INVESTIGATIONS INTO CROSS-PLATFORM AND LONG-TERM ROBUSTNESS OF A CCS METRIC

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OVERVIEW

- **The studies performed have utilised ion mobility (IM) to perform multi-class small molecule non-targeted screening assays to determine the long-term reproducibility of collision cross section (CCS) measurements.**
- **Travelling wave ion mobility (TWIM) CCS intra/inter-laboratory measurement and cross-platform CCS reproducibility has been compared.**
- **Several hundred substances including pesticides, flavonoid isomers, fluoroquinolone protomers and analytes relevant in forensic toxicology, have been compared over a period of up to 4 years.**
- **Using a single TWIM CCS calibration, QC mixes have been monitored for a period of up to 10 weeks in both positive/ negative electrospray ionization modes and for analysis of steviol glycosides over three orders of dynamic range in complex food extracts.**
- **When compared to CCS libraries, generated CCS delta values of < 2% have been obtained routinely (all within typical accepted MRM product ion ratios tolerances of 30%).**

INTRODUCTION

Since the first coupling of mass spectrometry and ion mobility in 1962, there has been a continuous increase in the research and utility of IM-MS over the last two decades (from 100 peer reviewed papers in 1995 to >1250 by 2014/2015), which has resulted from the commercialisation of IM instrumentation. [1-4]

UPLC-IM comprises ion mobility (gas phase separation prior to MS analysis) coupled with UPLC (neutral species separation). The timescale of UPLC (seconds), IMS (milliseconds) and time-of-flight MS (microseconds) are compatible with the requirement of high throughput analysis of complex samples. Ion mobility separation of compounds result from gas phase ions being separated within a gas filled (TWIM) RF ion guide of the mass spectrometer, prior to the mass analyser. Mobility separation is obtained by driving packets of ions through an inert buffer gas (nitrogen) using a relatively weak electric field. The number of collisions between ions and the buffer gas cause drift time differences. The time to traverse the device is mobility dependent on factors such as the mass of the ion, charge and shape. It provides an added dimension of separation to that of LC (hydrophobicity) and MS (*m/z*), in addition to CCS, a complementary identification metric.

The continuing evolution of ion mobility has been illustrated where a strategy to incorporate ion mobility CCS as an additional cumulative metric to enhance specificity in pesticide screening assays was developed.[5-7] The routine use of CCS for small molecule analysis has since increased across multiple areas of research including pharma (metabolism, metabolomics, lipids), food safety (veterinary drugs, mycotoxins, steroids, steviol glycosides, natural product screening, natural toxins). CCS searchable libraries have been generated, where use of a CCS metric can be used to increase cumulative specificity of identification as well decrease false detections.

We have investigated and present the results of multiple CCS applications, incorporating the use of the long-term reproducibility of CCS measurement (up to four years), TWIM CCS calibration robustness, intra/inter-laboratory measurement and cross-platform CCS reproducibility. The investigations included 100's of analytes relevant to forensic toxicology for which, a comparison has been performed where commonality in analyte investigation has occurred.[8] Additionally ion mobility investigations, into veterinary drugs (fluoroquinolone protomers),[9] medicinal plant extract analysis (flavonoids),[10] pesticide screening,[7] food analysis (steviol glycosides),[11] system performance monitoring (QC analytes) will be utilised to illustrate the robustness of routine collision cross section measurements.

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METHODS

LC Systems-Chromatographic Separation:

For the analysis of substances relevant to forensic toxicology, UPLC chromatographic separation was achieved using Waters ACQUITY UPLC HSS C18 column (150 mm × 2.1 mm, 1.8 µm). A reversed-phase gradient was used, comprising mobile phase A (5 mM aqueous ammonium formate buffer adjusted to pH 3 with formic acid) and mobile phase B (acetonitrile with 0.1% formic acid).[8]

For all other presented applications,[7,9-11] UPLC chromatographic separation was performed using a linear reversed-phase gradient of mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid), with both conventional UPLC (Waters ACQUITY UPLC I-Class chromatograph and a Waters ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 µm)) and micro-flow UPLC systems (Waters M-Class system connected to a Waters ionKey separation device - iKey PCA BEH C18 130Å (50 mm × 150 µm, 1.7 µm)).

MS Systems:

MS Source Parameters: Both positive (ESI+) and negative (ESI-) electrospray ionisation (ESI) modes were utilised with HDMS^E acquisition mode. Mass ranges varied between 50-2000 Da. Varying ESI capillary voltages were applied. Analytes included: forensic toxicology standards, steviol glycosides, fluoroquinolones, flavonoids and small molecule QC standards, full experimental details have previously been described.[7-11]

Calibration:
Instrument platform #1: (Waters Vion IMS Q-ToF-MS)
Instrument platform #2 (Waters SYNAPT G2-S*i*).

The working resolution of platforms #1 and #2 were 30,000 and 20,000 respectively (at *m/z* 556) full-width half-maximum (FWHM). The reference lockmass calibrant was leucine enkephalin ($C_{28}H_{37}N_5O_7$ (*m/z* 556.2766 for ESI+ and *m/z* 554.2620 for ESI-).
Calibration of the IM cell for $^{TW}CCS_{N2}$ calculations was performed using an IMS/ToF Calibration Kit (Waters Corp. UK). For platform #1 IM resolution was ≈ 30 Ω/ΔΩ (FWHM) and platform #2: IM resolution was ≈ 40 Ω/ΔΩ (FWHM). Default IMS screening parameters were utilised.

Cyclic ion mobility (cIM) research platform (Waters):
The mass spectrometer was mass calibrated ESI+ at 60,000 resolution FWHM over an *m/z* range of 50-1000 Da using an IMS/ToF Calibration Kit (Waters Corp.). The reference lockmass calibrant was sodiated raffinose ([M+Na]$^+$ *m/z* 527.1583). Ion mobility resolution was ≈ 65 Ω/ΔΩ (FWHM) for a single pass around the cIM device; resolution increases with the square root of the number of passes. Ion mobility parameters included: cIM T-wave velocity = 353m/s, T-wave pulse amplitude = 25V and gas flows of 120/25mL/min for the respective helium/IM cells resulting in an IM pressure of 2.7 mBar.

RESULTS AND DISCUSSION

Generation of CCS libraries and their application have been performed using three ion mobility platforms; two Q-ToF travelling wave ion mobility mass spectrometry platforms and a cyclic ion mobility (cIM) research platform. The cIM device in a modified SYNAPT platform that provides a longer mobility separation path length, where the standard linear IM cell is replaced by a multi-pass cyclic IM cell for increased mobility resolution.

An extensive comparison of two Q-ToF TWIM MS platforms has been performed. As part of an inter-laboratory study, a CCS library was generated for 500 forensic toxicology analytes, using Q-ToF TWIM MS instrument platform #1 (where the determined CCS measurements were within 2% of each other). The average CCS was used to create forensic toxicology CCS library.

The library produced was then used to screen CCS values obtained from an independent study where 600 forensic toxicology compounds were used to generate a CCS library using Q-ToF TWIM MS instrument #2; 435 of the forensic toxicology samples were common to CCS libraries generated using instrument platform #1 and #2, where the CCS values were determined to be within 2%. The comparison results are shown in Figure 1.

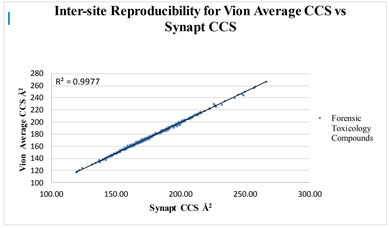

Figure 1. Comparison of collision cross section for forensic toxicology compounds. (I) Vion inter-site average versus SYNAPT and (II) corresponding frequency distribution for Vion library CCS delta. (III) Vion intra-site versus SYNAPT and (IV) corresponding frequency distribution for intra-site Vion library CCS delta.

Although frequency of instrument calibration is often determined by individual laboratory protocols, Figure 2 illustrates the long-term stability of applying an ion mobility CCS calibration strategy. Using a steviol glycoside CCS library, a screening assay to detect steviol glycosides (E960) in extracts of "off the shelf" complex food products, such as jam, yogurt, soft drinks and syrups, was performed. Detection over 3 orders of dynamic range was achieved for the sweetener food additive E960. When compared to the expected library CCS values, delta values of < 1% were achieved for 18974 detections with an RMS error of 0.26%, over 45 days. A second example of applying a single CCS calibration, and hence CCS metric robustness is presented, where QC standards were monitored for a period of ten weeks in ESI+ and ESI- modes. During the process of building a "small drug" molecules CCS library, positive (5360 measurements) and negative (3600 measurements) QC CCS delta values were maintained within 2%, of the expected QC mix analyte CCS values, as shown in Figure 3.

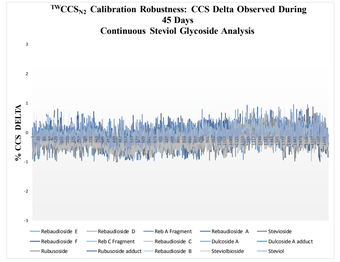

Figure 2. CCS stability for analysis of steviol glycosides detected in complex food extracts over a 45 day period (24/7) using a single IM calibration.

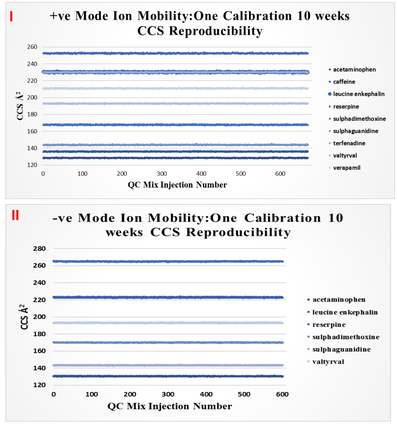

Figure 3. QC mix CCS reproducibility over 10 weeks, 24/7 acquisition in (I) ESI+ and (II) ESI- using a single ion mobility calibration.

Using a fluoroquinolone veterinary drugs protomers study, CCS values were compared using Q-ToF TWIM MS instrument platform #2 and the multi-pass cIM device, where the observed CCS values were within 2% of the fluoroquinolone CCS library values. Figure 4 presents the cross-platform (linear IM and cIM) and long-term fluoroquinolone protomer CCS reproducibility over a 3 year period. When comparing linear IM and cIM CCS, delta values of < 2% were obtained when compared to the fluoroquinolone protomer CCS library generated in 2015. Further long-term data is presented in Figure 5, for identification of isomer marker flavonoids determined to be present in medicinal plant extracts of *Passiflora* species where delta values of < 2% were obtained when compared to the flavonoid CCS library generated in 2015.

The extensive studies into TWIM CCS robustness are further illustrated in Figure 6, where the CCS errors for the 200 pesticides comprising multiple classes of analytes have also been determined over a period of 4 years (2000 measurements), where % CCS delta is < 2% compared to the pesticide library generated in 2013. The library comprised a range of *m/z* 123 to 859 Da.

The reliability of incorporation of a collision cross section metric into mass spectrometry libraries is illustrated through the reproducibility of long-term and cross-platform CCS measurements presented. When compared to CCS libraries generated, the vast majority of CCS delta values of < 1% have been obtained routinely (all within typical MRM product ion ratios tolerances of 30%)[12] which is acceptable when using MRM libraries. Combining product ion information and CCS information may provide enhanced cumulative specificity.

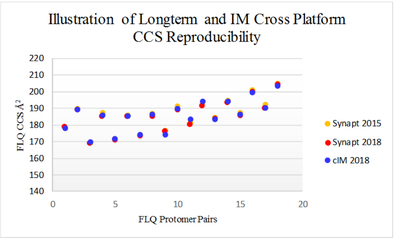

Figure 4. Linear IM (compared over a period of 3 years) and cIM for fluoroquinolone protomer CCS reproducibility.

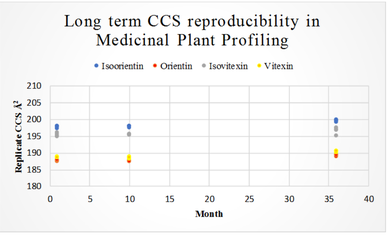

Figure 5. Linear IM (compared over a period of 3 years) for identification of isomer marker flavonoids determined to be present in medicinal plant extracts of *Passiflora* species.

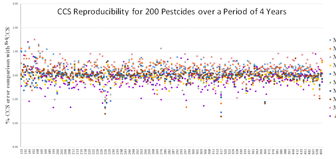

Figure 6. CCS reproducibility for 200 pesticides measured ten times over 4 years.

CONCLUSION

- **CCS library values have been shown to be routinely reproducible where studies have been performed over periods of time up to 4 years.**
- **CCS values can be measured routinely long-term using a single CCS ion mobility calibration.**
- **CCS values have been shown to be reproducible in intra/inter and cross-platform comparisons.**
- **A CCS metric can be routinely obtained < 2% of the expected library value.**
- **CCS reproducibility across multiple classes of small molecules has been shown for 1000's of analyte detections.**
- **Combined with retention time, *m/z*, a CCS metric is a reproducible metric that can be used to improve cumulative specificity.**

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