High Performance Ion Pair-Reverse Phase LC/Q-TOF Method for Profiling Diverse Classes of Endogenous Metabolites with Separation of Important Isomers

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Introduction

High resolution accurate mass LC/TOF or Q-TOF MS is routinely used in metabolomics for discovery work. Some analytical challenges remain, including retention of ionic metabolites, retention time (RT) reproducibility, chromatographic separation of biologically important isomers, and broad coverage of metabolite classes in a single analytical run.

To address these challenges, we have developed an ion pair-reverse phase (IP-RP) LC/TOF or Q-TOF MS method using a C18 column with tributylamine as an ion paring agent. This method enables simultaneous detection of several pairs of biologically significant isomers and diverse classes of endogenous metabolites. To further enhance signals of small, labile metabolites, we utilize an intelligent SWARM Autotune algorithm to optimize the ion transmission of the Q-TOF system. We demonstrate how this algorithm allows simplified customizable instrument optimization to meet

Experimental

Method

A set of metabolite standards covering different chemical classes and several pairs of biologically relevant isomers was selected for IP-RP LC/MS method development. The chromatographic separation was conducted on a rapid resolution C18 (2.1 x 150 mm, 1.8 micron) column using a non-linear mobile phase gradient from water to methanol containing the ion pairing agent tributylamine (TBA). The pH of the mobile phases was adjusted by varying the concentration of acetic acid. Tributylamine and acetic acid were added to both aqueous and organic mobile phases to ensure a constant concentration during the gradient chromatography.

Instrumentation

Agilent 1290 Infinity LC system coupled to either an Agilent 6230 TOF system or an Agilent 6545 Q-TOF system. Tables 1 and 2 summarize the optimized LC and MS conditions.

Table 1: Conditions for Agilent 1290 Infinity LC System

Analytical column	Zorbax Extend C18, 2.1 x 150 mm 1.8-Micron (p/n: 759700-902)
Guard column	Agilent Zorbax SB-C8, 2.1 mm x 30 mm, 3.5 µm (p/n: 873700-936)
Column temperature	40 °C
Injection volume	5 µL
Autosampler temperature	4 °C
Mobile phase	A: 97% water/3% methanol containing 5 mM TBA and 5.5 mM acetic acid B: Methanol containing 5 mM TBA and 5.5 mM acetic acid
Seal wash solvent	Isopropanol : H_2O (1:1 v/v)
Flow rate	0.25 mL/min
Gradient	A non-linear gradient from 0-99% B in 22 minutes
Stop time	22 min
Post time	5 min

Table 2: MS parameters	Agilent 6230 TOF	Agilent 6545 Q-TOF
Ion mode	Negative	Negative
Source	Agilent Dual ESI	Agilent Dual ESI
Capillary voltage	3500 V	3500 V
Dry gas temp.	250 °C	250 °C
Dry gas flow	13 L/min	13 L/min
Nebulizer pressure	35 psi	35 psi
Fragmentor	130 V	Optimized by SWARM Autotune technology
Skimmer	60 V	
Oct 1 RF Vpp	600 V	
MS range	60-1600 m/z	
MS acquisition rate	1.5 spectra/sec	
Reference mass	62.0327 and 983.0347	68.9958 and 983.0347
Reference pump flow	0.5 mL/min	
Reference delivery	Agilent 1100 isocratic pump with 100:1 splitter (p/n G1607-60000)	
Instrument mode	Extended dynamic range (2GHz)	

Data analysis software

- Agilent MassHunter Qualitative Analysis (Qual) B.07.00

Results and Discussion

Broad coverage of metabolite classes with separation of important isomers

A robust high performance IP-RP LC/MS method was developed using Agilent 1290 Infinity LC system coupled with an Agilent 6230 TOF. The RT reproducibility is excellent with CVs in the range of 0.01-0.45% for all 39 metabolites evaluated here. The separation of biologically important isomers was primary achieved by chromatographic means, in which the optimization of a stepwise non-linear gradient was the key to the success of the separation for citrate and isocitrate, D-glucose-6-phosphate and alpha-D-glucose-1-phosphate, leucine and isoleucine, maleic acid and fumaric acid. Figures 1 and 2 illustrate that this IP-RP LC/TOF MS method enables simultaneous detection of the multiple functional classes of 39 metabolites including amino acids, organic acids, sugars and sugar phosphates, nucleosides and nucleotides, energy and redox metabolites and Coenzyme A derivatives with separation of biologically sign

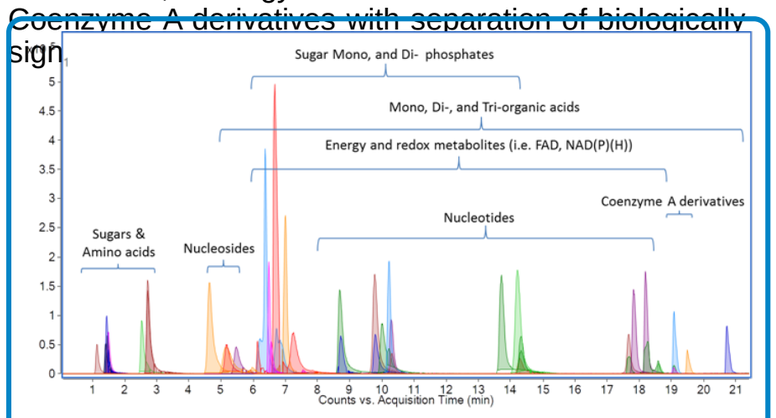

Figure 1. Overlaid EICs of 39 metabolite standards using the mobile phase at pH=5.8

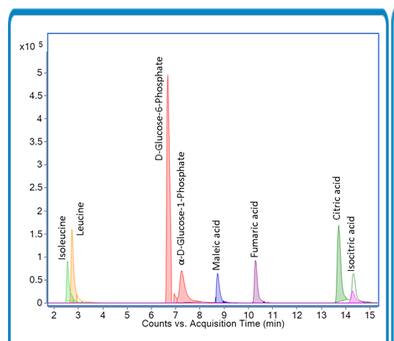

Figure 2. Separations of four pairs of the isomers

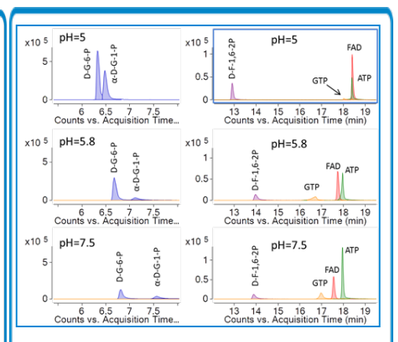

Figure 3. Effect of the mobile phase pH on the RT and selectivity

Effect of mobile phase pH on RT, selectivity and signal intensity

Changes to the pH of the mobile phase by moderating the concentration of acetic acid can alter the retention and selectivity of sugar phosphates and nucleotide triphosphates (Figure 3). However, the signal intensities of a wide range of metabolite classes were impacted with this change (Figure 4). The level of the signal intensity changes and the direction of retention time shifts are highly dependent on the chemical classes of the metabolites. Our findings suggest that we can improve selectivity and sensitivity for a specific class of metabolites by controlling the pH of mobile phases in IP-RP chromatography. In this study, the mobile phase at pH of 5.8 provides the overall best results in terms of the chromatographic separation for the isomers and acceptable sensitivity among the different classes of

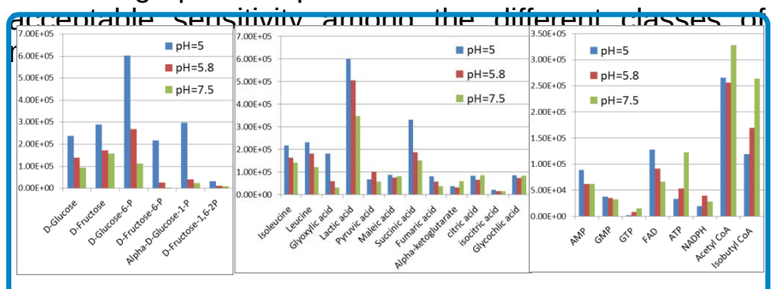

Figure 4: Effect of the mobile phase pH on the signal response of diverse classes of endogenous metabolites

Easy ion transmission optimization by SWARM Autotune

6545 Q-TOF system incorporated the SWARM Autotune technology provides faster tuning speed and enhanced sensitivity for the best small molecule performance. Figure 5 shows the results for a set of representative metabolites obtained under three different autotune conditions. It is evident that 50-750 m/z tuning provides better sensitivity for the metabolites evaluated here compared to the 50-1700 m/z tuning, and the fragile ion tuning further enhances

Results and Discussion

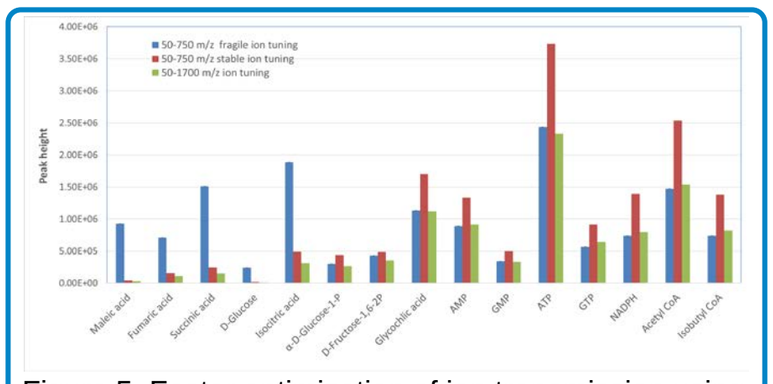

Figure 5. Faster optimization of ion transmission using Agilent SWARM Autotune algorithm

Excellent mass accuracy and isotopic fidelity

Mass accuracy and isotopic fidelity are important for reliable metabolite identification and comparative analyses in discovery metabolomics studies. Agilent 6545 Q-TOF LC/MS system offers excellent mass accuracy with mass errors within ±1 ppm for 54 out of 64 mass measurements under four different tuning conditions (Figure 6). Furthermore, exceptional isotope fidelity is also achieved with overall match scores of 97-100% calculated based on the mass accuracy, isotope abundance and isotope spacing matches.

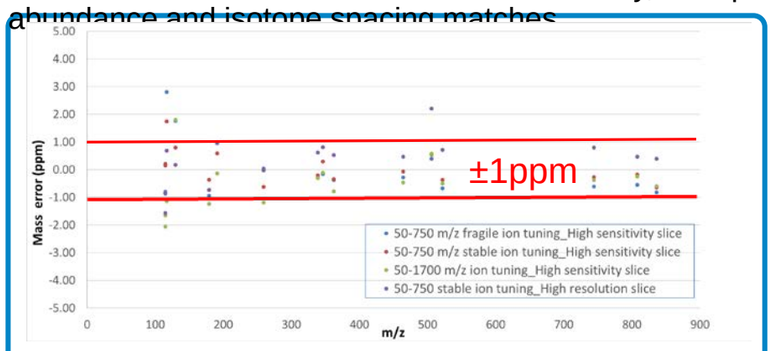

Figure 6. Excellent mass accuracy measured on 6545 Q-TOF

Application of the IP-RP LC/TOF method for cell extract analysis

The IP-RP LC/TOF method was implemented in analyzing the *M. tuberculosis* cell extracts (3 samples/group with total of 8 groups). The data were then processed using MassHunter Profinder software. As shown in the Figure 7, a total of 2156 compounds were found based on the settings of the untargeted feature extraction criteria. We can quickly review detailed information of each compound from the compound details table, the chromatogram results and mass spectrum results by clicking a compound in the up

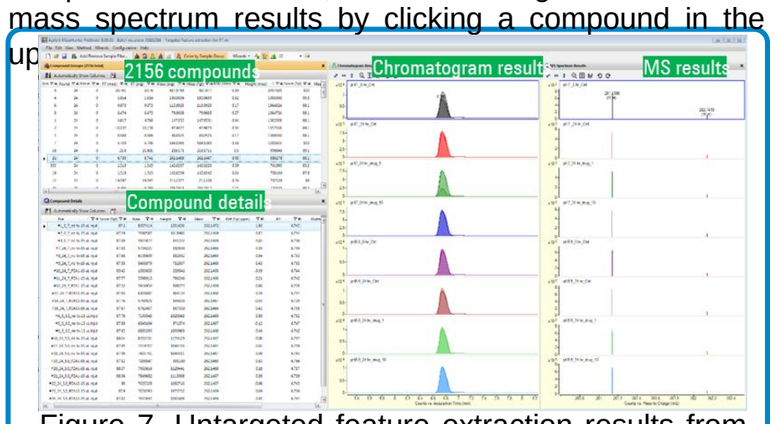

Figure 7. Untargeted feature extraction results from the cell extracts in the control and drug treated groups

Conclusions

A robust and reliable IP-RP LC/TOF or Q-TOF MS method was developed and successfully implemented to analyze the cell extracts in a metabolomics study. This method clearly demonstrates superior analytical performance:

- Provides broad coverage of metabolite classes
- Offers excellent chromatographic separation for biologically important isomers such as citrate/isocitrate, D-Glucose-6-phosphate/ α-D-Glucose-1-phosphate, fumeric acid/maleic acid and leucine/isoleucine.
- Achieves excellent reproducibility in RTs (%CV: 0.01~0.42%)
- Agilent dual ESI source delivers excellent mass accuracy and isotopic fidelity, which is achieved through real time mass calibration.
- Agilent SWARM Autotune technology enables faster ion transmission optimization and enhanced sensitivity for the best small, labile molecules performance.