Data and text mining

**AutoCCS: automated collision cross-section calculation software for ion mobility spectrometry–mass spectrometry**

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**Abstract**

**Motivation:** Ion mobility spectrometry (IMS) separations are increasingly used in conjunction with mass spectrometry (MS) for separation and characterization of ionized molecular species. Information obtained from IMS measurements includes the ion's collision cross section (CCS), which reflects its size and structure and constitutes a descriptor for distinguishing similar species in mixtures that cannot be separated using conventional approaches. Incorporating CCS into MS-based workflows can improve the specificity and confidence of molecular identification. At present, there is no automated, open-source pipeline for determining CCS of analyte ions in both targeted and untargeted fashion, and intensive user-assisted processing with vendor software and manual evaluation is often required.

**Results:** We present AutoCCS, an open-source software to rapidly determine CCS values from IMS-MS measurements. We conducted various IMS experiments in different formats to demonstrate the flexibility of AutoCCS for automated CCS calculation: (i) stepped-field methods for drift tube-based IMS (DTIMS), (ii) single-field methods for DTIMS (supporting two calibration methods: a standard and a new enhanced method) and (iii) linear calibration for Bruker timsTOF and non-linear calibration methods for traveling wave based-IMS in Waters Synapt and Structures for Lossless Ion Manipulations. We demonstrated that AutoCCS offers an accurate and reproducible determination of CCS for both standard and unknown analyte ions in various IMS-MS platforms, IMS-field methods, ionization modes and collision gases, without requiring manual processing.

**Availability and implementation:** https://github.com/PNNL-Comp-Mass-Spec/AutoCCS.

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**Supplementary information:** Supplementary data are available at *Bioinformatics* online. Demo datasets are publicly available at MassIVE (Dataset ID: MSV000085979).

**1 Introduction**

Ion mobility spectrometry (IMS) coupled with mass spectrometry (MS) is increasingly utilized in studies of biomolecules as it offers highly reproducible and extremely fast separations (milliseconds to seconds). IMS separates molecules in the gas phase based on their collision cross sections (CCS) with a neutral buffer gas, a property that represents the three-dimensional structure of the corresponding ion. IMS-MS provides high throughput, two-dimensional information (i.e. mass and mobility) enabling the identification and quantification of metabolites with greater confidence and effectiveness than approaches based on MS alone.

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Automated data processing tools are increasingly critical as instrumentation, data density and acquisition speed advance. Unfortunately, processing the large amounts of data generated in large-scale IMS analyses is still a bottleneck (Stow et al., 2017; Zheng et al., 2017). We previously developed PIXIE, which is a software for automated arrival time extraction and CCS calculation of standard analytes measured using the ‘stepped-field’ method in drift tube-based IMS (DTIMS)-MS instruments (Ma et al., 2017).

Here, we present a new IMS-MS software tool, AutoCCS, capable of calculating CCS from stepped- (i.e. multi-) field data and single-field analyses of unknowns where CCS determination is based on calibration against well-characterized ions measured under the same conditions. In contrast to PIXIE, which only performs a targeted extraction of predefined ions for stepped-field IMS-MS measurements, AutoCCS can utilize features detected from single-field IMS-MS measurements in an untargeted fashion to calculate CCS values for all measurable analytes and their various conformers. These functionalities enable CCS determination for unknown molecules, as well as for experiments performed on traveling wave based-IMS (TWIMS), including Waters Synapt and Structures for Lossless Ion Manipulations (SLIM) IMS devices (Ibrahim based-IMS (TWIMS), including Waters Synapt and Structures for molecules, as well as for experiments performed on traveling wave based-IMS (TWIMS), including Waters Synapt and Structures for Lossless Ion Manipulations (SLIM) IMS devices (Ibrahim et al., 2017). For single-field methods, AutoCCS supports internal or external calibration (with an arbitrary or configurable list of reference ions) and an ‘enhanced’ CCS calibration mode with external calibration that corrects for temperature and pressure variations that may be present in sample runs that are temporally distant from the calibrant run. Also, AutoCCS performs non-linear regression analysis with either a polynomial or linearized power function for TWIMS data. For use with in-house SLIM IMS-MS systems, we modified the typical non-regression methods by incorporating the accumulation time for the arrival time adjustment.

We demonstrate AutoCCS with data collected in various IMS systems and methods: DTIMS-MS, RapidFire-DTIMS-MS, TWIMS-MS, trapped IMS (TIMS)-MS and SLIM IMS-MS, for both authentic standard reference compounds and complex samples in various conditions (e.g. buffer gas and ionization mode).

2 Materials and methods

2.1 Overview of AutoCCS

AutoCCS is a freely available command-line tool to determine CCS values from various IMS-MS platforms and data acquisition modes. It is written in Python for greater accessibility and is complementary to other data processing layers, such as raw MS-data preprocessing [i.e. PNNL-PreProcessor (Bilbao, 2020)] and feature finding. The main input is the list of IMS features (i.e. tables of mass and arrival time), which can be generated with the user’s preferred software (Agilent proprietary Mass Profiler .cef and open-source MZmine .csv formats are currently supported).

AutoCCS allows users to change configurations as needed to calculate CCS in various use cases. Configuration parameters (e.g. Supplementary Fig. S1) and command-line options that users can control can be found in Supplementary Tables S1 and S2, respectively.

For the stepped-field DTIMS method (Stow et al., 2017), AutoCCS allows users to automatically determine CCS values for targeted ions by performing linear regression using various fields (Fig. 1a). For the single-field DTIMS method (Kurulugama et al., 2015), AutoCCS supports two modes: the standard method and also an ‘enhanced’ calibration method to improve CCS determination in high-throughput experiments by accounting for temperature and pressure variations between calibrant and complex sample analyses (Fig. 1b). For TWIMS methods, AutoCCS offers a polynomial function and linearized power function to apply to calibration curves.

2.2 Stepped-field DTIMS

A traditional, standardized stepped-field DTIMS method provides straightforward and highly reproducible accurate CCS determination, with very low relative standard deviations, even among different laboratories (Stow et al., 2017). The stepped-field method employed in DTIMS takes advantage of the linear relationship of arrival time (\(t_A\)) and reduced mobility (\(K_0\)), as follows:

\[
\frac{t_A}{K_0} = \frac{L^2}{273.15} \left( \frac{P}{T} \right) + t_0. \tag{1}\]

using the length of the drift tube (\(L\)), temperature (\(T\)), drift gas pressure (\(P\)), drift voltage (\(V\)) and \(t_0\) which encompasses time spent outside of the drift cell. Based on this linear regression analysis, the molecular CCS values can be directly computed as below (Revercomb and Mason, 1975):

\[
\Omega = \frac{3q}{16N_0} \left[ \frac{2\pi}{\mu kT} \right] \frac{1}{P} \frac{1}{T} m^2 = \frac{3q}{16N_0} \frac{1}{\mu kT K_0} \tag{2}\]

where \(q = Ze\) is the ionic charge (\(z\); charge state of the ion, \(e\); unit electronic charge), \(N_0\) is the number density of the drift gas, \(m\) is the reduced mass of the ion, \(k\) is the Boltzmann constant and \(E(=V/L)\) is the electric field.

AutoCCS allows users to automatically search for multiple adduct ions. A list of target ions with neutral exact masses (e.g. Supplementary Table S3) and metadata files with IMS acquisition conditions (e.g. electric fields, pressures and temperatures which are exported using the PNNL-PreProcessor) are also required. Based on the exact mass and configured adducts, the target ions are generated and selected from the measured mass-to-charge (\(m/z\)) ratio of the given IMS features and \(m/z\) tolerance as defined by users. For each combination of the selected features (i.e. arrival time) in all \(n\) fields, AutoCCS performs linear regression analysis between \(\left\{P_1/V_1, P_2/V_2, \ldots, P_n/V_n\right\}\) and \(\left\{t_1^2, t_2^2, \ldots, t_n^2\right\}\) where \(t_i^2\) indicates the arrival time of the \(i\)th features in the \(n\)th field, and \(P_n\) and \(V_n\) represent the pressure and temperature in the \(n\)th field, respectively. Among all combinations of selected features, AutoCCS identifies the best fit regressors by the \(R^2\) threshold value (e.g. \(R^2 \geq 0.99\) by default), which users can customize. To reduce the number of combinations, users can limit the number of selected features in each field based on peak intensity.
2.5 Calibration methods for TWIMS

For TWIMS instrumentation, such as for SLIM devices, CCS cannot be calculated directly using the mathematical function provided by the fundamental low-field ion mobility equation (Revercomb and Mason, 1975) due to the variable electric field used in TWIMS (Gabelica et al., 2019; Lanucara et al., 2014; Ruotolo et al., 2008). Unlike the single-field method in DTIMS, a non-linear relationship needs to be accounted for effective calibration in TWIMS. For this purpose, AutoCCS performs either a polynomial (Bush et al., 2012) or power (Gabelica et al., 2019; Ruotolo et al., 2008) regression analysis using the arrival time \( t_A \) and known CCS values of reference compounds to create a calibration function for determination of TWIMS CCS.

For polynomial regression, data for each calibrant run is fit using the Equation (5):

\[
\Omega = x_0 + x_1 t_A + x_2 t_A^2 + \cdots + x_D t_A^D + \text{and } t_A = t_{acc}
\]

where \( D \) denotes the degree of the polynomial function that users choose, \( t_{acc} \) represents the ion accumulation time in the SLIM system and \( \Omega \) represents the reduced CCS. For example, if \( D = 2 \) or 3, it becomes a quadratic function for the binomial regression or a cubic function for the trinomial regression, respectively.

In the power regression, the calibrant arrival times were corrected by Equation (6):

\[
t_A = t_{acc} - \frac{C \cdot \sqrt{T}}{1000} - t_{acc}
\]

where \( C \) is a constant coefficient designated as the EDC (Enhanced Duty Cycle) delay coefficient for the Synapt Q-IM-o-ToF instrument (Ruotolo et al., 2008), but was set to 0 for the SLIM system. Then the linear relationship between corrected arrival time \( t_A \) and the reduced CCS values \( \Omega \) is fit to the linearized power function as follows:

\[
\ln \Omega = X \times \ln t_A + \ln Y
\]

where \( X \) and \( Y \) denote the coefficients of the power function. From this regression analysis, we calculate the new corrected arrival time \( t_A \) from Equation (8):

\[
t_A = t_{acc} - \frac{C \cdot \sqrt{T}}{1000} - t_{acc}
\]

2.6 Sample preparation

Agilent ESI-L low concentration tuning mix solution (Agilent Santa Clara, CA; https://www.agilent.com/cs/library/certificateofanalysis/G1969-85000cofa872022-U-LB86189.pdf) was used without further purification, hereafter referred to as tune-mix. Tetraalkyl ammonium (TAA) salts were purchased from Sigma-Aldrich (Milwaukee, WI USA) and used without any further purification. The TAA salts, consisting of tetraethylammonium through tetraoctylammonium, were prepared as a mixture to a final concentration of 1 \( \mu \)M in 50/50 water/methanol with 0.5% acetic acid (v/v). Rotenone was spiked into a complex background of 3553 plant leaf extracts at 0.001 mg/mL. Briefly, leaf samples were harvested from a field located at the University of California Agriculture & Natural Resources (UC-ANR) Kearney Agricultural Research & Extension Center (KARE) in Parlier, CA for a large-scale plant study to assay the natural diversity of Sorghum bicolor. The leaves were placed into 50-ml conical tubes and frozen with liquid nitrogen in the field and then lyophilized. Dried samples were pulverized and ground. 60 mg aliquots were measured out of each leaf sample for metabolite extraction. 1 mL of 80:20 MeOH: H2O was added and samples were centrifuged. The supernatant was removed (~800 \( \mu \)L) and further
### Table 1. CCS values calculated for Agilent tune-mix samples measured in positive electrospray mode in nitrogen drift gas with four different IMS platforms

| Type               | Tune-mix calibrants | Adduct Ions m/z | AutoCCS CCS (Å²) | \(^1^{\text{CCS}}_{\text{Browser}}\) (Å²) | \(^1^{\text{ACC}}_{\text{Browser}}\) (%) | \(^2^{\text{CCS}}_{\text{PNNL}}\) (Å²) | \(^2^{\text{ACC}}_{\text{PNNL}}\) (%) | \(^3^{\text{CCS}}_{\text{McLean}}\) (Å²) | \(^3^{\text{ACC}}_{\text{McLean}}\) (%) |
|--------------------|---------------------|-----------------|------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Stepped-field      | TuneMix321          | 322.0481        | 153.6123         | 153.9                           | 0.19                            | 153.7                           | 0.06                            | 153.4(1)                        | 0.14(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix621          | 622.0290        | 202.9291         | 203.5                           | 0.28                            | 203.0                           | 0.03                            | 202.8(6)                        | 0.06(6)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix921          | 922.0098        | 244.9022         | 244.4                           | 0.21                            | 243.6                           | 0.53                            | 243.9(1)                        | 0.41(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1220         | 1221.9906       | 283.1977         | 282.9                           | 0.11                            | 282.2                           | 0.35                            | 283.1(1)                        | 0.03(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1520         | 1521.9715       | 317.0891         | 317.8                           | 0.22                            | 317.0                           | 0.03                            | 318.9(1)                        | 0.57(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
| Single-field       | TuneMix321          | 322.0481        | 153.9290         | 153.8                           | 0.08                            | 153.7                           | 0.15                            | 153.4(1)                        | 0.34(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix621          | 622.0290        | 203.0138         | 203.0                           | 0.01                            | 203.0                           | 0.01                            | 203.0                           | 0.01(8)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix921          | 922.0098        | 243.4948         | 243.6                           | 0.04                            | 243.6                           | 0.04                            | 243.9(1)                        | 0.17(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1220         | 1221.9906       | 281.7040         | 282.1                           | 0.14                            | 282.2                           | 0.18                            | 283.1(1)                        | 0.49(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1520         | 1521.9715       | 317.4272         | 317.0                           | 0.13                            | 317.0                           | 0.13                            | 318.9(1)                        | 0.46(1)                         |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
| TWIMS-MS (SLIM)    | TuneMix321          | 322.0481        | 154.1847         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix621          | 622.0290        | 203.1121         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix921          | 922.0098        | 242.8969         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1220         | 1221.9906       | 281.7227         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1520         | 1521.9715       | 316.9095         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
| TWIMS-MS (SYNAPPT) | TuneMix321          | 322.0481        | 154.1847         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix621          | 622.0290        | 203.0138         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix921          | 922.0098        | 242.8969         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1220         | 1221.9906       | 281.7227         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1520         | 1521.9715       | 316.9095         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
| TIMS-MS (TIMS-TOF) | TuneMix321          | 322.0481        | 153.8775         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix621          | 622.0290        | 202.7221         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix921          | 922.0098        | 243.5977         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1220         | 1221.9906       | 281.7227         | —                               | —                               | —                               | —                               | —                               | —                               |
|                    |                     |                 |                  |                                 |                                 |                                 |                                 |                                 |                                 |
|                    | TuneMix1520         | 1521.9715       | 316.6544         | —                               | —                               | —                               | —                               | —                               | —                               |

**Note:** All adduct ions are \([\text{M}+\text{H}]^+\). In \(^{\Delta}\text{CCS}_{\text{ref}}\) (%) columns: 100 x |AutoCCS CCS - CCS\text{ref}/CCS\text{ref}. \(^1^{\text{CCS}}_{\text{Browser}}\) is obtained from the Agilent MassHunter IMS Browser 10.0 with raw .d files. \(^2^{\text{CCS}}_{\text{PNNL}}\) is obtained from the PNNL CCS library (http://panomics.pnl.gov/metabolites/) (Stow et al., 2017; Zheng et al., 2017). \(^3^{\text{CCS}}_{\text{McLean}}\) is from the Unified CCS Compendium (https://mcleanresearchgroup.shinyapps.io/CCS-Compendium/, 2020/08/26 release) (Picache et al., 2019) (#) in \(^3^{\text{CCS}}_{\text{McLean}}\) and \(^{3}\text{ACC}_{\text{McLean}}\) (%) columns represents the different data sources used in the CCS Compendium repository. For example, CCS values of (6) in \(^3^{\text{CCS}}_{\text{McLean}}\) were sourced from the interlaboratory experimental study (Stow et al., 2017). For SLIM data, the quadratic function with polynomial calibration method was employed (Tune-Mix ion 321 was excluded due to low signal-to-noise). For Waters Synapt data, the linearized power function was used. For Bruker timsTOF data, the linear function was employed with IMS scan numbers used as substitute for drift time.
## Table 2. Results of CCS determination from stepped-field measurements in nitrogen drift gas using the demo dataset of the target compounds described in Supplementary Table S3

| Name                              | Type               | Adduct                  | Adduct m/z | CCS$_{avg}$ (Å$^2$) | %RSD  | $1^{\text{CCS}}_{\text{Browser}}$ (Å$^2$) | $1^{\Delta \text{CCS}}_{\text{Browser}}$ (%) | $2^{\text{CCS}}_{\text{PNNL}}$ (Å$^2$) | $2^{\Delta \text{CCS}}_{\text{PNNL}}$ (%) |
|-----------------------------------|--------------------|-------------------------|------------|----------------------|-------|------------------------------------------|--------------------------------------------|------------------------------------------|------------------------------------------|
| D-Biotin Metabolite [M+H]$^+$     | [M+H]$^+$         | 245.10                  | 154.0411   | 0.40                 | 154.0 | 0.03                                     | 154.0                                     | 154.16                                  | 0.08                                     |
|                                   | [M+Na]$^+$        | 267.08                  | 163.9419   | 0.63                 | 164.3 | 0.24                                     | 164.5                                     | 165.45                                  | 0.31                                     |
| Sucrose Metabolite [M+H]$^+$      | [M+H]$^+$         | 365.11                  | 174.7238   | 0.34                 | 174.7 | 0.01                                     | 173.9                                     | 173.93                                  | 0.46                                     |
| β-Nicotinamide adenine dimucleotide | [M-H]            | 666.12                  | 229.6815   | 0.41                 | 229.3 | 0.18                                     | 229.4                                     | 229.46                                  | 0.10                                     |
| Angiotensin I Peptide [M+H]$^+$  | [M+2H]$^+$        | 648.85                  | 386.06     | 0.42                 | 386.2 | 0.04                                     | 386.2                                     | 386.13                                  | 0.02                                     |
|                                   | [M+3H]$^+$        | 432.90                  | 474.32     | 0.12                 | 475.5 | 0.26                                     | 476.0                                     | 476.05                                  | 0.36                                     |
|                                   | [M+4H]$^+$        | 324.93                  | 548.9      | 0.32                 | 548.9 | 0.62                                     | 548.6                                     | 548.67                                  | 0.58                                     |
| Angiotensin II Peptide [M+H]$^+$ | [M+2H]$^+$        | 1046.54                 | 312.73     | 0.89                 | 313.4 | 0.22                                     | 314.2                                     | 314.43                                  | 0.41                                     |
|                                   | [M+3H]$^+$        | 523.78                  | 355.88     | 0.31                 | 354.1 | 0.49                                     | 354.4                                     | 354.43                                  | 0.41                                     |
| Bradykinin Peptide [M+H]$^+$     | [M+H]$^+$         | 349.52                  | 434.70     | 0.04                 | 435.3 | 0.14                                     | 435.3                                     | 435.63                                  | 0.21                                     |
|                                   | [M+2H]$^+$        | 1060.57                 | 315.35     | 1.10                 | 312.9 | 0.79                                     | 312.9                                     | 312.97                                  | 0.76                                     |
|                                   | [M+3H]$^+$        | 530.79                  | 343.14     | 0.53                 | 343.1 | 0.02                                     | 343.4                                     | 343.47                                  | 0.10                                     |
|                                   | [M+4H]$^+$        | 354.20                  | 447.70     | 0.12                 | 447.7 | 0.44                                     | 448.0                                     | 448.16                                  | 0.34                                     |
| Melittin Peptide [M+3H]$^+$      | [M+3H]$^+$        | 949.26                  | 718.65     | 0.28                 | 719.5 | 0.12                                     | 720.9                                     | 720.95                                  | 0.32                                     |
|                                   | [M+4H]$^+$        | 712.20                  | 756.02     | 0.23                 | 756.4 | 0.05                                     | 757.0                                     | 757.08                                  | 0.14                                     |
| Neurotensin Peptide [M+2H]$^+$   | [M+2H]$^+$        | 836.96                  | 433.72     | 0.39                 | 434.5 | 0.18                                     | 434.8                                     | 434.89                                  | 0.27                                     |
|                                   | [M+3H]$^+$        | 558.31                  | 524.65     | 0.26                 | 525.1 | 0.09                                     | 525.5                                     | 525.54                                  | 0.17                                     |
| Renin Peptide [M+3H]$^+$         | [M+3H]$^+$        | 586.98                  | 519.66     | 0.12                 | 519.0 | 0.12                                     | 519.5                                     | 519.58                                  | 0.02                                     |
|                                   | [M+4H]$^+$        | 440.49                  | 634.50     | 0.70                 | 635.9 | 0.21                                     | 636.3                                     | 636.34                                  | 0.29                                     |
| Substance P Peptide [M+H]$^+$    | [M+H]$^+$         | 1347.74                 | 357.83     | 1.11                 | 359.3 | 0.40                                     | 359.6                                     | 359.66                                  | 0.51                                     |
|                                   | [M+2H]$^+$        | 674.37                  | 399.02     | 0.15                 | 398.4 | 0.16                                     | 398.8                                     | 398.87                                  | 0.04                                     |
|                                   | [M+3H]$^+$        | 449.92                  | 497.19     | 0.20                 | 496.9 | 0.05                                     | 497.3                                     | 497.36                                  | 0.03                                     |

*Note:* %RSD (Percent relative standard deviation) = 100 × standard deviation/CCS$_{avg}$. In ΔCCS$_{avg}$ (%) columns: 100 × (AutoCCS CCS - CCS$_{avg}$/CCS$_{avg}$. $1^{\text{CCS}}_{\text{Browser}}$ is obtained from the Agilent MassHunter IM-MS Browser 10.0 with raw .d files. $2^{\text{CCS}}_{\text{PNNL}}$ for metabolites is obtained from the PNNL CCS library (http://panomics.pnnl.gov/metabolites/) (Stow et al., 2017; Zheng et al., 2017) and $2^{\text{CCS}}_{\text{PNNL}}$ for peptides is obtained from Supplementary Table S11 in Supplementary Material of the interlaboratory evaluation study paper (Stow et al., 2017).
aliquoted into conical bottom 96 well plates, analyzed via solid-phase extraction with IMS-MS (SPE-IMS-MS) (Zhang et al., 2016).

The IROA standards (Adenine, L-(-)-Trehalose and Pterin) were purchased from IROA Technologies (Sea Girt, NJ, USA), and all reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Standards were supplied as 5 μg dried weight and reconstituted using a combination of 0.1% acetic acid, 79.9% methanol and 19.9% water. Stock solutions of the standards were prepared at either 100 or 500 μM concentration and then further diluted to a concentration of 10 μM prior to analysis.

2.7 Data acquisition

2.7.1 Stepped-field DTIMS-MS measurements

For the experiments with nitrogen, the Agilent tune-mix solution was analyzed by direct infusion using an Agilent jet stream orthogonal electrospray ionization source maintained at the following parameters: nitrogen sheath gas, sheath gas temperature, drying gas, drying gas temperature, nozzle voltage and inlet capillary voltage of at 81/min, 275°C, 31/min, 325°C, 2 kV and 4 kV respectively. Data were acquired on an Agilent 6560 Ion Mobility QTOF MS system (Agilent Technologies, Santa Clara, CA) using a stepped-field method and the drift potential was varied between 850 and 1450 V by 100 V increments, and each drift potential was acquired for 30 s.

For the IROA standards and TAA mixture experiments with helium, a customized flow injection system (Orton et al., 2018) was coupled to an in-house built IMS-MS instrument that combines a 1 m drift tube with an Agilent 6538 QTOF MS (Ibrahim et al., 2015) and is comparable to the commercial Agilent 6560 system but providing increased IMS resolution. For these experiments, the drift potential was varied between 677 and 1198 V by 87 V increments, and each drift potential was acquired for 44 s. The IMS was pressurized with ultrahigh purity helium, and the trapping funnel pressure and drift tube pressure were maintained at 3.8 and 4.0 Torr, respectively.

2.7.2 SLIM IMS measurements

The SLIM TWIMS-MS platform used for these experiments has been described in detail elsewhere (Wojcik et al., 2019) and is comparable to an instrument that will soon be commercially available (https://mobionsystems.com). The Agilent tune-mix, TAA mixture and IROA standards were infused by a customized flow injection system (Orton et al., 2018) at 300 nL/min for nano-electrospray ionization at 3 kV into an inlet capillary heated to 130°C. Ions pass through an ion funnel (3.60 Torr, helium buffer gas) before entering the SLIM module (3.80 Torr, He buffer gas). As described previously, ions were accumulated in the SLIM module at an interface 9 m into the 13.5 m ion path (Deng et al., 2017). For these experiments, an accumulation period of 1000 ms was performed prior to separation in the remaining 4.5 m at a TW amplitude of 12 Vpp and TW speed of 180 m/s. An Agilent 6224 TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) was used for all experiments. All SLIM TWIMS-MS data was acquired using home-built software.

2.7.3 Synapt IMS measurements

For the IROA standards and TAA mixture experiments with helium, a customized flow injection system (Orton et al., 2018) at 300 nL/min for nano-electrospray ionization at 3 kV into an inlet capillary heated to 130°C. Ions pass through an ion funnel (3.60 Torr, helium buffer gas) before entering the SLIM module (3.80 Torr, He buffer gas). As described previously, ions were accumulated in the SLIM module at an interface 9 m into the 13.5 m ion path (Deng et al., 2017). For these experiments, an accumulation period of 1000 ms was performed prior to separation in the remaining 4.5 m at a TW amplitude of 12 Vpp and TW speed of 180 m/s. An Agilent 6224 TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) was used for all experiments. All SLIM TWIMS-MS data was acquired using home-built software.

2.7.4 Bruker timsTOF data

Agilent tune-mix data were kindly provided by Bruker Corporation. Data were acquired on a Bruker timsTOF Pro™ instrument equipped with trapped ion mobility coupled to a QTOF MS. Ions were generated in positive electrospray ionization mode using a scan range of 100–1600 m/z. Tune mix features were extracted using MetaboScape and IMS scan numbers were used as a substitute for drift times for CCS calibration.

2.7.5 Single-field DTIMS-MS measurements with RapidFire

Plant extracts were analyzed by SPE-IMS-MS using a RapidFire 365 (Agilent Technologies, Santa Clara, CA) coupled with an Agilent 6560 Ion Mobility QTOF MS system (Agilent Technologies, Santa Clara, CA). Samples were loaded onto Graphitic Carbon and C18 SPE cartridges. The IMS was pressurized with ultrahigh purity nitrogen. All data were acquired in positive electrospray ionization mode with a mass range of m/z 50–1700. A total of 7106 metabolite IMS runs were acquired. Agilent tune-mix was analyzed daily for single-field CCS calibration (28 calibrant runs).

2.8 IMS data pre-processing and feature finding

The Unified Ion Mobility Frame (UIMF) file format (https://github.com/PNNL-Comp-Mass-Spec/UIMF-Library) was used for in-house IMS/SLIM platforms to store both the raw data and the metadata associated with an IMS-MS experiment in a single cross platform file. Raw MS files (MassHunter ‘.d’ or UIMF format) were pre-processed in batch mode using the PNNL-PreProcessor v20200724 (https://omics.pnl.gov/software/pnnl-preprocessor) to apply smoothing and generate new raw files with all frames (ion mobility separations)
Table 3. Results of CCS determination using AutoCCS for 3 IROA standards measured in helium gas via the DTIMS-MS and SLIM IMS-MS platforms

| Compound name | Target adduct | Adduct $m/z$ | $^{DT}$CCS ($\AA^2$) | $^{SLIM}$CCS$_{bin}$ ($\AA^2$) | $^{SLIM}$CCS$_{power}$ ($\AA^2$) | $\Delta$CCS$_{bin}$ (%) | $\Delta$CCS$_{power}$ (%) |
|---------------|---------------|--------------|----------------------|-----------------------------|-----------------------------|-----------------------|-----------------------|
| Adenine       | [M+H]$^+$    | 136.0624     | 58.44                | 63.53                       | 58.51                       | 8.71                  | 0.12                  |
| D(+)-Trehalose| [M+Na]$^+$   | 365.1060     | 104.90               | 102.50                      | 104.50                      | 2.29                  | 0.38                  |
| Perin         | [M+H]$^+$    | 164.0574     | 63.85                | 65.90                       | 62.41                       | 3.21                  | 2.26                  |

Note: $^{DT}$CCS and $^{SLIM}$CCS represent the CCS values calculated by AutoCCS from DTIMS and SLIM IMS measurements, respectively. $^{SLIM}$CCS$_{bin}$ and $^{SLIM}$CCS$_{power}$ indicate the use of the two different regression methods in Supplementary Figure S8, a binomial regression and a linearized power regression, respectively. $\Delta$CCS$_{bin}$ (%) and $\Delta$CCS$_{power}$ (%) columns represent the CCS differences between DTIMS-MS and SLIM IMS-MS: $100 \times \frac{^{SLIM}$CCS$_{bin} - ^{DT}$CCS}{^{DT}$CCS}$ ($^{SLIM}$CCS$_{power} - ^{DT}$CCS ($reg$) / (bin, power)).
AutoCCS automatically generated all adduct ions for each molecule, performed all calculations, generated various visualization plots and generated a single output table with the CCS and all the information of all molecules, including statistics such as mean and RSD per technical replicates. Using AutoCCS, all processing for metabolites took 17 min in an untended fashion; in contrast, about 2 h were required to manually process the 15 raw .d files, including numerous mouse clicks as well as additional calculations in MS Excel to compare replicates.

3.3 Single-field DTIMS-MS with RapidFire sample injection

We then demonstrated CCS calibration for data acquired from real-world metabolomic analyses of plant samples using SPE-IMS-MS with the single-field method and external calibration; stepped-field measurements are not feasible in many cases where analysis time is constrained due to either a front-end separation stage, limited sample amount or acquisition throughput needed.

All 7106 SPE-IMS-MS runs were processed in two weeks, which includes the computational time for preprocessing, feature finding and AutoCCS calculations, the latter of which required just 15 min on a desktop computer (3.6 GHz Intel Core i7, 16 GB 2400 MHz DDR4, Windows 10). Importantly, AutoCCS automatically assigned the corresponding tune-mix calibrant run based on the ionization mode (positive or negative) and the acquisition time stamp extracted from the raw file metadata information. On the other hand, manual data processing for CCS calibration and feature finding in this massive dataset would take more than a month due to many user interactions required.

When analyzing this data, we observed that unexpected fluctuations of pressure and temperature occurred during IMS data acquisition (Supplementary Fig. S7) and were sufficiently large to reduce CCS accuracy. Such large variations may happen due to instrumental issues (e.g. a malfunctioning flow controller or rough pump), an improper setting of the flow regime which are difficult to monitor in real-time and correct during automated and large-scale studies.

We found that in cases where these fluctuations occur, the CCS error can be significantly reduced by taking into account the specific temperature and pressure values from each run as shown in Equation (3). Our enhanced single-field method incorporates the pressure and temperature well-recorded readings from each IMS-MS analysis, into the single-field CCS calibration (Fig. 2). Using this method, hourly fluctuations of temperature and pressure can be mitigated, beyond the daily fluctuations that are taken into account with the conventional single-field calibration. 7106 RapidFire runs were calibrated using the linear regressor derived from the following and subsequent closest tune-mix run in the acquisition batch. As shown in Figure 3, for protonated ions of the internal standard rotenone, the proposed enhanced single-field method provided a small variance (194.7 with 0.4% RSD) and almost a single distribution in the calibrated CCS values for rotenone ions despite an irregular distribution of arrival times due to temperature and pressure changes, while the conventional method provided a large variance (195.6 with 1.4% RSD) with the same set of features. This case demonstrates that the enhanced single-field method for DTIMS can correct any fluctuations due to changes in realized pressure and temperature, which could be encountered in real-world applications, and would be a benefit to the research community.

3.4 Calibration for SLIM IMS-MS (TAA, He)

We also conducted experiments to demonstrate the calibration methods for SLIM IMS-MS with measurements of TAA salts using helium as a buffer gas. The reference CCS values of the TAA used as external calibrant ions were obtained by the DTIMS stepped-field method. To compute the SLIM IMS-MS calibration curves, AutoCCS used the reference CCS values and the arrival times of the corresponding features detected by SLIM IMS-MS. The results of the two non-linear regression methods supported are shown in Supplementary Figure S8. High $R^2$ values of each calibration curve for the reference ions were observed for both modes, indicating that the two regression methods worked well and were comparable. Since IM-MS browser does not support non-linear calibration, we could not directly compare the CCS determination between the two software.

We demonstrated CCS determination for representative metabolites using three IROA standards measured by both DTIMS-MS and SLIM IMS-MS using helium gas. Table 3 shows that AutoCCS successfully calculates CCS values from measurements from both platforms. The linearized power regression provided more similar CCS values to DTIMS than the polynomial regression. However, the error for Pterin when using linearized power regression was notably higher at 2.26%. A possible explanation for this larger error could be that the measured arrival time of Pterin was slightly outside of the range of the TAA calibrants, and therefore CCS values calibrated in the extrapolation range would have a larger uncertainty level (Fig. 4). To improve the calibration, extrapolation uncertainty can be eliminated by measuring other calibrant ions in this range. The evaluation and optimization of different calibrants and experimental conditions is an active area of research for small molecules measured in SLIM systems.

3.5 Limitations

As demonstrated in various case studies, the current version of AutoCCS can be directly used for CCS determination from experimental data measured in two instruments (Agilent DTIMS and SLIM) and substantially reduce time-consuming and labor-intensive tasks, especially for large-scale experiments. We also demonstrated that AutoCCS can be used with other commercial vendors, e.g. Waters Synapt and Bruker timsTOF. However, for Waters and Bruker data, conversions of the raw data into open format (e.g. mzML) and file formatting for IMS features are required since AutoCCS currently supports only Agilent MassProfiler cef and MZmine output csv format as input files.

Another limitation is that AutoCCS determines the CCS of an ion based on a peak apex or centroid rather than accounting for the width of the arrival time distribution; therefore, CCS distributions driven by ion structural ensembles cannot be characterized. For identifying CCS distributions of analyte ions in a stepped-field experiment, FWHMstep (Marchand et al., 2017) can be utilized, which allows to characterize the analyte’s conformational diversity.

4 Conclusions

We present an open-source software, AutoCCS, for calculating the CCS of ions detected from various IMS platforms and data acquisition modes such as drift tube (Agilent), traveling wave (Waters Synapt and SLIM) and trapped (Bruker timsTOF) IMS-MS technologies. AutoCCS shows its effectiveness and utility to determine CCS values from various platforms, supporting different instruments, IMS-field methods, buffer gas (e.g. He or N2) and most importantly, an automatic workflow that generates reproducible results and is applicable for determining CCS in any omics study.

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Conflict of Interest: John C. Fjeldsted is employee of Agilent Technologies. All the other authors declare no conflict of interest.

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