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Improved Metabolite Structural Elucidation as a Result of Enhanced Resolution and Mass Accuracy Provided by the Select Series™ MRT

Adam King, Lee A. Gethings, Robert S. Plumb

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

For *in vitro* diagnostic use. Not available in all countries.

Abstract

This application brief highlights the benefit of the SELECT SERIES MRT in the analysis of human plasma samples, which demonstrates enhanced mass resolution and mass accuracy.

Benefits

Fast scanning, high mass resolution and stable, high mass accuracy for discovery LC-MS metabolomic studies.

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Introduction

Investigating the human metabolome can provide important information with regards to health and disease progression and can help determine potential targeted and personalized therapeutics. Identification of important novel biomarkers in discovery metabolomic studies routinely involves the utilization of high-resolution mass spectrometry, to provide reproducible, and accurate mass information for putative biomarker identification. The reliability of this mass information can be pivotal in the identification of unknown molecules where shifts in mass accuracy can result in missed identifications leading to errors in the eventual biological interpretation.

The achievable mass resolution of time-of-flight (Tof) mass spectrometers is dependent on the distance the ions of interest can travel through the vacuum and is proportional to the length of the instruments flight tube, therefore restricting the mass resolution of conventional Tof instruments due to available laboratory space and instrument design. In addition, the loss of ion transmission over a long flight path and the presence of reflectron grids can greatly impact the sensitivity of these instruments.

Experimental

Sample Preparation for Human Plasma

Plasma samples were obtained from six male patients diagnosed with COVID-19. The patients were classified into two groups (Mild and Severe) based on the severity of disease. Patients characterized 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 three samples collected across their hospital admission.

Each plasma sample underwent extraction for lipids and small molecules using the

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methyl-tert-butyl ether (MTBE) extraction method [1], briefly; 100 µL of sample was mixed with 800 µL of MTBE followed by 200 µL of methanol. The samples were then incubated at 2–8 °C for two hours before phase separation was performed by the addition of 300 µL of water. The samples were then vortex missed for two minutes before centrifugation at 4,500 g for ten minutes at 4 °C.

After centrifugation, the upper 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 analysis by a discovery hydrophilica interaction chromatography (HILIC) profiling method.

A pooled quality control (QC) sample was additionally prepared by combining a portion of each study sample prior to extraction as outlined above. This QC sample was injected after every tenth study sample to ensure analytical data quality. Liquid chromatography separation was performed by HILIC using the parameters outlined in Table 1.

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Discovery HILIC method	
LC system	Waters ACQUITY™ UPLC™ I-class system
Mobile phase A	5:95 acetonitrile:water 0.1% Formic acid, 10 mM ammonium formate
Mobile phase B	95:5 acetonitrile:water 0.1% Formic acid, 10 mM ammonium formate
Seal wash	10% Isopropanol in water
Weak wash	80:20 (v/v) water/acetonitrile
Strong wash	Isopropanol
Lockspray	Leucine enkephalin 200 pg/μL
Column	ACQUITY™ Premier BEH™ Amide, 1.7 μm, 2.1 × 100 mm (p/n 186009505)
Column temp.	40°C
Injection volume	2 μL
Run time	10 mins
Autosampler temp.	4 °C

Gradient	Time (min)	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	0.700	0	100	6

Table 1. ACQUITY I-Class instrument parameters.

All SELECT SERIES MRT data was acquired in continuum MSe 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 minutes.

Results and Discussion

The unique time-of-flight (Tof) design of the SELECT SERIES MRT allows the ability to extend the flight path of the ions compared to conventional Tof instruments, to 50 m without compromising on instrument size and loss of ion transmission. Figure 1 shows the schematic of the instrument with the gridless mirror reflectrons, pivotal in

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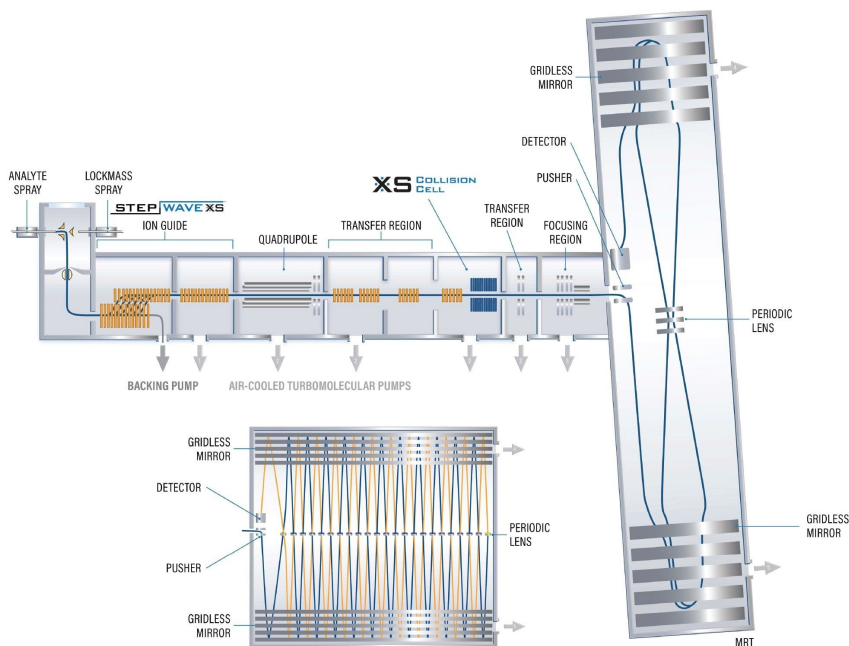enabling the multiple reflections without the loss of ions transiting through each pass of the tube.

Figure 1. Schematic of the SELECT SERIES MRT.

Table 2 shows a further five example endogenous compounds where the mass accuracy of the measured *m/z* value to the theoretical mass of the compound annotation was calculated. The MRT data showed considerable improvements in mass accuracy, with all compounds having sub ppm mass accuracy ranging from 21–134 ppb. This mass accuracy and high mass resolution provides greater confidence in determining the elemental composition of a molecule and therefore can improve the accuracy of compound annotation and identification.

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Compound	Formula	Adduct	Monoisotopic mass	Select series mrt		
				Measured mass	PPM error	PPB error
PC (36:3)	$C_{46}H_{80}NO_8P$	M+H	806.569431	806.56946	0.04	38
PC (38:3)	$C_{46}H_{86}NO_8P$	M+H	812.616382	812.61649	0.13	134
Taurochenodeoxycholate-7-sulfate	$C_{26}H_{45}NO_9S_2$	M+NH$_4$	597.287399	597.28739	-0.02	-21
L-Acetylcarnitine	$C_9H_{17}NO_4$	M+H	204.123034	204.12303	-0.03	-28
Iohexol	$C_{19}H_{26}I_3N_3O_9$	M+H	821.887586	821.88751	-0.09	-87

Table 2. Example mass accuracy for select endogenous compounds from human plasma extracts measured using the SELECT SERIES MRT.

For efficient, effective LC-MS based metabolomics it is critical that the mass spectrometer can maintain high MS resolution, whilst acquiring data at a sufficiently fast rate to correctly define the LC peaks being detected. This ensures that the LC resolution is maintained in the MS and that peaks can be accurately integrated. The SELECT SERIES MRT has an acquisition rate of up to 20 spectra/sec and thus can maintain high mass resolution and accuracy even during a fast-scanning data acquisition which is required for UPLC separations, when producing narrow analyte peaks. The extracted ion chromatogram of acetylcarnitine shown in Figure 2 highlights the number of data points achieved over the six second peak where the MRT collected the data across 28 scans. This demonstrates a potential for the SELECT SERIES to be used, not only for qualitative analysis but for compound quantitation. Furthermore, looking at the spectra from individual scans at different points across the chromatographic peak, we can see that the mass is stable across the peak at low and high intensities whilst maintaining high mass resolution.
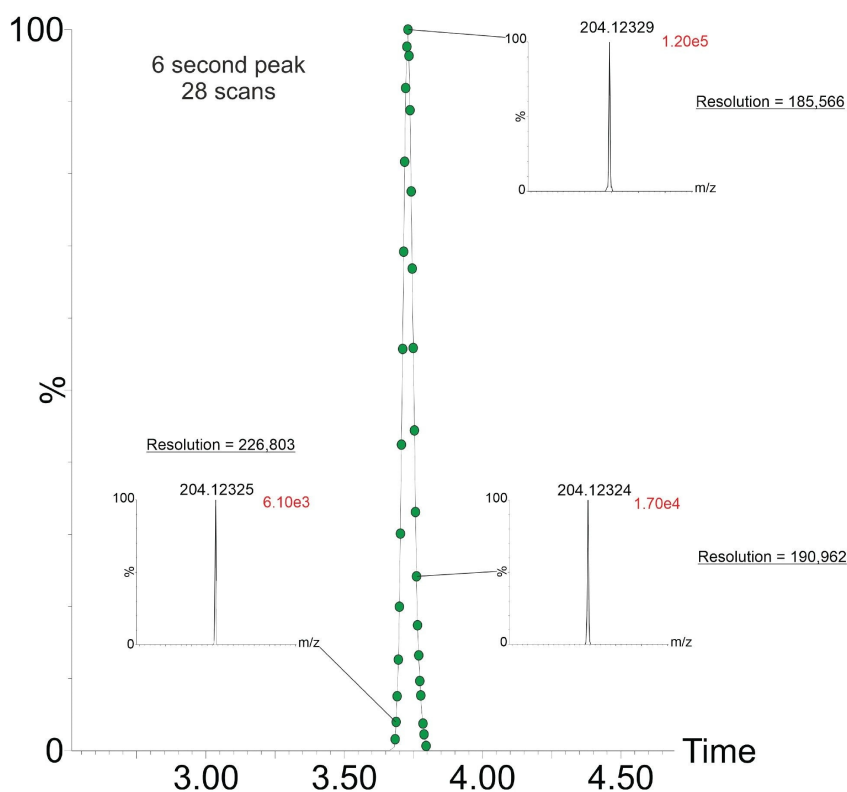
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Figure 2. Endogenous acetylcarnitine extracted ion chromatogram showing data points collected across the peak and inserted individual scan spectra.

Precise mass accuracy is not the only benefit of a high mass resolution capable instrument. The high mass accuracy provided by the MRT provides greater confidence in the identifications returned from database searching. Additionally, the increased mass resolution demonstrated by the MRT also allows for the detection of analytes with very similar accurate masses, a task which would be challenging with a conventional high-resolution platform. The example spectra shown in Figure 3 were obtained from a lipid peak at a retention time of t^R = 1.5 minutes from one of the plasma samples. The first spectra (Figure 3A) shows data from the MRT of a lipid precursor ion at *m/z* = 758.5705 with corresponding isotopic distribution of ions with an expanded section for the region round the ion at *m/z* = 760.5859. Here

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we can see two distinct ions ([1] *m/z* = 760.5779 & [2] *m/z* = 760.5859) due to the instruments mass resolution of 224,770 FWHM, whereas the same region examined at lower mass resolution (Figure 3B) shows only a single ion due to a resolution of 53,372 FWHM. Investigating the individual ion peaks, peak *(2)* from Figure 3 corresponds to a separate co-eluting lipid rather than being attributed to a third isotopic peak of the lipid at *m/z* = 758.5705 (Peak *(1)*). This is further highlighted in Figure 4 where the masses of the two co-eluting lipids were searched against the LIPID MAPS database [2]. The threshold was set to +/- 0.01 *m/z* with the results showing potential annotations of a) PC 34:2 (*m/z* = 758.5705 [M+H]$^+$) and b) PC 34:1 (*m/z* = 760.5859 [M+H]$^+$). The mass difference was measured between peaks a1 and a2 which was 1.003 Da, corresponding to the presence of a ^{13}C isotope present (elemental composition = $^{12}C_{41}$ ^{13}C $^1H_{80}$ ^{14}N $^{16}O_8$ ^{31}P). The mass difference between ions a2 and a3 was also 1.003 Da, corresponding to the third isotopic peak of PC (34:2) and the presence of two ^{13}C isotopes. Consequently, measuring the difference between ions a2 and b1 showed a difference greater than that of the presence of ^{13}C isotopes, and therefore can be attributed to a second co-eluting lipid. Additionally, further investigation of the spectra at t^R = 1.5 minutes and M+K$^+$ adducts of the lipids annotated from Figures 4 and 5, showed a detailed fine isotopic pattern (Figure 5) for PC (34:2) where the ^{41}K isotopic peak is easily resolved from the ^{13}C ^{39}K ion and PC (34:1) M+^{39}k$^+$ ion.

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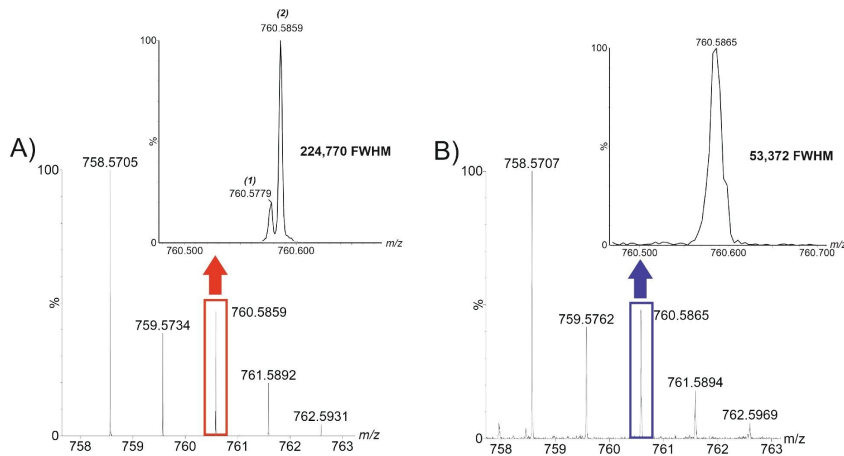
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Figure 3. Example spectra of a lipid peak at 1.5 minutes from a human plasma extract on the SELECT SERIES MRT with its higher mass resolution (A) and data acquired at lower resolution (B) showing the capabilities of the MRT in resolving a PC (34:2) isotope from a co-eluting mass for a PC (34:1).
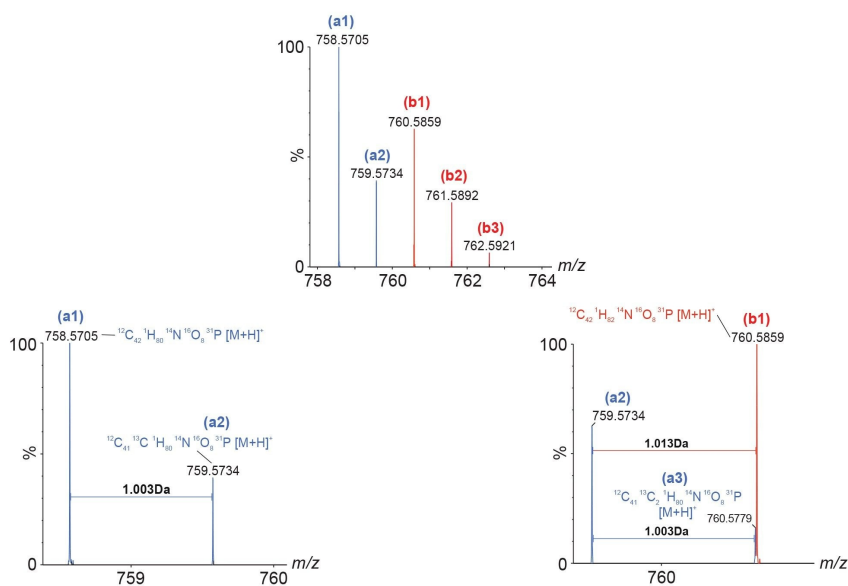
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Figure 4. Precursor ion and isotopic spectra of lipids PC (34:2) (a) and PC (34:1) (b) highlighting the corresponding $^{13}C_1$ ion (a2) and $^{12}C_2$ ion (a3) resolved from the molecular PC (34:1) ion (b1).

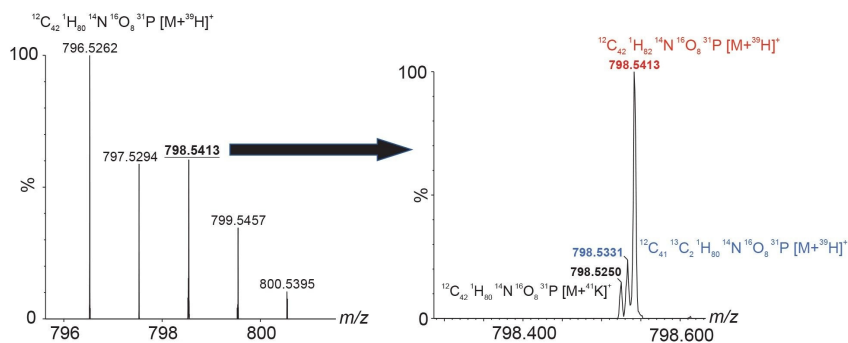Figure 5. Fine isotopic pattern of the PC (34:2) $M+K^+$ adduct showing peaks for ^{39}K, ^{41}K and $^{12}C_2$.

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Conclusion

Biomarker discovery studies require reproducible and highly accurate mass measurement. In particular, metabolomics relies heavily on reliable and accurate identifications as a result of database searching. The SELECT SERIES MRT instrument demonstrates high mass resolution whilst also providing mass accuracy at ppb levels, providing highly accurate compound identifications, and allowing for confident structural elucidation. A well-recognized advantage of Tof technology, is its ability to scan rapidly and thereby provide quantitative as well as qualitative datasets. The data outlined here, shows the ability to collect quantitative data with UPLC-based separations, typically providing >25 data points across peak profiles which are <10 seconds wide, whilst maintaining high mass accuracy and resolution.

References

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2. Fahy E., Subramaniam S., Murphy R., Nishijima M., Raetz C., Shimizu T., Spener F., Van Meer G., Wakelam M., and Dennis E. Update of the Lipid Maps® Comprehensive Classification System for Lipids. , *Journal of Lipid Research* 50, S9–S14 (2009).

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