Potential of ion mobility-mass spectrometry for both targeted and non-targeted analysis of phase II steroid metabolites in urine

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ABSTRACT

In recent years, the commercialization of hybrid ion mobility-mass spectrometers and their integration in traditional LC-MS workflows provide 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, \( \Omega \)), 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 (Rt) 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 (\( \Delta CCS < 1.3\% \) 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 epioandrosterone glucuronide, as well as 19-noretioccholanolone glucuronide and 19-norandrostosterone 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, 17a-boldenone glucuronide has been identified as one of the main metabolites resulted from boldione administration to calves.

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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 imple-
mented for this purpose [6,7]. LC-ESI-MS is especially recom-

dmended for the direct detection of conjugated steroids, such as those related to phase II metabolism (i.e. glucuronides, sulfates, 
cysteiny1, 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 enzym-
ic 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 [13–16]. These novel strategies 

not only provide a better understanding of steroid metabolism (i.e. 
steroidome) and the causes of its perturbations, but also can lead to 
the discovery of new biomarkers for detecting the illegal admin-
istration of steroids [17,18].

Advances in analytical instrumentation, mainly related to im-
provements 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 pre-
determined number of steroid metabolites (i.e. targeted metab-

olomics) 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 pre-

sent in the sample (non-targeted metabolomics) [2,9]. In this re-
gard, 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 ex-
posures [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 [22–24]. 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 tech-
nique that can be easily coupled to LC-MS systems and provides 
complementary information to mass spectra and Rt for the char-
acterization 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 [30–32]. 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, 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 car-
ried out by other IMS techniques. However, this structural param-
eter can also be measured by travelling wave ion mobility spectrometry (TWIMS), differential mobility analyzer (DMA) and 
trapped ion mobility spectrometry (TIMS) after instrument cali-
bration with compounds of known CCS under defined conditions 
[29,34,35].

Within this framework, several CCS databases have been re-
ported with the aim of integrating the CCS as an identification 
parameter in non-targeted metabolomics workflows [36–41].

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 com-
ounds 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 [43–46], a recent inter-laboratory study has 
shown that the tolerance for CCS measurements can be potentially reduced to ±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 ±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 
isorbic 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 demon-
strated the feasibility of IMS-MS for the separation of steroid iso-
mers 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-androstan-3,17-dione and 5α-androstan-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 calf 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-noretiocholanolone glucuronide, 19-norandrostenedione glucuronide, testosterone sulfate, epitestosterone sulfate, epiandrosterone sulfate, androsterone sulfate, DHEA sulfate, 5α-androstan-3α,17β-diol 17-sulfate, boldenone sulfate, androstenedione 3-sulfate, estrone 3-sulfate, and estradiol 3-sulfate were acquired from Steraloids (Newport, RI, USA). Each steroid stock solution was prepared at 100 µg mL⁻¹ in ethanol. Working standard solutions (10 µg 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 µg 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 calf 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 µg mL⁻¹ with working standard solutions and submitted to 10-fold dilution with 0.1% (v/v) aqueous formic acid. Initially, the 7TCCSN₂ of steroids was measured in different calf 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 µg 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 17β-glucuronide, 17β-estradiol 3-glucuronide, estradiol diglucuronide, 19-noretiocholanolone glucuronide, 19-norandrostenedione glucuronide), seven androgen sulfates (i.e. testosterone sulfate, epitestosterone sulfate, epiandrosterone sulfate, androsterone sulfate, DHEA sulfate, 5α-androstan-3α,17β-diol 17-sulfate, boldenone sulfate), and six estrogen sulfates (i.e. 19-nortestosterone sulfate, estradiol 17β-sulfate, 17β-estradiol 3-sulfate, 17α-estradiol 3-sulfate, estrone 3-sulfate, estriol 3-sulfate) at a concentration of 2 µg 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® System from Waters® was used to perform reversed phase liquid chromatography on a C18 column (Acquity UPLC® BEH C18, 2.1 × 100 mm, 1.7 µm; Waters®). Separations were carried out at 50 °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, 57:43 at 9.6 min, 100:0 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®) 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 °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 to 219 m s\(^{-1}\) and 4.0 V, respectively. IMS DC bias and trap DC bias were set at 3.0 and 470 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 μL 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- mode, 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 Å\(^2\). In negative conditions, CCS calibration curves covered a m/z range from 318 to 1082 and a CCS range from 130 to 322 Å\(^2\).

2.5. Data analysis

Chromatograms as well as mass and mobility spectra were analyzed using MassLynx (version 4.2, Waters\({\textsuperscript{\@}}\)) 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 TWCCSN\(_{9}\) 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 (ΔCCS = 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 begin now 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- mode. 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 theTWCCSN\(_{9}\) values reported in the database. In total, 297 CCS measurements were carried out taking into account positive and negative ionization conditions. Differences between the average measured TWCCSN\(_{9}\) values and theTWCCS\(_{9}\) 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, theTWCCSN\(_{9}\) of steroids was measured in different urine samples (n = 5) that were directly fortified at 2 μg 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 theTWCCSN\(_{9}\) 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 extends the current information available about the CCS characterization of steroids [38,39,54,55]. As previously observed for estrogen compounds [39], theTWCCS\(_{9}\) 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 TWCCSN\(_{9}\) much smaller than its related [M-H\(^{-}\)]\(^{-}\) ion (i.e. 206.0 Å\(^2\) and 218.5 Å\(^2\), 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 ±0.50% were observed for the averaged TWCCSN\(_{9}\) of steroid metabolites in urine samples compared to theTWCCSN\(_{9}\) values reported in the database (Table 1), except for DHEA sulfate (ΔCCS = +0.51%), 5α-androstan-3α,17β-diol 17-sulfate (ΔCCS = +0.64%) and estradiol 3-sulfate (ΔCCS = +0.84%) analyzed in positive mode. Therefore, urine matrix does not seem to have any relevant influence on the drift time and, consequently, 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.
| Compound | Chemical formula | Molecular weight (g/mol) | Rt (min) | ESI- m/z | **TWCCSN<sub>2</sub> (Å<sup>2</sup>)<sup>a</sup>** | **TWCCSN<sub>2</sub> (Å<sup>2</sup>)** | ESI<sup>+</sup> Ion | m/z | **TWCCSN<sub>2</sub> (Å<sup>2</sup>)<sup>a</sup>** | **TWCCSN<sub>2</sub> (Å<sup>2</sup>)** |
|----------|------------------|--------------------------|---------|---------|-----------------|-----------------|-----------------|----|-----------------|-----------------|
| **ANDROGENS** | | | | | | | | | | |
| testosterone glucuronide (4-androsten-17β-ol-3-one glucuronide) | C<sub>25</sub>H<sub>36</sub>O<sub>8</sub> | 464.555 | 6.7 | [M-H]<sup>-</sup> | 463.2326 | 218.5<sup>a</sup> | 218.4 | [M + H]<sup>+</sup> | 465.2483 | 219.8 | 220.4 |
| epitestosterone glucuronide (4-androsten-17α-ol-3-one glucuronide) | C<sub>25</sub>H<sub>36</sub>O<sub>8</sub> | 464.555 | 7.8 | [M-H]<sup>-</sup> | 463.2326 | 218.5<sup>a</sup> | 218.3 | [M + H]<sup>+</sup> | 465.2483 | 206.0 | 206.3 |
| DHEA glucuronide (5-androsten-3β-ol-17-one glucuronide) | C<sub>25</sub>H<sub>36</sub>O<sub>8</sub> | 464.555 | 7.1 | [M-H]<sup>-</sup> | 463.2326 | 221.2<sup>a</sup> | 221.2 | [M + Na]<sup>+</sup> | 487.2302 | 231.6 | 232.6 |
| etiocholanolone glucuronide (5β-androstan-3α-ol-17-one glucuronide) | C<sub>25</sub>H<sub>36</sub>O<sub>8</sub> | 466.571 | 8.5 | [M-H]<sup>-</sup> | 465.2483 | 207.2<sup>a</sup> | 206.9 | [M + Na]<sup>+</sup> | 489.2459 | 208.8 | 209.5 |
| epiaンドrostosterone glucuronide (5α-androstan-3β-ol-17-one glucuronide) | C<sub>25</sub>H<sub>36</sub>O<sub>8</sub> | 466.571 | 7.5 | [M-H]<sup>-</sup> | 465.2483 | 221.7<sup>a</sup> | 221.4 | [M + Na]<sup>+</sup> | 489.2459 | 232.0 | 233.1 |
| boldenone glucuronide (1,4-androstadien-17β-ol-3-one glucuronide) | C<sub>19</sub>H<sub>28</sub>O<sub>5</sub>S | 366.472 | 5.7 | [M-H]<sup>-</sup> | 365.1417 | 188.2 | 183.4 | [M + H]<sup>+</sup> | 367.1574 | 188.2 | 189.1 |
| **ESTROGENS** | | | | | | | | | | |
| 19-nortestosterone glucuronide (4-estren-17β-ol-3-one glucuronide) | C<sub>24</sub>H<sub>34</sub>O<sub>8</sub> | 450.528 | 6.2 | [M-H]<sup>-</sup> | 449.2170 | 214.0 | 214.9 | [M + H]<sup>+</sup> | 451.2266 | 217.5 | 217.8 |
| estradiol 17-glucuronide (1,3,5(10)-estratriene-3,17β-diol-17-glucuronide) | C<sub>18</sub>H<sub>24</sub>O<sub>5</sub>S | 488.512 | 6.2 | [M-H]<sup>-</sup> | 487.2013 | 214.9 | 215.1 | [M + Na]<sup>+</sup> | 471.1989 | 222.4 | 223.0 |
| estradiol 3-glucuronide (1,3,5(10)-estratrien-3,17β-diol-3-glucuronide) | C<sub>18</sub>H<sub>24</sub>O<sub>5</sub>S | 488.512 | 5.7 | [M-H]<sup>-</sup> | 487.2013 | 218.5 | 219.2 | [M + Na]<sup>+</sup> | 471.1989 | 217.0 | 216.9 |
| estradiol diglucuronide (1,3,5(10)-estratrien-3,17β-diol diglucuronide) | C<sub>18</sub>H<sub>24</sub>O<sub>5</sub>S<sub>4</sub> | 624.636 | 4.0 | [M-H]<sup>-</sup> | 623.2334 | 255.0 | 254.0 | [M + Na]<sup>+</sup> | 647.2310 | 264.4 | 263.7 |
| 19-noretiocholanolone glucuronide (5β-estrane-3α-ol-17-one glucuronide) | C<sub>24</sub>H<sub>30</sub>O<sub>8</sub> | 452.544 | 7.8 | [M-H]<sup>-</sup> | 451.2266 | 205.1 | 204.2 | [M + Na]<sup>+</sup> | 475.2302 | 205.4 | 206.2 |
| 19-norandrostosterone glucuronide (5z-estrane-3α-ol-17-one glucuronide) | C<sub>24</sub>H<sub>30</sub>O<sub>8</sub> | 452.544 | 8.0 | [M-H]<sup>-</sup> | 451.2266 | 214.1 | 213.5 | [M + 2H<sub>2</sub>O + H]<sup>+</sup> | 413.1065 | 212.3 | 214.1 |
| 19-nortestosterone sulfate (4-estren-17β-ol-3-one sulfate) | C<sub>19</sub>H<sub>28</sub>O<sub>5</sub>S | 384.461 | 5.5 | [M-H]<sup>-</sup> | 353.1417 | 185.9 | 185.6 | [M + H]<sup>+</sup> | 355.1574 | 187.4 | 187.8 |
| estradiol 17-sulfate (1,3,5(10)-estratrien-3,17β-diol-17-sulfate) | C<sub>18</sub>H<sub>24</sub>O<sub>5</sub>S<sub>2</sub> | 352.445 | 5.4 | [M-H]<sup>-</sup> | 351.1261 | 185.5 | 185.4 | n.d. | n.d. | n.d. | n.d. |
| estradiol 3-sulfate (1,3,5(10)-estratrien-3,17β-diol-3-sulfate) | C<sub>18</sub>H<sub>24</sub>O<sub>5</sub>S<sub>2</sub> | 352.445 | 6.0 | [M-H]<sup>-</sup> | 351.1261 | 189.4 | 189.4 | n.d. | n.d. | n.d. | n.d. |
| estrone 3-sulfate (1,3,5(10)-estratrien-3-ol-17-one 3-sulfate) | C<sub>18</sub>H<sub>24</sub>O<sub>5</sub>S<sub>2</sub> | 350.429 | 6.1 | [M-H]<sup>-</sup> | 349.1104 | 187.8 | 187.4 | n.d. | n.d. | n.d. | n.d. |
| estriol 3-sulfate (1,3,5(10)-estratrien-3,16,17-triol-3-sulfate) | C<sub>18</sub>H<sub>24</sub>O<sub>6</sub>S<sub>2</sub> | 368.444 | 3.3 | [M-H]<sup>-</sup> | 367.1210 | 193.2 | 192.3 | [M + 2Na]<sup>+</sup> | 413.1065 | 212.3 | 214.1 |

Abbreviations:
- n.d., not detectable
- a TWCCSN<sub>2</sub> 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 over four months (n = 9).
- b TWCCSN<sub>2</sub> values previously reported by the CCS database for steroids [39].
- c Averaged TWCCSN<sub>2</sub> values of phase II steroid metabolites in urine samples (i.e. adult bovine and calve urines) over four months.
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 $^{TW}_{\text{CCS}_{N_2}}$ of the vast majority of steroid ions. As indicated in Fig. 1A, more than 77% of CCS measurements matched the database $^{TW}_{\text{CCS}_{N_2}}$ values within ±0.50% error, whereas only 1.4% of the cases presented a CCS difference greater than ±1.00%. Hence, the threshold of ±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 ±1.30%, and one determination gave a false negative result even when applying the threshold of ±2% (Fig. 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 μg mL⁻¹ vs. 2 μg 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 ($\Delta_{\text{CCS max}} = -0.5\%$, 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 μg 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. Therefore, 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 ±2.0% to at least ±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 Fig. 1C for the deprotonated molecule of estradiol 17-glucuronide. Thus, it provides confidence in the $^{TW}_{\text{CCS}_{N_2}}$ values of the database for steroids. On the contrary, the precision related to the CCS measurement of other molecular ions

![Figure 1](image-url)
can still be improved if the TWCCS₀ values from the database are corrected by taking into account the additional measurements carried out through this work. For example, Fig. 1D shows that the TWCCS₀ value reported for the [M−H−2Na]⁺ species of estradiol 3-sulfate was slightly underestimated in comparison to these last measurements, although they matched the database value (i.e. TWCCS₀ = 212.3 Å²) within ±2.0% error.

**Table S1** (Supplementary Information, SI) includes the new TWCCS₀ values that should be applied in further studies for the identification of phase II steroid metabolites based on their TWCCS₀. In general, these new values differ by less than 1.0 Å² from published database values (i.e. in terms of absolute CCS differences).

**Table S2** (SI) includes the LODs that were calculated as the minimum analyte concentration yielding a S/N equal to three. S/N was estimated on its TWCCS₀ depending on the type of analyte: 25, 50, 100, 250, 375, 500 and 750 ng mL⁻¹ for androgen glucuronides and estrone 3-sulfate, the lowest concentration level (50 ng mL⁻¹) was out of the linear range. In the case of 17α-estradiol 3-sulfate, the lowest concentration level (50 μg 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 (Fig. 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 (Fig. 3 and Fig. S2 in 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 Fig. 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, requiring a minimum ΔCCS = 2.5% for compounds with 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 (TWCCS = 189.5 Å²) and epitestosterone sulfate (TWCCS = 191.1 Å²) could be potentially separated since both ions present a CCS difference greater than 0.5%. In our case, as shown by Fig. S4 (SI), only those isomeric steroids in urine samples presenting a large CCS difference (ΔCCS > 4%) were separated by TWIMS such as etiocholanolone glucuronide (TWCCS = 206.9 Å²) and epiaandrosterone glucuronide (TWCCS = 221.4 Å²) as well as 19-noretiocolanolone glucuronide (TWCCS = 204.2 Å²) and 19-norandrostenedione glucuronide (TWCCS = 213.5 Å²). Despite both steroid pairs were also separated by LC, 19-noretiocolanolone glucuronide and 19-norandrostenedione 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 Fig. 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 Rt 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 (Fig S5 in 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.

Fig. 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 calf urine samples by LC-ESI-TWIMS-TOF-MS. The following filters were applied for signal processing of 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.

3.4. Towards the implementation of IMS for the non-targeted analysis of steroids

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 Rt 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 (Fig. S7 in SI). Applying CCS criteria (i.e. TWCCSN2 ± 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 Rt (±0.1 min) since these steroid standards were available in our laboratory. Fig. S7 (SI) also shows the EIC \(m/z\) 465.2 obtained from the analysis of the urine sample fortified with both glucuronide conjugates (0.2 µg L\(^{-1}\)). The presence of epiandrosterone glucuronide in the sample was finally discarded based on its Rt, 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 Rt 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 (Fig. 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-C6H8O6+H]\(^+\) \(m/z\) 287.2006 and [M-H2O-C6H8O6+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 Å\(^2\), 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 epi-testosterone glucuronide, in which the glucuronide group is also in position 17α. Finally, samples were spiked with 17β-boldenone glucuronide (2 µg mL\(^{-1}\)) for reinforcing our results. As shown in Fig. 6, the peak related to 17β-boldenone glucuronide and the tentatively attributed to 17α-boldenone glucuronide present

![Fig. 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 µg mL\(^{-1}\)) and analyzed in positive mode.](image-url)
different Rt. In positive mode, both peaks are also separated in the mobility dimension based on their CCS difference (Fig. S8 in 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 Rt. 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 \( \Delta \text{CCS}_\text{N} \) values from a published database, high accuracy was obtained for CCS measurements over time (i.e. within four months), since \( \Delta \text{CCS} < 1.3\% \) 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 identifications before considering such parameter as additional new identifications.

Obvious, 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 applications. 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\( \alpha \)-bolenedione glucuronide in urine samples as one of the metabolites resulted from the administration of boldione.

Declaration of interest

The authors have no relevant interest(s) to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.acx.2019.100006.

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