DELVING DEEPER INTO THE METABOLOME WITH CYCLIC IMS, SUB PPM MASS ACCURACY AND HIGH RESOLUTION MASS SPECTROMETRY

Waters™

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INTRODUCTION

Metabolomic studies involve the analysis of complex matrices comprised of compounds from numerous different classes which possess different physiochemical properties. Deconvolving these complex mixes of compounds requires accurate instrumentation with the ability to fully separate all compounds in the sample (1,2). Combining multiple separation techniques, including liquid chromatography and ion mobility separations, with high mass resolution mass spectrometers (MS), contribute to increasing the number of compounds that can be accurately detected. Each of these techniques have their own obstacles to overcome, such as instability in mass accuracy when analysing large batches of samples and/or the co-elution of compounds during chromatographic separations (3,4). These instrumental issues are vital to address in order to improve compound identification and biomarker discovery in metabolomics studies. This has become ever more apparent during the recent global pandemic where the ability to rapidly obtain vital and accurate information with regards to the pathophysiology of a disease has been paramount (5). Here we describe the analysis of plasma sample obtained from a COVID-19 pilot study by two novel high resolution mass spectrometers, the SELECT SERIES™ Cyclic™ IMS and the SELECT SERIES™ MRT.

SAMPLE PREPARATION

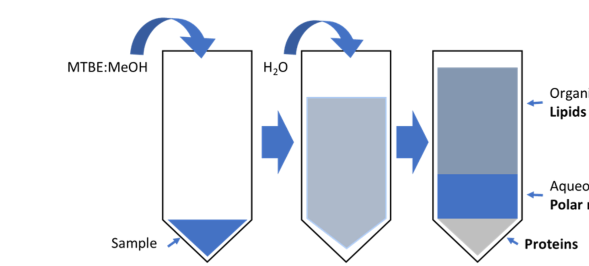

Figure 1. schematic of MTBE extraction procedure.

Plasma samples were obtained from 6 male patients diagnosed with COVID-19. The patients were characterised into two groups (Mild and Severe) based on the severity of disease. Patients characterised to the mild disease group had a single blood sample collected during their hospital admission whist two patients in the severe group each had a total of 3 samples collected across their hospital admission.

Each plasma sample underwent extraction for lipids and small molecules using the MTBE extraction method (6) (*Figure 1*). To 100 µL of sample 800 µL of MTBE was added followed by 200 µL of methanol. The samples were then incubated at 2-8 °C for 2 hours before phase separation was performed by the addition of 300 µL of water. The samples were then vortex missed for 2 minutes before centrifugation at 4,500 g for 10 minutes at 4 °C.

After centrifugation, the stop organic lipid phase of each extract was carefully removed without disturbing the lower phase and transferred to separate HPLC vials. This phase was dried under nitrogen then reconstituted using IPA and stored at –80 °C for future analysis. The lower aqueous phase was then carefully removed without disturbing the pellet, transferred to HPLC vials, dried under nitrogen and reconstituted in MeCN:water for HILIC analysis.

UPLC METHOD

Discovery HILIC metabolic profiling method

Chromatographic separation of the small molecule aqueous extraction was performed on a Waters ACQUITY™ I-class UPLC™ System by hydrophilic interaction chromatography (HILIC) using a Waters ACQUITY BEH™ Amide Column (1.7 µm, 2.1 x 100 mm). Mobile phase consisted of 5:95 acetonitrile:water with 10 mM ammonium acetate and 0.1 % formic acid (Mobile phase A) and 95:5 acetonitrile:water with 10 mM ammonium acetate and 0.1 % formic acid (Mobile phase B). Column temperature was maintained across the 10 minute gradient elution (Table 1) at 40 °C with sample injection volume set to 2 µL.

Table 1. Gradient elution composition for HILIC discovery metabolomic LC method.

#	Time (Mins)	Flow (mL/min)	% A	% B	Curve
1	Initial	0.700	0	100	Initial
2	0.1	0.700	0	100	6
3	5.0	0.700	20	80	6
4	6.0	0.700	50	50	6
5	6.5	0.700	50	50	6
6	7.0	0.700	0	100	6
7	10	0.700	0	100	6

SELECT SERIES CYCLIC IMS

Mass spectrometer settings

All MS data was acquired in positive and negative ESI polarities by CCS calibrated HDMS^e mode. HDMS^e data was acquired in two functions with precursor ions separated by a single pass round the Cyclic IMS cell (6) before Tof separation in the first function, and a second function producing fragmentation data by CID (CE Ramp: 20—40 eV) in a transfer region following the IMS separation (Figure 2). ESI capillary voltage was set to 2.0 kV with source temperature and desolvation temperature set to 120 °C and 600 °C respectively, and gas settings set to 50 and 800 L/hr for cone and desolvation flows. Data was acquired over the mass range of 50—1200 m/z with data acquired at a scan time of 0.3 s.

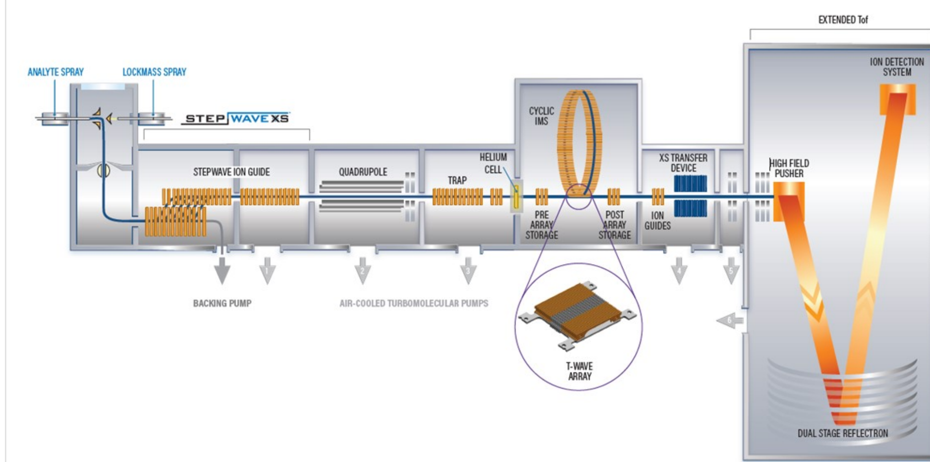

Figure 2. Instrument schematic of the SELECT SERIES Cyclic IMS

Cyclic IMS results

Raw data was pre-processed using Progenesis™ QI Software (Waters, UK) where data was aligned, normalised and underwent peak picking. The list of peaks was exported as a .csv file for import into Metaboanalyst (8) for statistical analysis. The PCA plot shown in Figure 3 shows good separation between the different patient samples with clear separation shown for severe COVID-19 suffering patient 1. The QC sample injections formed a tight cluster near the centre of the PCA plot, demonstrating good analytical reproducibility across the analysis. With the addition of the mobility separation, Progenesis QI identified 200 compounds separated by their drift time alone and an additional 1000 total features were detected on the Cyclic IMS compared to the MRT data of 5167 features. This is likely due to the resolution of co-eluting species and isomers following a single pass of the Cyclic IMS.

Database searching by HMDB (9) of a significant feature determined from patient 1, returned potential annotations of two conjugated sulphated bile acid isomers, taurochenodeoxycholic acid 7-sulphate and taurochenodeoxycholic acid 3-suphate. Accurate determination of which bile acid the feature could be is difficult by single pass IMS due to the resolution capabilities, but using the Cyclic IMS multipass functionality can provide the additional resolution required.

IMS^n multipass acquisition (6) of example isomeric compounds was achieved by direct infusion into the mass spectrometer. The quadrupole was focused on the corresponding negative ion (m/z = 391.28 [M-H$^-$) mass for the secondary bile acid isomers (chenodeoxycholic acid, hyodeoxycholic acid) which produced a single peak in the mobilogram after a single pass (Figure 5). The bile acids were sent round the Cyclic IMS cell 20 times before being ejected for Tof separation producing almost baseline resolution after < 200 ms separation and a IMS resolution of 290. Additionally, two methylated histidines (1-methylhistidine and 3-methlyhistidine, m/z = 168.07 [M-H$^-$) were separated by IMS^n after 5 passes in < 50 ms with a IMS resolution of 145.

Figure 3. PCA plot of COVID-19 pilot plasma samples following HILIC separation and analysis by HDMS^e on the Cyclic IMS.

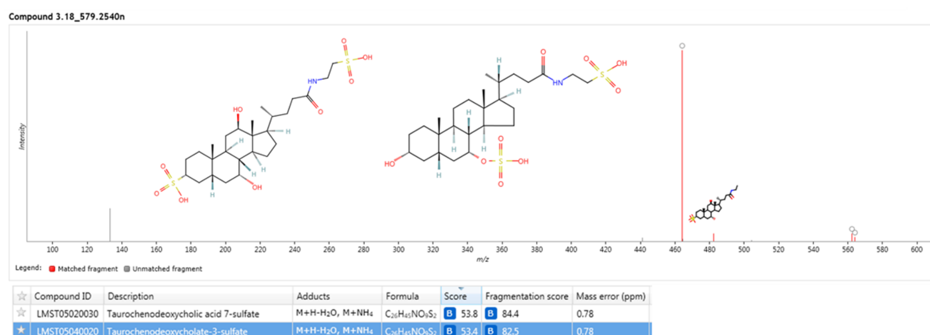

Figure 4. Potential compound annotations in Progenesis QI of two sulphated bile acids for a significant feature in Patient 1 .

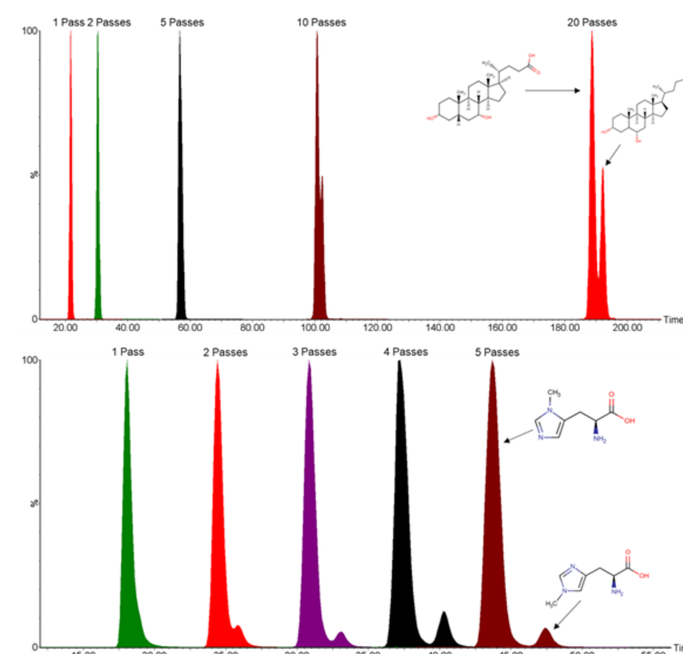

Figure 5. Mobilograms showing the separation of two conjugated bile acid isomers (top) and two methylated histidines (bottom) by IMS^n.

SELECT SERIES MRT

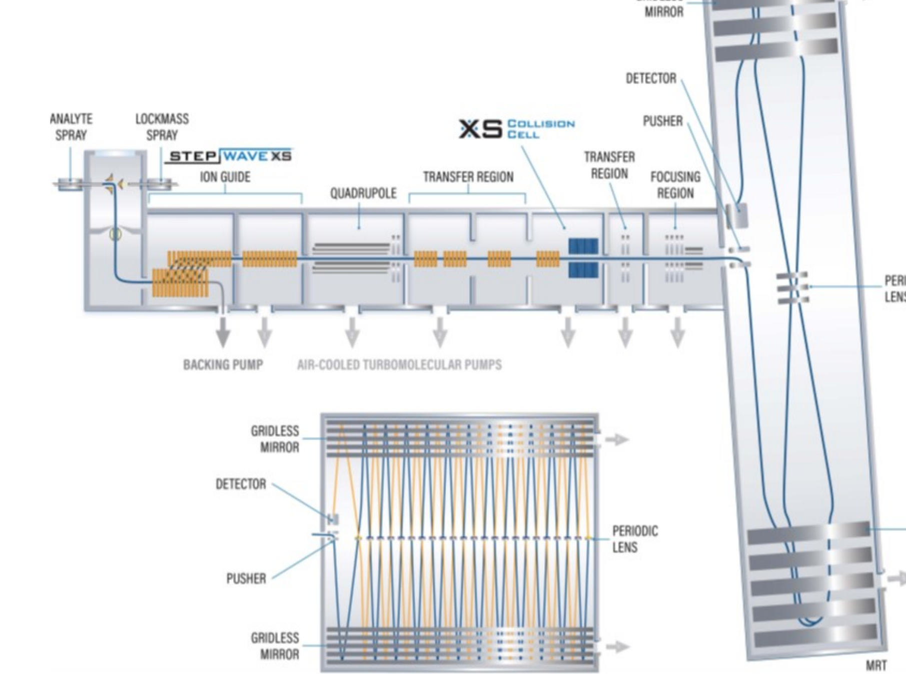

Figure 6. Instrument schematic of the SELECT SERIES MRT

Mass spectrometer settings

All MRT data was acquired in continuum MS^e mode in both positive and negative ESI polarities with a collision energy ramp from 20—40 eV. The MS was operated in EFP (extended flight path) mode to provide the best Tof resolution. ESI capillary voltage was set to 2.0 kV with source temperature and desolvation temperature set to 120 °C and 500 °C respectively, and gas settings set to 50 and 800 L/hr for cone and desolvation flows. Data was acquired over the mass range of 50—2400 m/z with data acquired at a scan time of 0.1 s.

MRT results

All raw data was processed using Progenesis QI Software, with statistical analysis performed using Metaboanalyst and compound annotation was achieved through searching against HMDB (9). Assessment of the data quality was performed by PCA which demonstrated good QC clustering and group separation comparable with the Cyclic IMS data.

The mass accuracy of example annotated compounds identified in both the Cyclic IMS and MRT data, was assessed. The mass error of these compounds from the Cyclic IMS data were all < 4 ppm (Table 2), but with the improved stable mass accuracy presented by the MRT, all annotated compounds possessing mass errors below 140 ppb, with 3 out of the 5 compounds below 40 ppb.

With the MRT's extended Tof flight path, the instruments MS resolution is greatly increased compared to conventional Tof instruments, which can improve the accuracy of compound annotations. The chromatogram and spectra shown in Figure 7 highlight this, using the example endogenous compound acetylcarnitine annotated from the HMDB database from raw data on the Cyclic IMS and the MRT. The mass resolution for acetylcarnitine (m/z = 204.1232 M+H$^+$) on the Cyclic IMS was determined to be ~37,000 whilst the calculated resolution on the MRT was over 160,000. This mass resolution combined with the stable mass accuracy in Table 2, greatly improves confidence in the accuracy of unknown compound identification. Furthermore this high Tof resolution at a low m/z was achieved at a fast scan speed of 10 spectra/second, compatible with narrow chromatographic peaks seen during UPLC separation. This allows for enough points across the peak for reproducible quantitation (15-20).

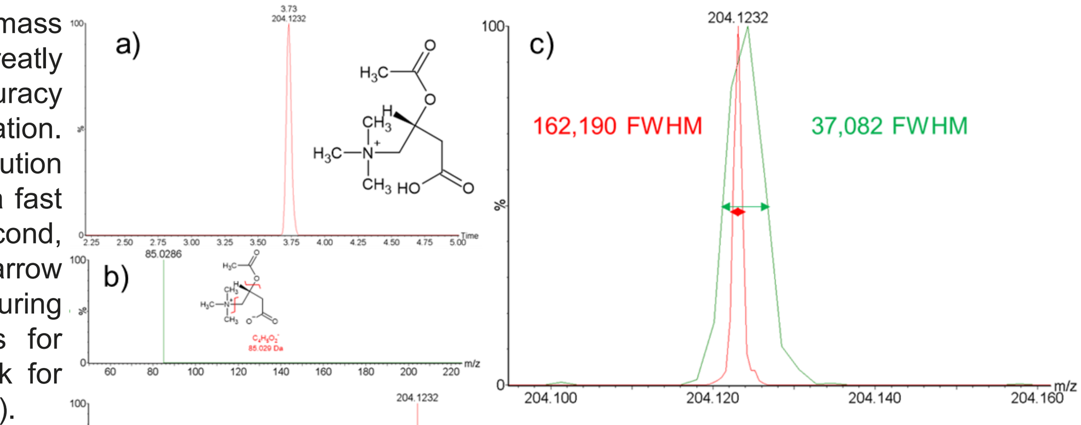

Figure 7. Extracted ion chromatogram of acetylcarnitine in human plasma (a) with the low and high CE spectra (b) and comparison of mass resolution between the Cyclic IMS (green) and MRT (red) (c).

Table 2. Summary of the mass accuracy for example endogenous annotated compounds from Progenesis QI on the Cyclic IMS and MRT .

Compound	Formula	Adduct	monoisotopic mass	SELECT SERIES Cyclic IMS			SELECT SERIES MRT		
				measured mass	*ppm error*	*ppb error*	*measured mass*	*ppm error*	*ppb error*
PC(36:3)	C46H80NO8P	M+H	806.569431	806.5683888	**-1.29**	**-1292**	806.569462	**0.04**	**38**
PC(38:3)	C46H86NO8P	M+H	812.616382	812.6131836	**-3.94**	**-3936**	812.6164909	**0.13**	**134**
Taurochenodeoxycholate-sulfate	C26H45NO9S2	M+NH4	597.287399	597.2878541	**0.76**	**762**	597.2873862	**-0.02**	**-21**
L-Acetylcarnitine	C9H17NO4	M+H	204.123034	204.1230918	**0.28**	**283**	204.1230283	**-0.03**	**-28**
Iohexol	C19H26I3N3O9	M+H	821.887586	821.8894527	**2.27**	**2271**	821.8875144	**-0.09**	**-87**

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CONCLUSION

- **Discovery metabolomic studies require accurate and reproducible instrumentation to produce reliable results.**
- **The complex matrices typically analysed for the study of human diseases contain many analytes across a broad mass range, including isomeric species.**
- **The SELEC SERIES Cyclic IMS reproducibly analysed a batch of COVID-19 plasma samples showing good group differentiation.**
- **The novel IMS^n acquisition enabled the separation of biologically important isomeric compounds.**
- **Analysis of the same samples on the MRT showed comparable statistics to that from the Cyclic IMS.**
- **Comparison of mass errors between the two platforms showed a dramatic improvement on the MRT.**
- **Comparison of the calculated resolution at 204 m/z showed over a 4 X increase in mass resolution on the MRT operating in EFP mode.**
- **This increase in accuracy and resolution can provide great confidence in compound identification for biomarker discovery.**
- **Analysis of these COVID-19 plasma sample on both platforms demonstrated the benefit mobility separation and high mass resolution at fast scan speeds can bring to metabolomic studies.**

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