Incorporating CCS Values to Enable 4-Dimensional Annotation of Metabolic Features

Reliable annotation of metabolites for LC-MS/MS based data requires the adept combination of many parameters. To extend our parameter portfolio we analyzed trapped ion mobility spectrometry (TIMS) data.

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Introduction

We investigated the reproducibility of CCS values for intra- and inter-lab measurements. Furthermore, we compared the measured CCS values to those from literature, and we applied these to a sample comparison. From the present results, we conclude that CCS values serve as an excellent additional filter for metabolite annotation.

Experimental

Sample preparation

Urine (Bremen): A sample was collected from a volunteer. The sample was centrifugated (20817 g for 10 min at 4°C) and filtered (0.22 µm sterile syringe filters with MCE membrane SLGS033SS from EMD Millipore).

NovaMT sample: Hydrophilized urine (www.novaMT.com, TRX-3178-R) was dissolved in 200 µl LC-MS grade water, vortexed and centrifugated (20817 g for 15 min at 4°C). After adding 250 µl methanol the sample was vortexed and centrifugated (20817 g for 5 min at 4°C). 600 µl LC solvent A was added to the supernatant.

Retention time standard (TRX-2101, NovaMT) prepared according to protocol from NovaMT.

MS	timsTOF Pro
Source	Apollo II ESI source
Ionization	ESI(+), 4500 V Capillary Voltage ESI(-), 4200 V Capillary Voltage
Scan range	20–1000 m/z
Calibration	Internal mass calibration through automation, sodium formate, Mobility calibration before sequence using Agilent Tunemix
PASEF	Collison energy 20/50 eV
LC	**Elute UHPLC (in Bremen), Waters I-Class (in Perth/AU)**
Column	LC column kit "T-ReX Elute M-column kit: RP", containing Bruker Intensity Solo 2 C18, 2.1 x 100 mm and Waters Acquity UPLC BEH C18, 1.7 µl Van Guard Pre-column 2.1 x 5 mm
Column Oven Temp.	35°C
Mobile phases	A: water with 0.1 % formic acid B: acetonitrile with 0.1 % formic acid
Wash solvents	Wash 1: Solvent A Wash 2: acetonitrile / methanol/ water (1:1:1)
Gradient	0 min 1% B, 0.25 mL/min 2 min 1% B, 0.25 mL/min 17 min 99 % B, 0.25 mL/min 20 min 99 % B, 0.25 mL/min 20.1 min 1 % B, 0.35 mL/min 22 min 1 % B, 0.35 mL/min 28 min 1 % B, 0.35 mL/min 28.1 min 1 % B, 0.25 mL/min 30 min 1 % B, 0.25 mL/min
Injection volume	2 µl

Table 1. LC-TIMS-MS conditions.

Data acquisition

See Table 1.

Data processing

Data were processed in MetaboScape® 2021 (preliminary version), resulting in a table of features, so called buckets. An Analyte List was created combining the retention times from the T-ReX LC-QTOF solution (https://www. bruker.com/products/mass-spectrometry-and-separations/ms-software/metabolomics-solution.html), CCS values from the Unified CCS Compendium (https://mcleanresearchgroup.shinyapps.io/CCS-Compendium/) and fragment spectra from the Bruker HMDB Metabolite Library 2.0 and the Bruker MetaboBASE® Personal Library 3.0 (https://www.bruker.com/products/mass-spectrometry-and-separations/ms-software/metabolomics-spectral-libraries/overview.html). For each system the retention times were checked and corrected using a retention time standard. Using MetaboScape, features in the bucket table were putatively annotated based on an Analyte List of known compounds.

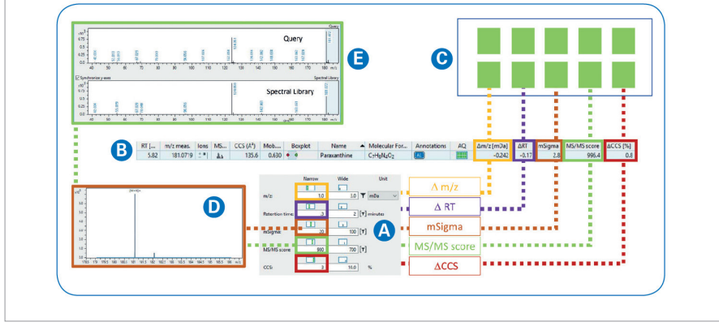

Figure 1. Annotation of paraxanthine. A: Narrow and wide filters set for annotation. B: Bucket table entry for paraxanthine. C: Visualisation of AQ scoring. D: TIMS cleaned MS spectra for precursor m/z 181.072. E: PASEF-MS/MS spectrum in comparison to Spectral Library spectrum.

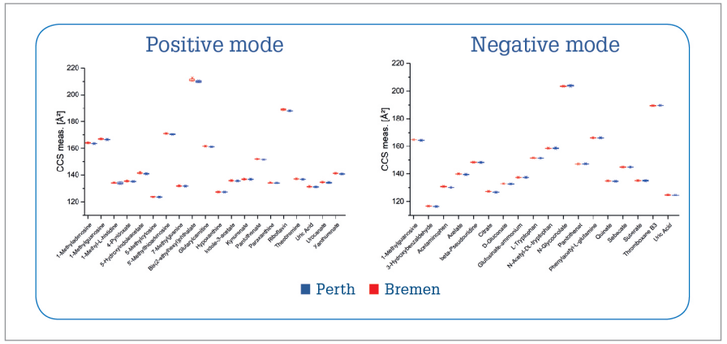

Figure 2. Reproducibility of measured CCS values. Box Plots CCS values obtained from data files.

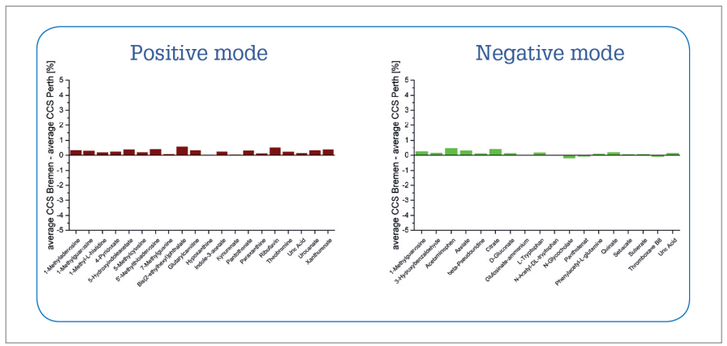

Figure 3. CSS variations across two laboratories in Bremen and Perth. The difference between the average CCS values for four replicate values is shown.

For this annotation five criteria were considered: precursor mass accuracy, retention time accuracy, isotopic pattern of the precursor (mSigma), MS/MS score and accuracy of the CCS value.

Statistical analysis (PCA and t-test) was performed in MetaboScape. For pathway mapping the caffeine and theobromine metabolism pathway (https://www.wikipathways.org/index.php/Pathway:WP3633) was imported in MetaboScape to illustrate differences in the urine samples.

Results

Investigation of CCS values

Data from one urine sample (NovaMT) was acquired on two timsTOF Pro instruments in Perth (ANPC, Australia) and Bremen (Bruker, Germany). These type of MS instruments combine advantages like high mass accuracy, sensitivity, dynamic range and mobility separation, and are thus highly suitable for this workflow. At both places, identical LC setup and methods for LC and data acquisition were used (Table 1). Data were processed using MetaboScape software. To ensure a high and reliable quality of annotation an approach combining filter criteria in five dimensions was selected. This was achieved by using the Unified CCS Compendium list, which has been extended by retention time from the Bruker HMDB Metabolite Library 2.0 and the corresponding MS/MS spectra. CCS values from the Compendium are now available for compounds searched for in PubMed and show good correlation with those observed using TIMS.

Annotation confidence is indicated in the Annotation Quality symbol (Figure 1). Each feature in the bucket table is categorized by applying criteria to measure the deviation in m/z, retention time, isotopic fit (calculated as mSigma), MS/MS score and CCS as compared with known values. Narrow filters indicate the highest possible fit, whilst wider filters can be applied to expand the number of possible annotations. The criteria can be refined by the user (Figure 1 A) and is visualized for each compound (Figure 1 C). MS and MS/MS data quality can be inspected within MetaboScape if required (Figures

1D and E). When TIMS or PASEF data are processed, the corresponding MS and MS/MS spectra shown are cleaned by mobility, allowing noise to be removed. For example as shown for paraxanthine, a bucket table results all information in detail (Figure 1 B), e.g. ΔCCS is 0.8 percent, and Δm/z is -0.242 mDa, for which both is excellent and is represented by two green bars in the AQ_ symbol. The quality of isotopic pattern of the precursor mass – compared to a theoretical pattern obtained for the resulting molecular formula and calculated as mSigma value – is also used. mSigma scores of <20 indicate high isotopic overlap. The MS/MS spectrum, which is clean due to mobility filtering applied during PASEF acquisition, overlays with a high score (max 1000) to the library spectra, which is also displayed.

For the investigation of CCS values described below, data from four technical replicates have been acquired. 20 buckets with high-quality annotation (positive and negative mode) from Perth and Bremen were selected.

CCS values in the range between 120 and 210 Å² have been determined. To confirm the reproducibility of CCS values, Box plots were created (Figure 2). For both instruments and polarities, the narrow boxes indicate the high stability of CCS values for each measurement. Small average standard deviations were determined:

Polarity/lab	*Perth*	*Bremen*
Positive	*0.27 Å²*	*0.37 Å²*
Negative	*0.21 Å²*	*0.18 Å²*

Also striking is the small absolute difference between the CCS values determined in Perth and in Bremen, for all compounds in positive and negative mode.

As shown before, the CCS values in Bremen differ only slightly from those obtained in Perth. The average |ΔCCS| for Bremen versus Perth is 0.25 percent for positive mode and 0.15 percent for negative mode. The absolute value for the difference in average CCS value is positive in most cases (Figure 3), which might arise from slightly different mobility calibration.

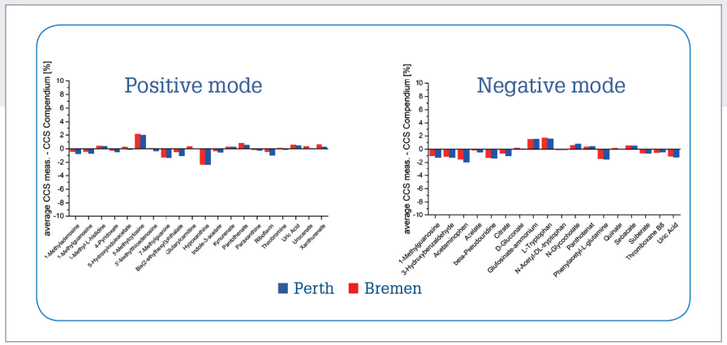

Figure 4. Differences between CCS values determined in Perth/Bremen and CCS Compendium.

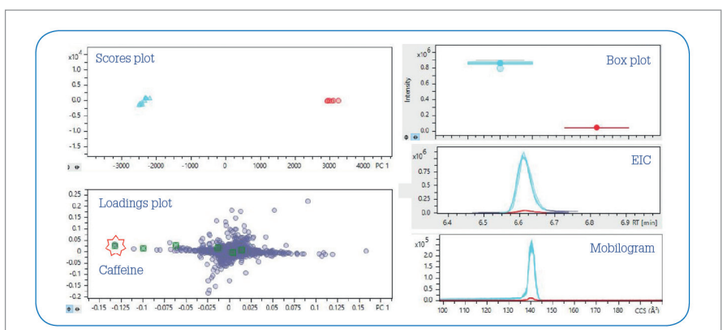

Figure 5. Comparison of two urine samples, eight technical replicates each. PCA results with Scores and Loadings plot (left). The metabolites investigated in Figure 6 are highlighted. Box Plot, Extracted Ion Chromatogram (EIC), and Mobilogram for caffeine (right). Red NovaMT, turquoise Bremen sample.

Comparison to reference CCS values

The measured CCS values were similar to the publicly available values from the Unified CCS Compendium as shown in Figure 4. Here, average differences between the measured and Compendium CCS value were plotted versus the metabolites for positive and negative mode data from Perth and Bremen. Small Average |ΔCCS| were determined:

Polarity/lab	*Perth*	*Bremen*
Positive	*0.94%*	*0.93%*
Negative	*0.88%*	*0.79%*

For all analyzed metabolites the deviations are <2 percent.

CCS values were compared to retention times in respect of usability for use as filter for metabolite annotation. One disadvantage of retention times is the low transferability from one system to the other. When acquiring data in Perth and in Bremen, no additional effort was required for the comparison CCS values. Calibration of CCS was applied automatically in MetaboScape, whereas retention times had to be adapted to accommodate different LC methods. We also observed that the average CV for CCS values (0.11 percent) was better than for retention times (0.25 percent), as determined for positive mode data.

The high stability of CCS values shows its advantage compared to retention time, demonstrating that CCS is a highly valuable characteristic for the annotation of the features. The availability of CCS values makes this a more desired attribute to characterize metabolites with more confidence.

Name	Position methyl group	p-value	Fold change NovaMT/Bremen	CV NovaMT [%]	CV Bremen [%]
Xanthine	none	9.05E-03	1.4	11.6	11.6
1-Methylxanthine	1	2.00E-06	-1.6	10	10.3
7-Methylxanthine	7	1.20E-04	1.5	9.1	14.8
Paraxanthine	1, 7	< 1E-08	-9.1	9.3	5.1
Theobromine	3, 7	< 1E-08	-4.6	5.5	10
Caffeine	1, 3, 7	< 1E-08	-19.6	5.9	3.3

Table 2. Fold changes and CV values for metabolites shown in Figure 6.

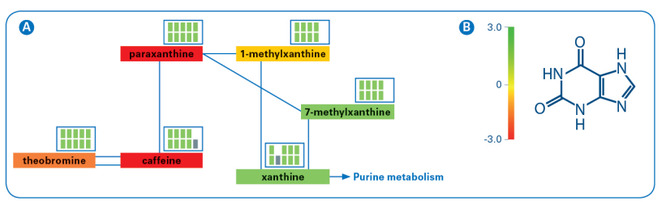

Figure 6. Caffeine and theobromine pathway, presented in a reduced form. Only xanthines are shown, which have been annotated with a high degree of confidence, represented by the Annotation Quality symbol. Two urine samples were compared "Bremen" and "NovaMT." The color coding of the metabolite name boxes indicates different intensities. Red color for higher intensity in the "Bremen" sample, green for the "NovaMT" sample A. B shows the structure of xanthine and the positions for potential methyl groups.

Comparing urine samples

The NovaMT and Bremen urine samples were investigated, and the Scores plot shows clear differences (Figure 5). The differences mainly arise from caffeine and other xanthines and are highlighted. For caffeine, the Box plot, the Extracted Ion chromatogram and the Mobilogram clearly indicate a big difference in intensity between the two urine samples.

Caffeine and theobromine pathway

For relative quantitative analysis, a t-test was performed to confirm suitable low CV values for all samples (Table 2). In Figure 6 we illustrate how two urine samples (NovaMT, Bremen) differ in respect of xanthines (with 0, 1, 2, and 3 methyl groups) in the Caffeine and Theobromine Pathway. To show the reliability of the annotation, the AQ_ symbols for the investigated metabolites are shown in the Figure. While the amount of xanthine, 1- and 7-methylxanthine are mostly comparable, the amounts for theobromine and caffeine and paraxanthine differ strongly by factors 5, 9 and 20 respectively (Table 1). This difference for theobromine, caffeine and paraxanthine in the Bremen sample clearly indicates consumption of coffee by the proband, which has not completely been degraded. In NovaMT urine, a smaller amount of caffeine was also detected (Figure 6 D and E). We further see that in NovaMT urine the amount of xanthine, a later degradation product of caffeine, is slightly higher than in the Bremen sample. This might result from sampling at a later stage of coffee consumption.

Conclusion

For metabolomics the reliable annotation of features is essential. In the current approach we demonstrated how ion mobility can support this:

- CCS values from the timsTOF Pro instruments show high intra-lab reproducibility for CCS values with a standard deviation <0.4 Å², low inter-lab differences in measured CCS values <0.3 percent, and high accordance of measured CCS values to those from Unified CCS Compendium with an average | ΔCCS | <1 percent. This allows to use CCS values as filter for 4D annotation – in addition to retention time, precursor mass, isotopic pattern and MS/MS spectrum.
- PASEF spectra benefit from ion mobility separation, since cleaner MS/MS spectra are obtained using an on-the-fly mobility filter. This improves ID in small molecule workflows.
- The application of using CCS values was shown for pathway mapping of metabolites of the Caffeine and Theobromine metabolism

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