Trace Phosphate Improves ZIC-pHILIC Peak Shape, Sensitivity, and Coverage for Untargeted Metabolomics

Jonathan L. Spalding[1,2,3,≠], Fuad J. Naser[1,≠], Nathaniel G. Mahieu[1], Stephen L. Johnson[2], and Gary J. Patti*[1,3]

1. Department of Chemistry, Washington University in St. Louis, St. Louis, USA
2. Department of Genetics, Washington University in St. Louis, St. Louis, USA
3. Department of Medicine, Washington University in St. Louis, St. Louis, USA

≠ These authors contributed equally to this work
* To whom correspondence should be addressed: gjpattij@wustl.edu

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Table S1. List of the 65 chemical standards and their suppliers.

Standard	Supplier
1,3-dicaffeoylquinic acid	Sigma Aldrich (St. Louis, MO, USA)
2-deoxyglucose	Sigma Aldrich (St. Louis, MO, USA)
2-deoxyglucose 6-phosphate	Sigma Aldrich (St. Louis, MO, USA)
2-hydroxyglutarate	Sigma Aldrich (St. Louis, MO, USA)
2'-deoxyguanosine	Santa Cruz Biotechnology (Dallas, TX, USA)
2-phosphoglycerate	Sigma Aldrich (St. Louis, MO, USA)
3'-deoxyguanosine	Cayman Chemical (Ann Arbor, MI, USA)
3-phosphoglycerate	Sigma Aldrich (St. Louis, MO, USA)
Acetylcholine	Santa Cruz Biotechnology (Dallas, TX, USA)
Adenine	Sigma Aldrich (St. Louis, MO, USA)
Adenosine	Sigma Aldrich (St. Louis, MO, USA)
Adipics acid dihydrazide	Sigma Aldrich (St. Louis, MO, USA)
Adenosine 5'-diphosphate	Sigma Aldrich (St. Louis, MO, USA)
α-Ketoglutarate	Sigma Aldrich (St. Louis, MO, USA)
Adenosine 5'-monophosphate	Cayman Chemical (Ann Arbor, MI, USA)
Arginine	Sigma Aldrich (St. Louis, MO, USA)
Adenosine 5'-triphosphate	Sigma Aldrich (St. Louis, MO, USA)
Biotin	Sigma Aldrich (St. Louis, MO, USA)
Butylamine	Sigma Aldrich (St. Louis, MO, USA)
Butyrate	Sigma Aldrich (St. Louis, MO, USA)
Chelidamic acid	Sigma Aldrich (St. Louis, MO, USA)
Choline	Sigma Aldrich (St. Louis, MO, USA)
Cidofovir	Sigma Aldrich (St. Louis, MO, USA)
Citrate	Sigma Aldrich (St. Louis, MO, USA)
Cyanocobalamin	Sigma Aldrich (St. Louis, MO, USA)
Cyclohexylammonium	Sigma Aldrich (St. Louis, MO, USA)
D-myoinositol 4-monophosphate	Sigma Aldrich (St. Louis, MO, USA)
Dopamine	Sigma Aldrich (St. Louis, MO, USA)
Ethylenediaminetetraacetic acid	Sigma Aldrich (St. Louis, MO, USA)
Epinephrine	Sigma Aldrich (St. Louis, MO, USA)
Ethanolamine	Sigma Aldrich (St. Louis, MO, USA)
Folic acid	Sigma Aldrich (St. Louis, MO, USA)
Fucose 1-phosphate	Sigma Aldrich (St. Louis, MO, USA)
Fumaric acid	Sigma Aldrich (St. Louis, MO, USA)
gamma-Aminobutyric acid	Sigma Aldrich (St. Louis, MO, USA)
Galactose	Sigma Aldrich (St. Louis, MO, USA)
Geneticin	Thermo Fisher Scientific (Waltham, MA, USA)
Glucose	Sigma Aldrich (St. Louis, MO, USA)
Glutathione	Sigma Aldrich (St. Louis, MO, USA)
Guanidine	Sigma Aldrich (St. Louis, MO, USA)

Guanine	Sigma Aldrich (St. Louis, MO, USA)
Histamine	Sigma Aldrich (St. Louis, MO, USA)
Iris 7-ws carboxylic acid	Sigma Aldrich (St. Louis, MO, USA)
Isocitrate	Sigma Aldrich (St. Louis, MO, USA)
Kanamycin	Sigma Aldrich (St. Louis, MO, USA)
L-Ascorbic acid 2-sulfate	Sigma Aldrich (St. Louis, MO, USA)
Lysine	Sigma Aldrich (St. Louis, MO, USA)
N,N,N-Trimethyphenylammonium	Santa Cruz Biotechnology (Dallas, TX, USA)
Neryl pyrophosphate lithium salt	Sigma Aldrich (St. Louis, MO, USA)
p-Aminohippuric acid	Sigma Aldrich (St. Louis, MO, USA)
Palmitoyl-CoA	Santa Cruz Biotechnology (Dallas, TX, USA)
Phosphoenolpyruvate	Sigma Aldrich (St. Louis, MO, USA)
Phenethylamine	Santa Cruz Biotechnology (Dallas, TX, USA)
Piperazine	Santa Cruz Biotechnology (Dallas, TX, USA)
Putrescine	Sigma Aldrich (St. Louis, MO, USA)
Pyruvate	Sigma Aldrich (St. Louis, MO, USA)
Serotonin	Sigma Aldrich (St. Louis, MO, USA)
Sodium-p-toluene-sulfinate	Santa Cruz Biotechnology (Dallas, TX, USA)
Spermine	Sigma Aldrich (St. Louis, MO, USA)
Streptomycin	Sigma Aldrich (St. Louis, MO, USA)
Taurocholic acid	Sigma Aldrich (St. Louis, MO, USA)
Toluene	Sigma Aldrich (St. Louis, MO, USA)
Uracil	Sigma Aldrich (St. Louis, MO, USA)
Uridine	Sigma Aldrich (St. Louis, MO, USA)
Vidarabine	Sigma Aldrich (St. Louis, MO, USA)

Table S2: Log*P* values and neutral charge states of 65 chemical standards. Isomeric compounds are highlighted in the same color. For our analyses, all standards were mixed such that each had a concentration of approximately 20 µM.

Compound	Neutral Charge State	Log P	Compound	Neutral Charge State	Log P
Geneticin	4	-1.37	Uracil	0	-0.71
Kanamycin	4	-4.81	Uridine	0	-1.61
Spermine	4	-0.96	Vidarabine	0	-1.02
Streptomycin	3	-2.53	1,3-dicaffeoylquinic acid	-1	1.64
Adipic acid dihydrazide	2	-2.67	Biotin	-1	0.12
Putrescine	2	-0.72	Butyrate	-1	0.78
Acetylcholine	1	-3.9	Cidofovir	-1	-3.37
Arginine	1	-1.79	Glutathione	-1	-0.87
Butylamine	1	0.93	p-Aminohippuric acid	-1	N/A
Choline	1	-3.7	Pyruvate	-1	-1.24
Cyclohexylammonium	1	1.39	Sodium-p-toluene-sulfinate	-1	0.93
Dopamine	1	0.12	Taurocholic acid	-1	0.05
Epinephrine	1	-0.63	2-deoxyglucose 6-phosphate	-2	-3.24
Ethanolamine	1	-1.31	2-hydroxyglutarate (2-HG)	-2	-1.45
Guanidine	1	-1.81	ADP	-2	-2.91
Histamine	1	-0.92	α-Ketoglutarate	-2	-1.43
Lysine	1	-1.04	AMP	-2	-0.22
Trimethyphenylammonium	1	-2.31	Chelidamic acid	-2	-1.43
Phenethylamine	1	1.46	D-myoinositol 4-monophosphate	-2	-1.4
Piperazine	1	-1.17	Folic acid	-2	-2.48
Serotonin	1	0.21	Fucose 1-phosphate	-2	-0.56
2-deoxyglucose (2-DG)	0	-1.46	Fumaric acid	-2	-0.01
2'-deoxyguanosine	0	-1.36	Iris 7-ws carboxylic acid	-2	N/A
3'-deoxyguanosine	0	-0.72	L-Ascorbic acid 2-sulfate	-2	N/A
Adenine	0	-2.12	Neryl pyrophosphate	-2	1.1
Adenosine	0	-1.02	2-phosphoglycerate (2-PG)	-3	-2.49
Cyanocobalamin	0	-6	3-phosphoglycerate (3-PG)	-3	-1.88
GABA	0	-0.64	ATP	-3	-4.18
Galactose	0	-3.17	Citrate	-3	-1.72
Glucose	0	-3.17	EDTA	-3	-0.43
Guanine	0	-0.98	Isocitrate	-3	-1.47
Toluene	0	2.68	Phosphoenolpyruvate (PEP)	-3	-1.23
			Palmitoyl-CoA	-4	3.54

Table S3. Chromatographic conditions tested for preliminary method optimization on ZIC-pHILIC. A total of 12 conditons were tested on 65 chemical standards.

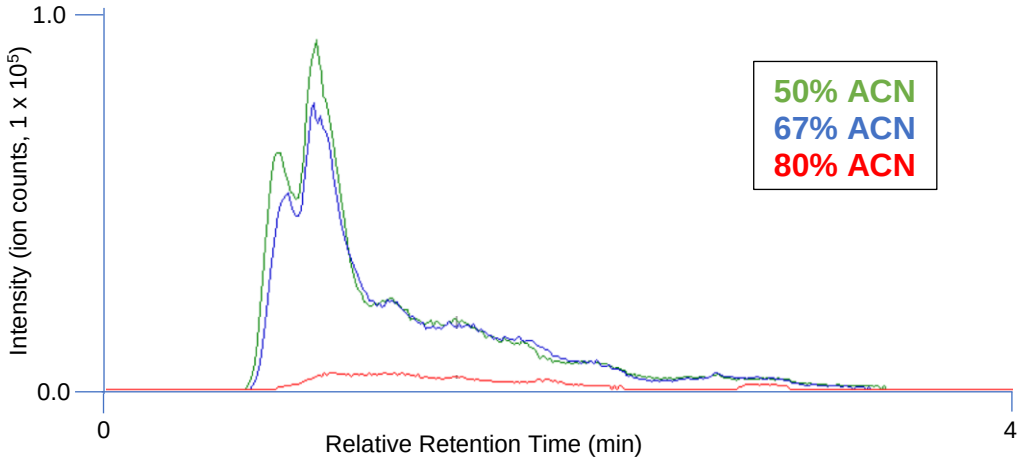
Salt Type	Salt Concentrations (mM)	pH
Ammonium Acetate	10, 20	4.0, 7.0
Ammonium Formate	10, 20	4.0, 7.0
Ammonium Bicarbonate	10, 20	7.0, 9.3

Figure S1. Heatmap of standard chromatographic performance on ZIC-pHILIC stationary phase with and without 5 µM ammonium phosphate in the aqueous fraction of the mobile phase (in addition to 20 mM ammonium acetate). Compounds that were analyzed in negative mode for all experiments contain a superscript (-) by their name while those that were analyzed in positive mode contain a superscript (+). Compounds were only analyzed with the ionization mode in which they ionized better so as to remove redundant data. The addition of phosphate to the mobile phase yields substantial improvements in separations. Refer to Figure 4 for details regarding color coding.

Standard	Neutral charge state	ZIC-pHILIC with phos	ZIC-pHILIC no phos	Standard	Neutral charge state	ZIC-pHILIC with phos	ZIC-pHILIC no phos
Geneticin$^+$	4			Uracil$^+$	0		
Kanamycin$^+$	4			Uridine$^+$	0		
Spermine$^+$	4			Vidarabine$^+$	0		
Streptomycin$^+$	3			1,3-dicaffeoylquinic acid$^-$	-1		
Adipic acid dihydrazide$^+$	2			Biotin$^-$	-1		
Putrescine$^+$	2			Butyrate$^-$	-1		
Acetylcholine$^+$	1			Cidofovir$^+$	-1		
Arginine$^+$	1			Glutathione$^-$	-1		
Butylamine$^+$	1			p-Aminohippuric acid$^+$	-1		
Choline$^+$	1			Pyruvate$^-$	-1		
Cyclohexylammonium$^+$	1			Sodium-p-toluene-sulfinate$^-$	-1		
Dopamine$^+$	1			Taurocholic acid$^-$	-1		
Epinephrine$^+$	1			2-deoxyglucose 6-phosphate$^-$	-2		
Ethanolamine$^+$	1			2-hydroxyglutarate (2-HG)$^-$	-2		
Guanidine$^+$	1			ADP$^-$	-2		
Histamine$^+$	1			α-Ketoglutarate$^-$	-2		
Lysine$^+$	1			AMP$^-$	-2		
Trimethyphenylammonium$^+$	1			Chelidamic acid$^-$	-2		
Phenethylamine$^+$	1			D-myoinositol 4-monophosphate$^-$	-2		
Piperazine$^+$	1			Folic acid$^-$	-2		
Serotonin$^+$	1			Fucose 1-phosphate$^-$			
2-deoxyglucose (2-DG)$^-$	0			Fumaric acid$^-$	-2		
2'-deoxyguanosine$^+$	0			Iris 7-ws carboxylic acid$^-$	-2		
3'-deoxyguanosine$^+$	0			L-Ascorbic acid 2-sulfate$^-$	-2		
Adenine$^+$	0			Neryl pyrophosphate$^-$	-2		
Adenosine$^+$	0			2-phosphoglycerate (2-PG)$^-$	-3		
Cyanocobalamin$^-$	0			3-phosphoglycerate (3-PG)$^-$	-3		
GABA$^+$	0			ATP$^-$	-3		
Galactose$^-$	0			Citrate$^-$	-3		
Glucose$^-$	0			EDTA$^-$	-3		
Guanine$^+$	0			Isocitrate$^-$	-3		
Toluene$^-$	0			Phosphoenolpyruvate (PEP)$^-$	-3		
				Palmitoyl-CoA$^-$	-4		

Figure S2. Effects of sample solvent composition on polar compound solubility. Metabolite extracts from *E. coli* were resuspended in acetonitrile:water with increasing proportions of acetonitrile (50%: green EIC, 67%: blue EIC, 80%: red EIC) to examine the effects of sample solvent composition on peak shape and polar analyte solubility. The EICs for two important polar compounds, citrate/isocitrate (top) and ADP (bottom) are shown. For citrate/isocitrate, acetonitrile proportions above 67% do not improve peak shape and significantly reduce its solubility, while ADP goes undetected at 80% acetonitrile. Samples were normalized by *E. coli* dry mass. Citrate and isocitrate could not be resolved and are therefore designated as citrate/isocitrate.

EIC of Citrate/Isocitrate



EIC of ADP

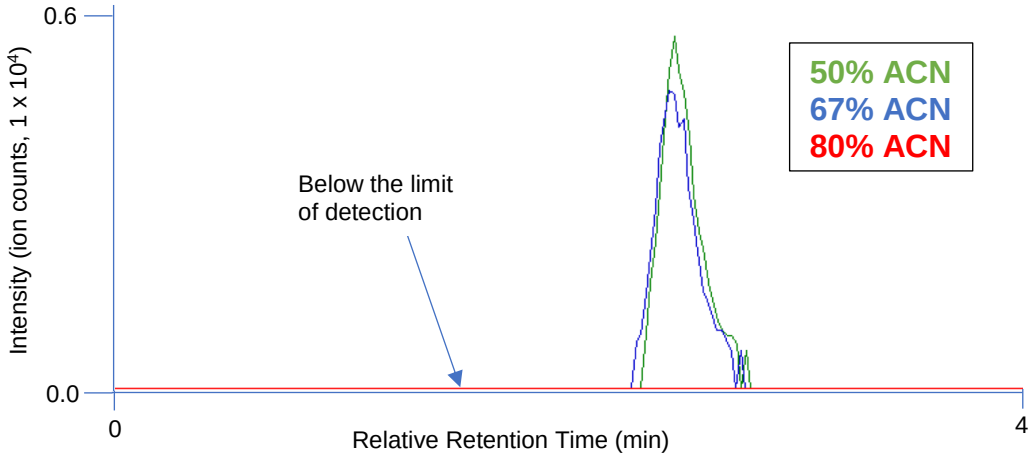
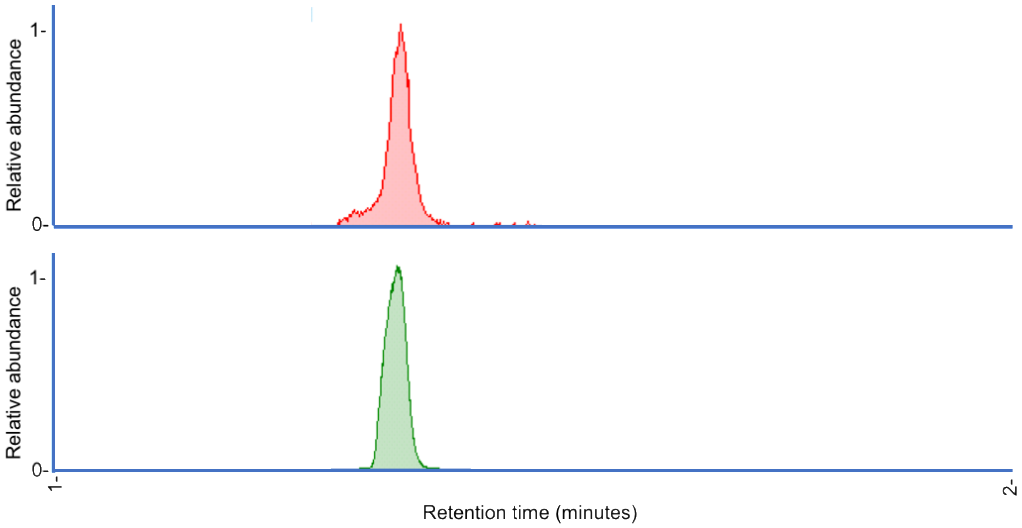
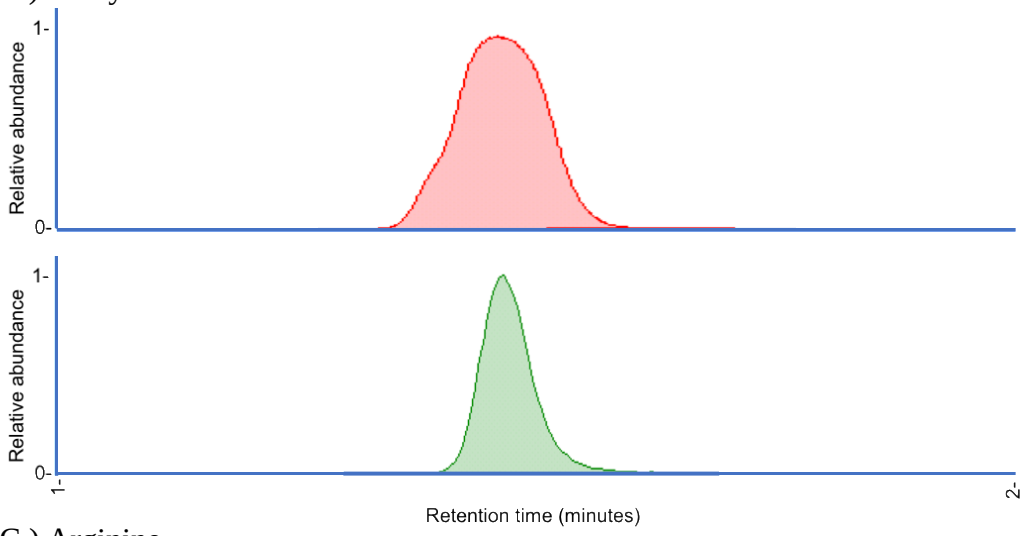


Figure S3. EICs of standards whose peak shape classification was changed by phosphate. Green peaks are those detected when 5 µM phosphate was present in the mobile phase, and red peaks are those detected without phosphate present.

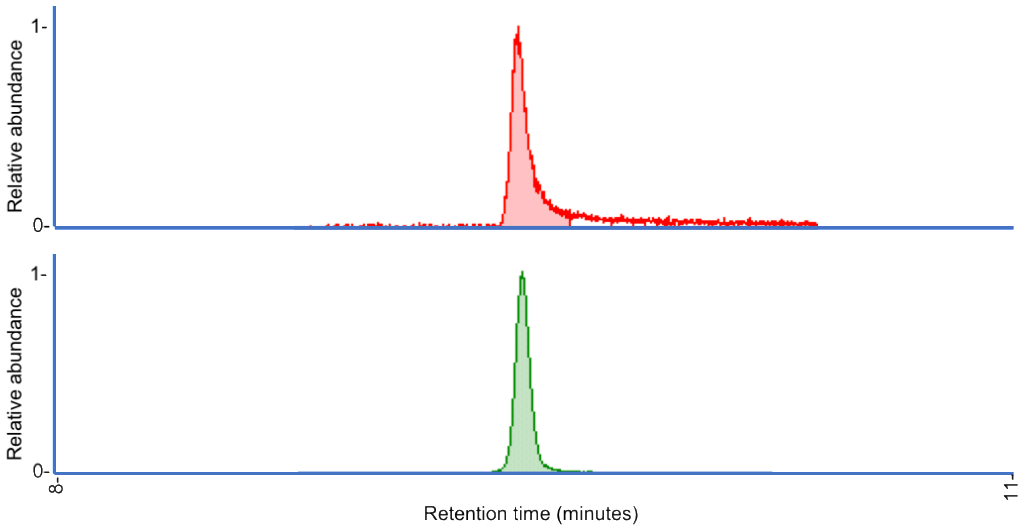
A.) Adipic acid dihydrazide



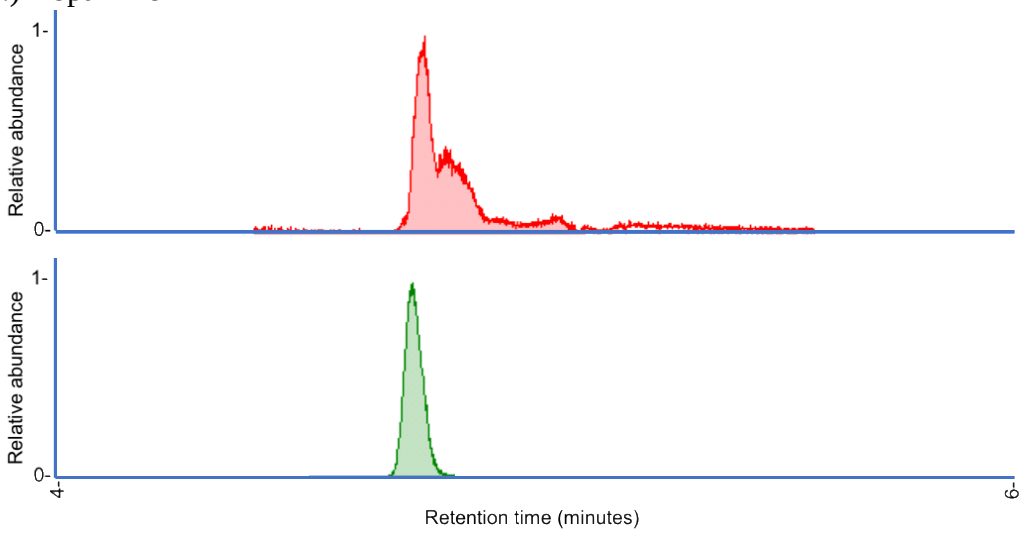
B.) Acetylcholine



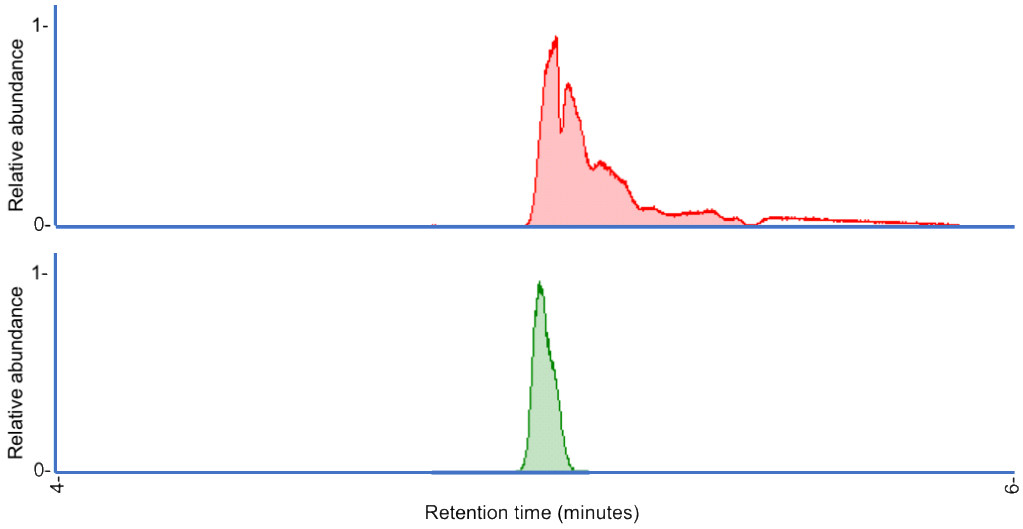
C.) Arginine



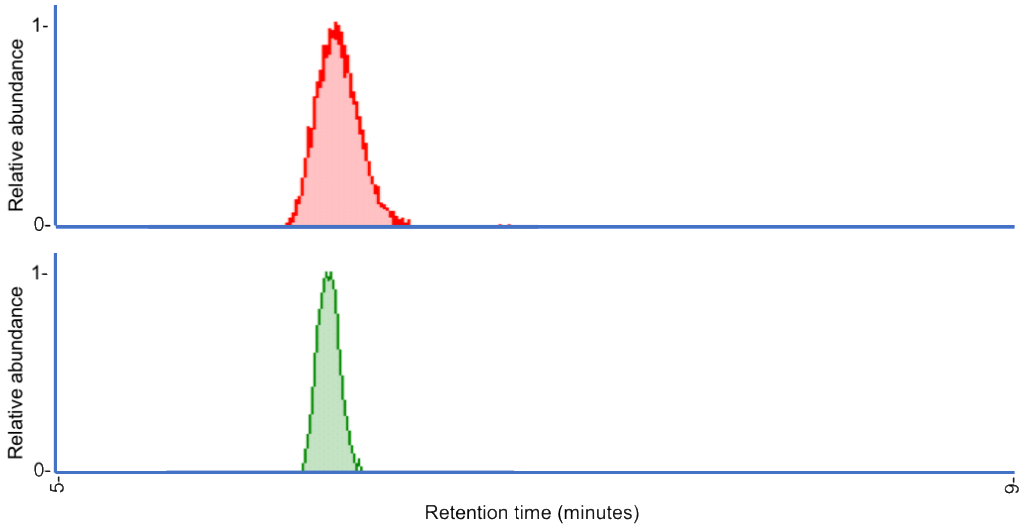
D.) Dopamine



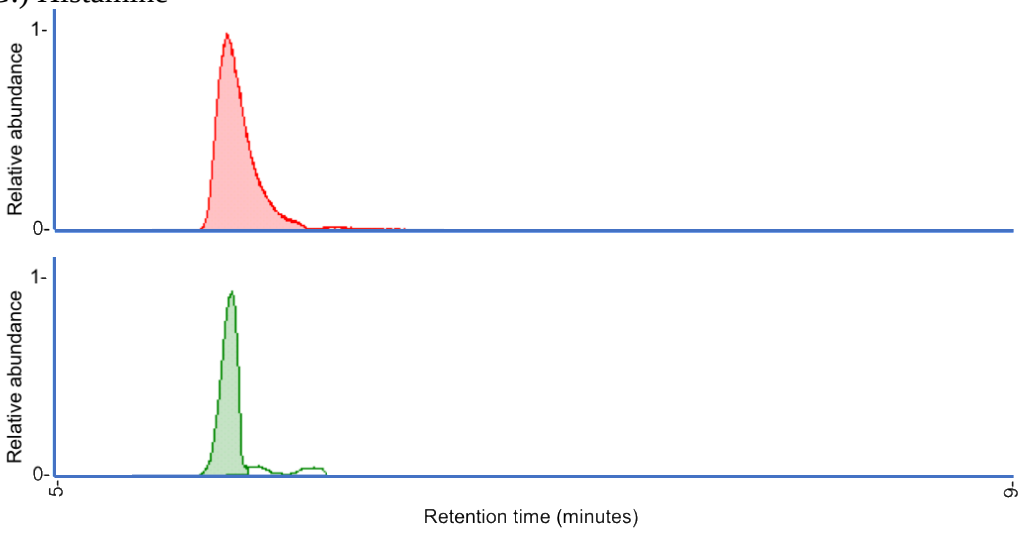
E.) Epinephrine



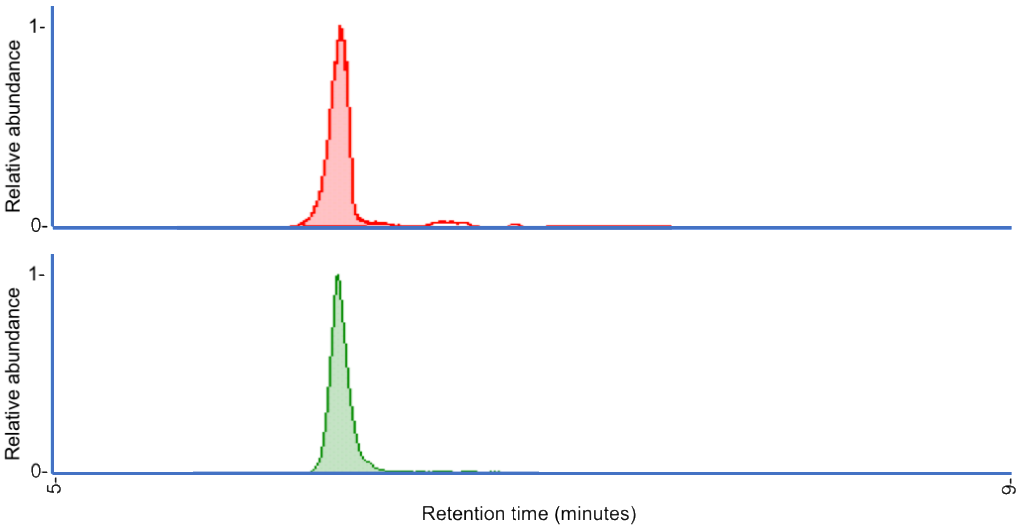
F.) Guanidine



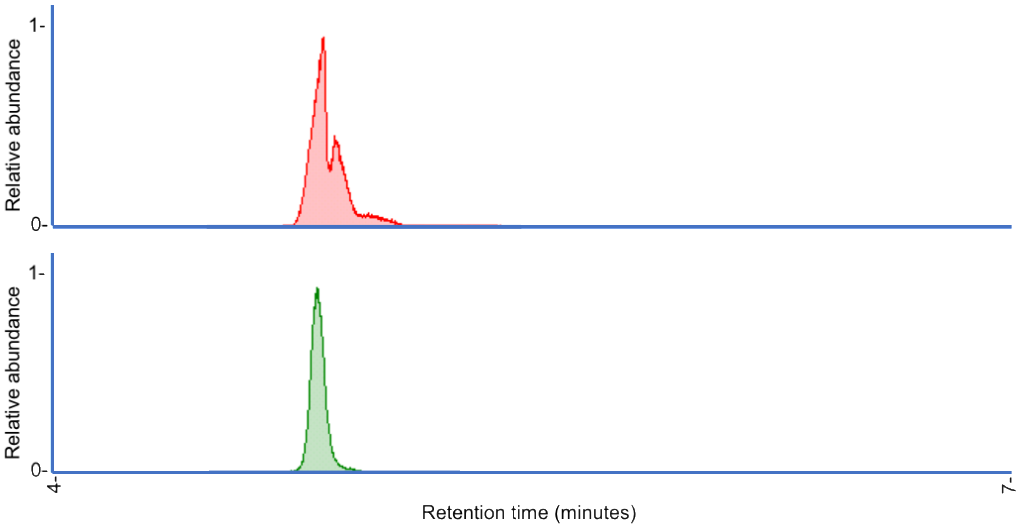
G.) Histamine



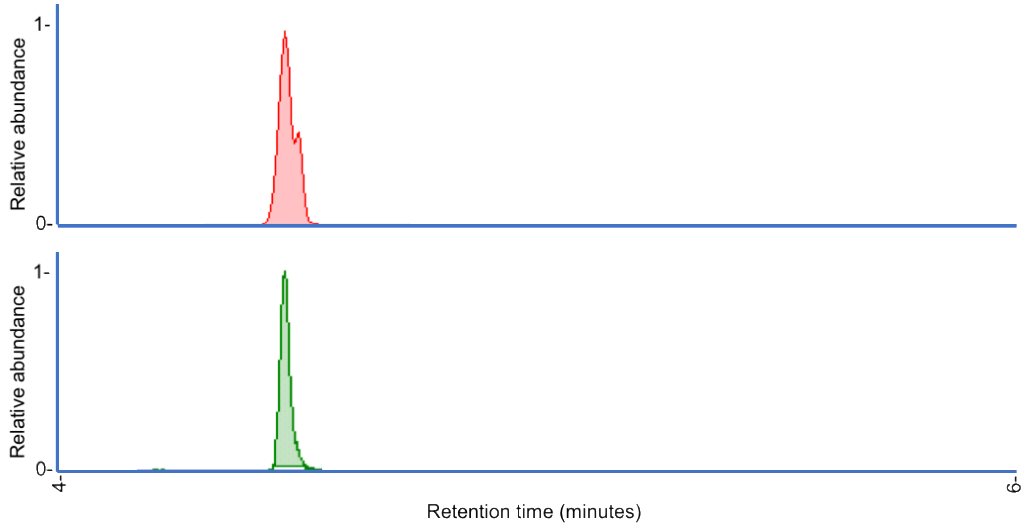
H.) Piperazine



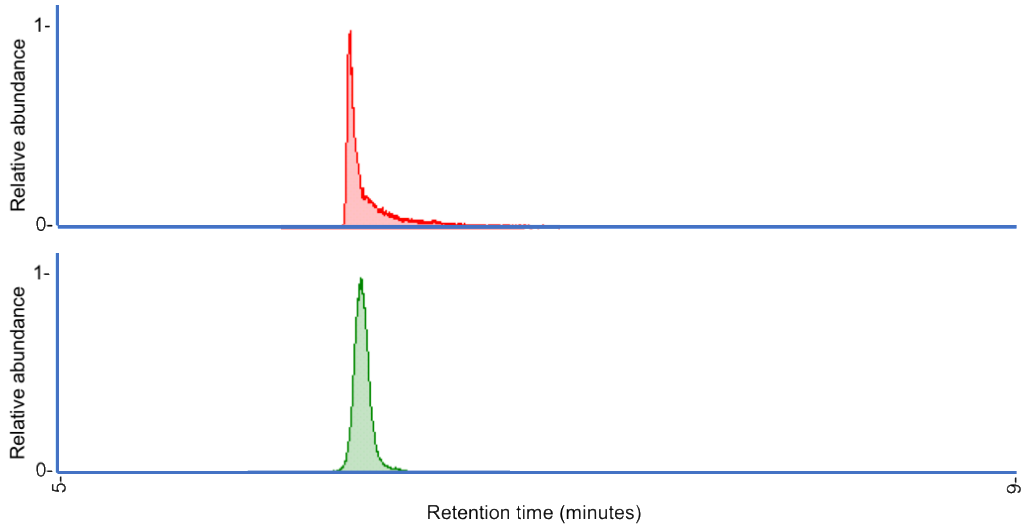
I.) Serotonin



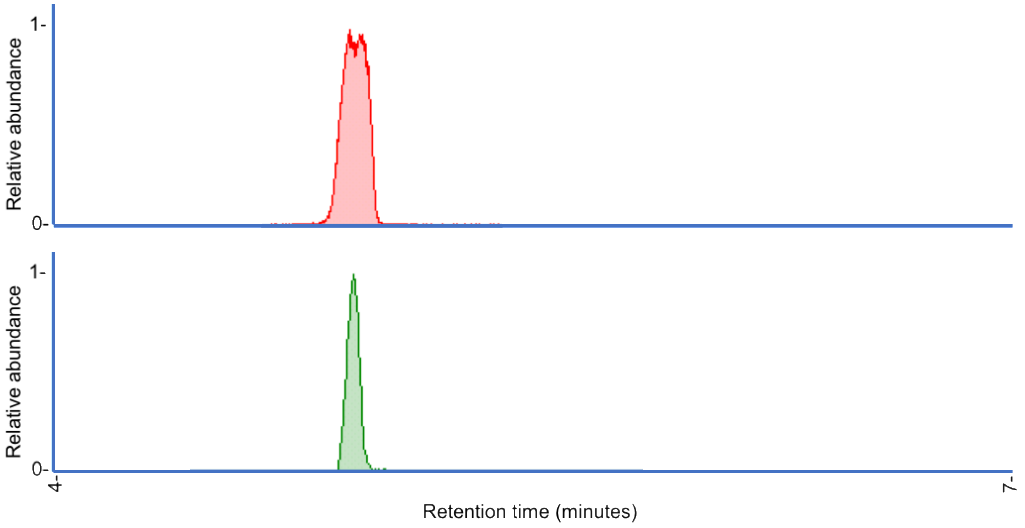
J.) Guanine



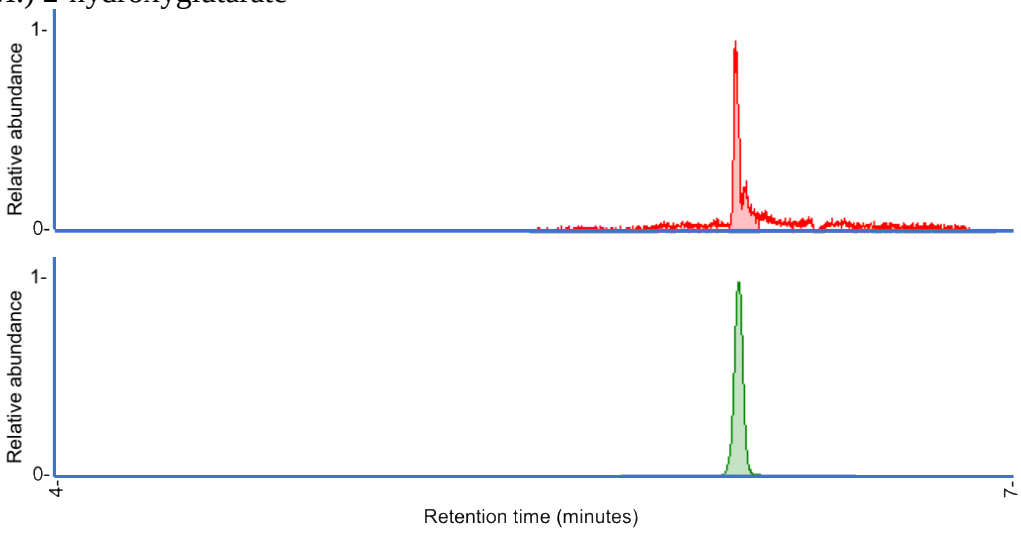
K.) Cidofovir



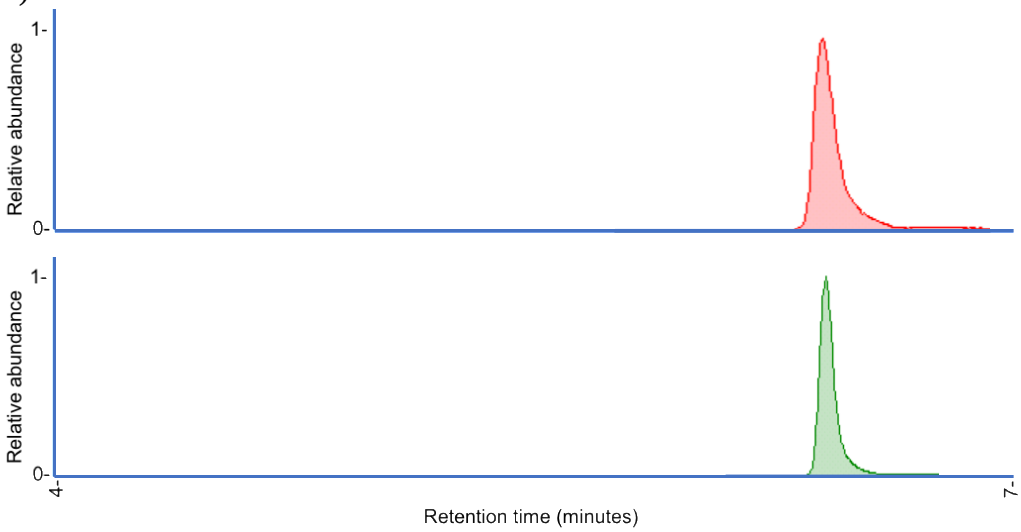
L.) 2-deoxyglucose 6-phosphate



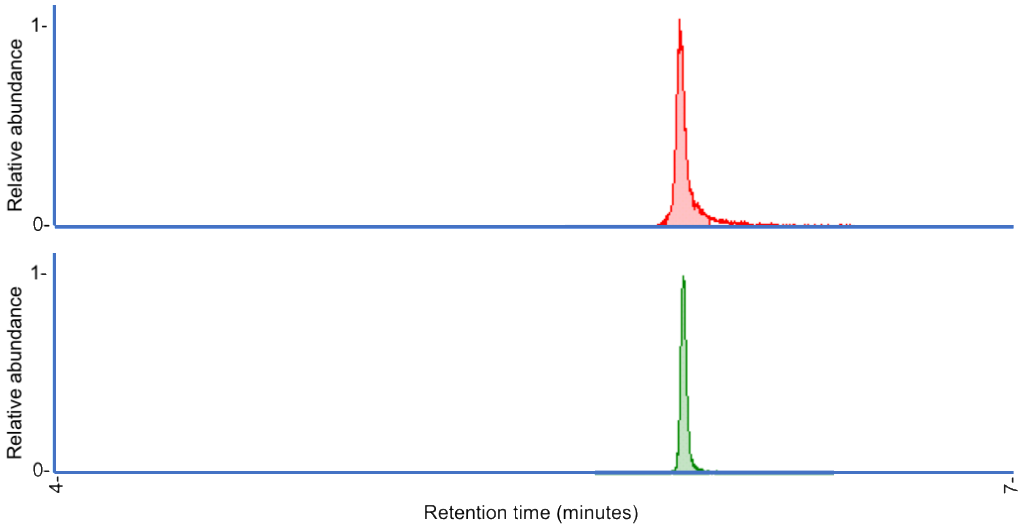
M.) 2-hydroxyglutarate



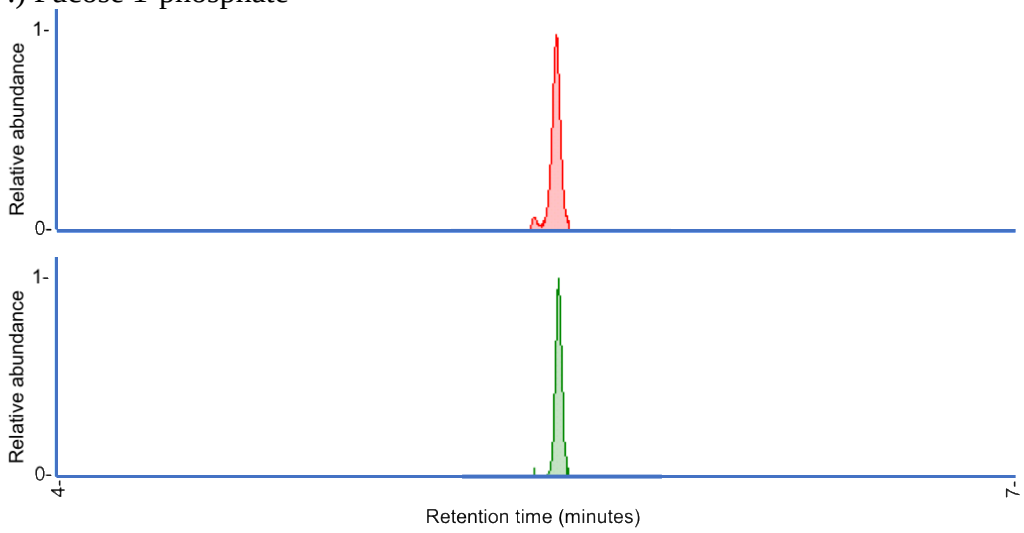
N.) ADP



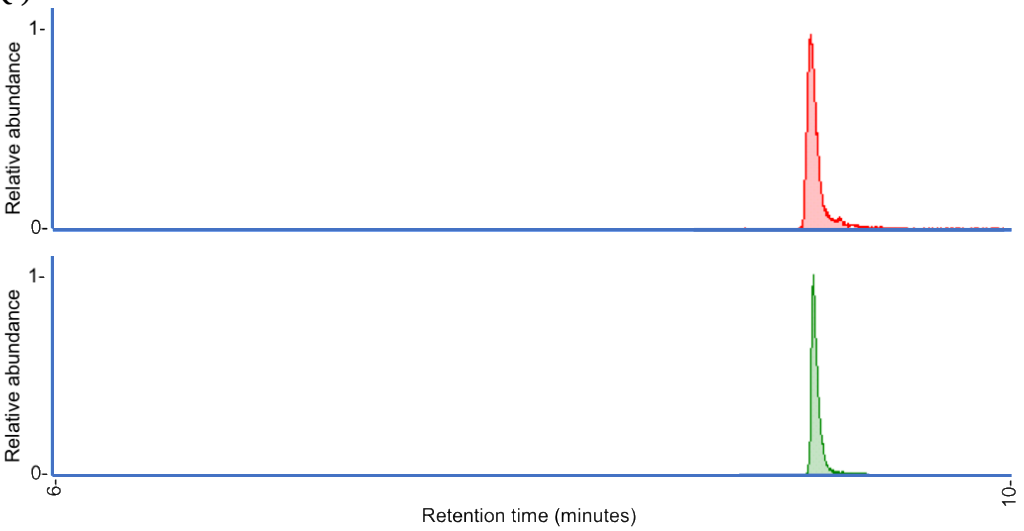
O.) D-myoinositol 4-phosphate



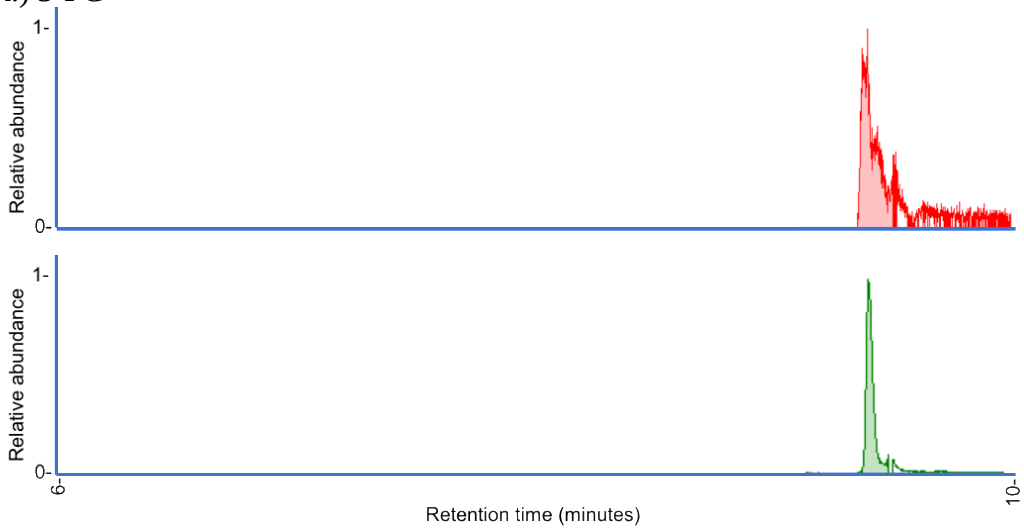
P.) Fucose 1-phosphate



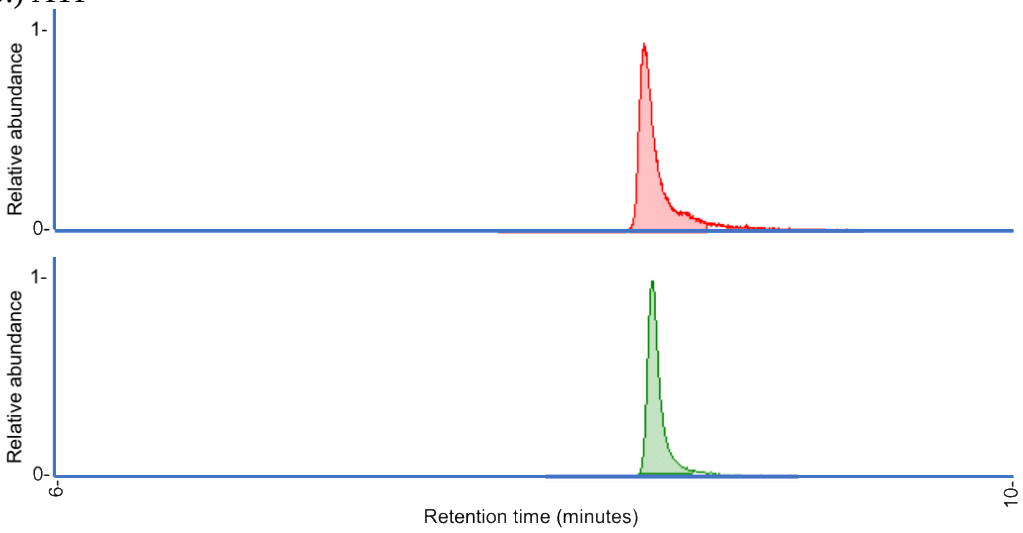
Q.) 2-PG



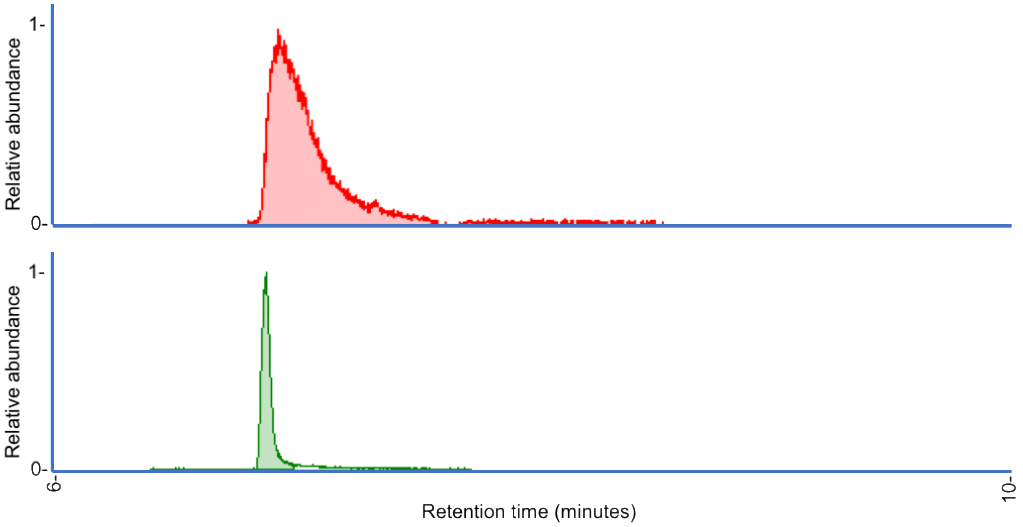
R.) 3-PG



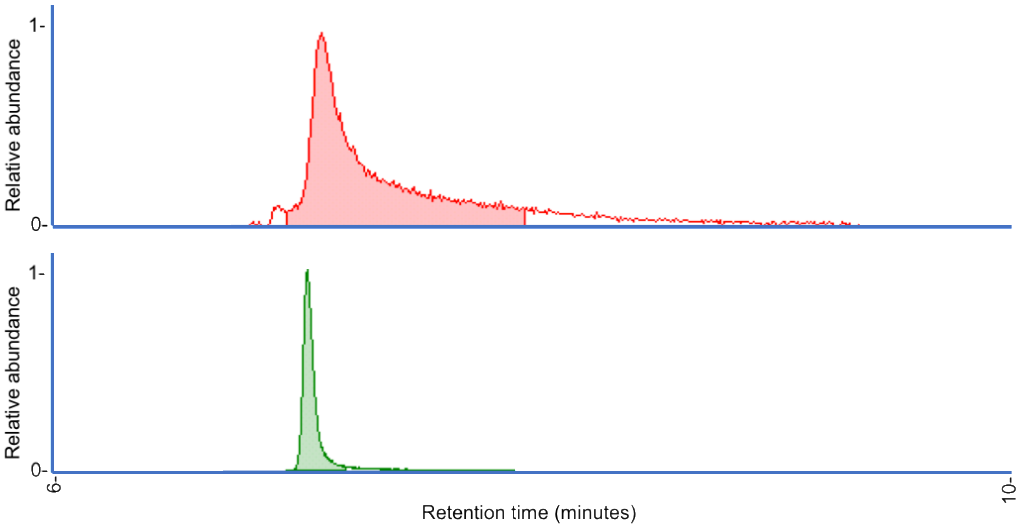
S.) ATP



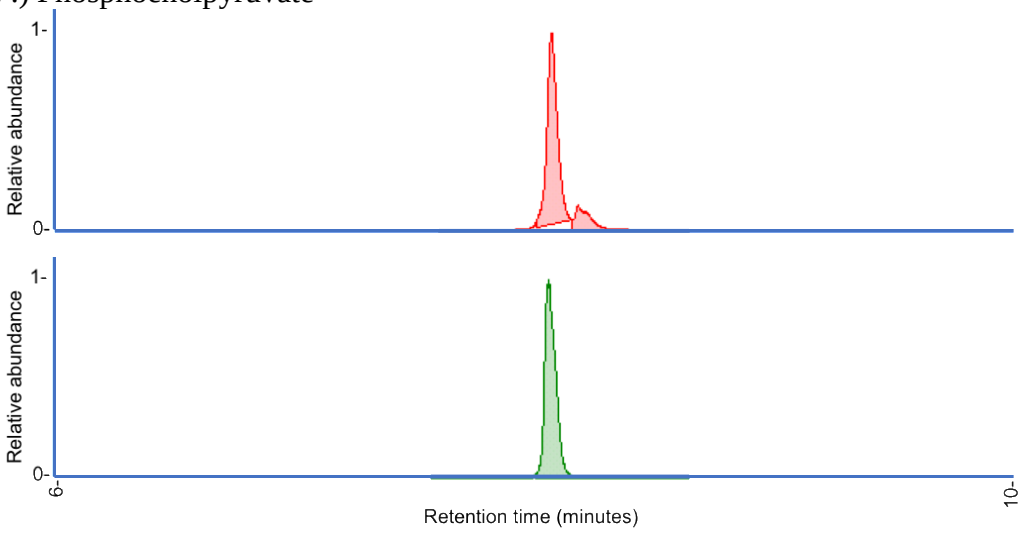
T.) Citrate



U.) Isocitrate



V.) Phosphoenolpyruvate



W.) Palmitoyl-CoA

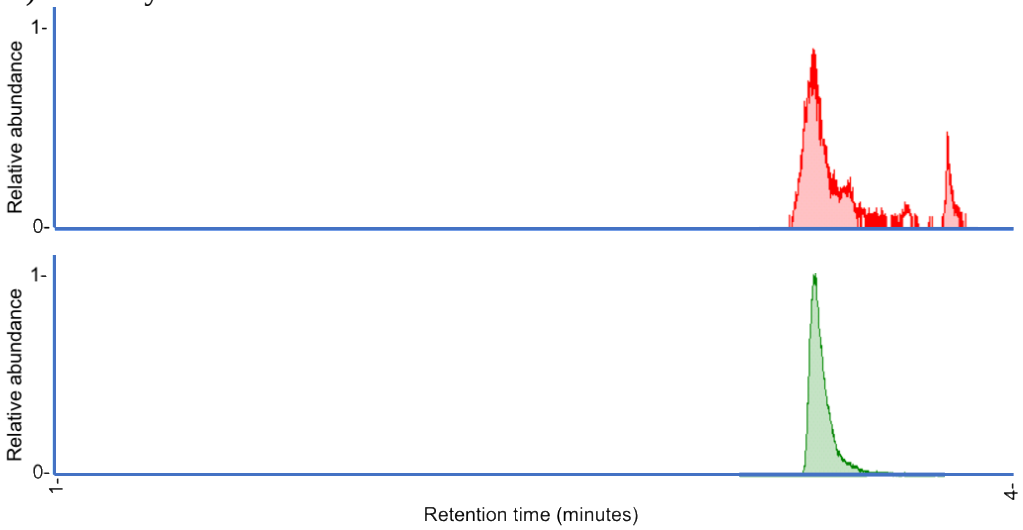


Figure S4. Heatmap of isomer resolution on ZIC-pHILIC stationary phase with and without 5 µM phosphate in the mobile phase. Red indicates isomers that were not resolved. Green indicates completely resolved isomers.

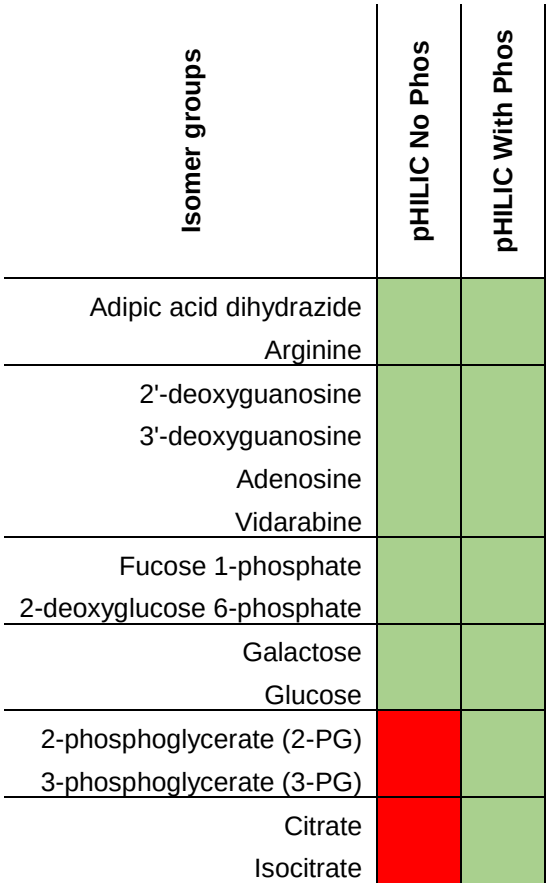


Figure S5. EICs of cidofovir when ammonium phosphate is in either the mobile phase or injection solvent. When phosphate is co-injected with cidofovir (bottom) instead of flowing in the mobile phase (top), peak tailing is reduced, and the peak is slightly more symmetrical.

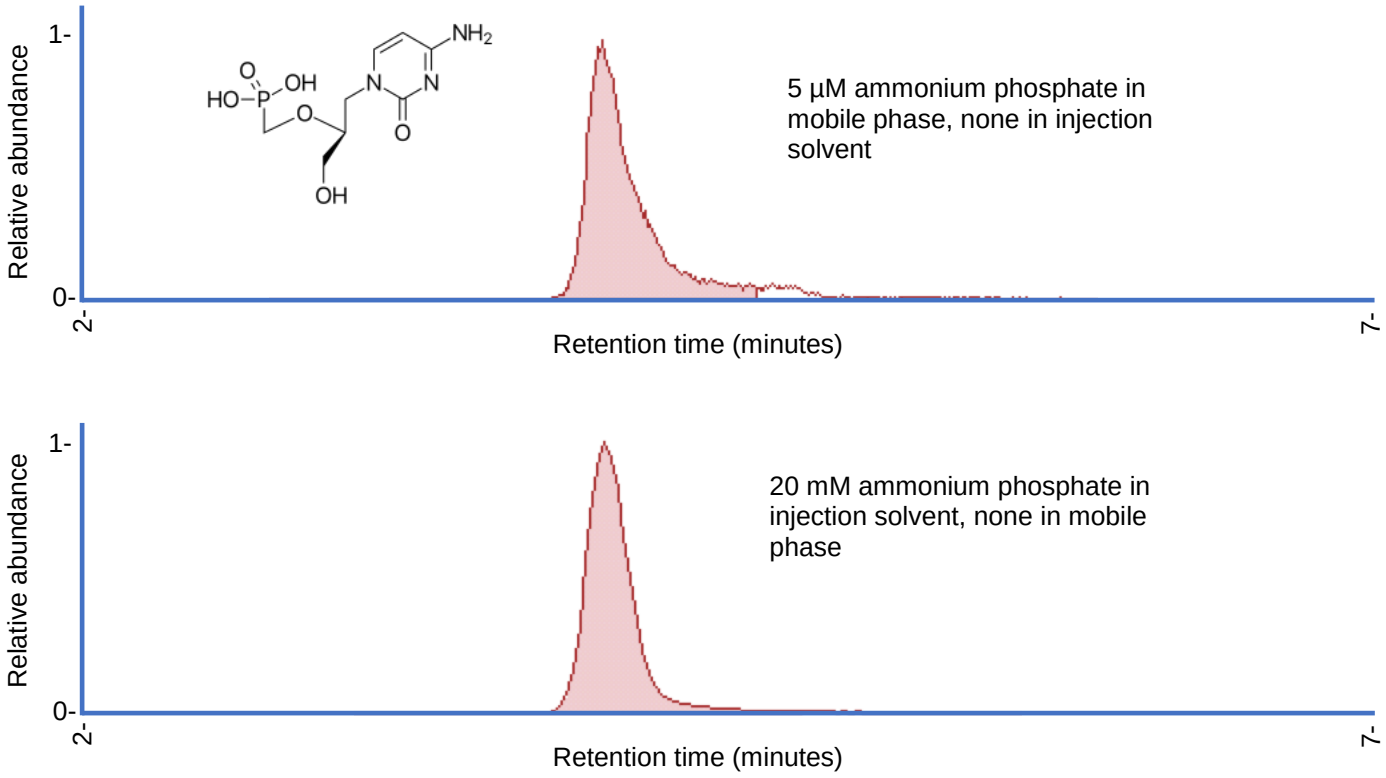


Figure S6: Effects of phosphate in the sample solvent on ZIC-pHILIC coverage, peak shape, and performance. Overall peak quality and coverage increased for the set of standards as detected using the ZIC-pHILIC stationary phase with and without 20 mM ammonium phosphate in the injection solvent. The results were nearly identical to using 5 µM phosphate in the mobile phase.

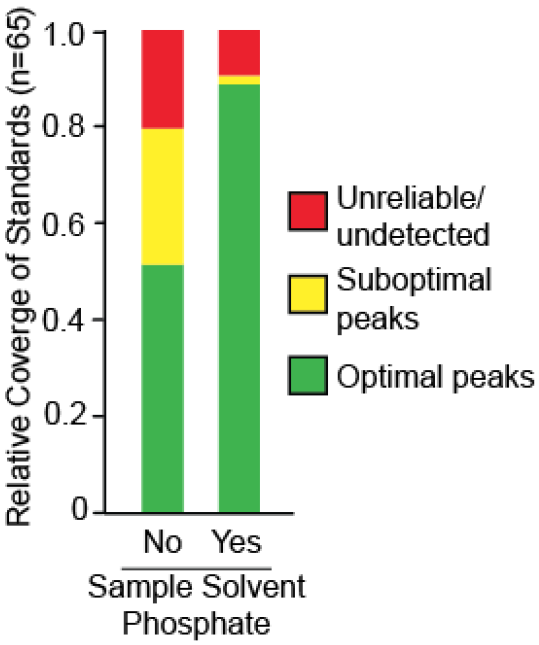


Figure S7: Ionization effects of trace phosphate on chemical standards. Standards were analyzed via direct infusion in the presence or absence of 5 μM ammonium phosphate in the mobile phase to isolate phosphate's effects on ionization from chromatography. Each standard was analyzed with the ionization mode in which it ionized best, so as to remove redundancy. Data are normalized to the no-phosphate condition. On average, 5 μM phosphate increased ionization efficiency by 6% for negatively ionizing compounds and 16% for positively ionizing compounds. These increases were not uniform. Phosphate increased signal by up to 50% for some compounds and decreased it by up to 25% for others. Higher concentrations of phosphate in the mobile phase (≥ 10 μM) showed more ion suppression. In our LC method, whether introduced in the mobile phase or the sample solvent directly, phosphate generally eluted in a large peak about halfway through the gradient, with sub-detectable elution before the peak and constant very low-level elution after. Considered collectively, our results suggest that trace phosphate slightly improves ESI efficiency throughout most of our gradient, independent of its chromatographic effects, except during the elution of the main phosphate peak, at which point its ESI effects may be more suppressive.

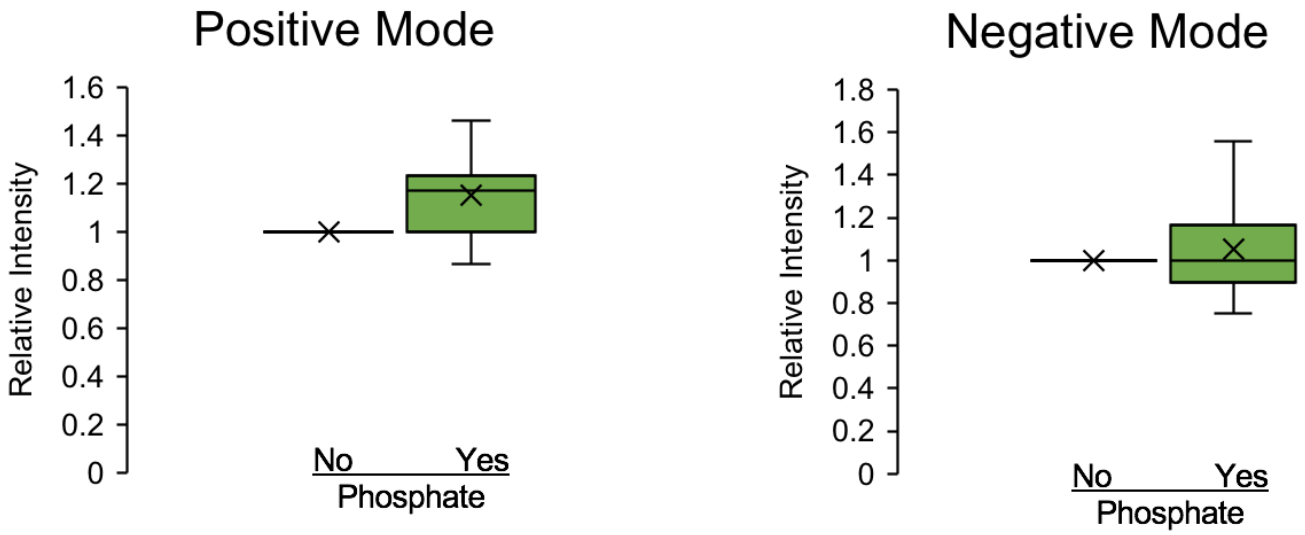


Figure S8. EICs of 2-PG from *credentialed E. coli* extracts. When 20 mM ammonium phosphate is included in the injection solvent (bottom), the 2-PG peak shape is improved. The peak width and shape of 2-PG with phosphate is within our *credentialing* parameters and was counted as a *credentialed* feature, whereas the wide, jagged peak shape without phosphate was not *credentialed*.

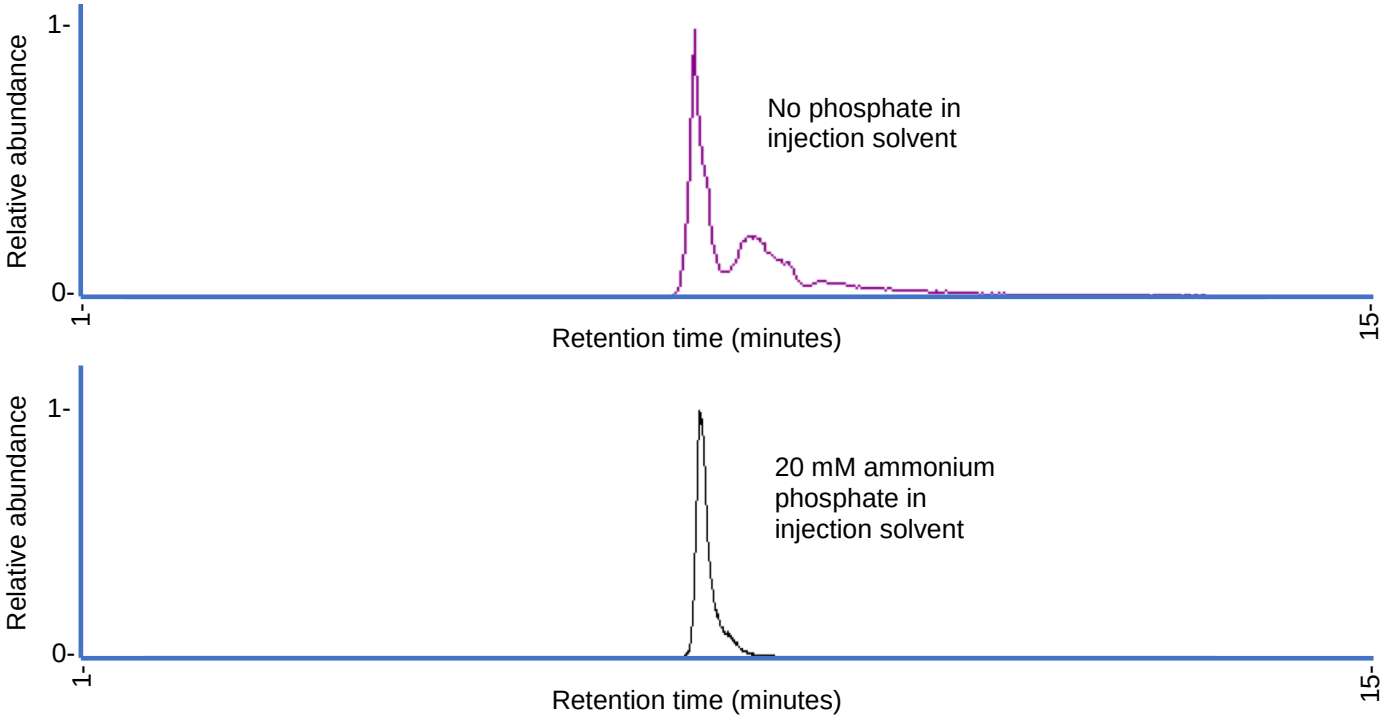
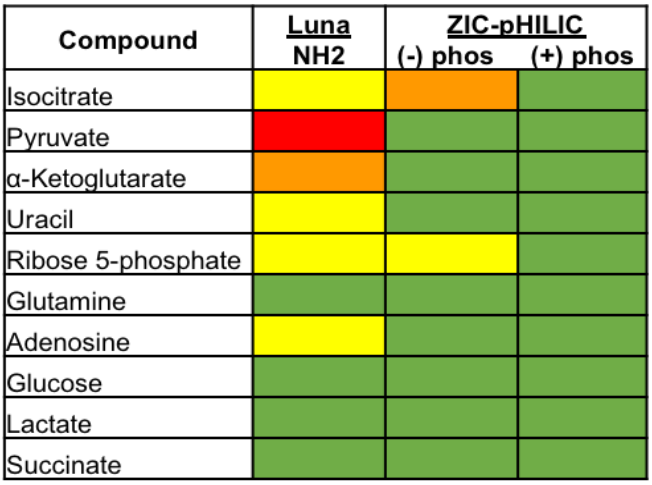


Figure S9. Heatmap of ten polar metabolites identified in *E. coli* extracts with excellent resolution on the ZIC-pHILIC when phosphate was present in the injection solvent. Inferior results were obtained when phosphate was omitted with the ZIC-pHILIC and when the Luna aminopropyl method was used. Red indicates that a standard was not detected above baseline noise. Orange indicates an unreliable peak with degradation in the form of significant band broadening, peak splitting, or asymmetry. Yellow indicates a quantitatively reliable peak with slight peak tailing, band broadening, or asymmetry. Green indicates an ideal peak shape.

Metabolites Detected in *E. coli*



Compound	Luna NH2	ZIC-pHILIC (-) phos	ZIC-pHILIC (+) phos
Isocitrate			
Pyruvate			
α-Ketoglutarate			
Uracil			
Ribose 5-phosphate			
Glutamine			
Adenosine			
Glucose			
Lactate			
Succinate			

Figure S10. EICs of 2-PG when analyzed without ammonium phosphate present (top) and with 20 mM ammonium phosphate injected onto the column prior to standard injection (bottom).

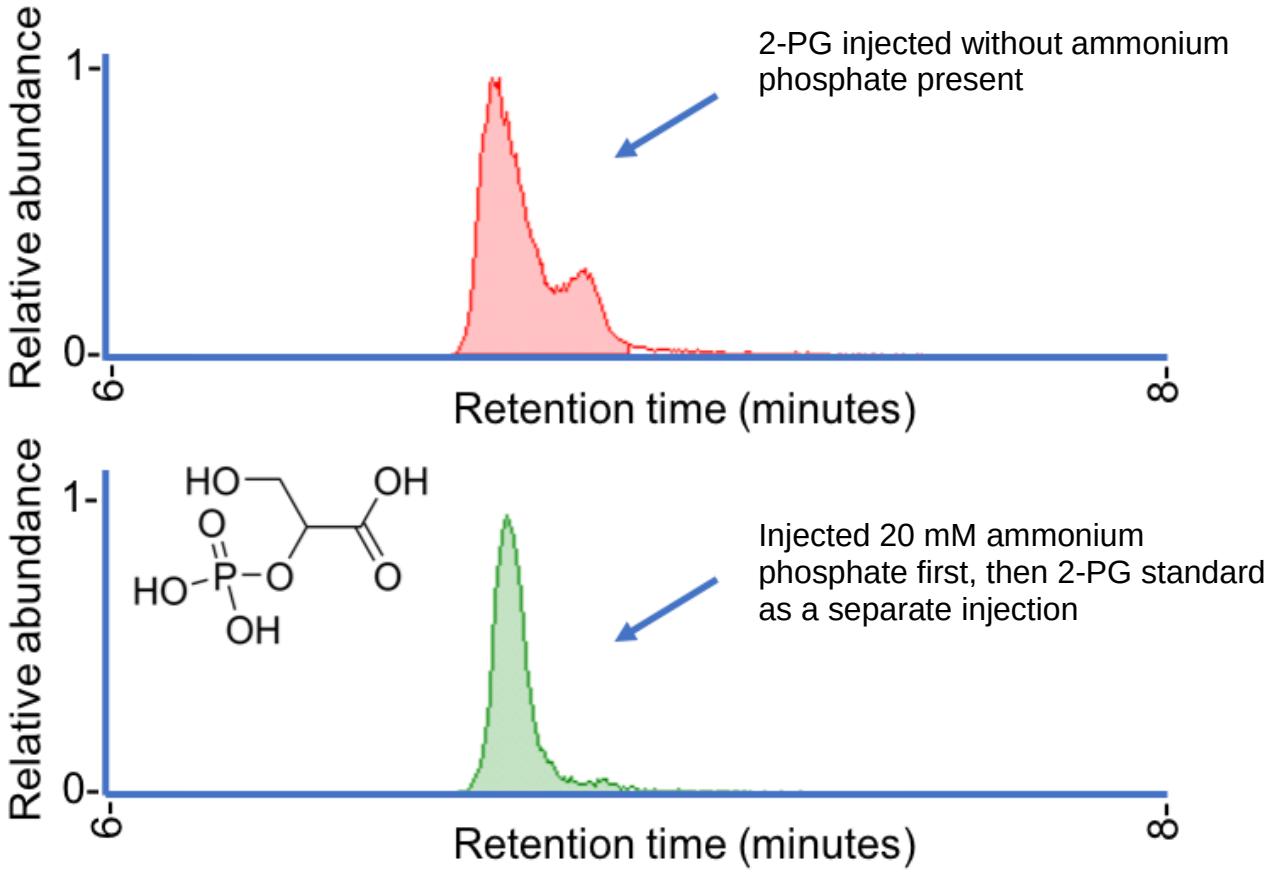


Figure S11. Effects of varying concentrations of mobile phase salt on ZIC-pHILIC performance**.** The bar graph shows that the relative peak height increases across the set of standards until 40 mM salt, which shows similar levels of ion suppression to the high salt conditions. The data shown are compiled from MS runs in positive and negative polarity. Compounds that eluted in the void volume were not considered in this analysis. Errors bars represent 95% confidence intervals.