Rapid, high-resolution isobaric separation of isotopically labeled metabolites using differential mobility separation

Improved speed and selectivity with the SCIEX TripleTOF® 6600 LC-MS/MS System and SelexION® Differential Mobility Separation Technology

Qiushi Sun[1], Richard M. Williams[1], Richard G. Kibbey[1]
[1]Yale University, New Haven, CT, USA

Mass spectrometry for metabolomics studies has become an important research tool for identifying and quantifying the vast diversity of small molecules present within biological systems. However, a major limitation of many metabolomics studies is that they provide a static picture of the components present at a specific point in time. In the past, stable isotope labeling has been used to provide a more dynamic picture by enabling the study of metabolic fluxes and the differentiation of metabolic pathways and networks in a variety of biological systems. They often only examine the mass of a metabolite, however, without identifying the location of the label, and they only extract a minimum of kinetic information.[1]

Liquid chromatography (LC) is typically coupled with mass spectrometry to provide separation of metabolites prior to mass spectrometry analysis. Due to the enormous diversity and complexity of the metabolome, complete separation of certain metabolites such as isomeric compounds can be challenging. differential mobility spectrometry (DMS) is a method of separating ions based on the difference between ion mobility in high and low electric fields at atmospheric pressure.[2,3] It adds a new dimension of selectivity and performance, which can reduce background noise and eliminate interferences.

In previous work, a targeted **M**ass **I**sotopomer **M**ulti-**O**rdinate **S**pectral **A**nalysis (**MIMOSA**) approach on a QTRAP® 5500 LC-MS/MS System equipped with a SelexION Differential Mobility Separation Device was developed to measure metabolic flow of central carbon metabolism of glycolysis and the TCA cycle after isotopic label (^{13}C) introduction.[4,5]

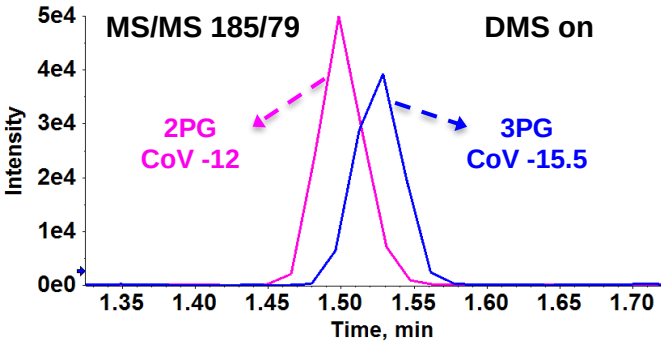

Figure 1. Separation of 3-phosphoglycerate (3PG) and 2-phosphoglycerate (2PG) using SelexION Technology. A separation voltage (SV) of 2000V and compensation voltages of -12V and -15.5V achieved separation of isotopically labeled 2PG and 3PG, respectively.

In this study, a TripleTOF 6600 LC-MS/MS System was used with the MIMOSA workflow.[6] The TripleTOF system has higher resolving power than the QTRAP® 5500 System, aiding in the differentiation of isotopomers. Additionally, the LC-DMS-MRM^{HR} method used on the TripleTOF 6600 System is fast and able to reduce the mass overlap issue (separate isobars) while simultaneously measuring all possible fragments from each metabolite.

Key features of LC-DMS-MRM^{HR} for MIMOSA fluxomics measurements

- Isobaric separation of isotopically labeled metabolites, that differ only in the location of the label, using differential mobility separation
- High-resolution MS/MS data for all fragments of the target isotopomers in one experiment, reducing cycle time
- Increased confidence in the identification of the isotope label position
- High resolution and reduced cycle time supports expanding analyses to a larger number of metabolites

Methods

Sample preparation: Labeled and unlabeled cells were cultured and seeded into 6-well plates. Cells were first washed quickly with 5 mM HEPES (2 mL/well), then harvested with quench buffer (150 µL/well). Cell lysates were transferred into a 96-well plate and then lyophilized. Samples were prepared by resuspending the cell pellet in 50 µL D_4-taurine water solution (used as the internal standard to normalize data) and then using 5 µL supernatant for analysis.

Chromatography: Separation was performed using an ExionLC™ system and a Thermo Scientific Hypercarb Carbon column (100x4.6 mm, 3 µm). Flow rate was 1 mL/min with a linear gradient as outlined in Table 1.

Mass spectrometry: Samples were analyzed using the TripleTOF 6600 System equipped with a SelexION Device. Data were collected using the MRM^{HR} workflow which consisted of a TOF MS scan (100 msec) and looped high resolution MS/MS scans (15 msec each) with a mass range of 15-550 for all scans. The ion source conditions were: Temp = 600 ºC, ISC = -1500V, GS1 = 55, GS2 = 65 and CUR = 40. Data were also collected on a QTRAP 5500 System, also equipped with a SelexION Device, using targeted MRM scans. The ion source conditions were: Temp = 600 ºC, ISC = -2000V, GS1 = 55, GS2 = 65 and CUR = 40. Compound dependent parameters for both systems are outlined in Table 2.

Table 1. Chromatographic separation.

Time	% A	%B
0	*100*	*0*
0.5	*100*	*0*
1	*60*	*40*
1.5	*60*	*40*
2	*100*	*0*
6	*100*	*0*

Mobile phase A: 15 mM ammonium formate, 10 µM EDTA, 0.03% acetyl acetone
Mobile phase B: 60% ACN, 35% IPA, 15 mM ammonium formate

SelexION Device parameters: The DMS separation of all targeted analytes was optimized using infusion and parameters were found to be: DMS temperature (DT) = low and DMS modifier (DM) = isopropanol (IPA) at low compensation for the TripleTOF 6600 System, or IPA at high compensation for QTRAP 5500 System. The optimized separation voltages (SV) and compensation voltages (CoV) are listed in Table 2. Note that values were selected to make sure there were no interferences among isobaric compounds.

Data processing: El-MAVEN software was used for peak picking and curation. Polly software was used for natural abundance correction and quantification.

Table 2. Parameter settings for the TripleTOF 6600 System and QTRAP 5500 System for all compounds in the MIMOSA experiment.

	TripleTOF 6600 System parameters					QTRAP 5500 System parameters					
Compounds	**DP**	**CE**	**CES**	**SV**	**CoV**	**DP**	**EP**	**CE**	**CXP**	**SV**	**CoV**
pyruvate	*-30*	*-3.7*	*0*	*1500*	*-9.5*	*-30*	*-6*	*-6*	*-7*	*2400*	*-30.75*
lactate	*-40*	*-4*	*0*	*1500*	*-8.5*	*-40*	*-10*	*-8*	*-5*	*2800*	*-44.5*
succinate	*-35*	*-13*	*0*	*2500*	*-39.5*	*-35*	*-13*	*-15*	*-8*	*2600*	*-41.25*
malate	*-15*	*-15*	*5*	*2500*	*-19.75*	*-40*	*-5*	*-15*	*-11*	*2400*	*-25.5*
glutamine	*-30*	*-20*	*10*	*3000*	*-49.25*	*-60*	*-5*	*-15*	*-8*	*3400*	*-57.5*
glutamate	*-20*	*-15.7*	*0*	*3000*	*-35*	*-40*	*-10*	*-15*	*-12*	*3400*	*-51.5*
PEP	*-20*	*-15.6*	*0*	*2600*	*-28.25*	*-40*	*-10*	*-14*	*-6*	*3000*	*-42.5*
DHAP	*-20*	*-55*	*0*	*2600*	*-37.5*	*-26*	*-4*	*-16*	*-8*	*3000*	*-47.25*
glycerol 3P	*-30*	*-22*	*0*	*2200*	*-21.75*	*-47*	*-8*	*-22*	*-13*	*2500*	*-25.25*
3-phosphoglycerate (3PG)	*-10*	*-50*	*0*	*2000*	*-15.5*	*-40*	*-12*	*-45*	*-9*	*3400*	*-44.25*
2-phosphoglycerate (2PG)	*-40*	*-50*	*0*	*2000*	*-12*	*-40*	*-9*	*-21*	*-10*	*3000*	*-32*
citrate	*-20*	*-31*	*0*	*3400*	*-1.3*	*-40*	*-10*	*-17*	*-10*	*3400*	*-8*
D_4-taurine	*-20*	*-35*	*0*	*2200*	*-31.5*	*-40*	*-10*	*-25*	*-8*	*2800*	*-48.25*

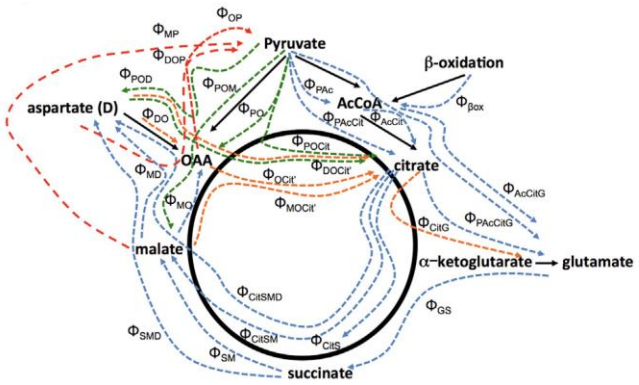

Figure 2. Isotopic steady state analysis. Graphical scheme of the 52 steady-state, precursor-product relationships between the metabolic intermediates contributing to the TCA cycle (Adapted from Alves *et al. 2015* [4]).

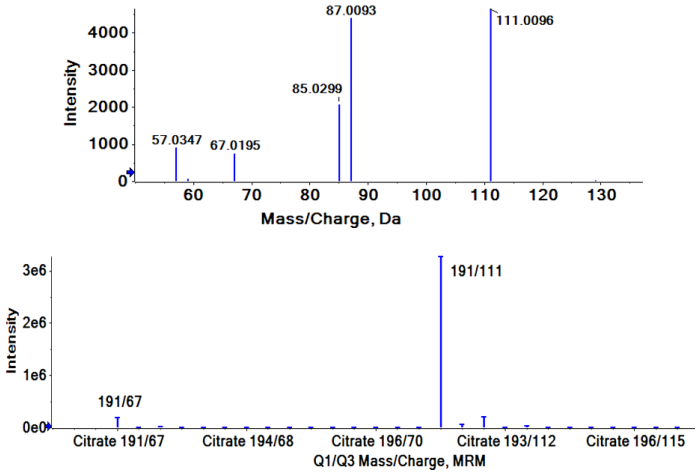

Figure 3. Comparison of scan methods. (A) MS/MS spectrum of citrate (*m/z* 191) from the TripleTOF 6600 System. All fragments of *m/z* 191, not only 191/67 and 191/111, are acquired on the TripleTOF 6600 System in a single MRM^{HR} experiment. In total, 7 MRM^{HR} experiments with precursor masses from 191 through 197 are required for full characterization of all isotopologues of citrate. (B) Q1/Q3 spectrum of citrate from the QTRAP 5500 System. Here, 27 MRM transitions of citrate parent/daughter combinations are required for full characterization of all citrate isotopologues.

Full scan MRM^{HR} workflow provides time and breadth of analysis advantage

The tricarboxylic acid (TCA) cycle contains many unique precursor-fragment relationships (Φ's) as shown in Figure 2. Discrete carbon entry points are identified by color: blue for oxidative reactions, green for **P**yruvate **C**arboxylase (PC)-driven anaplerosis, red for phosphoenolpyruvate (PEP)/pyruvate cycling and orange for subsequent turns of the TCA cycle measured by specific steady-state isotopomeric relationships across sequential and serial reactions of the TCA.[2] For example, two citrate parent/daughter combinations (191/111 and 191/67) need to be acquired to deconvolve citrate following $^{13}C_6$ glucose labeling.

Using the MRM^{HR} workflow, the TripleTOF 6600 System is able to acquire full scan high-resolution MS/MS data of all required fragments within one scan. Hence, instead of the 27 different MRM transitions required for the QTRAP 5500 System to cover all desired fragment ions of citrate, only 7 MRM^{HR} scans are required for the TripleTOF 6600 System for quantitative product ion scans from each citrate isotopologue (M+0 to M+6). This advantage of collecting full scan data for each precursor reduces the total cycle time from 1.62 sec to 0.97 sec, making it practical to expand MIMOSA to a larger number of metabolites on the TripleTOF 6600 System.

Shown in Figure 3 is a comparison of the spectra acquired for citrate that highlight the advantage of the MRM^{HR} approach for these studies. As shown, a single MRM^{HR} of *m/z* 191 from citrate is all that is required for quantification of this isotopologue on the TripleTOF 6600 System (A). That is, all fragments of *m/z* 191, not only 191/67 and 191/111, are acquired simultaneously on the TripleTOF 6600 System. In total, 7 MRM^{HR} experiments are necessary to fully quantify all required fragment ions from each of the precursor isotopologues. Basically a full scan MS/MS is collected for each precursor mass from 191 to 197. In contrast, multiple transitions are required on the QTRAP 5500 System in order to generate the same quantitative information (B). Here, the Q1/Q3 spectrum of citrate from the QTRAP 5500 System is shown for 191/111 and 191/67 along with all 27 transitions of citrate parent/daughter combinations for all isotopologues.

MRM acquisition is a discrete technique where the majority of mass space is not measured. This leads to the somewhat 'clean' background signal observed in Figure 4D except for spikes of signal when an interference elutes with one of the monitored precursor/product transitions. In contrast, the MRM^{HR} technique is acquiring full scan MS/MS data from each precursor ion. Hence, low level background signals are more uniformly observed throughout the run (Figure 4C). Despite these differences, extracted ion chromatograms of specific precursor/product combinations results in clean peaks that can be easily quantified using either method (Figure 4 A,B).

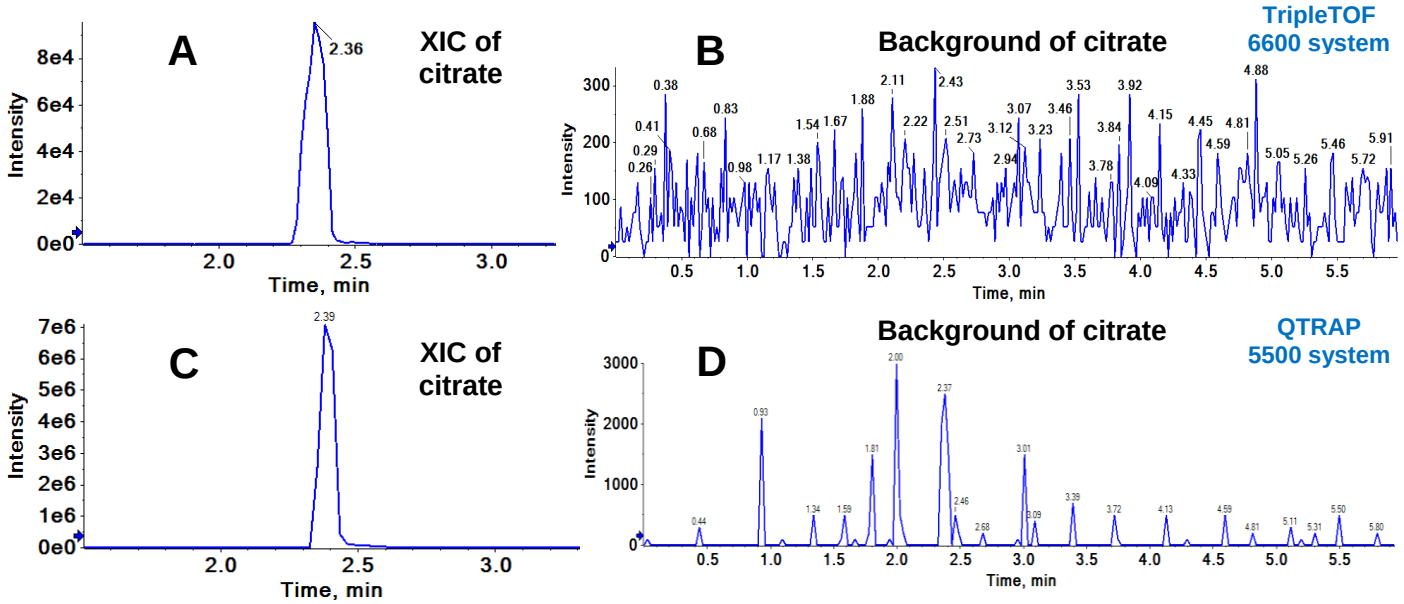

Figure 4. Comparison background and peaks for quantitation. (A) Extracted ion chromatogram (XIC) of fragment ion 111 from full scan MS/MS of citrate (*m/z* 191) from the TripleTOF 6600 System. (B) Background observed in the MS/MS for the citrate precursor for the TripleTOF 6600 System. (C) MRM data of citrate 191/111 from the QTRAP 5500 System. (D) Background observed at the citrate MRM transitions for the QTRAP 5500 System.

Resolve isobaric isomers with DMS

3-Phosphoglycerate (3PG) and 2-Phosphoglycerate (2PG) are the main metabolites in the glycolytic pathway. The only difference in their structures is the position of the phosphate group (Figure 5, top). They have the same precursor/fragment ions (185/79) and cannot be separated by LC which prevents accurate quantification (Figure 5, middle).

SelexION Technology uses differential mobility spectrometry (DMS) to separate ions based upon their differences in ion mobility in high and low electric fields in gases at or near atmospheric pressure. With DMS, the separation waveform (SV) radially displaces ions towards one or the other electrode, depending upon high and low field mobility characteristics. The compensation voltage (CoV) restores the trajectory for a given ion to allow them to transmit through the DMS device and enter the mass spectrometer.

When DMS was used to separate 3PG and 2PG, a separation voltage of 2000V was used. The compensation voltages were tuned for both 2PG and 3PG and were found to have quite different CoVs, -12 and -15.5, respectively (Figure 5, bottom). This allows complete separation of the two metabolites with DMS for accurate quantification with minimal overlap.

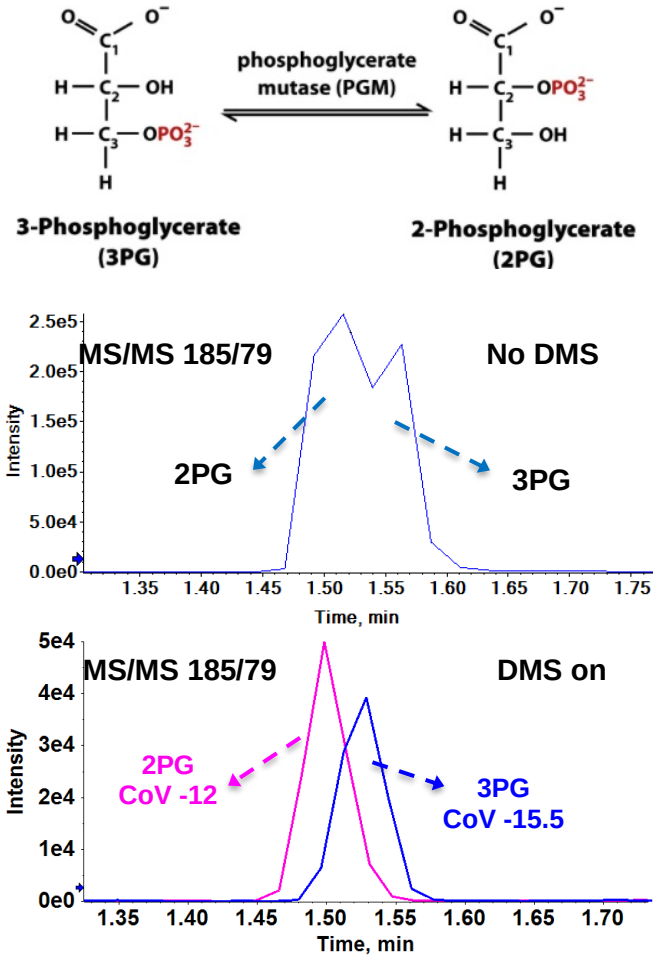

Figure 5. LC Separation of 2PG and 3PG. Incomplete separation using LC alone inhibits accurate quantification for phosphoglycerate isomers.

Conclusions

Resolving mass overlap between metabolites can be challenging using traditional LC-MS techniques. This can be further compounded during MIMOSA studies when ^{13}C isotopes are introduced. The unique selectivity of the SelexION Differential Mobility Separation Device and the high-resolution of the TripleTOF 6600 LC-MS/MS System can provide clear separation of the labeled isobars, resulting in increased confidence in isotope labeling studies. Additionally, because MRM^{HR} provides high resolution data for all fragment ions of an isotopologue simultaneously in a single scan, this reduces cycle time and provides an opportunity to expand analyses to larger numbers of metabolites. This increased efficiency also allows for exploring additional metabolic position information from other fragments without adding more transitions to the method.

The MIMOSA data generated here was consistent with previous ^{13}C-enrichment results for the central carbon metabolites[4] and provides the following advantages:

- SelexION Technology provides an additional level of separation and selectivity beyond LC
- The high-resolution MS/MS of the TripleTOF 6600 System provides clear separation of labeled isotopes for accurate identification and quantification
- The full scan high resolution MS/MS data generated using MRM^{HR} workflow decreases the total number of targeted experiments required to accurately track and quantify metabolite isotopologues allowing more metabolites and positional information to be tracked in a single experiment.

References

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Headquarters
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