Application Note

Forensics

Hydrogen Carrier Gas for GC/MS/MS Analysis of Steroids in Urine in Under 10 Minutes

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Abstract

This application note presents a developed and validated GC/MS/MS method for the quantitative analysis of 14 endogenous anabolic steroids in urine, using hydrogen (H_2) carrier gas. This method successfully reduced analysis time to under 10 minutes, a significant 40% reduction compared to the traditional 17-minute method using helium (He) carrier gas. Calibration performance was excellent across an extended range, meeting the limits of identification (LOIs) set by the World Anti-Doping Agency (WADA). Validation results showed that for all analyzed samples, z-scores were less than one, indicating high method accuracy. This robust and efficient method ensures reliable quantification of endogenous anabolic steroids in urine, providing a viable and environmentally sustainable alternative to helium in the face of global shortages and rising costs.

Introduction

Anabolic-androgenic steroids (AAS) are a class of steroidal hormones related to testosterone. Testosterone is naturally produced in the human body and is primarily excreted as a conjugate with glucuronide or sulfate. AAS exhibit both androgenic effects, associated with masculinization and virilization, and anabolic effects, linked to protein synthesis and muscle growth. Despite the ongoing debate about their efficacy and potential risks, the use of AAS is prevalent in power sports for muscle mass enhancement and in endurance sports for improved recovery. Furthermore, various indirect steroid doping strategies can similarly elevate testosterone levels, complicating the detection and regulation of doping practices.

The complexity of AAS detection is heightened by the fact that it must comply with the stringent requirements set by WADA. To maintain WADA accreditation, antidoping laboratories must achieve high sensitivity, selectivity, and reproducibility, along with quantitative capability for endogenous steroids. This entails accurate quantitation of AAS across a wide concentration range (ng/mL to µg/mL) for endogenous steroids. For exogenous steroids, where a zero-tolerance rule exists, laboratories must reach very low limits of detection (LODs) that are comparable to the minimum required performance levels (MRPLs) set by WADA.[1]

Exogenous AAS are not naturally present in a human body. Because there is a zero-tolerance policy in place for exogenous AAS, their mere identification leads to a doping rule violation, and hence, there is no need to quantitate them. For these compounds, WADA sets an MRPL, which is the minimum concentration of the substance in urine that a laboratory must be able to detect routinely. The LOD should be less than half of this value.

Endogenous AAS are all naturally present in the bodily fluids of both males and females. However, some endogenous AAS (for example, testosterone and DHEA) are also available as pharmaceutical drugs or marketed as "food supplements" in some countries. These drugs and supplements remain prohibited by WADA, but detecting their use is much more difficult due to the natural presence of endogenous AAS. Therefore, the detection of possible misuse is determined by evaluating AAS concentrations and ratios over a long period in the individual athlete's steroid passport. If significant fluctuations from the long-term mean appear, the sample can be forwarded to isotope ratio mass spectrometry (IRMS), which can unequivocally show the synthetic origin of the steroid. An alternative for IRMS analysis is the detection of steroid esters in the blood.

For both exogenous and endogenous steroids, anti-doping laboratories use initial testing procedures (ITPs) as a first stage to identify suspicious findings and a confirmation (CP) method to confirm these findings. This sample analysis process in an antidoping laboratory can be summarized in several steps:

1. An anonymous (coded) sample arrives at the lab (split into an A and B samples). The A sample is opened, and an aliquot is taken from it. The B sample remains sealed and is stored immediately in the freezer.
2. Sample extraction is performed using the aliquot from the A sample. Following extraction, the sample is injected into the GC/Q-TOF system. Further details can be found in a previous application note.[2] Quantification of endogenous AAS is completed using this system, and values are reported to the appropriate authorities.
3. The authorities responsible for sample collection have entered their data into the WADA database, allowing a link to be made between the sample code and the individual athlete. Next, an evaluation is made between the newly measured concentrations versus those obtained from previous doping control samples from this athlete. If these concentrations from ITP are not in-line with the expected ranges, the authorities warn the laboratory that the initially reported values require confirmation.
4. For confirmation, a second aliquot of the A sample is analyzed with the method as described in this application note.
5. If the second analysis confirms the ITP results, IRMS analysis will typically be performed on the A sample to see if the origin of the AAS is natural or from a pharmaceutical product.
6. If the athlete disagrees with a positive result from IRMS, a new analysis on the untouched B sample can be requested.

Our previous application note described a multitarget GC/Q-TOF method for ITP.[2] The newly developed hydrogen (H_2) method presented here is used during confirmation of the results from the ITP.

Triple quadrupole GC/MS/MS[3] and high-resolution GC/Q-TOF[4] are essential techniques, especially for the quantification of AAS and the separation of stereoisomers that LC/MS techniques often fail to resolve. Traditionally, helium (He) has been the carrier gas of choice for GC/MS analyses due to its optimal performance characteristics. However, recurring global helium shortages have necessitated the search for viable alternatives to ensure the continuity and reliability of analytical measurements.

Hydrogen could offer several advantages over helium as a carrier gas in GC/MS, including the fact that it is more readily available and significantly less expensive than helium. Hydrogen can also be produced sustainably through the electrolysis of water using green electricity, making it a more environmentally friendly option. Additionally, hydrogen keeps the electron ionization (EI) source cleaner during operation, reducing maintenance needs and increasing instrument uptime and lab productivity.

This application note presents a developed and validated GC/MS/MS method for the effective and reliable measurement of 14 endogenous AAS in urine, using electron ionization with hydrogen as the carrier gas. Method performance was maintained, meeting the same LOIs as the method using helium as the carrier gas. The feasibility of the transition to hydrogen as a carrier gas in GC/MS/MS applications provides antidoping laboratories with a robust alternative that ensures the continuity of high-quality analytical results in the face of helium supply challenges.

Experimental

Samples

A Biotage automated solid phase extraction (SPE) instrument was used for sample preparation and extraction from urine. Solvent evaporation under oxygen-free nitrogen (OFN) was performed with a Biotage TurboVap. Each analytical batch contained:

- Samples to be analyzed in duplicate (one aliquot with SPE and hydrolysis and one aliquot without SPE and hydrolysis)
- A system blank
- A calibration curve
- Quality control and quality assurance samples

Sample preparation steps are shown in Table 1. Samples were derivatized with an MSTFA/NH_4I/ethanethiol (500:4:2) derivatization mixture prior to GC/MS/MS analysis (Table 1).

Table 1. Sample preparation from urine.

Step	Description
Step 1: Sample Pipetting	1. Pipet 0.5 mL of urine into a test tube. 2. Add 25 µL of working solution of the internal standard (ISTD) and 1 mL phosphate buffer.
Step 2: Automated SPE Instrument Setup	1. Place the labeled tubes in the automated SPE instrument. 2. Place empty labeled tubes in the elution zone. 3. Insert the Agilent Bond Elut NEXUS cartridges (p/n 12103101T) into the instrument. 4. Check the volume of all solvent bottles. 5. Load the appropriate method into the software and input the number of samples. 6. Start the software, which will automate the following steps: a. Condition the SPE cartridges with 2 mL of methanol (MeOH) and 2 mL of aqua bidest. b. Load the samples onto the conditioned SPE cartridges. c. Elute the SPE cartridges with 2 × 1 mL of MeOH and transfer the eluent to a large screw-cap tube. d. Evaporate the eluent under oxygen-free nitrogen (OFN) using the TurboVap. 7. Add 1 mL of buffer (pH 7.0) and transfer to a large screw-cap test tube. 8. Add 25 µL of β-glucuronidase using an automated pipette. 9. Secure the caps on the tubes and check for leaks by turning the tubes. 10. Incubate the samples for at least 1 hour at 56 ± 5 °C. 11. Remove the samples from the oven and allow them to cool to room temperature.
Step 3: Extraction	1. Add 1 mL of $NaHCO_3$/K_2CO_3 buffer (pH 9.5). 2. Add 5 mL of methyl tert-butyl ether (MTBE) using a solvent dispensette. 3. Secure the caps and roll the tubes for 20 minutes. 4. Transfer the organic layer (top) into a small, labeled test tube. 5. Evaporate the organic solvent under oxygen-free nitrogen (OFN) at 40 ± 5 °C until dry.
Step 4: Derivatization	1. Add 50 µL of MSTFA/NH_4I/ethanethiol (500:4:2) derivatization mixture to the residue, and vortex for a few seconds. 2. Transfer the mixture into a GC vial. 3. Place the vials into an oven at 80 °C for 30 minutes. 4. Transfer the vials to the GC/TQ autosampler tray.

GC/TQ analysis

An Agilent 7000C triple quadrupole GC/MS (GC/TQ) system was used for the analysis. The instrument operating parameters are listed in Table 2. Additional considerations and best practices for using H_2 as a carrier gas can be found in the Helium to Hydrogen Carrier Gas Conversion User Guide.[5] Given that this was a targeted analysis and none of the targets demonstrated in-source reactivity with hydrogen carrier gas, a standard Agilent Inert Plus EI source was used. For analysis of unknowns or when analyzing compounds that are prone to interacting with hydrogen, an Agilent HydroInert source can be considered.

The previous positive chemical ionization (PCI) GC/MS/MS method using helium as the carrier gas used a 5:1 split.[6] However, it is common to observe a 2 to 5x decrease in sensitivity when converting the analysis to using hydrogen carrier gas. To compensate for decreased sensitivity, the injection split ratio or injection volume can be adjusted. In this newly developed method using hydrogen gas, splitless injection was employed to maintain sufficient sensitivity and comparability with the previous method.

Data acquisition and processing were performed using Agilent MassHunter acquisition software for GC/MS systems (version 10.2) and Agilent MassHunter Quantitative Data Analysis software (version 10.2).

Calibration curves for the 14 steroids included six points each and were prepared by spiking steroids into stripped urine. The calibration levels are provided in Table 3. Each calibration was run in triplicate, resulting in 18 calibration points at six levels. Deuterated internal standards (ISTDs) were used for internal standard calibration. Quadratic fit was used for all compound calibrations. The calibration curves had a 1/x weighting factor applied.

Table 2 . GC and MS conditions for forensic toxicological analysis.

Parameter	Value
Inlet	Split/splitless inlet
Mode	Splitless
Purge Flow to Split Vent	53 mL/min at 2 min
Injection Volume	1.5 µL
Inlet Temperature	280 °C
Inlet Liner	Agilent 4 mm Ultra Inert (UI) liner single taper with wool (part number 5190-2293)
Column	Agilent J&W Ultra 1, 12 m × 0.20 mm, 0.11 µm (p/n 19091A-005)
Column Temperature Program	120 °C (0.15 min hold) 75 °C/min to 170 °C (0 min hold) 40 °C/min to 185 °C (0 min hold) 2.5 °C/min to 199 °C (0 min hold) 10 °C/min to 213 °C (0 min hold) 60 °C/min to 300 °C (0 min hold) Run time: 9.64 min
Carrier Gas and Flow Rate	Hydrogen, 1 mL/min constant flow
Transfer Line Temperature	300 °C
Triple Quadrupole Mass Spectrometer	Agilent 7000C GC/TQ system with extractor EI source
Electron Energy	70 eV
Quench Gas Helium	2.25 mL/min **Note:** quench gas can be turned off and capped off when using hydrogen as a carrier gas
Collision Gas Nitrogen	1.5 mL/min
Ion Source Temperature	280 °C
Quadrupole Temperature	150 °C
Mode	Multiple reaction monitoring (MRM)
Tune	Etune: atunes.eiex.tune.xml

Method validation

To assess external bias, 18 matrix-matched urine samples from the WADA external quality assessment scheme (EQAS) were also analyzed, and z-scores for six compounds were calculated.

Results and discussion

In the method presented here, using hydrogen as a carrier gas reduced the analysis time to less than 10 minutes without compromising the chromatographic resolution for the isomers. Notably, the original GC/MS/MS method with helium carrier gas resulted in an analysis time of 17 minutes.[6] Therefore, by transitioning to hydrogen carrier gas and using suitable column dimensions, analysis time can be decreased by more than 40%. Figure 1 shows the total ion chromatogram in multiple reaction monitoring (MRM) mode for both methods, using either helium carrier gas (A) or hydrogen carrier gas (B).

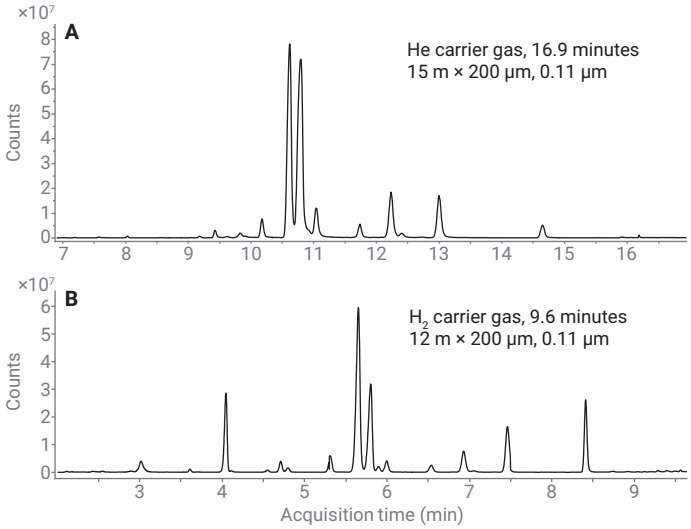

Figure 1. MRM chromatogram with (A) helium and (B) hydrogen.

EI source with hydrogen carrier gas

An important consideration when switching from helium to hydrogen carrier gas in GC/MS is spectral fidelity. Some active compounds may react with hydrogen, resulting in spectral changes. This is crucial not only for GC/MS in scan data acquisition mode, but also for GC/MS/MS in MRM mode, because the precursor ion might be affected by these spectral changes. This can lead to sensitivity loss and calibration problems. If such issues occur, they can effectively be addressed through the use of a HydroInert source, which minimizes interactions between hydrogen and the analytes, maintaining spectral integrity and ensuring reliable, accurate results.[7] Not all compounds react with hydrogen, and therefore, many do not show spectral changes with hydrogen carrier gas even when a standard EI source is used. For best performance when using a conventional EI source with hydrogen, the lens can also be replaced with a 9 mm lens.

In this work, the target compounds did not demonstrate in-source reactions with hydrogen when using an Inert Plus EI source with the standard 3 mm extractor lens. To confirm the absence of undesired chemical interactions between target analytes and the hydrogen carrier gas, spectra were acquired with helium and hydrogen carrier gas using the standard Inert Plus EI source equipped with a 3 mm lens. Figure 2 shows the mass spectra for 5b-androstanediol-d_5, one of the deuterated internal standards, acquired using either helium or hydrogen carrier gases. Spectra for four other deuterated internal standards are shown in the Appendix Figures A1 to A4. For all the evaluated compounds, no spectral changes are observed, suggesting that the standard Inert Plus EI source with 3 mm extractor lens can be used for this analysis.

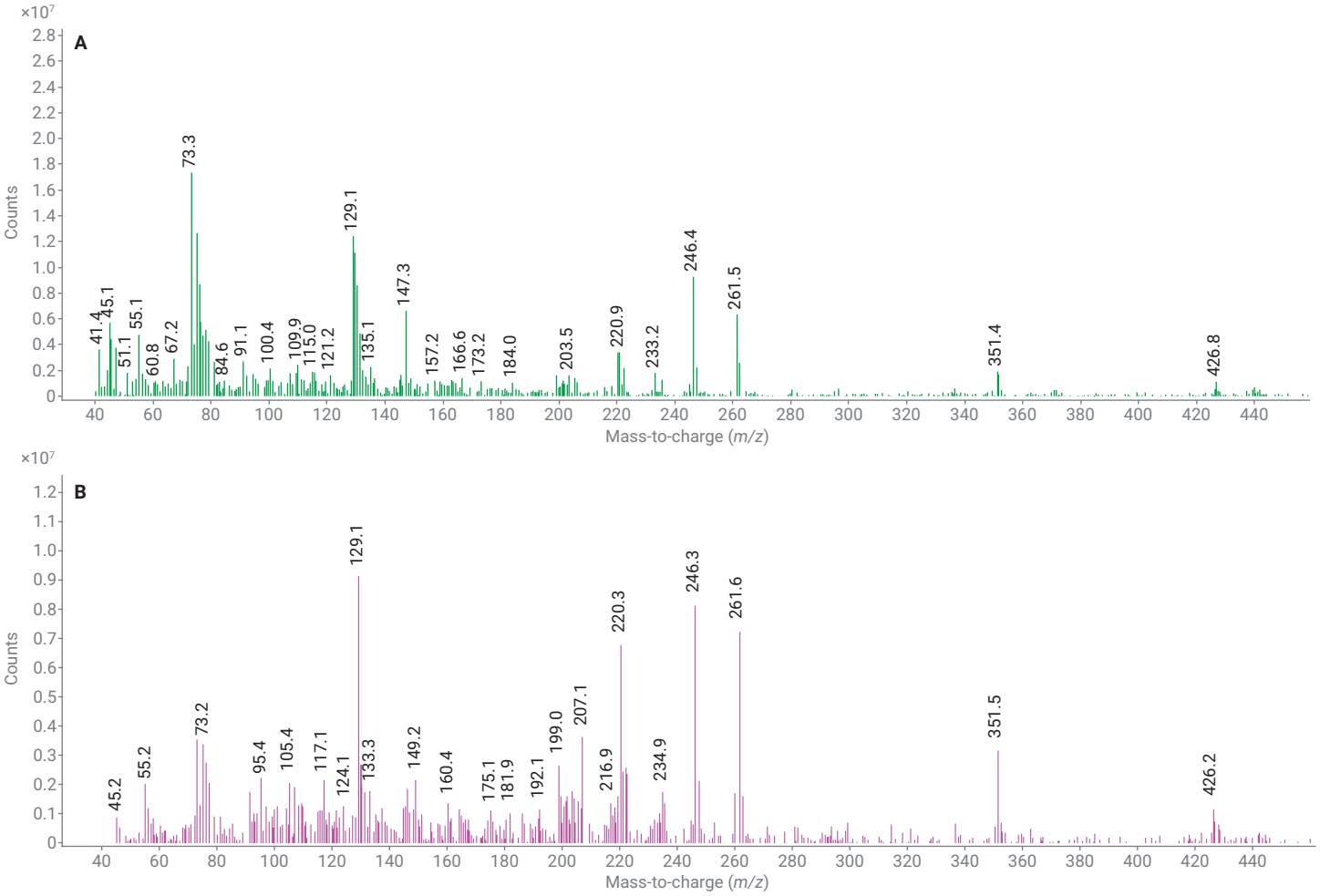

Figure 2. EI mass spectra acquired for 5b-androstanediol-d_5 (deuterated derivatized internal standard) using an Agilent Inert Plus EI source with (A) helium carrier gas or (B) hydrogen carrier gas.

It was determined that the derivatized steroids analyzed in this study did not show noticeable spectral changes even when a conventional Inert Plus source was used with hydrogen carrier gas. This could be attributed to the derivatization process, as the derivatizing agent converts the analytes into more stable derivatives, masking functional groups that might otherwise interact with the hydrogen carrier. This reduces the likelihood of decomposition or side reactions.

Quantitation of 14 steroids in urine

Table 3 shows calibration ranges and correlation coefficients for 14 steroids that require quantitation according to WADA. The endogenous anabolic steroids quantitated in this work are some of the most analytically challenging compounds. On one side, this is due to their low LOIs, but on the other side, challenges can arise from the need to accurately quantify them at high concentrations. Using this method, the correlation coefficients (R^2) were greater than 0.997 for all 14 targets.

Figure 3 shows MRM chromatograms at the lowest calibration point for epitestosterone, testosterone, 6α-OH-androstenedione, androsterone, and etiocholanolone. At their lowest calibration points, all compounds have a signal-to-noise ratio greater than three. Additionally, androsterone and etiocholanolone are fully resolved at their lowest calibration levels.

To illustrate observed peak fronting, chromatograms for androsterone and etiocholanolone at the highest calibration level are also shown (Figure 3, bottom). This peak fronting is a result of GC column overloading at 9,600 ng/mL. Despite the peak fronting, excellent calibrations with R^2 values of 0.999 were achieved for both compounds.

Table 3 . Retention time, calibration coefficients, R^2 values, and internal standards (ISTDs) used for 14 steroids requiring quantitation according to WADA.

Substance	Retention Time (min)	Calibration in Urine (ng/mL)	Coefficient of Determination (R^2)	ISTD Used
Testosterone	7.507	1, 3, 10, 30, 100, 400	0.9978	Testosterone-d_3
Epitestosterone	6.98	1, 3, 10, 30, 100, 400	0.9978	Epitestosterone-d_4
Androsterone	5.702	24, 72, 240, 720, 2,400, 9,600	0.9991	Androsterone-d_4
Etiocholanolone	5.862	24, 72, 240, 720, 2,400, 9,600	0.9996	Etiocholanolone-d_5
Dihydrotestosterone	7.103	0.5, 1.5, 5, 15, 50, 200	0.9993	Dihydrotestosterone-d_3
Dehydroepiandrosterone	6.605	2, 6, 20, 60, 200, 800	0.9992	Dehydroepiandrosterone-d_6
4-Androstene-3,17-dione	7.295	0.5, 1.5, 5, 15, 50, 200	0.9994	Dehydroepiandrosterone-d_6
5α-Androstane-3α,17β-diol	6.021	2, 6, 20, 60, 200, 800	0.9984	5α-Androstane-3α,17β-diol-d_5
5β-Androstane-3α,17β-diol	6.063	2, 6, 20, 60, 200, 800	0.9996	5β-Androstane-3α,17β-diol-d_5
5α-Androstane-3,17-dione	6.857	0.5, 1.5, 5, 15, 50, 200	0.9991	Dehydroepiandrosterone-d_6
5β-Androstane-3,17-dione	4.882	0.5, 1.5, 5, 15, 50, 200	0.9988	Dehydroepiandrosterone-d_6
6α-OH-Androstenedione	8.754	0.25, 0.75, 2.5, 7.5, 25, 100	0.9992	Dehydroepiandrosterone-d_6
4OH-Androstenedione	8.848	0.25, 0.75, 2.5, 7.5, 25, 100	0.9993	Dehydroepiandrosterone-d_6
5β-Pregnanediol	8.591	2, 6, 20, 60, 200, 800	0.9978	5β-Androstane-3α,17β-diol-d_5

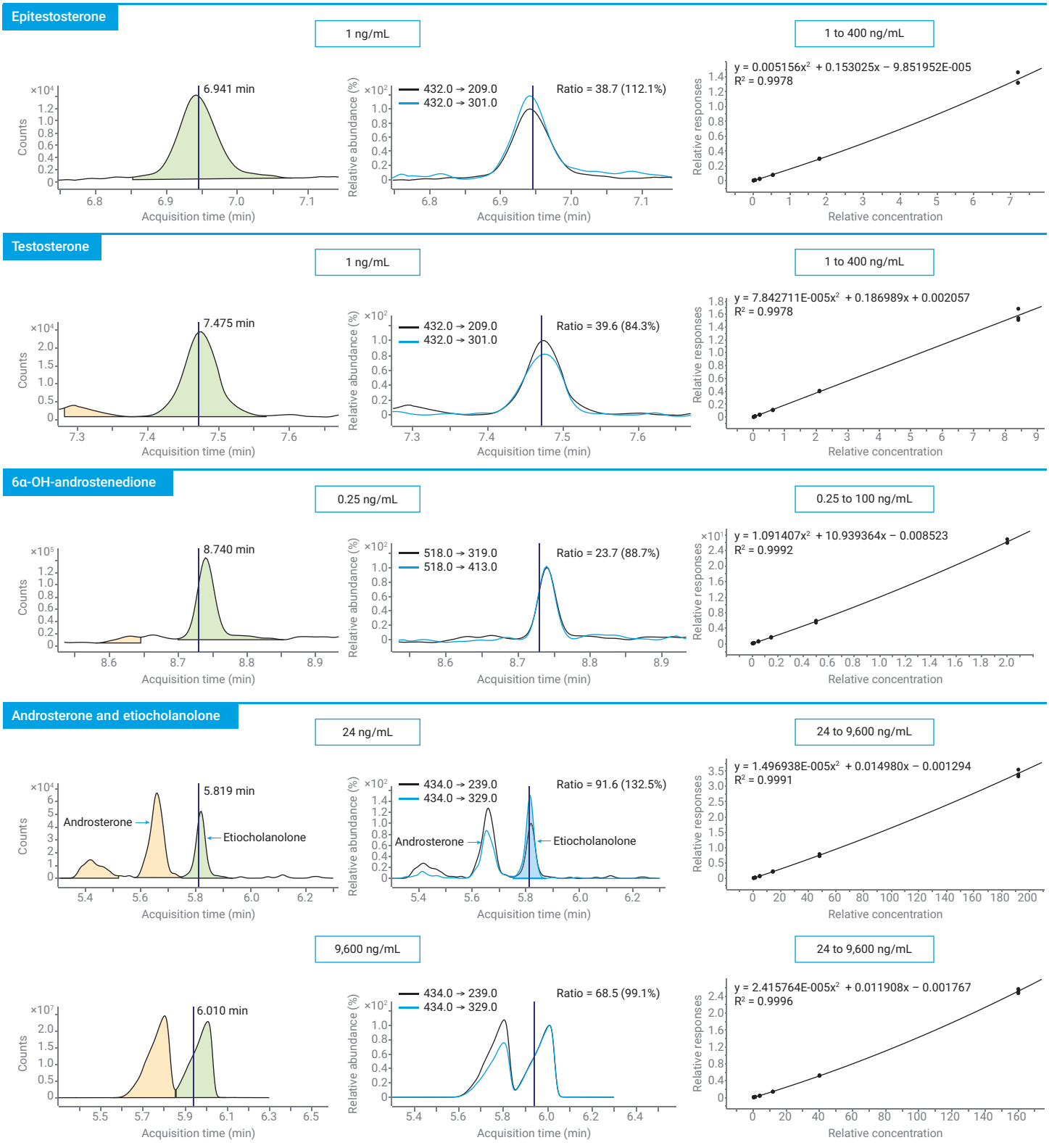

Figure 3. Chromatograms (left) and calibration curves (right) for selected anabolic steroids. MRM chromatograms at the lowest calibration levels are shown for epitestosterone, testosterone, 6α-OH-androstenedione, androsterone, and etiocholanolone. At the bottom, chromatograms for androsterone and etiocholanolone at the highest calibration level are shown.

Validation study

Within the WADA EQAS, urine samples are frequently sent to accredited laboratories.[4] For this purpose, six targets were analyzed in 18 EQAS samples, and measured values were compared to the reported consensus values. For all 108 values, the z-scores were less than 1, indicating that this method provides sufficient accuracy.

Conclusion

A GC/MS/MS method with electron impact ionization (EI) using hydrogen carrier gas was developed for the analysis of 14 anabolic steroids in urine. Excellent calibration performance was shown across an extended calibration range covering the minimum required performance levels (MRPLs). This analysis could be performed in under 10 minutes, which is 40% faster than the original positive chemical ionization (PCI) GC/MS/MS method. The WADA validation demonstrated that for all analyzed samples, z-scores were less than 1, indicating acceptable method accuracy.

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Appendix

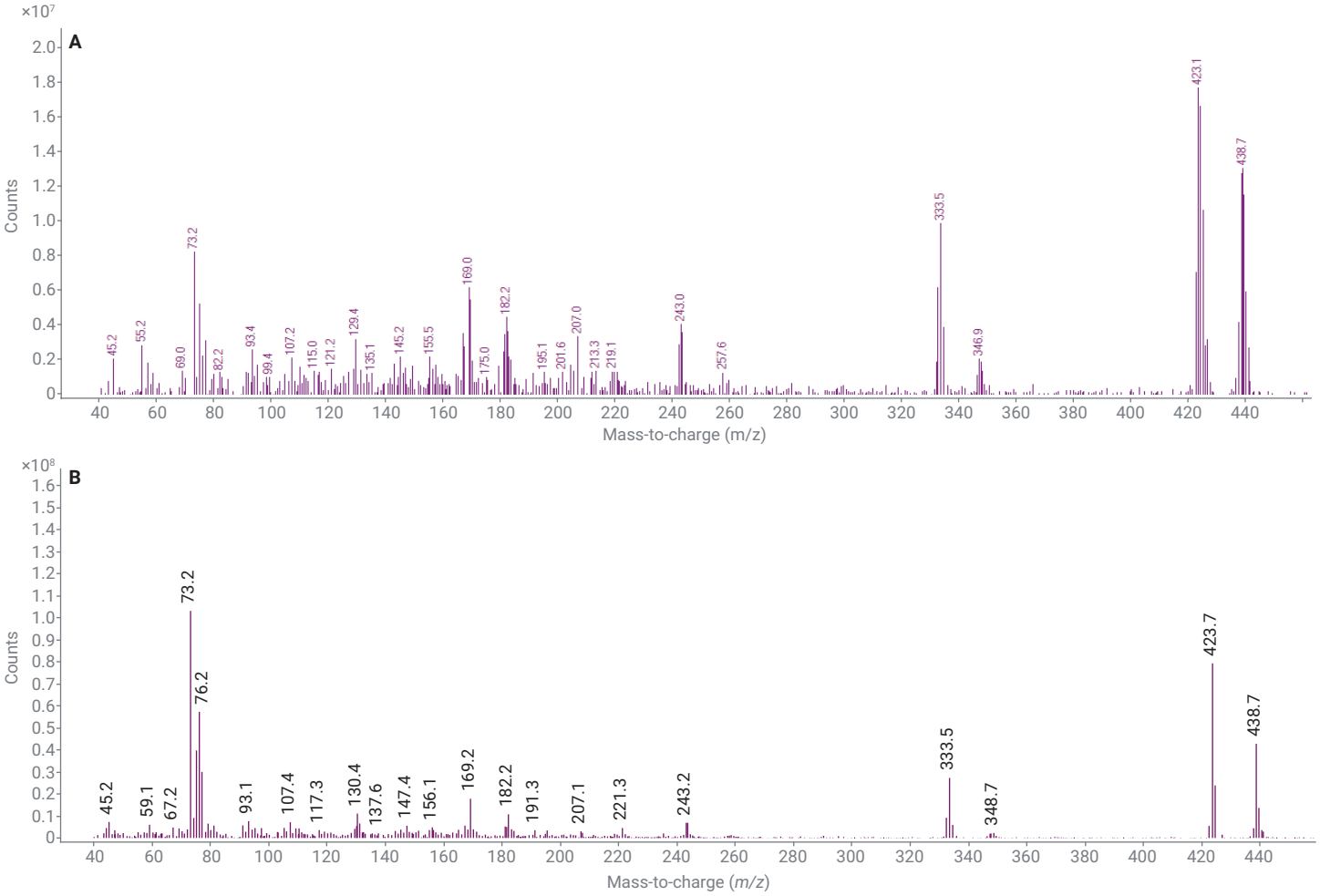

Figure A1. EI mass spectra acquired for androsterone-d_4 (deuterated derivatized internal standard) using the Inert Plus EI source with (A) helium carrier gas or (B) hydrogen carrier gas.

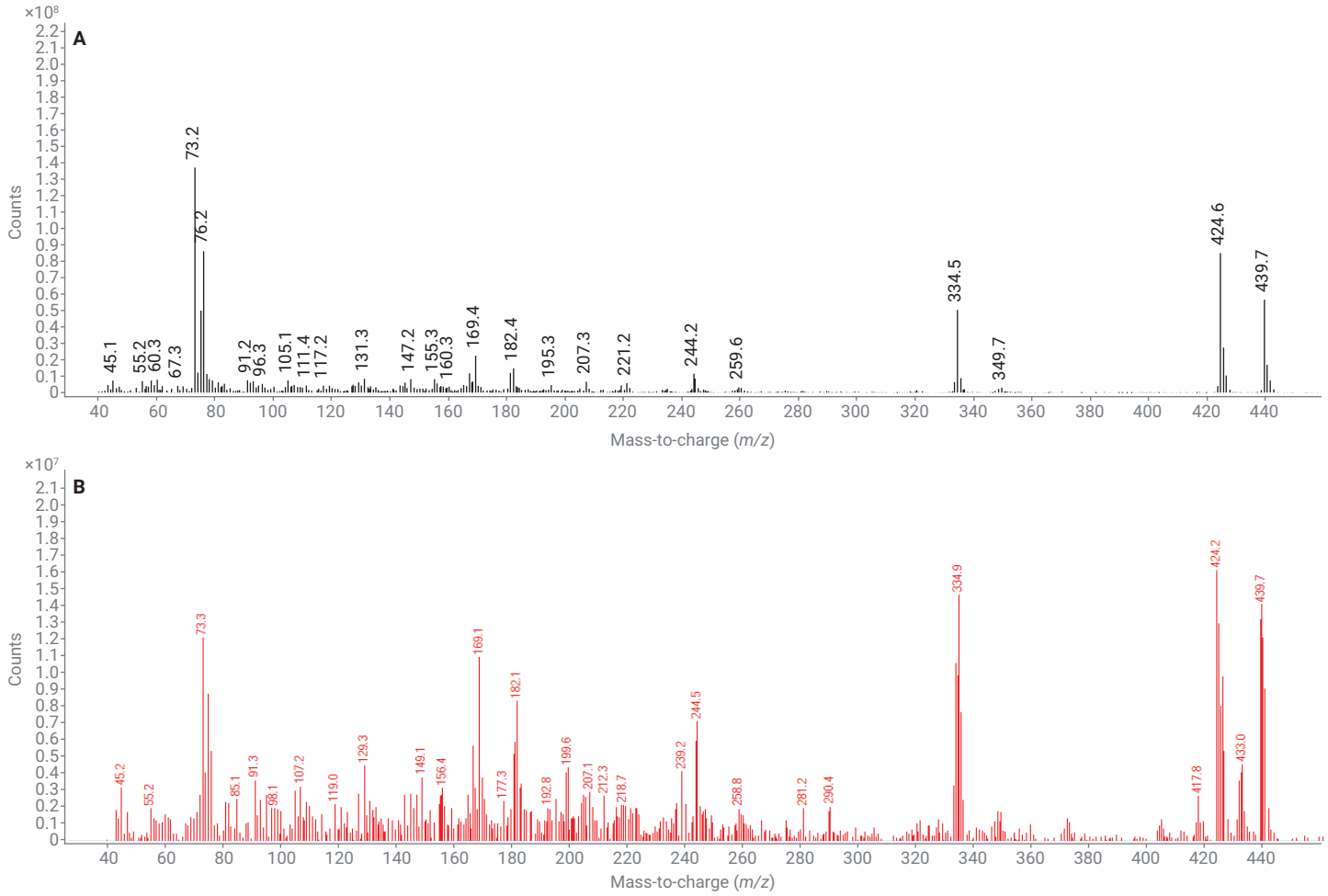

Figure A2. EI mass spectra acquired for etiocholanolone-d_5 (deuterated derivatized internal standard) using the Inert Plus EI source with (A) helium carrier gas or (B) hydrogen carrier gas.

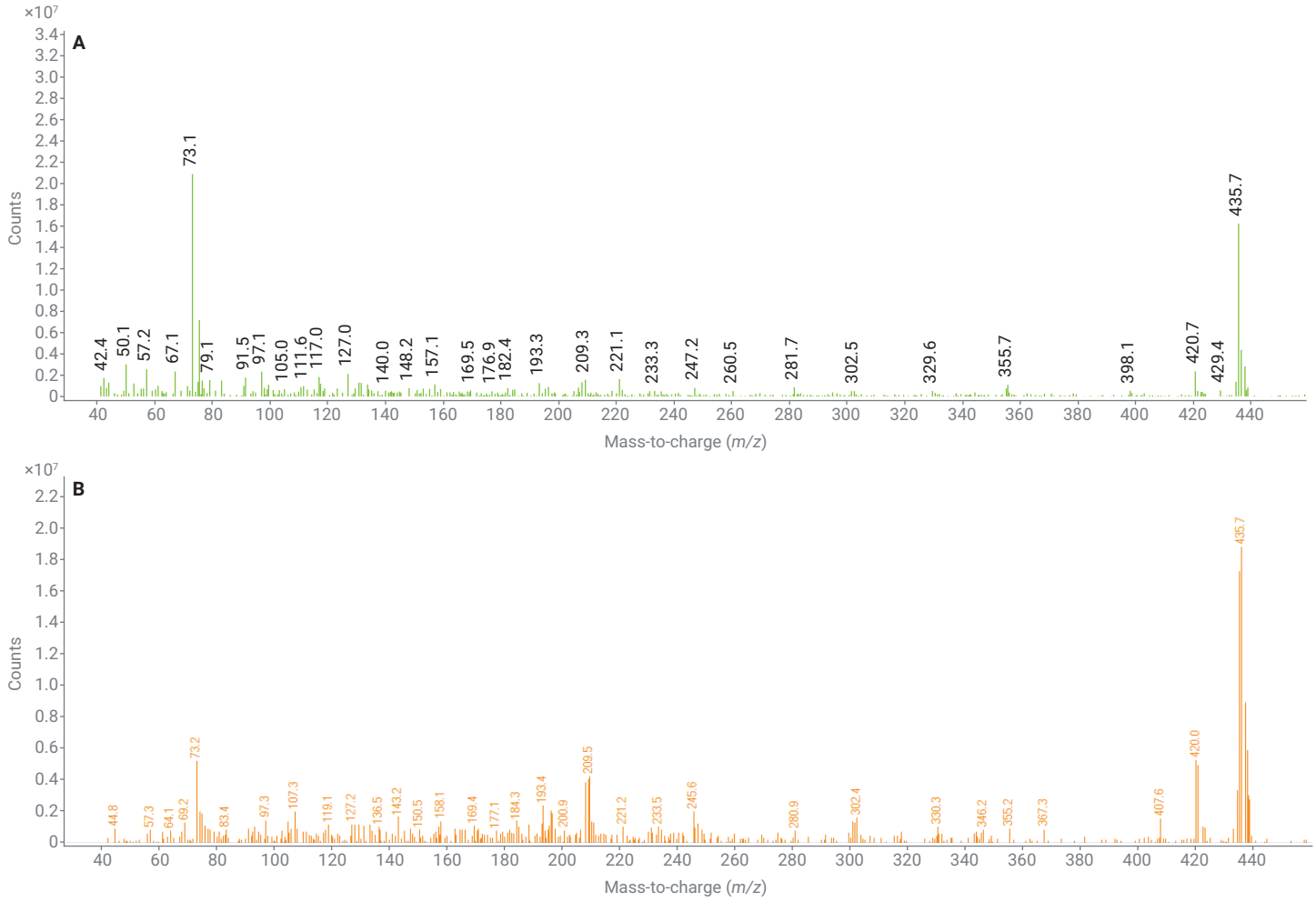

Figure A3. EI mass spectra acquired for testosterone-d_3 (deuterated derivatized internal standard) using the Inert Plus EI source with (A) helium carrier gas or (B) hydrogen carrier gas.

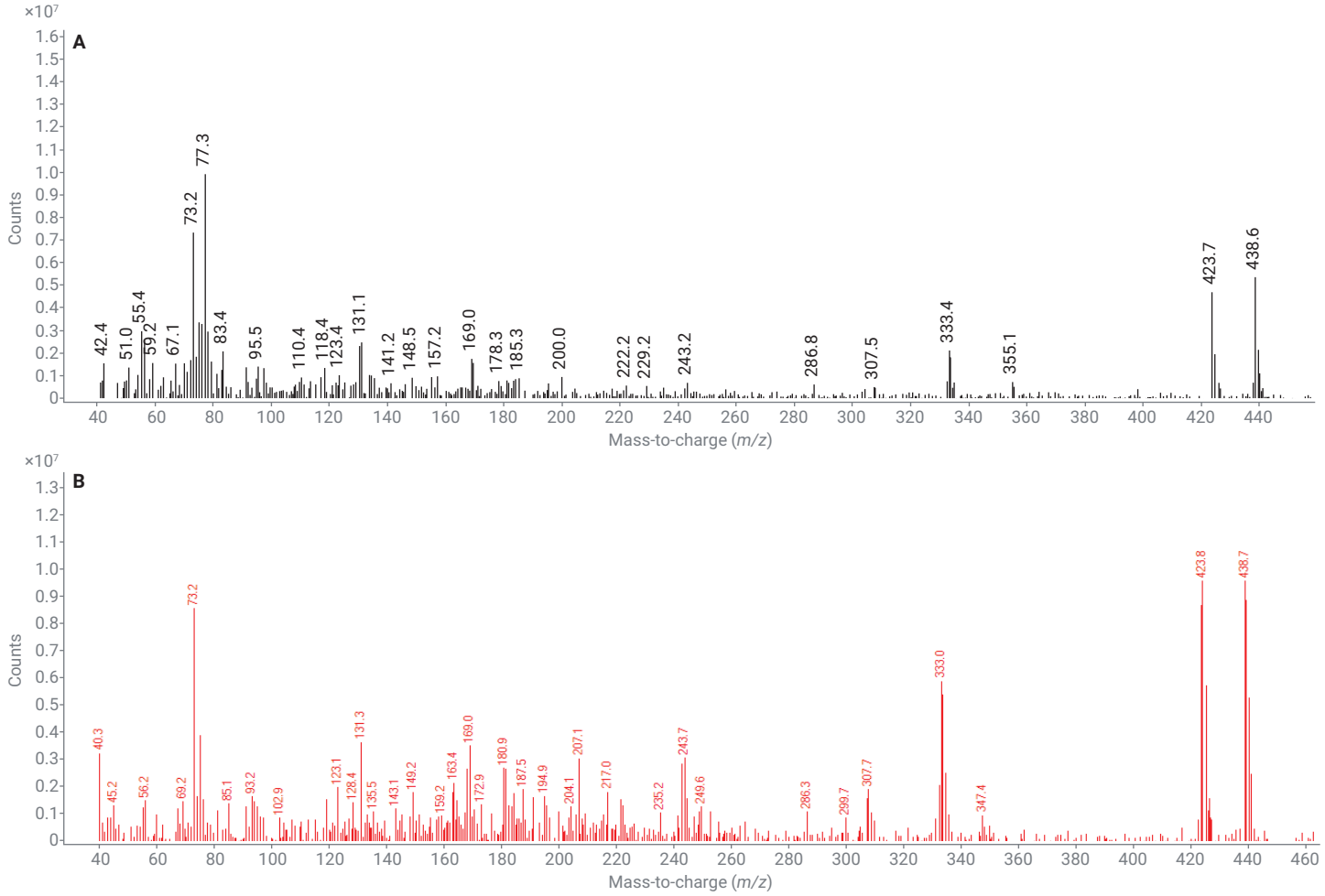

Figure A4. EI mass spectra acquired for DHEA-d_6 (deuterated derivatized internal standard) using the Inert Plus EI source with (A) helium carrier gas or (B) hydrogen carrier gas.

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