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AN INTER-LABORATORY AND INTER-PLATFORM STUDY OF STEROIDS COLLISION CROSS SECTION BY TRAVELING WAVE ION MOBILITY SPECTROMETRY

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ABSTRACT: Collision cross section (CCS) databases based on single-laboratory measurements must be cross-validated to extend their use in peak annotation. This work addresses the validation of the first comprehensive $^{TW}CCS_{N_2}$ database for steroids. First, its long-term robustness was evaluated (i.e. a year and a half after database generation; Synapt G2-S instrument; bias within ±1.0% for 157 ions, 95.7% of the total ions). It was further cross-validated by three external laboratories, including two different TWIMS platforms (i.e., Synapt G2-Si and two Vion IMS QToF; bias within the threshold of ±2.0% for 98.8, 79.9 and 94.0% of the total ions detected by each instrument, respectively). Finally, a cross-laboratory $^{TW}CCS_{N_2}$ database was built for 87 steroids (142 ions). The cross-laboratory database consists of average $^{TW}CCS_{N_2}$ values obtained by the four TWIMS instruments in triplicate measurements. In general, lower deviations were observed between $^{TW}CCS_{N_2}$ measurements and reference values when the cross-laboratory database was applied as reference instead of the single-laboratory database. Relative standard deviations below 1.5% were observed for inter-laboratory measurements (< 1.0% for 85.2% of ions) and bias between average values and $^{TW}CCS_{N_2}$ measurements was within the range of ±1.5% for 96.8% of all cases. In the context of this inter-laboratory study, this threshold was also suitable for $^{TW}CCS_{N_2}$ measurements of steroid metabolites in calve urine. Greater deviations were observed for steroid sulfates in complex urine samples of adult bovines, showing a slight matrix effect. The implementation of a scoring system for the application of the CCS descriptor in peak annotation is also discussed.

Nowadays, ion mobility spectrometry (IMS) is attracting more and more attention from researchers in various fields (e.g., clinical, environmental, food analysis, etc.),[1-4] due to its compatible hyphenation with mass spectrometry (MS) and the commercialization of ion mobility-mass spectrometry (IM-MS) platforms.[5] Specifically, IMS has been widely applied in metabolomics studies, where a large chemical diversity of molecules (i.e., lipids, steroids, peptides, amino acids, nucleotides, etc.) must be determined and characterized.[6-8] In non-targeted metabolomics, the analysis of biological samples can be achieved by liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) coupled to MS. From an analytical perspective, the high complexity of biological samples represents an important challenge because sample preparation is generally kept to a minimum to avoid loss of information during non-targeted metabolomics workflows. Consequently, sample analysis frequently leads to complex mass spectra, and the determination of molecules at low concentration levels can be hindered by the presence of major components. Furthermore, MS-based measurements may be ineffective for the identification of isobaric and isomeric compounds that co-elute in the chromatographic separation. In this sense, the identification of compounds, which is mainly based on retention indices (i.e., retention or migration times) and mass spectra, is still the main bottleneck of metabolomics since many metabolites remain unidentified.[9] In this context, IMS represents a powerful and complementary strategy to overcome these limitations and expand the current boundaries of metabolomics and, in general, reinforce targeted and non-targeted approaches.[10]

IMS is a post-ionization separation technique in which ions are separated according to their size, shape and charge in a gaseous phase under the influence of an electric field. There are different types of IMS technologies commercially available, which operate under different principles and show different advantages and disadvantages.[5,11-14] The new trends are towards the integration of IMS in liquid chromatography-mass spectrometry (LC-MS) workflows (although hyphenation with other separation techniques is also possible), providing a third sepa-

ration dimension.[15] As a result, peak capacity is increased, isomers are distinguished in the mobility dimension, and additional information (i.e., mobility of ions, or drift time) is provided to support compounds identification.[16]

In addition to the mobility of ions and the related drift time, the reporting of ion-neutral Collision Cross Section (CCS) as output data of IMS measurements is becoming popular for feature annotation in metabolomics.[17-19] This parameter can be related to the mobility of ions according to the Mason-Schamp equation, and describes the momentum transfer between ions and drift gas particles.[20] Therefore, it is considered a structural property of ionized molecules, which depends on experimental conditions such as drift gas composition, temperature, and reduced field strength (E/N, where E represents the electric field and N is the gas number density). However, unlike drift time, CCS values are not instrument-dependent, so they should be comparable between instruments and laboratories operating under the same experimental conditions.[21] Based on this principle, a growing number of CCS databases have been reported in recent years with the aim of supporting the identification of metabolites, including exogenous chemicals.[22-25]

CCSs values can be obtained by different IMS technologies, including drift tube ion mobility spectrometry (DTIMS), traveling wave ion mobility spectrometry (TWIMS), trapped ion mobility spectrometry (TIMS) and differential mobility analyzers (DMA). At least to date, CCS databases have been created based on DTIMS measurements and, to a lesser extent, TWIMS (i.e. ^{DT}CCS and ^{TW}CCS databases, respectively).[22] In the case of DTIMS, drift times are measured directly and converted into CCS values (i.e. stepped field CCS method). In contrast, calibration curves are applied when performing CCS measurements by TWIMS, as well as by TIMS.[20] It must be highlighted that calibration curves are also required when DTIMS operates in single-field conditions, which is the operating mode of LC-DTIMS-MS methods.[13,21]

Because the generation of large CCS databases is in its early stage, standardized methods for CCS measurements (including calibration) on IM-MS platforms and reference values for this purpose have not been established or scarcely investigated.[21,26,27] In this context, databases refer to apparent CCS values, so the evaluation of measurement trueness is not applicable. However, an error threshold of ±2% is currently accepted for CCS measurements compared to the values in databases.[27] This tolerance has been shown to be suitable for the internal use of CCS databases,[28,29] even in long-term studies.[30,31] On the contrary, inter-laboratory reproducibility has been studied to a lesser extent. Paglia *et al.* have evaluated the reproducibility of CCS measurements for 125 metabolites in three different laboratories equipped with TWIMS (Synapt G2 instruments, Waters Corporation).[17] Relative standard deviations (RSDs, %) below 5% were observed for 99% of the $^{TW}CCS_{N_2}$ values (209 values for 125 common metabolites, including positive and negative ions) and, in 80.3% of the cases, they were less than 2%. In a subsequent work, the same authors evaluated the reproducibility of CCS measurements for 244 lipids, observing RSDs below 3% for 98% of the measurements.[32] In addition, recent inter-laboratory studies involving TWIMS technology have shown that an error threshold of ±2% for CCS measurements can be considered as acceptance criterion to confirm the identity of molecules.[31,33] This tolerance may be potentially more restrictive for CCS measurements in DTIMS. Inter-laboratory reproducibility of $^{DT}CCS_{N_2}$ values for 120 ions has been evaluated by three different laboratories equipped with DTIMS-MS instruments (6560, Agilent Technologies).[26] Stepped and single field methods were investigated, and an absolute bias of 0.34 and 0.54% to reference $^{DT}CCS_{N_2}$ values (obtained by a fourth DTIMS platform) were observed, respectively.

Some inter-laboratory studies have also included the comparison of CCS measurements carried out by different IMS technologies (i.e. DTIMS, TWIMS, TIMS).[34,35] However, although theoretically possible, it is not so obvious that a single CCS database can be applied to commercial platforms based on different IMS technologies. Hinnenkamp *et al.* have compared the $^{DT}CCS_{N_2}$ and $^{TW}CCS_{N_2}$ of 124 protonated molecules and related sodium adducts. Although the absolute deviations were below 2% for most CCS measurements, both values differed by more than 2% for 7 and 13% of $[M+H]^+$ and $[M+Na]^+$ ions, respectively. This fact cannot be neglected if CCS databases are implemented for peak annotation. Particularly for small isomeric molecules that typically have CCS differences of less than 2%.[36] In these cases, high measurement precision is required to correctly assign their identity.

Taking into account that the majority of CCS databases are based on single-instrument/platform measurements, instrument-to-instrument variation is considered a limitation for their application in peak annotation workflows. The use of cross laboratory-derived CCS values has been proposed to greatly improve compound identification in metabolomics.[37] Moreover, the use of the CCS descriptor for specific analytical applications regulated by guidelines requires a comprehensive assessment of reproducibility across different platforms and laboratories.

Within this framework, the present study proposes the first cross-validation of a large $^{TW}CCS_{N_2}$ database previously reported, in this case for steroids (i.e. androgens, estrogens, progestagens and corticosteroids),[38] which exhibit great relevance in the study of metabolism processes and disorders.[39,40] This work also evaluates the inter-laboratory reproducibility and, for the first time, the inter-platform reproducibility between two different TWIMS-MS platforms (i.e., Synapt and Vion IMS QToF from Waters) involving four instruments [i.e. Synapt G2-S, Synapt G2-Si, and Vion IMS QToF (n = 2)] located in different laboratories. It discusses the improvements in precision achieved when cross-laboratory or standardized CCS databases are implemented. In order to apply the CCS as criterion in peak annotation, this study also proposes the development of a scoring system similar to those applied for compound identification by MS fragmentation patterns. Finally, as a proof of concept, the standardized CCS database is applied to the determination of phase II steroid metabolites in urine samples.

EXPERIMENTAL SECTION

Chemicals and reagents. Standards of 97 steroids were supplied by Steraloids (Newport, RI, USA), Sigma Aldrich (St. Louis, MO, USA), and National Measurement Institute (NMI, Pymble, Australia). Stock standard solutions of each steroid (1 mg mL^{-1}) in ethanol were prepared and stored in amber glass vials at -20°C. Working standard solutions (10 µg mL^{-1}) were prepared by the dilution of stock standard solution mixtures with methanol and kept at -20°C.

Methanol and acetonitrile (LC-MS Chromasolv® grade) were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Loughborough, UK). Propan-2-ol was provided by Sigma-Aldrich. Ethanol (Promochem©, HPLC grade)

was acquired from LGC Standards GmbH (Wesel, Germany). Formic acid (eluent additive for LC-MS or UPLC-MS grade) was supplied by LGC Standards GmbH and Biosolve (Valkenswaard, Netherlands). Formic acid (Promochem®) and sodium hydroxide (Fisher Chemical™) were provided by LGC Standards and Fisher Scientific, respectively. Water (HiPerSolv Chromanorm®, HPLC grade) was purchased from VWR International (West Chester, PA, USA) and Fisher.

LC methods. Chromatographic separations were carried out on an Acquity UPLC® Systems (Waters®, Milford, USA) using a C-18 column (Acquity UPLC® BEH C18, 2.1 × 100 mm, 1.7 µm, from Waters®) equipped with an in-line filter kit (0.2 µm; Waters®). The same column was employed in the four laboratories for LC separation. Mobile phase consisted of 0.1% (v/v) formic acid in water (solvent A), and acetonitrile with 0.1% (v/v) formic acid (solvent B). In general, mobile phase flow rate was established at 0.6 mL min^{-1} but, to avoid pressures higher than 750 bar, it was limited to 0.5 mL min^{-1} in the UPLC system coupled to the Synapt G2-Si. Column temperature was maintained at 50 °C. The following concentration gradient program was established for the analysis of steroid standard solutions (except for steroid esters and progestagens) and urine samples: 95/5 (A/B, v/v) between 0 and 0.3 min, 57/46 at 9.6 min, 0/100 from 13.5 to 15.5 min, and 95/5 from 16 to 19.5 min. In the case of steroid esters and progestagens standards, concentration gradient program consisted of: 50/50 (A/B, v/v) between 0 and 2 min, 90/10 at 9.6 min, 0/100 from 13.5 to 15.5 min, and 50/50 from 16 to 19.5 min. Sample injection volume was set to 5 µL.

IMS-MS instrumentation and conditions. Four TWIMS-MS instruments (Waters®) were involved in this inter-laboratory study. They included two different electrospray ionization (ESI)-quadrupole (Q)-TWIMS-time of flight (ToF)-MS platforms, i.e. Synapt G2-S and Synapt G2-Si instruments located at LABERCA and INRAE-BIA platform BIBS (Nantes, France), respectively, and two benchtop ESI-TWIMS-Q-ToF-MS instruments, i.e. two Vion IMS QToF (hereinafter 'Vion') systems located at the University of Geneva (Geneva, Switzerland) and Waters Corporation (Wilmslow, UK), respectively. Campuzano and Giles have recently discussed the evolution of TWIMS technology and the differences between these two TWIMS platforms.[4 1]

In all these four instruments, the ToF analyzer was operated in high-resolution mode. Analyses were performed in ESI+ and ESI- modes, acquiring continuum data in the range of 50 – 1000 *m/z* with a scan time of 0.2 s. Capillary voltage was set at +3.0 kV in ESI+ mode and at -2.5 kV in ESI- mode. Source temperature was set at 150 °C, desolvation temperature at 500 °C, and desolvation gas flow at 800 L h^{-1}. For Vion instruments, cone voltage and source offset were set at 40 and 80 V, respectively, whereas in Synapt systems, both parameters were set at 31 and 40 V, respectively.

Regarding IMS parameters, gas (i.e. nitrogen) flow of Vion instruments was adjusted to 1.60 L min^{-1} for trap gas and 25 mL min^{-1} for buffer gas. Velocity and height of StepWave1 and StepWave2 were set at 300 m s^{-1} and 5 V, and at 200 m s^{-1} and 15 V, respectively. Other High Definition Mass Spectrometry (HDMS; system operating in ion mobility mode) settings consisted of trap wave velocity at 100 m s^{-1}; trap pulse height A at 10 V; trap pulse height B at 5 V; IMS wave velocity at 250 m s^{-1}; IMS pulse height at 45 V; wave delay set at 20 pulses and gate delay at 0 ms. In Synapt instruments, the experimental conditions were similar to those previously reported for steroid analysis.[30] Drift and trap gas consisted of nitrogen and were supplied at 100 and 0.2 mL min^{-1}, respectively. The flow rate of gas in the helium cell was 180 mL min^{-1}. Velocity and height of both StepWave were set at 300 m s^{-1} and 5 V, respectively. IMS wave velocity and height were fixed at 1000 m s^{-1} and 40 V for positive ionization conditions, and at 550 m s^{-1} and 40 V for negative mode, respectively. Other HDMS settings were adjusted as follows: trap DC bias, 47 V; IMS DC bias, 0 V; trap wave velocity, 311 m s^{-1}; trap wave height, 4 V; transfer wave velocity, 219 m s^{-1}; transfer wave height, 4 V.

Mass and CCS calibration. Major Mix IMS/ToF Calibration Kit (Ref. 186008113, Waters®, Wilmslow, UK) was used for mass and CCS calibration of Vion instruments. In the case of Synapt instruments, this kit was only used for CCS calibration, while sodium formate (0.5 mM) in 90/10 (%, v/v) propan-2-ol/water was used as mass calibrant.

Leucine-enkephalin (0.2-2 µg mL^{-1} in 50/50 (%, v/v) water/acetonitrile solution containing 0.1-0.2% (v/v) of formic acid; Waters®) was used as lock mass standard for accurate mass measurements. Leucine-enkephalin signal was acquired each 15 s for 0.3 s (3 scans to average) in Synapt instruments, while it was infused in intervals of 5 min for Vion systems.

Software and data analysis. DriftScope V.2.8 included in MassLynx 4.2 software (Waters®) was used for the analysis of mass and mobility spectra obtained with Synapt instruments. UNIFI V.1.9.3 (Waters®) was used for the acquisition and processing of data obtained with Vion instruments. Statgraphics centurion 18.1.12 software (Statgraphics Technologies, Inc., The Plains, Virginia, USA) was used for statistical data processing.

Sample preparation. In this work, urine samples from adult bovine animals (n = 2) and calves (n = 2), which were stored at LABERCA's biobank at -20°C, were analyzed. Samples were thawed at room temperature and subsequently filtered by centrifugation (polyethersulfone membrane, molecular weight cut-off of 10 kDa; VWR International) for 10 min at 7800 g and 15 °C. After filtration, samples were spiked with a mixture of 20 phase II steroid metabolites (i.e., estradiol diglucuronide, estradiol 17-sulfate, estradiol 3-glucuronide, 19-nortestosterone sulfate, estradiol 3-sulfate, boldenone sulfate, 19-nortestosterone glucuronide, testosterone sulfate, estrone 3-sulfate, boldenone 17β-glucuronide, epitestosteorne sulfate, testosterone glucuronide, DHEA sulfate, DHEA glucuronide, epiandrosterone sulfate, androsterone sulfate, epiandrosterone glucuronide, epitestosterone glucuronide, 19-norandrosterone glucuronide, and etiocholanolone glucuronide; 2 µg mL^{-1} each). Finally, samples were diluted 10-fold with 0.1% (v/v) formic acid in water and submitted to analysis.

RESULTS AND DISCUSSION

We developed the first large $^{TW}CCS_{N_2}$ database for 300 steroids in 2017 and published this work in March 2018.[38] As is often the case with CCS databases, it was based on single laboratory measurements and was not cross-validated at that time. In this context, the $^{TW}CCS_{N_2}$ of 97 steroids selected from this database has been investigated in more detail to validate our previous results, evaluate the inter-laboratory reproducibility of

CCS measurements by TWIMS, and establish the basis for reporting CCS databases based on multiple laboratories measurements.

From the original $^{TW}CCS_{N_2}$ database that includes 300 steroids,[38] different androgens (n = 42), estrogens (n = 28), progestagens (n = 8) and corticosteroids (n = 19) were selected. Compounds were chosen to cover a wide range of steroids, including phase I and II metabolites, and exogenous steroids (e.g. steroid esters). Only molecules that produce protonated/deprotonated ions or sodium adducts under ESI conditions were selected. Mixtures of these steroid standards at 10 µg L^{-1} each were submitted to LC-TWIMS-ToF-MS analysis in four laboratories (i.e. LABERCA, INRAE-BIA platform BIBS, University of Geneva and Waters Corporation) equipped with different TWIMS-ToF-MS instruments (i.e. Synapt G2-S, Synapt G2-Si, and Vion) as above mentioned. Positive and negative ESI conditions were applied for the analysis of corticosteroids, estrogens, and phase II androgen metabolites. Other steroids were only analyzed in ESI+ mode.

In general, $^{TW}CCS_{N_2}$ measurements were carried out for 199 ions of 97 steroids (Figure 1A), including $[M+H]^+$, $[M-H]^-$, $[M+Na]^+$, $[M-H+2Na]^+$ and $[M+HCOO^-]^-$ adducts (see '$^{TW}CCS_{N_2}$ data set' in Supporting Information). These ions involved *m/z* from 271 to 647 and presented $^{TW}CCS_{N_2}$ values between 165.0 and 266.4 $Å^2$ (Figure 1B). Only 167 of these ions were previously characterized in terms of $^{TW}CCS_{N_2}$ by a Synapt G2-S instrument at LABERCA.[30,38] However, the 167 ions were not detected by all four TWIMS instruments (Figure S1). Ion formation generally depends on the instrumental configuration and ionization conditions (e.g. the formation of sodium adducts can sometimes be favored at the expense of protonated ions), so different ions can be generated by different LC-ESI-TWIMS-MS systems. In addition, in this case, the four TWIMS platforms did not detect some steroid ions because they had low signal intensity or eluted under column re-conditioning conditions. Finally, 142 ions, related to 87 steroids and already included in the initial $^{TW}CCS_{N_2}$ data set, were detected by the four platforms (Figure 1C).

$^{TW}CCS_{N_2}$ database for steroids over time. Before discussing the validation of the $^{TW}CCS_{N_2}$ steroids database, its applicability was investigated over time. In this context, additional $^{TW}CCS_{N_2}$ measurements were carried out a year and a half after the development of the initial database and using the same TWIMS instrument (i.e. Synapt G2-S located at LABERCA). Of the total of 167 ions, the $^{TW}CCS_{N_2}$ of 164 ions related to 96 steroids was measured in triplicate (RSDs < 0.7% for all ions; < 0.2% for 97.0% of the ions). In comparison to $^{TW}CCS_{N_2}$ values in the database, deviations were below 1.0% for 95.7% of the ions (Figure S1). Moreover, only one error (or bias) greater than 1.3% was observed for the $^{TW}CCS_{N_2}$ measurement of an ion (i.e. protonated ion of 14α-hydroxytestosterone; $\Delta CCS/CCS_{database}$ = 1.33%). These CCS measurements are in accordance with current studies that accept an error threshold of ±2% of the values in the CCS database,[29,31] and demonstrate the applicability of the $^{TW}CCS_{N_2}$ database for steroids over time.

Regarding the acceptable error of ±2% for CCS measurements, recent studies have shown that this criterion may be more restrictive, especially in the case of CCS measurements by DTIMS.[26] Based on our results of intra-laboratory precision over a 1.5-year period, a tolerance threshold of ±1.0% can potentially be investigated as a new criterion for CCS measurements by TWIMS when a same instrument is used. Between the thresholds of ±1.0 and ±2.0%, an error threshold of ±1.5% could be considered as a more conservative approach to be evaluated.

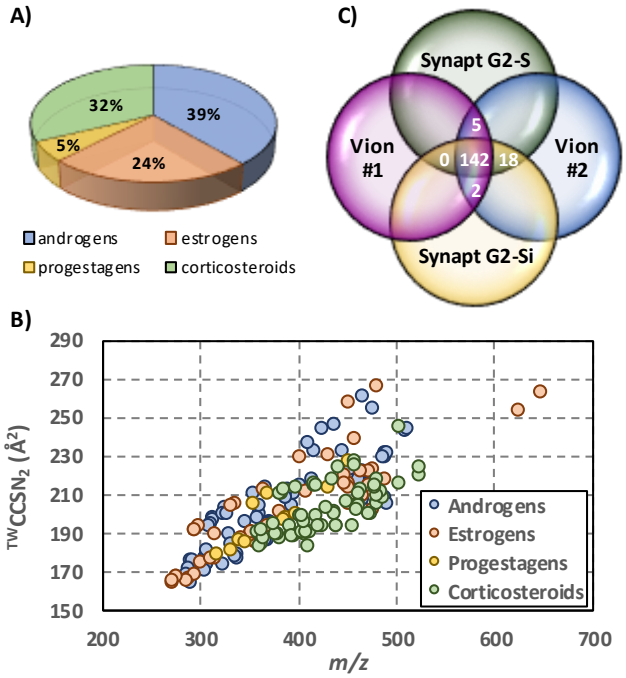

Figure 1. A) Percentage of ions of the total 199 ions detected in this work according to the steroid family. B) $^{TW}CCS_{N_2}$ vs *m/z* for the 199 steroid ions observed in this work. C) Number of ions detected by the four TWIMS instruments (only refers to ions whose $^{TW}CCS_{N_2}$ has been previously reported by Hernández-Mesa *et al.*[30,38]).

Validation of the $^{TW}CCS_{N_2}$ database for steroids and inter-laboratory reproducibility. Three external laboratories, i.e. INRAE-BIA platform BIBS (Synapt G2-Si), University of Geneva (Vion #1) and Waters Corporation (Vion #2), participated in the validation of the $^{TW}CCS_{N_2}$ database for steroids. In all cases, the same CCS calibrant (i.e. Major Mix IMS/ToF Calibration Kit from Waters®) was used for TWIMS calibration to minimize its effect on CCS measurements, as different CCS values may result when using different CCS calibrants.[20,42] All $^{TW}CCS_{N_2}$ values were measured in triplicate. Of the total of 167 ions, the Synapt G2-Si system detected 162 steroid ions, showing a RSD for $^{TW}CCS_{N_2}$ measurements below 0.4% for triplicates (< 0.2% for 96.3% of the ions). Vion platforms showed different performance in terms of precision. Vion #1 only detected the 142 common ions, but provided high precision in accordance with the performance of both Synapt systems (RSDs < 0.5% and < 0.3% for 97.2% of the ions). On the contrary, the 167 ions were detected by Vion #2 but greater RSDs were observed. More precisely, RSDs greater than 0.3% were obtained only for 17.4% of the ions and only a value greater than 0.7% was observed for protonated cortisol 21-acetate (RSD = 1.6%). Since both Vion instruments operated under identical experimental conditions and because the same software was used for data processing, the difference in precision performance was attributed to the intrinsic performance of each instrument.

In comparison to $^{TW}CCS_{N_2}$ values in the database, all three TWIMS instruments provided similar values. In general, 98.8, 79.9 and 94.0% of the $^{TW}CCS_{N_2}$ measurements for the ions detected by Synapt G2-Si, Vion #1 and Vion #2 instruments, respectively, were within the error threshold of ±2.0% currently accepted (Figure S1). Although greater deviations were observed for measurements with Vion #1, errors were always within the range of ±3.0% (except for the protonated ion of prednisolone 21-hemisuccinate and epitrenbolone, $\Delta CCS/CCS_{database}$ = -3.4 and -3.1%, respectively). In addition, as can be seen in Figure S2, Vion systems as well as Synapt instruments present the same deviation pattern. Synapt G2-Si provided similar results to those recently obtained by the Synapt G2-S and are in accordance with the database. In contrast, $^{TW}CCS_{N_2}$ values measured by Vion #1 and #2 were slightly lower, but still within the threshold of ±2.0%.

Although both Synapt and Vion instruments present a difference in their configuration, which is the quadrupole position, before or after the ion mobility cell, respectively;[41] this fact should not have any influence on the conformation of molecules. In all these TWIMS platforms, ion injection into the TWIMS cell is preceded by trap and helium cells that guarantee similar injection conditions, and ion conformation is not altered. In the same vein, different wave velocity and height were established on Synapt and Vion platforms, but it has been shown that these parameters do not significantly affect the conformation of steroids.[38] On the other hand, these IM-MS platforms do not allow the user to directly measure temperature and pressure in the drift cell, so the same drift conditions cannot be guarantee on different platforms. However, the bias between $^{TW}CCS_{N_2}$ values measured by the different platforms is limited (a more exhaustive discussion is carried out in the next section), which shows that these differences are not significant enough to compromise the validity of the database.

Taking into account the current criterion applied to CCS measurements and the results obtained in terms of bias by the three instruments ($\Delta CCS/CCS_{database}$ < ±2.0% for 91.4% of $^{TW}CCS_{N_2}$ measurements), we can confirm that the $^{TW}CCS_{N_2}$ database for steroids[38] is still valid on other TWIMS instruments and platforms, therefore it is validated. Nevertheless, a bias greater than ±2.0% of the database values questions the use of single-laboratory CCS databases for peak annotation if they are applied in other laboratories. This also reflects that, when CCS is applied to molecular libraries for identification purposes, the real identity of the molecule can be discarded if potential candidates are rejected based on the threshold of ±2.0% currently accepted for CCS measurements.

Standardization of the $^{TW}CCS_{N_2}$ database for steroids. It is clear that the implementation of IMS as a routine analysis tool still requires a consensus of the entire community (i.e. both academic and industrial investigators) regarding several issues.[13,21] Standardization of CCS calibration procedures (because they can influence the results of CCS measurements)[42] and CCS databases to be universally used, are some of the topics that should be discussed in greater depth to reach a consensus.

In an attempt to evaluate the improvements achieved by cross-laboratory versus single-laboratory CCS databases, this work includes a $^{TW}CCS_{N_2}$ database for 142 ions of 87 steroids based on twelve measurements by TWIMS (i.e. three replicate measurements in four TWIMS instruments) (see 'standardized $^{TW}CCS_{N_2}$ database' in Supporting Information). The repeatability and reproducibility of $^{TW}CCS_{N_2}$ measurements were quantified by the variance decomposition as described in many validation guidelines (ANOVA). In this work, most of the variability observed could be attributed to repeatability (57.8%) rather than reproducibility (42.2%). As mentioned before, a small bias was observed for measurements carried out by Vion #1 compared to the other instruments (Figure S3 and S4) but, in general, high reproducibility was observed for $^{TW}CCS_{N_2}$ measurements of all ions (Figure S4). Therefore, an average of all measurements for each ion was accepted as $^{TW}CCS_{N_2}$ reference. In addition, in the context of CCS and ion mobility, it is not possible to use the terms 'true' or 'absolute' values that allow trueness evaluation. CCS values result from mathematical models based on the best understanding of the physical phenomenon and some simplifications, as the property being measured is the mobility constant (K) or drift time (t_D).[21] In the case of TWIMS technology, CCS calibration is required, which can unequivocally introduce errors in the measurements. Thus, terms like 'error', 'trueness' or 'accuracy' should be used with caution when comparing experimentally derived CCS values.

RSDs below 1.5% were observed for the $^{TW}CCS_{N_2}$ measurement of all ions, with low variability (RSD < 0.5%) for 21.1% of cases. RSD values were within the range of 0.5 and 1.0% in most cases (i.e. 64.1% of the 142 $^{TW}CCS_{N_2}$ values). Furthermore, small deviations were observed between all measurements and reference $^{TW}CCS_{N_2}$ values (Figure 2), so none of them were discarded. Only three measurements in Vion #1 resulted in a bias greater than ±2.0% (Figure S5A). In general, deviations were observed within the range of ±1.5% for 96.8% of the measurements in the four instruments, with a high percentage of measurements (i.e. 83.5%) showing a bias within the range of ±1.0%. Although greater deviations were observed for $^{TW}CCS_{N_2}$ values provided by Vion #1, most of its measurements (i.e. 90.1%) were at a threshold of bias of ±1.5%. According to Figures S5A and 2, a threshold of ±1.5% can be implemented for $^{TW}CCS_{N_2}$ measurements compared to cross-laboratory databases, reducing the threshold currently accepted for peak annotation in analytical workflows including TWIMS (i.e. ±2%). It is clear that most confidence can be gained if this threshold is narrowed (e.g. 1.0%), but there exists a high risk to discard the correct molecular candidate as shown by Vion #1 results.

Following the path of previous inter-laboratory CCS databases,[17,26,32] these average $^{TW}CCS_{N_2}$ values for steroids constitute one of the first cross-laboratory or standardized ^{TW}CCS databases accessible for feature annotation (related to steroids) in metabolomics and other studies. In this context, the reporting of standardized rather than single-laboratory CCS databases should be pursued because they reduce the uncertainty of CCS measurements. Figure S5 shows that the bias between $^{TW}CCS_{N_2}$ measurements and reference values was always reduced when the average values (cross-laboratory database) were selected as reference rather than the single-laboratory CCS values, except for Synapt G2-S. In this case, lower deviations were found when the single-laboratory $^{TW}CCS_{N_2}$ database was applied because, as expected, the possible instrument effect due to calibration and experimental conditions (i.e. temperature and pressure) was avoided or reduced (i.e. single-laboratory $^{TW}CCS_{N_2}$ database for steroids was generated by this Synapt G2-S).[30,38] Therefore, single-laboratory CCS databases may be suitable for

internal use, but cross-laboratory CCS databases are recommended if extended application is desired.

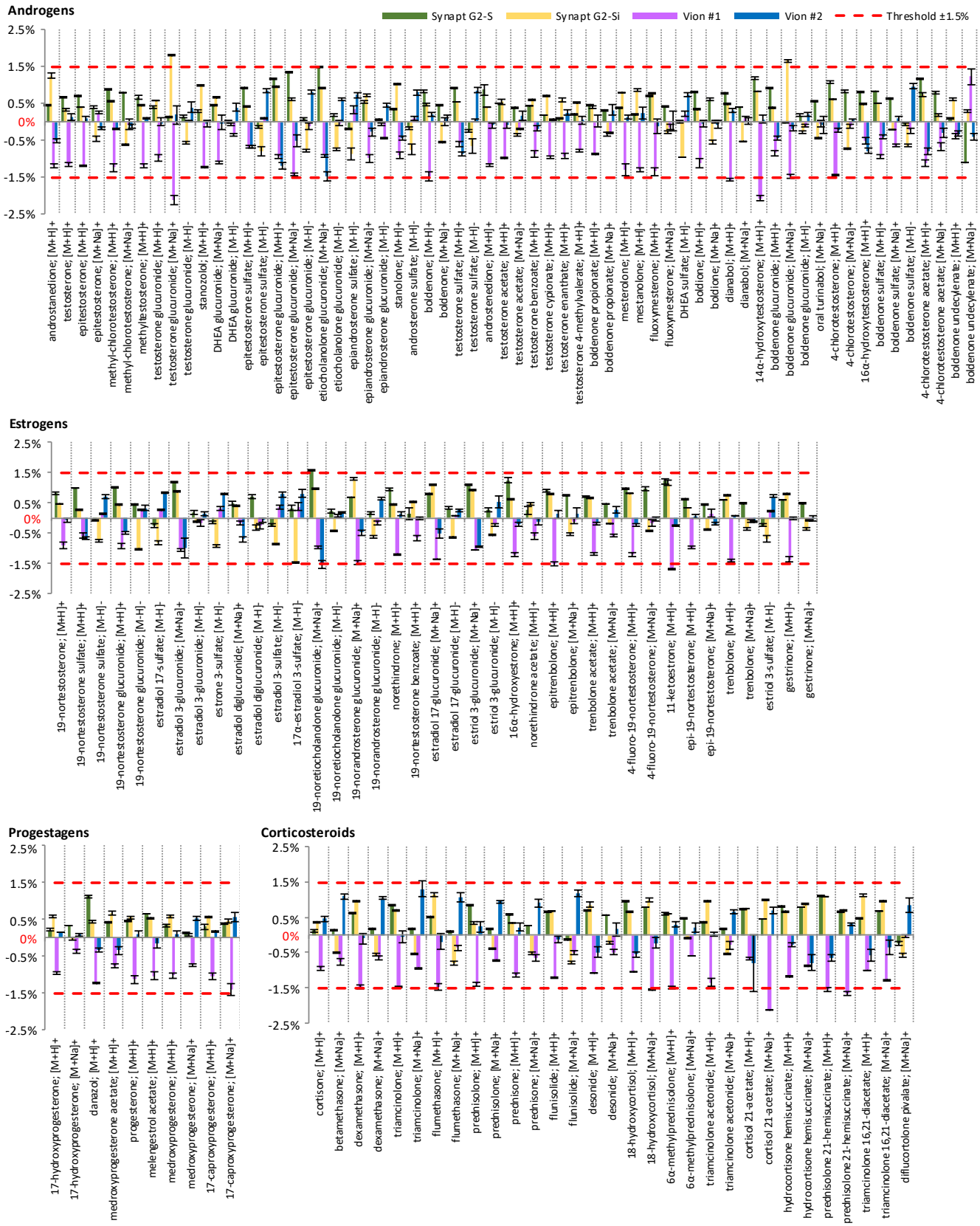

Figure 2. Bias between average $^{TW}CCS_{N_2}$ values and those measured by each TWIMS instrument in triplicate (n = 142 ions).

On the other hand, the importance of reporting the uncertainty in CCS measurements in addition to CCS values has been highlighted.[21] For instance, measuring ^{DT}CCS, Stow *et al.* have recently estimated the uncertainty of drift time measurements in

stepped field by considering the main parameters (i.e. drift tube length, pressure, temperature, and voltage) that describe the relationship between the arrival time (t_A) and the CCS.[26] Drift gas pressure and temperature were both found to contribute the most to the uncertainty in DTIMS measurements (48 and 44%, respectively) by applying the Monte Carlo simulation method. However, the uncertainty of ^{TW}CCS measurements cannot be evaluated in a such straightforward manner. The arrival time distribution of an analyte in TWIMS depends on the wave height, wave velocity, buffer gas pressure, and length of the drift cell, which potentially contributes to the uncertainty of measurement. The effect of each instrumental setting is thus very complex to be estimated because the relationship between the CCS and experimental separation parameters in TWIMS has not yet been completely understood. Although significant advances have recently been made to better understand the theoretical treatment of travelling wave ion mobility,[41] an extensive investigation into the engineering and theory of TWIMS is still needed.

Furthermore, since CCS calibration is applied to carry out ^{TW}CCS measurements, the fit of the calibration curve to the experimental data, the error associated with the standards and the reproducibility of the measurements contribute to the overall uncertainty of ^{TW}CCS measurements.[43] The present work provides knowledge about the reproducibility of ^{TW}CCS measurements, but there are two open questions that need to be investigated in more detail. First and foremost, it is needed to provide knowledge about the uncertainty associated with calibrant values in order to establish the uncertainty of output values.[21] In this sense, IMS reference compounds for calibration have not been universally accepted by the IMS community, although it would be useful to investigate the uncertainty related to secondary IMS methods such as TWIMS. Regarding the uncertainty related to the calibration curve, it has been shown that the wave velocity and buffer gas pressure have a relevant impact on it.[43] Therefore, additional studies are expected to support these previous findings, since they will contribute to establish the experimental parameter values that reduce uncertainty.

Application of the standardized ^{TW}CCS$_{N_2}$ steroids database to the analysis of urine samples. To evaluate the applicability of the cross-laboratory ^{TW}CCS$_{N_2}$ database, the ^{TW}CCS$_{N_2}$ of twenty phase II steroid metabolites (2 µg mL^{-1}) in urine samples (n = 4, two calves and two urine samples from adult bovines) was investigated by the four TWIMS instruments (Figure 3 and S6).

As previously discussed, a threshold of ±1.5% was considered for ^{TW}CCS$_{N_2}$ measurements compared to reference values in the cross-laboratory database. This threshold was suitable for the measurement of steroid ^{TW}CCS$_{N_2}$ in calve urines (Figure 3A and S5A) by the four TWIMS instruments. On the contrary, when ^{TW}CCS$_{N_2}$ values from the single-laboratory database were selected as reference, this threshold did not allow the detection of estradiol 3-sulfate by Vion #2 (Figure 3B). This example shows how the implementation of cross-laboratory CCS databases can reduce the bias between measurements and database values.

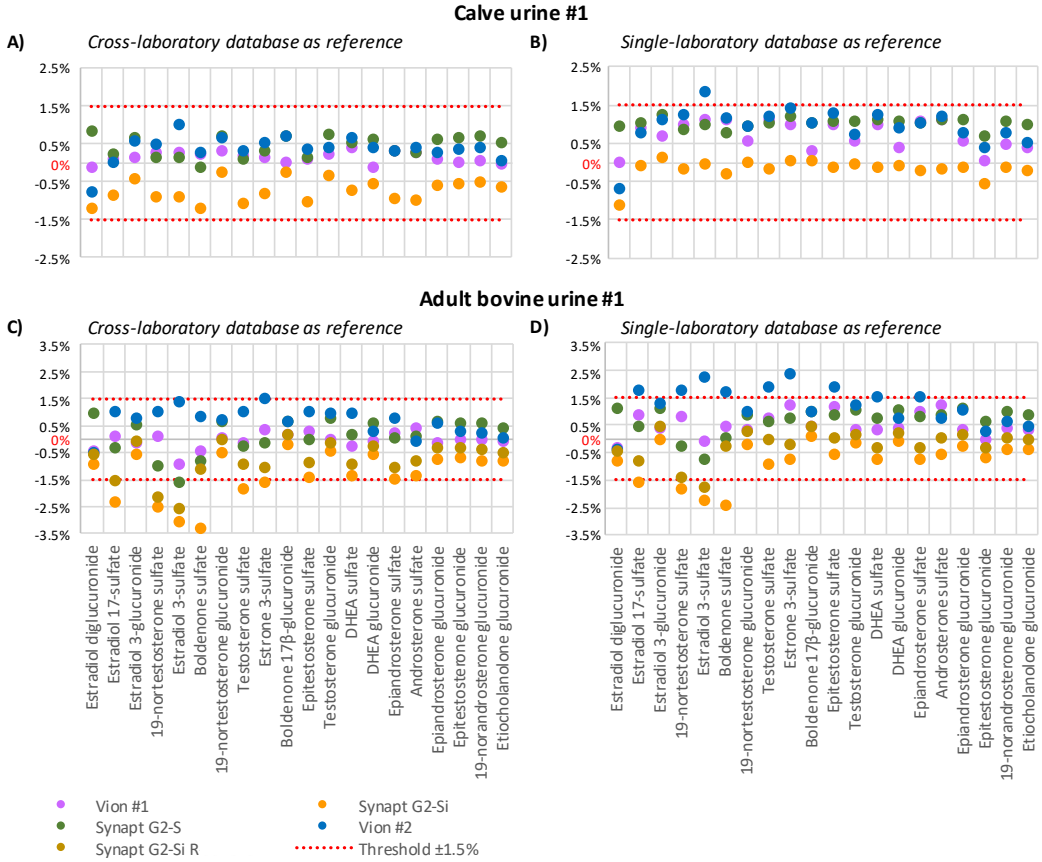

Figure 3. Bias between ^{TW}CCS$_{N_2}$ measurements in each TWIMS instrument and (A and C) the cross-laboratory database or (B and D) the single-laboratory database. Phase II steroid metabolites were investigated in calve and adult bovine urine samples. ^{TW}CCS$_{N_2}$ measurements are related to the [M-H]$^-$ ion of each molecule and were performed in triplicate by each instrument.

In the case of adult bovine urine samples, this observation was even more obvious (Figure 3C-D and S6C-D). When applying the cross-laboratory database, 90.0% of measurements were within the threshold of ±1.5% (75% within the range of ±1.0%), whereas this score was reduced (i.e. 84.4%) using the single-laboratory database as reference. Bias reduction was especially noteworthy for $^{TW}CCS_{N_2}$ measurements performed by Vion #2, since a large number of measurements were in compliance with the threshold of ±1.5% only when the cross-laboratory database was applied. In addition, a bias greater than ±1.5% was observed for the $^{TW}CCS_{N_2}$ measurement of several sulfate metabolites in adult bovine urines by the Synapt G2-Si platform. To confirm these results, these steroid metabolites were re-analyzed six months after the first measurements (see Synapt G2-Si R in Figures 3C-D and S6C-D). Although the results improved in terms of bias, they were still outside the range of ±1.5% for estradiol 17-sulfate, estradiol 3-sulfate and 19-nortestosterone sulfate, confirming the previous findings. In this sense, we have previously reported that complex urine samples [adult bovine urine samples were more complex than those of calves according to the total ion chromatogram (TIC)] can induce a slight effect on $^{TW}CCS_{N_2}$ measurements, specifically for sulfate steroids.[30] As the most plausible hypothesis, this effect was attributed to Coulomb repulsion,[44] which can lead to the contraction of the molecule and could be more significant for sulfate steroids.

Although the study of the effect of urine matrix on $^{TW}CCS_{N_2}$ values was beyond the scope of this work, our results suggest that it should be studied in more details, involving a larger sample size and more compounds (especially sulfate and similar metabolites). The probable matrix effect is also appointed by the RSDs observed for $^{TW}CCS_{N_2}$ measurements according to the compound and urine sample. RSDs between 0.4 and 0.9% were observed for all steroids in calve urines while RSDs were lower or equal to 0.9% for steroid glucuronides in adult bovine urines. However, RSDs ranged from 1.0 to 1.9% for sulfate steroids in adult bovine urines. Therefore, this matrix effect cannot be considered negligible for peak annotation, as it seems to induce a consequence on measurement precision as well as on the bias from reference values. In this context, a scoring system for the application of CCS descriptor and databases in peak annotation seems to be more appropriate than a specific threshold (either ±1.5% or ±2%) for comparing CCS measurements with databases.

Scoring system for the application of CCS descriptor in peak annotation. Within the framework of using CCS libraries for peak annotation, the deviation of CCS measurements from reference values can be applied to score molecules from large molecular databases (as already widely implemented for peak annotation according to fragmentation patterns).[45] Obviously, as with other aspects related to the implementation of IMS technology, the development of a scoring system intended to the application of CCS descriptor in peak annotation requires the consensus of the IMS community. As a first approach, we propose that such a scoring system can be based on different ranges of bias between CCS measurements and CCS values in databases. According to the results from urine samples, there is a very high probability that $^{TW}CCS_{N_2}$ measurements will result in a deviation below |1.0%| from cross-laboratory $^{TW}CCS_{N_2}$ database values. Consequently, a high scoring weight can be attributed to molecular candidates showing a $^{TW}CCS_{N_2}$ deviation within the range of ±1.0%. A low scoring weight can be given to candidates presenting a bias between |1.0%| and |1.5%|, but this interval should also be considered to find suitable candidates with high probability. Finally, candidates with a bias greater than |1.5%|, or even greater than |2.0%|, should not be discarded completely, and still other annotation information (e.g. fragmentation pattern) should be considered in more detail for greater annotation confidence.

As an example, if a scoring weight of 20% is attributed to the CCS descriptor in peak annotation, as indicated by Dodds *et al.*,[13] this score could be applied as follows: the maximum score (i.e. 20%) for candidates presenting a bias within ±1.0%, 10-15% for those with greater bias but lower than |1.5%|, and a scoring weight of only 5-10% for candidates with a bias between |1.5%| and |2.0%|. In this regard, it should also be taken into account that, although unusual, $^{TW}CCS_{N_2}$ measurements are not exempt from leading to biases greater than |2.0%| as observed for testosterone glucuronide in a urine sample measured by Vion #2 (Figure S6C). These unusual measurements would probably lead to false annotation or no annotation. Therefore, although a 0% can be attributed to candidates with greater CCS bias (> |2.0%|), other molecular descriptors (i.e. retention time, accurate mass, etc.) will have a relevant weight on the final score for peak annotation (up to 80% of the total). Consequently, the risk of discarding the good candidate merely based on a CCS deviation threshold is reduced.

CONCLUSIONS

CCS is an important distinguishing characteristic of ions in the gas phase that provides specific information about ions configuration and possible structural confirmation. The use of the CCS descriptor for peak annotation is becoming relevant in several research areas like metabolomics, since CCS can be used in addition to traditional molecular identifiers of precursor ion accurate mass, isotopic pattern, fragment ions, and retention time for the confirmation of compound identity. Moreover, the inclusion of the CCS parameter in high resolution mass spectrometry (HRMS) libraries also provides greater confidence on compound identification in screening analysis of, for example, residues and contaminants. As a result, large sets of CCS values have been reported in the last years to be accessible to the entire IMS community and related fields. Due to the lack of standardized procedures for the generation of CCS databases and, taking into account that most of them are based on single-laboratory measurements, cross-validation is required for an extended implementation. Herein we have successfully cross-validated the first $^{TW}CCS_{N_2}$ database for steroids, which was previously generated on a Synapt G2-S instrument, on three external laboratories involving different TWIMS platforms (Synapt G2-Si and two Vion IMS QToF instruments).

In general, this inter-laboratory study was in compliance with the threshold of ±2% currently accepted for CCS measurements compared to databases. Nevertheless, the observed bias of $^{TW}CCS_{N_2}$ measurements was reduced when the cross-laboratory CCS database was used as reference instead of the single-laboratory database. In this context, it is strongly recommended to report CCS databases based on multiple-laboratory measurements rather than on single-laboratory measurements as is generally done. Moreover, this work shows that $^{TW}CCS_{N_2}$ values

obtained by different TWIM-MS platforms (i.e. Synapt and Vion systems) are comparable, and the same $^{TW}CCS_{N_2}$ database can be implemented on all of them. Although it is clear that cross-laboratory CCS databases can and should be based on different TWIMS-MS platforms, future inter-laboratory studies related to CCS descriptor should also involve different IMS technologies (i.e. DTIMS, TWIMS and TIMS). According to the literature, there is a high correlation between CCS values of steroids measured by TWIMS and DTIMS.[38] However, a large set of steroids have not been investigated by DTIMS (neither by TIMS), and deeper study about this topic is still required. It is very important to understand if cross-laboratory CCS databases can be universally applied in all commercial IM-MS platforms or, on the contrary, they must remain exclusive to each technology to achieve the real implementation of this molecular parameter in routine laboratories.

Based on our results, a threshold of ±1.5% can be considered without assuming a high risk of false negative results when applying cross-laboratory $^{TW}CCS_{N_2}$ databases. This is of special interest for the global implementation of CCS databases in peak annotation because precision CCS thresholds for candidate acceptance can be reduced. Consequently, the number of molecules to be interrogated can be decreased. In this sense, the development of a scoring system seems to be more suitable for the application of CCS descriptor for compound identification rather than an acceptance threshold (i.e. 2%) as currently applied. As observed for steroid sulfates in bovine adult urine samples, matrix effect on $^{TW}CCS_{N_2}$ measurements cannot be totally negligible if the acceptance criteria are highly restrictive. As a first approach, a scoring system based on different ranges of bias between CCS measurements and values in databases seems logical. Nevertheless, the development of a scoring system must ultimately rely on the entire IMS community. Finally, understanding and explaining the uncertainty related to ^{TW}CCS measurements from a theoretical point of view, as already studied for ^{DT}CCS,[26] would further strengthen the robustness of the measured parameter.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

$^{TW}CCS_{N_2}$ measurements and database (XLSX)

Bias between $^{TW}CCS_{N_2}$ measurements and reference values (databases) for steroid standards and urine samples (DOCX)

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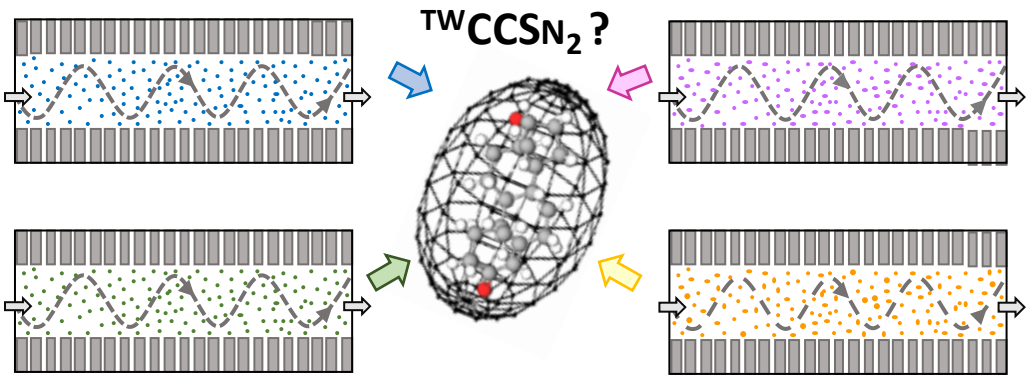
"for TOC only"



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