delta^{13}C$</th>
<th>$\delta^{13}C$</th>
<th>$\Delta\delta^{13}C$</th>
<th>$\Delta\delta^{13}C$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CU/USADA 33-1</td>
<td>CU/USADA 34-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>5α-A-AC</td>
<td>A-AC</td>
<td>11k-AC</td>
<td>Cne</td>
</tr>
<tr>
<td>Dev(Ampoule Set 1)</td>
<td>-0.10</td>
<td>-0.02</td>
<td>-0.04</td>
<td>-0.09</td>
</tr>
<tr>
<td>Dev(Ampoule Set 2)</td>
<td>0.10</td>
<td>0.01</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Dev(master sol'n)</td>
<td>0.05</td>
<td>-0.09</td>
<td>-0.05</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SIS</th>
<th>$\Delta\delta^{13}C$</th>
<th>$\Delta\delta^{13}C$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CU/USADA 33-1</td>
<td>CU/USADA 34-1</td>
</tr>
<tr>
<td>Dev(Ampoule Set 1)</td>
<td>0.05</td>
<td>-0.03</td>
</tr>
<tr>
<td>Dev(Ampoule Set 2)</td>
<td>-0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Dev(master sol'n)</td>
<td>0.14</td>
<td>0.14</td>
</tr>
</tbody>
</table>
Table 2.8. Final calibrated values of CU/USADA 33-1 and 34-1 based on the means and standard deviations of determinations of 8 ampoules as presented in Tables 2.1 and 2.3.

<table>
<thead>
<tr>
<th></th>
<th>5α-A-AC</th>
<th>A-AC</th>
<th>11k-AC</th>
<th>Cne</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU/USADA 33-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-30.61±0.14</td>
<td>-33.04±0.02</td>
<td>-16.70±0.06</td>
<td>-24.77±0.13</td>
</tr>
<tr>
<td>CU/USADA 34.1</td>
<td>E</td>
<td>A</td>
<td>5βP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-28.91±0.09</td>
<td>-27.06±0.19</td>
<td>-31.49±0.06</td>
<td></td>
</tr>
</tbody>
</table>
effect is well within acceptable bounds defined by the typical precision of the measurement.

**Use of SIS for isotope calibration.** Table 2.8 summarizes the final, best \( \delta^{13}C_{VPDB} \) figures calculated as a mean of the two single day determination means in Tables 2.1 and 2.3. SD (\( \delta^{13}C \)) for the two standards average 0.09‰ and 0.12‰ for the two standards. CU/USADA 33-1 \( \delta^{13}C \) values from -33.04 to -16.70 bracket the range normally encountered in antidoping applications. CU/USADA 34-1 \( \delta^{13}C \) values bracket a much smaller range closer to the values known for C3 carbon.

A suitable procedure for using these standards is similar to the manner in which they were calibrated themselves. At the beginning of the day, SIS isotope ratios can be used to assign an apparent isotope ratio for an external CO2 volume. The isotope ratio for CO2 can then be used to calibrate unknowns, again avoiding all hypothetical sources of bias in flow paths.

**2.4. CONCLUSIONS**

In summary, a protocol is demonstrated here for the creation of uniform steroid isotopic standards for use in calibration of GCC-IRMS and harmonization of results from the carbon isotopic analysis for the detection of synthetic steroid use in sport doping. The protocol used to prepare the steroid isotopic standards in ampoule containers maintains isotopic integrity to uniformity within 0.09‰ from ampoule to ampoule. A limited quantity of the standards reported here is available for distribution to other interested laboratories.
REFERENCES

CHAPTER THREE

Fast Gas Chromatography/Combustion Isotope Ratio Mass Spectrometry

3.1. INTRODUCTION

The utility of gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS) for high precision compound-specific isotope analysis (CSIA) is well documented in the recent literature [1-3]. In comparison to Elemental Analysis (EA)-IRMS and Dual Inlet (DI)-IRMS, GCC-IRMS requires lower levels of analyte (nanomoles of C vs. micromoles) and provides the convenience of on-line isolation of components [3]. Commercial GCC-IRMS systems for $^{13}$C/$^{12}$C analyses first became available in the late 1980s, and the next decade saw several papers investigating fundamental analytical questions such as the effects of instrumental stability [4], standard introduction strategies [5-7], chromatography parameters [8], data processing algorithms [9-11], and the combustion interface [12-14] on precision and accuracy. In recent years, GCC-IRMS for carbon isotope analyses has matured and the majority of literature has focused on applications rather than fundamental aspects [1, 15]. The transition of GCC-IRMS from specialized to routine brings with it the demands inherent to other routine analyses, e.g. faster run times, higher throughput, and lower costs. Most GCC-IRMS analyses are performed with conventional capillary GC columns (0.25-0.32 mm i.d.), which require run times of 20-40 minutes. Narrow bore fast GC columns (0.10-0.20 mm i.d. columns) routinely achieve run times <10 min when coupled to FID or time-of-flight (TOF) MS detection, but there is little in the literature to suggest whether coupling fast GC to

---

IRMS is appropriate or feasible.

The shorter run times associated with fast GC are due to the smaller total internal volumes of the columns which result in short hold-up times and permit higher linear velocities and faster ramp rates [16]. Fast GC typically generates peak widths of 200-1000 ms (FWHM) in comparison to >1s wide peaks associated with conventional GC [17, 18]. In addition, fast GC can yield improved sensitivity due to the diminished band broadening of the eluting peak; a fast GC peak will be narrower and higher than conventional GC peaks, which results in a proportional improvement in sensitivity when used with detectors such as an FID.

While the coupling of fast GC to IRMS could offer considerable time savings, the concept presents several analytical challenges. Accurate determination of isotope ratios in GCC-IRMS demands that components be baseline resolved [19], which means that the separation of the narrow, fast GC peaks must be preserved post-column. For any fast GC application, the physicochemical nature and the mechanical design of the detector must be amenable to narrow peaks and not introduce broadening or distortion, and a sufficiently fast data logger must be employed [20]. In the case of GCC-IRMS, it is appropriate to consider the entire post-GC interface (combustion, water trap, transfer lines, open split, IRMS) as the detector. The physicochemical mechanism of GCC-IRMS detection (combustion and electron impact-mass spectrometry (EI-MS) should be appropriate for fast GC peaks. The gas-phase combustion process occurs on the millisecond time scale and has proven appropriate for fast GC detection in the form of the FID[20], and EI-MS is appropriate for peaks <50 ms wide (FWHM) for non-scanning detectors, e.g. TOF-MS [18].

A greater concern is that the design of a typical post-GC interface has an internal volume approaching that of a fast GC column, and thus may cause significant extra-column broadening. Most commercial interfaces are similar to that described by
Werner and colleagues [14] based on earlier work by Merritt, et.al. [13], and use a 0.5 mm i.d. ceramic tube for combustion and a backflush system to divert the solvent. Goodman compared this approach to a continuous capillary design that used a rotary valve to divert the solvent [12], using GC-FID and GCC-IRMS chromatograms of alkanes to evaluate peak broadening. Careful evaluation of those data shows that a 1200 ms (FWHM) chromatographic peak detected by FID was broadened to 1450 ms when measured by continuous capillary GCC-IRMS and to 1800 ms when measured by the commercial system. Assuming that the additional components resulted in Gaussian broadening, thus the variances describing the interaction of the various components are additive, we calculated that the minimum attainable peak widths for the continuous capillary and conventional interfaces are ~800 and ~1340 ms, respectively. Both of these approaches would result in noticeable broadening for fast GC peaks (FWHM < 1s). There are no studies addressing which components in each system are responsible for the observed broadening, and it is not clear if further improvements are feasible.

A secondary concern associated with coupling fast GC to IRMS is that the sample capacity of narrow bore columns is lower than conventional columns. Column capacity is proportional to the column cross-sectional area and the film thickness. Narrow bore typically use thin films (df = 0.10-0.15 μm) such that column capacity for most 0.10 mm i.d. columns is around 10 ng per compound, 30-fold less than comparable 0.32 mm columns. High precision isotope ratio measurements are well-known to have much higher sample size demands than GC-MS; a typical benchmark is 1 nmol of C on column to achieve a precision of SD (δ¹³C)=0.2‰. Therefore, compromises must be made in the choice of fast GC column to ensure that there is adequate capacity for the analyte.

We report the construction of a high precision fast GCC-IRMS interface from
conventional GC components of reduced diameter, optimized for retention of fast GC peak widths and shapes. We evaluate minimal peak widths possible with the interface and test the system with mixtures of fatty acid methyl esters (FAME) and underivatized steroids. Finally, we investigate the ability of the system to achieve high precision at lower sample sizes compared to conventional interfaces.

3.2. EXPERIMENTAL

Chemicals and Standard Mixtures. High purity and UHP He, UHP N₂, air and CO₂ (99.9%) were purchased from Airgas East (Salem, NH). The FAME mixture was a commercial standard “68A” (Nu-Chek-Prep; Elysian, MN) containing 19 FAME (16:0 at 10% by weight; 14:0, 14:1, 16:1, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 20:4, 22:0, 22:1, 22:6, 24:0, 24:1 at 5% by weight). To this was added 17:0 and 23:0 FAME (Nu-Chek-Prep; Elysian, MN), and the standard was prepared in heptane to yield approximate final concentrations of: 17:0, 23:0 = 6000 ng/μL, 16:0 = 600 ng/μL, all other FAME = 300ng/μL.

For the steroid analyses, a mixture of arbitrary steroids with a range of retention times was made, with the following abbreviations: 3β-hydroxy-5α-androstane, OH-Andro; 19-noretiochonanolone, 19-NA; Etiocholandiol (5β-androstan-3β, 17β-diol), Etio; Dehydroepiandrosterone, DHEA; Androsterone, Andro; 11-ketoetiocholanolone, 11-keto; 5β-pregnane-3α, 20α-diol, 5βP; 5α-Cholestane, Chln; 5β-pregnane-3α, 17, 20α-triol, Preg; Cholesterol, Ch-OH. All steroids were at 99% purity, and were purchased from Steraloids (Newport, RI) with the exception of 5βP which was purchased from Acros Organics USA (Morris Plains, NJ). The standard mixture was prepared in 2-propanol (Mallinckrodt Baker) with 100 ng/μL of each component.

Conventional GCC-IRMS system. The conventional GCC-IRMS system
has been described in detail previously [21]. Briefly, an HP 5890 GC with split/splitless injector and a Varian 8200 autoinjector was coupled via a combustion furnace to a Finnigan MAT 252 (Bremen, Germany) run in high linearity mode. The GC effluent entered a 4-way rotary valve which permitted The effluent from the capillary column was connected via Valco (Houtson, TX) stainless steel ZDV fittings to a 30 cm × 0.5 mm i.d. alumina combustion tube hand-packed with a double strand of 20 cm × 0.1 mm Cu wire. The combustion tube was resistively heated to 920 °C by a 30 cm Thermcraft furnace (Winston Salem, NC), and periodically recharged with oxygen via the auxiliary line of the rotary valve. Following combustion, the effluent was dried in a Nafion® water trap (dimensions = 10 cm × 0.8 mm i.d.). The dried effluent was admitted to the IRMS through a 2 m × 0.1 mm 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. The IRMS was operated at a source pressure of 1×10^{-6} Torr and had an absolute sensitivity of ~3000 molecules/ion as estimated from CO_2 injections. All connections were made with Press-Tight® connectors (Restek; Belafonte, PA).

**Fast GCC-IRMS System.** The optimized fast GCC-IRMS system also used the FMAT 252 IRMS and was similar to the conventional system with the following modifications. (1) The GC was a Varian 3800 with programmable temperature vaporization (PTV) injector; (2) No post-column rotary valve was employed; (3) The combustion tube was a 40 cm × 0.25 mm i.d. FS tubing packed. A single strand of 20 cm × 0.1 mm i.d. Cu wire was positioned in the center of the combustion tubing. The combustion tubing was inserted into a 30 cm × 0.5 mm i.d. ceramic tube and connected on either side for mechanical stabilization, as described by Goodman[12]. (4) The post furnace transfer line was 2 m × 0.15 mm; (5) The Nafion water trap was
replaced with a cold trap (dry ice in acetone) with the cooled zone of the transfer line ~30 cm in length; (6) Oxygen recharging of the combustion furnace required the physical removal of the furnace capillary to GC effluent Press-Tight connection, and attachment of the furnace capillary to an oxygen flow.

Data Collection and Processing. Data was collected from the FMAT 252 using SAXICAB [22], a home-built LabVIEW-based [23] data acquisition system. Three 24-bit National Instruments (Austin, TX) NI4351 digitizers were used to simultaneously monitor the m/z = 44, 45, and 46 cups after the head amplifiers with >99% duty cycle. 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 25 Hz. For both fast and conventional GC-FID experiments, FID data was collected by a NI4351 digitizer at 60 Hz, and processed by SAXICAB.

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[24]. In our CO₂ analyses, 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 3, 30, and 100 ms, respectively. Noticeable broadening for 200-1000 ms fast GC peaks would be a problem only for the 46 cup. To avoid this, the 46 cup feedback resistor was replaced with a 30 GΩ resistor. All data sets were processed using SAXICAB using the individual summation method, adapted from Ricci et al. [11]. The data point before and after the peak start were used to define the “beginning background” and “end background” data points, and a straight line was drawn between the two points to define the background.

A minor modification to the usual data reduction algorithm was necessary in order to correctly account for the time shift between the ⁴⁴CO₂ and ⁴⁵CO₂ peaks. In
conventional GC, the difference in retention times between the two peaks, $\Delta t_R$, is determined by least-square parabolic fit of points around the peak maxima. The integrated 44 area is then corrected by addition of the term, $\Delta t_R \left( ^{44}I_{n+1} - ^{44}I_1 \right)$, where $^{44}I_n$ is the signal intensity at time point n, and the peak is integrated from $^{44}I_1$ to $^{44}I_n$. This approach relies on a linear interpolation of the signal on either side of the peak, and also requires $\Delta t_R$ be less than the bin size (i.e. (sampling rate)$^{-1}$). In our fast GC work, the time between points was 40 ms, and the typical time shift was 40-80 ms (1-2 data points). Time correction using the Ricci approach resulted in poor precision and accuracy. Instead, we shifted the integration and background windows on the 45 trace by $\Delta t_R$. In the typical case where $\Delta t_R$ was fractional, the signal intensity on the 45 trace at a fractional data point was determined by linear interpolation between the adjacent data points.

High precision isotope ratios are expressed in the delta ($\delta^{13}C$, ‰) notation. $\delta^{13}C$ of the CO$_2$ injections were calculated using pulses of standard CO$_2$ gas that had been indirectly calibrated to the PDB reference via isotopically calibrated condensed phase FAME standards[5]. The contribution of $^{17}O$ to the $^{45}$CO$_2$ signal was corrected for by the method of Santrock, et.al. [25]. No outliers were excluded from the reported data.

**Precision as a Function of Peak Area for Varying Peak Widths.** Peak widths of 250, 2500, and 7500 ms on the fast GCC-IRMS system were generated by using FS capillaries of differing dimensions, 2 m × 0.1 mm, 30 m × 0.32 mm, and 30 m × 0.53 mm, respectively, between the injector and reactor. The flow rate measured before the open split was adjusted to ~1 mL/min for the 250 and 2500 ms peaks, and ~0.5 mL/min for the 7500 ms peak. CO$_2$ injections were performed by hand, and the split ratio (20:1-500:1) and injection size (0.5-50 μL) were varied to yield peak areas
between 0.01 and 80 nC. Between 50 and 100 injections were performed for each peak width.

**Comparison of FAME Analyses by Conventional and Fast GCC-IRMS.**

The FAME standard was prepared from the commercial 68A standard and the 17:0 and 23:0 FAME in heptane to yield approximate final concentrations of: 17:0, 23:0 = 6000 ng/μL, 16:0 = 600 ng/μL, all other FAME = 300 ng/μL. Injections were 1μL, split 15:1.

For conventional GCC-IRMS, a 30 m × 0.32 mm × 0.25 μm BPX-70 (70% cyanopropyl) column was used (SGE; Australia). The oven program was 50 °C (initial, hold 2 min) to 170 °C (50 °C/min, no hold) to 250 °C (4 °C/min, hold 4 min). The injector was held at 250 °C. The FAME mixtures were injected split at 15:1. The GC was operated in constant pressure mode, and the head pressure was adjusted such that the flow rate at 200 °C was 1 mL/min.

For fast GCC-IRMS and fast GC-FID of FAMEs, a 15 m × 0.15 mm × 0.15 μm Varian VF-23ms (70% cyanopropyl) was used. The oven parameters were: 50 °C (initial, hold 3 min) to 150 °C (80 °C/min, no hold) to 230 °C (30 °C/min, hold 2 min). The PTV injector parameters were 50 °C (initial, hold 0.5 min) to 250 °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 reduced to 15:1 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. At the end of the run, the PTV injector was cooled by a CO₂ jet that was turned on and off by an electronically actuated valve. The GC was operated in constant flow mode (1 mL/min). Because of the multiple changes in column diameter after the GC, we found it necessary to input column dimensions that would achieve a particular flow rate as measured at the post-GC interface outlet, rather
than the actual column dimensions. Also, the Varian 8200 autosampler syringe (SGE; Australia) leaked at column head pressures over ~30 psi. Therefore, we performed injections with the head pressure at 25 psi, and ramped the head pressure immediately after the injection.

**Comparison of Underivatized Steroid Analyses by Conventional and Fast GCC-IRMS.** A steroid standard mixture was prepared with 100 ng/μL per component in 2-propanol. One microliter of the mixture was injected on column for both conventional and fast analyses.

For conventional GCC-IRMS, a Varian 25 m × 0.25 mm × 0.25 μm VF-5ms (5% phenyl) column was used. The oven program was 80 °C (initial, no hold) to 265 °C (70 °C/min, hold 4 min) to 330 °C (2 °C/min). The injector was held at 310 °C. Injections were performed splitless, and the purge was turned at 0.89 min. The GC was operated in constant pressure mode, and the head pressure was adjusted such that the flow rate at 80 °C was 1.1 mL/min.

For fast GCC-IRMS and fast GC-FID of the underivatized steroids, a 20 m × 0.15 mm × 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), with similar considerations for the column pressure as for fast GCC-IRMS of FAMEs.
3.3. RESULTS AND DISCUSSION:

Minimizing Band Broadening Effects in Combustion Interface. The components that interface the GC to the IRMS are (a) solvent diversion, (b) combustion reactor, (c) transfer lines, (d) water trap, and (e) open split. Other than considerations associated with the single capillary interface, there is no extant literature on the postcolumn band broadening effects of the GCC-IRMS interface effects.

The components used in three interface designs are summarized in Table 3.1: A conventional commercial design, the continuous capillary design, and finally a fast GCC-IRMS interface developed in the present work by optimization of all of the listed components. Because many of the interface components have open-tubular capillary geometries, the minimal attainable peak width could in principle be calculated by determining the plate height, \( H \), from the Golay equation for an unretained component: [26]

\[
H = \frac{2D_m}{u} + \frac{r^2}{24D_M} u
\]  

(1)

where \( D_m \) is the diffusion coefficient of the analyte, \( u \) is the linear velocity of the carrier gas, and \( r \) is radius of the capillary. \( H \) can then be substituted into:

\[
w_{1/2} = 2.355 \left( \frac{H}{L} \right)^{1/2} t_0
\]  

(2)

to yield \( w_{1/2} \), the full width half-maximum (FWHM) of the resultant peak, where \( t_0 \) is the hold up time, and \( L \) is the length of the capillary. The initial peak width of the analyte is assumed to be zero. For simple systems in which temperature and band composition is constant, it is possible to calculate the broadening associated with any
Table 3.1. Comparison of GCC-IRMS combustion interface components used in commercial, continuous capillary and fast GCC-IRMS systems. The components used in the fast GCC-IRMS interface are described in this paper, and are capable of 250 ms peak widths (FWHM).

<table>
<thead>
<tr>
<th>Function</th>
<th>Commercial GCC-IRMS</th>
<th>Continuous-capillary</th>
<th>Fast GCC-IRMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent diversion</td>
<td>Backflush system</td>
<td>Rotary valve</td>
<td>PTV injector</td>
</tr>
<tr>
<td>Combustion Reactor</td>
<td>0.5 mm i.d. ceramic tube with oxidized metal (e.g Cu, Ni)</td>
<td>0.32mm i.d. FS packed with 0.1mm (× 2) Cu strand</td>
<td>0.25 mm i.d. FS packed with 0.1mm (×1) Cu strand</td>
</tr>
<tr>
<td>Transfer lines</td>
<td>2 m × 0.32 mm i.d. FS</td>
<td>2m × 0.32mm i.d. FS</td>
<td>2 m × 0.10 mm i.d. FS</td>
</tr>
<tr>
<td>Water trap</td>
<td>Nafion trap</td>
<td>Cold trap</td>
<td>Cold trap</td>
</tr>
<tr>
<td>Open split</td>
<td>Partially closed glass capillary and 1.5 m × 0.1 mm FS tubing to IRMS</td>
<td>2 m× 0.1 mm FS inserted into column effluent</td>
<td>0.7 m × 0.075 mm FS inserted into column effluent</td>
</tr>
<tr>
<td>Minimum Peak Width (FWHM)</td>
<td>&gt; 1s</td>
<td>~0.8 s</td>
<td>0.25 s</td>
</tr>
</tbody>
</table>
interface design from theoretical considerations alone. However, accurate calculations of this nature are difficult because $D_M$ is dependent on many factors, including the system temperature and the analyte [27]: calculating $D_M$ under combustion conditions, where both the chemical nature of the analyte and the temperature are not constant over the length of the reactor, would be nontrivial and require detailed experimental results. Similar difficulties exist for other system components. It was therefore necessary to determine the individual impact of each system component empirically. A summary of the broadening associated with each component is shown in Table 3.2 at a column flow rate of 1 mL/min. We consider features in this table as follows.

**Solvent diversion:** Typical GCC-IRMS injection sizes are 1 μL solvent (~50 μmol C). If the entire solvent load is allowed to enter the combustion furnace, then the oxygen will be rapidly exhausted. In most commercial systems, solvent is diverted by means of a backflush system [13], with T-connectors before and after the combustion furnace. As a simpler alternative, Goodman proposed the use of a 4-way rotary valve to direct the column effluent to either a vent or to the combustion interface and IRMS.

Recently, Flenker proposed another option for solvent diversion that requires no extra connections [28]. The sample is injected onto a cooled programmable temperature vaporization (PTV) injector in split mode to purge the solvent. The split is turned off and the PTV temperature ramped to transfer the analytes to the column. The solvent load is thus vented out the septum purge, and no connections to components prior to the combustion reactor are required. To test the broadening associated with the rotary valve, we performed injections of $CO_2$ into the IRMS via a transfer line or via a transfer line interrupted by a Valco 4-position rotary valve. Peak widths (FWHM) of 242 ms were achievable without the rotary valve, compared
Table 3.2. Summary of effects of post-GC components on minimal attainable peak widths, $\sigma$.  

$^a$ Measured with either FID or IRMS detection, and $\sigma$ was calculated by assuming Gaussian broadening.  

$^b$ The minimal attainable peak width with our fast GCC-IRMS system was $\sim$250 ms. FS = fused silica capillary.

<table>
<thead>
<tr>
<th>Component</th>
<th>Peak Width (ms)$^a$</th>
<th>s, min. FWHM attainable with component (ms)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Furnace (CH$_4$ detected at FID)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No furnace</td>
<td>174</td>
<td>N/A</td>
</tr>
<tr>
<td>+ 30 cm x 0.25 mm FS with 0.1 mm Cu wire (1)</td>
<td>246</td>
<td>174</td>
</tr>
<tr>
<td>+ 30 cm x 0.32 mm FS with 0.1 mm Cu wire (2)</td>
<td>345</td>
<td>298</td>
</tr>
<tr>
<td><strong>Solvent Diversion (CO$_2$ detected at IRMS)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No rotary valve (PTV injector)</td>
<td>242</td>
<td>N/A</td>
</tr>
<tr>
<td>+ Rotary valve</td>
<td>296</td>
<td>159</td>
</tr>
<tr>
<td><strong>Water Trap (CO$_2$ detected at IRMS)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Nafion trap (Cold trap)</td>
<td>242</td>
<td>N/A</td>
</tr>
<tr>
<td>20 cm x 0.8 mm Nafion trap</td>
<td>1650</td>
<td>1630</td>
</tr>
<tr>
<td><strong>Transfer Lines (CH$_4$ detected at FID)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 m x 0.1 mm FS</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>2 m x 0.32 mm FS</td>
<td>760</td>
<td>760</td>
</tr>
<tr>
<td><strong>Open Split</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No open split (CH$_4$ detected at FID)</td>
<td>246</td>
<td>N/A</td>
</tr>
<tr>
<td>Inserted 0.1 m x 0.05 mm FS (CO$_2$ at IRMS)</td>
<td>277</td>
<td>127</td>
</tr>
<tr>
<td>Inserted 2 m x 0.10 mm FS (CO$_2$ at IRMS)</td>
<td>307</td>
<td>183</td>
</tr>
</tbody>
</table>
to 296 ms with the rotary valve in place. Back calculating the broadening associated with the rotary valve using an equation analogous to eq 1, the minimum obtainable peak width with the rotary valve in place is thus 159 ms. Peak broadening from Valco rotors has been observed before in fast GC [29] presumably due to the internal volume of the rotor channels. We did not have access to a commercial backflush system; however, the T-connections are expected to result in noticeable broadening.

The PTV injector approach for diverting solvent is thus advantageous because it adds no extra components. However, there are a number of caveats associated with its use. This approach can only be used for analytes with substantially higher boiling points than the solvent, so that the analyte is quantitatively retained in the cool injector while the solvent is purged. Minor analyte losses are likely to induce an unacceptable degree of isotopic fractionation in the remaining analyte loaded on the column. In addition, in our design, recharging the combustion reactor with O2 is more inconvenient, as the combustion capillary must be physically disconnected from the GC effluent and attached to an auxiliary O2 source.

**Combustion Reactor.** Commercial systems typically employ 20-40 mm long by 0.5 mm or wider i.d. ceramic or quartz tubes loaded with CuO, NiO or ZnO, which are then connected in series to the column effluent [13]. The single capillary design replaces the ceramic tube with a continuous 0.32 mm FS capillary, where the combustion region was loaded with 2 strands of 0.1 mm Cu wire and oxidized in situ. Further improvements in peak width were achieved by using 30 cm × 0.25 mm i.d. FS capillary loaded with a single strand of 20 cm × 0.1 mm Cu wire for the combustion zone. Hand-loading the narrow capillary requires some degree of dexterity, patience, and eyesight. The narrow-bore furnace was more fragile than the 0.32 mm i.d. capillaries typically used in our lab upon heating, but this property was
not investigated rigorously.

The furnace temperature was set at 950 °C, while this temperature has been recommended for quantitative combustion of CH$_4$. It also results in more rapid reduction of the CuO. This was particularly noticeable for the fast GCC-IRMS system, likely because it had only a single strand of Cu and thus lower O$_2$ storage capacity. To prevent depletion of the oxygen source, the furnace was recharged every 24-48 h.

We performed injections of CH$_4$ (detected by FID) with a transfer line connected (a) directly to the FID; (b) via a 30 cm × 0.32 mm i.d. FS with two Cu wire strands; or (c) via a 30 cm × 0.25 mm i.d. FS loaded with one Cu wire strand. The minimal attainable peak width is 174 ms using 0.25 mm i.d. reactor, and 298 ms using a 0.32 mm i.d. reactor, as shown in Table 3.2.

**Water Trap:** Water must be removed from the post-combustion effluent stream to prevent protonation of CO$_2$ in the source and loss of accuracy or precision; this is most commonly achieved through a Nafion or cryogenic trap. We have been successful in quantitatively removing water with 20 cm × 0.8 mm Nafion tube. In our hands, injections of CO$_2$ (detected at the IRMS) showed that addition of a 20 cm × 0.8 mm i.d. Nafion trap results in a minimal peak width of >1600 ms. In contrast, a length of narrow bore capillary inserted into a cryotrap would not require connections or i.d. changes and should optimally retain chromatographic quality. Considerations for improvements are similar to those discussed for transfer lines.

**Transfer Lines and Connectors.** Typically, 1-3 m of 0.32 mm i.d. FS capillary is used for transfer lines between the furnace and the water trap and open split. Two meters of 0.32 mm i.d. FS capillary resulted in peak widths of 760 ms and likely accounts for most of the broadening observed previously with the continuous capillary system. Two meters of 0.1 mm i.d. FS capillary resulted in peak widths of
120 ms, although this number likely reflects broadening associated with the injector, too. Narrower tubing could in principle be used but would require undesirably high back pressures. Because the furnace diameter is larger than the optimal transfer line diameter and the GC column, connections must be used on either side of the furnace. A potential advantage of the continuous capillary system is that there are fewer connections and fewer changes in column diameter, both of which could cause peak broadening. However, in our work, we have observed no measurable broadening from FS press-tight connectors. Matisova, et.al. have reported similar results [30].

**Open Split Design and IRMS.** The open split design described previously [12, 13] employs a narrow diameter piece of FS capillary that acts as a capillary leak into the IRMS. The o.d. of the capillary leak line is narrow enough to permit its insertion directly into the combustion effluent transfer line. The commercial ConFlo [14] system directs the effluent into a partially closed glass tube, with the capillary leak positioned alongside the interface transfer line. This arrangement enables facile mixing, variable dilution, and admission of standard and is advantageous in applications such as EA-IRMS where band broadening is not a serious consideration. However, our measurements indicate that the relatively large mixing volume of the glass tube results in standard pulses requiring 400 ms to reach half-maximal intensity, which would limit GC peaks passing through this interface to FWHM of about 800 ms.

For many years, we have employed an open split consisting of a small capillary that fits inside a large one, jacketed with dry He gas. Although the open split capillaries are typically narrow i.d. (0.10 mm), the low flow rates through the leak (~0.2 mL/min) exacerbate broadening. Therefore, we compared peak widths on the IRMS for two open split capillaries (12.5 cm × 0.05 mm and 2 m × 0.10 mm) which should yield similar leak rates. Minimal attainable peak widths were calculated by comparing these widths to GC-FID were determined to be 127 ms for
the 0.05 mm i.d. leak and 183 ms for the 0.10 mm i.d.. Because the residence time in the 0.05 mm i.d. capillary leak is <75 ms, it seems unlikely that it is singularly responsible for this broadening. More likely, we think the broadening results from the internal volume of the IRMS inlet prior to the source, as the open split flow passes through a changeover valve in our current system. In practice, we found the 0.05 mm i.d. capillary leak too short to be practical, since cutting short lengths of the capillary would significantly change the inlet flow rate. We were able to achieve comparable peak widths with a 60 cm × 0.075 mm open split.

**Minimal Broadening GCC-IRMS Interface and Quantitative Conversion of CH₄.** With all these considerations, our minimal broadening GCC-IRMS was constructed as follows: A PTV injector connected to a 2 m × 0.1 mm FS capillary (w₁/₂ = 124 ms), simulated a column. The capillary was connected to a 30 cm × 0.25 mm furnace (w₁/₂ = 174 ms), and then to a 2 m × 0.1 mm FS capillary (w₁/₂ = 124 ms) with an in-line cold trap. Connections on both sides of the furnace were made by press-tight connection. The 60 cm × 0.075 mm capillary leak was used at the open split as an inlet (w₁/₂ = 127 ms). We observed peak widths as narrow as 250 ms for both CO₂ and CH₄ injections, as shown in Figure 3.1. Taking the square of the summed variances, the predicted peak width was w₁/₂(total) = 277 ms, very close to our observed value. This best case resolution was obtained with the PTV used for solvent diversion; replacing this optimal solution with a rotary valve for solvent diversion degraded peak widths to a minor degree, with the minimal attainable FWHM of < 300 ms. Injections of 1 nmol CO₂ and CH₄ on column resulted in similar peak areas, (13 ± 2 nC) for both gases, indicating that the combustion process was quantitative. Furthermore, the combustion process does not cause noticeable broadening, as the CO₂ and CH₄ peak shapes and widths are quite similar. This is
Figure 3.1. $^{44}$CO$_2$ and $^{45}$CO$_2$ traces of unretained CH$_4$ peak in fully optimized system Fast GCC-IRMS system, showing 250 ms wide peaks (FWHM). Data acquisition was at 25Hz (40 ms per data point). System parameters: 1 m $\times$ 0.10 mm fused silica (FS) column to 30 cm $\times$ 0.25 mm FS furnace (CuO, 950 °C) to 1.5 m $\times$ 0.10 mm FS transfer line with in-line cold-trap (dry ice + acetone). Open split (70 cm $\times$ 0.075 mm FS) was inserted directly into transfer line effluent. Flow rate = 1 mL/min.
not surprising, as combustion of methane occurs on the low millisecond timescale, and FID is known to be compatible with ultrafast GC peaks as narrow as 3 ms [29].

**Effects of Peak Width on GCC-IRMS Accuracy and Precision.** To ascertain that high precision and good accuracy could be achieved with fast GC peaks over a wide dynamic range, we performed injections of a CO\(_2\) standard gas with three columns of different i.d.’s and lengths to generate three different peak widths: 250, 2500, and 7500 ms. The injection size and injector split ratio were varied to generate peak areas of 0.01-80 nC. Taking into account the absolute sensitivity of the instrument under our high linearity mode operating conditions (~3000 molecules/ion), this corresponds to 0.3-2500 pmol C entering the IRMS or 1.5-12500 pmol C on column, considering the open split ratio (0.2:1.0). A potential drawback to fast GCC-IRMS is that the amplifiers are saturated at relatively low signal areas. With the 10V maximum of the FMAT 252 cups, the maximum peak area for the 44 trace that maintains the 45 trace on scale for our fastest GCC-IRMS peaks (250 ms) is ~2 V·s, or 6.6 nC. This limitation can be overcome by replacing the feedback resistors with lower impedance values. Also, newer commercial IRMS systems have Faraday cups that can measure up to 50 V.

\(\Delta\delta^{13}C\) values were normalized with respect to the mean value for all injections, which was taken as \(\delta^{13}C=0\%\). Plots of \(\Delta\delta^{13}C\) vs peak area for the three peak widths are shown in Figure 3.2. At sufficiently high sample levels (>4 nC), the precision converges on 0.2-0.3% for all three peak widths. This limit to minimal achievable precision in isotope ratio measurements has been termed “signal-independent noise” by others [4]. However, at low signal levels, we achieved better precision from the narrow 250 ms peaks than for the wider peaks. This result is more easily appreciated in the histogram plot in Figure 3.2. The counting statistics limit to precision[4], \(\sigma_{\delta}\),
Figure 3.2. At low signal levels, narrow peaks result in improved precision. (left) For CO₂ injections normalized δ^{13}C vs. peak area as a function of peak width (250, 2500, and 7500 ms). (right) Histogram showing precision, SD(∆δ^{13}C), as a function of peak area for the three peak widths. The theoretical limit based on counting statistics is also plotted. For peak areas over 4 nC (~600 pmol C on column), precision was about 0.2‰ for all peak widths. For peak areas < 1 nC, the narrow peaks (250 ms) were within a factor of 3 of the counting statistics limit, much better than the broader peaks.
was also included for each histogram category, given as,

\[ \sigma = \left( \frac{q \cdot 2 \cdot 10^6 (1 + R)^2}{Area \cdot R} \right)^{1/2} \]  

where \( R \) is the natural abundance isotope ratio of C (\( \sim 0.011 \)), \( Area \) is the average peak area of the histogram category (in Coulombs), and \( q \) is the fundamental unit of charge (\( 1.6 \times 10^{-19} \text{C/ion} \)). At peak areas <1 nC, the narrow 250 ms peaks provide superior precision to wider peaks, and are within a factor of 3 or less of the counting statistics limit. For example, for the peak area range of 0.03-0.10 nC (~1-3 pmol C to the IRMS), the counting statistics limit is 0.7‰ and the experimentally measured precisions are 0.9‰, 12.0‰, and 40.4‰ for the 250 ms, 2500 ms, and 7500 ms peaks, respectively. For the peak area range of 0.2-0.5 nC, the counting statistics limit is 0.3‰ and the experimentally measured precisions are 0.4‰, 2.2‰, and 14.6‰ for the same peaks. In general, for low-levels of signal, we observe that the precision scales inversely to the peak width, e.g., the 250 ms peak achieves ~10-fold better precision than the 10-fold wider 2500 ms peak, and ~30-fold better precision than the 30-fold wider 7500 ms peak.

The observation that narrow GCC-IRMS peaks yield better precision than wider peaks at very low signal levels is previously unreported to our knowledge. The current literature on GCC-IRMS precision at sub nmol levels C is sparse. Merritt, et.al. reported precisions within a factor of 2 of the counting statistics limit for serial dilutions of CH\(_4\) down to 10 pmol to the IRMS [4]. This would translate to 1 nC at the reported absolute sensitivity of 700 molecules/ion. In the present work, conventional 2500 ms GC peaks were within a factor of 4 of the counting statistics limit for peak areas 1-8 nC, and < 1 nC marks the transition where we start to observe a steep decrease in precision for conventional GC peaks. The mechanism for
degradation of precision for wider peaks is not entirely clear. This is not likely because of overlapping interferences from column bleed or other sources, because the wide peaks are still accurate even at low signal levels. We also considered whether the loss in precision may be due to less reproducible start and stop times for the broader peaks. However, manually changing the peak start and stop times in the vicinity around peaks did not change precision significantly. Ricci, et.al., observed that while auto-defining peaks results in better precision compared to manual definition, the improvement was modest [11], and would not explain >10-fold differences in precision.

Another possibility is that the loss of precision is due to uncorrelated baseline noise in the 44 and 45 traces. We encountered similar circumstances to this in our investigation of the effects of quantization error (bit noise) on GCC-IRMS precision [31]. We derived the following expression to calculate the error associated with the background, $\sigma_A$, for cases when the noise on the isotopomer traces is uncorrelated:

$$\sigma_A = \frac{W}{2} \sqrt{\sigma_{y1}^2 + \sigma_{y2}^2}$$

(4)

where $\sigma_{y1}$ and $\sigma_{y2}$ are the errors associated with the points selected to define the background, and $W$ is the total integrated peak width between time points $t_1$ and $t_2$. This treatment was designed for cases where the peak areas are calculated by summation and the subtracted background area is a trapezoid defined by the points $[(t1, 0), (t2, 0), (t2, y2), (t1, y1)]$. If the errors for the 44 and 45 traces are uncorrelated, then we can write:

$$^{45}R_{obs} = \frac{A_{45} \pm \sigma_{45}}{A_{44} \pm \sigma_{44}} = ^{45}R_{act} \pm \sigma_{obs}$$

(5)
where,

$$\sigma_{\text{obs}} = 44_{\text{act}} \sqrt{(45_{\text{act}})^2 + (44_{\text{act}})^2}$$  \hspace{1cm} (6)$$

and $A_{44}$, $A_{45}$ and $\sigma_{44}$, $\sigma_{45}$ are the background corrected areas and errors of the 44 and 45 traces, respectively.

The standard error can be rearranged into terms of parts per thousand, which will correlate approximately to per mil at C natural abundance:

$$\sigma_{\text{ppt}} = 1000 \ast \frac{\sigma_{\text{obs}}}{44_{\text{act}}} = 1000 \ast \sqrt{(45_{\text{act}})^2 + (44_{\text{act}})^2}$$  \hspace{1cm} (7)$$

Assuming that the error at the beginning and end of the background definition are equal ($\sigma_{yi} = \sigma_{y2}$), then substitution of eq 4 into eq 7 yields:

$$\sigma_{\text{ppt}} = 1000 \ast \frac{W \sqrt{2}}{2} \sqrt{(y_{45})^2 + (y_{44})^2}$$  \hspace{1cm} (8)$$

where $y_{44}$ and $y_{45}$ are the uncorrelated errors of their respective traces.

Intriguingly, the error associated with background correction is predicted to scale linearly with the integration window ($W$), as we observed in Figure 3.2, as opposed to the square root of the integration as is seen in many other analytical circumstances. This is because any imprecision made in defining the GCC-IRMS peak background is multiplied through the entire length of the integration window, without the signal averaging associated with summing the peak area. While this error will be imperceptible at high signal-to-noise levels, it may dominate for sufficiently wide peaks at lower $S/N$.

In summary, uncorrelated noise between the isotopomer traces in the points
Comparison of FAME Analysis by Fast and Conventional GCC-IRMS. We ran replicates of FAME standards to test the performance of the fast GCC-IRMS system under real conditions. FAME are a common subject of GCC-IRMS analyses, and are typically run on very polar columns (e.g. BPX-70, DB-23) with 4-5 °C/min temperature ramps [32]. For the FAME most commonly of interest (C14-C22) this results in analysis times of 20-40 minutes on conventional columns.

We prepared two concentrations of a FAME mixture, containing 14:0, 14:1, 16:0, 16:1, 17:0, 18:0, 18:1, 18:2n6, 18:3n3, 20:0, 20:1, 20:2, 20:3, 20:4, 20:3, 22:0, 22:1, 23:0, 24:0, 24:1, and 22:6. The FAME are listed in their chromatographic retention order. For the first concentration, the 18 bolded FAMEs were at 300 ng/μL, and were the analytes of interest. The methyl 17:0 and methyl 23:0 were at ~10-fold higher concentration and used as internal isotopic standards. The second mixture was a 15-fold dilution of the first mixture. For both conventional and fast analyses, the 1μL injection was split 15:1. This results in about 1.5 and 0.1 nmol C on column for each bolded component in the first and second mixtures, respectively.

Polar (70% cyanopropyl) columns were used for both fast (VF-23ms, 15 m × 0.15 mm × 0.15 μm) and conventional (BPX-70, 30 m × 0.32 mm × 0.25μm) runs. Temperature programs for the two columns were similar, but the fast GC column was programmed at 30 °C/min vs. 4 °C/min for the conventional column. The GCC-IRMS chromatograms are shown in Figure 3.3. Because the columns were produced using the same conditions, by two different manufacturers, slight differences were
Figure 3.3. Comparison of conventional GCC-IRMS (top), fast GCCC-IRMS (middle), and fast GC-FID chromatograms of FAME mixture separated on 70% cyanopropyl columns. Fast GCC-IRMS runs achieve comparable separation to conventional runs in 1/3 the time. Retention order of the FAMEs was 14:0, 14:1, 16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, 20:3, 22:0, 20:4, 22:1, 20:5, 23:0, 24:0, 24:1, 22:6. The peaks used for the precision comparison are in Figure 3.4 and Table 3.3 and are in bold and had a mean peak width by fast GCC-IRMS of 720 ms FWHM, compared to 650 ms FWHM for fast GC-FID using the same conditions.
observed in the retention factors for analytes, but the retention order was identical. The fast GCC-IRMS run required only 450 s, about 1/3 the time of the conventional analysis (1400 s). The separation achieved in both runs was comparable: in fast GC, 21 FAMEs were separated from \( t_i = 320 \) s to \( t_f = 445 \) s with an average peak width of 720 ms; in conventional GC, the FAMEs were separated over from \( t_i = 575 \) s to \( t_f = 1375 \) s with an average peak width of 4.7 s. Defining peak capacity, \( k \), as \( k = (t_f-t_i) / (\text{FWHM}_{\text{avg}}) \), we note that both chromatograms have identical capacities of 170 peaks.

Comparison of the fast GCC-IRMS chromatogram to fast GC-FID (Figure 3.3, bottom) run under the same conditions reveals that the optimized combustion interface causes minor but noticeable broadening. The average peak width for the subject FAME is 720 ms with GCC-IRMS, and 650 ms with GC-FID. Assuming the broadening is Gaussian in nature, we can calculate \( \sigma_c = 310 \) ms for the combustion interface, slightly worse than the \( \sigma_c = 250 \) ms measured with CO\(_2\) injections. Despite the minor loss of resolution for the GCC-IRMS interface, separation for these narrow peaks would have been completely lost with a conventional interface.

\( \delta^{13}\text{C} \) values and precisions (SD(\( \delta^{13}\text{C} \), \( n=3 \)) were determined for 15 FAME on both systems at the high and low concentrations and plotted in Figure 3.4. Mean precisions are reported in Table 3.3. 22:0, 22:4, and 22:1 were not used because they were at higher concentration than the other FAME. At the higher concentration (1.5 nmol C of each FAME), the agreement between the fast (○) and conventional (●) GCC-IRMS systems was good. For 14 of 15 FAME, \( \delta^{13}\text{C} \) values were within ±1.5‰ and were not significantly different (\( p > 0.05 \)) as calculated by Student’s t-test. The mean precision was SD (\( \delta^{13}\text{C} \)) = 0.4‰ for fast GC and SD (\( \delta^{13}\text{C} \)) = 0.5‰ for conventional GC. The precisions are slightly poorer than benchmark precisions for GCC-IRMS of standards (SD (\( \delta^{13}\text{C} \)) = 0.2‰) but are acceptable considering the low concentration of analyte (~1.5 nmol C on column) and modest absolute sensitivity of
Figure 3.4. Comparison of $\delta^{13}C$ values of fifteen FAME, 1.5 or 0.1 nmol (circle) or 0.1 nmol (square) as C on column run by fast GCC-IRMS (open) and conventional GCC-IRMS systems (closed). The legend for this figure is reported in Table 3.3.
**Table 3.3.** Comparison of mean precisions, SD(δ\(^{13}\)C), and accuracies for fifteen FAME runs by conventional and fast GCC-IRMS at two different concentrations. The symbols correlate to the symbols used in Figure 3.4.

<table>
<thead>
<tr>
<th></th>
<th>nmol C</th>
<th>Precision (‰)</th>
<th>Accuracy(^a)</th>
<th>Accuracy(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD(δ(^{13})C)</td>
<td>δ(^{13})C(1.5) - δ(^{13})C(0.1)</td>
<td>rms = (1.4)‰</td>
</tr>
<tr>
<td><strong>Fast GC(^b)</strong></td>
<td>○</td>
<td>1.5</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>□</td>
<td>0.1</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td><strong>Conventional GC</strong></td>
<td>●</td>
<td>1.5</td>
<td>0.5</td>
<td>rms = 2.9‰</td>
</tr>
<tr>
<td></td>
<td>■</td>
<td>0.1</td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>
our 15-year old IRMS tuned for high linearity.

At the lower concentration (0.1 nmol C on column per FAME), fast GCC-IRMS (□) performance was superior to conventional GCC-IRMS (■). At this concentration, the average precision was SD ($\delta^{13}$C) = 1.4‰ for fast GC and SD ($\delta^{13}$C) = 2.2 ‰ for conventional GC. We also observed a greater degradation in accuracy for the conventional GCC-IRMS peaks. We calculated a single accuracy parameter as the root-mean-squared differences between the $\delta^{13}$C values measured for the high and low concentrations of each FAME, or:

$$Accuracy(\delta^{13}C, rms) = \sqrt{\frac{\sum_{i=1}^{n} (\delta^{13}C(high) - \delta^{13}C(low))^2}{n}}$$  \hspace{1cm} (11)

The root-mean-squared accuracy was 1.4 ‰ for fast GCC-IRMS and 2.9 ‰ for conventional GCC-IRMS. The lower concentration (0.1 nmol C on column, or 20 pmol C to the IRMS) generated ~0.5-1.0 nC peak areas, around the signal level associated with improved precision for fast GC compared to conventional GC. The cause of the increased degradation of precision on conventional GC compared to fast GC conditions is consistent with that proposed for the CO2 injections, i.e., uncorrelated noise between the $^{44}$CO2 and $^{45}$CO2 traces.

**Analyses of underivatized steroids by fast and conventional GCC-IRMS.** High precision $\delta^{13}$C measurements of urinary steroids are of forensic interest in antidoping science for the detection of exogenous testosterone (T) administration in competitive sports [1]. The $^{13}$C/$^{12}$C ratio of T metabolites in the urine (e.g. androsterone) is compared to “endogenous reference compounds”, ERCs, (e.g. 5β-pregnene-3α, 20α-diol) i.e. steroids that are not endogenously synthesized via T.
Typical run lengths for both derivatized and underivatized steroids are 20-40 min.

We analyzed a mixture of 10 steroids (OH-Andro, 19-NA, Eto, DHEA, Andro, 11-keto, 5βP, Chln, Preg, and Ch-OH) that have been used in doping analysis by both fast and conventional GCC-IRMS. The mixture, containing 100 ng of each steroid, was injected splitless. Fast GCC-IRMS using a 20 m × 0.15 mm × 0.60 μm VF-5ms column achieved near baseline separation of the 10 steroids in ~10 minutes (Figure 3.5, middle). The peak widths ranged from 890 to 1700 ms. In comparison, conventional GCC-IRMS with a 25 m × 0.25 mm × 0.25 μm VF-5ms column required 20 minutes to achieve the same level of separation (Figure 3.5, middle). Also, because of the broadening associated with the conventional combustion interface, the conventional GCC-IRMS is not able to resolve two of the steroids (DHEA and Andro) that were resolved in the FID trace. δ¹³C values and precisions are listed in Table 3.4. The mean precision for both fast and conventional GC was SD (δ¹³C) = 0.2 ‰. A comparison of values between the two systems shows that differences in δ¹³C are ≤ 2‰ for all steroids, and ≤1 ‰ for six of the eight steroids. In summary, fast GCC-IRMS provides better resolution in less time than conventional analyses, with comparable accuracy and precision.

**Sample size and fast GCC-IRMS.** A necessary caveat in discussing GCC-IRMS is the limits of narrow columns on sample capacity. In our experiments with FAME using a 0.15 mm ID column with a 0.15 μm coating, we experienced fronting with > 30-50 ng analyte (2.0-3.5 nmol C) on column. The 0.10 mm i.d. capillary favored in fast GC-FID applications would have even lower capacity. By comparison, a 0.32 mm i.d. column with 0.5 μm coating can accommodate ~10-fold more analyte before fronting is noticeable. Because the typical specification for a modern GCC-IRMS system is 1 nmol C to achieve SD (δ¹³C) = 0.2 ‰, it may
Table 3.4. Comparison of values for a steroid mixture between conventional and fast GCC-IRMS corresponding to the chromatograms in Figure 3.5.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Steroid</th>
<th>Conventional δ(^{13})C ± 1 SD (%)</th>
<th>Fast δ(^{13})C ± 1 SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>OH-Andro</td>
<td>-30.3 ± 0.1</td>
<td>-30.2 ± 0.1</td>
</tr>
<tr>
<td>B</td>
<td>19-NA</td>
<td>-29.7 ± 0.3</td>
<td>-30.2 ± 0.1</td>
</tr>
<tr>
<td>C</td>
<td>Etio</td>
<td>-33.1 ± 0.1</td>
<td>-32.1 ± 0.1</td>
</tr>
<tr>
<td>D</td>
<td>DHEA</td>
<td>n.d.</td>
<td>-26.0 ± 0.4</td>
</tr>
<tr>
<td>E</td>
<td>Andro</td>
<td>n.d.</td>
<td>-26.6 ± 0.4</td>
</tr>
<tr>
<td>F</td>
<td>11-keto</td>
<td>-13.7 ± 0.3</td>
<td>-14.7 ± 0.2</td>
</tr>
<tr>
<td>G</td>
<td>5βP</td>
<td>-30.3 ± 0.1</td>
<td>-30.8 ± 0.2</td>
</tr>
<tr>
<td>H</td>
<td>Chln</td>
<td>-23.8 ± 0.2</td>
<td>-25.8 ± 0.1</td>
</tr>
<tr>
<td>I</td>
<td>Preg</td>
<td>-31.6 ± 0.2</td>
<td>-31.4 ± 0.2</td>
</tr>
<tr>
<td>J</td>
<td>Ch-OH</td>
<td>-20.7 ± 0.2</td>
<td>-22.3 ± 0.4</td>
</tr>
</tbody>
</table>
Figure 3.5. Conventional (top) and fast (middle) GCC-IRMS chromatograms of the 10-steroid mixture on a 95% PDMS/5% phenyl columns. The letters correspond to Table 3.3. The 45/44 ratio trace for the fast GCC-IRMS required about 50% of the run length of a conventional analysis while achieving comparable precision. The inset demonstrates that peak widths of < 1 s (FWHM) are achievable.
appear that using fast GC effectively cuts the already narrow dynamic range of GCC-IRMS by 10-fold. However, there are some mitigating factors.

First, we demonstrate in this chapter that while fast and conventional GCC-IRMS achieve comparable precision at high levels of signal, at low signal levels the precision scales inversely with the peak width. For example, fast GC peaks can achieve SD ($\delta^{13}C$) < 1.0 ‰ for peak areas down to 0.03 - 0.1 nC (5-15 pmol C on column), more than 10-fold better than conventional GC peaks. For many natural abundance applications, SD ($\delta^{13}C$) < 1.0 ‰ is acceptable, and for tracer experiments SD ($\delta^{13}C$) < 100 ‰ can be still be useful[10]. Therefore, in addition to shorter run times, fast GCC-IRMS may provide better dynamic range for low level analytes despite sacrificing dynamic range for high concentration analytes. Also, in cases where precision of SD ($\delta^{13}C$) ~1.0 ‰ is acceptable, concentrated samples can be split to achieve resolution in faster run times.

Second, it is possible to modestly compromise the speed enhancement of the fast GCC-IRMS by using high phase ratio (thick film, narrow i.d.) columns to increase column capacity. For our fast steroid analyses, we used a 0.15 mm i.d. with 0.60 µm film thickness; this allowed us to inject ~100 ng on each steroid on column with minimal fronting. The higher phase ratio requires higher temperatures and results in slightly worse column efficiency and slower run times than comparable thin film columns but still results in faster run times than conventional columns. Another possibility would be to investigate “multicapillary columns” which bundle several microbore GC columns in parallel, increasing the column capacity while retaining the benefits of a narrow-bore column. [34]

Finally, we have not observed that overloaded peaks result in changes in $\delta^{13}C$ values in $\delta^{13}C$ values if the ion currents remain on-scale, nor are we aware of any such reports. While fronting peaks mean a loss in peak capacity, we were able to achieve
good precision even for grossly overloaded and distorted peaks, e.g. 500 ng of FAME on the fast 0.15 mm i.d. VF-23 ms column.

3.4. CONCLUSIONS

We have demonstrated that a carefully optimized GCC-IRMS system is capable of measuring 250 ms wide chromatographic peaks (FWHM), one-fifth that of any previously reported GCC-IRMS peak. Using FAME and steroid standard mixtures, we show that this fast GCC-IRMS system can achieve equivalent or better precision (SD ($\delta^{13}\text{C}$) = 0.2-0.5‰) and resolution than conventional GCC-IRMS with a 2- to 3-fold improvement in run time. $\delta^{13}\text{C}$ values measured by fast and conventional systems were within ±1.5‰ for 21 of 23 components measured. Although the narrow i.d. fast GC columns had lower column capacity, at low signals we observed that narrower peaks yielded better precisions than conventional GC peaks with comparable peak areas. For peak areas <0.5 nC, the improvement in precision scaled inversely with the peak width. Therefore, fast GCC-IRMS may be ideal for isotopic analyses of trace components where modest precision (SD ($\delta^{13}\text{C}$) ≥ 1.0‰) is acceptable. Finally, the fast GCC interface we reported may also be used for conventional columns to preserve chromatographic quality.

The fast GCC interface described in this report likely does not represent the absolute limit in GCC-IRMS peak widths. In particular, we believe that the current furnace design can be optimized considerably. The current furnace is 40 cm × 0.25 mm i.d.; shrinking the dimensions to 0.10 mm i.d. would reduce broadening, but presents technical challenges when one considers how to include an oxygen source (CuO, ZnO) in such a narrow capillary. One possibility is to devise a means to coat the inside of a 0.10 mm i.d. FS capillary with a thin layer of an appropriate metal. Further improvements could be realized by minimizing broadening caused by the
IRMS inlet; we found that the minimal attainable peak width with our IRMS inlet system was 124 ms. Our data do not address the possibility that this is a fundamental property of the IRMS ion source or whether residual volumes and other modifications could yield even sub-200 ms peaks amenable to even faster GCC-IRMS applications.
REFERENCES

CHAPTER FOUR

Comprehensive Two-Dimensional Gas Chromatography Combustion
Isotope Ratio Mass Spectrometry (GC × GCC-IRMS)

4.1. INTRODUCTION

High precision gas isotope ratio mass spectrometry (IRMS) is the established
technique for determination of the fractionation of the natural isotopic variability of
the organic elements, C, N, O, S and H [1-3]. For $^{13}$C/$^{12}$C analysis, the analyte must
be converted to CO$_2$ for analysis; thus, most analytes must be chemically transformed
by combustion prior to admission to the IRMS. The integration of gas
chromatography (GC) with IRMS via an online combustion interface, GC-combustion
IRMS (GCC-IRMS), enables compound-specific isotope analysis [4].

It has long been known that accurate isotope ratio analysis by continuous-flow
methods required baseline separation of adjacent chromatographic peaks using
conventional data reduction algorithms [5] and that minimal analyte levels, in the 10-
ng range, are required to achieve high-precision results [6]. Although high resolution
and peak capacity are possible with long, narrow-bore columns, such as those with 80
m × 0.10 mm inner diameter (i.d.), minimal analyte requirements typically dictate that
GCC-IRMS analyses employ 30-60 m × 0.25-0.53 mm i.d. columns that have modest
chromatographic resolution and peak capacity [3]. As a result, analyses of real-world
samples can often require cumbersome sample cleanup and preconcentration, such as
with high-performance liquid chromatography (HPLC), prior to GCC-IRMS
analysis[8].

3 Based on the reference of Analytical Chemistry 2008, 80, 8613-8621
Alternatively, some chromatographic limitations of a single GC column (1DGC) can be overcome through the use of multiple GC columns, referred to as multidimensional GC (MDGC) or 2DGC, an approach that has long been used to improve separation of closely eluting components in difficult to separate mixtures [9]. The traditional configuration for MDGC diverts a small section of a chromatogram ("Heart-cut") from the first GC column (GC1) and introduces it onto a second GC column (GC2). Multiple targeted cuts can be made for analysis and undesirable regions, such as large tailing peaks, can be avoided. MDGC configurations have previously been coupled to IRMS, such as for flavor analysis and authentication [10,11], environmental pollutant tracing [12], and analysis of non-methane hydrocarbons emitted from biomass burning [13]. However, MDGC techniques require prior knowledge of the sample mixture, and they result in decreased peak capacity in cases where more than 10 components are transferred from GC1 to GC2 [14]. MDGC is appropriate for improving the resolution of a few closely eluting analytes, but is usually poorly suited for complex mixtures with multiple target analytes.

Unlike heart-cut MDGC approaches, comprehensive two-dimensional gas chromatography (GC × GC) separates the entire sample mixture in two dimensions with continuous detection and is thus more suited to nontargeted separation of all components in complex mixtures [15]. The technique also provides information about elution of related chemical entities that facilitates identification. In GC × GC technology over the past decade have enabled practical commercial instrumentation [16-22]. GC × GC employs two chromatographic columns with orthogonal properties operating in tandem. A modulator, commonly using cryogenics, between the first and second columns allows a plug of eluting components from the first column to be trapped and continuously transferred as a narrow band onto the second
column in 2-10-s intervals, generating an entire secondary chromatogram every 2-10 s. The first column is usually a conventional capillary column that separates components in minutes, while the second column is short and operated very rapidly, achieving fast isothermal or nearly isothermal separations on the order of seconds within each modulation interval. GC × GC has been successfully used to separate and classify hundreds of chemical species in many different types of complex mixtures such as petroleum [23], ambient air [24], and lipid [25-27] samples. The major advantages of GC × GC over 1DGC and MDGC include enhanced nontargeted component resolving power on the order of a factor of 10, the formation of chemically similar compound patterns in the 2D chromatogram, and up to 1 order of magnitude enhanced sensitivity through solute band reconcentration onto the second GC column.

Key to interfacing GC × GC to IRMS is to maintain the chromatographic resolution of the fast GC2 effluent signal through the combustion interface. Commercial GCC-IRMS combustion interfaces cause band broadening on the order of 1 s, and the response of IRMS detectors is also of this order, thus making fast GC response impossible without extensive redesign [3]. We recently reported a system capable of fast GCC-IRMS supporting full width at half-maximum (FWHM) peaks of >250 ms [28]. Here, we report the construction of the first prototype GC × GC-C-IRMS system and adaptation to 2D analysis. As test compounds, we use steroids relevant to antidoping applications [29-36]. Carbon isotope ratio (CIR) testing is the currently accepted tool for unambiguously detecting synthetic steroids used in performance enhancement, most notably testosterone, and has been described extensively [37].

4.2. EXPERIMENTAL

Chemicals. Air, H2 (for the FID), N2, and high purity He gases and high-
purity CO$_2$ gases, plus siphon-type liquid CO$_2$, were purchased from Airgas East (Salem, NH). The steroids 5α-androstane-3β-ol acetate (5α-A-AC), 5α-androstane-3α-ol-17-one acetate (A-AC), 5β-androstane-3α-ol-11,17-dione acetate (11k-AC), and 5α-cholestane (Cne) were acquired from Steraloids (Newport, RI). The steroid 5β-pregnane-3α-20α-diol (5βP) was acquired from Acros Organics (Morris Plains, NJ). All steroids were of >99% purity and used without further purification. HPLC grade 2-propanol was obtained from Mallinckrodt Baker (Phillipsburg, NJ). Two steroid mixtures were prepared for analysis. Steroid mixture 1 (SM1) contained an approximately equal mass mixture of 5α-A-AC, A-AC, 11k-AC, and Cne. SM1 contained steroids that are well separated in GC1 and was prepared to investigate the characteristics of modulated peaks. Steroid mixture 2 (SM2) contained an approximately equal mass mixture of 5α-A-AC, 5βP, 11k-AC, and Cne. SM2 was prepared to investigate the characteristics of steroids that coelute in GC1. The steroid mixtures were prepared in 2-propanol at concentrations ranging from 6 ng/μl to 25 ng/μl, as specified for experiments outlined below. These steroids were calibrated relative to the methane and ethane contained in the National Institute of Standards and Technology (NIST) natural gas reference material RM 8559 (>80% methane of coal origin, Gaithersburg MD)[38] to determine their CIR ($\delta^{13}$C$_{VPDB}$) and are described in detail elsewhere [39].

**GC × GCC-IRMS System.** The schematics of the GC × GCC-IRMS system is depicted in Figure 4.1. An HP6890A GC (Agilent Technologies, Menlo Park, CA) was interfaced to a MAT 252 IRMS (Thermo Finnigan, Bremen, Germany). A longitudinal modulated cryogenic system (LMCS) developed by Marriott et. al.[17] (Chromatography Concepts, Sandringham Australia) was installed between GC column 1 (GC1) and GC column 2 (GC2). A 0.3 mm x 0.1 mm i.d. deactivated
Figure 4.1. Schematic of the GC × GCC-IRMS system. A LMCS, developed by Marriott et al., was retrofitted to a GC. A PTV inlet was used for solvent diversion and the GC2 was directly coupled to an IRMS. For PTV inlet optimization, effluent was directed to a FID after GC2.
fused-silica capillary was used in the modulator to bridge GC1 and GC2, which were in the same GC oven at the same temperature. During operation, the LMCS was maintained at 100 °C, using liquid CO2 at 9 min and modulation was started at 10 min into the GC × GC run. N2 continuously purged the outside of the LMCS capillary to flush away condensation. A Programmable Temperature Vaporization (PTV) inlet (Agilent Technologies, Menlo Park, CA) was used to divert solvent away from the column. The PTV inlet parameters were optimized as discussed later. During PTV characterization, GC effluent was directed to a Flame Ionization Detector (FID) after GC2. For GC × GCC-IRMS analyses, the PTV inlet was held at 50 °C for 1 min and then ramped to 300 °C at 600 °C/min. The PTV vent time was 0.9 min, vent pressure was 5 psi, vent flow was 100 ml/min, and purge time was 2.5 min.

A home-built combustion interface was constructed. On-line conversion of sample effluent to carbon dioxide gas was accomplished using a narrow i.d. capillary combustion reactor held at 925 °C using an open tubular glass fiber furnace (Thermcraft Inc., Winston-Salem NC) containing ~0.15-m length of a radiatively heated zone. The combustion reactor was composed of a 0.45 m × 0.25 mm i.d. × 0.36 mm o.d. deactivated fused-silica capillary (Polymicro Technologies, Phoenix, AZ) containing two wires: one 0.19 m × 0.10 mm diameter Cu/Mn/Ni (84/12/4%) wire (Alfa Aesar, Ward Hill, MA) and one 0.19 m × 0.10 mm diameter Pt (>99%) wire (Alfa Aesar).

The Cu/Mn/Ni wire was ultimately oxidized using pure oxygen at 600 °C flowing for one day and at 925 °C for a second day before use of the reactor. The upstream of the wires were carefully set in the capillary so that they were well within the furnace hot zone to prevent effluent peak tailing due to metal and metal oxides acting as significant active sites at temperatures below 850 °C. The capillary reactor was mechanically supported in a 0.30 m × 0.5 mm i.d. × 1.5 mm o.d. alumina tube.
(AC Technologies Inc., Yonkers, NY) with metal fittings (Valco Instruments Inc., Houston, TX) on both ends as previously described [28, 40].

A 1-m deactivated fused-silica capillary with a 0.10-mm i.d. was used as a transfer capillary between the combustion reactor and the open split. Water vapor generated from the combustion process was removed from the system before the IRMS by immersing 10 cm of the transfer capillary into a dry ice and acetone-water trap at -78 °C. Press-tight fittings (Restek, Bellefonte PA) were used for connections between GC1, the modulator capillary, GC2, the combustion reactor, and the transfer capillary. A press-tight fitting was also attached at the downstream end of the transfer capillary. A portion of the dry effluent was sampled into the IRMS by placement of the upstream end of 1 m × 0.075 mm i.d. IRMS sampling capillary flush with the end of the transfer capillary within the press-tight, thereby operated as an open split.

**GC Parameters.**  Samples analyzed in this study are referred to as “modulated” or “nonmodulated”, where all samples were run through the GC × GCC-IRMS system (i.e., through GC1 and GC2) with the LMCS cryogenic modulator on, or off, respectively. The nonmodulated analyses are equivalent to GCC-IRMS analyses. All analyses were performed with He carrier gas using a PTV inlet in solvent vent mode. A 1-µl aliquot of 20 ng/µl SM1 solution was injected for non modulated analysis, and 1-µl of 8 ng/µl solution of SM1 was injected for 4-sec modulated analysis in each run. GC1 was a 30 m × 0.25 mm i.d. × 0.25 µm film DB5 (5% phenyl 95% dimethyl polysiloxane, J&W Scientific, Menlo Park, CA) and GC2 was a 1 m × 0.10 mm i.d. × 0.10 µm film DB17 (50% phenyl 50% dimethyl polysiloxane, J&W Scientific). The head pressure was set to 36 psi at 50 °C with a flow rate of 1.2 ml/min measured at the open split and run in constant-flow mode.
The GC oven was initially held at 50 °C for 2.5 min, then ramped at 30 °C/min to 270 °C, where it was held for 17.3 min, and then ramped at 10 °C/min to 300 °C, where it was held for 1 min.

A 1.2-μl aliquot of 25 ng/μl SM2 solution was injected for nonmodulated analysis, and 0.5 μl of 6 ng/μl solution of SM2 was injected for 8-s modulated analysis. GC1 was a 30 m × 0.25 μm i.d. × 0.25 mm film DB5 (J&W Scientific), and GC2 was a 2 m × 0.10 mm i.d. × 0.10 μm film DB17 (J&W Scientific). The head pressure was set to 42 psi at 50 °C with a flow rate of 1.2 ml/min measured at the open split and run in constant-flow mode. The GC oven was initially held at 50 °C for 2.5 min, then ramped at 50 °C/min to 250 °C, where it was held for 24 min, and then ramped at 20 °C/min to 300 °C, where it was held for 2 min.

Data Acquisition and Analysis. SAXICAB, a home-built LabView (National Instruments, Austin, TX) based IRMS data acquisition and reduction system, was used to monitor the m/z 44, 45, and 46 signals, for 44CO2, 45CO2, and 46CO2, from the MAT 252 head amplifiers using three 24-bit National Instruments NI4351 digitizers. The data were collected at 50 Hz and consecutive points were averaged to halve the size of files, resulting in an effective data acquisition rate of 25 Hz. The standard RC time constants of the faraday cup detectors were reduced and matched by exchanging appropriate resistors (Thermo Finnigan) and capacitors (Just Radios, Scarborough, Ontario, Canada) in order to prevent broadening of fast GC2 peaks for the m/z 46 signal, an effect described previously for IRMS detectors. [28, 41] The pre-exchange standard arrangements were m/z 44 R (3e8 Ω) × C (470 pF) = τ (141 ms), m/z 45 R (3e10 Ω) × C (5 pF) = τ (150 ms), and m/z 46 R (1e11 Ω) × C (2 pF) = τ (200 ms). All three m/z arrangements were changed to m/z 44 R (3e8 Ω) × C (100 pF) = τ (30 ms), m/z 45 R (3e10 Ω) × C (1 pF) = τ (30 ms), and m/z 46 R (3e10
\( \Omega \times C \) (1 pF) = \( \tau \) (30 ms).

All data sets were processed for isotopic analysis through SAXICAB using the individual summation method with an individual background definition, using the mean of five points prior to peak start and five points after the peak stop to define the baseline [42]. Also, a minor modification to the conventional time correction was applied due to fast GC2 peaks, detailed previously [28]. The contribution of \(^{17}\)O to the \(^{45}\)CO\(_2\) signal was taken into account by the method of Santrock et al [43]. SAXICAB software was additionally modified to allow for summation of peak slices prior to isotope ratio calculation. For carbon isotopes, the CIR is expressed as

\[
\delta^{13}C_{VPDB} = \frac{(R_{SPL} - R_{VPDB})}{R_{VPDB}} \times 1000 \text{‰},
\]

where \( R_{SPL} \) is the \(^{13}\)C/\(^{12}\)C of the sample and \( R_{VPDB} \) is the \(^{13}\)C/\(^{12}\)C of the international standard Vienna PeeDee Belemnite (\( R_{VPDB} = 0.0112372 \)).

In general, the steroids analyzed with no modulation were used to calibrate CO\(_2\) gas, which in turn was used to calculate the \( \delta^{13}C_{VPDB} \) of modulated GC x GCC-IRMS analytes. This procedure takes into account any minor isotopic fractionation due to the difference in flow paths traversed by the CO\(_2\) calibrant gas and the steroids, as well as any fractionation associated with imperfect combustion. For each set of analyses, a fresh aliquot of CO\(_2\) gas was expanded into a static volume. Three CO\(_2\) pulses were admitted from this volume near the beginning of each run. The known \( \delta^{13}C_{VPDB} \) values was assigned to one of the steroid components in the nonmodulated runs and used to calculate an “apparent” \( \delta^{13}C_{VPDB} \) for the CO\(_2\) gas [39]. Since multiple steroids were used in this process, a best fit (i.e. the mean) was calculated and used as the final apparent \( \delta^{13}C_{VPDB} \). The CO\(_2\) gas was calibrated for its apparent \( \delta^{13}C_{VPDB} \) using the four steroids \( 5\alpha\)-A-AC, A-AC, 11k-AC, and Cne for SM1 analysis and the two steroids \( 5\alpha\)-A-AC and Cne for SM2 analysis. The apparent \( \delta^{13}C_{VPDB} \) was then assigned to the CO\(_2\) gas and used to calculate the \( \delta^{13}C_{VPDB} \) values.
for the analytes in the modulated sample runs and to recalculate the $\delta^{13}C_{VPDB}$ values for the non-modulated sample runs.

The following procedure was used for the reconstruction of modulated peaks for the calculation of their $\delta^{13}C_{VPDB}$. The peak start and stop times were manually chosen for the first modulation of the analyte(s). The $\delta^{13}C_{VPDB}$ was calculated for the peak slice in this time bin relative to the apparent $\delta^{13}C_{VPDB}$ of CO$_2$ gas pulses admitted to the IRMS during the beginning of the GC $\times$ GCC-IRMS runs. For each additional peak slice, the modulation frequency (i.e., 8 s) was added to the start and stop times of the previous slice automatically through the software. The $\delta^{13}C_{VPDB}$ was then calculated based on the sum of the isotopomer signal areas of the current slice and the previous slice(s). The same time bins were used for all GC $\times$ GCC-IRMS runs in a data set. Although the data are collected and presented as a 1D chromatogram in the software, this procedure mimics the practice of boxing a region for a compound on a 2D contour plot, as shown later.

In order to enable multidimensional visual representations of the data, raw text files were exported from SAXICAB and modified into appropriate comma separated value (CSV) format data files containing retention times and $m/z$ 44 signal levels or calculated $m/z$ 45/44 levels. The CSV files were then processed to create the appropriate modulation matrix CSV files using the program 2D converter version 2.2 (Fortner Research LLC, Sterling, VA). The 2D contour and 3D surface plots were then generated from these CSV files using Transform version 3.3 (Fortner Research LLC), with smoothing and interpolated raster imaging of the raw data.

4.3. RESULTS AND DISCUSSION

**GC and Home-Built Combustion Interface.** In previous work developing fast GCC-IRMS,[28] critical components were evaluated for minimizing peak
broadening effects in the GC and combustion interface. The major components that were considered and optimized were the solvent diversion method, combustion reactor design, transfer lines, water trap, and the open split to reduce GC effluent pathway inner dimensions, dead volumes, and connections. Additional refinements for GC × GCC-IRMS are as follows.

**Solvent Diversion.** The diversion of solvent is required in GCC-IRMS to prevent solvent from passing through the on-line combustion reactor and rapidly depleting its combustion capacity. Normally, in GCC-IRMS systems, a rotary valve or backflush system is used for this purpose. In this work, a PTV inlet was used in solvent vent mode to purge the solvent; therefore, it did not require additional connections between the GC and combustion reactor. The use of a PTV was previously introduced by Flenker et al. [44] as an alternative method for use in GCC-IRMS and was also adopted in our fast GCC-IRMS work [28]. In this method, the sample is injected onto a cooled inlet while a large purge flow, known as the solvent vent flow, diverts the solvent to waste. After a set time period (i.e., 1 min), the solvent vent flow is turned off and the inlet is rapidly heated to focus the analytes onto the top of the GC column. In principle, the PTV carries the advantage of improved peak shapes over a rotary valve or backflush system [28,44] by avoiding solvent-induced peak broadening inherent in split/splitless injectors. In addition, the PTV inlet does not require the additional connections necessary for a rotary valve or backflush system, thereby avoiding additional tubing i.d. changes and dead volumes that can broaden peaks and cause peak tailing.

The PTV inlet temperature and the solvent vent flow must be optimized to clear solvent, while quantitatively retaining analyte in order to avoid the risk of isotopic fractionation, as well as, comprised sensitivity. PTV parameter optimization using FID detection is depicted in Figure S-1 provided in the Supporting Information.
The analysis performed using a solvent vent flow of 0 ml/min represents the signal level of 100% analyte retention in the PTV inlet. The optimal parameters determined for the steroids here were an initial PTV inlet temperature of 50 °C, and a solvent vent flow of 100 ml/min.

**Combustion Reactor.** Ceramic or quartz tubes that are 0.5-mm i.d. or larger and loaded with CuO, NiO, and Pt wire are typically used in traditional and commercially available GCC-IRMS systems. A single capillary design is better suited to minimization of peak broadening where large i.d. changes and dead volumes can be reduced and at some points eliminated [40]. For fast GCC-IRMS work, we developed a narrow i.d. combustion reactor that yielded peak widths of < 250 ms for unretained methane [28]. That reactor was composed of a continuous 0.25-mm-i.d. FS capillary, where the combustion region was loaded with one strand of 0.1-mm Cu wire and oxidized in situ.

The hand-loading of 0.1-mm-diameter Cu (>99%) wire into a 0.25-mm i.d. capillary is difficult due to its softness and high malleability and very easily kinks upon handling, and only a single strand can be loaded in practice. Here, we investigated alternative capillary combustion reactor designs by qualitatively investigating a limited number of 0.1-mm-diameter wires with more suitable loading properties. In contrast to malleable Cu, Ni (>99%) wire is relatively rigid and springy, retaining its coiled shape from the wire spool. Other wires qualitatively fell between the properties of Cu (>99%) and Ni (>99%) in the following order: Cu (>99%), Cu/Mn/Ni (84/12/4%), Pt (>99%), Cu/Ni (55/45%), and Ni (>99%). All the other wires apart from Cu were easy to load because they were rigid enough to avoid kinking. However, the moderately rigid and springy metals such as Cu/Ni (55/45%) and Ni (>99%) appear to induce capillary cracking upon heating up to 925 °C, possibly because they slightly bent/stressed the capillary, and probably scratched the inside
wall of the capillary during loading, both compromising the capillary integrity at high
temperatures. We found 0.1-mm Cu/Mn/Ni (84/12/4%) and 0.1-mm Pt (>99%) to
have the best properties for 0.25-mm capillary hand-loading and capillary robustness
at high temperatures. We were also easily able to load a 0.25-mm-i.d. capillary with
two strands of either of these wires, which was not possible with pure Cu. This allows
the construction of a reactor with greater oxidation capacity or addition of Pt catalyst.
In this work, we used a total of two wires, one 0.1-mm Cu/Mn/Ni (84/12/4%) and one
0.1-mm Pt (>99%) wire. Though narrow capillary reactors are fragile, in our hands a
carefully built and maintained reactor can have a physical lifetime of weeks during
regular operation of the GC GCC-IRMS system. For each data set, the combustion
reactor was charged with oxygen before analysis, and thus, the combustion capacity of
a single oxygen charge beyond 12 or so analyses was not investigated in this work.

*Water Trap.* Removal of water generated from the combustion process is
required to prevent protonation of $\text{CO}_2$ in the IRMS that leads to loss in $\delta^{13}\text{C}_{\text{VPDB}}$
precision and accuracy. Here we employed a continuous 1 m x 0.10 mm i.d.
deactivated FS capillary as a transfer capillary between the combustion reactor and the
open split. A portion of this capillary was immersed in a dry ice/acetone bath to
freeze out water. In our previous work, we experienced sample signal loss after a
series of fast GCC-IRMS runs using a water trap of these dimensions, where enough
water condenses over a short time causing capillary obstruction. In this work, the
water trap was always purged between sample runs to prevent this phenomenon, and
no decrease or loss in signal over time was ever observed due to the water trap. The
water trap was manually flushed out by removing the capillary from the bath for $\sim$5
min and heating it to $>100\,^\circ\text{C}$ for $\sim1\,\text{min.}$
Modulated and Non Modulated Analysis. The LMCS that was used in this work was originally developed and refined by Marriott et al [16-18]. Advantages of the LMCS are simplicity for retrofitting onto an existing GC and robustness in operation. In each modulation with the LMCS, a cryogenic trap focuses a portion of the eluting solute band from GC1 over 2-10 s. Then the cryogenic trap rapidly moves upstream of the focused band promoting band remobilization into GC2 by capillary exposure to heat in the GC oven. Finally, the cryogenic trap moves back to its original position to focus the next band. The low thermal mass of the FS capillary allows for quick cooling and heating. In the LMCS modulation process, a balance is required between effective solute focusing and efficient solute remobilization. Relative to the volatility of the solute, focusing is dependent on the temperature of the oven. The thermodynamic considerations of this process are discussed in detail elsewhere [18]. Briefly, for the steroids analyzed in this work, a very low modulator trap temperature, -20 °C, contributed to first-dimension broadening where there was an increase in the number of peak slices generated from a solute peak due to incomplete remobilization between modulations. On the other hand, when the temperature of the modulator trap was set too high, 200 °C, some components partially leaked through (“breakthrough”) during the focusing step, due to incomplete trapping. Therefore, we settled upon an LMCS temperature of 100 °C during GC × GCC-IRMS analysis.

Modulated Peak Shapes. SM1 (5α-A-AC, A-AC, 11k-AC, and Cne) was analyzed by GC × GCC-IRMS to investigate the characteristics of modulated peaks that pass through a 1-m GC2 and home-built combustion interface. Figure 4.2A shows the nonmodulated, and Figure 4.2B shows the 4-s modulated m/z 44 chromatograms of SM1, where all steroids in Figure 4.3C and 4.3D for nonmodulated and modulated peaks, respectively, show the expected rise and fall in isotope ratio
indicative of the arrival of $m/z$ 45 prior to $m/z$ 44 at the detectors.

When not modulated, the steroids had FWHM peak widths increase over a very large range (2640-6777 ms) as compound GC1 retention times increased. Figure 4.3 is an expanded view of the chromatograms in Figure 4.2. Figure 4.3A shows the nonmodulated peak shape of 11k-AC with a FWHM of ~ 5260 ms. Figure 4.3B shows that modulation at 4-s intervals resulted in reduced widths for peak slices at FWHM of 240-340 ms, with an average of 276 ms ± 9‰. These peak slice widths are within the domain required for GC × GC and approach the 250-ms FWHM peak widths achieved for the unretained methane in our earlier work on fast GCC-IRMS.[28] Moreover, the peak signal intensity obtained from the modulated 11k-AC is ~ 2.3 V for 8 ng on column, compared to 0.29 V for 20 ng on column for the nonmodulated peak. The isotope ratio traces in Figure 4.3C and 4.3D compare the rise and return to baseline for the ratio traces of the nonmodulated and modulated peaks, respectively. Figure 4.3D demonstrates the rapid return to a flat baseline between the peak slices, as well as highlighting a peak slice barely noticeable in the $m/z$ 44 trace. The relative positive/negative deflection is reduced in consecutive GC2 runs, reflecting the change in $m/z$ 45/44 as the GC1 peak is sliced into GC2.

SM2, containing 11k-AC and 5βP, was analyzed by GC × GCC-IRMS in order to investigate the characteristics of steroids that coelute in GC1. The mass of each component was approximately 30 ng for nonmodulated analysis and 3 ng for modulated analysis at 8-s intervals. The non modulated $m/z$ 44 chromatogram shown in Figure 4.4A demonstrates that 11k-AC and 5βP completely coeluted in GC1. Figure 4.4B shows that the 8-s modulated $m/z$ 44 GC2 chromatograms achieved baseline separation of 11k-AC and 5βP. The isotope ratio traces in Figure 4.4C show the unresolved doublet and in Figure 4.4D show baseline resolution of all peak slices, as well as the shift toward lower $^{13}$C/$^{12}$C in consecutive GC2 runs.
GC × GC data are normally plotted in a two-dimensional format, with the GC1 signal plotted against the GC2 signals. The data of Figure 4.4B were transformed into a contour plot and a 3D surface plot, presented in Figure 4.5A and 4.5B, respectively. When modulated at 8-s intervals, the FWHM sliced peak widths of these steroids averaged 660 ms \(\pm\) 8%, eluting from a 2-m GC2 and combustion interface. These peak widths are wider than the ones presented for analyses of SM1 above, in part because the GC2 column used for SM2 analyses was double in length.

Three-dimensional isotope ratio traces from the data in Figure 4.4D are presented as a contour plot in Figure 4.6A and as 3D surface plot in Figure 4.6B, depicting for the first time isotope ratio traces in 4.3D. In Figure 4.6A, the enrichment toward the beginning of the peaks and depletion toward the end of the peaks is seen in both the first and the second dimensions. The effect is that the depleted section of the peak is positioned diagonally to bottom and right of the enriched early part of the peak. In Figure 4.6B, the negative going trough is more prominent than the positive going peak for the \(5\beta P\) compared to those of the relatively \(^{13}\)C enriched 11k-AC.

**Isotopic Analysis of Modulated Peaks.** The calculation of \(\delta^{13}\)CVPDB for the reconstructed GC1 peaks from their cryogenically sliced peaks was done according to the routine described in the Experimental section. For the modulated analyses, results for the \(\delta^{13}\)CVPDB from consecutively summed peak slices in the analysis of SM1 are plotted in Figure S-2 provided in the Supporting Information. As expected from the foregoing figures, the earlier peak slices are highly enriched by have large errors. This is primarily due to shifts in GC1 retention times, run to run, resulting in arbitrary time slices of peaks with continuously changing instantaneous isotope ratios. As more peak slices are summed, the \(\delta^{13}\)CVPDB values rapidly decrease, as is expected from the
Figure 4.2. GC × GCC-IRMS chromatograms of SM1. The (A) nonmodulated $m/z$ 44, (B) 4-s modulated $m/z$ 44, (C) nonmodulated $m/z$ 45/44, and (D) 4-s modulated $m/z$ 45/44 chromatograms. Here, GC1 = DB5 (30 m × 0.25 mm i.d. × 0.25 μm film); GC2 = DB17 (1 m × 0.10 mm i.d. × 0.10 μm film).
Figure 4.3. Expanded view of 11k-AC in the chromatograms presented in Figure 4.2, from the GC × GCC-IRMS analysis of SM1. The (A) nonmodulated m/z 44, (B) 4-s modulated m/z 44, (C) nonmodulated m/z 45/44, and (D) 4-s modulated m/z 45/44 chromatograms. Here, GC1 = DB5 (30 m × 0.25 mm i.d. × 0.25 μm film); GC2 = DB17 (1 m × 0.10 mm i.d. × 0.10 μm film).
Figure 4.4. GC × GCC-IRMS chromatograms of SM2, where 11k-AC and 5βP coeluted in GC1, but separated in GC2. The (A) nonmodulated $m/z$ 44, (B) 8-s modulated $m/z$ 44, (C) nonmodulated $m/z$ 45/44, and (D) 8-s modulated $m/z$ 45/44 chromatograms. Here, GC1 = DB5 (30 m × 0.25 μm i.d. × 0.25 μm film); GC2 = DB17 (2 m × 0.10 mm i.d. × 0.10 μm film).
Figure 4.5. $m/z$ 44 2D contour and 3D surface plots of the GC × GCC-IRMS analysis of 11k-AC and 5βP in SM2 using 8-s modulation depict the baseline separation of the two components. The 1D representation is presented in Figure 4.4B.
Figure 4.6. $m/z$ 45/44 ratio trace 2D contour and 3D surface plots of the GC × GCC-IRMS analysis of 11k-AC and 5βP in SM2 using 8-s modulation depict for the first time 2D and 3D representations of an isotope ratio trace. The 1D representation is presented in Figure 4.4D.
isotope ratio trace, and the errors reduce, as is expected as more of the peak is included in the calculation. The analysis of SM2 was similar (data not shown). Moreover, for the SM1 analysis, as each steroid’s GC1 retention time increase, the width of the GC1 peak increases, resulting in an increased number of peak slices upon modulation.

The final numerical results for the SM1 analysis are presented in Table 4.1, with no outliers excluded. The integration of peak slices produced $\delta^{13}C_{VPDB}$ values with average standard deviations of SD ($\delta^{13}C$) = 0.30 ‰ and average accuracies within 0.34 ‰, compared with average standard deviations of SD ($\delta^{13}C$) = 0.26 ‰ and average accuracies within 0.29 ‰ for non-modulated analysis. Modulated results were obtained using 2.5-fold less steroid mass on column (8 vs 20 ng). These data show that modulated peaks can be accurately re-integrated at precision and accuracy acceptable for IRMS analysis.

In work preliminary to the results presented here, the SM1 analysis was analyzed using a split/splitless inlet and a rotary valve instead of a PTV inlet alone. The results from those analyses were similar to the qualitative and quantitative results presented here for SM1 analysis with one exception. The GC1 peaks had obvious tailing, due to dead volume in the valve, which manifested as numerous additional peak slices upon modulation. This made peak stop definition less clear, but we noted that the number of slices did not significantly affect the precision and accuracy of the $\delta^{13}C_{VPDB}$ values (unpublished work).

For SM2 analysis, Figure 4.4 shows the non-modulated analysis resulted in complete coelution of 11k-AC and 5$\beta$P, whereas 8-s modulated analysis resulted in complete separation of 11k-AC and 5$\beta$P. The final numerical results for two data sets are presented in Table 4.2, where the two data sets were acquired identically on
Table 4.1. Comparison of the isotopic analysis of 5α-A-AC, A-AC, 11k-AC, and Cne from SM1 by the GC × GCC-IRMS system using no modulation and using a 4-s modulation rate with peak slice reconstruction\(^a\).

<table>
<thead>
<tr>
<th>Component</th>
<th>calibrated</th>
<th>nonmodulated (20 ng; (n = 4))</th>
<th>4-s modulated (8 ng; (n^b = 6))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\delta^{13}C_{VPDB}) (%)</td>
<td>(\delta^{13}C_{VPDB}) (%)</td>
<td>SD (±%)</td>
</tr>
<tr>
<td>5α-A-AC</td>
<td>-30.61</td>
<td>-30.90</td>
<td>0.18</td>
</tr>
<tr>
<td>A-AC</td>
<td>-33.04</td>
<td>-32.69</td>
<td>0.12</td>
</tr>
<tr>
<td>11k-AC</td>
<td>-16.70</td>
<td>-15.58</td>
<td>0.36</td>
</tr>
<tr>
<td>Cne</td>
<td>-24.77</td>
<td>-25.16</td>
<td>0.39</td>
</tr>
</tbody>
</table>

\(^a\) Peak slice data shown in Figure S-2 in the Supporting Information. No outliers were excluded.
Table 4.2. Comparison of the isotopic analysis of 11k-AC and 5βP contained in SM2 that coeluted in the GC×GCC-IRMS system with no modulation and separated using an 8-s modulation rate with peak slice reconstruction.a

<table>
<thead>
<tr>
<th>Component</th>
<th>Calibrated 6°CVPDB (%)</th>
<th>Calibrated 6°CVPDB (%)</th>
<th>Nonmodulated (30 ng; n = 6, 95% conf = 3)</th>
<th>Nonmodulated (30 ng; n = 6, 95% conf = 3)</th>
<th>8-s modulated (3 ng; 95% conf = 3)</th>
<th>8-s modulated (3 ng; 95% conf = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>δ¹³CVPDB (%)</td>
<td>δ³²VPDB (%)</td>
<td>SD (±%)</td>
<td>95% conf (±%)</td>
<td>Accuracy (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>Data set 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11k-AC/5βP</td>
<td>N.A.</td>
<td>-22.42</td>
<td>0.42</td>
<td>0.34</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>11k-AC</td>
<td>-16.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5βP</td>
<td>-31.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Data set 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11k-AC/5βP</td>
<td>N.A.</td>
<td>-22.94</td>
<td>0.58</td>
<td>0.51</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>11k-AC</td>
<td>-16.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5βP</td>
<td>-31.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Two data sets were acquired identically on two separate days. No outliers were excluded.
two separate days, with no outliers excluded. The non-modulated analyses result in δ^{13}C_{VPDB} values with a standard deviation of SD (δ^{13}C) = 0.50 ‰, but as a weighted mean of the isotope ratios of the two components due to the coelution, the δ^{13}C_{VPDB} is of little meaning. However, the modulated analyses result in δ^{13}C_{VPDB} values that are accurate for the individual steroids, with average SD (δ^{13}C) = 0.86 ‰ and average accuracies within 0.26 ‰. Modulated results were obtained using 10-fold less steroid mass on column (3 vs 30 ng). This reveals that second-dimension separation of compounds in GC × GC generates accurate and precise isotope ratio measurements acceptable for IRMS analysis.

One of the future challenges to making GC × GCC-IRMS a routine technique is the development of methodology and software for automated detection and integration of modulated peaks. Currently, our data analysis software is not fully automated for this task. As described in detail in the Experimental Section, the peak start and stop times for the first modulation of the analyte(s), and the total the number of peak slices representative of the compound were chosen manually by inspection. The software does automatically determine the remaining peak slice start and stop times based on the modulation frequency used. Once chosen, the same parameters were applied to all runs in a data set to minimize any bias. This was appropriate here because in all analyses the slices of an entire peak eluted isothermally; thus, there were no changes in retention times in the second dimension over subsequent modulation periods. It was also generally observed that small changes in the peak start and stop times associated with a modulated peak did not significantly change the isotope ratio values. Nevertheless, isothermal elution in GC2 is not always possible, highlighting the importance of developing more advanced data processing. In addition, repeatability in GC × GC separation can be compromised by uncontrollable factors such as the fluctuation of pressure and temperature, sample matrix effects, and
stationary-phase degradation and tends to be more important than for 1DGC analysis due to the small time scales and the complexity of the 2D chromatograms. Algorithms have been developed to help align or “warp” 2D chromatograms to achieve better repeatability and allow chemometric analyses of the data, but are still undergoing refinement [45]. These issues are even more important for isotope ratio measurements, as the integration of isotopologue signals can be very sensitive to inaccuracies of peak start and stop definitions. Generalized, data analysis of C-IRMS measurements coupled with complex GC × GC separations must overcome these challenges.

4.4. CONCLUSIONS

A prototype GC × GCC-IRMS system was developed with a configuration consisting of a PTV inlet for sample introduction and solvent diversion, a LMCS enabling the coupling of two GC columns for GC × GC capabilities, and a low dead volume and low inner diameter combustion interface to an IRMS. GC2 slices of steroids peaks well separated in GC1 were successfully reconstructed for carbon isotope ratios resulting in $\delta^{13}C_{VPDB}$ values with average precisions of SD ($\delta^{13}C$) = 0.30 ‰ and average accuracies within 0.34 ‰. In addition, cryogenically sliced peaks of steroids, which coeluted in GC1 but were well separated in GC2, were successfully reconstructed for carbon isotope ratios resulting in $\delta^{13}C_{VPDB}$ values with average precisions of SD ($\delta^{13}C$) = 0.86 ‰ and average accuracies within 0.26 ‰. The successful analysis of simple standard test mixtures investigated in this work unequivocally demonstrate the feasibility of using GC × GC separations with online C-IRMS. Future work will entail further combustion furnace design optimization to help achieve smaller GC2 peak widths allowing better separations in more complex sample analyses. Also, data analysis software and methodology will be advanced to
enable more automated procedures required for the larger data sets that come with more complex samples.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org
REFERENCES


33. Ferchaud, V.; Le Bizec, B.; Montan, F.; Andre, F. Analyst, 1998, 123, 2617-
20.
CHAPTER FIVE

Comprehensive Two-Dimensional GC (GC × GC)-TOF-MS Detection of Urinary Steroids

5.1. INTRODUCTION

Many anti-doping tests target specific endogenous urinary steroids, such as testosterone/epitestosterone (T/E) ratio measurement using GC-MS [1,2,3] or carbon isotope ratio analysis of testosterone and its metabolites using gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS) [4,5,6]. These techniques are limited by the compound separation capability of 1D GC and require extensive and tedious urine sample clean-up procedures, such as high pressure liquid chromatography (HPLC) purification of targeted endogenous urinary steroids before GC analysis.

Comprehensive two-dimensional gas chromatography (GC × GC) coupled to time of flight mass spectrometry (TOF-MS) has been shown recently to be a powerful technique with advantages over conventional GC-MS. GC × GC employs two columns with orthogonal stationary phases resulting in much improved compound separations, over 1D GC, of complex mixtures, such as urinary steroid extracts. In addition, TOF-MS provides full mass spectral information across the whole chromatogram, with detection limits comparable with single ion monitoring GC-MS, allowing non-targeted analysis. While, GC × GC coupled with Flame Ionization detection (GC × GC-FID) has been used as a tool for routine analysis to classify the compounds based on their 1D and 2D retention times, the use of GC × GC-TOF-MS has been demonstrated as an excellent tool for both classifying of compounds and the detailed identification of individual components in complex urinary steroid mixtures.
GC × GC should allow for urine extract analysis without extensive sample cleanup. Most steroids and their metabolites are polar compounds containing one or more polar groups (-OH, >C=O, -COOH). Derivatization is usually applied before GC analysis in order to decrease polarity and to increase volatility and thermal stability. The derivatization reagents most commonly used are, pyridine and acetic anhydride for acetylation [5,6], and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) [9,10-13] and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA)[14-17] for trimethylsilyl (TMS) derivatization. However, derivatized steroids are not ideal for GCC-IRMS analysis because of the addition of at least three external carbons, possibility of isomerizations, and the contamination of the combustion reactor with silicon from TMS derivatives. Therefore, development of chromatography allowing separation of the complex mixture of native urinary steroids is required to ensure the measurements of carbon isotope ratios with good accuracy and precision.

In this work, three column sets, polar-nonpolar, nonpolar-polar, and polar-medium polar, were evaluated for the best dispersion of steroid acetates in 2D separation space. In addition, a thick film polar first dimension (GC1) column (ZB-50 from Phenomenex) and a nonpolar second dimension (GC2) column (DB-5 from Agilent) for reduction of interaction between underivatized, active steroids and capillary walls were selected and interfaced to TOF-MS. Urinary steroids extracts without any HPLC sample cleanup were analyzed by GC × GC- FID and GC × GC - TOF-MS.

5.2. EXPERIMENTAL

Chemicals and Standards. High purity He, N_2, O_2, and CO_2 were purchased from Airgas East (Salem, NH). A mixture of nine steroids (SM9) contained the
following components: 5β-androstan-3α-ol-17-one (etiocholanolone, E), 5α-androstan-3α-ol-17-one (andosterone, A), 5-androsten-3β-ol-17-one (DHEA), 5β-androstan-3α,17β-diol(5βA), 5α-androstan-3α,17β-diol(5αA), 4-androsten-17α-ol-3-one(EpiT), 4-androsten-17β-ol-3-one(T), 5β-pregnane-3α, 20α-diol (5βP), 5α-androstan-3α, 11β-diol-17-one(11-OHA). All steroids were of 99% purity, and were purchased from Steraloids (Newport, RI) with the exception of 5βP which was purchased from Acros Organics USA (Morris Plains, NJ). Chromabond® C18 cartridges (500mg, 6mL) were obtained from Macherey-Nagel (Bethlehem, PA). HPLC grade 2-propanol and methanol were obtained from Mallinckrodt Baker (Phillipsburg, NJ). The nine steroid mixture was prepared in 2-propanol at a concentration of 10 ng/μl for each steroid in the mixture. Pyridine, acetic anhydride, tert-butylmethyleneether (TBME), β-glucuronidase from Escherichia coli, sodium phosphate buffer (0.2M, PH=7), and potassium carbonate buffer (K₂CO₃/KHCO₃ 1:1, w/w, 200 g/L) were purchased from Sigma-Aldrich (St. Louis, MO). All solvents and reagents were of analytical grade.

A standard containing a set of 70 native anabolic steroids and drugs (SMRTL70), normally targeted for doping analysis, was obtained from the Sports Medicine Research & Testing Laboratory (SMRTL). The concentrations spanned two orders of magnitude and are shown in Table 5.1.

**Sample Preparation for Nine Steroid Acetate Mixture.** A mixture of nine steroids (SM9) was prepared by dissolving a known equal amount of each steroid (~1mg) in 1 mL HPLC grade isopropanol and diluted to a concentration of about 10 ng/μL each. Solutions were stored at 4°C when not in use. A 200 microliter aliquot of SM9 (10ng/μL) was dried under nitrogen, acetylated by adding 100μL pyridine and 100μL acetic anhydride and heating at 60 °C for 1 hour, and then evaporated to dryness under nitrogen. The derivatized steroids were reconstituted in 200 μL 2-
Table 5.1. The SMRTL standard mixture (SMRTL70).

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Conc. (ng/μL)</th>
<th>Compound Name</th>
<th>Conc. (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norethandrolone</td>
<td>1.2</td>
<td>Epimetendiol</td>
<td>0.24</td>
</tr>
<tr>
<td>Dromostanolone</td>
<td>1.2</td>
<td>Dehydrochlormethyltestosterone</td>
<td>1.2</td>
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<tr>
<td>Metenolone</td>
<td>1.2</td>
<td>Calusterone</td>
<td>1.2</td>
</tr>
<tr>
<td>Bolasterone</td>
<td>1.2</td>
<td>Calusterone Met.</td>
<td>1.2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.2</td>
<td>6a-Hydroxyandrostenedione</td>
<td>1.2</td>
</tr>
<tr>
<td>Methylandrostenediol</td>
<td>1.2</td>
<td>1-Testosterone</td>
<td>1.2</td>
</tr>
<tr>
<td>Oxandrolone</td>
<td>1.2</td>
<td>Oxymetholone Met.</td>
<td>1.2</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>1.2</td>
<td>Morphine</td>
<td>24</td>
</tr>
<tr>
<td>Fluoxymesterone</td>
<td>1.2</td>
<td>Carboxy-THC</td>
<td>1.2</td>
</tr>
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<td>Boldenone</td>
<td>1.2</td>
<td>Buprenorphine</td>
<td>1.2</td>
</tr>
<tr>
<td>Epitestosterone</td>
<td>0.24</td>
<td>Hydromorphone</td>
<td>24</td>
</tr>
<tr>
<td>Mibolerone</td>
<td>1.2</td>
<td>4-Hydroxytestosterone</td>
<td>1.2</td>
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<tr>
<td>Salbutamol</td>
<td>12</td>
<td>Oxycodeone</td>
<td>24</td>
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<tr>
<td>Clenbuterol</td>
<td>0.24</td>
<td>Oxymorphine</td>
<td>24</td>
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<tr>
<td>19-Noretiococholanone</td>
<td>1.2</td>
<td>Codeine</td>
<td>24</td>
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<tr>
<td>19-Norandrostosterone</td>
<td>0.12</td>
<td>Zeranol</td>
<td>1.2</td>
</tr>
<tr>
<td>Mesterolone Met.</td>
<td>1.2</td>
<td>19-Norclosterol</td>
<td>1.2</td>
</tr>
<tr>
<td>Ethylestrenol &amp; Norethandrolone</td>
<td>1.2</td>
<td>Tamoxifen</td>
<td>1.2</td>
</tr>
<tr>
<td>Methyltestosterone &amp; Methandriol</td>
<td>0.24</td>
<td>Fentanyl</td>
<td>24</td>
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<td>Cyclofenil</td>
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<td>Boldenone Met.</td>
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<td>Formestane</td>
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<tr>
<td>6b-Hydroxymethandienone</td>
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<td>Formosterol</td>
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<tr>
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<td>Salmeterol</td>
<td>12</td>
</tr>
<tr>
<td>3'-Hydroxystanozolol</td>
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<td>Fenoterol</td>
<td>12</td>
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<td>16b-Hydroxyfurazabol</td>
<td>1.2</td>
<td>Letrozole</td>
<td>3.6</td>
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<tr>
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<td>Hydrocodone</td>
<td>24</td>
</tr>
<tr>
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<td>1.2</td>
<td>Oxabolone</td>
<td>1.2</td>
</tr>
<tr>
<td>Methenolone Met.</td>
<td>1.2</td>
<td>1-Androstendione</td>
<td>1.2</td>
</tr>
<tr>
<td>Epioxandrolone</td>
<td>1.2</td>
<td>4-OH-norethisterone</td>
<td>1.2</td>
</tr>
<tr>
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<td>1.2</td>
<td>Hydroxybromantane</td>
<td>4.8</td>
</tr>
<tr>
<td>4b-Hydroxystanozolol</td>
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<td>Bambuterol</td>
<td>1.2</td>
</tr>
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<td>1.2</td>
<td>Finasteride</td>
<td>1.2</td>
</tr>
<tr>
<td>Norbolethone Met. 2</td>
<td>1.2</td>
<td>Ethisterone</td>
<td>1.2</td>
</tr>
<tr>
<td>Oxymesterone</td>
<td>1.2</td>
<td>Terbutaline</td>
<td>12</td>
</tr>
<tr>
<td>Dehydrochlormethyltestosterone</td>
<td>1.2</td>
<td>Pemoline</td>
<td>60</td>
</tr>
</tbody>
</table>
Urine Samples. Free steroids were extracted from a 20 mL urine sample obtained from a 38 year old healthy male subject. The flow chart of urine extraction procedure is shown in Figure 5.1. Chromabond® C18 cartridges (500mg, 6mL) were conditioned with 2 mL of MeOH and 2 mL of water. 20 mL of urine was applied to the column. After washing with 2 mL of water, the residue was eluted with 2 × 1 mL of MeOH and evaporated to dryness under nitrogen. The dried eluate was dissolved in 1 mL of sodium phosphate buffer (0.2 M, PH=7) and 5 mL of TBME) was added. After shaking for 5 min and centrifugation at 1200 g for 5 min, the organic layer was discarded. Then 100 uL of β-glucuronidase was added and the sample was incubated at 50 °C for 1 hour. After cooling to room temperature 500 uL of potassium carbonate buffer (K₂CO₃/KHCO₃ 1:1, w/w, 200 g/L) was added. The aqueous layer was extracted with 2 × 5 mL of TBME, shaken for 5 min and centrifuged (1200 g, 5 min), and the organic layers were combined in a conical test-tube and evaporated to dryness under nitrogen. The residue was redissolved in 2 × 100 uL of 2-isopropanol, and transferred to an auto-sampler vial for GC analysis, as shown in Figure 5.1. Before the urinary steroid extract was loaded onto GC × GC-TOF-MS, the urinary steroid extract was further diluted [19]. For GC × GC-FID analysis, urinary steroids were acetylated using the same procedure as SM9. No HPLC cleanup was performed on the steroid extract samples.

GC × GC-FID System. An HP 6890 GC (Plo Alto, CA), equipped with a flame ionization detector (FID), was coupled to a Longitudinal Modulation Cryogenic System (LMCS, Chromatographic Concepts, Sandringham, Australia) for GC × GC-FID analysis of SM9-AC [20]. All analyses are performed with He carrier gas using a programmable temperature vaporization (PTV) inlet (Agilent Technologies, Menlo Park, CA).
Figure 5.1. Flow diagram for the sample preparation procedure of urinary steroid metabolites. Note: no HPLC sample cleanup was performed.
Park, CA) in solvent vent mode to divert solvent away from column. The PTV inlet was held at 50 °C for 1 min and then ramped to 300 °C at 725 °C/min. The PTV vent time was 0.9 min, vent pressure was 5 psi, vent flow was 100 ml/min, and purge time was 2.5 min.

A 1-uL aliquot of 10 ng/uL SM9-AC solution was injected with a 6-s modulation for each GC × GC-FID analysis. Three column sets were evaluated; a nonpolar-polar (NP-P) column set with a BPX-5 (30 m × 0.25 mm × 0.25um) for GC1 and BPX-50(2 m × 0.1 mm × 0.1 um) for GC2; polar-nonpolar (P-NP) column set with BPX-50 (30 m × 0.25 mm × 0.25um) for GC1 and BPX-5(2 m × 0.1 mm × 0.1 um) for GC2; and polar-medium polar (P-midP) column set with BPX-50 (30 m × 0.25 mm × 0.25um) for GC1 and BPX-35 (2 m × 0.1 mm × 0.1 um) for GC2. All columns were from SGE (Austin, TX). The head pressure was set to 33 psi at 80 °C with a flow rate of 1.0 mL/min run in constant-flow mode. The GC1 oven was held at 80 °C for 2 min, ramped to 240 °C at 40 °C /min, then ramped to 255 °C at 10 °C /min, then ramped to 270 °C at 1 °C /min, where it was held for 5 min, and finally ramped to 300 °C at 1.5 °C /min, where it was held for 2 min. During operation, the LMCS was maintained at 130 °C, using liquid CO₂. N₂ continuously purged the outside of the LMCS capillary to flush away any condensation. A home-built system and software (Saxicab) was used for data acquisition and 2D contour plots were then generated using Transform version 3.3 (Fortner Research LLC, Sterling, VA).

**GC × GC-TOF-MS System.** A LECO Pegasus 4D (LECO Corp., St. Joseph, MI) GC × GC-TOF-MS was used for the analysis of a native urinary steroid extract, a SMRTL70 standard, and a urinary steroid extract spiked with SMRTL70 at a data collection rate of 50 Hz. The same PTV inlet and parameters were as described in the GC × GC-FID system for all analyses.
A 1-uL aliquot of SMRTL70 (~1.2 ng/uL), 1-uL of 1:8 dilution of urinary steroid extract from 20 mL urine, and 1-uL of urinary steroid extract (1:8 dilution from the stock solution) spiked with ~1.2 ng/uL of SMRTL70 solution was injected with a 3-s modulated analysis. GC1 was a 30 m × 0.25 mm i.d. × 0.50 um film ZB-50 (50% phenyl 50% dimethyl polysiloxane, Phenomenex), and GC2 was a 1 m × 0.10 mm i.d. × 0.10 um film DB-5 (5% phenyl 50% dimethyl polysiloxane, Agilent). The head pressure was set to 28 psi at 45°C with a flow rate of 1.0 mL/min run in constant-flow mode. The GC1 oven temperature program was: 45°C for 1 min, ramped to 80°C at 40°C /min for 1 min, then ramped to 280°C at 20°C /min, then ramped to 300°C at 0.5°C /min, where it was held for 3 min, and finally ramped to 310°C at 5°C /min, where it was held for 10 min. During operation, the modulator was operated at a trapping temperature of 50°C, using liquid N2. Data acquisition and processing were performed using ChromaTOF version 4.21 (LECO Corp.).

5.3. RESULTS AND DISCUSSION

**GC × GC-FID for SM9-AC Analysis.** Figure 5.2 shows separation of steroid acetates in SM9 using three different GC × GC column combinations; NP-P (nonpolar-polar), P-NP (polar-nonpolar), and P-midP (polar-midpolar). Peaks are not specifically identified. For peaks that are partially or completely overlapped on the GC1 column, such as compounds 4, 5 and 6 in Figure 5.2(A) and compounds 3 and 4 in Figure 5.2 (B), are well separated on the GC2 column. It can be seen that the NP-P set provides the greatest dispersion of steroids, while the P-NP and P-midP sets does not provide much dispersion in GC2, and P-NP has better dispersion than P-midP. However, the 2D peak width at full width half maximum (FWHM) of SM9-AC for NP-P column set (~440 ms) is more than 2 fold broader than the P-midP column set (~180 ms). The column set P-NP achieved 2D peak width at FWHM of
about 260 ms. Due to its moderate dispersion capacity and suitable 2D peak widths, the P-NP set is an appropriate choice for steroid analysis.

Figure 5.3 is a repeat of the NP-P analysis using two different samples; SM9-AC shown in Figure 5.3 (A) and U-AC, an acetylated extract of urine shown in Figure 5.3 (B). Individual steroid acetate in the mixture of SM9-AC was run at the same GC × GC-FID conditions as the SM9-AC for compound identification. Retention time matching enabled tentative assignment of all 9 steroids. Baseline separation was achieved for 7 of the 9 steroids in the standard mix. For example, 5βA-AC labeled as 4, 5αA-AC (5), and EpiT-AC (6) are overlapped at 22 min on GC1 column for both SM9-AC and urine spiked with SM9-AC samples, but are well separated on GC2 column, as shown in Figure 5.3 (A) and 5.3 (B). These data demonstrate GC × GC separations for urinary steroids in a form that approaches potential use in GC × GCC-IRMS, which requires good peak resolution for isotope ratio measurements with suitable precision and accuracy. More importantly, these data suggests that GC × GCC-IRMS may be used to eliminate time consuming HPLC fraction collections, which are also prone to isotopic fractionation if entire peaks are not collected. Note that the concentration range of the steroids in the urine sample varies by 100-fold.

**GC × GC-TOF-MS of Urinary Steroid Extract Analysis.** Figure 5.4 shows single ion monitoring (SIM) GC × GC contour plots of native (underivatized) steroids for (A) the standard mixture of SMRTL70(average ~ 1.2 ng/μL); (B) urinary steroid extract from urine; and (C) urinary steroid extract spiked with SMRTL70(∼1.2 ng/μL), which were all analyzed by GC × GC-TOF-MS. A partial total ion chromatogram was plotted using m/z 54, 55, 67, 83, 84, 95, 97-101, 108-111 signals in order to eliminate signal due to column bleed. The results show that the GC peak shapes are symmetric in both GC1 and GC2 dimensions. It can be seen that some
Figure 5.2. Comparison of separations with three different column sets applied to nine endogenous steroids as acetates(SM9). (A) NP-P; (B) P-NP; (C) P-midP. Dispersion is best with the NP-P, but 2D peak width is worst.
Figure 5.3. GC × GC-FID of SM9-AC. (A) Mixture consisting of 9 steroid acetate standards at approximately equal concentration. (B) Urinary steroids extracted and prepared in the Ithaca Lab. Color scale is adjusted to emphasize the major, relevant steroid peaks; lower intensity peaks are present but not visible. 1, E-AC; 2, A-AC; 3, DHEA-AC; 4, 5βA-AC; 5, 5αA-AC; 6, EpiT-AC; 7, T-AC; 8, 5βP-AC; 9, 11-OHA-AC.
groups of components in the SMRTL70 standard are partially or completely overlapped on the GC1 column (Figure 5.4A), such as S9, S10, & S11, but are well separated on the GC2 column. However, the chromatogram of the urinary steroid extract spiked with SMRTL70 standard sample shown in Figure 5.4(C) reveals that some compounds in SMRTL70 standard coelute with those in the urinary steroid extract, but can be resolved with mass spectrometry.

Figure 5.5 depicts a flow chart for the GC × GC-TOF-MS data analysis. The mass spectra and the GC1 and GC2 retention times of compounds in SMRTL70 standard were used to create a database. The database was used to search for SMRTL70 compounds in the urine extract and the urine extract spiked with the standard. The right-side panels in Figure 5.4 show an expanded region where twenty-six SMRTL70 compounds appear, designated S-8 through to S-33. The results also show that the urinary steroid extract contains one detected SMRTL70 peak named S-12. The mass spectra (MS) of the labeled peaks reveal four peaks with overlapping retention times for endogenous urine peaks and the spiked SMRTL70 peaks: S-11, S-14, S-24, and S-32. For example, the MS at the retention time for S-11 in urine extract does not match the SMRTL70 S-11 mass spectrum, indicating an endogenous overlap. These preliminary data demonstrate the feasibility of using GC × GC-TOF-MS of urinary steroid extracts, without extensive sample cleanup, for the detection of targeted and unknown steroids.

5.4. CONCLUSIONS

In summary, a polar-nonpolar column set was shown to achieve sufficient peak separation for native (underivatized) steroids and other drugs in a complex urine matrix using GC × GC-TOF-MS. The exogenous steroids and drugs spiked in the urinary steroid extract have a mostly unique separation space compared to the
**Figure 5.4.** GC × GC-TOF-MS 2D SIM* (A) SMRTL70, (B) urinary steroid extract, and (C) urinary steroid extract spiked with SMRTL70.  * Single ion monitoring of m/z = 54, 55, 67, 83, 84, 95, 97-101, 108-111, chosen to eliminate column bleed.
**Figure 5.5.** Flow chart for GC × GC-TOF-MS data analysis.
endogenous substances. The coupling of GC × GC to TOF-MS, which meets the requirement of fast detection for GC × GC, offers a good opportunity to explore the GC × GC separations of complex samples, and also identify the non-targeted steroid components of all urinary steroids that are different from the conventional targeted compounds. This technique will enable the detection of previously uncharacterized or unknown designer steroids in urine.

5.5. ACKNOWLEDGEMENT

This work was supported by the Partnership for Clean Competition (PCC) and the United States Anti-doping Agency (USADA). I thank Dr. Jonathan P. Danaceau of the Sports Medicine Research & Testing Laboratory (Salt Lake City, UT) for providing us the SMRTL70 standard of native anabolic steroids and drugs. I also thank Dr. Larry Bowers for many helpful discussions, and Professor Gavin L. Sacks and Dr. Bruce Pan for assistance in performing the GC × GC-TOF-MS steroid analyses.
REFERENCES


CHAPTER SIX

Conclusions and Future Directions for Carbon Isotope Ratio Analysis of Steroids

6.1. CONCLUSIONS

Procedures to create steroid isotopic standards and to standardize methodology for commonly analyzed steroids by on GCC-IRMS in doping test to achieve more uniform results between antidoping laboratories have been developed in this thesis. The calibration traceability procedure created in the uniform steroid isotopic standard analysis for use in calibration of GCC-IRMS has ensured there is no significant differential isotopic fractionation associated with the different flow path for steroid samples and reference CO₂ gas during the measurement of carbon isotope ratios of the steroids. The protocol used to prepare the steroid isotopic standards in ampoule containers maintains isotopic integrity to uniformity within 0.09‰ from ampoule to ampoule. These standards were distributed to twelve antidoping laboratories worldwide for Interlaboratory Comparison Tests. Eventually, they would be used daily as working standards for the calibration of GCC-IRMS data between antidoping laboratories.

The steroid studies in doping tests are to deal with complex real-world samples, which often require cumbersome sample cleanup and preconcentration, such as with HPLC, prior to GCC-IRMS analysis. The alternative way to solve the chromatographic limitations of the single GC (1D) C-IRMS is to build up a GC × GCC-IRMS system. The prototype GC × GCC-IRMS system with optimized parameters has been created and demonstrated for the feasibility of successful carbon isotope ratio analysis in a simple standard test mixture. To apply this technique to a
complex urine extract, the molecular MS, GC × GC-TOF-MS has been explored, as a prelude study for GC × GCC-IRMS, to provide not only structural information of non-targeted steroids in urine, but also good peak resolution and detection for isotope ratio measurements.

6.2. FUTURE DIRECTIONS

IRMS differs from the more extensively used molecular MS in that IRMS is unable to elucidate the structure of a compound. GC × GC-TOF-MS is a good complement for IRMS. Future work of coupling the GC × GC-TOF-MS to the IRMS via a home built combustion interface needs to be implemented for synthetic steroid detection.

As mentioned in Chapter 4, future work needs to be done to help achieve smaller GC2 peak widths allowing better separations in complex urine samples, such as further optimization of combustion furnace. The data analysis software and methodology need to be further advanced and updated to enable more automated procedures allowing requiring larger data sets resulting from complex urinary steroid analyses.