

## Separation of Pentose Phosphate Pathway, Glycolysis, and Energy Metabolites Using an ACQUITY Premier System With an Atlantis Premier BEH Z-HILIC Column

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### Abstract

Analyses of phosphorylated metabolites are challenging because of their interactions with metal surfaces in conventional LC systems and columns. Here, we describe a targeted UPLC-MS/MS method for 27 pentose phosphate, glycolysis, and energy metabolites in plasma and tissue extracts. The method leverages the benefits of MaxPeak High Performance Surfaces Technology used in the ACQUITY Premier System and the Atlantis Premier BEH Z-HILIC Column to mitigate interactions of the metabolites with metal surfaces. The stability of the column to high pH mobile phases is also key, as the best peak sharpness, peak symmetry, and sensitivity was achieved using a pH 9 ammonium bicarbonate buffer in the mobile phase. The results demonstrate that high efficiency, UPLC-pressure tolerant 1.7  $\mu\text{m}$  Atlantis Premier BEH Z-HILIC Columns provide excellent separations for these challenging analytes.

### Benefits

- A targeted UPLC-MS/MS method that provides sharp, symmetric peaks for 27 challenging metabolites

- The base-stable Atlantis Premier BEH Z-HILIC Column allows the use of an optimal pH 9 buffer
- MaxPeak High Performance Surfaces Technology in the UPLC system and column enables excellent peak sharpness, peak symmetry, and sensitivity

## Introduction

Central carbon metabolism is comprised of several enzyme-mediated pathways which work together to provide energy, precursors, and reduction of cofactors for cell growth and homeostasis. Two important pathways within central carbon metabolism are glycolysis and the pentose phosphate pathway. Glycolysis consists of a sequence of reactions that convert glucose into pyruvate under oxygen-rich conditions, and further into lactate under oxygen-poor conditions. These metabolites may then feed into the tricarboxylic acid (TCA) cycle. Through these reactions, ATP is formed and NADH is oxidized to NAD<sup>+</sup>. Alternative to glycolysis, the pentose phosphate pathway ultimately converts glucose into ribose 5-phosphate as well as NADPH. Ribose 5-phosphate undergoes further reactions into ribose sugars that are used as DNA and RNA building blocks.<sup>1</sup> Perturbations in the relative concentrations of central carbon metabolites arising from disease are studied to understand their underlying mechanisms.<sup>2</sup>

The analysis of the components of the pentose phosphate pathway and glycolysis is fraught with challenges due to their polar nature, with most containing phosphorylated moieties, as well as a number of isomers. The presence of phosphate groups on the metabolites contributes to the adsorption of these analytes to metal surfaces in the analytical system. This causes the measurements to be unreliable as the metal adsorption results in poor peak shapes, high variability, and poor sensitivity.

We present a method for the analysis of pentose phosphate pathway, glycolysis, and energy metabolites using a zwitterionic HILIC Column, the Atlantis Premier BEH Z-HILIC Column. This column contains a sulfobetaine stationary phase on ethylene-bridged hybrid (BEH) particles.<sup>3</sup> This material is packed into MaxPeak High Performance Surfaces (HPS) column hardware to mitigate interactions of metal sensitive compounds with metal surfaces.<sup>4</sup> The column was used on an ACQUITY Premier System, which also incorporates MaxPeak HPS Technology, and a Xevo TQ-S micro Tandem Quadrupole Mass Spectrometer.

## Experimental

## Mobile Phase Preparation

To ensure reproducibility of preparation, the optimized mobile phase was prepared gravimetrically every 3 days. A 150 mM ammonium bicarbonate pH 9.00 stock solution was prepared by weighing out 2.96 g of ammonium bicarbonate (Sigma 09830) on a top loading balance and adding water to 240 g. The solution was then adjusted to pH 9.00 (+/- 0.02) by adding aqueous 28% ammonium hydroxide (Sigma 338818) and returned to the balance for accurate weighing to 250 g. 100 g of this buffer concentrate was added to two LDPE mobile phase bottles. 900 g of water was weighed and added to one bottle to make mobile phase A, and 707.6 g (900 mL) of acetonitrile (ACN) was weighed and added to the second bottle to make mobile phase B. The mobile phases were capped tightly and sonicated for 20 minutes before use.

The pH screening mobile phases were prepared in a similar fashion, however the final proportions of mobile phases A and B were measured volumetrically. Briefly, 0.976 g of ammonium acetate (Sigma 431311) was weighed and water added to ~100 g. The pH was adjusted by adding either acetic acid (Sigma 338826) or 28% ammonium hydroxide, then the solution was returned to the balance for accurate weighing to 125 g to make a 100 mM solution. 50 mL of this solution was added to two mobile phase bottles. 450 mL of water was added to one bottle to make mobile phase A and 450 mL of acetonitrile was added to the other to make mobile phase B, which was then capped tightly and sonicated for 20 minutes.

## Stock Solution Preparation

High purity analyte standards were obtained from Sigma Aldrich. Glyceraldehyde 3-phosphate was purchased as a 10.7 mg/mL solution in water and all others as solids. Stock solutions were prepared individually at 20 mM free acid in 50%ACN/50%H<sub>2</sub>O and subsequently combined into three equal mixes at 1 mM in 50%ACN/50%H<sub>2</sub>O for the working stock solutions. Stable labelled internal standards (Cambridge Isotope Laboratories and Sigma Aldrich) were each prepared at 1 mM free acid in 50%ACN/50%H<sub>2</sub>O. A 5x concentrated working internal standard mix of 100 µM lactic acid-<sup>13</sup>C and 10 µM each of glucose 6-phosphate-<sup>13</sup>C, AMP-<sup>13</sup>C<sup>15</sup>N, ADP-<sup>15</sup>N, ATP-<sup>13</sup>C, GMP-<sup>13</sup>C<sup>15</sup>N, and GTP-<sup>13</sup>C was prepared in 50%ACN/50%H<sub>2</sub>O. All stock solutions were stored at -20 °C.

Standards for the calibration curves were prepared in 50%ACN/50%H<sub>2</sub>O by serial dilution from the working stock solution to make stock standards of 250, 125, 50, 25, 12.5, 5, 2.5, 1.25, 0.50, 0.250, 0.125, and 0.050 µM. 5 µL of each standard as well as 5 µL of the internal standard mixture was added to 15 µL of 50%ACN/50%H<sub>2</sub>O in a silanized vial. This represents calibration solutions of 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, and 0.01 µM respectively in 25 µL. Seventy five microliters of 50%ACN/50%H<sub>2</sub>O was then added to give a final volume of 100 µL. Thus, the final concentrations for the calibration curve were 12.5, 6.25, 2.5, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.025, 0.0125, 0.00625, and 0.0025 µM.

## Liver and Brain Extraction

Sample preparation was based on a previously described method<sup>5</sup>, with modifications. Female Sprague Dawley Rat Liver and Male Sprague Dawley Rat brain were from BioIVT (Westbury, NY). 25 mL of liver/brain were added to a 2 mL Precellys Tissue homogenization Tube (p/n 10409) followed by 750  $\mu$ L of 80%MeOH/20%H<sub>2</sub>O (chilled on ice). Samples were homogenized on the Precellys Evolution using a program of 3 x 15 s cycles at 5800 rpm, with a 30-second pause between cycles. Homogenate was pipetted into new 1.5 mL microcentrifuge tubes and samples were placed at -20 °C for 60 min. Samples were centrifuged at 18407 xg for 10 minutes at 4 °C. The supernatant was transferred to a 1.5 mL microcentrifuge tube and dried using a TurboVap at room temperature using 1 L/min for 100 min and then 2.5 L/min for 30 minutes. Samples were reconstituted in 50  $\mu$ L of 50%ACN/50%H<sub>2</sub>O, briefly vortex mixed and placed at 4 °C for 10 minutes followed by centrifugation at 18407 xg for 10 minutes at 4 °C. Samples were pipetted into total recovery silanized vials and analyzed immediately.

## Plasma Extraction

Sprague Dawley K2EDTA pooled gender plasma was purchased from BioIVT (Westbury, NY). 25  $\mu$ L of plasma was added to a 1.5 mL microcentrifuge tube. 125  $\mu$ L of 80%MeOH/20%H<sub>2</sub>O (chilled on ice) was then added. Samples were vortexed for 1 minute, and then covered and placed on a shaker at 250 rpm for 20 minutes at room temperature. The samples were then placed in a -20 °C freezer for 60 minutes. Samples were centrifuged at 18407 xg for 10 minutes at 4 °C. Supernatant was transferred to a 1.5 mL microcentrifuge tube and dried using a TurboVap at room temperature using 1 L/min for 100 minutes and then 2.5 L/min for 30 minutes. Samples were reconstituted in 50  $\mu$ L of 50%ACN/50%H<sub>2</sub>O, briefly vortex mixed and placed at 4 °C for 10 minutes followed by centrifugation at 18407 xg for 10 minutes at 4 °C. Samples were pipetted into total recovery silanized vials and analyzed immediately.

## LC Conditions

LC system:	ACQUITY Premier BSM System
Vials:	Waters Total Recovery Vials, Deactivated (p/n: 186000385DV)
Mobile phase containers:	Waters Certified LDPE Container, 1 L (p/n: 186009110)

Column(s):	Atlantis Premier BEH Z-HILIC 1.7 $\mu$ m, 2.1 x 100 mm
Column temp.:	30 $^{\circ}$ C
Sample temp.:	8 $^{\circ}$ C
Injection volume:	3 $\mu$ L
Flow rate:	0.5 mL/min
Mobile phase A:	15 mM Ammonium bicarbonate, pH 9.00 in H <sub>2</sub> O
Mobile phase B:	15 mM Ammonium bicarbonate, pH 9.00 in 90%ACN/10%H <sub>2</sub> O v/v

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
initial	0.5	10	90	
5.00	0.5	35	65	6
6.00	0.5	35	65	6
6.50	1.0	10	90	6
9.50	1.0	10	90	6
9.51	0.5	10	90	6

## MS Conditions

MS system:	Xevo TQ-S micro Tandem Quadrupole Mass Spectrometer
Ionization mode:	Positive/Negative
Capillary voltage:	1 kV/1 kV
Desolvation temperature:	500 °C
Desolvation gas:	1000 L/hr
Cone gas:	50 L/hr
Source temperature:	150 °C

## Data Management

MS software	MassLynx v4.2 SCN1017
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## Results and Discussion

Previous studies have reported that the best peak sharpness, symmetry, and sensitivity for phosphorylated metabolites is obtained using mobile phases containing *ca* pH 9 ammonium acetate or ammonium bicarbonate buffers.<sup>6-9</sup> However, few HILIC columns are stable at this pH, the primary exceptions being those based on organic polymer or ethylene-bridged hybrid (BEH) particles. Columns containing polymeric particles have very limited pressure ranges and relatively low efficiencies compared to those packed with hybrid particles. Atlantis Premier BEH Z-HILIC Columns combine stability to pH 10 with high efficiencies<sup>3</sup>, making them ideal for this application. In addition, these columns use MaxPeak High Performance Surfaces (HPS) for the column hardware to mitigate interactions between the analytes and metal surfaces, resulting in improved peak shape, peak areas, and peak area reproducibility for metal-sensitive compounds.<sup>4,10,11</sup>

Three different buffer pH values were evaluated to determine the effect on peak sharpness, symmetry, and

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sensitivity: all contained 10 mM ammonium acetate, with the pH adjusted to pH 5.0, 6.8 or 9.0. The results for four of the analytes are shown in Figure 1. For 3-phosphoglyceric acid (3PG), guanosine triphosphate (GTP), and adenosine triphosphate (ATP), the best results were obtained using the pH 9.0 buffer. This is in agreement with previous studies that used different columns.<sup>6-9</sup> In contrast, narrow symmetric peaks were observed for nicotinamide adenine dinucleotide (NAD) with all three buffers. To understand the effect of buffer choice and concentration on the peak shape and retention of the analytes, ammonium bicarbonate at pH 9.0 was also investigated. Although retention decreased, peak shape for the majority of compounds improved using ammonium bicarbonate, pH 9.0 (Figure 2). Notable improvements in the separation of ribose 5-phosphate (5P) from the isobaric ribulose 5P/xylulose 5P as well as a dramatically sharper peak for 3PG were observed using ammonium bicarbonate, pH 9.0. Finally, the effect of buffer concentration on peak shape and retention was studied. Separations were performed using ammonium bicarbonate buffer, pH 9.0 at 5, 10, 15, and 20 mM in both mobile phase A and B. Figure 3 shows the results of this study using as an example the separation of 2PG and 3PG. We can see that increasing the buffer concentration increases the retention time of the analytes. In fact, this phenomenon was observed for all the analytes in this study. 15 mM ammonium bicarbonate pH 9.0 buffer gave the best peak shape for 3PG as well as for other analytes and was chosen for the final method.

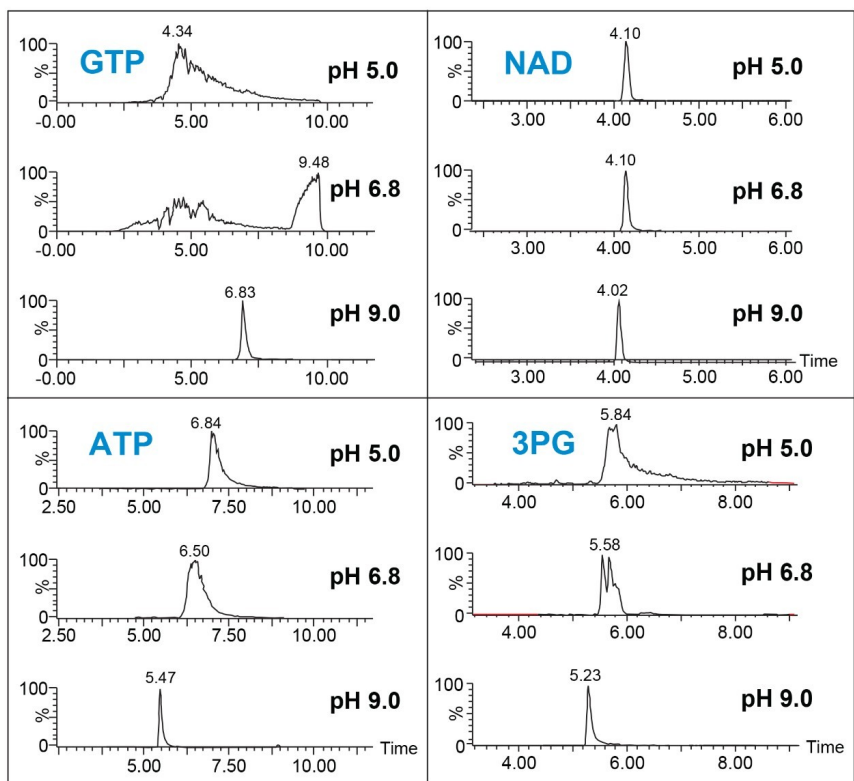


Figure 1. Influence of mobile phase pH on the peak shape of phosphorylated analytes.

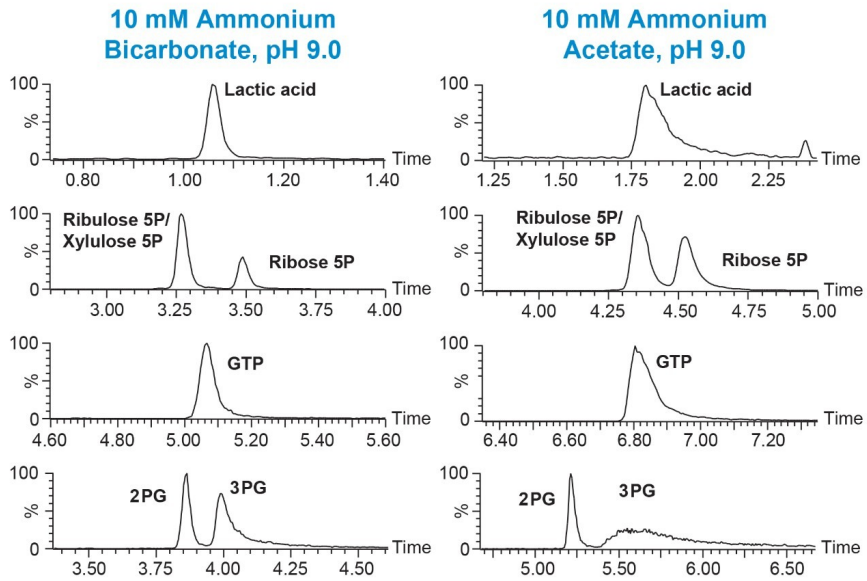
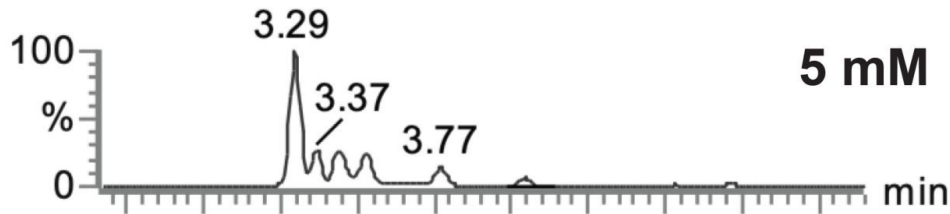


Figure 2. Influence of mobile phase buffer on peak shape and resolution of analytes.

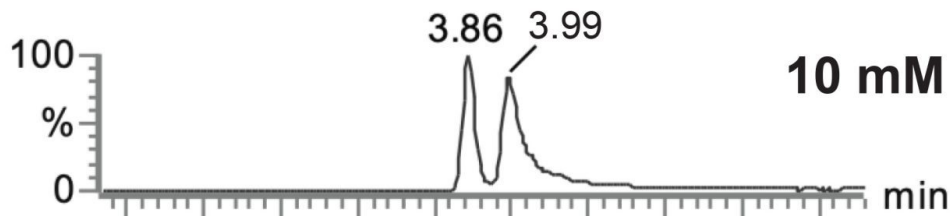


## Ammonium Bicarbonate, pH 9.0

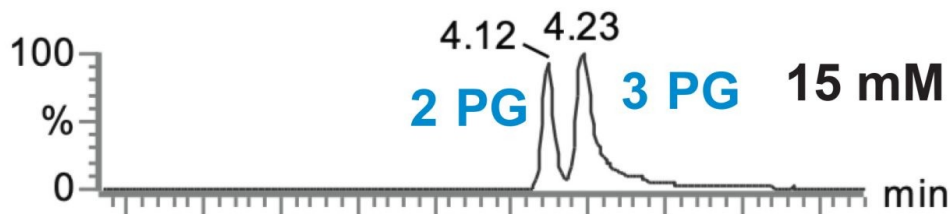
27G21\_026



27G21\_054



28G21\_016



28G21\_048

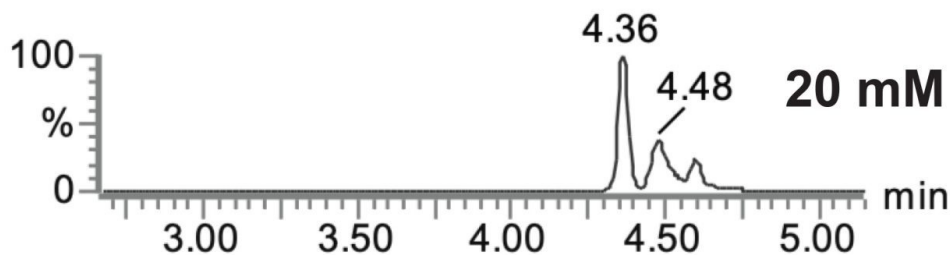


Figure 3. Influence of

mobile phase buffer concentration on the separation of 2PG and 3PG.

The MS conditions were optimized by infusing each analyte into the mass spectrometer. The optimal parameters are shown in Table 1. A representative separation of twenty-seven analytes dissolved in 50:50 acetonitrile/water is shown in Figure 4. A 2.1 x 100 mm, 1.7  $\mu$ m Atlantis Premier BEH Z-HILIC Column was used at a flow rate of 0.5

mL/min. Chromatograms detected using positive and negative ion electrospray (ES) ionization are shown separately. Sharp symmetric peaks were observed for nearly all the analytes. The isobaric pairs ribulose 5P/ribose-5P and 2PG/3PG were well resolved. Calibration curves were created for all 27 analytes, using peak areas ratioed to stable isotope labelled internal standards. The correlation coefficients ranged from 0.9904 for glyceraldehyde-3-phosphate to 0.9996 for ribulose5P/xylulose5P and ADP (see Table 2). The lower limits of quantification (LLOQ) ranged from 0.0063 to 1.25  $\mu$ M.

#	Name	Mode	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (V)
1	Pyruvic acid	ES-	87	43	15	7
2	Lactic acid	ES-	89	43	20	12
3	Deoxyribose 5-phosphate	ES-	213	97	15	10
4	Dihydroxyacetone phosphate	ES-	169	97	20	10
5	Ribulose 5-phosphate/ Xylulose 5-phosphate	ES-	229	97	10	14
6	Ribose 5-phosphate	ES-	229	97	10	14
7	Glyceraldehyde 3-phosphate	ES-	169	97	20	10
8	Sedoheptulose 7-phosphate	ES-	289	97	20	17
9	Fructose 6-phosphate	ES-	259	97	25	14
10	2-Phosphoglycerate	ES-	185	97	25	15
11	3-Phosphoglycerate	ES-	185	97	25	15
12	Phosphoenolpyruvate	ES-	17	79	20	10
13	Glucose 6-phosphate	ES-	259	97	25	14
14	Fructose 1,6 bisphosphate	ES-	339	97	15	20
15	6-Phosphogluconic acid	ES-	275	97	20	12
16	FAD	ES+	786	348	25	25
17	Acetyl CoA	ES+	810	303	25	32
18	NADH	ES+	666	649	15	15
19	AMP	ES+	348	136	25	16
20	NAD	ES+	664	524	25	15
21	ADP	ES+	428	136	30	20
22	GMP	ES+	364	152	25	15
23	ATP	ES+	508	136	25	26
24	NADPH	ES+	746	729	25	18
25	NADP	ES+	744	604	25	18
26	GDP	ES+	444	152	25	22
27	GTP	ES+	524	152	30	24
	Erythrose 4P	ES-	199	97	25	10
	Lactic Acid-C13	ES-	92	45	20	12
	AMP-C13N15	ES+	363	146	25	16
	AMP-C13N15 neg	ES-	361	144	25	32
	ADP-N15	ES+	433	141	30	20
	ADP-N15 neg	ES-	431	139	25	20
	GMP-C13N15	ES+	379	162	25	15
	GMP-C13N15 neg	ES-	377	79	25	45
	G6P-C13	ES-	265	97	15	9
	ATP-C13	ES+	518	141	25	26
	ATP-C13 neg	ES-	516	139	25	37
	GTP-C13	ES+	534	157	30	24
	GTP-C13 neg	ES-	532	79	25	37

Table 1.

MS Parameters.

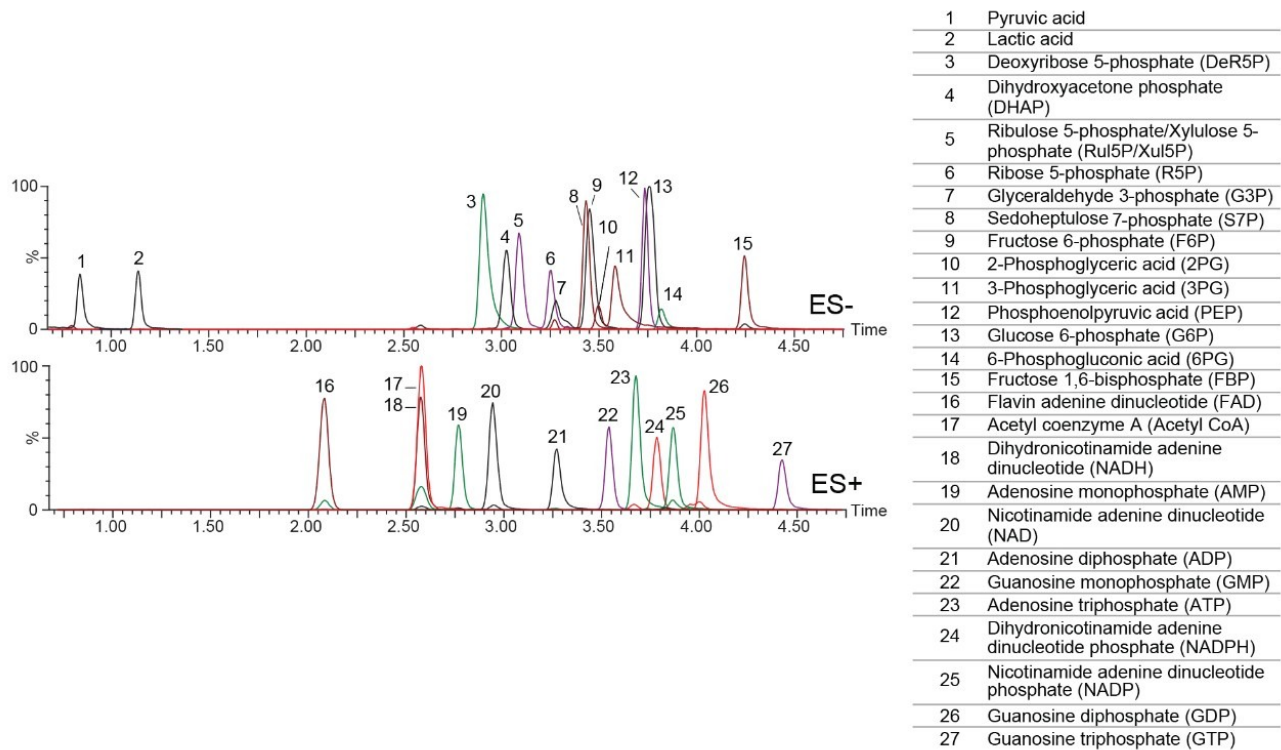


Figure 4. Representative chromatograms of analyte standards in 50/50 ACN/water.

#	Name	IS	RT min	LLOQ, $\mu\text{M}$	ULOQ, $\mu\text{M}$	R <sup>2</sup>
1	Pyruvic acid	Lactic Acid-C13	0.9	0.625	6.25	0.9941
2	Lactic acid	Lactic Acid-C13	1.2	1.25	12.5	0.9987
3	Deoxyribose 5-phosphate	ADP-N15 neg	2.8	0.0063	6.25	0.9993
4	Dihydroxyacetone phosphate	ADP-N15	3.0	0.0625	6.25	0.9983
5	Ribulose 5-phosphate/ Xylulose 5-phosphate	ADP-N15 neg	3.1	0.0125	12.5	0.9996
6	Ribose 5-phosphate	ADP-N15 neg	3.3	0.0125	2.5	0.9978
7	Glyceraldehyde 3-phosphate	ADP-N15 neg	3.3	1.25	12.5	0.9904
8	Sedoheptulose 7-phosphate	ADP-N15 neg	3.4	0.0063	6.25	0.9985
9	Fructose 6-phosphate	ADP-N15 neg	3.4	0.0625	6.25	0.9974
10	2-Phosphoglycerate	GMP-C13N15 neg	3.5	0.125	2.5	0.9990
11	3-Phosphoglycerate	G6P-C13	3.6	0.125	2.5	0.9965
12	Phosphoenolpyruvate	ATP-C13 neg	3.7	0.025	2.5	0.9980
13	Glucose 6-phosphate	G6P-C13	3.7	0.0125	6.25	0.9997
14	Fructose 1,6 biphosphate	GTP-C13 neg	4.3	0.025	6.25	0.9980
15	6-Phosphogluconic acid	ATP-C13 neg	3.9	0.025	6.25	0.9972
16	FAD	AMP-C13N15	2.1	0.25	12.5	0.9969
17	Acetyl CoA	AMP-C13N15	2.6	0.0063	6.25	0.9985
18	NADH	AMP-C13N15	2.6	0.0063	2.5	0.9990
19	AMP	AMP-C13N15	2.8	0.0125	6.25	0.9990
20	NAD	ADP-N15	2.9	0.0625	12.5	0.9952
21	ADP	ADP-N15	3.3	0.0125	6.25	0.9996
22	GMP	GMP-C13N15	3.5	0.125	12.5	0.9976
23	ATP	ATP-C13	3.7	0.0125	6.25	0.9995
24	NADPH	ATP-C13	3.8	0.0625	12.5	0.9993
25	NADP	ATP-C13	3.9	0.0125	2.5	0.9960
26	GDP	ATP-C13	4.0	0.0125	12.5	0.9982
27	GTP	GTP-C13	4.4	0.025	2.5	0.9987

Table 2. Calibration Curve Results.

This method was used for targeted analysis of the twenty-seven analytes in rat plasma, rat liver, and rat brain extracts. Representative chromatograms are shown in Figure 5. The sharp, symmetric peaks observed for the standards prepared in solvent were also seen in these tissue extracts. Phosphoenol pyruvate (PEP) was detected at a high concentration in the plasma sample, as was 3PG, ADP, GMP, fructose-6-phosphate (F6P), and glucose-6-phosphate (G6P). The three latter metabolites were also observed in both the liver and brain tissue samples. In addition, a high concentration of nicotinamide adenine dinucleotide phosphate (NADP) was seen in the liver

extract, while the brain extract had a high level of acetyl coenzyme A (Acetyl CoA).

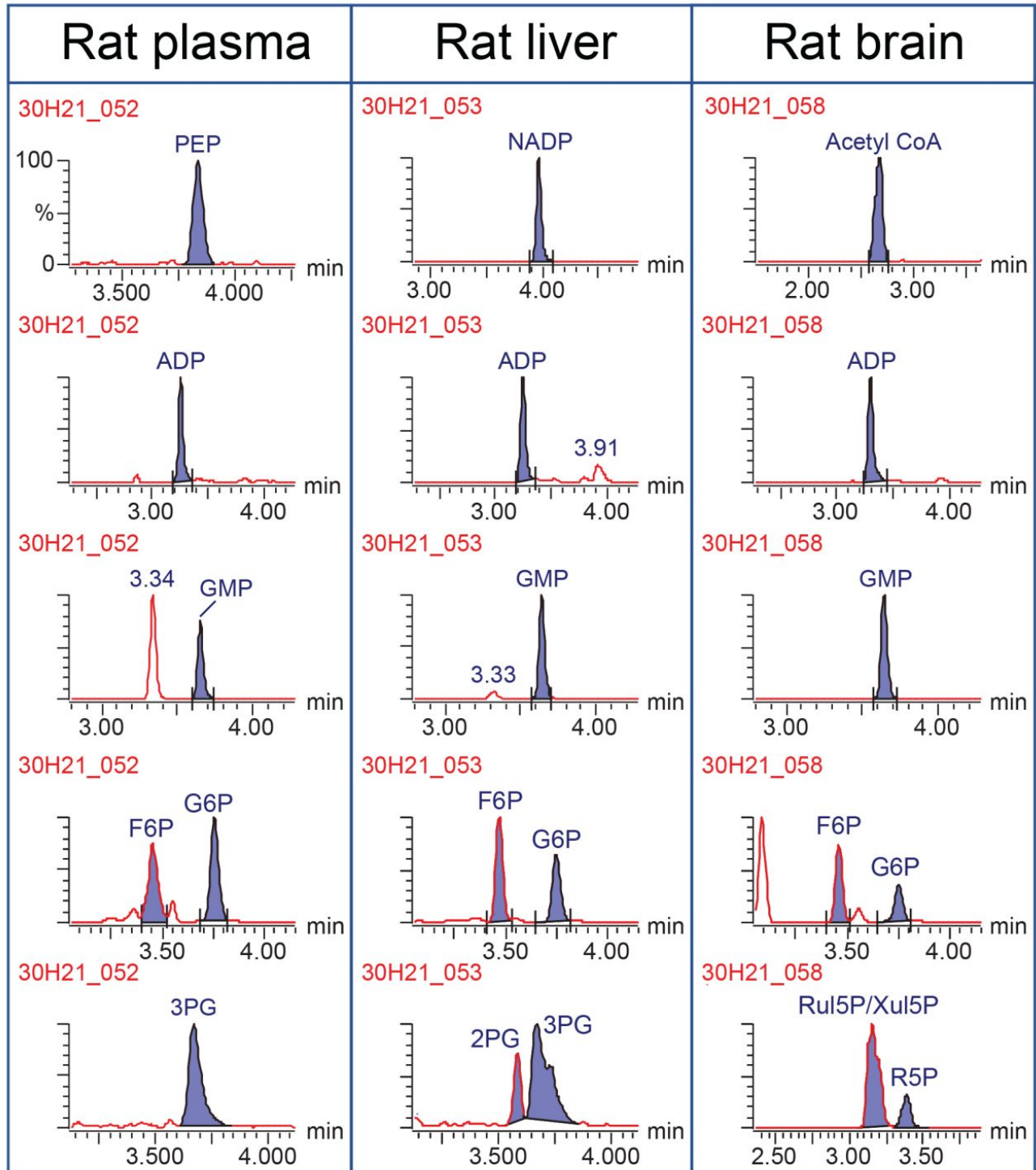


Figure 5. Representative chromatograms of analytes in rat plasma and tissue extracts.

## Conclusion

We developed a sensitive UPLC-MS/MS method for the targeted analysis of twenty-seven pentose phosphate pathway, glycolysis, and energy metabolites in plasma and tissue extracts. Using an ACQUITY Premier System and an Atlantis Premier BEH Z-HILIC Column we achieved sharp, symmetric peaks for these challenging analytes. The stability of the BEH Z-HILIC column to basic mobile phases was essential for this method, since the best peak sharpness, peak symmetry, and sensitivity was achieved for many of the metabolites when using a pH 9.0 ammonium bicarbonate buffer. These results suggest that the ACQUITY Premier System and Atlantis Premier BEH Z-HILIC Columns will be useful for polar metabolomics assays, particularly for metabolites that interact with metal surfaces.

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