COMPARISON AND EVALUATION OF CCS VALUES OBTAINED VIA DIRECT INFUSION IM-MS AND LC-IM-MS FOR THE CHARACTERIZATION OF RAT URINE METABOLITES

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INTRODUCTION

There is increasing interest in the application of IMS in metabolic phenotyping studies. The additional separation provided by IMS can enable the resolution of LC co-eluting species. This separation not only increases the number of detected features, but also provides better quality MS data by reducing spectral overlap. Moreover, since CCS is a ion-specific physicochemical measurement under a set of given experimental conditions, it can greatly aid identification, or confirmation of identity. Test metabolites were analyzed by DI-IM-MS on multiple instruments to determine CCS measurement precision and the results additionally validated using external resources. The same set of compounds was then analyzed by microcolumn LC-IM-MS to obtain retention time data to further aid metabolite identification in a test set of rat urine samples run under the same conditions.
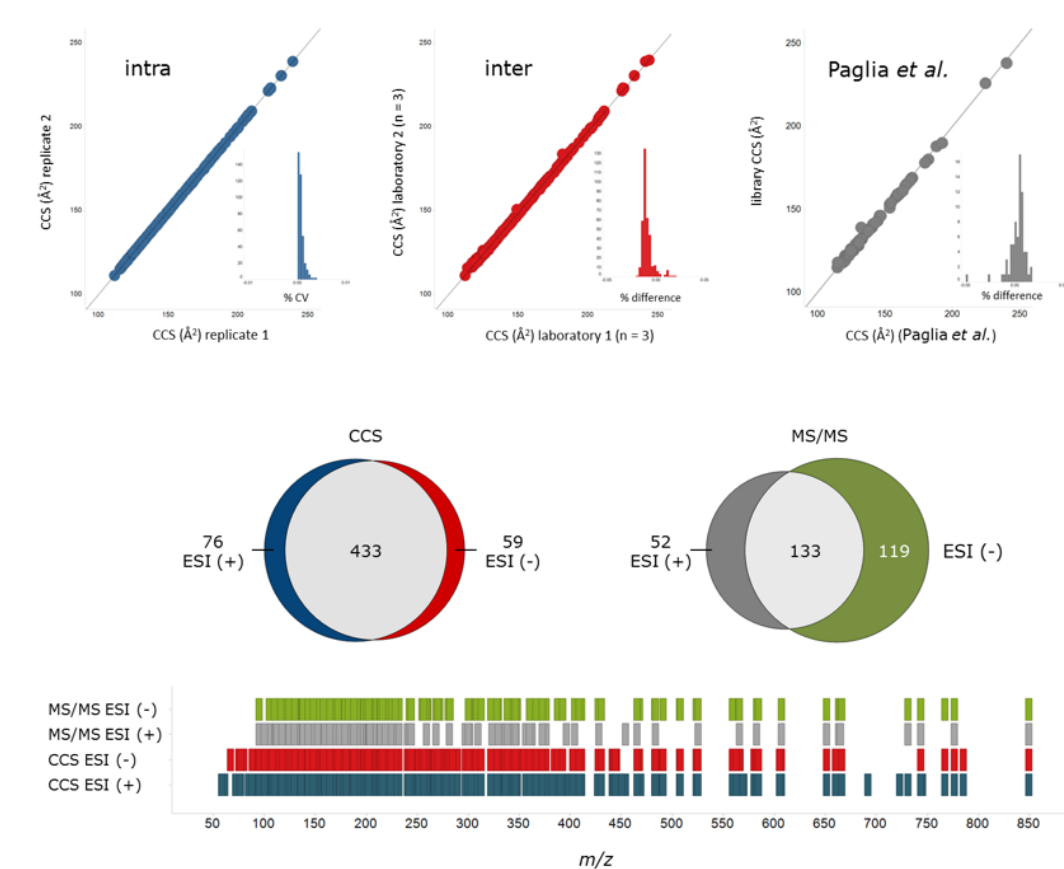

Figure 1. Intra and inter (internal and external Paglia et al. [1]) DI-IM-MS $^{TW}CCS_{N2}$ measurement precision (top) and $^{TW}CCS_{N2}$ and MS/MS library coverage (bottom). CID fragmentation data/results not shown (publically available at http://nonlinear.com/progenesis/qi/v2.2/download/ccs-libraries or https://marketplace.waters.com/apps/177290/metabolic-profiling-ccs-library#!/overview).

METHODS

Library standards
The library was created from compounds purchased from Sigma-Aldrich, IROA Technologies. The Mass Spectrometry Metabolite Library of Standards are a collection of small biochemical molecules that span a broad range of primary metabolism and compound classification.

IM-MS conditions
MS: Synapt G2-S*i*
Mode: ESI (+ve/-ve)
Calibrant: MajorMix (power fit)
+ve mode: *m/z* 152 - 1013 (130 - 306 Å^2)
-ve mode: *m/z* 150 - 1082 (131 - 322 Å^2)

Direct infusion conditions
For CCS and MS/MS library generation, each IROA sample was diluted to 10 ng/µL and loop injected into a solvent flow (50 µL/min) consisting of 50% aqueous ACN containing 0.1% FA (+ve) / 0.05% NH$_3$ (-ve) using an Acquity I-Class System.

LC-IM-DIA-MS conditions
Reversed-phase separations were conducted with an AQUITY I-Class system and a short 2.5 min gradient, including re-equilibration, using a 1 x 50 mm HSS T3 1.8 µm column .

Informatics workflows
IM-MS, MS/MS and LC-IM-DIA-MS were used to measure $^{TW}CCS_{N2}$ and fragmentation data of the standards and rat urine samples. The data were acquired using MassLynx, which was further processed using UNIFI and Skyline for the generation of libraries and data analysis. $^{TW}CCS_{N2}$ predictions were conducted using a machine-learning approach and internally acquired $^{TW}CCS_{N2}$ measurements to fit an appropriate model using the XGBoost algorithm [2]. Multivariate analysis was conducted with SIMCA-P+.

Table 1. Average inter and intra CV $^{TW}CCS_{N2}$ measurement values. * = intersection, ** = combined, † outliers not removed and multiple adducts allowed, Δ site 3 = Waters Corporation, Beverly, MA, ‡ reference [1]. Inter and intra statistical outliers exceeding a 95% confidence level (see text for details) were not included.

site/instrument	$^{TW}CCS_{N2}$ %CV (n) †	
	ESI (+ve)	ESI (-ve)
intra		
site/instrument 1	0.2 (349)	0.1 (433)
site/instrument 2	0.1 (419)	0.1 (428)
intra (m/z > 150)		
site/instrument 1	0.2 (260)	0.1 (303)
site/instrument 2	0.1 (309)	0.1 (290)
inter		
sites 1 and 2*	0.5 (280)	0.8 (378)
sites 1 and 2**	0.5 (493)	0.7 (484)
inter (m/z > 150)		
sites 1 and 2*	0.4 (214)	0.8 (262)
sites 1 and 2**	0.4 (359)	0.7 (331)
inter/external		
site/instrument 3Δ	0.5 (155)	--
Paglia et al.‡	1.4 (36)	0.7 (84)

RESULTS

IM-MS
The results of the direct infusion IM-MS experiments, following curation and statistical outlier removal, the inter or intra sample standard deviation, or both, exceeding a 95% confidence level, are graphically summarized in Figures 1 and Table 1. The CCS and MS/MS coverage and intersection for both ionization modes are shown in Figure 1 as well.

The detection frequency and chemical/MeSH classification are summarized in Figures 2 and 3. Three chemical classes were found to be underrepresented in negative electrospray mode and the observed $^{TW}CCS_{N2}$ data, in general, correlated very well with class-specific *m/z* values.
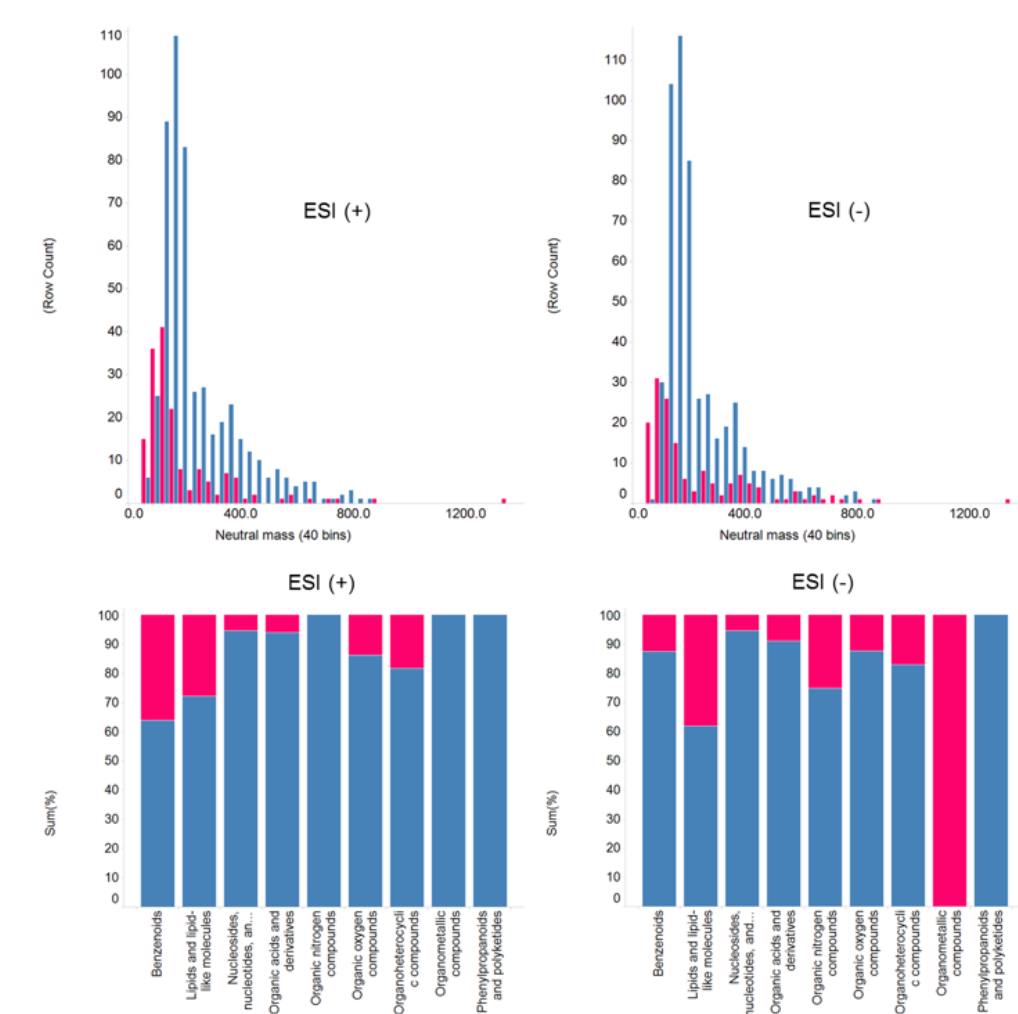

Figure 2. Detection frequency as a function of m/z (top) and relative (%) chemical class annotation of the library compounds (m/z 150 - 800) (bottom). Blue = detected; red = not detected.
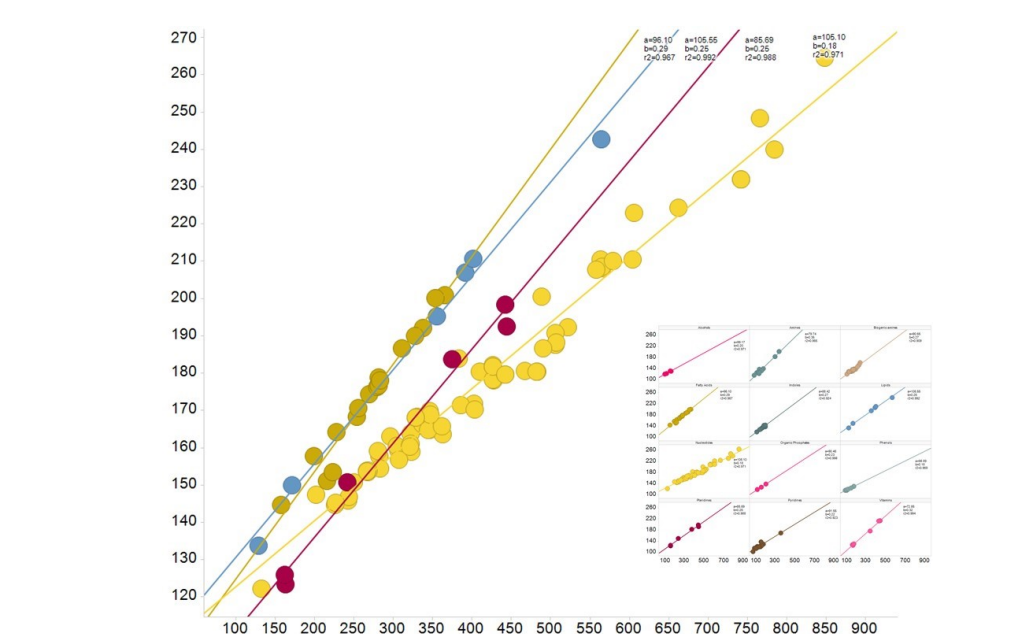

Figure 3. Class centric m/z vs. $^{TW}CCS_{N2}$ relationship based on MeSH (Medical Subject Headings) classification.

$^{TW}CCS_{N2}$ predictions
The observed $^{TW}CCS_{N2}$ results were found to be in good agreement with $^{DT}CCS_{N2}$ data as show in Figure 4 (results are a subset of the displayed data) for both modes of ionization. Moreover, the measured values also showed good correlation with machine-learning based CCS predictions.

However, as illustrated in Figure 5, a model based accuracy was noted, which most likely stems from the differences in training data sets, applied model, and/or instrument configuration used to generate the training data.
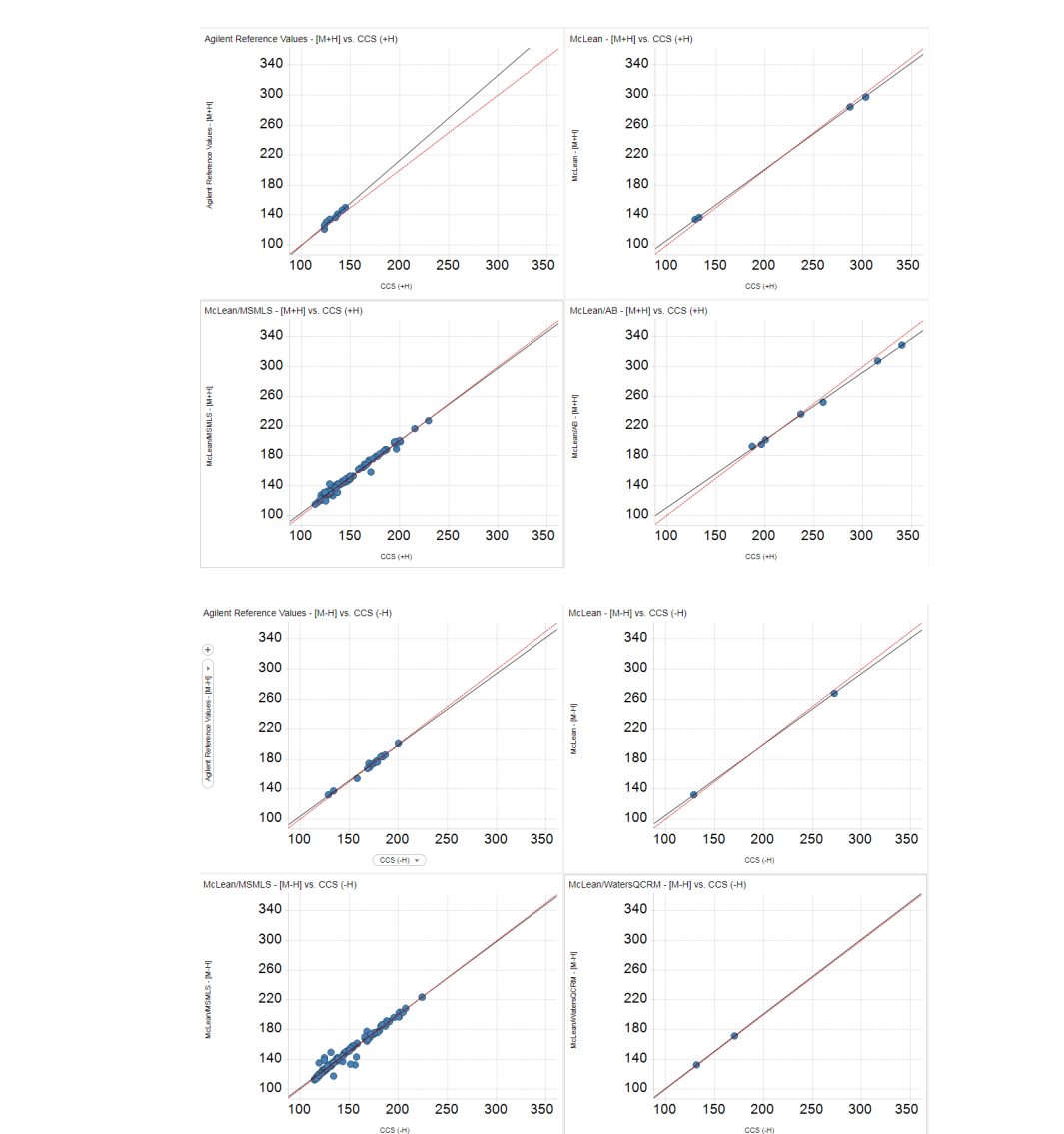

Figure 4. Experimental $^{TW}CCS_{N2}$ data /results vs. $^{DT}CCS_{N2}$ (https://mcleanresearchgroup.shinyapps.io/CCS-Compendium/) [3]. Top = +ve; bottom = -ve. Black = regression line; red = $^{TW}CCS_{N2}$ = $^{TW}CCS_{N2}$ curve.
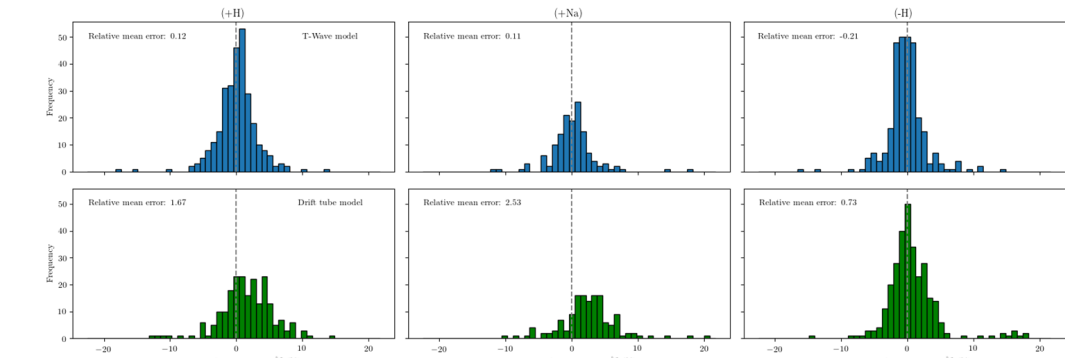

Figure 5. $^{TW}CCS_{N2}$ machine-learning prediction (blue) and MetCCS [4] $^{DT}CCS_{N2}$ based prediction (green) values vs. observed $^{TW}CCS_{N2}$ data.

LC-IM-DIA-MS
The application of the IM and MS/MS library is demonstrated in Figures 6 to 8. Shown are the profiling based on t$_r$, IM, and MS/MS and two tentative screening based identification examples, respectively. Classification of the samples was feasible based on a subset of the library compounds.

In addition, comparison of the LC-derived $^{TW}CCS_{N2}$ values against the averaged values found from direct infusion showed that 90.0% of the LC-IM-MS detected compounds were within ±1% and 97.0% were within ±2% of the values obtained from M-MS.
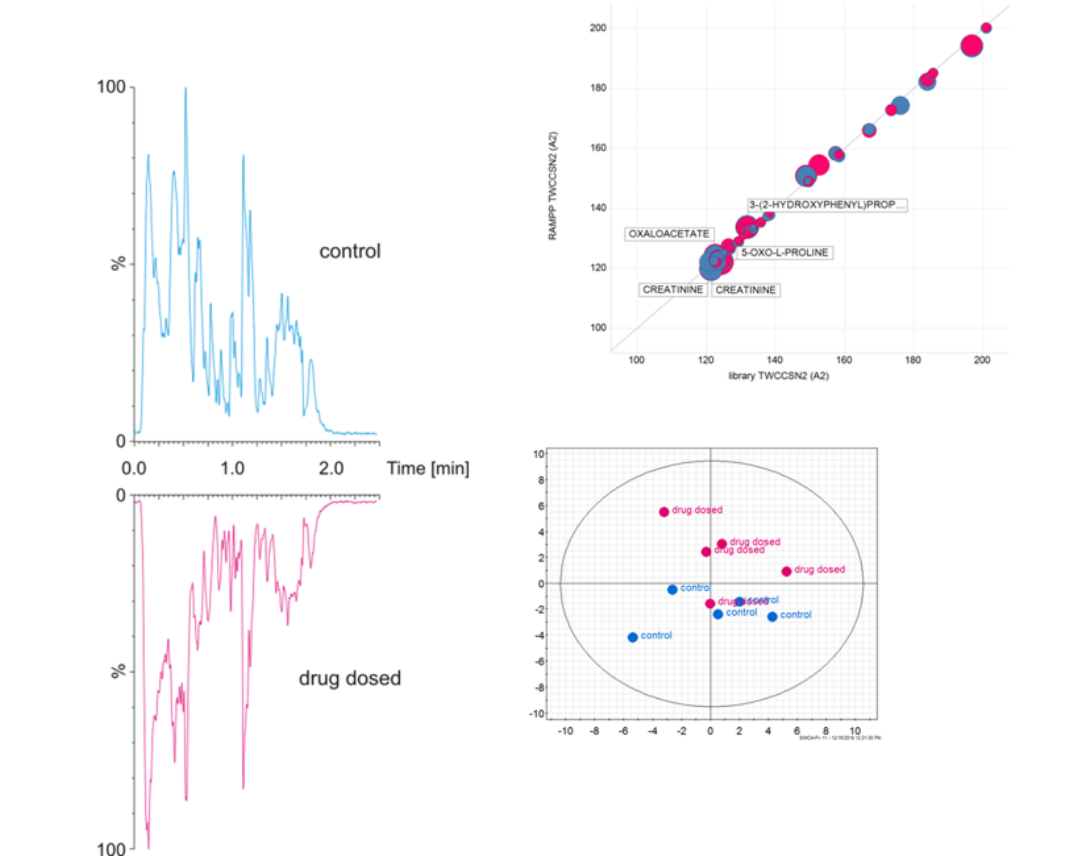

Figure 6. RAMMP LC-IM-MS BPI chromatograms (left), LC-IM-DIA-MS derived vs. IM-MS $^{TW}CCS_{N2}$ values (top right; the biological replication rate is represented by size and the sample by color, blue = control, red = drug dosed) and unsupervised PCA on the abundances of the detected metabolites in control and MTX dosed rat urine.
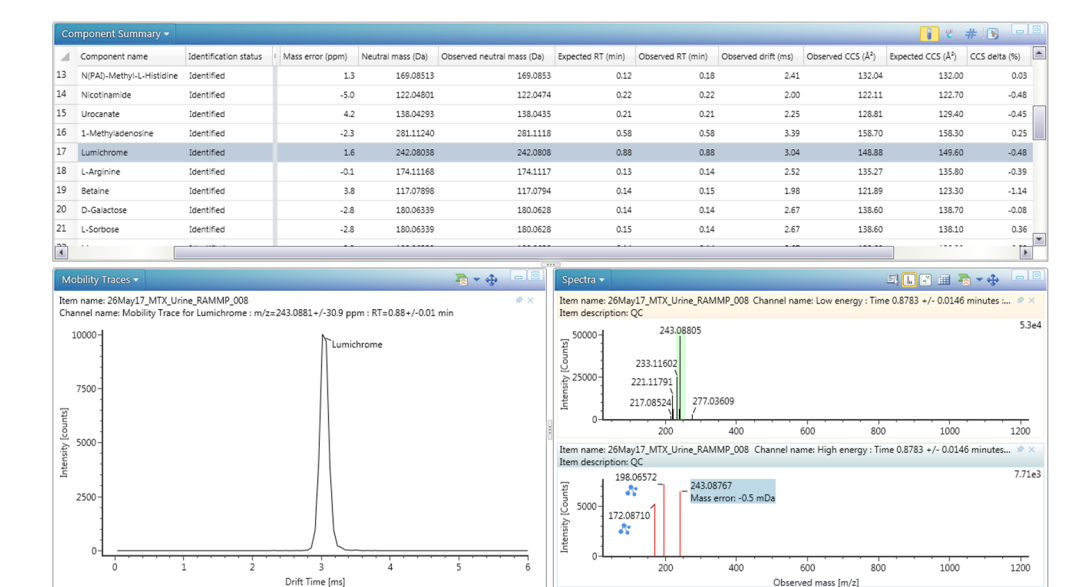

Figure 7. Tentative identification example of native lumichrome (6,7-dimethylalloxazine) in a study pool QC rat urine sample based on MS, t$_r$, CCS and IM resolved CID MSn using data independent analysis LC-IMS-DIA-MS. Shown clockwise are the tentatively identified compounds, an IM resolved MS1 spectrum, an IM resolved MSn spectrum, and an arrival time distribution.
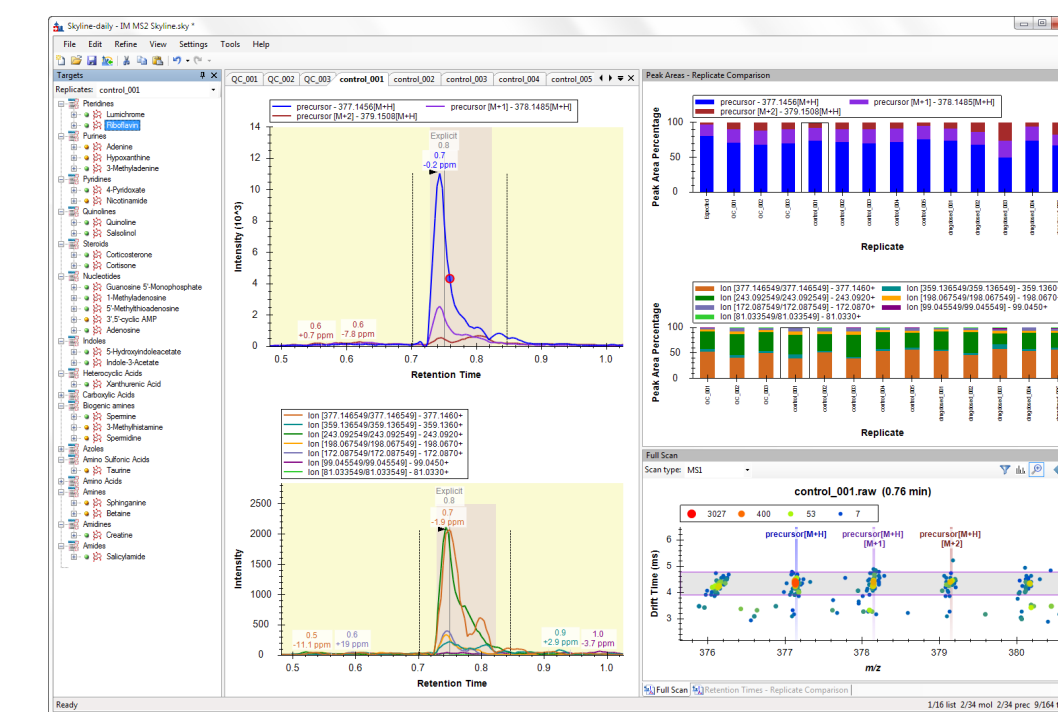

Figure 8. Tentative identification example of native riboflavin in a control rat urine sample based on MS, t$_r$, $^{TW}CCS_{N2}$ and IM resolved CID MS2 using data independent analysis LC-IM-DIA-MS. Shown from left to right are the tentatively identified compounds, MS1 and MSn XICs, normalized relative precursor isotope and product ion abundances (top) and a precursor drift vs. m/z distribution (bottom).

CONCLUSION

- The $^{TW}CCS_{N2}$ values for a range of metabolite standards, determined in triplicate using standardized settings, measured on two separate instruments (located on different sites) were found to be very similar
- For DI-IM-MS it was possible to obtain $^{TW}CCS_{N2}$ values that were well within 1% of each other on two different sites and within 2% of two external reference sources. Application of reversed-phase LC separation prior to IM-MS, gave results that were within 2% of those measured with IM-MS
- Analysis of rat urine samples by U(H)PLC-IM-MS enabled 65 compounds to be identified using the combination retention time, $^{TW}CCS_{N2}$ and MS data. This demonstrates the potential utility of adding IMS with CCS values to metabolite identification studies

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