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Quantitative confirmation of testosterone and epitestosterone in human urine by LC/Q-ToF mass spectrometry for doping control

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Abstract

Testosterone (T) is the primary male sex hormone. In addition to the development of secondary sex characteristics, testosterone has anabolic effects including increases in muscle size and strength and increases in lean body mass, making it an attractive candidate to enhance athletic performance. In the case of exogenous administration of testosterone, the ratio of testosterone to its isomer, epitestosterone (E), is elevated. WADA has set a standard for T/E ratios of 4.0 as indicative of possible exogenous testosterone administration. Typically, a sample that screens for a T/E ratio above that threshold is then subjected to quantitative confirmation by GC/MS. This methodology, however, can be limited due to sensitivity issues as well as a limited number of qualifier ions that can be used for unambiguous identification. We have developed a confirmation method which uses liquid/liquid extraction, followed by room temperature Girard P derivatization, and analysis using LC/MS-QToF. We observe a number of advantages over conventional GC/MS analysis. Analysis time is decreased. Sensitivity is increased, resulting in limits of detection of 2 and 0.5 ng/ml for testosterone and epitestosterone, respectively. The number of diagnostic qualifier ions is also increased allowing more confident identification of the analytes. Finally, while this method has been developed on a QToF instrument, it should be easily transferable to any tandem LC/MS/MS system.

Materials and Methods

Sample Preparation

Three mL of synthetic urine (Dynatek, Lenexa, KS, USA) was added to a silanized glass test tube containing 300 ng of d_3-testosterone and 75 ng of d_3-epitestosterone. After incubation with β-glucuronidase from *E. coli* K12 (Roche, Indianapolis, IN, USA) and buffering, the

samples were extracted with 6 mL of methyl tert-butyl ether (MTBE). The organic layer was transferred to a silanized 13 x 100 mm glass tube and dried under a gentle stream of air at 40°C. Samples were reconstituted in 20 µL of methanol followed by 80 µL of 1M Girard's Reagent P in 50 mM ammonium acetate buffer (pH 4.2). Samples were then transferred to auto sampler vials and 20 µL was injected after a 1-hr incubation time at room temperature.

LC-MS-MS

LC-MS-MS equipment consisted of an Agilent 1100 series HPLC connected to an Applied Biosystems QStar-XL tandem MS equipped with a Turboionspray$^®$ (heated electrospray) ion source. The HPLC column used was a Phenomenex Luna C18(2) column with 2.0 x 100 mm dimensions and 3 micron particles with 1.0×10^2 Å pore size. The column compartment was heated to 50°C during the analysis. HPLC mobile phase A consisted of 0.1% formic acid in MilliQ water and mobile phase B consisted of 0.1% formic acid in methanol. A mobile phase flow rate of 350 µL/min was employed with the following gradient: After equilibration at 75% A, the mobile phase composition was ramped up to 95% B over 9 min and held for 1.5 min, followed by a return to 75% A over 0.5 min.

The mass spectrometer was operated in positive ion mode with source and fragmentation conditions optimized for the analysis. For the first 2.5 min of the analytical run, flow was diverted to waste via an automatic post-column switching valve to avoid excessive contamination of the ion source with Girard's Reagent P. The quasi molecular ions at m/z 422.3 (GRP derivatized testosterone and epitestosterone) and 425.3 (GRP derivatized d$_3$-testosterone and d$_3$-epitestosterone) were fragmented at -46 V, which resulted in approximately 10% of the precursor ion remaining after fragmentation.

Results

The structure of GRP derivatized testosterone is shown in Fig. 1A. Q-ToF mass spectra for GRP derivatized testosterone and epitestosterone are shown in Figs. 2A and 2B. Characteristic fragment ions dominate the mass spectra, such as, m/z 343 (loss of 79) representing the loss of the pyridinium moiety (C$_5$H$_5$N) and resulting cyclization at C$_4$, and m/z 315 (loss of 107) representing the loss of the pyridinium plus a carbon and an oxygen (C$_6$H$_5$NO) with cyclization at the same C$_4$ position. On the lower MW end of the spectrum, ions 80.05, 94.07, 109.08, 120.05, and 137.07 all represent typical fragments of the GP hydrazone group.[1]
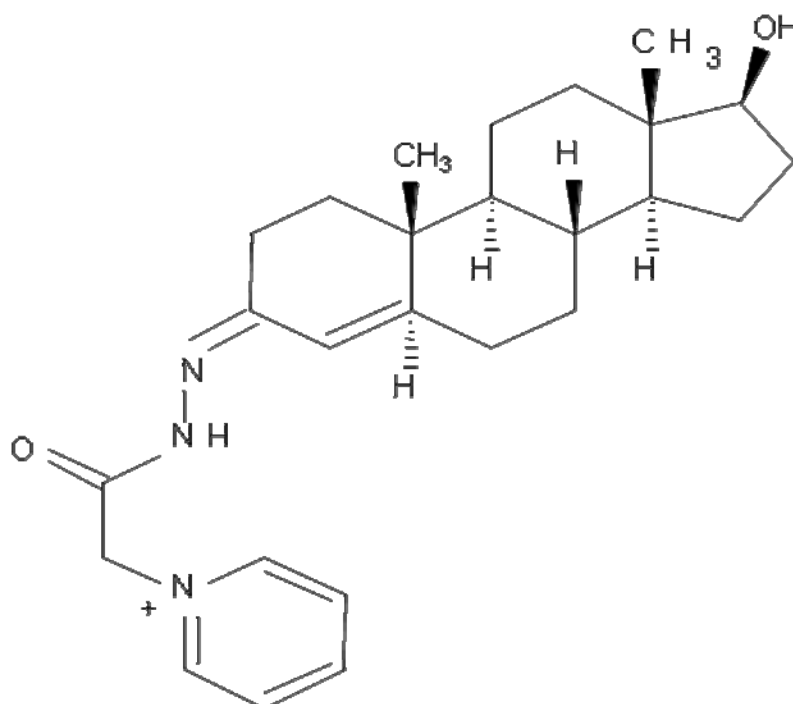
Fig. 1. A. Chemical structure of GRP derivatized testosterone.

Fig. 3A shows an extracted ion chromatogram (m/z 315) of a low standard (5 ng/mL E and 20 ng/mL T). This shows clear chromatographic separation of the two isomers and baseline resolution of each peak. The double peak at 6.88 and 7.23 is most likely the result of cis and trans isomers of GRP derivatized testosterone, a phenomenon we have seen with other GRP derivatized steroids.[2] An extracted real urine sample is shown in Fig. 3B. Endogenous peaks at 7.68 and 7.95 are present, but show baseline separation from the analytes of interest

Method Validation

For method validation purposes, we evaluated linearity, specificity in relation to other endogenous compounds, accuracy, and precision. To determine linearity, calibration curves of 1-100 ng/mL E and 4-400 ng/mL T, respectively, were extracted and analyzed. In each case, a 1/x weighted quadratic equation was used, resulting in r values of 0.999 for testosterone and epitestosterone over 2 orders of magnitude.

Even though limits of detection (LODs) are typically defined by a 3:1 signal to noise ratio, for this analysis, the LOD was reported as the lowest dilution that gave a signal to noise of 5:1 and met the WADA ion ratio criteria for qualitative confirmations[3] when compared to the 25E/100T standard. This resulted in an LOD of 0.5 ng/mL for E and 2.0 ng/mL for T. The

limit of quantitation (LOQ) was determined to be the lowest point on the calibration curve, or 1.0 ng/mL for E and 4.0 ng/mL for T.

Accuracy was assessed by fortifying quality control samples at three concentrations for each analyte. Synthetic urine was fortified with epitestosterone at 2, 10, and 50 ng/mL. Testosterone was fortified at 8, 40, and 200 ng/mL. Mean quantitation results of all quality control samples were within 15% of target for all concentrations of testosterone and epitestosterone.

In addition to calibration standards and quality control samples, we wanted to determine the specificity of this method in relation to other endogenous compounds that could interfere with T and E. The sample depicted in Fig. 3B was extracted and analyzed in triplicate to compare this method to traditional GC/MS analysis. The results from this experiment revealed T and E concentrations of 30.4 ± 0.9 ng/mL (S.D.; N=3) and 29.0 ± 0.2 ng/mL (S.D.; N=3), respectively, resulting in a T/E ratio of 1.05 ± 0.03 (S.D.; N=3). This compares quite favorably with the T/E ratio of 1.03 for this urine pool established by traditional GC/MS analysis. We have also analyzed approximately 15 athlete samples using this method and none of them have shown any confounding effects due to either the urine matrix or endogenous, related compounds.
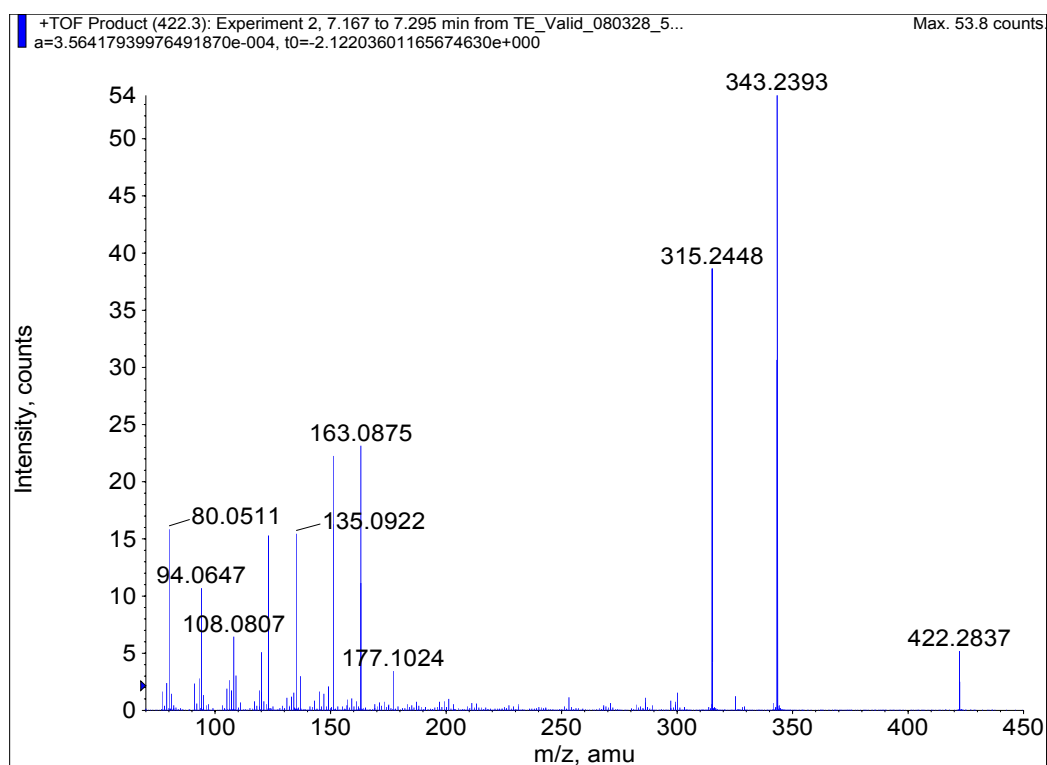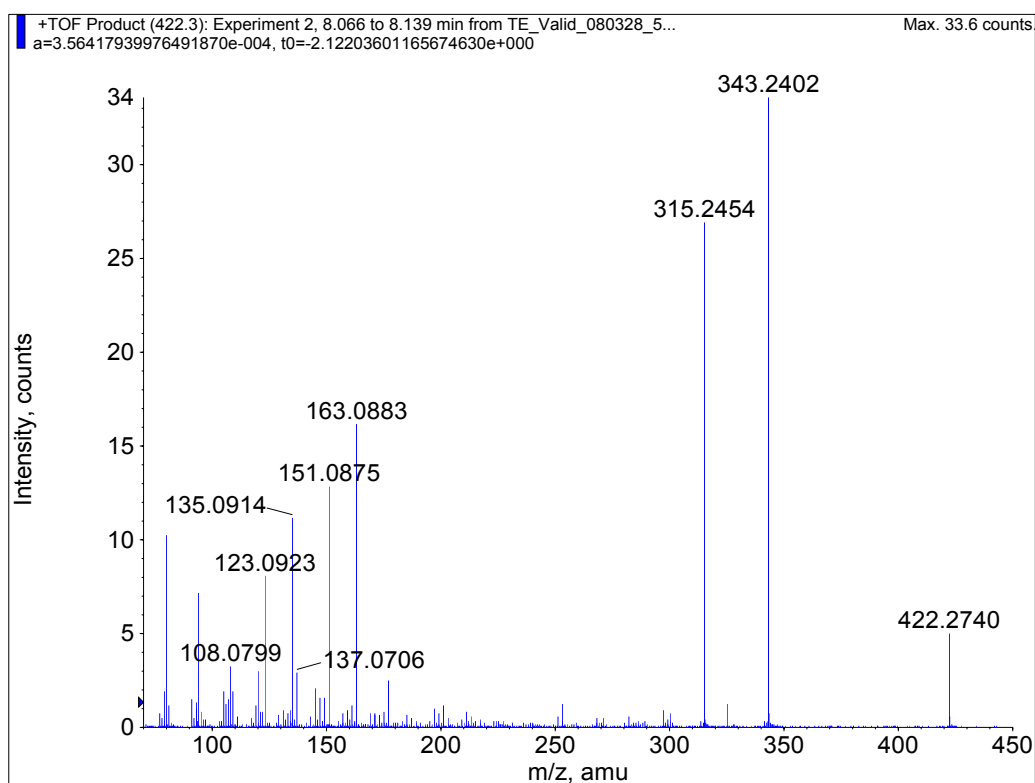
Fig. 2. A. ToF product mass spectra of testosterone at 7.23 min.

Discussion

We have developed an LC/MS/MS method for the quantitative determination of testosterone to epitestosterone ratios that utilizes Girard Reagent P derivatization and quadrupole/time of flight mass spectrometric analysis. It makes use of the previously seldom utilized GRP reagent as a derivatization tool for dramatically increasing the ability to identify and quantify poorly ionizing compounds such as steroids.[1,4,5] This method offers several advantages over traditional confirmation by TMS derivatization and analysis by GC/MS, including analysis speed, improved limits of detection and quantification, ease of derivatization, and the number of unambiguous diagnostic ions in the mass spectra.

B. ToF product mass spectra of epitestosterone at 8.1 min.

Two of the most significant advantages attributable to this derivatization are the high sensitivity of the assay and the quality of the mass spectra afforded by both the tandem LC/MS analysis and the GRP derivatization. As mentioned above and seen in Figs. 2A and 2B, the number of diagnostic qualification ions is also very useful. While additional peaks are present in Fig. 3B at 7.68 and 7.95 min., both of these have ion ratios that differ significantly from T and E. Unlike a quadrupole instrument, the QToF mass spectrometer detection system generates full "scan" data with high mass acuracy, while at the same time maintaining relatively high sensitivity, eliminating the need to choose between the sensitivity afforded by selected MRM transitions and the information provided by a full product scan.

Other improvements include the reduction in analysis time, from 25 min. to 15 min, and the chromatography of this method compared to traditional GC/MS analysis. In our experience, epitestosterone in particular can be subject to interference from other endogenous steroids, especially when it is present in low concentrations. In addition to affecting quantitation, the ion ratios can also be impacted, making it difficult to achieve the requirements set forth by WADA for qualitative confirmations.[3]
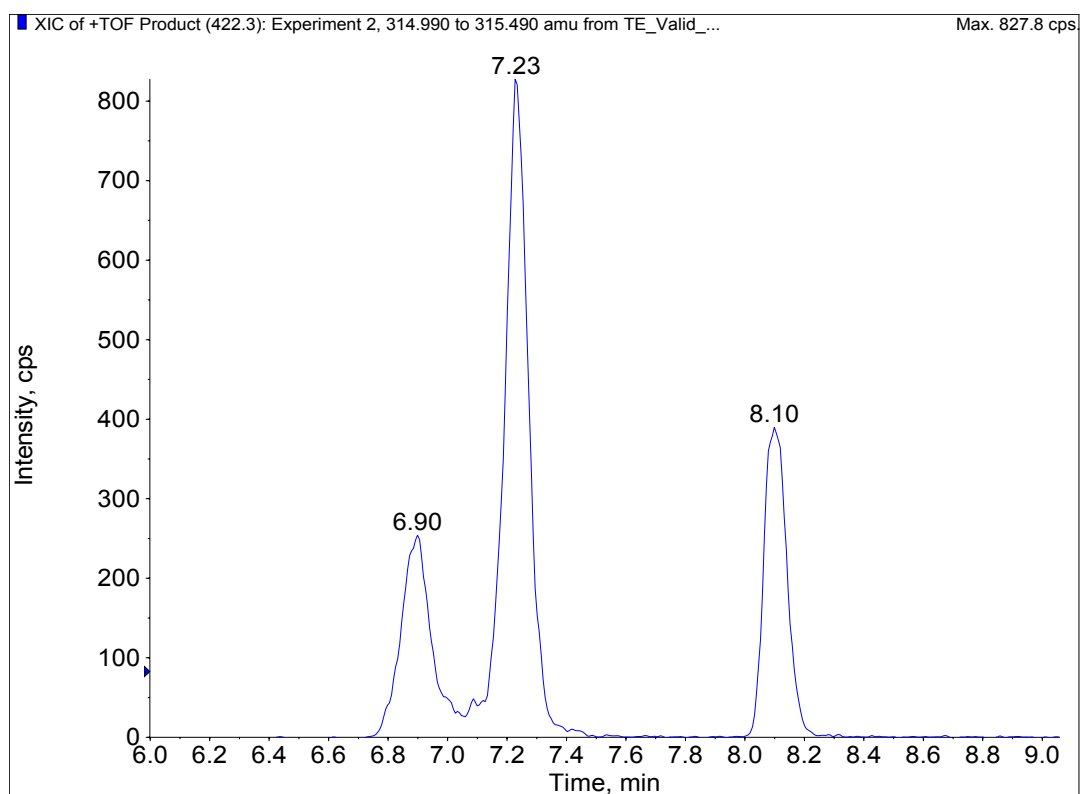
Fig. 3. A. Extracted product ion chromatogram at m/z 315.2 of a calibration standard extracted from synthetic urine containing 5 ng/mL epitestosterone and 20 ng/mL testosterone. GRP derivatized testosterone and epitestosterone elute at 7.23 and 8.10 min., respectively. Note the minor peak at 6.9 min due to a stereoisomer of derivatized testosterone.

Finally, limits of detection and quantification are improved in this method. With LODs of 2 and 0.5 and LOQs of 4 and 1 for T and E, respectively, this method clearly outperforms the GC/MS method as we use it in this lab for both quantitation at these low concentrations as well as unambiguous qualitative identification.

This method should contribute to the growing use of LC/MS for the identification of steroids for doping control.[6-9] Although this method was developed using a QToF instrument, we believe that this method should be readily applicable to other tandem LC/MS systems, as the dynamic range and sensitivity of tandem quadrupole systems should at least equal and possibly exceed the results presented in this manuscript.
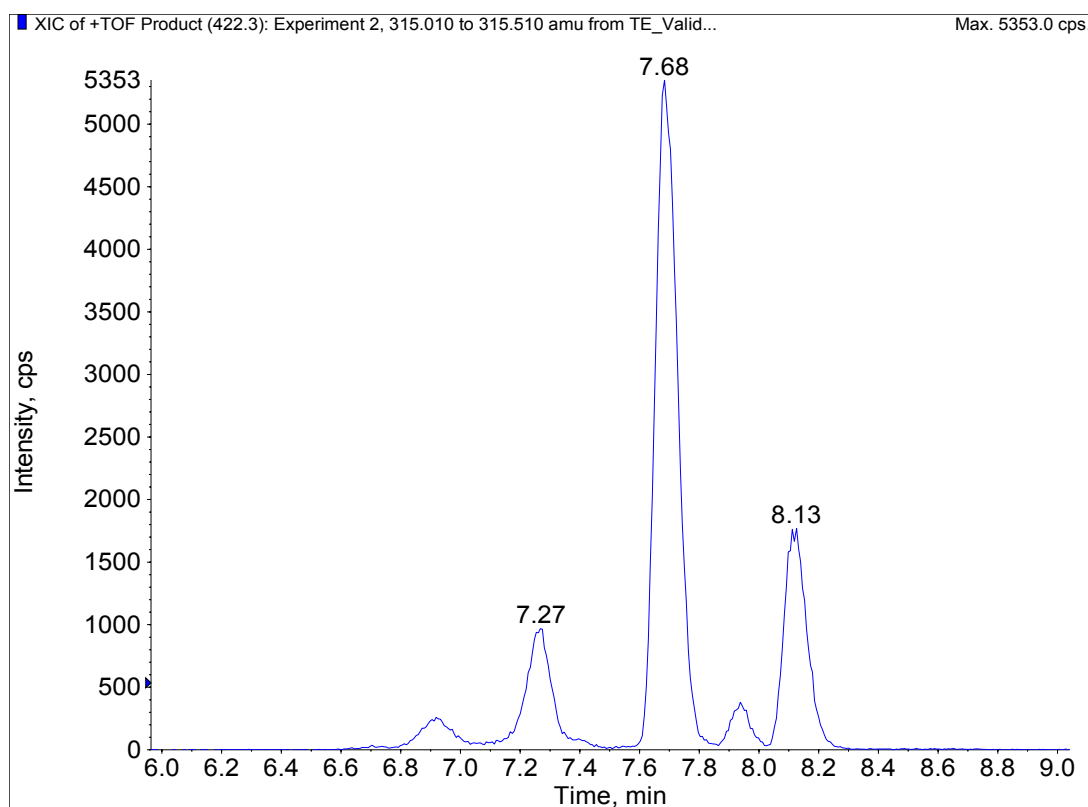
Fig. 3. B. Extracted product ion chromatogram at m/z 315.2 of an extracted, derivatized pooled urine sample. Testosterone and Epitestosterone elute at 7.27 and 8.13 min., respectively. Again, note the minor peak at 6.9 min. representing the stereoisomer of derivatized testosterone. Endogenous peaks at 7.68 and 7.95 are baseline separated from testosterone and Epitestosterone and have different major product ion ratios (data not shown).

Further details are available in the original publication of this material in:

Danaceau J, Morrison M, Slawson M. (2008) Quantitative confirmation of testosterone and epitestosterone in human urine by LC/Q-ToF mass spectrometry for doping control. J. Mass Spectrom 43, 993-1001.

References

1.   Griffiths WJ, Wang Y, Alvelius G, Liu S, Bodin K, Sjovall J.(2006) Analysis of oxysterols by electrospray tandem mass spectrometry. *J Am. Soc. Mass Spectrom.* **17**: 341-362.

2.   Borges C, Miller N, Shelby M, Hansen M, White C, Slawson M, Monti C, Crouch DJ.(2007) Analysis of a challenging subset of World Anti-Doping Agency-banned steroids and antiestrogens by LC-MS-MS. *J. Analyt. Tox.* **31**: 125-131.

3.   World Anti-Doping Agency. WADA Technical Document - TD2003IDCR. Identification Criteria for Qualitative Assays Incorporating Chromatography and Mass Spectrometry (2003) http://www.wada-ama.org/rtecontent/document/criteria_1_2.pdf (25.04.2008)

4.   Khan MA, Wang Y, Heidelberger S, Alvelius G, Liu S, Sjovall J, Griffiths WJ.(2006) Analysis of derivatised steroids by matrix-assisted laser desorption/ionisation and post-source decay mass spectrometry. *Steroids* **71**: 42-53.

5.   Wang Y, Hornshaw M, Alvelius G, Bodin K, Liu S, Sjovall J, Griffiths WJ.(2006) Matrix-assisted laser desorption/ionization high-energy collision-induced dissociation of steroids: Analysis of oxysterols in rat brain. *Anal. Chem.* **78**: 164-173.

6.   Thevis M, Bommerich U, Opfermann G, Schanzer W.(2005) Characterization of chmically modified steroids for doping control purposes by electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **40**: 494-502.

7.   Thevis M, Geyer H, Mareck U, Schanzer W.(2005) Screening for unknown synthetic steroids in human urine by liquid chromatography-tandem mass spectrometry. *J. Mass Spectrom.* **40**: 955-962.

8.   Williams TM, Kind AJ, Houghton E, Hill DW.(1999) Electrospray collision-induced dissociation of testosterone and testosterone hydroxy analogs. *J. Mass Spectrom.* **34**: 206-216.

9.   Kim Y, Lee Y, Kim M, Yim Y-H, Lee W.(2000) Determination of the metabolites of gestrinone in human urine by high performance liquid chromatography, liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry. *Rapid Comm. Mass Spectrometry* **14**: 1717-1726.