A COMPARISON OF CONVENTIONAL AND RAPID GRADIENT MICROBORE LC CYCLIC™ ION MOBILITY MASS SPECTROMETRY FOR NON-TARGETED SCREENING OF BIOLOGICAL MATRICES

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OVERVIEW

- Ion mobility-enabled mass spectrometry libraries afford additional specificity compared to conventional mass spectrometry libraries.
- Collision cross section (CCS) values acquired using high resolution cyclic ion mobility (cIM) facilitate reduced false detection rates and increased confidence of identification in complex matrices.
- Increased analysis flexibility.
- CCS values and precursor/product ions generated simultaneously.

INTRODUCTION

High resolution mass spectrometers (HRMS) such as quadrupole time of flight mass analyzers (Q-TOF), have become more prevalent as screening tools in clinical, forensic toxicology and metabolite identification, where the constituents of interest are present in complex biological matrices such as urine and blood.[1,2] Using non-targeted "full scan" data acquisition thousands of detections can be made in a single analysis, and can be followed by retrospective targeted data analysis. The drive for higher sample throughput is global, requirement for improved time efficiency and cost reduction has resulted in movement towards multiclass compound analysis. This approach has been used to analyse pesticides, mycotoxins, natural plant toxins[3] and organic contaminants[4,5], which also reside within a variety of complex sample matrices, ranging from food[6] to environmental samples such as water effluent.[7,8] The purpose of a screening method is to rapidly detect and identify target compounds in the sample under investigation, with false detection rates being kept as low as possible. Using measured properties of a compound, such as the accurate mass, isotope pattern, and product ion spectrum, appropriate filters can be applied to determine the presence of a compound in a sample. However, for compounds of interest which are present only at low concentration, within complex biological matrices, using these properties alone to achieve matrix or analyte identification may prove to be more challenging and additional method development strategies need to be employed. For such complex analyses, the extra dimension of cIM separation can help to mitigate such analytical challenges, as well as generate additional identification specificity via the collision cross section.

Using a previously reported mass spectrometry library generation strategy[9], a set of commercially available FDA approved drugs was characterized using liquid chromatography ion mobility mass spectrometry (LC-cIM-MS) and rapid gradient microbore liquid chromatography ion mobility mass spectrometry (RGM-cLC-IM-MS). The strategy employed enables retention time (t_r), precursor ions, product ions and CCS to be determined. Analytical strategies utilising CCS as an additional endpoint to aid identification specificity have been developed, for example pesticide screening assays.[10,11] The routine use of CCS for small molecule analysis has increased across multiple areas of research including pharma (metabolism, metabolomics, lipids) and food safety (veterinary drugs, mycotoxins, steroids, natural product screening, natural toxins). CCS-searchable libraries have been generated whereby use of a CCS metric can be used to increase cumulative specificity of identification as well decrease false detections.

Ion mobility separation of compounds result from gas phase ions being separated within the gas filled Cyclic IM Separator, prior to the mass analyser (see Figure 1). Mobility separation is obtained by driving packets of ions through an inert buffer gas (nitrogen) using a relatively weak electric field and is a function of factors such as the ion mass, shape, charge differentiation as well as on its dipole moment in cases where polarizable buffer gas is used.[12,13]

IM-MS provides a third dimension of separation to that of LC (hydrophobicity) and MS (*m/z*) in addition to CCS values, a complimentary physiochemical descriptor. The combined peak capacity of LC-IM-MS and CCS can be used to produce unequivocal xenobiotic identification. A non-targeted urinary screen using conventional LC-cIM-MS (total cycle time of 12 min) and RGM-LC-cIM-MS (total cycle time of 2.5 min) have been compared as a strategy to identify administered pharmaceutical xenobiotics in the urine of a healthy volunteer patient.

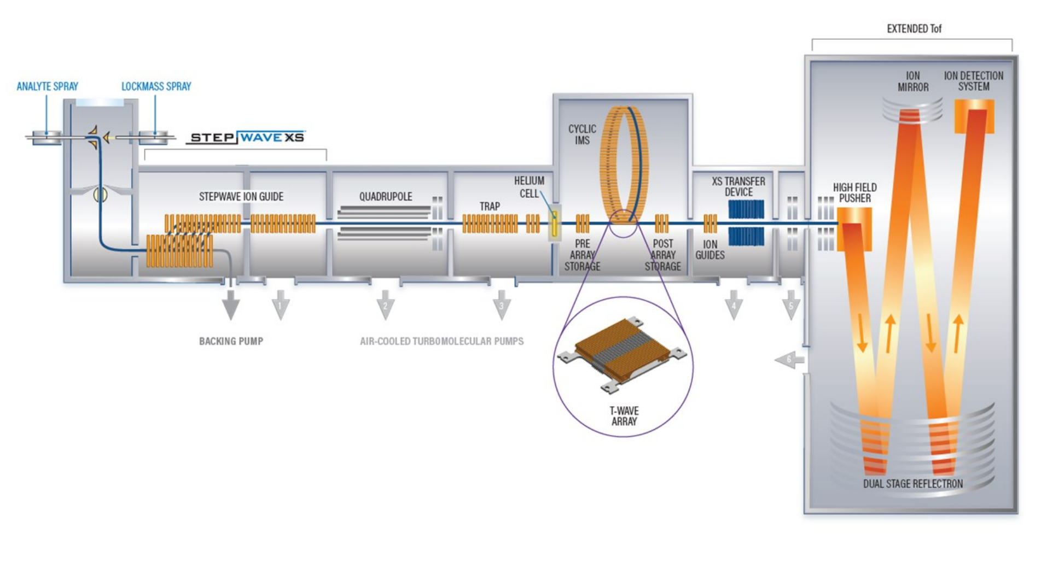

Figure 1. Schematic of Cyclic IMS, with the cIM device.

METHODS

SAMPLE DESCRIPTION
Human urine sample diluted 10:1 (H_2O).
Sample taken 5hrs after medication was administered.
Carbamazepine Dosage: 2 x 200mg tablets.
Acetaminophen Dosage: 2 x 500mg tablets.
Naproxen Dosage: 1 x 500mg tablets.

Positive ion electrospray with precursor/product ion data acquisition was performed using a quadrupole-cIM-time-of-flight (ToF) mass spectrometer (IMS Resolution (R)~65). Human urine samples were analysed, using conventional and RGM reverse phase separation liquid chromatography. Chromatographic Method 1 (LC): 12-minute (0.1% *v/v* formic acid in H_2O) and (0.1% *v/v* formic acid in acetonitrile) reverse phase gradient at 0.5 mL/min, using a C_{18} (100 mm x 2.1 mm, 1.8 µm) column. Chromatographic Method 2 (RPG LC): 2.5-minute (0.1% *v/v* formic acid in H_2O) and (0.1% *v/v* formic acid in acetonitrile) reverse phase gradient at 0.4 mL/min, using a C_{18} (50 mm x 1.0 mm, 1.8 µm) column. Column temperatures: 40ºC. Injection volumes 10 µL and 2 µL.

RESULTS AND DISCUSSION

A set of FDA-approved small molecule pharmaceuticals were used to produce conventional LC and RGM-LC-IM-MS libraries, comprising retention time t_r, precursor ion, product ions and CCS values (LC:1343 and RGM-LC: 1206 entries) in ES^+ mode. The rationale for the generation of a LC-IM-MS library is two-fold. Primarily the library facilitates a high degree of specificity to detect the presence or absence of therapeutic xenobiotics. The library specificity also provides a route to distinguishing components of interest from the exogenous/endogenous components of complex biological matrices such as urine. The complexity of human urine matrix is illustrated in Figure 2, where the extracted base peak ion chromatogram is comprised of 1000's of major and minor intensity components (20003 candidate masses detected (response> 1800 counts intensity)). The corresponding ion mobility separation, illustrating the combined peak capacity of LC-IM, is also shown where chromatographically coeluting components are separated in the IM dimension. This facilitates generation of non-targeted single component precursor ions with corresponding product ion spectra, from the drift time and retention time aligned species. The libraries were used to perform a human urine drug screen of a healthy volunteer to identify administered pharmaceutical compounds and distinguish them from the endogenous compounds of the complex biological matrix.

The volunteer patient urine sample was taken 5hrs after medication was administered and diluted 10:1 (H_2O). Using non-targeted screening LC-cIM-MS with typical post processing tolerances (t_r (0.1 min) and mass accuracy (+/-5ppm), 86 identifications were observed, inclusion of an additional identification point (product ion count ≥1) reduced the detection count to 13 (see Figure 3. Finally, a ΔCCS (<2%) tolerance was applied to reduce the detection count to 6 (see Figure 4). Alternatively using tolerances (t_r (0.1 min), mass accuracy (+/-5ppm) and ΔCCS (<2%) 43 false detections could be removed.

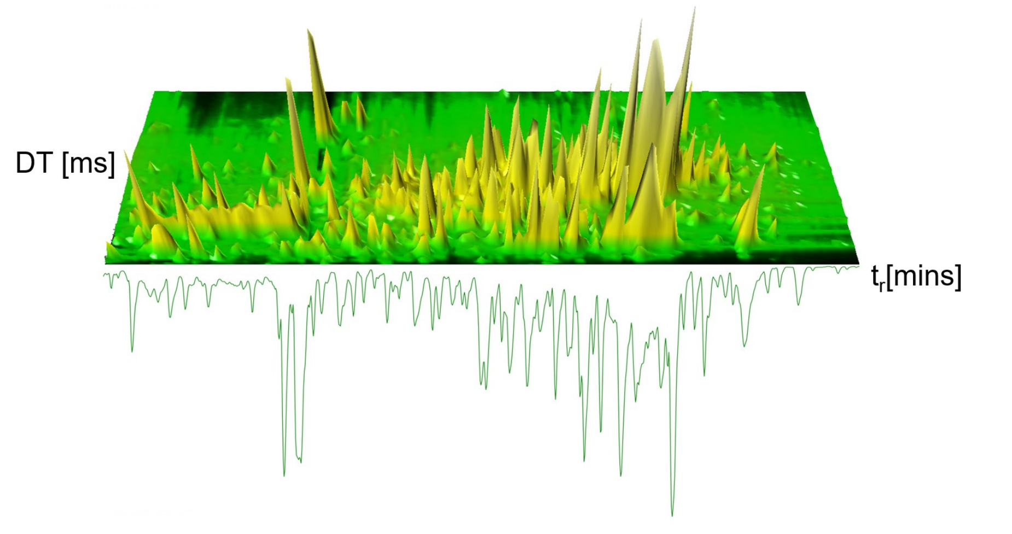

Figure 2. LC-cIM-MS separation obtained for non-targeted urinary screening.

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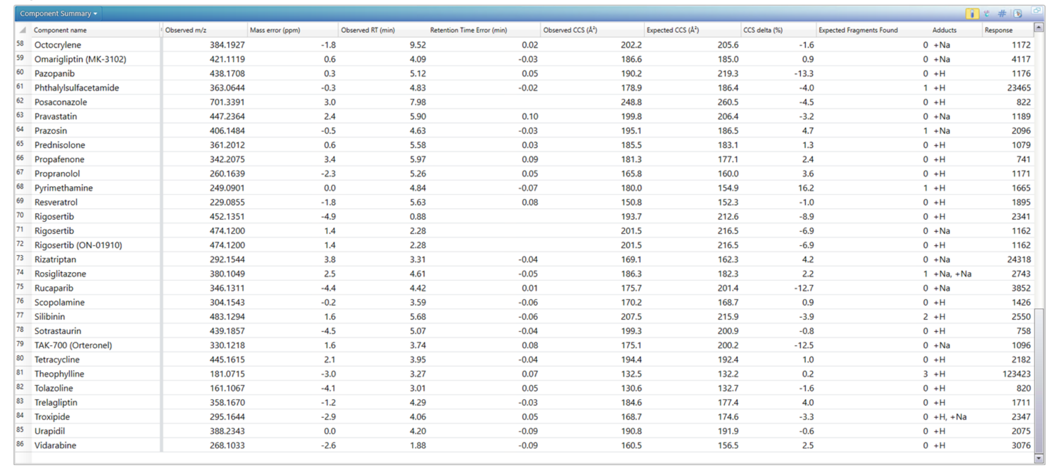

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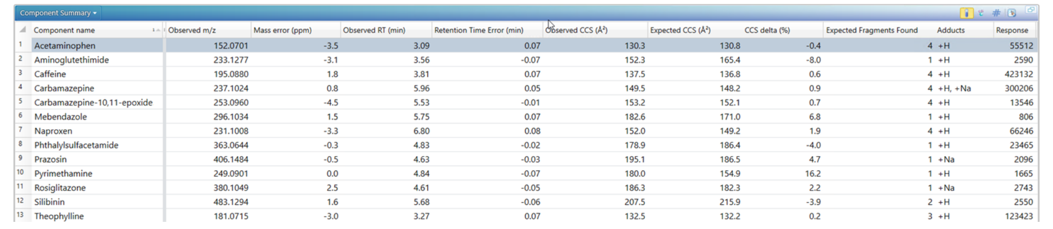

	Component name	Observed m/z	Mass error (ppm)	Observed RT (min)	Retention Time Error (min)	Observed CCS (Å²)	Expected CCS (Å²)	CCS delta (%)	Expected Fragments Found	Adducts	Response
1	Acetaminophen	152.0701	-3.5	3.09	0.07	130.3	130.8	-0.4	4	+H	55512
2	Aminoglutethimide	233.1277	-3.1	3.56	-0.07	152.3	165.4	-8.0	1	+H	2590
3	Caffeine	195.0880	1.8	3.81	0.07	137.5	136.8	0.6	4	+H	423132
4	Carbamazepine	237.1024	0.8	5.96	0.05	149.5	148.2	0.9	4	+H, +Na	300206
5	Carbamazepine-10,11-epoxide	253.0960	-4.5	5.53	-0.01	153.2	152.1	0.7	4	+H	13546
6	Mebendazole	296.1034	1.5	5.75	0.07	182.6	171.0	6.8	1	+H	806
7	Naproxen	231.1008	-3.3	6.80	0.08	152.0	149.2	1.9	4	+H	66246
8	Phthalylsulfacetamide	363.0644	-0.3	4.83	-0.02	178.9	186.4	-4.0	1	+H	23465
9	Prazosin	406.1484	-0.5	4.63	-0.03	195.1	186.5	4.7	1	+Na	2096
10	Pyrimethamine	249.0901	0.0	4.84	-0.07	180.0	154.9	16.2	1	+H	1665
11	Rosiglitazone	380.1049	2.5	4.61	-0.05	186.3	182.3	2.2	1	+Na	2743
12	Silibinin	483.1294	1.6	5.68	-0.06	207.5	215.9	-3.9	2	+H	2550
13	Theophylline	181.0715	-3.0	3.27	0.07	132.5	132.2	0.2	3	+H	123423

Figure 3. I) LC-cIM-MS component summary for post-acquisition processing workflow filtered detection results for screening using an FDA approved drug small molecule library, applied tolerance t_r 0.1 min and mass accuracy +/-5ppm (identified count 89). II) Applied tolerance t_r 0.1 min, mass accuracy +/-5ppm and ≥1 product ion (identified count 13).

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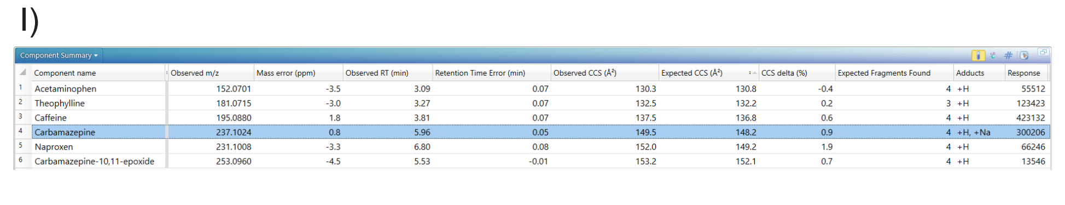

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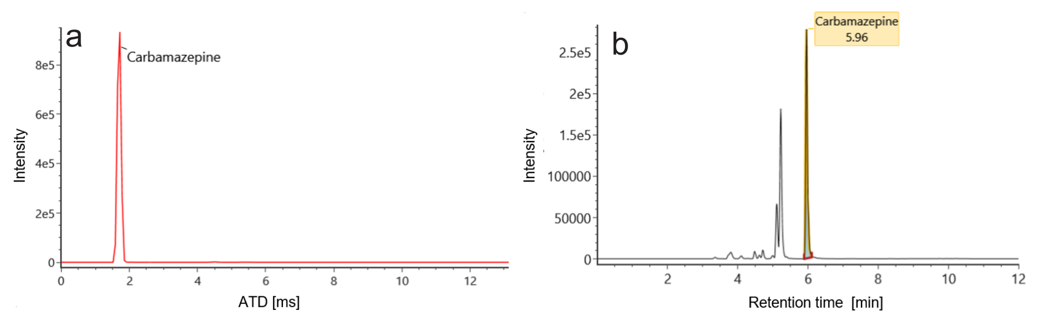

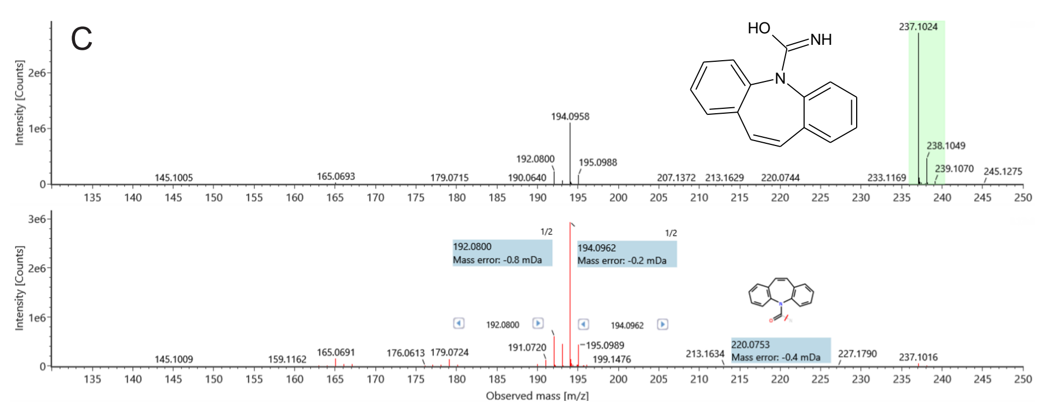

Figure 4. I) LC-cIM-MS component summary for post-acquisition processing workflow filtered detection results for screening using an FDA approved drug small molecule library, applied tolerance t_r 0.1 min, mass accuracy +/-5ppm, ≥1 product ion and ΔCCS <2% (identified count 6). II) (a) Carbamazepine ion arrival time distribution (ATD), (b), m/z 237 extracted mass chromatogram and (c) precursor/product ion spectra.

The MS libraries utilised comprise a diverse range of small molecule pharmaceuticals, as a result using conventional LC-cIM-MS, it was determined that the volunteer patient had been administered, carbamazepine, naproxen, and acetaminophen. For carbamazepine, accurate mass measurement (0.8 ppm), retention time error (0.05 min), product ions (4) and ΔCCS (0.92%) were obtained, , also shown are the arrival time distribution (ATD), extracted mass chromatogram, and the precursor/product ion spectra obtained for carbamazepine.

Using rapid gradient strategies have been shown to reduce peak capacity by ~66%.[14] However, utilising ion mobility, a high throughput strategy with a fivefold increase in sample throughput can be employed, but with increased peak capacity (compared to conventional MS). Combining the RGM-LC method with IMS affords the opportunity to enhance peak capacity, specificity and analysis flexibility, while retaining the time efficiency of a strategy that provides a 5-fold increase in sample throughput.[15] The feasibility of the approach has been assessed, via comparison of the application of conventional LC-IM-MS and RGM-LC-IM-MS libraries.

For RGM-LC-cIM-MS, using tolerances (t_r (0.1 min) and mass accuracy (+/-5ppm), 140 identifications were observed. Inclusion of a product ion count ≥1, reduced the detection count to 11 (See Figure 5). Applying a final ΔCCS (<2%) tolerance produced a detection count of 8 (see Figure 6). Alternatively using tolerances (t_r (0.1 min), mass accuracy (+/-5ppm) and ΔCCS (<2%) 79 false detections could be removed. The precursor/product ion spectra obtained for acetaminophen, caffeine and theophylline are shown in Figure 6, (ΔCCS<0.6% and mass error<3ppm). Endogenous melatonin has also been identified. Ultimately using a rapid screening approach, only one false detection was observed and identified as ketoprofen, however the observed product ion spectra are characteristic of naproxen and likely to be a naproxen biotransformation product. Results obtained with RGM-LC-IM-MS approach also determined indicate that the subject patient, was administered acetaminophen, naproxen and carbamazepine. The metabolite carbamazepine-10, 11-epoxide was also detected as well as caffeine and its biotransformation product theophylline, illustrating CCS values of biotransformation products can be utilised to provide secondary confirmation in a non-targeted xenobiotic screening strategy.

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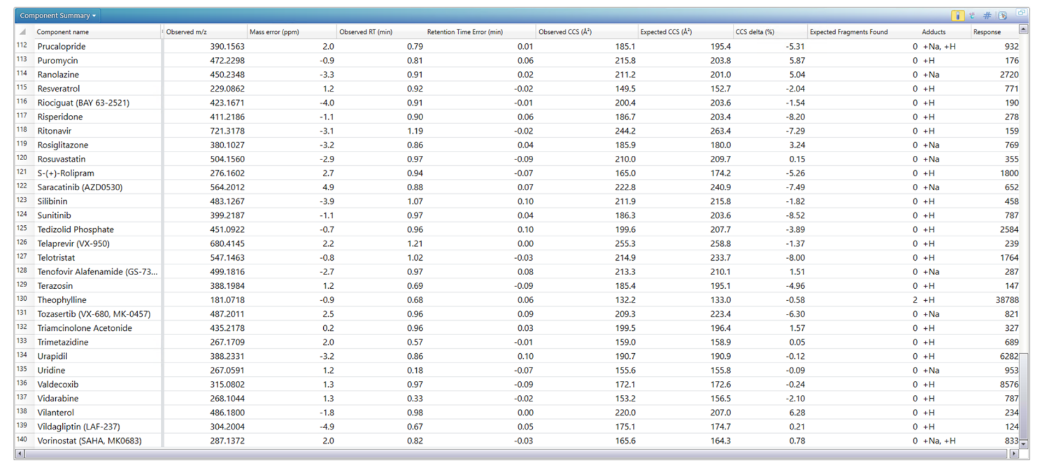

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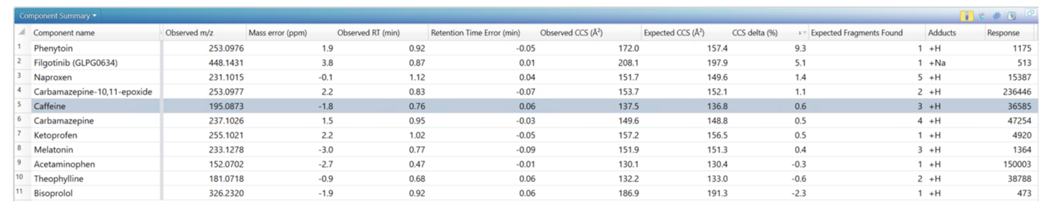

	Component name	Observed m/z	Mass error (ppm)	Observed RT (min)	Retention Time Error (min)	Observed CCS (Å²)	Expected CCS (Å²)	CCS delta (%)	Expected Fragments Found	Adducts	Response
1	Phenytoin	253.0976	1.9	0.92	-0.05	172.0	157.4	9.3	1	+H	1175
2	Filgotinib (GLPG0634)	448.1431	3.8	0.87	0.01	208.1	197.9	5.1	1	+Na	513
3	Naproxen	231.1015	-0.1	1.12	0.04	151.7	149.6	1.4	5	+H	15387
4	Carbamazepine-10,11-epoxide	253.0977	2.2	0.83	-0.07	153.7	152.1	1.1	2	+H	236446
5	Caffeine	195.0873	-1.8	0.76	0.06	137.5	136.8	0.6	3	+H	36585
6	Carbamazepine	237.1026	1.5	0.95	-0.03	149.6	148.8	0.5	4	+H	47254
7	Ketoprofen	255.1021	2.2	1.02	-0.05	157.2	156.5	0.5	1	+H	4920
8	Melatonin	233.1278	-3.0	0.77	-0.09	151.9	151.3	0.4	3	+H	1364
9	Acetaminophen	152.0702	-2.7	0.47	-0.01	130.1	130.4	-0.3	1	+H	150003
10	Theophylline	181.0718	-0.9	0.68	0.06	132.2	133.0	-0.6	2	+H	38788
11	Bisoprolol	326.2320	-1.9	0.92	0.06	186.9	191.3	-2.3	1	+H	473

Figure 5. I) RGM-LC-cIM-MS component summary for post-acquisition processing workflow filtered detection results for screening using FDA approved drug small molecule library, applied tolerance t_r 0.1 min and mass accuracy +/-5ppm (identified count 140). II) Applied tolerance t_r 0.1 min, mass accuracy +/-5ppm and ≥1 product ion (identified count 11).

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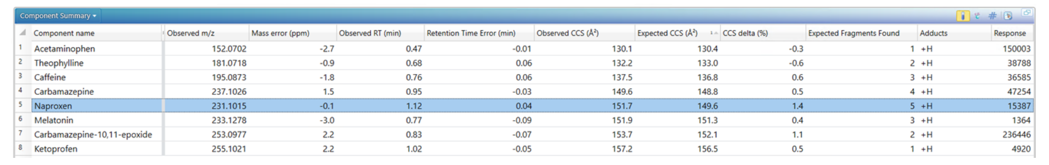

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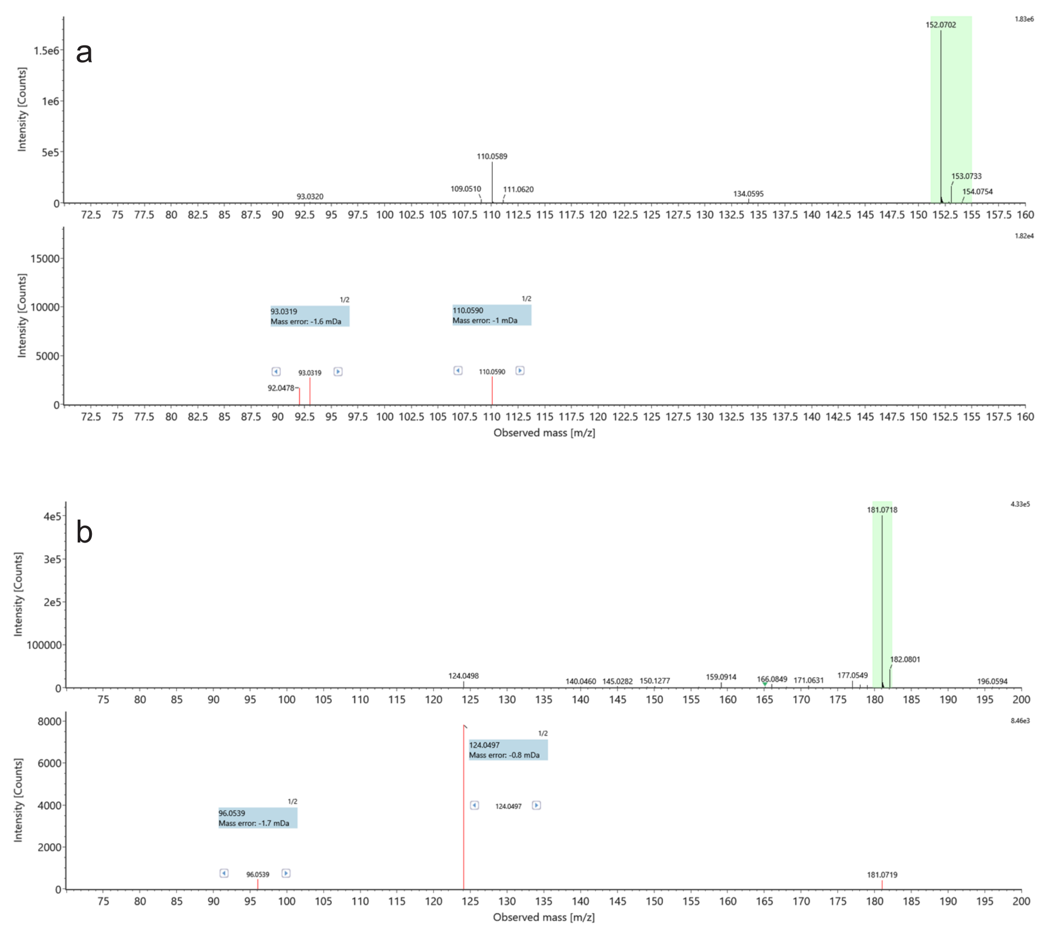

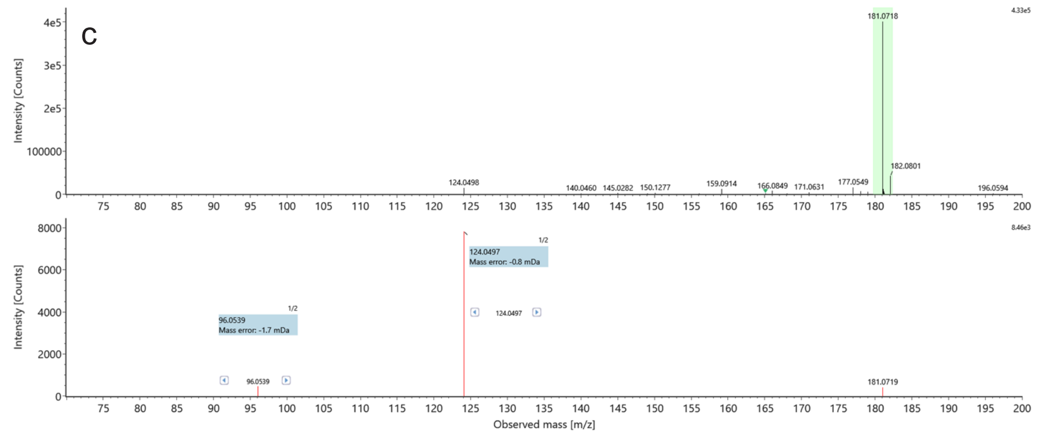

Figure 6. I) RGM-LC-cIM-MS component summary for post-acquisition processing workflow filtered detection results for screening using an FDA approved drug small molecule library, applied tolerance t_r 0.1 min, mass accuracy +/-5ppm, ≥1 product ion and ΔCCS <2% (identified count 8). II) Precursor/product ion spectra for a) acetaminophen, b) caffeine and c) theophylline.

CONCLUSION

- The incorporation of CCS into mass spectrometry libraries and the use in non-targeted screening workflows affords the opportunity to increase specificity of identification, whilst simultaneously increasing acquisition strategy flexibility.
- The complex biological matrix human urine was screened for administered xenobiotic pharmaceuticals, using retention time, mass accuracy, product ion count and CCS values. Comparable detection rates were observed using LC-cIM-MS and RGM-LC-cIM-MS methods.
- The research presented illustrates only one detection requiring further investigation, where ketoprofen was observed, which could be rationalized as a naproxen biotransformation product. Exogenous xenobiotics and natural endogenous species have been identified.
- Inclusion of biotransformation products into mass spectrometry libraries can provide additional confirmatory evidence when performing pharmaceutical xenobiotic screening assays.

References

1. Sundström M, Pelander A and Ojanperä I. Comparison Between Drug Screening by Immunoassay and Ultra-High Performance Liquid Chromatography/High-Resolution Time-Of-Flight Mass Spectrometry in Post-Mortem Urine. Drug Test. Anal. 2015;7:420-427.
2. Mollerup CB, Dalsgaard PW, Mardal M, Linnet K. Targeted and non-targeted drug screening in whole blood by UHPLC-TOF-MS with data-independent acquisition. Drug Test. Anal. 2016;DOI: 10.1002/dta.2120.
3. Dzumana Z, Zachariasovaa M, Veprikovaa Z, Godulab M, Hajslovaa J. Multi-analyte high performance liquid chromatography coupled to high resolution tandem mass spectrometry method for control of pesticide residues, mycotoxins, and pyrrolizidine alkaloids. Anal. Chim. Acta. 2015;863:29–40.
4. Pérez-Ortega P, Lara-Ortega FJ, García-Reyes JF, Gilbert-López B, Trojanowicz M, Molina-Díaz, A. A feasibility study of UHPLC-HRMS accurate-mass screening methods for multiclass testing of organic contaminants in food, Talanta 160 (2016) 704-712.
5. Pérez-Ortega P, Lara-Ortega FJ, Gilbert-López B, Moreno-González D, García-Reyes JF, Molina-Día A. Screening of Over 600 Pesticides, Veterinary Drugs, Food-Packaging Contaminants, Mycotoxins, and Other Chemicals in Food by Ultra-High Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-QTOFMS). Food Anal. Methods. 2017;10:1216–1244.
6. Romero-González R. Food safety: how analytical chemists ensure it. Anal. Methods. 2015;7:7193-7201.
7. Coscollà C, León N, Pastor A, Yusà V. Combined target and post-run target strategy for a comprehensive analysis of pesticides in ambient air using liquid chromatography-Orbitrap high resolution mass spectrometry. J. Chromatogr. *A*. 2014;1368:132-142.
8. Sjerps RMA, Vughs D, van Leerdam JA, ter Laak TL, van Wezel AP. Data-driven prioritization of chemicals for various water types using suspect screening LC-HRMS. Water Research. 2016;93:254–264.
9. Goshawk J, Barknowitz G, and McCullagh M. A Workflow for Automatic MS Library Creation from Time-of-Flight Full-Spectra Data Processed in UNIFI. 720006783EN. 2020 Feb.
10. Goscinny S and McCullagh M. A novel approach to the reduction of false positive and negative identifications in screening of pesticide residues in food analysis. Proceedings of the 61st ASMS Conference on Mass Spectrometry and Allied Topics, Minneapolis, Minnesota. June, 2013.
11. McCullagh M, Far J, De Pauw E and Eppe G. Towards the use of ion mobility mass spectrometry derived collision cross section as a screening approach for unambiguous identification of targeted pesticides in food. S. Goscinny, Rapid Commun Mass Spectrom. 2019;33(S2):34-48.
12. Giles K, Pringle SD, Worthington KR, Little D, Wildgoose J, Bateman RH. Applications of a travelling wave-based radio-frequency only stacked ring ion guide. Rapid Commun Mass Spectrom. 2004;18:2401-2414.
13. Pringle SD, Giles K and Wildgoose J. An investigation of the mobility separation of some peptide and protein ions using a new hybrid quadrupole/travelling wave IMS/oa-ToF instrument. Int J Mass Spectrom. 2007;261(1):1-12.
14. Gray N, Adesina-Georgiadis K, Chekmeneva E, Plumb RS, Wilson ID, Nicholson JK. Development of a Rapid Microbore Metabolic Profiling Ultraperformance Liquid Chromatography−Mass Spectrometry Approach for High-Throughput Phenotyping Studies. Anal. Chem. 2016, 88, 5742−5751.
15.Letertre M, Munjoma NC, Slade SE, Plumb RS, Swann J, Coen M, Nicholson JK, Wilson ID. Metabolic phenotyping using UPLC–MS and rapid microbore UPLC–IM–MS: Determination of the effect of different dietary regimes on the urinary metabolome of the Rat. Chromatographia volume 83, pages853–861(2020).