Travelling Wave Ion Mobility-Derived Collision Cross Section for Mycotoxins: investigating interlaboratory and interplatform reproducibility

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Abstract

Parent and modified mycotoxins analysis remain a challenge due to their chemical diversity, the presence of isomeric forms, and the lack of analytical standards. The creation and application of Collision Cross Section (CCS) database for mycotoxins may bring new opportunities to overcome these analytical challenges. However, it is still an open question whether common CCS databases can be used independently from the instrument type and Ion Mobility Mass Spectrometry (IM-MS) technologies, which utilize different methodologies for determining the gas-phase mobility. Here, we demonstrated the reproducibility of CCS measurements for mycotoxins in an interlaboratory study (average RSD 0.14% ±0.079) and across different Travelling Wave IM-MS (TWIMS) systems commercially available (ΔCCS% < 2). The separation in the drift time dimension of critical pairs of isomers for modified mycotoxins was also achieved. In addition, the comparison of measured and predicted CCS values, including regulated and emerging mycotoxins, was addressed.

Keywords: Mycotoxins; food residues; Travelling wave ion mobility separation; CCS database; Interlaboratory comparison; Interplatform.

INTRODUCTION

Over the past decade, the hyphenation of ion mobility spectrometry (IMS) with high resolution mass spectrometry (HRMS) has risen as a powerful technique for the separation, identification, and structural elucidation of analytes across diverse fields of science. The addition of a new dimension of separation to the common workflow will benefit both targeted and non-targeted analysis. On the one hand, when profiling a target class of analytes, IMS enhances the performance characteristics in terms of sensitivity, peak capacity, and compound identification, reducing the false detections [1]. On the other hand, IMS-MS expands the analyte coverage and increases confidence in the metabolite annotation, which represents the bottleneck of untargeted omics [2–4].

This is possible because IMS-MS allows the determination of the collision cross section (CCS), that is considered as a structural property of ionized molecules. As a result of these advantages, several research groups have used IMS-MS to build CCS libraries [1,5–9] in which the measured values serve as additional molecular descriptors for assigning identities to unknown analytes or gain more confidence in the identification of known molecules.

The implementation of IMS within the food analytical field is quite new and its applicability in routine food safety analysis has been slow down by the lack of CCS database for contaminants and residues. Very recently, a few contaminant databases have been proposed (e.g. mycotoxins, pesticides, veterinary drugs environmental contaminants) [1,6,7] but they are far away from covering the varied range of contaminants present in food samples.

CCS have been demonstrated to be a good molecular descriptor being independent from the concentration and the complexity of the matrix [1,4] and highly reproducible in inter- and intra-day studies (variation < 1%) [6,8]. There is a consensus that the precision of drift time measurements and with these CCS is relatively high thus these values can certainly be used with an in-house database [6,8,10]. There is also evidence that CCS reproducibility is within the range of ±2% (which is normally considered the acceptable error) between identical instruments across different laboratories equipped with traveling wave (TWIMS) [8,11] and drift tube (DTIMS) [10]. Based on the high reproducibility

71 reported across DTIMS instruments (RSD 0.29%) [10] and TWIMS (RSD <1%)[6,8], some authors
72 proposed to narrow the tolerance threshold to ±1.5% when a same instrument is used.
73 However, the challenge is to demonstrate whether common CCS databases can be used independently
74 from the instrument type and IMS technologies, which utilize different methodologies for
75 determining the gas-phase mobility. DTIMS relies on the fundamental ion mobility relationship that
76 directly correlates the measured arrival time of an ion to the CCS [12,13], whereas in the case of other
77 IM technologies (i.e. TWIMS, ion trapping (TIMS), and structures for lossless ion manipulation
78 (SLIM)) , the CCS value is obtained indirectly, by the use of a calibration equation [12,13] based on
79 universally accepted DTIMS-derived CCS as reference values [13].
80 So far, few studies have investigated the comparability of the CCS determined by different platforms,
81 and the comparison of DTIMS with non-DTIMS still poses the greatest challenge when attempting
82 to use a common database. This is an emerging issue, and an in-depth discussion around the proposal
83 of using CCS information obtained from different IM technologies is ongoing, and reported by the
84 Ion Mobility community [12].
85 One of the most comprehensive study [14] reported deviations lower than ±1% for most of the
86 considered analytes when comparing CCS obtained using DTIMS and TWIMS. However, some
87 compounds showed deviations of up to 6.2%, which drove the authors to the conclusion that CCS
88 databases cannot be used without care independently from the instrument type. Although more data
89 would be needed, when creating a database it is good practice to clearly indicate the instrument type
90 used for the CCS determination.
91 Furthermore, when building a Travelling Wave CCS (^{TW}CCS) database, the calibrant mixture used
92 should also be indicated, being the CCS derived through calibration equation and not directly
93 measured. There is currently no consensus regarding the CCS calibration procedure or the type of
94 calibration compounds to be used [12]. Originally, TWIM calibration was based on Poly-DL-Alanine
95 (mass range: 151.1-1154.6 Da; CCS: 130.4-333.6 Å^2), which was then implemented by the addition
96 of a number of small molecules, which include perfluorinated compounds in the range *m/z* 1000-

2000, and organic acids for a more comprehensive coverage at low masses in negative ion mode (Major Mix IMS/TOF Calibration Kit - mass range: 151.1-1966.9 Da; CCS: 130.4-372.6 $Å^2$). Some research groups build their own calibration mixtures, or complemented the Major Mix with the analytes of interest [8]. However, by doing so a further bias is introduced.

Recently, Hernandez-Mesa et al., [6] reported a TWIMS interplatform study, demonstrating deviation within the range of ±1.5% between Synapt and Vion for most of the CCS measurement for steroids, when using the same calibration mixture. However, some compounds, showed deviations greater than this threshold. In light of this findings, the authors suggested for targeted-screening purposes, the use of a score system in which CCS will have a weight on the final score for peak annotation depending on the CCS bias ranges, together with the other molecular descriptor, named retention time, accurate mass and fragmentation pattern. The application of a score system would reduce the risk of discarding a good candidate only based on a CCS deviation threshold.

We recently reported the first $^{TW}CCS_{N2}$ database for mycotoxins, showing its applicability and utility in screening of mycotoxins in real food samples [7]. The present study aims to extend our previous investigation by evaluating the reproducibility of CCS measurements in an interlaboratory study and across different TWIM-MS systems commercially available. The separation in the drift time dimension of critical pairs of isomers for masked mycotoxins is addressed. In addition, the comparison of measured and predicted CCS values for 53 compounds, including regulated and emerging mycotoxins, will be discussed.

MATERIAL AND METHODS

Chemicals and reagents.

LC-MS-grade methanol and LC-MS grade water were purchased from Honeywell (Riedel-de Haen, Germany). Acetic acid 99.99% (Sigma Aldrich, Germany) and ammonium acetate (Fischer Chemicals, UK) were used as mobile phase modifiers. Leucine Enkephalin [186006013] used as lock mass solution and Major Mix IMS/TOF Calibration Kit [186008113] for masses and CCS calibration were purchased from Waters (Manchester, UK).

Fifty-three analytical standards of mycotoxins were purchased from different manufactures including Sigma-Aldrich (Taufkirchen, Germany) and Biopure (Tulln, Austria). Zearalenone-14-glucoside (ZEN14Glc) was chemically synthesized and purified in our laboratory. T-2 toxin glucosides were kindly provided by Dr. Susan P. McCormick (National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, United States). Standards of partially hydrolysed (pHFB) and hydrolysed (HFB) fumonisins were prepared by alkaline hydrolysis of FB standard solutions. Further details on the synthesis of these mycotoxins are reported in Note 1, Supplementary Information. Mixtures containing different standards were prepared in acetonitrile or methanol, depending on their chemical stability, at a concentration of 2 mg/L and stored in glass vials at -20°C. From the stock solutions, three different solutions were prepared (1, 10, 100 µg/L) and diluted in an appropriate solvent, matching the initial conditions of the LC gradient.

UPLC-IMS-MS analysis.

$^{TW}CCS_{N2}$ values were determined employing three commercial TWIM-MS instruments: two Vion IMS QTOF (resolution ~20 Ω/ΔΩ FWHM) located in two different laboratories and one Synapt G2-Si (resolution ~40 Ω/ΔΩ FWHM). UPLC was coupled to each MS system for chromatographic separation prior to ionization. The IMS-MS systems consist of hybrids quadrupole orthogonal

146 acceleration time-of-flight mass spectrometers, in which a stacked ring ion guide, that is, the mobility
147 cell, is positioned before the quadrupole mass filter (Vion configuration), or after the quadrupole and
148 between trap and transfer regions (Synapt configuration). Campuzano and Giles have discussed the
149 evolution of TWIMS technology and the differences between these two TWIMS platforms in detail
150 [15]. Furthermore, CCS calibration procedure for the TWIMS technology has been reported [16] and
151 briefly summarized in Supplementary Information (Note 2).
152 Nitrogen was used as buffer gas in the three instruments.

153 • **Vion UK (Vion #1)**

154 The instrument was located at Waters Corporation, Wilmslow, Cheshire, UK. Mycotoxins standard
155 mixes prepared at different concentration levels (1, 10, 100 µg/L) were injected in triplicate, thus
156 obtaining the $^{TW}CCS_{N2}$ from the average of n = 9, n = 6 or n= 3 values depending on the differences
157 in ionization efficiency.
158 Data was acquired on an ACQUITY UPLC® I-Class System coupled to an ion mobility mass
159 spectrometer Vion IMS QTOF operating in electrospray mode ($ESI^{+/-}$).
160 For the chromatographic separation, a reverse phase C18 BEH column (Waters, UK) with 2.1 x 100
161 mm and particle size of 1.7 µm heated at 35°C was used. LC solvents were 1 mM ammonium acetate
162 in water (solvent A) and methanol (solvent B) both acidified with 0.5% acetic acid. Initial conditions
163 (0.0-0.5 min) were set to 10% solvent B increased to 90% B in 3 min followed by 1 min at 90% B.
164 Reconditioning was achieved by 1.10 min using initial conditions. The total run time was 6 min.
165 The mass spectrometry detection was conducted in both positive and negative electrospray ionization
166 modes in the mass range of *m/z* 50 – 1000 under the following source conditions: capillary voltage
167 0.5 kV for positive and 0.5 kV for negative ion modes, cone voltage 50 V, source temperature 150
168 °C, desolvation temperature 450 °C, desolvation gas flow 600 L/h. Nitrogen was used as collision
169 gas. Two independent scans with different collision energies (CE) were alternatively acquired during
170 the run ($HDMS^E$ acquisition mode): a low-energy scan (CE 6 eV), to monitor the

protonated/deprotonated molecules and other potential adducts, whilst a high-energy scan (CE ramp 28 − 42 eV), to fragment the ions traveling through the collision cell.

The TOF analyzer was operated in sensitivity mode with the following settings: IMS gas (nitrogen) flow rate 25 mL/min, wave velocity 250 m/s, IMS pulse height 45 V. The acquisition rate was 10 Hz. Data acquisition and analysis were performed using UNIFI software (Waters, UK).

- **Vion Spain (Vion #2)**

The instrument was located at the Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain. The mycotoxin standards were diluted to different concentrations (1, 10, 100 µg/L) and 5 µl were injected, in triplicates per standard, on a CORTECS® C18 2.1 x 100 mm, 2.7 µm fused core column (Waters) kept at 40º C. Obtained CCS values were averaged over the replicates detected (n = 9, 6 or 3).

Data was acquired on an ACQUITY UPLC® I-Class System coupled to an ion mobility mass spectrometer Vion IMS QTOF, (Waters, UK) in electrospray mode ($ESI^{+/-}$).

LC solvents were 0.01% formic acid in water (solvent A) and methanol (solvent B) acidified with 0.01% formic acid. Initial conditions (0.0 min) were set to 10% solvent B increased to 90% B in 14 min followed by 2 min at 90% B. Reconditioning was achieved by 2.0 min using initial conditions. The total run time was 18 min with a flow rate of 0.3 mL/min.

The mass spectrometry detection was conducted in electrospray mode in the mass range of *m/z* 50 – 1000. Collision energy ramp 28 - 56 eV (Vion IMS QTOF, fitted with nitrogen as collision gas). The capillary voltage was 0.7 kV for positive ESI mode and 2.5 kV for negative ESI mode. The cone voltage was set at 40 V, the source temperature kept at 120ºC, the desolvation gas at 550ºC with a flow of 1000 L/h.

The TOF analyzer was operated in sensitivity mode with the following settings: IMS gas (nitrogen) flow rate 25 mL/min, wave velocity 250 m/s, IMS pulse height 45 V. The acquisition rate was 10 Hz. Data acquisition and analysis were performed using UNIFI software (Waters, UK).

- **Synapt UK (Synapt #3)**

The instrument was located at Waters Corporation, Wilmslow, Cheshire, UK.

Triplicate injections were performed for each mycotoxin standard mix (100 µg/L). The chromatographic separation was achieved on an ACQUITY UPLC® I-Class System with an FTN sample manager. A reverse-phase C_{18} BEH column (Waters) with 2.1 x 100 mm and particle size of 1.7 µm, heated at 35°C was used. The injection volume was 10 µL, and the flow rate was 0.4 mL/min. LC solvents were 1 mM ammonium acetate in water (aqueous mobile phase, A) and methanol (organic mobile phase, B) both acidified with 0.5% acetic acid. A binary gradient method was used as follows: 3 – 40% B in 4 min with no initial isocratic holding time, 40 – 90% B in 6 min, hold for 2 min at 90% B, re-equilibration at 3% B for 3 min prior to next injection. The total run time was 15 min.

The chromatographic system was interfaced with a Synapt G2-S*i* operating in electrospray mode ($ESI^{+/-}$). The capillary voltage was set to +2.5 kV and -1.5 kV; the sampling cone voltage was 30 V for both polarities, the cone gas flow 50 mL/min and the source temperature 150 °C. Desolvation gas temperature was 550 °C with a flow rate of 1000 L/h. Prior to use, the ion mobility cell settings were standardized for by setting the following values: 2 mL/min gas flow for the Trap cell, 180 mL/min for the helium cell, and 90 mL/min nitrogen flow in the mobility cell, giving an IM cell pressure of ~3.2 mBar. The IM wave velocity linearly ramped from 1000 to 300 m/s with a constant pulse height of 40 V. Data were acquired over the mass range of *m/z* 50–1200, at 10 spectra per second in data-independent $HDMS^E$ mode whereby after the separated precursor ions exit the IM cell, they are fragmented in one scan function and transmitted intact in another. Low-energy spectra were acquired at CE 3 eV, whilst high-energy spectra were acquired with a ramp of the transfer CE from 20 to 35

eV. Argon was used as collision gas. Mass and CCS calibration was performed with Major Mix, using the same reference points as for Vion. Prior to CCS calibration, the system was switched to mobility mode and left to equilibrate for 1 h. Leucine-Enkephalin was employed as the LockSpray solution at a concentration of 200 pg/µL (infusion rate 10 µL/min) acquired every 30 s to provide a real-time single-point mass and CCS calibration. The instrument was controlled with MassLynx v. 4.2 SCN 983. Raw data were processed on UNIFI software v. 1.9.4.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8.4.2, GraphPad Software San Diego, CA). Data correlation was evaluated by Pearson's correlation test ($\alpha = 0.05$).

Prediction of the theoretical CCS values

Theoretical CCS were obtained with two different models trained with machine learning approaches, the one proposed by Zhou et al [17], namely AllCCS (http://allccs.zhulab.cn/) and the recently published by Ross et al.[18], CCSbase (https://ccsbase.net/). Briefly, using a training set of experimentally measured CCS, the software employs a machine-learning algorithm able to predict CCS values for novel structures. To calculate the predicted CCS for $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$ and $[M-H]^-$ adducts, the SMILES string of each mycotoxins were imported to both web interfaces, AllCCS Predictor and CCSbase.

RESULTS AND DISCUSSION

In the present work we extended our previous investigation [7], by complementing and validating our mobility derived $^{TW}CCS_{N2}$ database of mycotoxins. We assessed the reproducibility of CCS measurement by means of an interlaboratory test. Furthermore, since different types of TWIM-MS systems are commercially available, it is necessary to validate the comparability of different

instrument types, when CCS databases are used independently from the instrument. For this purpose, CCS values were determined and compared for a total of 53 mycotoxins and different adduct states in both positive ($[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M+K]^+$) and negative ionization modes ($[M-H]^-$, $[M+CH_3COO]^-$).

CCS repeatability and interlaboratory reproducibility

At first the mycotoxins database was built using the Vion #1. Mycotoxins standard mix prepared at different concentration levels (1, 10, 100 µg/L) were injected per triplicate, therefore the $^{TW}CCS_{N2}$ values were average over n = 9 values (for some cases 6 or 3 because the lowest levels could not be observed). The $^{TW}CCS_{N2}$ values, average, standard deviation, and relative standard deviation (RSD) are summarized in **Table S1**. On the total of 225 $^{TW}CCS_{N2}$ values considered for both positive and negative ionization modes, the minimum RSD was 0.018%, the average RSD was 0.14% (±0.079%), and the maximum RSD was 0.61%. The majority of ions were within the strictest range of highly reproducible measurements (see **Figure 1S**) recently published by Stow et al. [10]. Indeed, 97% of measurements reported an RSD < 0.3%. The high precision of the measured $^{TW}CCS_{N2}$ led us to confidently state that these values can certainly be used with an in-house database for mycotoxin screening.

The $^{TW}CCS_{N2}$ obtained with Vion #1 were then compared with those experimentally derived in a second laboratory (Vion #2). Overall, 100 compounds were detected by both instruments at both sites, with a further 125 ions only detected by either the first or the second site. Such differences are not unexpected given that differences in ionization efficiency between different instruments are frequently observed as reported in previous interlaboratory validation studies [6,8]. Also stability issues during transportation of standard mixtures across laboratories should be considered as a source of differences in the compounds detected.

Results from the two Vion instruments demonstrated high precision for the $^{TW}CCS_{N2}$ measurements, showing an overall average interlaboratory RSD of 0.25 ± 0.17% for instruments located in two different laboratories.

The percentage deviation (ΔCCS%) between the two instruments was calculated keeping the Vion #1 as "reference". All the $^{TW}CCS_{N2}$ values for the ions detected by Vion#1 and Vion #2 were within the currently accepted error threshold of ±2.0%. In particular, deviations were observed within the range of ±1.5% for 100% of the measurements, within a high percentage of measurements (93%) showing a bias within the range of ±1%, as represented in **Figure 1**.

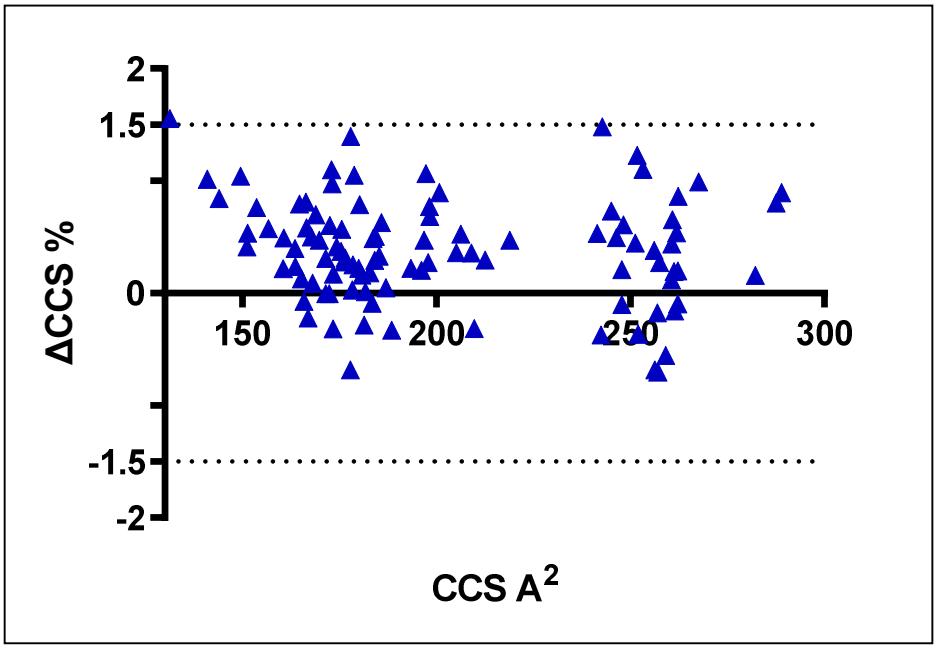


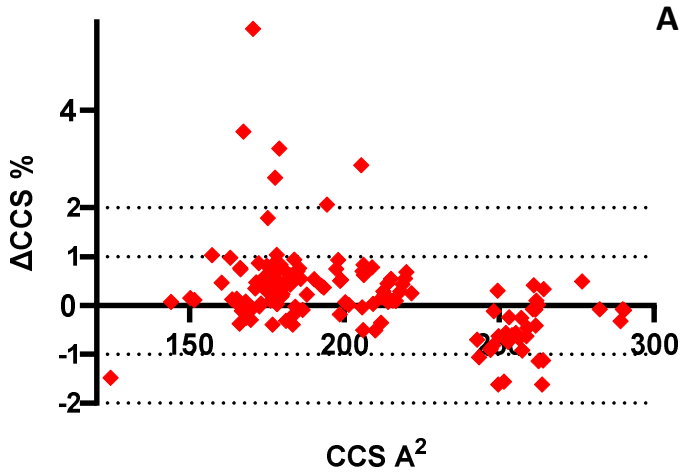
Figure 1. Bland-Altman plot displaying the spread of $^{TW}CCS_{N2}$ percent deviation (ΔCCS%) of values taken from replicate experimental acquisitions on two Vion TWIM-MS instruments located in two different laboratories.

Based on these results, when using the same TWIMS instrument type (including the same calibration standards), a threshold of ±1.5% can be considered without assuming high risk of false negatives when applying cross-laboratory $^{TW}CCS_{N2}$. Narrowing the acceptance error window below 2% in screening analysis will allow higher precision to be achieved in the annotation of molecular

candidates. This outcome is in agreement with the result reported recently [6,8] on the $^{TW}CCS_{N2}$ reproducibility across different laboratories.

Inter-platform CCS reproducibility

After demonstrating repeatability and reproducibility of the $^{TW}CCS_{N2}$ when the same instrument type is used, we carried out further studies to understand whether a common mycotoxins database can be used independently from the instrument type. To this purpose the mycotoxins standard mixes were analyzed using a Synapt G2-Si. Overall, 139 common ions were detected by both instrument types (Vion and Synapt) and compared in terms of bias against the database. A graphical comparison of the CCS means for single laboratory (Vion #1, Vion #2 and Synapt G2-Si) is reported in **Figure 2S**. Synapt G2-Si platform showed high precision, in accordance with the performance of both Vion and Synapt instruments. The average RSD of triplicate measurements was 0.113 ±0.11%, the minimum RSD was 0.006%, while the maximum RSD was 0.70%. Bar charts displaying the spread of relative standard deviation for both instrument types are depicted in **Figure 1S**.



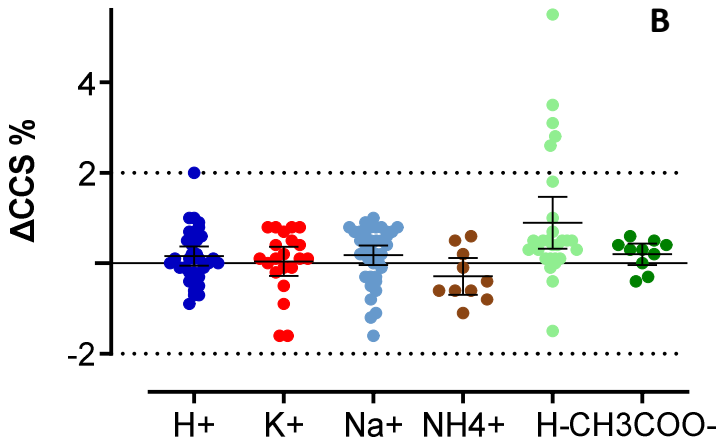


Figure 2. $^{TW}CCS_{N2}$ percent deviation of values taken from two different TWIM-MS instruments (Vion vs Synapt) located in two different laboratories. (A) Bland-Altman plot displaying the spread of $^{TW}CCS_{N2}$ percent deviations and (B) their trend according to the adduct ions monitored.

When evaluating the bias between the two T-Wave systems, different performance in terms of reproducibility were found for positive and negative ionization modes. In general, 96.4% of the $^{TW}CCS_{N2}$ measurements, were within the error threshold of ±2.0% and interestingly, 89.2% of the ions were within the narrowed error threshold of ±1.0% (see **Figure 2A**). Very few compounds (n = 5) showed deviations greater than the threshold of ±2.0%. The highest deviations were observed for the deprotonated ion of nivalenol (ΔCCS% = 5.5%). The other ions reporting error % higher than ±2.0% were the deprotonated deoxynivalenol (DON) (ΔCCS% = 3.5%), 3-acetyl-DON (ΔCCS% = 3.6%), DON-3-glucoside (ΔCCS% = 2.8) and fusarenon X (ΔCCS% = 3.1%).

Indeed, by further elaborating the data, a trend according to the adduct monitored and the mycotoxin chemical classes was observed (**Figure 2B**). The highest deviations from the database were observed for the $[M-H]^-$ adduct of the type B trichothecenes class. These compounds are sesquiterpene epoxides, characterized by multiple protonation and de-protonation sites. Therefore, differences in the CCS might be expected considering the formation of charged isomers depending on the loss of a proton from different molecule sites.

Further investigations are needed to confirm this hypothesis, including the use of high-resolution IMS with improved resolving power, such as cyclic-IMS.

Finally, the database generated within this study was compared with the previously published $^{TW}CCS_{N2}$ data [7] which were derived from arrival time measurements using a previous generation travelling wave IM-MS instrument, the Synapt HDMS Q-TOF Mass Spectrometer (from Waters Corporation). It is important to note that the original database obtained from the previous generation TWIM system was created using a different calibrant (i.e. Poly-DL-Alanine mix, monitoring [M-H_2O] ions) compared to the calibrant employed in the present work (Major Mix, containing Poly-DL-Alanine, Ultramark 1621, low-MW acids, and nine additional small molecules, commonly used as QC standards). The exact composition of the different calibration solutions is reported in **Table S3** and **Table S4**. Moreover, the first generation TWIMS technology included different informatics analysis tool, comprising an older peak detection algorithm. Because of the different calibration profiles, slightly higher deviations are to be expected, however, the reported values were still found to be within the common error distribution range. Indeed, for 84.2 % of the measurements, deviations were within the threshold of ±2.0%, while the higher errors were found for trichothecenes and aflatoxins monitored as potassium, sodium and ammonium adducts.

These findings showed that the choice of the calibrants can have an impact, as already discussed elsewhere [12], but not as high as it might be expected. A systematic error on CCS measurements can be attributed to the intrinsic difference of chemical structure between Poly-Alanine (linear conformation) and the diversified groups of mycotoxins, which in many cases, share a cyclic-base structure (e.g. trichothecenes, zearalenone and its derivatives, enniatins).

The results presented in this study empirically confirmed the recommendations for reporting ion mobility mass spectrometry measurements recently published [12], which suggest that when building a ^{TW}CCS database, the calibration mixture used should also be indicated, being the CCS derived through a calibration equation and not directly measured.

Mycotoxin isomers separation

Several isomers have been included in the database, mainly modified mycotoxins, including positional isomers (3- or 15-Ac-DON) or conformational isomers (T-2 α/β-glucoside).

In particular, the drift time separation of acetylated derivatives of DON, was investigated considering the challenge of their chromatographic separation. 3 and 15-Ac-DON were detected as protonated, potassium, sodium and ammonium adducts in positive and as deprotonated and acetate adduct in negative mode. Only the sodiated and potassiated species resulted in $^{TW}CCS_{N2}$ values that are significantly different, and whose percentage difference is > ±2%. **Figure 3A** shows the separation of the $[M+Na]^+$ adduct at *m/z* 361.1258 for 3-Ac-DON (CCS 183.4 $Å^2$ and 4.01 ms arrival time) and 15-Ac-DON (CCS 176.7 $Å^2$ and 3.74 ms arrival time), suggesting a different shape of the ions, which is intensified by the coordination of a sodium atom within the molecular structure.

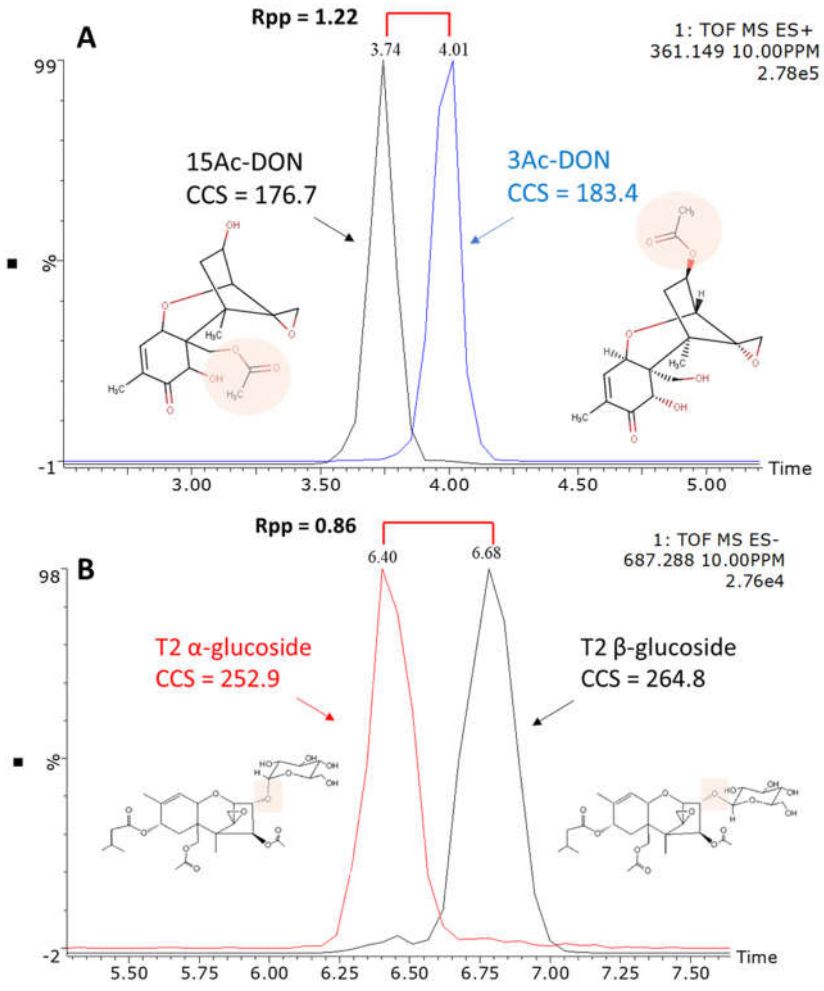


Figure 3. Arrival time distribution (ms) of (A) acetylated forms of DON $[M+Na]^+$ and (B) T2 α/β glucoside $[M+CH_3COO]^-$ obtained using the Synapt G2-Si. Rpp: two-peak resolution.

372 The separation efficiency was calculated in terms of two-peak resolution (Rpp) using the equation
373 from Dodd et al., [19], resulting in a Rpp > 1 (1.22) and thus indicating that 3- and 15-Ac-DON isomers
374 are resolved in the drift time dimension when the sodium adduct is considered.
375 Even more challenging is the separation of conformational isomers, as for the different configuration
376 of the anomeric carbon in T-2 α- and β-glucoside. In this case the drift time separation was achieved
377 in negative ionization mode, by monitoring the acetate adduct, as shown in **Figure 3B**. The resolution
378 between the two peaks was Rpp < 1 (0.86) mainly because of a distortion of the peak at half height
379 (not Gaussian), thus the two isomers cannot be considered fully resolved, even though the valley is <
380 10% of the peak height.
381 However, since their CCS percent difference is higher than ±2% (ΔCCS% = 4.3%) T-2 α- and β-
382 glucoside will not be aligned in the drift time dimension, and they will be processed as two different
383 ions. The separation of isomers aids with an increased confidence in the identification process when
384 screening for real samples.

385 The separation of additional pairs of mycotoxin isomers was further investigated. Although a broad
386 and splitting peak shape was observed, the resolving power of the employed technique was not
387 sufficient to resolve the positional (i.e. zearalenone 14/16 glucoside) and conformational isomers (i.e.
388 α/β zearalenol) analyzed herein. With the improvements in IMS technology and enhanced resolving
389 power of cyclic IMS, their separation could potentially be possible.
390

391 **CCS prediction**

392 The experimentally derived $^{TW}CCS_{N2}$ can also be compared with the theoretical values allowing a
393 higher degree of confidence in the identification process. New mycotoxins and modified forms may
394 be discovered and characterized by matching theoretical and experimental rotationally averaged
395 cross-sectional areas, despite the lack of analytical standards.

396 Theoretical CCS values can be obtained via computational chemistry tools like MOBCAL [17,20], as it
397 was more recently developed, by machine-learning based mathematical methods to predict drift times
398 or CCS [20].
399 Here, the theoretical CCS were predicted using machine learning based on AllCCS [17] and CCSbase
400 [18] on-line tools. Overall, 155 and 189 ions were considered for AllCCS and CCSbase, respectively.
401 The difference is due to the prediction of potassium adducts, that was not available in AllCCS.
402 Predicted CCS values were found to be highly correlated (Pearson r > 0.98, see Supplementary
403 Information) with the experimentally observed values ($^{TW}CCS_{N2}$ - Vion#1), as depicted in **Figure 3S**.
404 Despite the power of artificial intelligence, high deviations were found, with prediction errors within
405 ±2 % only for 39% of the analytes, while 91% of the compounds fell in the range ±5 % of percentage
406 difference when AllCCS prediction model is considered. Interestingly, greater deviations were found
407 for the protonated adducts when compared with sodium, ammonium and potassium adducts (see
408 **Figure 3S)**.
409 A possible explanation of the bias observed could be that the CCS data used to build the training set
410 were indeed $^{DT}CCS_{N2}$ using the stepped field method [17]. To test the real suitability of the model
411 algorithm for the prediction of TW-derived CCS, a training set composed by $^{TW}CCS_{N2}$ would be
412 needed.
413 On the other hand, CCSbase[18] prediction model provides much more comprehensive coverage of
414 structures that include also measurements on TWIM platforms. Indeed, lower deviations were found,
415 with half of the analytes (50.3%) displaying prediction errors within ±2 % (see **Figure 3S and Table**
416 **S5).**
417 Because a percentage deviation > 5 % would not be acceptable due to unlikely applicability, the
418 results obtained in the present study confirmed that prediction models are not completely universal
419 for small molecules [21]. At least for mycotoxins, building a theoretical CCS database is not reliable
420 when using machine learning approaches based on a training model that was not constructed with the
421 same class of chemical compounds and experimentally derived using the same IMS technology.

However, research is ongoing and preliminary data are highly encouraging [21]. This highlights the importance of creating and validating reliable databases which ultimately can aid with improved validation of predicted CCS for natural toxins as well as for other food contaminants. The final, and perhaps holistic goal being the ability to predict the CCS of compounds for which standards are not readily available, thus bringing about great benefit for future applications in food safety.

In conclusion, the mycotoxin CCS database can be used independently for TWIMS instruments (Vion and Synapt), since 96.4% of the $^{TW}CCS_{N2}$ measurements, were within the error threshold of ±2.0%. The remaining 4% was due to a specific class of mycotoxins and further studies are already ongoing to investigate the presence of eventual charge isomers, whose effect is impactful in the measurement of CCS for de-protonated species.

Regarding the theoretical CCS, even though results collected so far are highly promising, we are far from relying on predicted CCS values, and further studies are required before proposing the use of CCS as molecular parameter as such that can be universally applied on all commercial IM-MS platforms. On the other hand, the implementation of a score system based on different ranges of bias between CCS measurements and values in databases seems to be a preferable approach which does not compromise the validity of the databases developed so far.

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Abbreviations Used

CCS: Collision Cross Section

IM-MS: Ion Mobility Mass Spectrometry

RSD: Relative Standard deviation

HRMS: High Resolution Mass Spectrometry

TWIMS: traveling wave Ion Mobility Spectrometry

DTIMS: drift tube Ion Mobility Spectrometry

$^{TW}CCS_{N2}$: Collision Cross Section derived using traveling wave Ion Mobility Spectrometry and nitrogen as buffer gas

DON: deoxynivalenol

3- or 15-Ac-DON: acetyl-deoxynivalenol

Supporting Information

Note 1 - Chemical synthesis of zearalenone-14-glucoside and hydrolysed fumonisins.

Note 2 - CCS Calibration of TWIMS-MS.

Figure 1S. Bar charts displaying the spread of relative standard deviation (%) of CCS values taken from replicate experimental acquisitions on (A) Vion IMS QTof and (B) Synapt G2-Si instruments.

Figure 2S. Representation of the $^{TW}CCS_{N2}$ values ($Å^2$) measured for each ion by the three TWIMS instruments.

Figure 3S. (A, C) CCS based prediction values vs. observed $^{TW}CCS_{N2}$ obtained with AllCCS (A) and CCSbase (C) on-line tools.

Table S1. Mycotoxin database built using TWIM-MS Vion IMS QTof, nitrogen as buffer gas and Major Mix IMS/TOF as calibrants.

Table S2. Mycotoxin database built using TWIM-MS Synapt G2-Si, nitrogen as buffer gas and Major Mix IMS/TOF as calibrants.

Table S3. Composition of CCS calibration solution used for positive ion mode.

Table S4. Composition of CCS calibration solution used for negative ion mode.

Table S5. Theoretical CCS ($Å^2$) for mycotoxins obtained using AllCCS and CCSbase prediction models.

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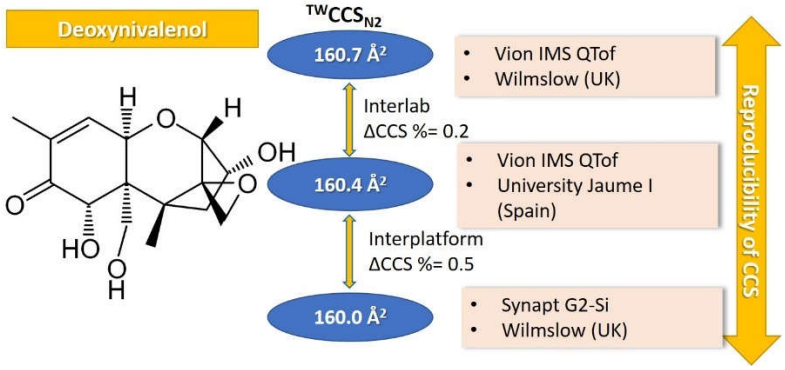
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Interplatform
ΔCCS %= 0.5
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Synapt G2-Si
Wilmslow (UK)
Reproducibility of CCS

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