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POTENTIAL OF ION MOBILITY-MASS SPECTROMETRY FOR BOTH TARGETED AND NON-TARGETED ANALYSIS OF PHASE II STEROID METABOLITES IN URINE

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Ion mobility-mass spectrometry; Collision cross section; Steroids; Phase II metabolites; Urine

Abbreviations:

DHEA – dehydroepiandrosterone

DMA – differential mobility analyzer

DTIMS – drift tube ion mobility spectrometry

DMS – differential ion mobility

EDCs – endocrine disrupting chemicals

EIC – extracted ion chromatogram

EU – European Union

FAIMS – high-field asymmetric waveform ion mobility spectrometry

HMDB – Human Metabolome Database

IMS – ion mobility spectrometry

m/z – mass-to-charge ratio

TIMS – trapped ion mobility spectrometry

R_t – retention time

TIC – total ion chromatogram

TWIMS – travelling wave ion mobility spectrometry

WADA – World Anti-Doping Agency

ABSTRACT

In recent years, the commercialization of hybrid ion mobility-mass spectrometers and their integration in traditional LC-MS workflows provides new opportunities to extend the current boundaries of targeted and non-targeted analyses. When coupled to LC-MS, ion mobility spectrometry (IMS) provides a novel characterization parameter, the so-called averaged collision cross section (CCS, Ω), as well as improves method selectivity and sensitivity by the separation of isobaric and isomeric molecules and the isolation of the analytes of interest from background noise. In this work, we have explored the potential and advantages of this technology for carrying out the determination of phase II steroid metabolites (i.e. androgen and estrogen conjugates, including glucuronide and sulfate compounds; $n = 25$) in urine samples. These molecules have been selected based on their relevance in the fields of chemical food safety and doping control, as well as in metabolomics studies. The influence of urine matrix on the CCS of steroid metabolites was evaluated in order to give more confidence to current CCS databases and support its use as complementary information to retention time (R_t) and mass spectra for compound identification. Samples were only diluted 10-fold with aqueous formic acid (0.1%, v/v) prior analysis. Only an almost insignificant effect of adult bovine urine matrix on the CCS of certain steroid metabolites was observed in comparison with calve urine matrix, which is a less complex sample. In addition, high accuracy was achieved for CCS measurements carried out over four months ($\Delta\text{CCS} < 1.3\%$ for 99.8% of CCS measurements; $n = 1806$). Interestingly, it has been observed that signal-to-noise (S/N) ratio could be improved at least 2 or 7-fold when IMS is combined with LC-MS. In addition to the separation of isomeric steroid pairs (i.e. etiocholanolone glucuronide and epiandrosterone glucuronide, as well as 19-noretiocholanolone glucuronide and 19-norandrosterone glucuronide), steroid-based ions were also separated in the IMS dimension from co-eluting matrix compounds that presented similar mass-to-charge ratio (m/z). Finally, based on CCS measurements and as a proof of concept, 17α -boldenone glucuronide has been identified as one of the main metabolites resulted from boldione administration to calves.

1. Introduction

Steroids are biomolecules derived from cholesterol that play essential roles in several biological processes such as growth and reproduction. Consequently, the analysis of steroids is of high relevance in numerous areas such as doping control and the public health field including chemical food safety. The analysis of this type of compounds is relevant for the identification of metabolic disorders, including those related to exposures to endocrine disrupting chemicals (EDCs), and ultimately for disease diagnosis [1,2]. Moreover, the use of anabolic steroids for improving sport performance is forbidden by the World Anti-Doping Agency (WADA) [3], so their analysis is crucial to detect any misuse. In the same vein, the exogenous administration of steroids to food producing animals and, more specifically, the application of substances with hormonal actions as growth promoters (e.g. anabolic steroids) has been banned within European Union (EU) countries since 1988 [4,5]. Under this context, the analysis of steroids represents a great challenge since they encompass a wide range of compounds, including isobaric and isomeric molecules. These compounds are usually present at low concentration levels in biological samples so highly selective and sensitive analytical methods are required for their determination. Although GC-MS is the technique typically employed for the analysis of steroids and is used in most food, anti-doping and clinical laboratories, LC-MS methods have also been implemented for this purpose [6,7]. LC-ESI-MS is especially recommended for the direct detection of conjugated steroids, such as those related to phase II metabolism (i.e. glucuronides, sulfates, cysteinyl, glycine and taurine conjugates) [8,9].

Steroids are extensively metabolized and are mainly excreted in urine as phase II metabolites, mostly in the form of glucuronide and sulfate metabolites and which comprise more than 90% of the excreted steroidal urinary pool [10]. Phase II steroid metabolites have traditionally been determined as free steroids by LC-MS and GC-MS after deconjugation, which can be accomplished by enzymatic or chemical reactions [11,12]. However, new approaches based on the analysis of intact conjugated steroids are currently emerging because the monitoring of phase II steroid metabolites gives a more complete picture of urinary steroid profiles than the measurement of total free steroids [13,14,15,16]. These novel strategies not only provide a better understanding of steroid metabolism (i.e. steroidome) and the causes of its perturbations, but can also lead to the discovery of new biomarkers for detecting the illegal administration of steroids [17,18].

Advances in analytical instrumentation, mainly related to improvements in resolution and sensitivity of MS (e.g. TOF-MS, Orbitrap-MS, etc.), have also contributed to better knowledge of the steroidome. Due to these instrumental enhancements, classical steroid profiling strategies based on the detection of a predetermined number of steroid metabolites (i.e. targeted metabolomics) are currently being replaced by steroid fingerprinting approaches that allow the determination of a non-predetermined set of molecules giving a deeper insight of the metabolites present in the sample (non-targeted

metabolomics) [2,9]. In this regard, steroid fingerprinting has already demonstrated to be a powerful strategy for biomarkers discovery in the case of doping control [19,20] and metabolic perturbations due to xenobiotic exposures [21]. However, compound identification still remains as the major bottleneck of non-targeted analysis, not only because it is a time-consuming process, but also due to the current low number of metabolites that are unequivocally identified [22,23,24]. Compound identification mainly relies on the comparison between the observed chromatographic retention time (Rt) and mass spectra, as well as on the information included in metabolite databases such as the Human Metabolome Database (HMDB) or METLIN [25,26]. Nevertheless, a wide number of the detected features are finally not identified [2,27]. Compound identification based on mass spectra can be difficult if only a monoisotopic peak is detected or if the formation of adducts prevents fragmentation, as well as MS/MS data may be insufficient to distinguish between different structural isomers and/or stereoisomers. Furthermore, Rt is highly dependent on experimental conditions (i.e. column chemistry and dimensions, mobile phase, and elution gradient) and is affected by matrix shifts, column loading and LC configuration (i.e. dead volume from tubing lengths and valves).

In the recent years, IMS has re-emerged as an analytical technique that can be easily coupled to LC-MS systems and provides complementary information to mass spectra and Rt for the characterization and/or identification of metabolites [28,29]. IMS is a gas-phase technique in which ionized molecules are separated under an applied voltage according to their size, shape and charge. Drift tube ion mobility spectrometry (DTIMS) is considered the traditional and simplest IMS form, but other IMS technologies are already mature or are under development [30,31,32]. In DTIMS, the time employed by the ions for passing through the mobility cell (i.e. drift time) can be related to their rotationally average collision cross section (CCS, Ω) according to the Mason-Schamp equation [33]. The CCS is an intrinsic characteristic of each compound that represents the effective area of interaction between an individual ion and the molecules of buffer gas employed in the drift cell (e.g. N₂, He or CO₂). Unlike in DTIMS, CCS measurements cannot be directly carried out by other IMS techniques. However, this structural parameter can also be measured by travelling wave ion mobility spectrometry (TWIMS), differential mobility analyzer (DMA) and trapped ion mobility spectrometry (TIMS) after instrument calibration with compounds of known CCS under defined conditions [29,34,35].

Within this framework, several CCS databases have been reported with the aim of integrating the CCS as an identification parameter in non-targeted metabolomics workflows [36,37,38,39,40,41]. Nevertheless, consensus about CCS values is still required in order to build reference CCS libraries that can be used globally in metabolomics studies [29]. In this sense, it is also necessary to extend the existing databases to a wider number of parent compounds and metabolites. Threshold criteria for CCS measurements must also be established before this parameter may be accepted as an identification parameter to support metabolomics or other type of analysis. Paglia et al. have originally proposed an acceptable deviation of 2% between CCSs measured in samples and reference CCS

values in databases [34,42]. Although this criterion has been applied in current LC-IMS-MS workflows intended for food or biological analysis [43,44,45,46], a recent inter-laboratory study has shown that the tolerance for CCS measurements can be potentially reduced to $\pm 0.5\%$ when using DTIMS [47]. Thus, more studies are still needed, not only to ensure that complex sample matrices do not influence the drift time and, as a consequence, affect CCS measurements [48,49], but also to validate or reduce the threshold of $\pm 2\%$ currently accepted for CCS measurements.

In addition to CCS values, IMS also involves a third separation dimension when analyses are carried out in LC-IMS-MS systems [29]. IMS has shown to be very effective for the separation of isobaric and isomeric compounds based on their CCS as well as for the isolation of analyte signals from background noise [50]. Therefore, method selectivity and sensitivity are improved. In this sense, only a few studies have reported the advantages of IMS for the analysis of steroids. For example, Ahonen et al. have demonstrated the feasibility of IMS-MS for the separation of steroid isomers after their derivatization with *p*-toluenesulfonyl isocyanate [51]. Although the separation of native steroid isomers was not achieved because they exhibited similar CCSs, the further study of a larger set of steroids has highlighted that some pairs of steroid isomers can be separated based on their CCS differences, such as 5β -androstane-3,17-dione and 5α -androstane-3,17-dione [39]. IMS was also shown to enhance the sensitivity achieved by classical LC-MS methods intended for the analysis of testosterone and epitestosterone glucuronides in urine samples [52]. Cleaned-up chromatograms, and consequently greater S/N, were obtained when applying LC-IMS-MS instead of merely using LC-MS.

Based on the considerations above, this work presents the potential of IMS for improving the analysis of phase II steroid metabolites by LC-MS in adult bovine and calve urine samples. The reproducibility of the measured CCS of 25 glucuronide and sulfate metabolites of androgens and estrogens was studied over four months in order to evaluate the effect of the matrix on this molecular characteristic and establish threshold criteria for applying the CCS as identification parameter in metabolomics. The clean-up effect achieved on chromatograms by the integration of TWIMS in the LC-MS workflow is also discussed in terms of sensitivity and selectivity improvement. As a proof of concept, the advantages of using IMS in non-targeted metabolomics are also demonstrated by the use of the CCS for the identification of one of the main metabolites resulted from the exogenous administration of boldione to calves.

2. Experimental

2.1 Chemicals and reagents

All reference steroids including testosterone glucuronide, epitestosterone glucuronide, dehydroepiandrosterone (DHEA) glucuronide, etiocholanolone glucuronide, epiandrosterone glucuronide, boldenone glucuronide, 19-nortestosterone glucuronide, estradiol 17β -glucuronide, 17β -estradiol 3-glucuronide, estradiol diglucuronide, 19-noretiocholanole glucuronide, 19-norandrosterone

glucuronide, testosterone sulfate, epitestosterone sulfate, epiandrosterone sulfate, androsterone sulfate, DHEA sulfate, 5 α -androstane-3 α ,17 β -diol 17-sulfate, boldenone sulfate, 19-nortestosterone sulfate, estradiol 17 β -sulfate, 17 β -estradiol 3-sulfate, 17 α -estradiol 3-sulfate, estrone 3-sulfate, and estriol 3-sulfate were acquired from Steraloids (Newport, RI, USA). Each steroid stock solution was prepared at 100 $\mu\text{g mL}^{-1}$ in ethanol. Working standard solutions (10 $\mu\text{g mL}^{-1}$) were prepared by the dilution of stock standard solutions in methanol. Standard solutions were stored in amber glass vials at -20°C.

Acetonitrile and propan-2-ol (LC-MS Chromasolv® grade) were supplied by Sigma-Aldrich (St. Louis, Mo, USA). Water (HiperSolv Chromanorm® for HPLC) was provided by VWR International (West Chester, PA, USA). Formic acid (eluent additive for LC-MS) was acquired from LGC Standards GmbH (Wesel, Germany). A solution of sodium formate (0.5 mM in 90/10 (% v/v) propan-2-ol/water) was used for mass calibration. MS calibration solution was prepared from sodium hydroxide (1 M, Fisher Chemical™) and formic acid (Promochem®) supplied by Fisher Scientific (Loughborough, UK) and LGC Standards (Wesel, Germany), respectively. CCS calibration was carried out using the Major Mix IMS/TOF Calibration Kit from Waters® (Manchester, UK). A solution of leucine-enkephalin (2 $\mu\text{g mL}^{-1}$) in 50/50 (% v/v) water/acetonitrile solution containing 0.2% (v/v) of formic acid was used as a lock mass standard. Leucine-enkephalin standard was acquired from Waters®.

2.2 Sample preparation

Bovine (i.e. adult animals) and calve urine samples (n = 4 and 5, respectively) were analyzed throughout this work. These urine samples were already stored at the biobank of LABERCA, so this research work did not imply any animal experiment. Samples were defrosted at room temperature and subsequently prepared according to ‘dilute-and-shoot’ procedures [53]. Briefly, urine samples were submitted to centrifugal filtration for 10 min at 9000 rpm and 15°C using centrifugal filters (polyethersulfone membrane, molecular weight cut-off of 10 kDa), which were acquired from VWR International. After filtration, samples were spiked at 2 $\mu\text{g mL}^{-1}$ with working standard solutions and submitted to 10-fold dilution with 0.1 % (v/v) aqueous formic acid. Initially, the TMCCSN₂ of steroids was measured in different calve urine samples (n = 5) that were directly fortified prior to their dilution and further analysis. After the first week of experiments, urine samples from four calves and one adult bovine were filtered, spiked at 2 $\mu\text{g mL}^{-1}$ with the standard solution mixture, homogenized and kept at -20°C. Over the following four months, aliquots (n = 7) of these samples were brought to room temperature, submitted to 10-fold dilution and directly injected into the LC-IMS-MS system. In addition, other adult bovine urine samples (n = 3) were punctually processed as described above and analyzed. In general, samples were spiked with a standard solution containing six androgen glucuronides (i.e. testosterone glucuronide, epitestosterone glucuronide, DHEA glucuronide, etiocholanolone glucuronide, epiandrosterone glucuronide, boldenone glucuronide), six estrogen

glucuronides (i.e. 19-nortestosterone glucuronide, estradiol 17 β -glucuronide, 17 β -estradiol 3-glucuronide, estradiol diglucuronide, 19-noretiocholanole glucuronide, 19-norandrosterone glucuronide), seven androgen sulfates (i.e. testosterone sulfate, epitestosterone sulfate, epiandrosterone sulfate, androsterone sulfate, DHEA sulfate, 5 α -androstane-3 α ,17 β -diol 17-sulfate, boldenone sulfate), and six estrogen sulfates (i.e. 19-nortestosterone sulfate, estradiol 17 β -sulfate, 17 β -estradiol 3-sulfate, 17 α -estradiol 3-sulfate, estrone 3-sulfate, estriol 3-sulfate) at a concentration of 2 $\mu\text{g mL}^{-1}$ in urine.

2.3 Liquid chromatographic separation

A LC method previously developed in our laboratory was applied in this work [18]. An Acquity UPLC \textcircledR System from Waters \textcircledR was used to perform reversed phase liquid chromatography on a C18 column (Acquity UPLC \textcircledR BEH C18, 2.1 x 100 mm, 1.7 μm ; Waters \textcircledR). Separations were carried out at 50 $^{\circ}\text{C}$ under gradient elution conditions. Mobile phase was supplied at a flow rate of 0.6 mL min^{-1} and consisted of 0.1% (v/v) aqueous formic acid (solvent A) and acetonitrile containing 0.1% (v/v) of formic acid (solvent B). The following gradient program was established for mobile phase composition (A:B, v/v): 95:5 between 0 and 0.3 min, 57:46 at 9.6 min, 0:100 from 10.5 to 12.5 min, and 95:5 from 13 to 16.5 min.

2.4 Ion mobility-mass spectrometry operation conditions and calibration

IMS-MS analyses were performed on a hybrid quadrupole-TWIMS-TOF-MS instrument (Synapt G2-S HDMS, Waters \textcircledR) equipped with an ESI interface. Samples were analyzed under both ESI+ and ESI- modes. Nitrogen was used as both cone and desolvation gases at flow rates of 50 and 1000 L h^{-1} , respectively. Nebulizer pressure was fixed at 6.0 bar. Source and desolvation temperatures were established at 150 and 350 $^{\circ}\text{C}$, respectively. Cone voltage and source offset were set at 31 and 40 V, respectively. Capillary voltage was fixed at 2.5 and 3.0 kV for ESI- and ESI+ mode, respectively. Regarding IMS conditions, nitrogen was used as trap and IMS buffer gas at flow rates of 0.2 and 100 mL min^{-1} , respectively. The flow rate of the helium cell was set at 180 mL min^{-1} . In the trap cell, wave velocity and height were established at 311 m s^{-1} and 4.0 V, respectively. In the case of the transfer cell, these parameters were set at 219 m s^{-1} and 4.0 V, respectively. IMS DC bias and trap DC bias were set at 3.0 and 47.0 V, respectively. For analyses carried out under ESI+ mode, IMS wave velocity and height were fixed at 1000 m s^{-1} and 40.0 V, respectively, whereas these parameters were set at 550 m s^{-1} and 40.0 V when the system was operated in ESI- mode. Quadrupole resolution was established to 12.5 for MS/MS analyses.

MS data were acquired in the range m/z 150-1200 at 2.5 Hz. The TOF analyzer was operated in high resolution mode. Lock mass standard was supplied at 20 $\mu\text{L min}^{-1}$ and MS data was acquired each 15 s at 5 Hz (3 scans to average). LockSpray capillary voltage was fixed at 3 kV and 2.5 kV for ESI+ and ESI- modes, respectively. A maximum tolerance of 10 ppm was established for the identification of ions based on mass accuracy. CCS calibration was performed as previously described [34]. In positive

ionization conditions, CCS calibration curves covered a m/z range between 195 and 1013, and a CCS range from 138 to 306 Å². In negative conditions, CCS calibration curves covered a m/z range from 318 to 1082 and a CCS range from 130 to 322 Å².

2.5 Data analysis

Chromatograms as well as mass and mobility spectra were analyzed using MassLynx (version 4.2, Waters®) software that includes Drifscope (version 2.8) software and allows to obtain data related to the CCS of ions.

3 Results and discussion

In 2017, we developed the first large TMCCSN₂ database for steroids because the lack of databases is view as the main drawback for the integration of this molecular characteristic in metabolomics workflows for peak annotation [39]. For the creation of this database, steroid standards were analyzed by ESI-TWIMS-TOF-MS and the CCS of detected molecular ions was measured in triplicate. As a proof of concept, the CCS of the protonated molecule of nandrolone was also measured in urine samples. In this sense, urine matrix did not show any effect on its CCS ($\Delta\text{CCS} = 0.2\%$). Deeper insight is still needed to discard any effect of the matrix on CCS measurements taking into consideration that first studies about this topic are now beginning to be reported [43,48]. In addition, more studies are required before being confident in the use of CCS databases for the identification of compounds, especially for those molecules that may involve legal actions such as the analysis of steroids in the fields of antidoping and chemical food safety. In order to reinforce the application of our CCS database for steroids identification, the CCS of glucuronides and sulfate conjugates ($n = 25$) was examined in presence of different urine samples over four months ($n = 9-10$). Under this context, it is also shown the improvement on sensitivity and selectivity achieved by the implementation of TWIMS in a LC-ESI-TOF-MS method intended for the analysis of phase II steroid metabolites. Finally, a practical approach is presented in order to highlight the benefits of including IMS in non-targeted workflows.

3.1 Robustness of CCS measurements in urine samples

In this work, different urine samples were selected in order to evaluate the influence of this matrix on the CCS of steroid conjugates. Samples were treated and fortified with a steroid mixture standard solution at 2 µg mL⁻¹ in urine according to the procedure described in **Section 2.2**. This concentration level ensured that all steroids were detected under both ESI+ and ESI- modes. Despite conjugated steroids are usually analyzed in negative ionization conditions because it provides higher signal sensitivity [15,18], sample analysis was also performed in positive mode with the aim of carrying out a more comprehensive study of the CCS of steroids in urine matrices. Moreover, the published CCS database for steroids does not include information related to the negative ionization of androgens. On

the other hand, urine samples were diluted 10-fold before their analysis by LC-ESI-TWIMS-TOF-MS in order to reduce and/or avoid the matrix effect affecting the ionization of steroids. In addition to fortified samples, blanks of each urine sample and standard solutions were also analyzed.

Standard solutions were used as quality control and as a part of internal reproducibility studies for in-house validation of the CCS database for steroids [39]. Selected steroid standards have been characterized in terms of CCS over four months (9 measurements for each steroid) and compared with the $^{TW}CCSN_2$ values reported in the database. In total, 297 CCS measurements were carried out taking into account positive and negative ionization conditions. Differences between the mean measured $^{TW}CCSN_2$ values and the $^{TW}CCSN_2$ values in the database were lower than 0.9%. These results fully fall within the threshold of 2% widely applied to CCS measurements [34,49], showing the robustness of the database over the time. Consequently, the reported CCS database was used with confidence for the CCS characterization of steroids in presence of biological matrices. Initially, the $^{TW}CCSN_2$ of steroids was measured in different calve urine samples ($n = 5$) that were directly fortified at $2 \mu\text{g mL}^{-1}$ prior to their dilution and further analysis. After the first week of experiments, urine samples from four calves and one adult bovine were filtered, spiked at the same concentration level, homogenized and kept at -20°C . Aliquots ($n = 7$) of these samples were treated and analyzed within the following four months with the aim of obtaining a more detailed vision of the interactions between the matrix and these metabolites as a function of time. In this sense, some interactions between phase II metabolites and other urine components cannot be evident in samples that are spiked just before analysis. Moreover, the performance of CCS measurements over time was also pursued in order to take into account the influence of the system calibration on the analytical response when using TWIMS for the analysis of real samples as well as any variation related to instrument/laboratory conditions.

Table 1 shows the $^{TW}CCSN_2$ value of the most intense ion identified for each steroid under positive and negative ionization conditions. The CCS of phase II androgen metabolites analyzed in negative mode is reported for the first time, which involves extending the current information available about the CCS characterization of steroids [38,39,54,55]. As previously observed for estrogen compounds [39], the $^{TW}CCSN_2$ is similar for the protonated and deprotonated molecules of androgens presenting both species under ESI+ and ESI- conditions, respectively. Surprisingly, the $[M+H]^+$ ion of epitestosterone glucuronide possesses a $^{TW}CCSN_2$ much smaller than its related $[M-H]^-$ ion (i.e. 206.0 \AA^2 and 218.5 \AA^2 , respectively). In comparison to its epimer (i.e. testosterone glucuronide, **Table 1**), it seems that this molecule compacts when is protonated. Consequently, it provides evidence justifying why the protonated species of epitestosterone glucuronide is more compact than other steroids presenting similar m/z [39].

CCS differences within the range $\pm 0.50\%$ were observed for the averaged $^{TW}CCSN_2$ of steroid metabolites in urine samples compared to the $^{TW}CCSN_2$ values reported in the database (**Table 1**),

except for DHEA sulfate ($\Delta\text{CCS} = +0.51\%$), 5α -androstan- $3\alpha,17\beta$ -diol 17-sulfate ($\Delta\text{CCS} = +0.64\%$) and estriol 3-sulfate ($\Delta\text{CCS} = +0.84\%$) analyzed in positive mode. Therefore, urine matrix does not seem to have any relevant influence on the CCS of phase II steroid metabolites. Such conclusion was also previously reported for other compounds and matrices [43,48]. Nevertheless, important results can be obtained when the CCS measurements for each compound in urine are examined in detail. In total, 1806 CCS measurements were carried out taking into account the number of analytes studied, the number and type of urine samples that were analyzed within four months, and both negative and positive ionization modes. High accuracy was generally achieved for the measurement of the $^{\text{TW}}\text{CCS}_{\text{N}_2}$ of the vast majority of steroid ions. As indicated in **Figure 1A**, more than 77% of CCS measurements matched the database $^{\text{TW}}\text{CCS}_{\text{N}_2}$ values within $\pm 0.50\%$ error, whereas only 1.4% of the cases presented a CCS difference greater than $\pm 1.00\%$. Hence, the threshold of $\pm 2\%$ currently accepted for CCS measurements may be quite conservative as suggested by Regueiro et al. [48], and it could potentially be decreased. In our case, only two determinations over the total number of CCS measurements led to CCS differences greater than $\pm 1.30\%$, and one determination gave a false negative result even applying the threshold of $\pm 2\%$ (**Figure 1B**). It was related to the analysis in negative mode of estradiol 3-glucuronide in one adult bovine urine sample spiked at lower concentration level than the fortification level applied to the other studied samples (i.e. $0.2 \mu\text{g mL}^{-1}$ vs. $2 \mu\text{g mL}^{-1}$). Since leucine-enkephalin was used as lock mass and can potentially be used as lock CCS, its drift time/CCS was monitored during the analysis and no variability was observed ($\Delta\text{CCS}_{\text{max}} = -0.5\%$, $\text{RSD} = 0.4\%$). Consequently, the application of the lock CCS did not avoid the false negative result related to estradiol 3-glucuronide. The further analysis of other adult bovine urine samples ($n = 3$) spiked with estradiol 3-glucuronide at $0.2 \mu\text{g mL}^{-1}$ confirmed that the observed CCS deviation (i.e. -3.0%) was the result of a specific analysis rather than due to any influence of the matrix or the concentration level evaluated. Based on our results it can be stated that false negative results for CCS measurements are almost negligible ($< 1\%$) but they can occur even when applying a wide threshold such as 2% .

Based on our results, we propose that the threshold currently accepted for CCS measurements can be reduced from $\pm 2.0\%$ to at least $\pm 1.5\%$ as a first attempt to implement the CCS as determination parameter, although further decisions taken should be widely adopted by the scientific community. In this regard, more studies are required, not only for identifying and avoiding any potential not yet described matrix effects on the CCS of analytes, but also for guaranteeing the reliability and precision of the CCS databases reported. Long-term and inter-laboratory assays are highly needed for achieving this purpose. High precision has been observed for the majority of CCS measurements of steroid ions as discussed above and shown by **Figure 1C** for the deprotonated molecule of estradiol 17-glucuronide. Thus, it provides confidence in the $^{\text{TW}}\text{CCS}_{\text{N}_2}$ values of the database for steroids. On the contrary, the precision related to the CCS measurement of other molecular ions can still be improved if the $^{\text{TW}}\text{CCS}_{\text{N}_2}$ values from the database are corrected by taking into account the additional

measurements carried out through this work. For example, **Figure 1D** shows that the ${}^{\text{TW}}\text{CCSN}_2$ value reported for the $[\text{M-H}+2\text{Na}]^+$ species of estriol 3-sulfate was slightly underestimated in comparison to these last measurements, although they matched the database value (i.e. ${}^{\text{TW}}\text{CCSN}_2 = 212.3 \text{ \AA}^2$) within $\pm 2.0\%$ error.

Table S1 (in Supplementary Information, SI) includes the new ${}^{\text{TW}}\text{CCSN}_2$ values that should be applied in further studies for the identification of phase II steroid metabolites based on their ${}^{\text{TW}}\text{CCSN}_2$. In general, these new values differ by less than 1.0 \AA^2 from published database values (i.e. in terms of relative CCS differences, see equation in Figure 1). Differences equal or greater than 1.0 \AA^2 are observed in some cases, such as for the deprotonated molecule of estradiol diglucuronide ($\Delta\text{CCS} = -1.0 \text{ \AA}^2$), the sodium adduct of DHEA glucuronide and epiandrosterone glucuronide ($\Delta\text{CCS} = 1.0$ and 1.2 \AA^2 , respectively), the protonated molecule of boldenone sulfate ($\Delta\text{CCS} = 1.0 \text{ \AA}^2$) and the $[\text{M-H}+2\text{Na}]^+$ species of DHEA sulfate, 5α -androstan- $3\alpha,17\beta$ -diol 17-sulfate and estriol 3-sulfate ($\Delta\text{CCS} = 1.1, 1.4$ and 1.8 \AA^2 , respectively). For compounds presenting a CCS of 200 \AA^2 , a difference of 1.0 \AA^2 involves a CCS deviation of $\pm 0.5\%$. As a consequence, CCS values reported by current and further databases have to be as precise as possible in order to narrow the thresholds applied to CCS measurements. It must be taken into account that small differences normally exist between the CCS of two molecules presenting equal or similar m/z , so a wide acceptance threshold could probably lead to wrong peak assignments either in targeted or non-targeted analysis.

Furthermore, despite the influence of the matrix on the CCS of analytes seems to be negligible, their characterization in terms of CCS in a broad variety of matrices is required for improving the accuracy and confidence of the CCS values reported by databases. As shown by the protonated molecule of boldenone sulfate, slight differences are observed on its ${}^{\text{TW}}\text{CCSN}_2$ depending on the type of urine (**Figure 1E**). Smaller ${}^{\text{TW}}\text{CCSN}_2$ is obtained in presence of adult bovine urine than in calve urines. The same effect is also observed for other sulfate conjugates (**Figure S1**, SI). In these cases, peak saturation was not shown and neither peak intensity was lower in one matrix than in the other. Adult bovine urine is a more complex matrix than calve urine based on their total ion chromatogram (TIC). We hypothesize that the presence of an increased ion population in the drift cell for adult bovine urine could lead to Coulombic repulsion, affecting ion mobility measurements [56].

3.2 Sensitivity enhancement by ion mobility spectrometry

One of the main advantages of the integration of IMS in LC-MS workflows is that applying drift time/CCS filter to the acquired data enables analytes to be isolated from the chemical background noise, thus improving method sensitivity [32]. Despite differential ion mobility (DMS) and high-field asymmetric waveform ion mobility spectrometry (FAIMS) act as real signal filters, TWIMS has also demonstrated to reduce the background noise when combined with LC-MS [52]. As a result, cleaned-up ion chromatograms are obtained, peak integration is facilitated and S/N is generally improved.

Figure 2 and **Figure S2** (SI) include several examples in which a clean-up effect is achieved in the extracted ion chromatograms (EICs) when the mobility region of the analyte is applied as a signal filter. In general, signals close to the LOD ($S/N = 3$) were obtained for the analysis of estradiol diglucuronide (**Figure 2A**), boldenone glucuronide (**Figure 2C**), estriol 3-sulfate (**Figure 2E**), and boldenone sulfate (**Figure S2A**, SI) in urine samples when the entire drift time range or mobility region was selected for the EICs. However, S/N was improved from 2 to 7-fold by selecting the mobility region of targeted analytes.

In addition, calve urine samples were spiked with glucuronide and sulfate conjugates at different concentration levels ($n = 7$). The following concentration levels were selected depending on the analyte: 25, 50, 100, 250, 375, 500 and $750 \mu\text{g L}^{-1}$ for androgen glucuronides and 50, 100, 200, 500, 750, 1000 and $1500 \mu\text{g L}^{-1}$ for the other phase II steroid metabolites. Samples were analyzed by LC-ESI-TWIMS-TOF-MS and LC-ESI-TOF-MS in negative mode. The same LC and ESI conditions were established for both analytical methods. On the contrary, some parameters related to the IMS-MS system were modified. When the system was operated in MS instead of IMS-MS, the flow rate of the trap buffer gas was increased from 0.2 to 2.0 mL min^{-1} for achieving ion transmission. Moreover, trap DC bias was decreased from 47.0 to 3.0 V in order to reduce ion fragmentation. Lower sensitivity is normally attributed to IMS-MS methods in comparison to MS methods due to low ion transmission [52], although in the last years several instrumental improvements has been done for overcoming this inconvenience [57]. In our case, sensitivity was not decreased when steroids were analyzed by LC-IMS-MS in comparison to the results obtained by LC-MS. In some cases, lower sensitivity was unexpectedly achieved by LC-MS than by LC-IMS-MS (**Figure S3**, SI). The further optimization of other instrumental parameters would probably improve the sensitivity provided by the LC-MS method, but this was beyond the scope of this work.

All analytes were characterized applying the following criteria: $R_t \pm 0.1 \text{ min}$, accurate $m/z \pm 10 \text{ ppm}$, and $\text{CCS} \pm 1.5\%$. When comparing to the $^{\text{TW}}\text{CCSN}_2$ values included in **Table S1** (SI), a CCS deviation within a range of $\pm 1.1\%$ was obtained for all molecular ions characterized. It highlights the robustness of the CCS as identification parameter regardless compound concentration. In general, a linear response between signal intensity and concentration level was observed in LC-ESI-TWIMS-TOF-MS for all analytes, showing wide dynamic ranges. **Table S2** (SI) includes the regression curve obtained for each steroid metabolite. Smaller dynamic range was obtained for 19-nortestosterone glucuronide, estradiol diglucuronide, estradiol 17-sulfate, and 17α -estradiol 3-sulfate. In these cases, the highest concentration level ($1500 \mu\text{g L}^{-1}$) was out of the linear range. In the case of 17α -estradiol 3-sulfate, the lowest concentration level ($50 \mu\text{g L}^{-1}$) resulted in an analytical signal below the LOQ ($S/N = 10$), but above the LOD when the mobility region of this compound was selected as signal filter. Thus, a clean-up effect on the chromatogram was achieved and sensitivity was improved by the integration of IMS in the LC-MS workflow (**Figure 3**). Due to the same clean-up effect, the signals obtained for 19-

nortestosterone glucuronide, estradiol diglucuronide and estrone 3-sulfate at 50 ng mL⁻¹ were improved and, as a consequence, S/N greater than 10 (i.e. LOQ) was reached in these cases (**Figure 3** and **Figure S2**, SI). Signals above the LOQ were generally achieved for the other metabolites at the lowest concentration level assayed even without selecting the mobility region. However, S/N was always increased at least 2-fold when the selection of the mobility region of the analyte was applied as signal filter as shown for boldenone sulfate in **Figure 3**.

Despite a more exhaustive validation is still required for the implementation of the LC-ESI-TWIMS-TOF method proposed for the determination of phase II steroid metabolites in urine (i.e. repeatability and reproducibility studies, etc.), **Table S2** also includes the LODs that were calculated as the minimum analyte concentration yielding a S/N equal to three. S/N was estimated based on peak height. LODs ranged between 1.7 and 12.5 µg L⁻¹, except for 17α-estradiol 3-sulfate (LOD = 60.0 µg L⁻¹), which shows the potential of this method for the detection of these substances at biological levels [52]. In this work, samples were only submitted to a 'dilute-and-shoot' protocol. Nevertheless, sample treatment methods such as SPE, which are usually applied in steroid analysis and usually involve sample concentration [15,18], can be applied for increasing method sensitivity and reaching lower LODs. In addition, for improving signal sensitivity, TOF system can also be operated in sensitivity mode instead of high resolution mode as it was operated.

3.3 Selectivity enhancement by ion mobility spectrometry

In addition to the sensitivity improvement achieved by TWIMS, its integration in the LC-MS workflow also provides a third separation dimension in which compounds are separated based on their CCS. The main drawback of current TWIMS technology is related to its low resolving power (max. CCS/ΔCCS ≈ 40; max. ΔCCS = 2.5% for CCS = 200 Å²). However, advances are continuously taking place in the field, and other IMS instrumentation currently allows to accomplish separations of analytes differing by 0.5% in CCS [58]. Under this context, the deprotonated molecules of testosterone sulfate (^{TW}CCSN₂ = 189.5 Å²) and epitestosterone sulfate (^{TW}CCSN₂ = 191.1 Å²) could be potentially separated since both ions present a CCS difference greater than 0.5%. In our case, as shown by **Figure S4** (SI), only those isomeric steroids in urine samples presenting a large CCS difference (ΔCCS > 4%) were separated by TWIMS such as etiocholanolone glucuronide (^{TW}CCSN₂ = 206.9 Å²) and epiandrosterone glucuronide (^{TW}CCSN₂ = 221.4 Å²) as well as 19-noretiocholanolone glucuronide (^{TW}CCSN₂ = 204.2 Å²) and 19-norandrosterone glucuronide (^{TW}CCSN₂ = 213.5 Å²). Despite both steroid pairs were also separated by LC, 19-noretiocholanolone glucuronide and 19-norandrosterone glucuronide presented a difference in Rt lower than 0.2 min. It must take into account that the LC method implemented in this work was specifically developed for the separation and detection of phase II steroid metabolites [18]. Therefore, it may be expected that both metabolites could not be chromatographically separated whenever a more generic LC method as those used in metabolomics

would be applied, thus hindering their identification. This fact justifies the need to explore orthogonal and complementary tools to LC for improving analyte separation and method selectivity.

The improvement on selectivity provided by TWIMS also allows to separate targeted analytes from co-eluting matrix compounds that present similar m/z , thus improving peak integration and quantification process. As shown in **Figure 4**, testosterone glucuronide co-elutes with an intrinsic urine component which also presents a m/z within the m/z 463.2 - 463.3 range. Nevertheless, this matrix peak is avoided when the mobility range of the deprotonated molecule of testosterone glucuronide is selected. Furthermore, a high intense peak related to urine matrix (m/z 413.2299) presents a similar R_t than testosterone sulfate. Consequently, the $[M-H+2Na]^+$ species of this analyte (m/z 413.1369) can barely be detected due to its low intensity (**Figure 5S**, SI). In this case, the selection of its mobility region allows to isolate this ion from other molecular species and, as a result, achieving a cleaned-up chromatogram where its related chromatographic peak can be perfectly identified. **Figure 5** and **Figure 6S** (SI) represent the two-dimensional IMS-MS spectrum resulted from the analysis of androgen glucuronides in adult bovine urine in negative and positive mode, respectively. As can be observed, analytes are separated from matrix compounds in the ion mobility dimension as well as some isomeric pairs (e.g. testosterone and epitestosterone glucuronides in positive ionization conditions, **Figure 6S**, SI).

3.4 Towards the implementation of IMS for the non-targeted analysis of steroid

As discussed above, the integration of IMS in LC-MS systems increases detection sensitivity and selectivity as well as provides a novel parameter for compound identification (i.e. CCS). These advantages may enhance the performance characteristics of analytical methods intended for the targeted analysis of steroids but, undoubtedly, will improve non-targeted approaches outcome. In non-targeted methods, compound identification is usually carried out based on m/z spectra from public databases. However, compound identification cannot rely on R_t if standards are not available because LC methods can suffer slight variations within laboratories. Within this framework, CCS has huge potential for supporting compound identification. It is an intrinsic characteristic of each molecule and, in general, does not depend on experimental conditions, except from the drift buffer gas. As an example, a non-spiked adult bovine urine sample was analyzed according to our LC-TWIMS-MS workflow for steroid analysis. Under the context of steroid analysis, three peak signals, which presented a S/N greater than 3 (i.e. LOD), could initially be assigned to etiocholanolone and/or epiandrosterone glucuronides since these signals matched their exact monoisotopic mass (m/z 465.2483) within ± 5 ppm error (**Figure S6**, SI). Applying CCS criteria (i.e. $^{TW}CCS_{N_2} \pm 1.5$), only one signal could be attributed to etiocholanolone glucuronide whereas any of the other two peaks could be assigned to epiandrosterone glucuronide. In our case, peak assignment was finally supported by R_t (± 0.1 min) since these steroid standards were available in our laboratory. **Figure S7** (SI) shows the EIC

(m/z 465.2) obtained from the analysis of the urine sample fortified with both glucuronide conjugates ($0.2 \mu\text{g L}^{-1}$). The presence of epiandrosterone glucuronide in the sample was finally discarded based on its R_t , whereas the presence of etiocholanolone glucuronide was confirmed. At this point, other information such as fragmentation and isotopic patterns could also be applied for a more confident peak assignment.

As a real application, urine samples collected from calves exposed *per os* to boldione were analyzed by LC-TWIMS-MS. Boldione is an active precursor of boldenone, which is a popular steroid for misuse [59]. Concluding on boldenone misuse in livestock requires metabolism investigations to highlight relevant markers such as phase II metabolites [60]. In urine sample collected one day after boldione administration, at R_t of 7.2 min, a peak signal presenting m/z 461.2181 and m/z 463.2328 was detected when samples were analyzed in negative and positive mode, respectively (**Figure 6**). Both signals were attributed to the deprotonated and protonated molecules of boldenone glucuronide, respectively, with a mass accuracy tolerance of 5 ppm. MS/MS experiments in positive mode were carried out for confirming the presence of this molecule in urine samples. For these analysis, m/z 463 was selected in the quadrupole and 20 V was established as transfer cell voltage. In addition to the precursor ion (i.e. $[M+H]^+$), two fragments were also identified, $[M-C_6H_8O_6+H]^+$ (m/z 287.2006) and $[M-H_2O-C_6H_8O_6+H]^+$ (m/z 269.1900), which were also previously detected in MS experiments. The loss of the glucuronide group confirmed the presence of a boldenone conjugate but this information was not enough to confirm if this compound was the 17β - or 17α -boldenone glucuronide form. Based on CCS measurements, the presence of 17β -boldenone glucuronide was discarded because its $[M+H]^+$ and $[M-H]$ species present similar CCS (217.0 and 218.3 \AA^2 , respectively). On the contrary, a CCS difference of 4.9% was observed between the CCS of the ions detected under both positive and negative mode. Consequently, the chromatographic peak was tentatively assigned to 17α -boldenone glucuronide, mainly based on mass spectra, but also supported by CCS measurements. This metabolite has previously been pointed as a suspicious biomarker of the illegal use of boldenone [9,61]. Furthermore, it is not surprising that the protonated molecule of 17α -boldenone glucuronide could be more compact than its deprotonated molecule. As mentioned above, the same effect has been observed for epitestosterone glucuronide, in which the glucuronide group is also in position 17α . Finally, samples were spiked with 17β -boldenone glucuronide ($2 \mu\text{g mL}^{-1}$) for reinforcing our results. As shown by **Figure 6**, the peak related to 17β -boldenone glucuronide and the tentatively attributed to 17α -boldenone glucuronide present different R_t . In positive mode, both peaks are also separated in the mobility dimension based on their CCS difference (**Figure S8, SI**).

4 Concluding remarks

The recent commercialization of IMS-MS instruments is providing new opportunities to extend the current boundaries of targeted and non-targeted analysis. Nevertheless, more studies about the

robustness and advantages provided by this technology are still required before it can be fully implemented in analytical laboratories, especially for those applications that can involve legal consequences.

In this context, this work shows that CCS can be used with confidence for the identification of phase II steroid metabolites in addition to m/z and R_t . In general, this molecular characteristic has not shown to be influenced by urine matrix. If any effect was observed, it did not lead to CCS deviations above the threshold currently accepted for CCS measurements (i.e. $CCS \pm 2\%$). Considering $^{TW}CCS_{N_2}$ values from a published database, high accuracy was obtained for CCS measurements over time (i.e. within four months), since $\Delta CCS < 1.3\%$ was observed in the majority of the cases. Based on our results, the feasibility of reducing the threshold from 2% to at least 1.5% for CCS measurements increases. Obviously, this issue requires a deep discussion within the ion mobility community, and several aspects such as the related increase of false negative results should be considered before considering such parameter as additional new identification criteria, in the current context of Dec 2002/657/EU revision. On the contrary, keeping a wide threshold such as 2% can involve high number of false positive results since molecules with the same m/z normally present similar CCS. Normalized CCS databases are required for a deeper evaluation of the accuracy of CCS measurements that should lead to a consensus decision about reducing this threshold. The CCS characterization of compounds in different matrices by different IMS technologies, and involving inter-laboratory studies, is view as the first step to create normalized CCS databases. Consequently, it brings new opportunities of collaboration within the ion mobility community and its related application areas. In our case, the validation of our CCS database for steroids by DTIMS and TIMS is within the framework of our current perspectives.

In addition, the implementation of TWIMS in LC-MS workflows is a potential strategy to improve method sensitivity. The selection of the mobility region of targeted analytes reduces background noise, providing cleaned-up chromatograms and, consequently, greater S/N. In the case of phase II steroid metabolites in urine samples, sensitivity was improved between 2 and 7-fold. TWIMS also provided higher selectivity, not only by improving the separation of isomeric steroids but also by allowing the separation of analytes and co-eluting matrix compounds. Therefore, TWIMS has shown that its integration in LC-MS methods can improve analytical performance characteristics such as peak capacity or LODs, without being extremely limited by the dynamic range provided. This approach is very useful in the case of steroid analysis since these compounds are present at low physiological concentration levels and are constituted by a wide range of isobaric and isomeric compounds.

From our point of view, we are still in the early stages of the implementation of IMS-MS in routine analysis either in targeted or non-targeted methods. Nevertheless, it offers great opportunities such as a novel identification parameter as well as sensitivity and selectivity improvements. Evidently, non-

targeted approaches such as metabolomics will obtain higher benefit from this technology. The number of detected peaks can be increased and peak assignment can be carried out with more confidence as shown by the tentative identification of 17 α -boldenone glucuronide in urine samples as one of the metabolites resulted from the administration of boldione.

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Figure captions

Figure 1. A) Accuracy of CCS measurements of phase II steroid metabolites in urine samples (n = 1806). Evaluation of the CCS accuracy within four months for: B) estradiol 3-glucuronide (i.e. [M-H]⁻), C) estradiol 17-glucuronide (i.e. [M-H]⁻), D) estriol 3-sulfate (i.e. [M-H+2Na]⁺), and E) boldenone sulfate (i.e. [M+H]⁺).

Figure 2. EICs resulted from the analysis of: I) estradiol diglucuronide (E₂-DiG; 2 µg mL⁻¹; [M+Na]⁺), and II) boldenone glucuronide (Bold-G; 0.2 µg mL⁻¹; [M-H]⁻) in adult bovine urine samples, and III) estriol 3-sulfate (E₃-3S; 2 µg mL⁻¹; [M-H+2Na]⁺) in calve urine samples by LC-ESI-TWIMS-TOF-MS. The following filters were applied for the signal processing of the related total ion chromatograms: A) *m/z* 647, B) *m/z* 647.0, and drift time range between 11.3 and 11.7 ms, C) *m/z* 461, and D) *m/z* 461, and drift time range between 4.9 and 5.2 ms, E) *m/z* 413, F) *m/z* 413, and drift time range between 7.3 and 7.7 ms.

Figure 3. EICs resulted from the analysis of: I) 17α-estradiol 3-sulfate (aE₂-3S; 50 µg L⁻¹; [M-H]⁻), II) 19-nortestosterone glucuronide (19NT-G; 50 µg L⁻¹; [M-H]⁻) and III) boldenone sulfate (Bold-S; 50 µg L⁻¹; [M-H]⁻) in calve urine samples by LC-ESI-TWIMS-TOF-MS. The following filters were applied for the signal processing of the related total ion chromatograms: A) *m/z* 351, B) *m/z* 351, and drift time range between 3.8 and 4.2 ms, C) *m/z* 449, D) *m/z* 449, and drift time range between 4.7 and 5.1 ms, E) *m/z* 365, and F) *m/z* 365, and drift time range between 3.8 and 4.3 ms.

Figure 4. EICs (*m/z* 463.2) resulted from the analysis of: A) and B) adult bovine urine samples spiked with testosterone glucuronide (0.2 µg mL⁻¹), and C) and D) non-spiked adult bovine urine samples. Analyses were performed by LC-ESI-TWIMS-TOF-MS in negative mode. In B) and D), the mobility range of the deprotonated molecule of testosterone glucuronide (i.e. between 4.7 and 5.2 ms) was selected.

Figure 5. Two-dimensional IMS-MS spectrum showing the separation of androgen glucuronides (0.2 µg mL⁻¹) in adult bovine urine under ESI- conditions. Peaks identification: A, etiocholanolone glucuronide; B, boldenone glucuronide; C, epitestosterone glucuronide; D, testosterone glucuronide; E, DHEA glucuronide; and F, epiandrosterone glucuronide. Other red points indicate matrix compounds presenting a signal intensity similar to the intensity of the targeted compounds. Peak selection was limited to a Rt range between 0 and 10 min, and a *m/z* range between 460 and 466.

Figure 6. EICs resulted from the analysis of urine samples from calves exposed to boldione by LC-ESI-TWIMS-TOF-MS in: A) negative mode (*m/z* 461) and B) positive mode (*m/z* 463). Mass spectra and analytical information of the chromatographic peak detected at 7.2 min are also shown. C) EIC

(m/z 463) of urine samples spiked with 17β -boldenone glucuronide ($2 \mu\text{g mL}^{-1}$) and analyzed in positive mode.

Table 1. ^{TW}CCSN₂, accurate *m/z* and Rt of phase II steroid metabolites analyzed by LC-ESI-TWIMS-TOF-MS in urine samples.

Compound	Chemical formula	Molecular weight (g/mol)	Rt (min)	ESI-				ESI+			
				Ion	<i>m/z</i>	^{TW} CCSN ₂ (Å ²) ¹	^{TW} CCSN ₂ (Å ²) ¹ in urine samples ² (n = 39)	Ion	<i>m/z</i>	^{TW} CCSN ₂ (Å ²) ¹	^{TW} CCSN ₂ (Å ²) ¹ in urine samples ² (n = 40)
ANDROGENS											
testosterone glucuronide (4-androsten-17β-ol-3-one glucuronide)	C ₂₅ H ₃₆ O ₈	464.555	6.7	[M-H] ⁻	463.2326	218.5*	218.4	[M+H] ⁺	465.2483	219.8	220.4
epitestosterone glucuronide (4-androsten-17α-ol-3-one glucuronide)	C ₂₅ H ₃₆ O ₈	464.555	7.8	[M-H] ⁻	463.2326	218.5*	218.3	[M+H] ⁺	465.2483	206.0	206.3
DHEA glucuronide (5-androsten-3β-ol-17-one glucuronide)	C ₂₅ H ₃₆ O ₈	464.555	7.1	[M-H] ⁻	463.2326	221.2*	221.2	[M+Na] ⁺	487.2302	231.6	232.6
etiocholanolone glucuronide (5β-androstan-3α-ol-17-one glucuronide)	C ₂₅ H ₃₈ O ₈	466.571	8.5	[M-H] ⁻	465.2483	207.2*	206.9	[M+Na] ⁺	489.2459	208.8	209.5
epiandrosterone glucuronide (5α-androstan-3β-ol-17-one glucuronide)	C ₂₅ H ₃₈ O ₈	466.571	7.5	[M-H] ⁻	465.2483	221.7*	221.4	[M+Na] ⁺	489.2459	232.0	233.1
boldenone glucuronide (1,4-androstadien-17β-ol-3-one glucuronide)	C ₂₅ H ₃₄ O ₈	462.539	6.2	[M-H] ⁻	461.2170	217.1*	217.0	[M+H] ⁺	463.2326	217.6	218.3
testosterone sulfate (4-androsten-17β-ol-3-one sulfate)	C ₁₉ H ₂₈ O ₅ S	368.488	6.1	[M-H] ⁻	367.1574	189.5*	189.3	[M+H] ⁺	369.1730	190.5	190.7
epitestosterone sulfate (4-androsten-17α-ol-3-one sulfate)	C ₁₉ H ₂₈ O ₅ S	368.488	6.3	[M-H] ⁻	367.1574	191.1*	190.9	[M+H] ⁺	369.1730	191.6	192.0
epiandrosterone sulfate (5α-androstan-3β-ol-17-one sulfate)	C ₁₉ H ₃₀ O ₅ S	370.504	7.0	[M-H] ⁻	369.1730	195.2*	194.9	[M-H+2Na] ⁺	415.1526	225.5	225.9
androsterone sulfate (5α-androstan-3α-ol-17-one sulfate)	C ₁₉ H ₃₀ O ₅ S	370.504	7.3	[M-H] ⁻	369.1730	194.1*	193.8	[M-H+2Na] ⁺	415.1521	221.0	220.7
DHEA sulfate (5-androsten-3β-ol-17-one sulfate)	C ₁₉ H ₂₈ O ₅ S	368.488	6.7	[M-H] ⁻	367.1574	193.9*	193.7	[M-H+2Na] ⁺	413.1369	223.4	224.5
5α-androstan-3α,17β-diol 17-sulfate	C ₁₉ H ₃₂ O ₅ S	372.52	7.2	[M-H] ⁻	371.1887	193.6*	193.4	[M-H+2Na] ⁺	417.1682	219.3	220.7
boldenone sulfate (1,4-androstadien-17β-ol-3-one sulfate)	C ₁₉ H ₂₆ O ₅ S	366.472	5.7	[M-H] ⁻	365.1417	188.5*	188.2	[M+H] ⁺	367.1574	188.2	189.1

ESTROGENS											
19-nortestosterone glucuronide (4-estren-17 β -ol-3-one glucuronide)	C ₂₄ H ₃₄ O ₈	450.528	6.2	[M-H] ⁻	449.2170	214.0	214.9	[M+H] ⁺	451.2326	217.5	217.8
estradiol 17-glucuronide (1,3,5(10)-estratriene-3,17 β -diol-17-glucuronide)	C ₂₄ H ₃₂ O ₈	448.512	6.2	[M-H] ⁻	447.2013	214.9	215.1	[M+Na] ⁺	471.1989	222.4	223.0
estradiol 3-glucuronide (1,3,5(10)-estratrien-3,17 β -diol 3-glucuronide)	C ₂₄ H ₃₂ O ₈	448.512	5.7	[M-H] ⁻	447.2013	218.5	219.2	[M+Na] ⁺	471.1989	217.0	216.9
estradiol diglucuronide (1,3,5(10)-estratrien-3,17 β -diol diglucuronide)	C ₃₀ H ₄₀ O ₁₄	624.636	4.0	[M-H] ⁻	623.2334	255.0	254.0	[M+Na] ⁺	647.2310	264.4	263.7
19-noretiocholanolone glucuronide (5 β -estran-3 α -ol-17-one glucuronide)	C ₂₄ H ₃₆ O ₈	452.544	7.8	[M-H] ⁻	451.2326	205.1	204.2	[M+Na] ⁺	475.2302	205.4	206.2
19-norandrosterone glucuronide (5 α -estran-3 α -ol-17-one glucuronide)	C ₂₄ H ₃₆ O ₈	452.544	8.0	[M-H] ⁻	451.2326	214.1	213.5	[M-2H ₂ O+H] ⁺	417.2272	194.6	195.5
19-nortestosterone sulfate (4-estren-17 β -ol-3-one sulfate)	C ₁₈ H ₂₆ O ₅ S	354.461	5.5	[M-H] ⁻	353.1417	185.9	185.6	[M+H] ⁺	355.1574	187.4	187.8
estradiol 17-sulfate (1,3,5(10)-estratrien-3,17 β -diol 17-sulfate)	C ₁₈ H ₂₄ O ₅ S	352.445	5.4	[M-H] ⁻	351.1261	185.5	185.4	n.d.			
17 β -estradiol 3-sulfate (1,3,5(10)-estratrien-3,17 β -diol-3-sulfate)	C ₁₈ H ₂₄ O ₅ S	352.445	5.6	[M-H] ⁻	351.1261	189.4	189.4	n.d.			
17 α -estradiol 3-sulfate (1,3,5(10)-estratrien-3,17 α -diol-3-sulfate)	C ₁₈ H ₂₄ O ₅ S	352.445	6.0	[M-H] ⁻	351.1261	189.4	189.4	n.d.			
estrone 3-sulfate (1,3,5(10)-estratrien-3-ol-17-one 3-sulfate)	C ₁₈ H ₂₂ O ₅ S	350.429	6.1	[M-H] ⁻	349.1104	187.8	187.4	n.d.			
estriol 3-sulfate (1,3,5(10)-estratrien-3,16,17-triol-3-sulfate)	C ₁₈ H ₂₄ O ₆ S	368.444	3.3	[M-H] ⁻	367.1210	193.2	192.3	[M-H+2Na] ⁺	413.1005	212.3	214.1

Notes:

* ^{TW}CCSN₂ values of steroid standards that have been experimentally measured and reported for the first time. They are averaged values resulted from CCS measurements that were done within four months (n = 9).

¹ ^{TW}CCSN₂ values previously reported by the CCS database for steroids [39].

² Averaged ^{TW}CCSN₂ values of phase II steroid metabolites in urine samples (i.e. adult bovine and calve urines) within four months.

Abbreviations:

n.d., not detectable

Figure 1

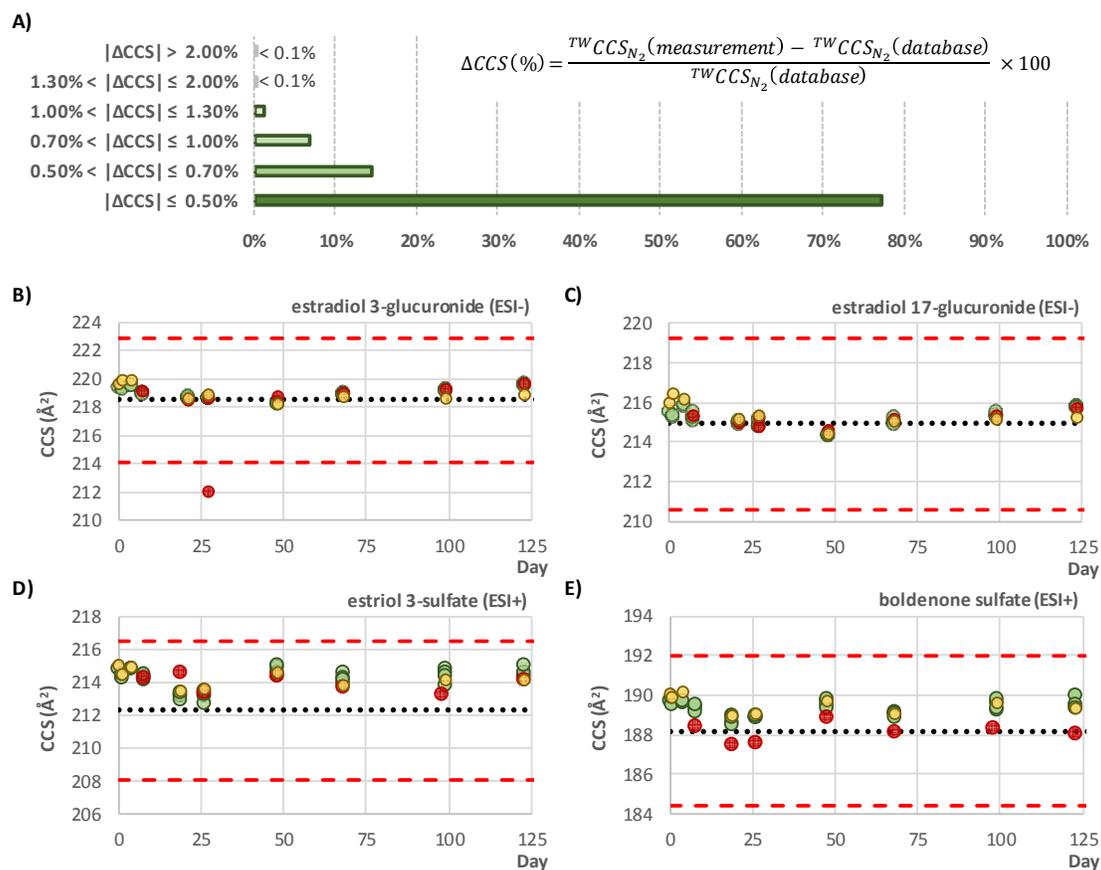


Figure caption:

- Steroids in adult bovine urine samples
- Steroids in calve urine samples
- Steroid standards

- $^{TW}CCS_{N_2}$ values reported in the CCS database for steroids (in Hernández-Mesa et al., *Anal. Chem.* 2018, 90, 4616-4625.
- Threshold of $\pm 2\%$ from the $^{TW}CCS_{N_2}$ reported in the CCS database for steroids.

Figure 2

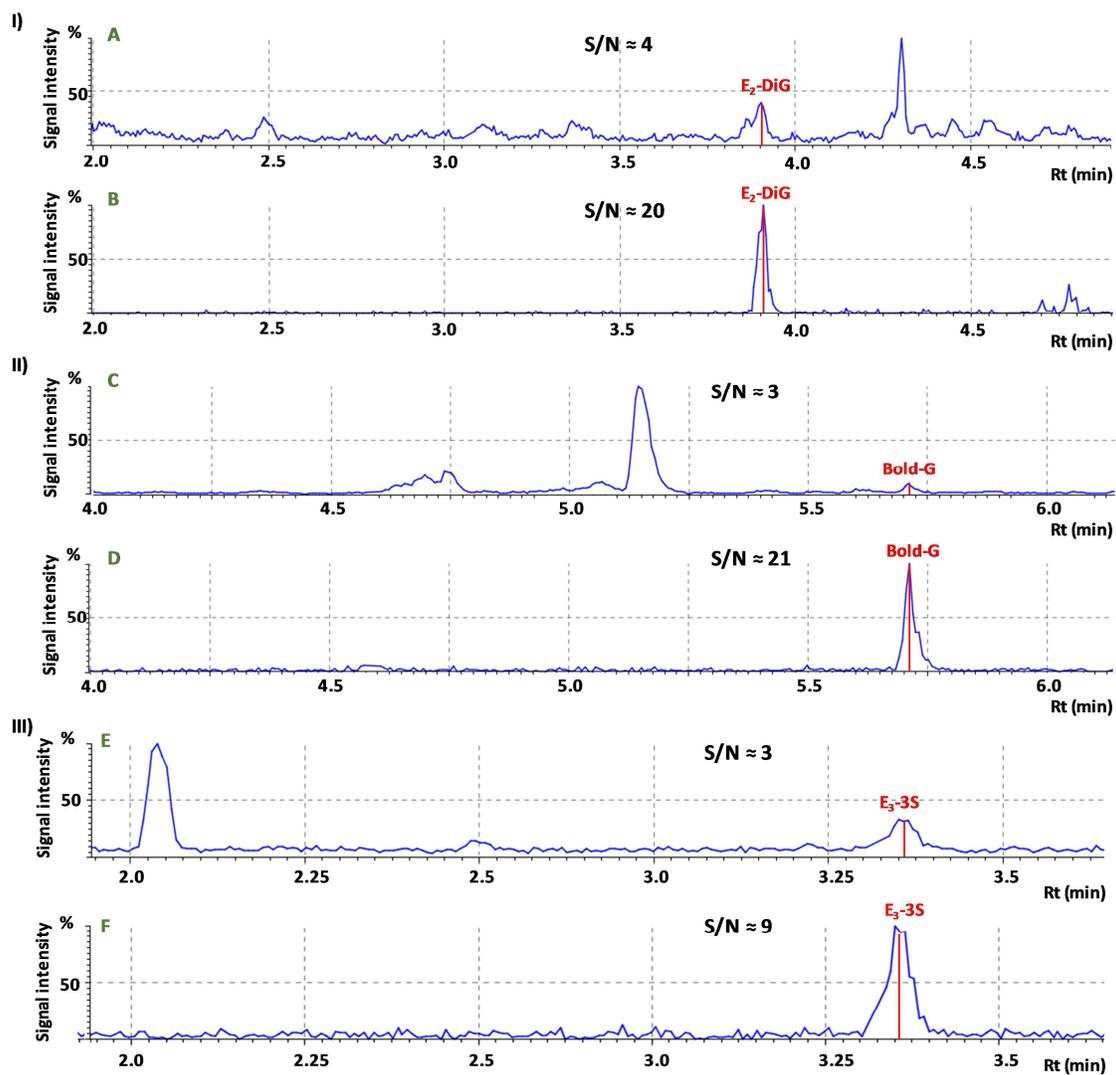


Figure 3

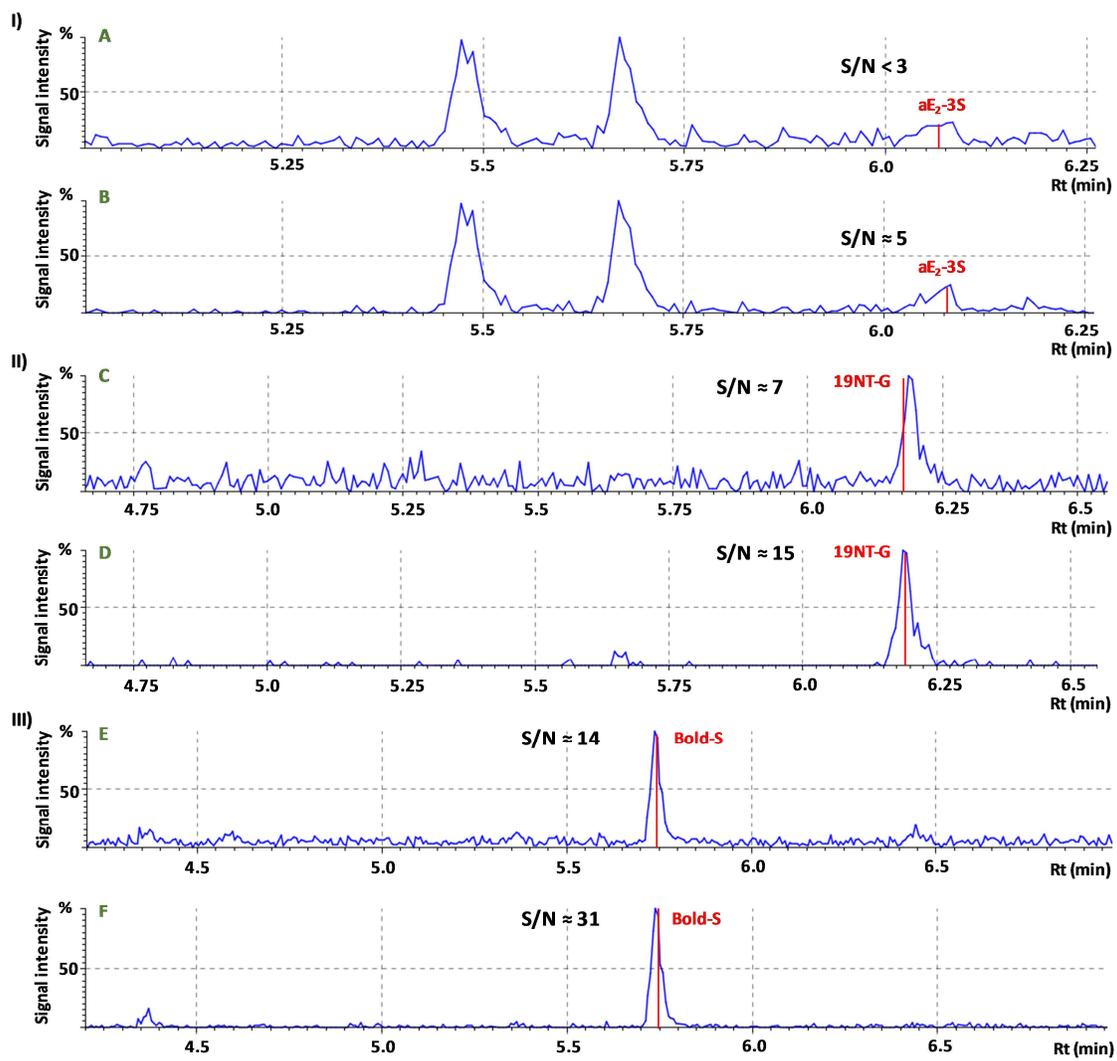


Figure 4

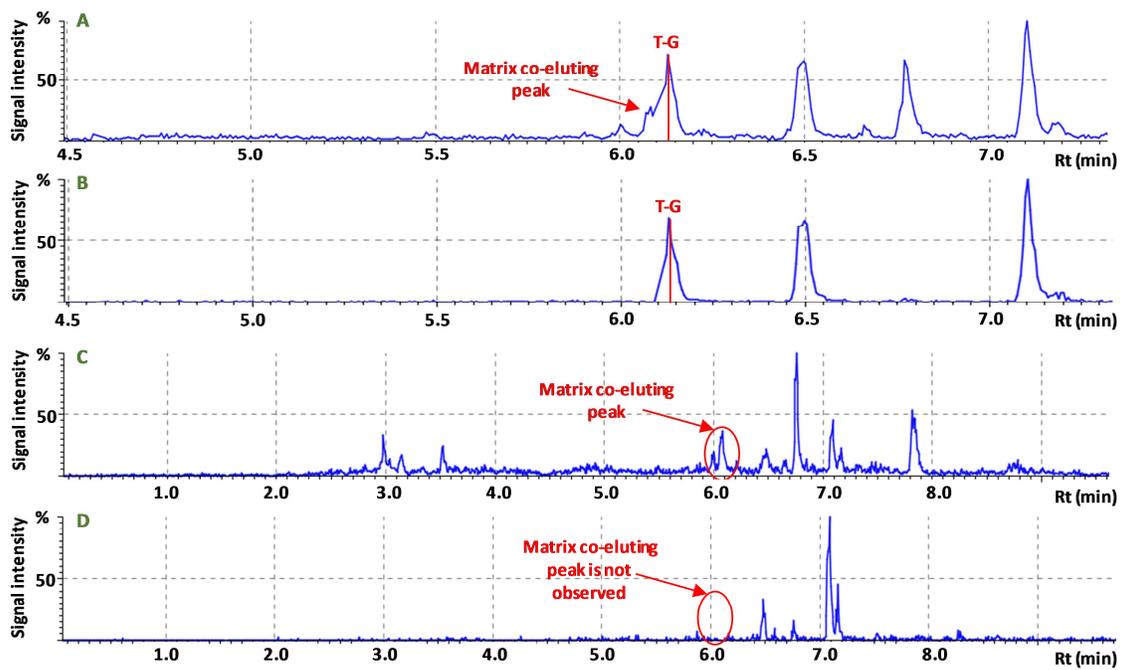


Figure 5

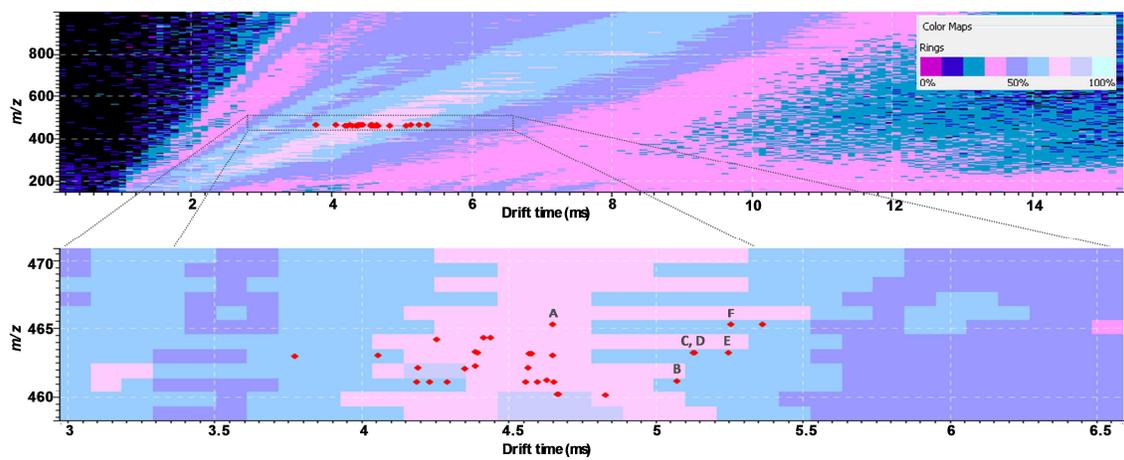
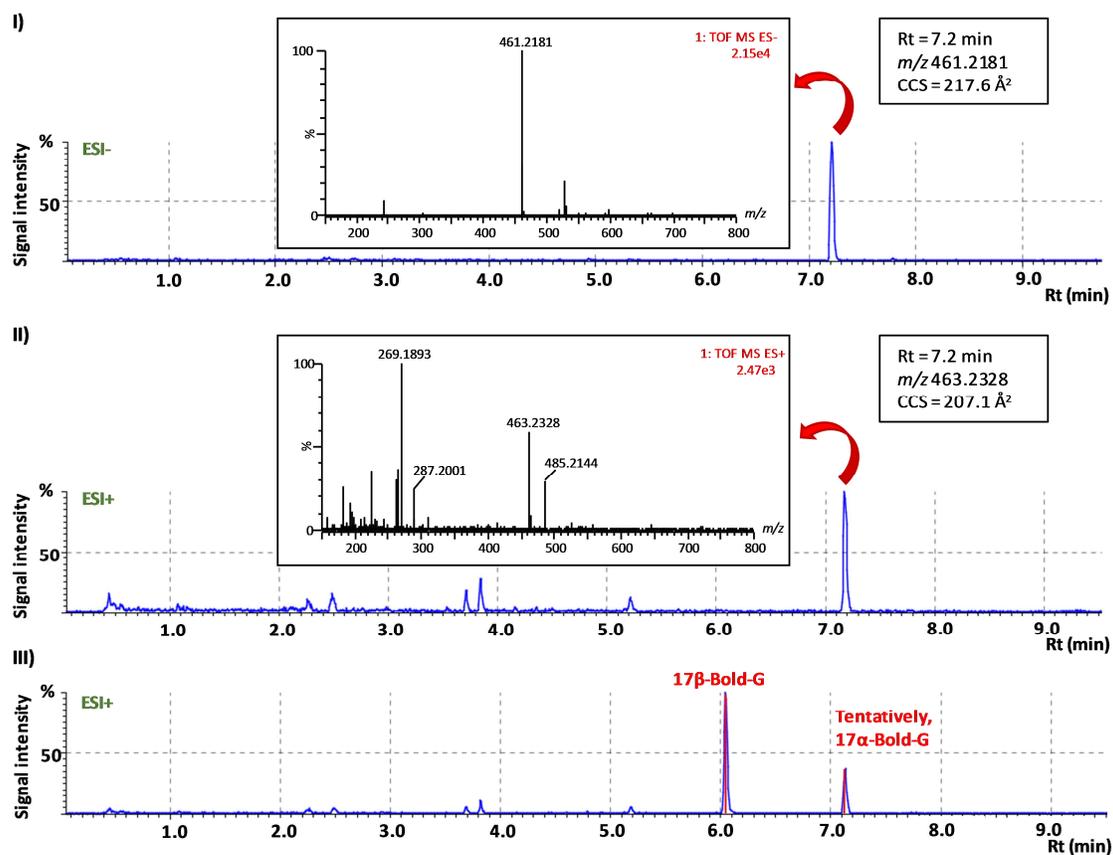


Figure 6



Graphical abstract

