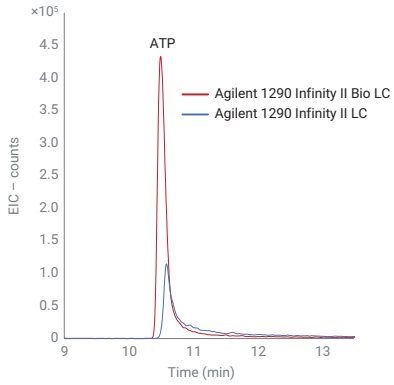
Application Note

Biopharma/Pharma

Improved Metabolomic Analysis Using an Iron-Free Flow Path

Comparing the Agilent 1290 Infinity II Bio LC System and Agilent 1290 Infinity II LC System for metabolomics



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Abstract

Metabolomics is a tool to decipher and understand the physiological state of a cell or organism. Recently, LC/MS emerged as the prevalent analytical technique of choice. However, there is still significant potential for improved robustness and ease of use. This application note demostrates that a biocompatible flow path has significant advantages over stainless steel. These advantages were proven by comparing the Agilent 1290 Infinity II Bio LC and Agilent 1290 Infinity II LC. The 1290 Infinity II Bio LC showed improved peak shape and resolution for phosphorylated compounds such as nucleotides and sugar phosphates without apparent adsorption effects, compared to the 1290 Infinity II LC. Excellent retention time RSD values of 0.1% were generated for intracellular metabolite extracts derived from Saccharomyces cerevisiae. These data suggest that essential metabolites for physiological parameters such as the adenylate energy charge could be analyzed consistently. Together, these results show that the 1290 Infinity II Bio LC is the perfect choice for a seamless and robust analysis in metabolomics.

Introduction

The scientific field called metabolomics describes the chemical processes involving metabolites in a biological cell, tissue, organ, or organism. As one of the holistic systems biology relevant omics techniques, such as genomics and proteomics, metabolomics covers the intersection of all regulatory mechanisms and represents the physiological state and phenotype of the cell as close as possible.[1] State-of-the-art analytical tools range from nuclear magnetic resonance spectroscopy (NMR) to electrospray ionization mass spectrometry (ESI-MS) coupled to gas (GC) or liquid chromatography (LC). LC/MS methods based on hydrophilic interaction chromatography (HILIC) are regarded as the gold standard for comprehensive metabolomics studies.[2] However, classical HPLC systems based on stainless steel can cause severe peak tailing and adsorption for metabolites such as nucleotides or phosphorylated sugars. These unwanted side effects were historically mitigated by passivation of the whole LC system or by adjusting the mobile phase with iron-chelating compounds.[3,4] Unfortunately, these solutions also have disadvantages. The passivation must be re-applied regularly and can lead to reduced system lifetime, and mobile additives can cause unwanted MS ion suppression. Therefore, the LC system of choice for metabolomics is completely iron-free, rendering any special treatments unnecessary. In this application note, the biocompatible Agilent 1290 Infinity II Bio LC will be evaluated for use in metabolomics compared to an equivalent stainless-steel-based LC system. The iron-free flow path of the 1290 Infinity II Bio LC will demonstrate superior performance for the analysis of phosphorylated compounds.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System contained the following modules:

- Agilent 1290 Infinity II Bio Flexible Pump (G7131A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Agilent InfinityLab Sample Thermostat (option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with an Agilent Quick Connect Bio Heat Exchanger Std. (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 6546 LC/Q-TOF (G6546A)

The Agilent 1290 Infinity II LC System contained the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler (G7167A) with Agilent InfinityLab Sample Thermostat (option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with an Agilent InfinityLab Quick Connect Heat Exchanger Std. (G7116-60015) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 6546 LC/Q-TOF (G6546A)

Software

- Agilent MassHunter Workstation Software (B.09.00 or later)
- Agilent MassHunter Qualitative Analysis (10.0 or later)

Column

Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm, PEEK-lined (part number 675775-924)

Chemicals

Agilent InfinityLab Ultrapure LC/MS acetonitrile (part number 5191-4496) and Agilent InfinityLab Ultrapure LC/MS water (part number 5191-4498) were used. Ammonium acetate and ammonium hydroxide were obtained from VWR (Darmstadt, Germany). All metabolites standards (see abbreviations table in the appendix) were purchased from Sigma-Aldrich (Steinheim, Germany).

Cultivation and sampling

The haploid, prototrophic Saccharomyces cerevisiae model strain CEN.PK 113-7D was cultivated aerobically in a 3 L stainless steel benchtop bioreactor (Bioengineering, Wald, Switzerland) in a synthetic medium. The process was operated as a continuous fermentation under glucose limitation with a dilution rate of 0.1 h^{-1}. A steady-state reference sample (time point 0 s) was drawn when the off-gas signals and the biomass concentration were constant for five residence times. Next, a starvation stimulus was imposed on the culture by switching off the glucose feed for 120 seconds, and six samples were drawn during this time window (20 to 130 s).

Sampling was realized with a custom-made, semi-automated sampling device allowing for rapid withdrawal of precise volumes (error was less than 2% for 1 to 5 mL volumes). In this experiment, samples were quenched by directly collecting 1.5 mL broth into 10 mL of –40 °C methanol within 1 second. Next, samples were subjected to a centrifugation step at 5,000 g for 5 minutes at –11 °C without further washing. The resulting pellets were directly flash frozen.

For extraction, the frozen pellets were resuspended in 1 mL of –28 °C extraction buffer consisting of 50% (v/v) methanol, 100 mM ammonium acetate (pH 9.2, adjusted with ammonium

hydroxide), 100 µM L-norvaline, and 2.5 mM 3-mercaptopropionic acid. The sample temperature was kept below –20 °C by constant vortexing and intermediate chilling in a –40 °C cryostat. Entirely resuspended samples were then mixed with 1 mL of –28 °C chloroform, vortexed vigorously, and placed in a rotary overhead shaker for 3 hours. Extracts were centrifuged for 10 minutes at 20,000 g and 4 °C. The upper aqueous methanol phase with all polar metabolites was collected and stored at –70 °C.

Sample preparation

Metabolite samples were prepared in 60% acetonitrile and 10 mM ammonium acetate pH 9. Yeast-derived metabolite extracts were additionally diluted 1:10.

Table 1. LC method for metabolomics analysis with the Agilent 1290 Infinity II Bio LC and Agilent 1290 Infinity II LC.

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm, PEEK-lined
Solvent	A) Acetonitrile B) Water C) 500 mM ammonium acetate, pH 9
Gradient	Time (min) A (%) B (%) C (%) 0.00 80 18 2 0.66 80 18 2 13.50 60 38 2 13.51 10 88 2 14.50 10 88 2 14.51 80 18 2 20.00 80 18 2
Flow Rate	0.300 mL/min
Temperature	40 °C with Agilent Thermal Equilibration Devices (G7116-60013) installed
MS Detection	See Table 2
Injection	Injection volume: 1 µL Sample temperature: 4 °C Wash: 3 s with acetonitrile:water 9:1 (flush port)

Table 2. Source and MS parameters for metabolomics analysis with the Agilent 1290 Infinity II Bio LC and Agilent 1290 Infinity II LC.

Parameter	Value
Instrument	Agilent 6546 LC/Q-TOF
Gas Temperature	320 °C
Drying Gas Flow	8 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
VCap	3,500 V
Nozzle Voltage	1,000 V
Fragmentor	125 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Negative, low (*m/z* 1,700) mass range
Mass Range	*m/z* 25 to 1,700
Acquisition Rate	2 spectra/s
Reference Mass	*m/z* 112.985587, *m/z* 1,033.988109

Results and discussion

Using a stainless steel HPLC system for metabolomics can have adverse effects due to adsorption of the analytes on the iron surface and building iron complexes, which can impair MS data. Adsorption takes place immediately after injection of the sample. Therefore, the injection needle, needle seat, and sample loop are most susceptible to adsorption. To investigate the differences in adsorption between the 1290 Infinity II Bio LC and the Infinity II 1290 LC, a brand new needle, needle seat, and sample loop were installed on both systems to mitigate injection passivation effects. The PEEK-lined InfinityLab Poroshell 120 HILIC-Z column was used to exclude any iron interaction in the column, leaving only the LC system for possible adsorption effects. Immediately after replacement, two consecutive injections of various metabolite standards were performed on both systems using the exact same column (see list of all metabolites in the abbreviations table in the appendix). Figure 1 shows the chromatograms of those injections. Strikingly, there was an area difference of 33% (ATP) and 38% (GTP) between the first and second injection on the 1290 Infinity II LC, pointing towards heavy adsorption on the metal surface. In comparison, the 1290 Infinity II Bio LC only showed 2% (ATP) and 3% (GTP) area deviations between the injections, which is in the range of the method precision.

To further investigate adsorption differences between these systems, 30 metabolites were also analyzed with this experimental layout. The differences are depicted in percentage in Figure 2 for all metabolites. The results demonstrate that the 1290 Infinity II Bio LC was superior. No adsorption was observed on the 1290 Infinity II Bio LC,

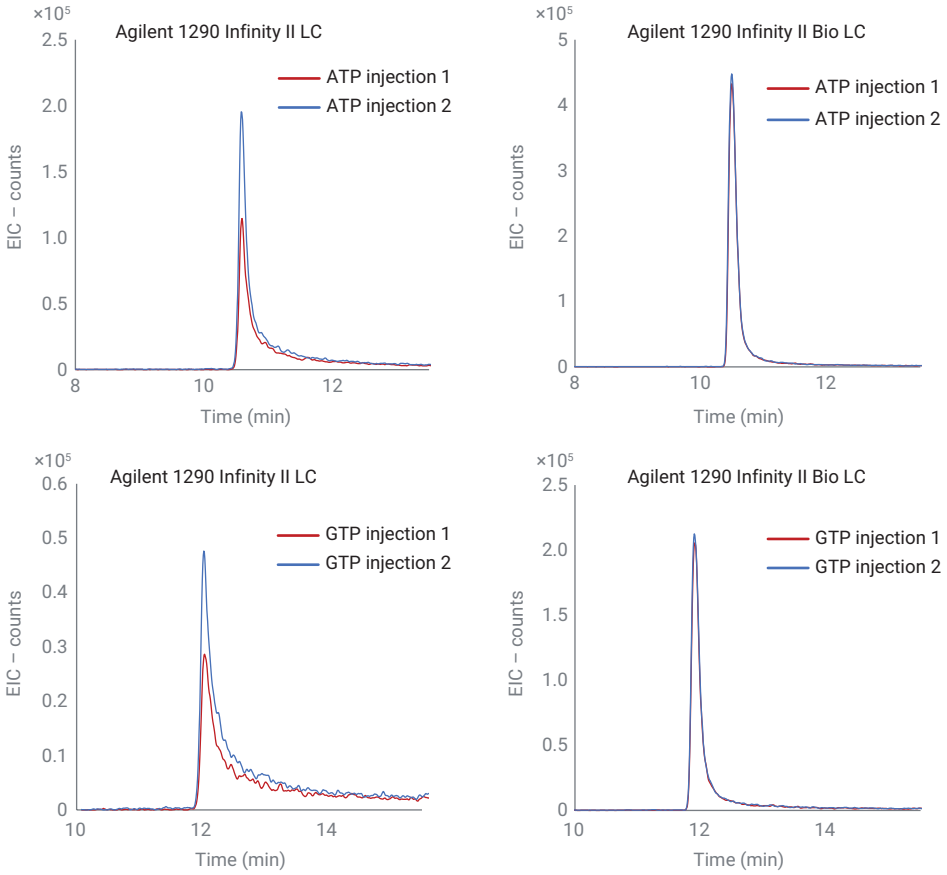

Figure 1. Chromatograms from ATP adsorption experiments (upper), and chromatograms from GTP adsorption experiments (lower).

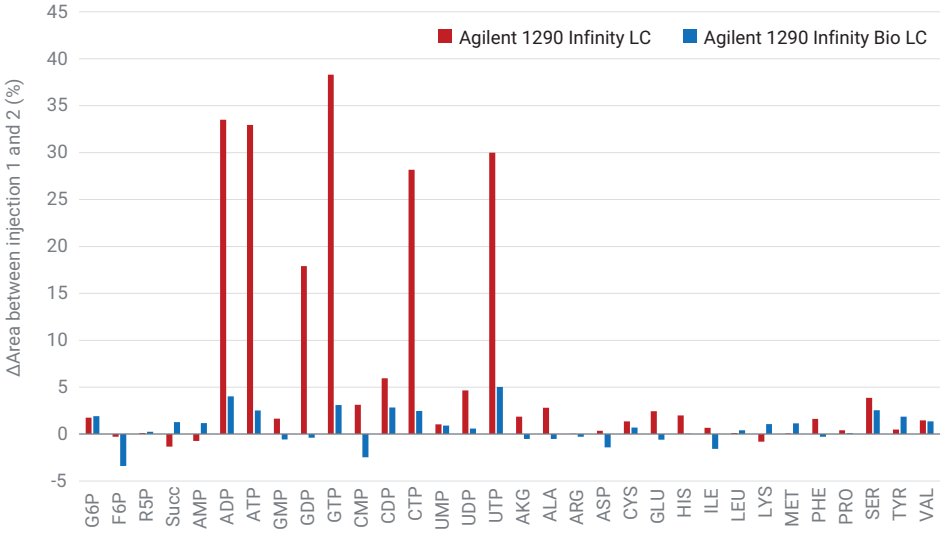

Figure 2. Area differences between the first and second injections on a new injection flow path for 32 metabolites.

especially for highly phosphorylated nucleotides such as ATP, GTP, CTP, and UTP, rendering it the best choice for metabolomics analysis.

In addition to the adsorption of analytes due to the iron surface, tailing and bad peak shape caused by secondary interactions with the LC system can also be common problems in metabolomics. To better understand and compare peak shape, the previously shown chromatograms of ATP were scaled and depicted in Figure 3.

The side-by-side comparison of the 1290 Infinity II LC (SST LC) and the 1290 Infinity II Bio LC (Bio LC) shows significant tailing using the stainless steel LC compared to the biocompatible version. The adsorption described in the previous graphs increases tailing even though the column is PEEK lined. Next, tailing factors (T_f) for the phosphorylated metabolites were calculated to quantify the differences between the LC systems, which can be seen in Table 3. The 1290 Infinity II Bio LC has better tailing factors for all the analyzed metabolites, with deviations between 0.1 to 2.7 T_f units.

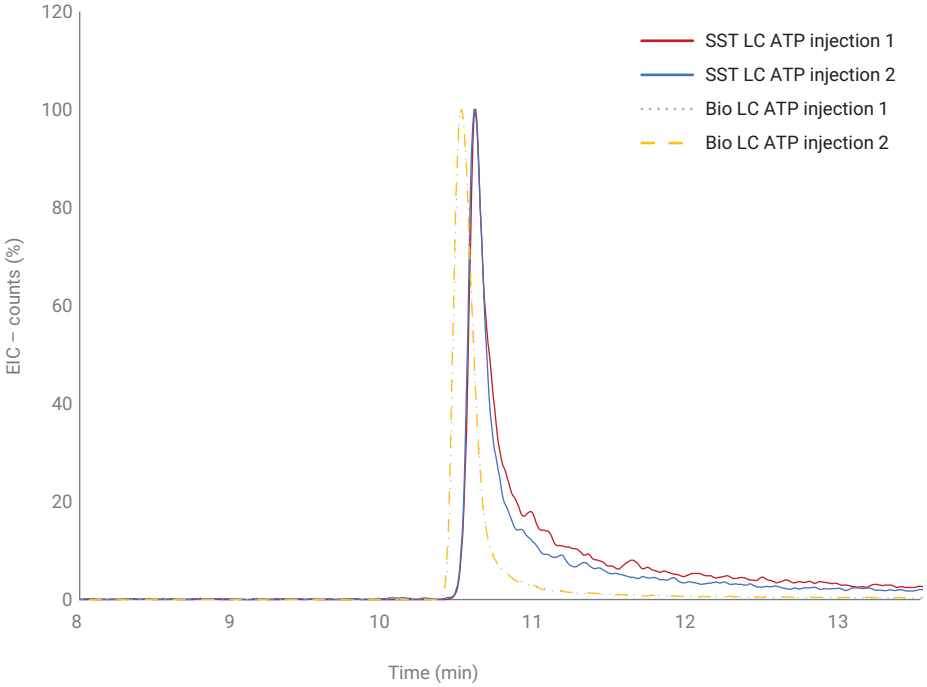

Figure 3. Scaled chromatograms of ATP on the Agilent 1290 Infinity II Bio LC and Agilent 1290 Infinity II LC.

Table 3. Tailing factors for phosphorylated metabolites on the Agilent 1290 Infinity II Bio LC and Agilent 1290 Infinity II LC.

Metabolites	Tailing Factor (T_f)		
	Agilent 1290 Infinity II LC	Agilent 1290 Infinity II Bio LC	ΔT_f
G6P	2.8	2.5	0.4
F6P	3.5	3.0	0.5
R5P	3.1	2.7	0.4
AMP	3.1	2.6	0.5
ADP	3.4	2.2	1.2
ATP	4.0	2.3	1.7
GMP	2.6	2.2	0.4
GDP	5.3	2.6	2.7
GTP	4.3	2.5	1.8
CMP	3.0	2.4	0.6
CDP	3.2	2.8	0.4
CTP	3.4	2.3	1.1
UMP	3.4	2.4	1.1
UDP	2.4	2.3	0.1
UTP	2.3	1.9	0.4

Metabolomics relies on MS detection in most analytical cases. So, peak shape and resolution are not always the top priorities for chemists, as there is usually another level of selectivity through mass detection. However, several isobaric metabolites such as G6P and F6P or leucine and isoleucine rely on proper LC separation for comprehensive analysis. For example, Figure 4 shows the two isobaric sugar phosphates, G6P and F6P, as standards analyzed with both systems.

The 1290 Infinity II LC cannot baseline separate the two sugar phosphates due to the adsorption and peak tailing caused by the iron-based flow path. In comparison, the 1290 Infinity II Bio LC can separate them with sufficient resolution for metabolomics analysis.

All previous data were generated with analytical standards for controlled in-depth analysis. However, real-life metabolomics analysis uses one of the most complex samples in chemical analysis, intra- or extracellular metabolite extracts. These extracts can consist of thousands of different metabolites, e.g., the laboratory *Escherichia coli* K-12 strain contains around 1,500 intracellular small molecules.[5] To investigate the ability of the Agilent 1290 Infinity II Bio LC to tackle complex real-life matrices, authentic intracellular *Saccharomyces cerevisiae* metabolite extracts were analyzed. An overview of all chromatograms for G6P, F6P, AMP, ADP, and ATP can be seen in Figures 5 and 6.

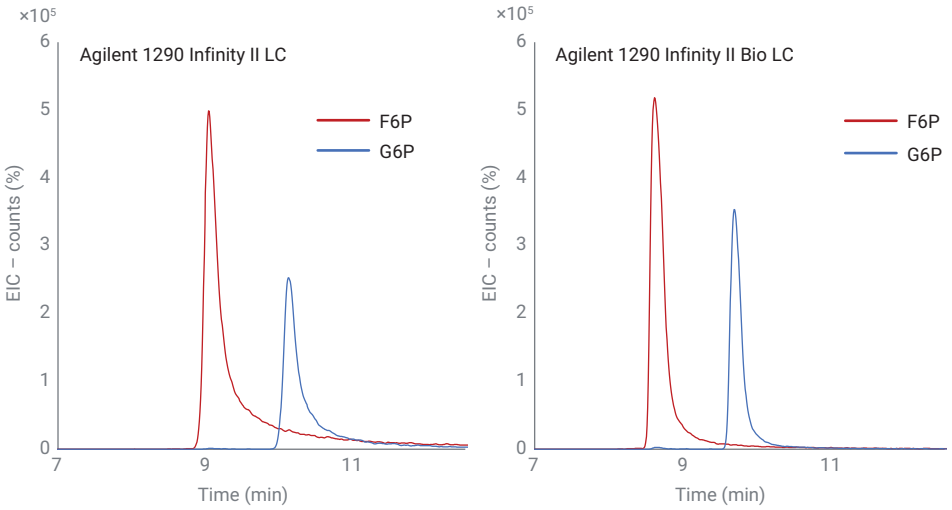

Figure 4. Separation of the isobaric sugar phosphates G6P and F6P on the Agilent 1290 Infinity II LC and Agilent 1290 Infinity II Bio LC.

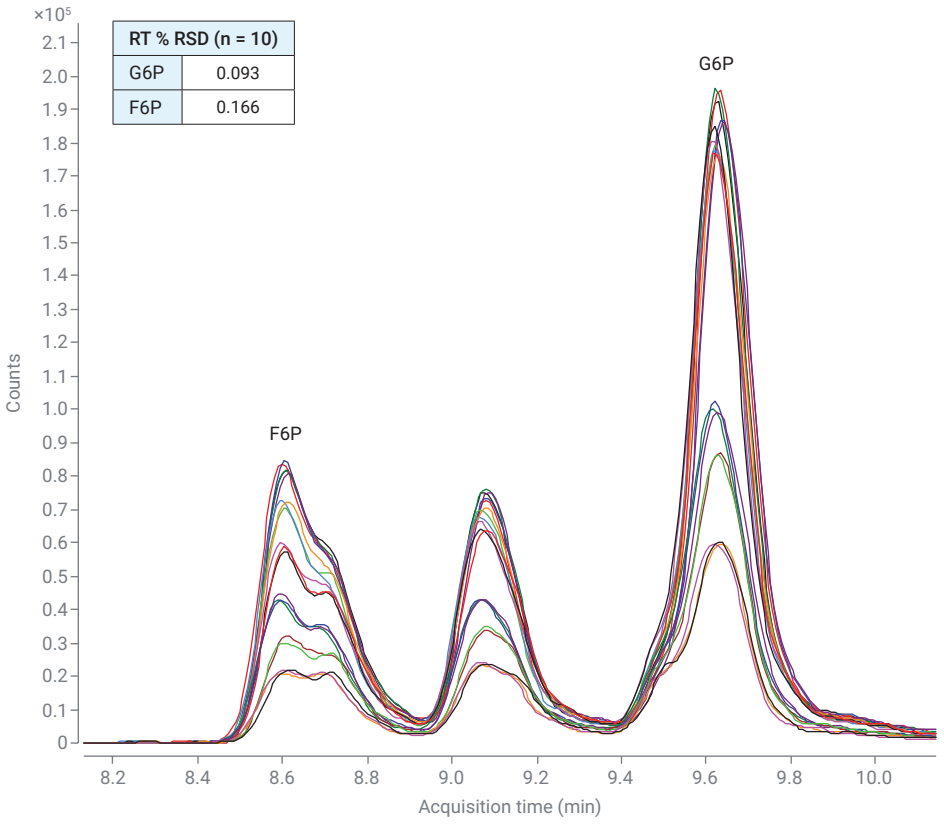

Figure 5. Overlay of the dynamic EICs for G6P and F6P on the Agilent 1290 Infinity II Bio LC, showcasing the excellent retention time (RT) precision.

Based on 10 consecutive injections, relative standard deviations (RSDs) were calculated and are depicted in the corresponding tables within the figures. Excellent RSD values around 0.1% deviation were determined, proving the exceptional performance of the 1290 Infinity II Bio LC for metabolomics analysis in complex matrices. Also, isobaric peaks for G6P and F6P could be resolved thanks to the excellent peak shape and resolution, enabling an even more pronounced analysis of intracellular hexose phosphates. This analysis is especially important since those metabolites constitute the first crossroad of metabolism after sugar consumption.

All AxP species could be analyzed with similar peak shapes no matter the phosphorylation extent. ATP, ADP, and AMP reflect the energetic status of the cell through the adenylate energy charge.[6] Therefore, robust analysis of these analytes is mandatory for high-quality data concerning the cell's physiology.

Since the metabolite extracts were taken from continuous yeast cultivation under glucose limitation/starvation, characteristic dynamic metabolite profiles could be identified in Figure 7. Due to the glucose starvation, the area intensity of G6P and F6P declines over time, as well as the amount of ATP, as the cell's energy equivalent. However, AMP and ADP increase as rephosphorylation slows down due to glucose starvation.

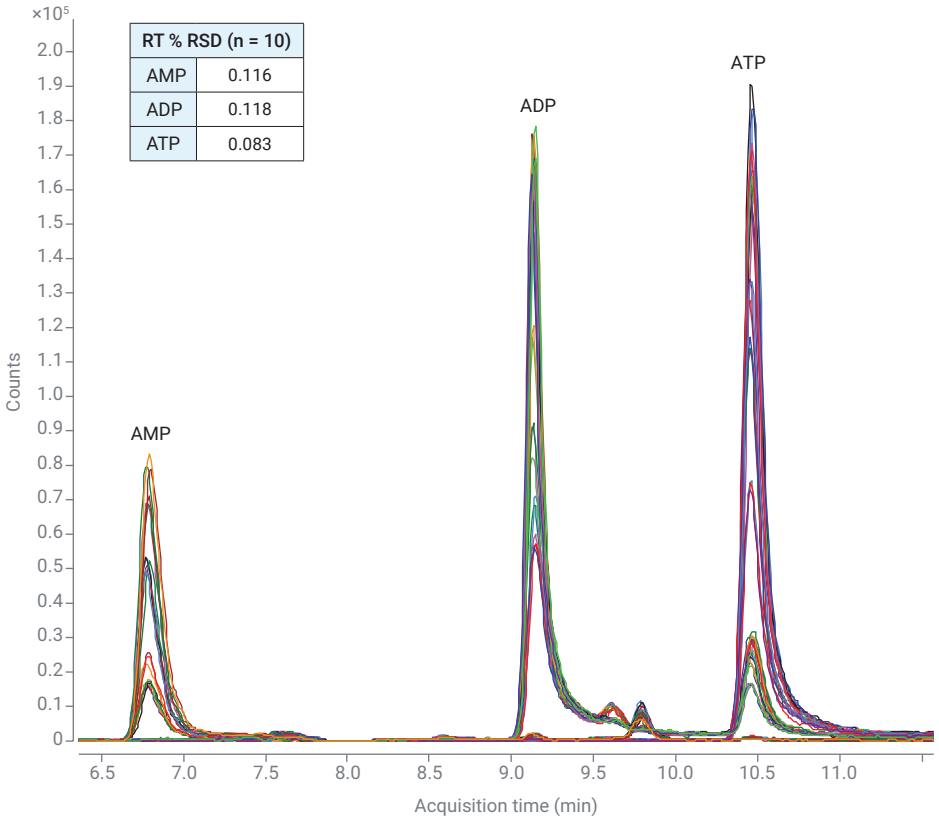

Figure 6. Overlay of the dynamic EICs for AMP, ADP, and ATP on the Agilent 1290 Infinity II Bio LC, showcasing the excellent retention time (RT) precision.

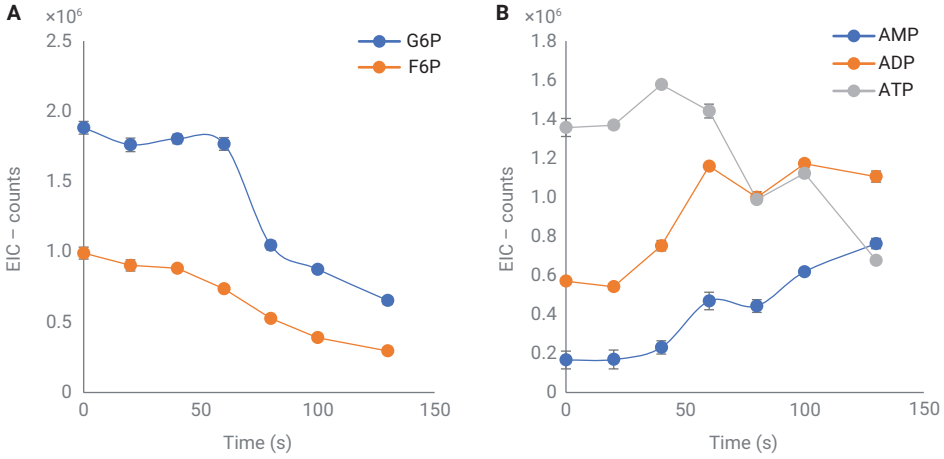

Figure 7. Area intensities for G6P and F6P (A) and AMP, ADP, and ATP (B) during the glucose starvation experiment.

Conclusion

The analysis of complex metabolomics samples can be tedious and error prone due to unstable and demanding analytes. Stainless steel LC systems must be passivated, or specific mobile phase additives should be used. This application note shows that the Agilent 1290 Infinity II Bio LC is the premier choice for metabolomics. No adsorption, good peak shape, and high resolution for challenging phosphorylated compounds was shown, rendering previous treatments unnecessary due to the biocompatible flow path. Combined with the robust performance in complex matrices and excellent precision, the Agilent 1290 Infinity II Bio LC is the perfect front end for any LC/MS-based metabolomics analysis.

Appendix

Metabolite abbreviations

Abbreviation	Compound
G6P	Glucose 6-phosphate
F6P	Fructose 6-phosphate
R5P	Ribose 5-phosphate
Succ	Succinic acid
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
GMP	Guanosine monophosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
CMP	Cytidine monophosphate
CDP	Cytidine diphosphate
CTP	Cytidine triphosphate
UMP	Uridine monophosphate
UDP	Uridine diphosphate
UTP	Uridine triphosphate
AKG	α-Ketoglutaric acid
ALA	L-Alanine
ARG	L-Arginine
ASP	L-Aspartic acid
CYS	L-Cystine
GLU	L-Glutamic acid
HIS	L-Histidine
ILE	L-Isoleucine
LEU	L-Leucine
LYS	L-Lysine
MET	L-Methionine
PHE	L-Phenylalanine
PRO	L-Proline
SER	L-Serine
TYR	L-Tyrosine
VAL	L-Valine

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