Profiling of Endogenous Metabolites Using Time-of-Flight LC/MS with Ion-Pair Reverse Phase Chromatography

Application Note

Metabolomics

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Abstract

A high performance ion-pair reverse-phase (IP-RP) chromatographic method has been developed for use with a time-of-flight (TOF) mass spectrometer (MS). This negative ionization method provides comprehensive coverage of many endogenous metabolite classes including amino acids, organic acids, sugars and sugar phosphates, nucleosides and nucleotides, energy and redox metabolites, and coenzyme A derivatives. Optimization of mobile phase pH and the LC gradient was critical in obtaining good chromatographic separation of isomers such as citric acid and isocitric acid, and impacted peak shape and optimal signal response. The IP-RP LC/TOF MS method was evaluated with 19 representative metabolite standards covering the entire chromatographic region. All metabolites showed good retention time reproducibility.

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Introduction

High resolution accurate mass LC/TOF or Q-TOF MS is routinely used in metabolomics for discovery work[1-2]. TOF MS is preferred due to spectral acquisition speed, high mass accuracy, high isotopic ratio fidelity, and sensitive detection of metabolites. Some analytical challenges remain[3-4], including retention of ionic metabolites, reproducibility in retention time (RT), chromatographic separation of biologically important isomers, and broad coverage of metabolite classes in a single analytical run.

To address these challenges, a robust and reproducible ion-pair reverse phase (IP-RP) LC/TOF MS method was developed using tributylamine as the ion-pairing agent. As part of the chromatographic optimization, the effect of both pH and concentration of tributylamine in aqueous and organic mobile phases was investigated. This method enables simultaneous detection of different classes of polar and anionic endogenous metabolites with separation of several pairs of biologically relevant isomers such as citric acid and isocitric acid.

Experimental

Method

A set of metabolite standards covering different chemical classes and several pairs of biologically relevant isomers was selected for IP-RP LC/TOF MS method development. Using a nonlinear water/methanol gradient, the chromatographic separation was performed on an Agilent ZORBAX RRHD Extend 80Å C18, 2.1 × 150 mm, 1.8 µm column (p/n 759700-902) with an Agilent ZORBAX SB-C8, 2.1 mm × 30 mm, 3.5 µm (p/n 873700-936) guard column. Tributylamine (Sigma, p/n 90781-50mL) was added to both aqueous and organic mobile phases to ensure a constant concentration during gradient elution. The pH of the aqueous mobile phase was adjusted using acetic acid (Fluka, p/n 49199-50mL-F), and the same amount of acetic acid was added to the organic mobile phase. All analyses were performed using negative ion electrospray on an Agilent LC/TOF MS system.

Instrumentation

LC/MS analysis was performed using an Agilent 1290 Infinity LC coupled to an Agilent 6230 TOF with an Agilent Dual ESI Source. The LC consisted of an Agilent binary pump with seal wash (G4220A), an Agilent sampler (G4226A) with thermostat (G1330B), an Agilent thermostatted column compartment (G1316C) and an Agilent 1100 isocratic pump (G1310A) with a 100:1 splitter (G1607-60000) for reference mass addition. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution. Tables 1 and 2 summarize the optimized LC and MS conditions.

Data acquisition and analysis was done using the Agilent MassHunter software suite. The TOF MS provided sensitive, full spectrum data, which facilitated method development of the chromatographic conditions.

Table 1. The optimal LC parameters.

Agilent 1290 Infinity LC System	
Analytical column	Agilent ZORBAX RRHD Extend 80Å C18, 2.1 × 150 mm, 1.8 µm (p/n 759700-902)
Guard column	Agilent ZORBAX SB-C8, 2.1 mm × 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 with pH adjusted by acetic acid B) Methanol containing 5 mM TBA with addition of acetic acid
Seal wash solvent	Isopropanol : H_2O (1:1 v/v)
Flow rate	0.25 mL/min
Gradient	A nonlinear gradient from 0–99 % B in 22 minutes
Stop time	22 minutes
Post time	5 minutes

Table 2. The optimal MS parameters.

Parameter	Agilent 6230 TOF system
Ion mode	Negative
Source	Agilent Dual ESI
Capillary voltage	3,500 V
Dry gas temperature	250 °C
Dry gas flow	13 L/min
Nebulizer pressure	35 psi
Fragmentor	130 V
Skimmer	60 V
Oct 1 RF Vpp	600 V
MS range	60–1,600 *m/z*
MS acquisition rate	1.5 spectra/sec
Reference mass	62.0327 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 (2 GHz)

Results and Discussion

A robust, high-performance IP-RP LC/TOF MS method was developed using an Agilent Extend column designed for robust performance at high pH. The addition of tributylamine improved retention of anionic metabolites, allowing separation of both anionic and more hydrophobic metabolites in a single method. The separation of biologically relevant isomers was achieved by a combination of a stepwise nonlinear gradient and pH optimization of the mobile phase. This resulted in the separation of citric acid and isocitric acid, D-glucose-6-phosphate and α-D-glucose-1-phosphate, leucine and isoleucine, and maleic acid and fumaric acid. Maintaining the same concentration of tributylamine in both aqueous and organic mobile phases helped control the pH. Furthermore, the concentration of tributylamine was decreased by half without causing any significant impact on the signal or separation. Generally, a lower concentration of buffer in the mobile phase will improve source cleanliness and system robustness.

Figure 1 illustrates that this method enabled detection of 38 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 relevant isomers (Figure 2) in a 22-minute analytical run. Table 3 summarizes the chemical formula, RT, and *m/z* values for these metabolites. The chromatographically separated isomers are highlighted with four different colors.

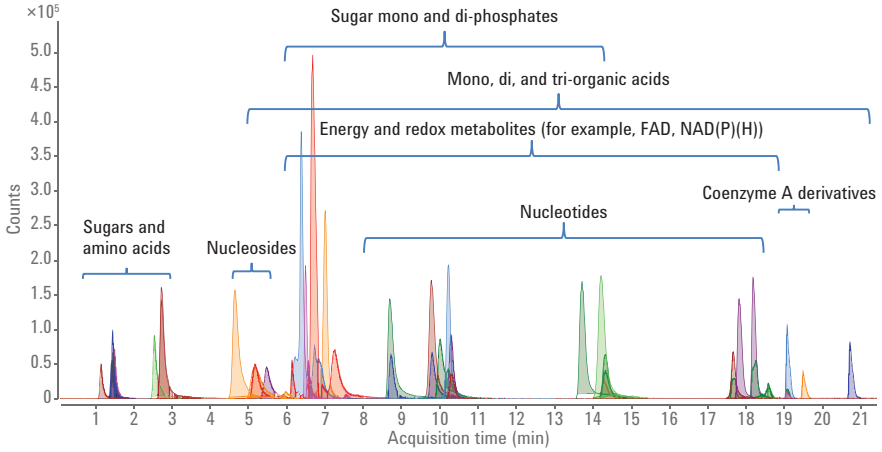

Figure 1. Overlaid extracted ion chromatograms (EICs) of 39 metabolite standards using a mobile phase at pH = 5.8.

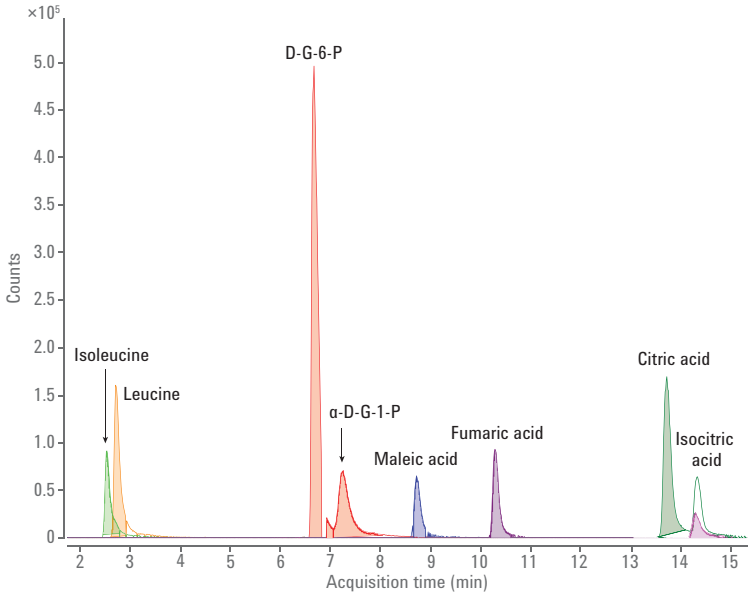

Figure 2. Overlaid EICs showed chromatographic separations of four pairs of biologically relevant isomers.

Table 3. A list of 38 metabolites with chemical formula, RT, and $(M-H)^-$ information.

Compound	Chemical formula	$(M-H)^-$	RT (min) (Ave) (n = 3)	
L-Arginine	$C_6H_{14}N_4O_2$	173.1044	1.15	
Glycine	$C_2H_5NO_2$	74.02423	1.42	
L-Glutamine	$C_5H_{10}N_2O_3$	145.0619	1.45	
D-Glucose	$C_6H_{12}O_6$	179.0561	1.47	
D-Fructose	$C_6H_{12}O_6$	179.0561	1.50	
Isoleucine	$C_6H_{13}NO_2$	130.0874	2.53	Isomer pair
Leucine	$C_6H_{13}NO_2$	130.0874	2.72	
Inosine	$C_{10}H_{12}N_4O_5$	267.0735	4.64	
Guanosine	$C_{10}H_{13}N_5O_5$	282.0839	5.17	
L-Phenylalanine	$C_9H_{11}NO_2$	164.0717	5.48	
Glyceric acid	$C_3H_6O_4$	105.0188	6.13	
Glyoxylic acid	$C_2H_2O_3$	72.9931	6.38	
Lactic acid	$C_3H_6O_3$	89.0244	6.49	
NAD	$C_{21}H_{27}N_7O_{14}P_2$	662.1013	6.55	
D-Glucose-6-phosphate (D-G-6-P)	$C_6H_{13}O_9P$	259.0224	6.67	Isomer pair
D-Fructose-6-Phosphate (D-F-6-P)	$C_6H_{13}O_9P$	259.0224	6.72	
Pyruvic acid	$C_3H_4O_3$	87.0088	7.00	
α-D-Glucose-1-phosphate (α-D-G-1-P)	$C_6H_{13}O_9P$	259.0224	7.25	
AMP	$C_{10}H_{14}N_5O_7P$	346.0558	8.69	
Maleic acid	$C_4H_4O_4$	115.0037	8.72	Isomer pair
Succinic acid	$C_4H_6O_4$	117.0193	9.78	
GMP	$C_{10}H_{14}N_5O_8P$	362.0507	9.79	
S-Malate	$C_4H_6O_5$	133.0137	10.01	
N-acetylglutamic acid	$C_7H_{11}NO_5$	188.0564	10.22	
Fumaric acid	$C_4H_4O_4$	115.0037	10.29	
α-Ketoglutarte	$C_5H_6O_5$	145.01373	10.31	
Citric acid	$C_6H_8O_7$	191.0197	13.73	Isomer pair
Salicylic acid	$C_7H_6O_3$	137.0239	14.21	
Isocitric acid	$C_6H_8O_7$	191.0197	14.28	
D-Fructose-1,6-bisphosphate (D-F-1,6-2P)	$C_6H_{14}O_{12}P_2$	338.9888	14.33	
GTP	$C_{10}H_{16}N_5O_{14}P_3$	521.9834	17.68	
FAD	$C_{27}H_{33}N_9O_{15}P_2$	784.1499	17.83	
ATP	$C_{10}H_{16}N_5O_{13}P_3$	505.9885	18.19	
NADPH	$C_{21}H_{30}N_7O_{17}P_3$	744.0828	18.60	
Succinyl CoA	$C_{25}H_{40}N_7O_{19}P_3S$	866.124	19.06	
Acetyl CoA	$C_{23}H_{38}N_7O_{17}P_3S$	808.1185	19.08	
Isobutyl CoA	$C_{25}H_{42}N_7O_{17}P_3S$	836.1498	19.49	
Glycocholic acid	$C_{26}H_{43}NO_6$	464.3018	20.72	

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

Changes to the pH of the mobile phase by adjusting the concentration of acetic acid can alter the RT and selectivity for sugar phosphates and nucleotide triphosphates (Figure 3). However, the signal intensities of a number of metabolite classes were impacted with this pH change (Figure 4).

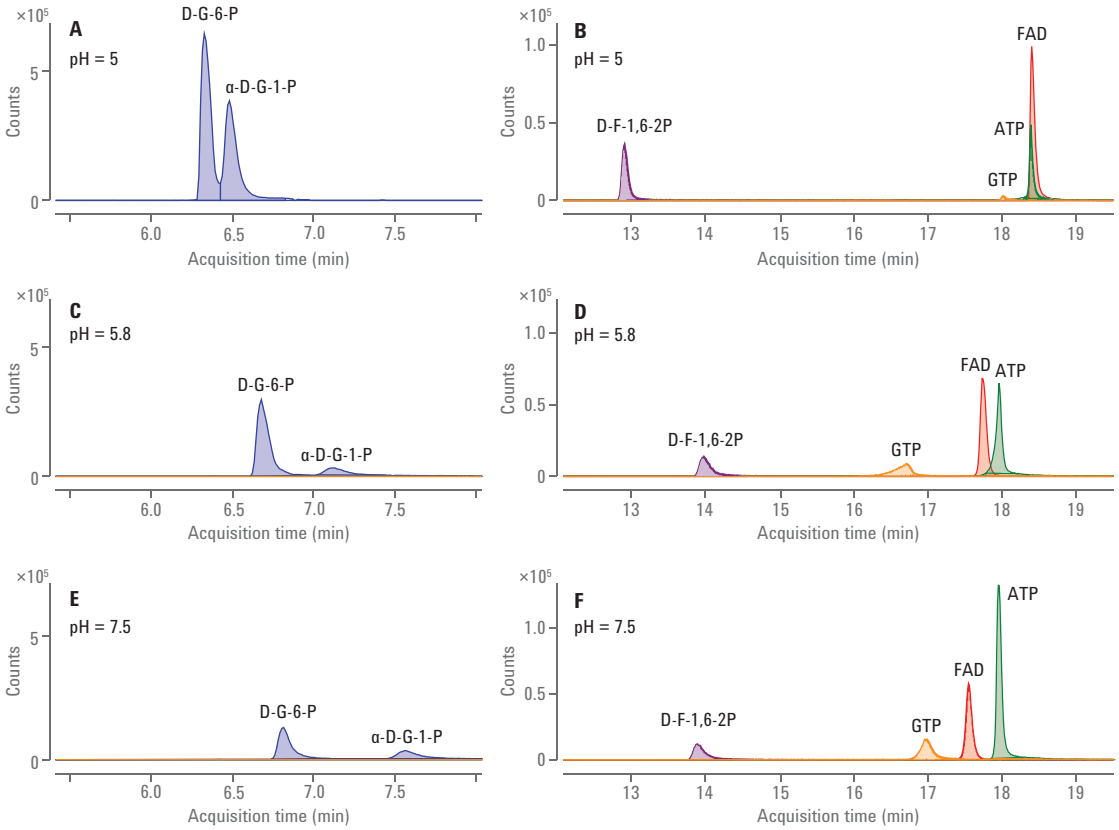

Figure 3. Effect of three mobile phase pH conditions on the RT and selectivity of some phosphorylated metabolites.

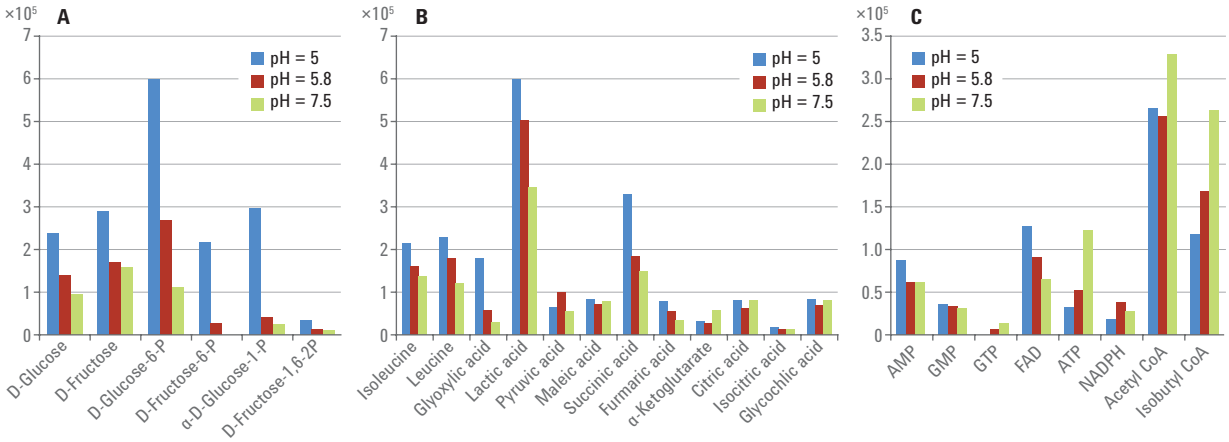

Figure 4. Effect of three mobile phase pH conditions on the signal responses of broad classes of endogenous metabolites.

The magnitude of the signal intensity changes and the direction of RT shifts are highly dependent on the metabolite chemical class. Results suggest that analytical selectivity and sensitivity for a specific class of metabolites can be improved by controlling the pH of the mobile phase in IP-RP chromatography. In this study, a mobile phase at pH 5.8 provided the best overall results for chromatographic separation of the isomers, with acceptable sensitivity among the different classes of metabolites.

Good RT reproducibility

Reproducibility is important for reliable differential analysis and confident identification in metabolomics studies. To ensure that the developed IP-RP LC/TOF MS method is robust and reliable, 19 representative metabolites covering the entire chromatographic region were selected for further evaluation. Table 4 shows that good reproducibility was achieved for RT. The Agilent UHPLC binary pump with seal wash increases robustness when using buffered mobile phases by flushing the back side of the seal, thus maintaining the seal lifetime.

Table 4. Reproducibility of RTs for the 19 representative metabolites.

Compound	Chemical formula	$(M-H)^-$	RT (Ave) (n = 3)	RT (%CV)) (n = 3)
D-Glucose	$C_6H_{12}O_6$	179.0561	1.47	0.42
D-Fructose	$C_6H_{12}O_6$	179.0561	1.50	0.29
Inosine	$C_{10}H_{12}N_4O_5$	267.0735	4.64	0.10
Guanosine	$C_{10}H_{13}N_5O_5$	282.0839	5.17	0.27
Glyoxylic acid	$C_2H_2O_3$	72.9931	6.38	0.08
Lactic acid	$C_3H_6O_3$	89.0244	6.49	0.08
NAD	$C_{21}H_{27}N_7O_{14}P_2$	662.1013	6.55	0.07
D-Fructose-6-phosphate	$C_6H_{13}O_9P$	259.0224	6.72	0.02
Pyruvic acid	$C_3H_4O_3$	87.0088	7.00	0.02
AMP	$C_{10}H_{14}N_5O_7P$	346.0558	8.69	0.11
GMP	$C_{10}H_{14}N_5O_8P$	362.0507	9.79	0.10
α-Ketoglutarte	$C_5H_6O_5$	145.01373	10.31	0.06
Citric acid	$C_6H_8O_7$	191.0197	13.73	0.09
GTP	$C_{10}H_{16}N_5O_{14}P_3$	521.9834	17.68	0.09
FAD	$C_{27}H_{33}N_9O_{15}P_2$	784.1499	17.83	0.03
ATP	$C_{10}H_{16}N_5O_{13}P_3$	505.9885	18.19	0.03
NADPH	$C_{21}H_{30}N_7O_{17}P_3$	744.0828	18.60	0.02
Acetyl CoA	$C_{23}H_{38}N_7O_{17}P_3S$	808.1185	19.08	0.03
Isobutyl CoA	$C_{25}H_{42}N_7O_{17}P_3S$	836.1498	19.49	0.01

Conclusions

These results demonstrate that this robust and reliable IP-RP method on the Agilent 6230 TOF LC/MS provides excellent analytical performance for metabolite profiling across a broad range of metabolite classes. Excellent chromatographic separation was achieved for four pairs of biologically relevant isomers: citrate/isocitrate, D-glucose-6-phosphate/α-D-glucose-1-phosphate, fumeric acid/maleic acid, and leucine/isoleucine. The Agilent 1290 Infinity LC system combined with the 6230 TOF system delivered excellent performance, making it an ideal system for metabolomics profiling.

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