Application Note

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Discovery Metabolomics LC/MS Methods Optimized for Polar Metabolites

Authors

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Abstract

High pH and low pH hydrophilic interaction chromatography (HILIC) methods were developed to provide comprehensive coverage of many common endogenous metabolite classes. The methods were developed for use with a high-resolution, accurate mass LC/Q-TOF mass spectrometer (MS). The metabolite classes targeted include vitamins, amino acids, polyamines, sugars, sugar phosphates, organic acids, nucleotides, and coenzyme A (CoA) derivatives. The LC gradient and ion source were systematically optimized to obtain good chromatographic separation of isomers and yield optimal signal response for all metabolite classes investigated. To demonstrate the robustness of this approach, the HILIC LC/Q-TOF MS methods were used to evaluate yeast extracts across 25 hours of run time.

Introduction

LC/Q-TOF MS is routinely used in metabolomics for discovery work with the goal of comprehensively characterizing small molecules within an organism. Untargeted mass spectrometry-based metabolomics yields a large number of molecular features. However, high-confidence annotation and identification of these features may not be made due to the challenges of the metabolomic identification process. The detected mass-to-charge (*m/z*) ratio signal can often be assigned to several proposed metabolites in curated metabolite databases. Thus, confident metabolite identification requires additional physical information such as retention time or MS/MS fragmentation spectra from an authentic standard.[1] Chromatography challenges for polar metabolites remain, including retention of ionic metabolites, chromatographic separation of biologically relevant isomers, and broad coverage of metabolite classes.

To address these challenges, high-pH and low-pH HILIC LC/Q-TOF MS methods were developed to improve the analytical performance for metabolome profiling. A wide range of metabolites (including vitamins, amino acids, polyamines, mono- and disaccharides, sugar phosphates, organic acids, nucleotides, CoA derivatives, and redox metabolites) were used as representative analytes for chromatographic method development. Moreover, the Agilent Jet Stream (AJS) source conditions were optimized to yield the best analytical sensitivity for the broad classes of metabolites investigated. Lastly, yeast extracts were used to demonstrate analytical performance such as reproducibility, mass accuracy, and isotopic fidelity of this method.

Experimental

Method

Metabolite standards covering different chemical classes and 20 pairs of biologically relevant isomers were selected for HILIC LC/Q-TOF MS method development. Chromatographic separation was performed on an Agilent InfinityLab Poroshell 120 HILIC-Z column, 2.1 mm × 150 mm, 2.7 µm, PEEK-lined (p/n 673775-924) using two different solvent systems, as described in Table 1.

LC/MS-grade acetonitrile and Milli-Q purified water were used for the LC/MS analysis. Detailed steps for solvent buffer preparation are shown in Table 1.

To minimize the binding of polar ionic metabolites to trace levels of metal in the system, InfinityLab Deactivator Additive (p/n 5191-4506) was added to the high-pH mobile phase.[2]

Table 1. Mobile phase preparation for LC/MS analysis.

	Ionization Polarity	10X Stock Buffer	Mobile Phase A	Mobile Phase B	Mobile Phase Additive
High pH Method	Negative ion mode (organic acids, sugar phosphates, nucleotides, CoA derivatives, etc.)	100 mM ammonium acetate, pH 9.0, adjusted with ammonium hydroxide	100 mL stock buffer was mixed with 900 mL water	100 mL stock buffer was mixed with 50 mL water and 850 mL ACN	Deactivator Additive was added to both mobile phase A and B to have a final 2.5 µM concentration
Low pH Method	Positive ion mode (amino acids, vitamins, polyamines)	100 mM ammonium formate	100 mL stock buffer was mixed with 900 mL water	100 mL stock buffer was mixed with 900 mL ACN	Formic acid was added to both mobile phase A and B to have a final 0.1% (v/v) concentration

Instrumentation

LC/MS analysis was performed using an Agilent 1290 Infinity II LC coupled to an Agilent 6545 LC/Q-TOF with an Agilent Jet Stream source. The LC consisted of:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler with Thermostat (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (MCT) (G7116B)

An isocratic pump (G1310A) with a 100:1 splitter (G1607-60000) was used for reference mass addition. Dynamic mass axis correction was achieved by continuous infusion of a reference mass solution. Tables 2 and 3 summarize the optimized LC and MS conditions. Data acquisition and analysis was done using the Agilent MassHunter software suite.

Table 2. Optimal LC parameters.

Agilent 1290 Infinity II LC System		
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 mm × 150 mm, 2.7 µm, PEEK-lined (p/n 673775-924)	
	Positive Ion Mode	**Negative Ion Mode**
Solvent	A) 10 mM ammonium formate in water with 0.1% FA B) 10 mM ammonium formate in water/ACN 10:90 (v:v) with 0.1% FA	A) 10 mM ammonium acetate in water with 2.5 µM InfinityLab Deactivator Additive, pH = 9 B) 10 mM ammonium acetate in water/acetonitrile 15:85 (v:v) with 2.5 µM InfinityLab Deactivator Additive, pH = 9
Nonlinear Gradient	Time (min) %B 0 98 3 98 11 70 12 60 16 5 18 5 19 98 20 98	Time (min) %B 0 96 2 96 5.5 88 8.5 88 9 86 14 86 17 82 23 65 24 65 24.5 96 26 96
Post Time	4 minutes	3 minutes
Column Temperature	25 °C	50 °C
Flow Rate	0.25 mL/min	
Injection Volume	3 µL	
Autosampler Temperature	4 °C	

Table 3. Optimized MS parameters.

Agilent 6545 LC/Q-TOF		
Ionization Polarity	Positive	Negative
Ionization Mode	Dual AJS	Dual AJS
Gas Temperature	225 °C	225 °C
Drying Gas	6 L/min	13 L/min
Nebulizer Pressure	40 psi	35 psi
Sheath Gas Temperature	225 °C	350 °C
Sheath Gas Flow	10 L/min	12 L/min
Capillary Voltage	3,000 V	3,500 V
Nozzle Voltage	0 V	0 V
Fragmentor	125 V	125 V
Skimmer	65 V	45 V
Octopole 1 RF Voltage	450 V	750 V
Acquisition Range	*m/z* 50 to 1,000	*m/z* 60 to 1,600
MS acquisition Rate	1 spectrum/sec	1 spectrum/sec
Reference Mass	*m/z* 922.009798	*m/z* 68.9957 *m/z* 980.0163

Results and discussion

Two robust and high-performance HILIC LC/MS methods were developed on the Agilent HILIC-Z column. The advantages of using the HILIC-Z column include excellent analyte peak shape, high salt tolerance, good chromatographic retention time reproducibility, and wide pH stability.[3] To ensure comprehensive coverage of broad metabolite classes, positive ion and negative ion mode methods were developed. A low-pH mobile phase buffer was optimized for positive ion mode to facilitate the detection of three major metabolite classes encompassing vitamins, amino acids, and polyamines (Figure 1). Similarly, a high-pH mobile phase buffer was optimized for negative ion mode using seven major metabolite classes including amino acids, sugars, sugar phosphates, organic acids, nucleotides, energy and redox metabolites, and CoA derivatives (Figure 2). To explore the option of using a single mobile phase condition for both positive and negative ion mode analyses, we investigated the use of high-pH mobile phase buffer for the detection of amino acids, vitamins, and polyamines in positive ion mode. The results agreed with previously published data showing that amino acids, vitamins, and polyamines yielded better peak shape and signal intensity with low-pH mobile phase.[3] Thus, the use of high- and low-pH methods maximizes the coverage of metabolite classes and analytical sensitivity.

These optimized methods chromatographically separate 15 pairs of biologically relevant isomers (examples are shown in Figure 3). These included the separation of leucine (Leu) and isoleucine (Ile), 2-phosphoglycerate (2-PG) and 3-phosphoglycerate (3-PG), isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), fructose, glucose, and inositol, glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P), and 5'-ADP and 3',5'-ADP (Figure 3). Tables 4 and 5 show the complete list of structural isomers and metabolites with similar masses that were investigated.

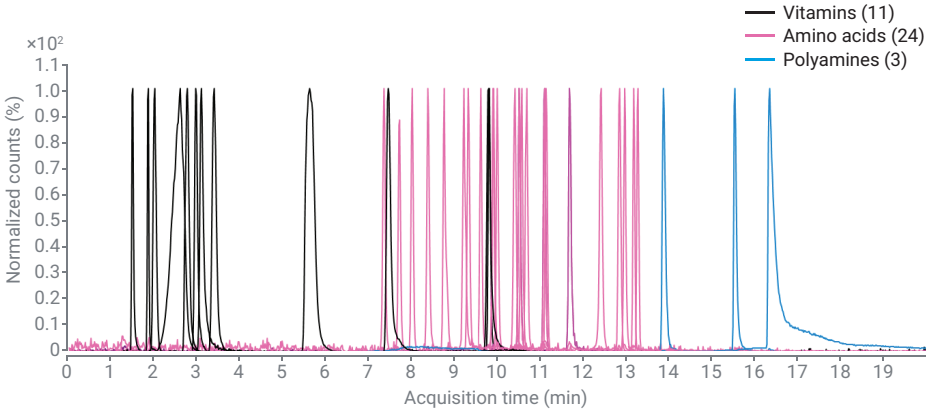

Figure 1. Overlaid extracted ion chromatograms (EICs) of 38 metabolite standards using the low-pH mobile buffer in positive analysis mode.

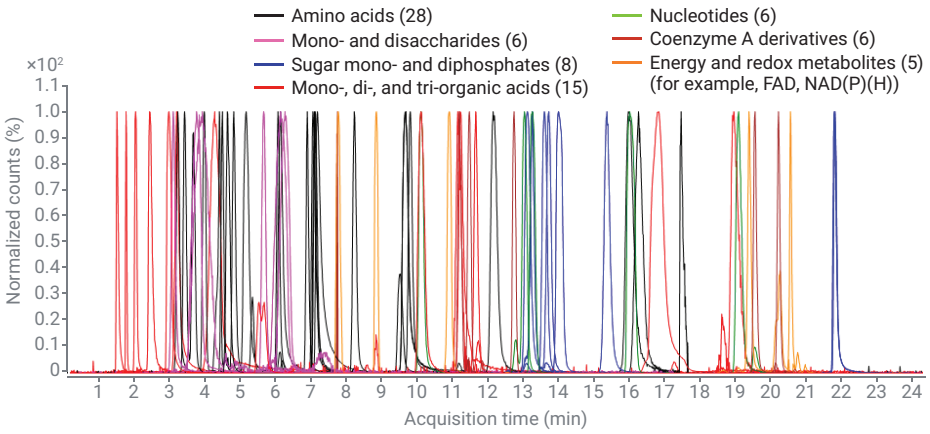

Figure 2. Overlaid EICs of 74 metabolite standards using the pH 9.0 mobile phase buffer in negative analysis mode.

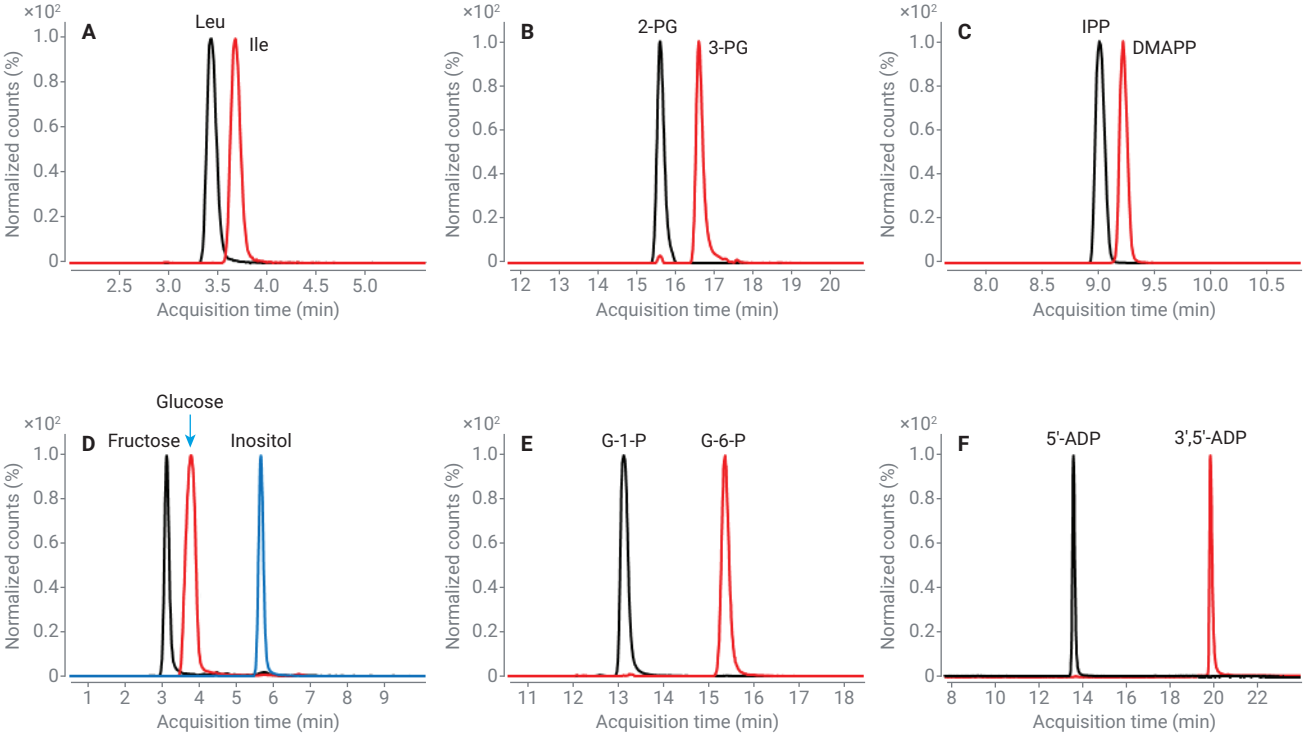

Figure 3. Overlaid EICs showed chromatographic separations of six groups of biologically relevant isomers.

Table 4. Separation of structural isomers.

No.	Metabolites	Chemical Formula	Mol. Wt.	Polarity	Separation
1	β-Alanine/L-alanine/L-sarcosine	$C_3H_7NO_2$	89.0477	Pos/Neg	Separated
2	L-α-Amino-*n*-butyric acid/D, L-β-aminoisobutyric acid	$C_4H_9NO_2$	103.0633	Pos/Neg	Separated
3	Maleic acid/fumaric acid	$C_4H_4O_4$	116.0110	Neg	Separated
4	Methylmalonic acid/succinic acid	$C_4H_6O_4$	118.0266	Neg	Separated
5	L-Leucine/L-isoleucine	$C_6H_{13}NO_2$	131.0946	Pos/Neg	Separated
6	O-Acetyl-L-serine/L-glutamate	$C_5H_9NO_4$	147.0532	Neg	Separated
7	Dihydroxyacetone phosphate/glyceraldehyde-3-phosphate	$C_3H_7O_6P$	169.9980	Neg	Separated
8	*cis*-Aconitate/*trans*-aconitate	$C_6H_6O_6$	174.0164	Neg	Separated
9	Fructose/glucose or galactose/inositol	$C_6H_{12}O_6$	180.0634	Neg	Separated
10	Glucose/galactose	$C_6H_{12}O_6$	180.0634	Neg	Coeluted
11	2-phosphoglycerate/3-phosphoglycerate	$C_3H_7O_7P$	185.9929	Neg	Separated
12	Citric acid/iso-citric acid	$C_6H_8O_7$	192.0270	Neg	Partially separated
13	Isopentenyl pyrophosphate (IPP)/dimethylallyl pyrophosphate (DMAPP)	$C_5H_{12}O_7P_2$	246.0058	Neg	Separated
14	α-Glucose-1-phosphate/D-glucose-6-phosphate	$C_6H_{13}O_9P$	260.0297	Neg	Separated
15	Fructose-6-phosphate/fructose-1-phosphate	$C_6H_{13}O_9P$	260.0297	Neg	Separated
16	α-D-Mannose-1-phosphate/D-mannose-6-phosphate	$C_6H_{13}O_9P$	260.0297	Neg	Coeluted
17	Fructose-1,6-bisphosphate/fructose-2,6-bisphosphate	$C_6H_{14}O_{12}P_2$	339.9960	Neg	Coeluted
18	Maltose/α-lactose	$C_{12}H_{22}O_{11}$	342.1162	Neg	Coeluted
19	Adenosine-5'-diphosphate (5'-ADP)/adenosine-3',5'-diphosphate (3'5'-ADP)	$C_{10}H_{15}N_5O_{10}P_2$	427.0294	Neg	Separated
20	Methylmalonyl CoA/succinyl CoA	$C_{25}H_{40}N_7O_{19}P_3S$	867.1313	Neg	Separated

As part of the method optimization, the AJS conditions were optimized to achieve the best overall analytical sensitivity for metabolites representing each chemical class. Figure 4 shows that changes to the drying gas and sheath gas temperatures had the largest impact on the detection of the standard mixes. For example, in positive ion mode analysis with low-pH mobile phase buffer, the peak height for all analytes was reduced as drying gas

Table 5. Separation of metabolites with near isobaric masses.

Group	Metabolite	Chemical Formula	Mol. Wt.	Polarity	Separation
1	Glutaric acid	$C_5H_8O_4$	132.0423	Neg	Separated
	L-Asparagine	$C_4H_8N_2O_3$	132.0535	Neg/Pos	Separated
	L-Ornithine	$C_5H_{12}N_2O_2$	132.0899	Neg/Pos	Separated
2	α-Ketoglutarate	$C_5H_6O_5$	146.0215	Neg	Separated
	L-Glutamine	$C_5H_{10}N_2O_3$	146.0691	Neg/Pos	Separated
	L-Lysine	$C_6H_{14}N_2O_2$	146.1055	Neg/Pos	Separated
3	*cis*-Aconitate	$C_6H_6O_6$	174.0164	Neg	Separated
	L-Arginine	$C_6H_{14}N_4O_2$	174.1117	Neg/Pos	Separated
	N-Acetyl-ornithine	$C_7H_{14}N_2O_3$	174.2000	Pos	Separated

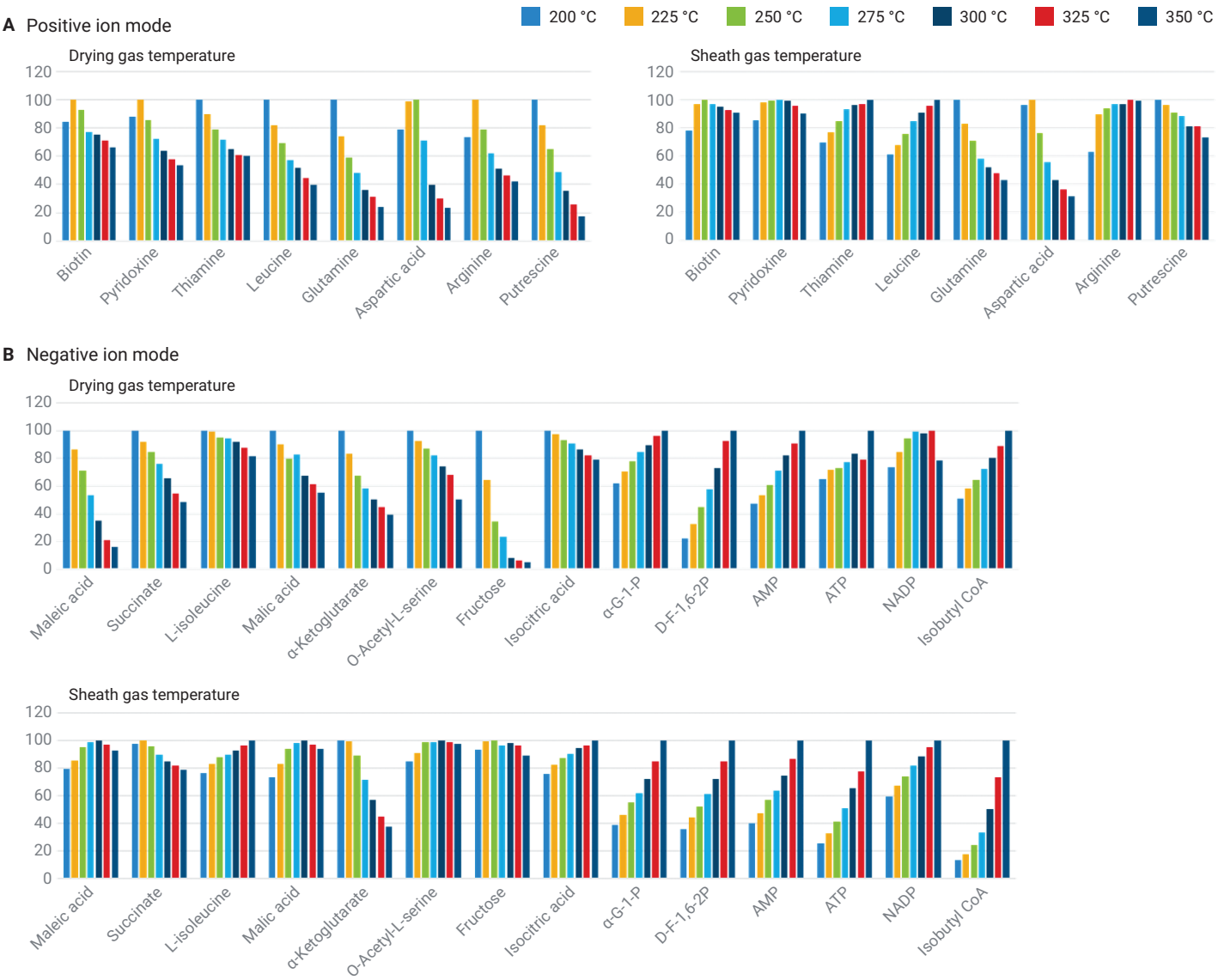

Figure 4. Optimization of AJS source parameters for HILIC LC/MS methods in both (A) positive and (B) negative ion mode. Examples of drying gas and sheath gas temperature optimization results are shown.

temperature increased from 225 to 350 °C (Figure 4A, left panel). Conversely, the response for most analytes improved as sheath gas temperature increased, except for glutamine, aspartic acid, and putrescine (Figure 4A, right panel). In negative ion mode analysis with pH 9 mobile phase buffer, the response for organic acids, amino acids, and sugars was significantly reduced as drying gas temperature increased from 200 to 350 °C (Figure 4B). However, phosphate-containing molecules including sugar phosphates, nucleotides, redox metabolites, and CoA derivatives showed improved response as drying gas temperature increased (Figure 4B). Modifications to the sheath gas temperature also yielded similar trends for the metabolites analyzed with the negative ion mode method. The final drying and sheath gas temperatures shown in Table 3 were chosen for the optimized methods to encompass all metabolite classes that were tested, but users may choose to adjust their source parameters based on the specific metabolite classes of interest.

To demonstrate the robustness of this HIILC LC/MS method for analyzing biological samples, yeast extracts were evaluated with the optimized pH 9 method in negative ion mode as this method covers the major metabolite classes commonly studied. Figure 5 shows EICs of representative metabolites from yeast extracts.

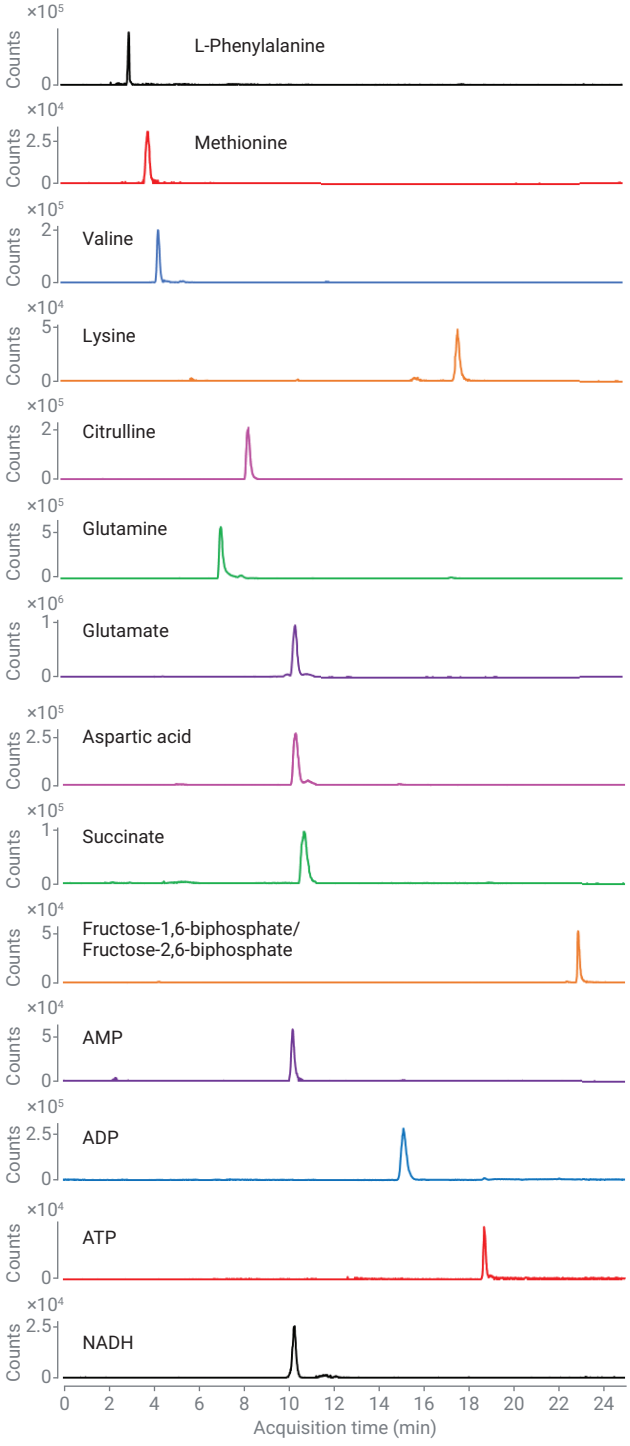

Figure 5. EICs of 14 representative metabolites from yeast extract using the optimized pH 9 method in negative ion mode.

The stability of chromatographic retention time, signal response, and mass accuracy were evaluated for a targeted list of 45 metabolites (Figures 6 and 7). The results demonstrated excellent reproducibility for both retention time (%RSD <1.1%) and signal response (%RSD <15%) across 25 hours of run time for 45 representative endogenous metabolites detected in the yeast extract (Figure 6). Moreover, excellent mass accuracy and precision over this time were achieved with the average absolute mass error ≤1.6 ppm and RSD of ≤1.2 ppm (Figure 7), demonstrating the stability of the instrument and the HILIC LC/MS method over this analytical timeframe.

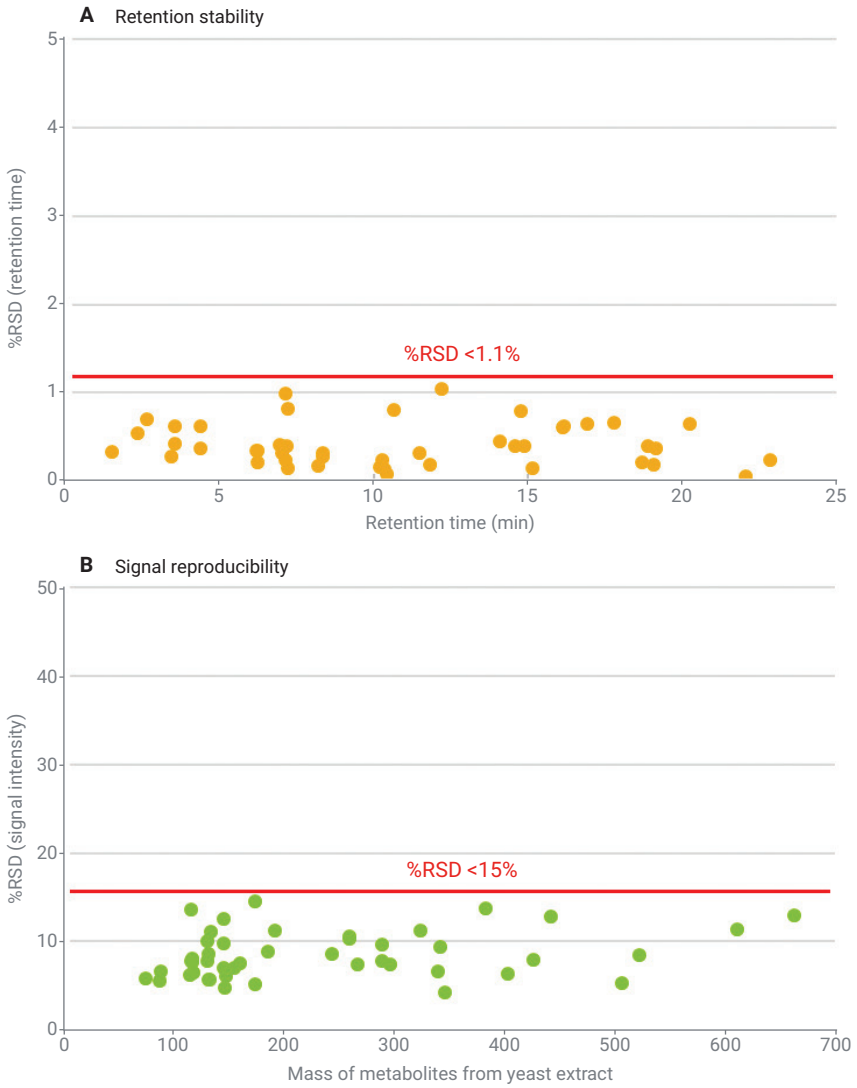

Figure 6. Excellent reproducibility of retention time and signal intensity across 25 hours of run time for metabolites extracted from yeast. The (A) % RSD of retention time was <1.1% and (B) %RSD of signal intensity was <15% (n = 45 metabolites) across 50 analytical runs in negative ion mode.

Conclusion

By optimizing the chromatographic conditions, superior analytical selectivity was achieved for important biological isomers, and broad classes of metabolites were detected. Baseline separation was achieved for 15 pairs/groups of biologically relevant isomers and three groups of metabolites with similar masses. The AJS parameters were also systematically optimized to yield the best overall analytical sensitivity. Lastly, the robustness of the chromatographic method and the LC/MS instrument was demonstrated by analyzing biological samples across 50 injections covering a span of 25 hours of analysis time. The Agilent LC/MS system delivered excellent performance, making it an outstanding system for metabolomics profiling.

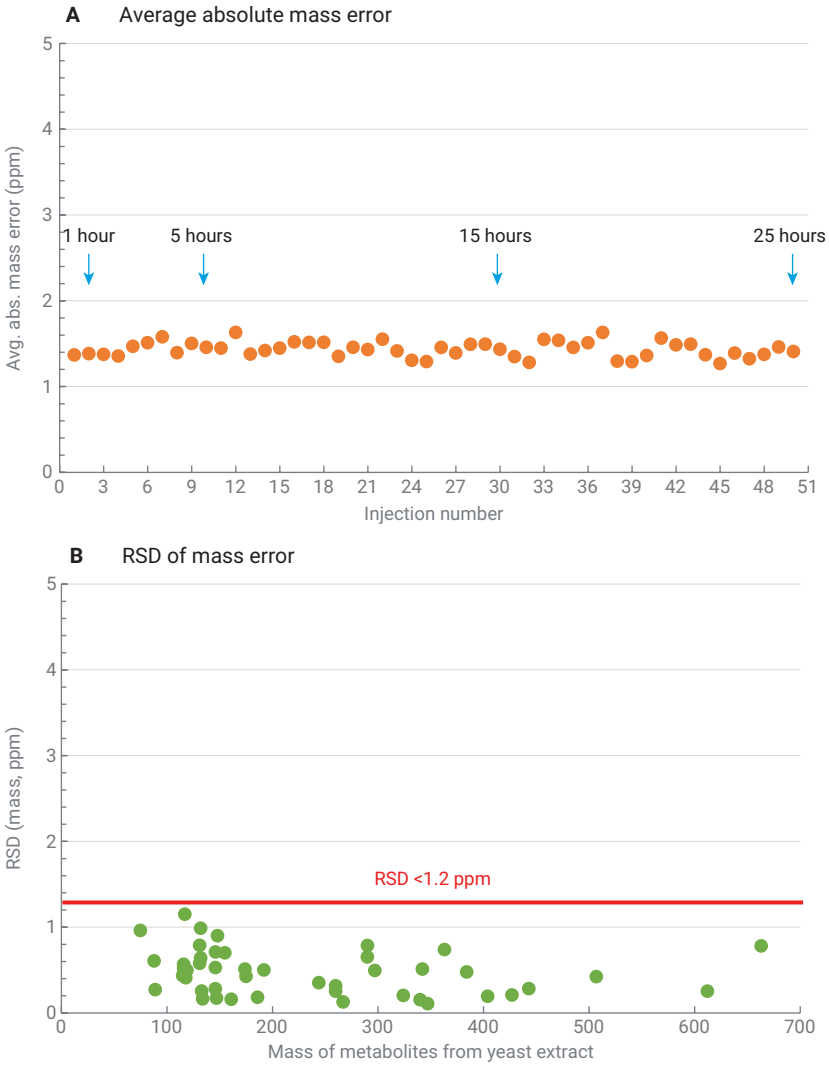

Figure 7. Excellent mass accuracy stability across 25 hours of run time for metabolites extracted from yeast. The (A) average absolute mass error was ≤1.6 ppm and (B) RSD of mass error was ≤1.2 ppm (n = 45 metabolites) across 50 analytical runs in negative ion mode.

References

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