Improved LC/MS Analysis of Polar Metabolites

Emerging Omics Webinar Series
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Agilent CrossLab
From Insight to Outcome

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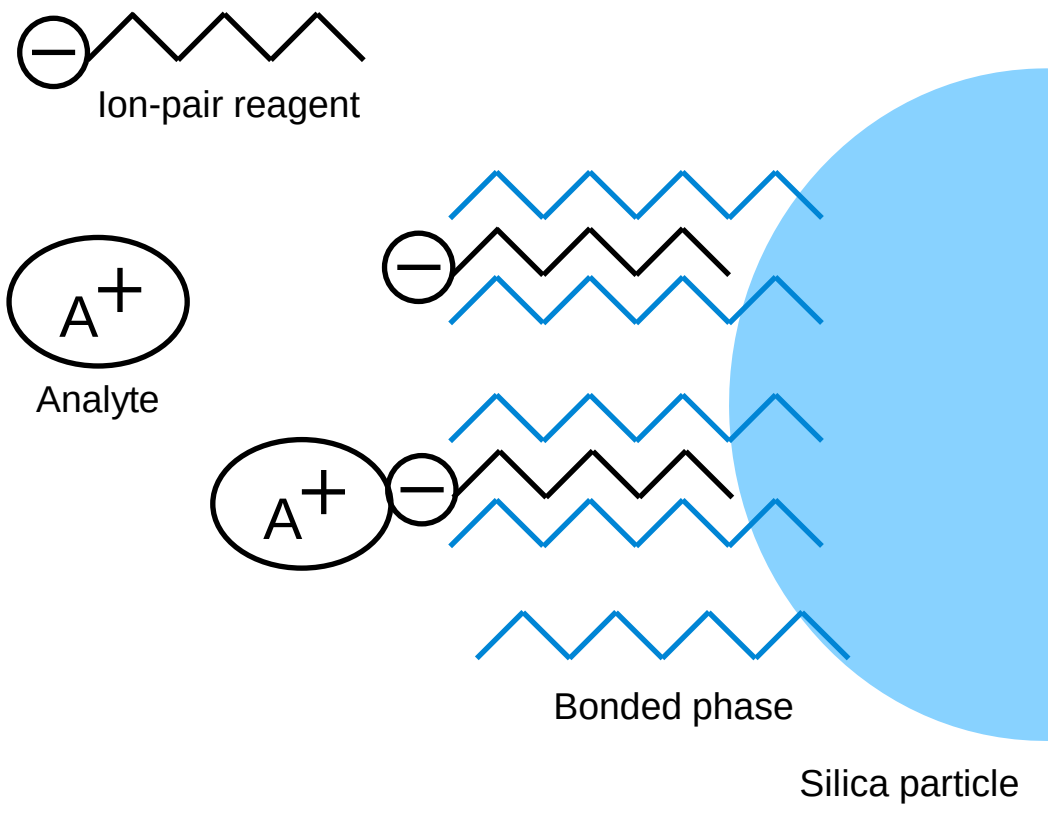
Current Methods for Metabolite Analysis

- Polar metabolites are not retained in reversed phase chromatography
- Could use ion pair reversed phase chromatography with reagents like tributylamine
- This improves retention and peak shape for polar acidic metabolites
- It is a very a robust method

Challenges of Ion Pairing:

- Doesn't work for all polar metabolites
- Ion-pairing reagents can stick in the LC system and column
- Trace levels of ion pair reagents change selectivity and create MS signals
- Therefore, users typically dedicate an entire LC system for ion pairing experiments

Schematic of ion pairing*:



* Image adapted from Figure 1 of Dolan JW. Ion Pairing – Blessing or Curse? LCGC Eur. 2008 (21):5, 258-263.

New Zwitterionic HILIC Column for Metabolite Analysis

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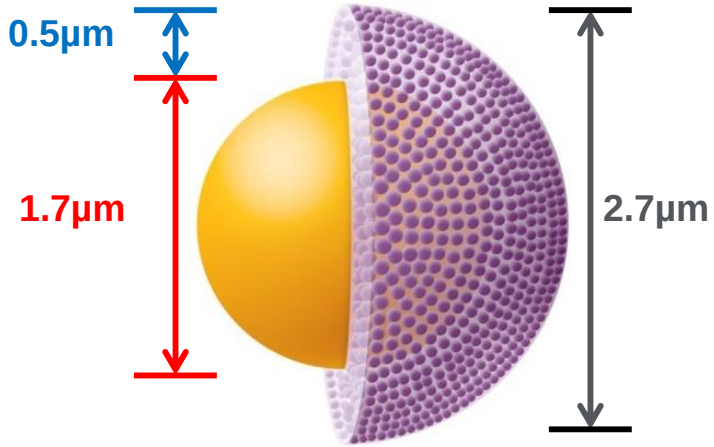
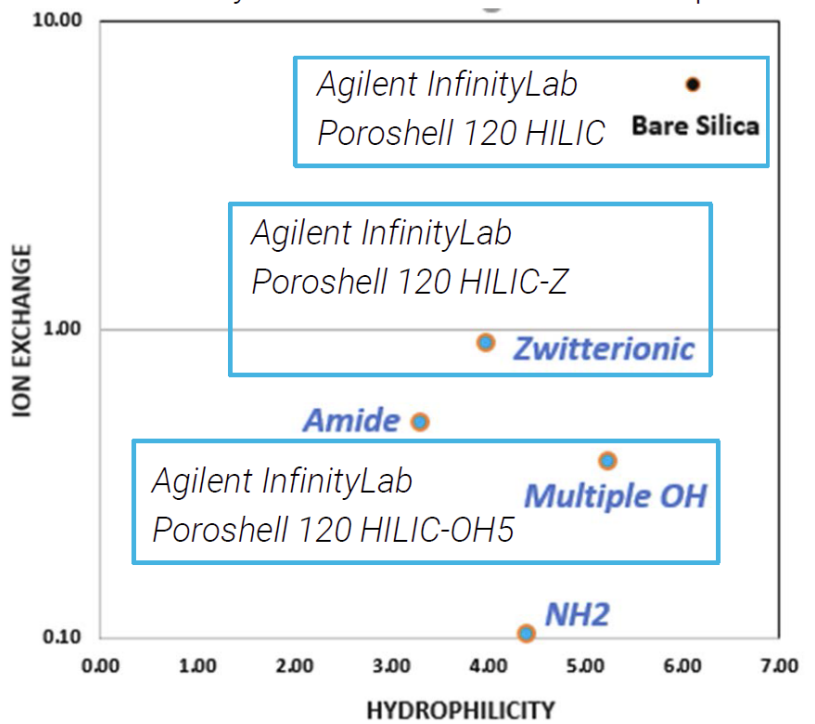
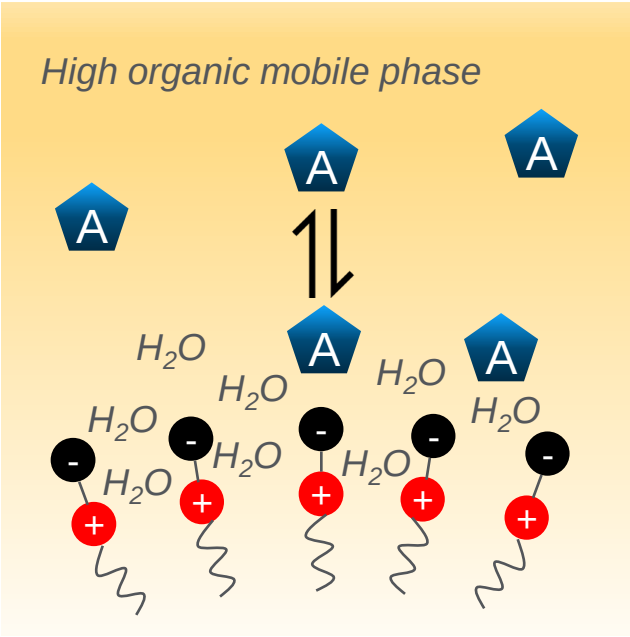
Based on Poroshell technology



The availability of orthogonal HILIC phases, allows for varied selectivity and facilitates method development.



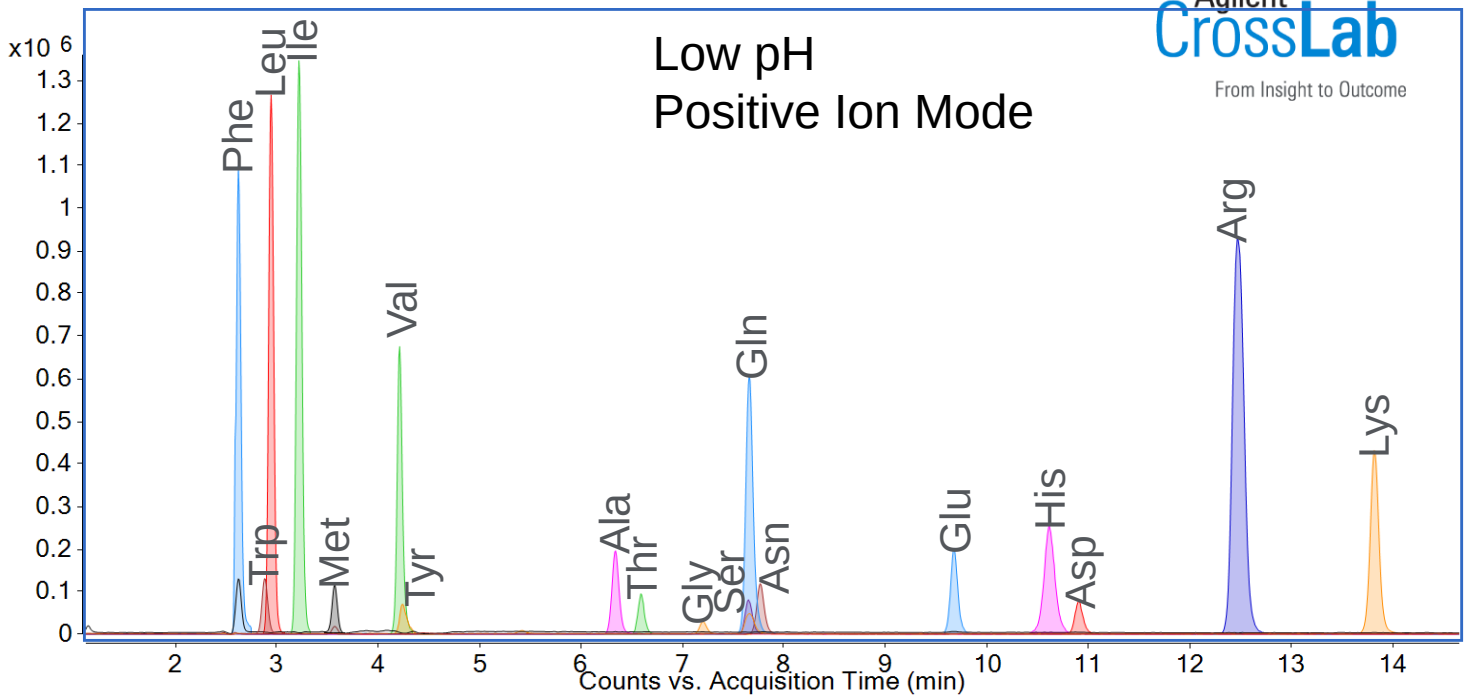


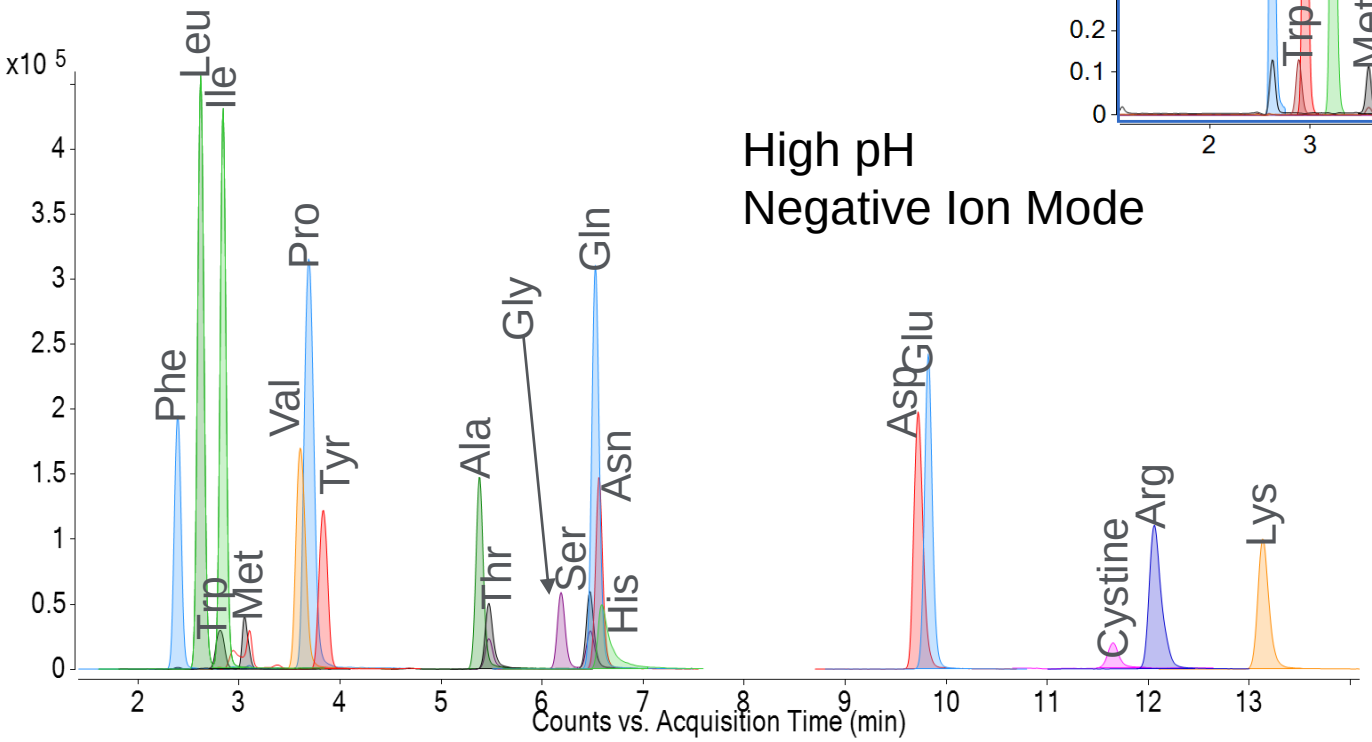
Zwitterionic,
high pH coating added

Best performance for
polar acidic metabolites

Amino Acids

Amino acids can be analyzed in either positive or negative ion mode.





- Low pH, positive ion is generally more sensitive
- Isoleucine and leucine are well separated
- Column is QA'ed with amino acids

What About Other Analytes?

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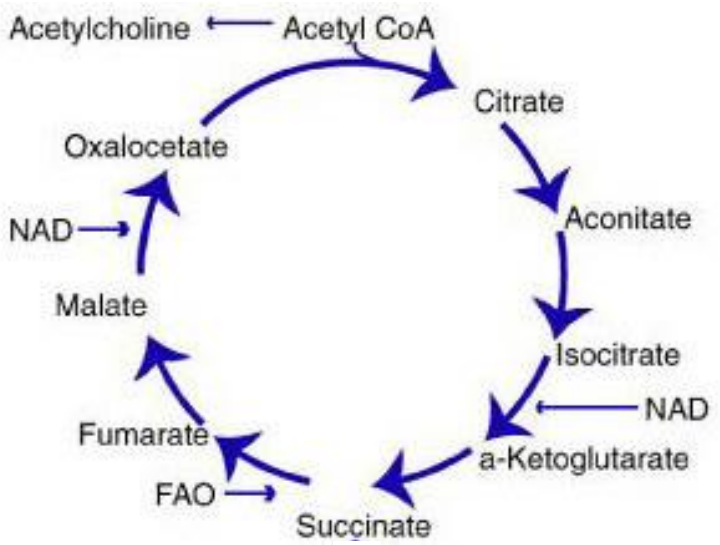
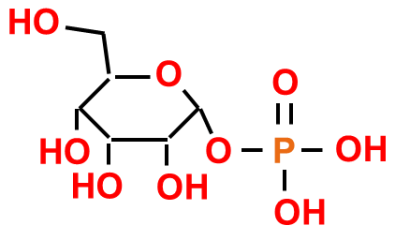
Some people need to analyze metabolites that present extra challenges:

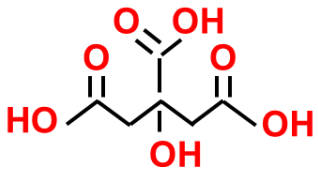
- Phosphorylated metabolites require negative mode MS and higher pH
- Some acidic metabolites are sensitive to trace metal in LC flow path[1-2]

TCA Cycle

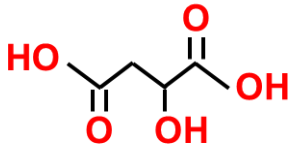




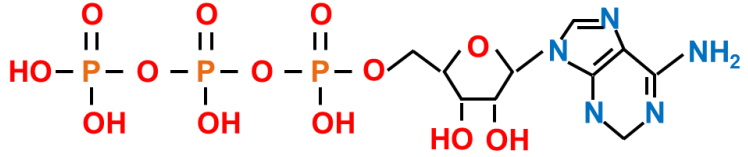
Glucose 1-phosphate



Citrate



Malate



ATP

[1] Myint KT et al, Anal Chem, 2009
[2] Pesek JJ et al, J Sep Sci, 2011

Initial Experimental Setup

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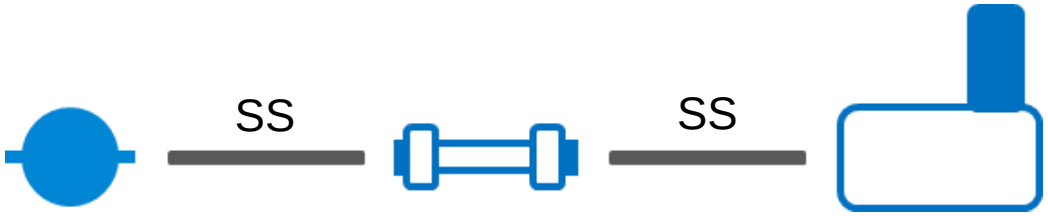
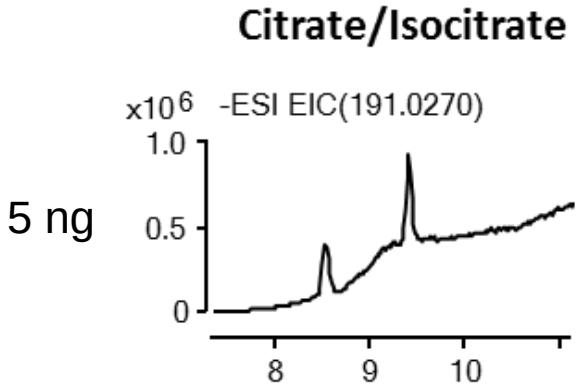
LC/MS setup:

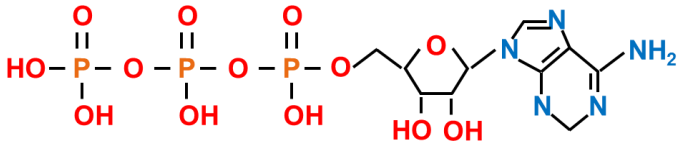


Results:

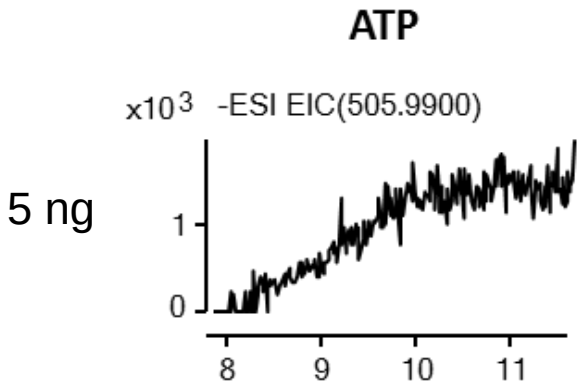


Samples:

- Phosphorylated Metabolites (e.g., ATP, sugar phosphates, etc.)



- Organic acids (e.g., malate, citrate, isocitrate, etc.)



Initial Column Testing Couldn't Detect Sub-10ng of Samples

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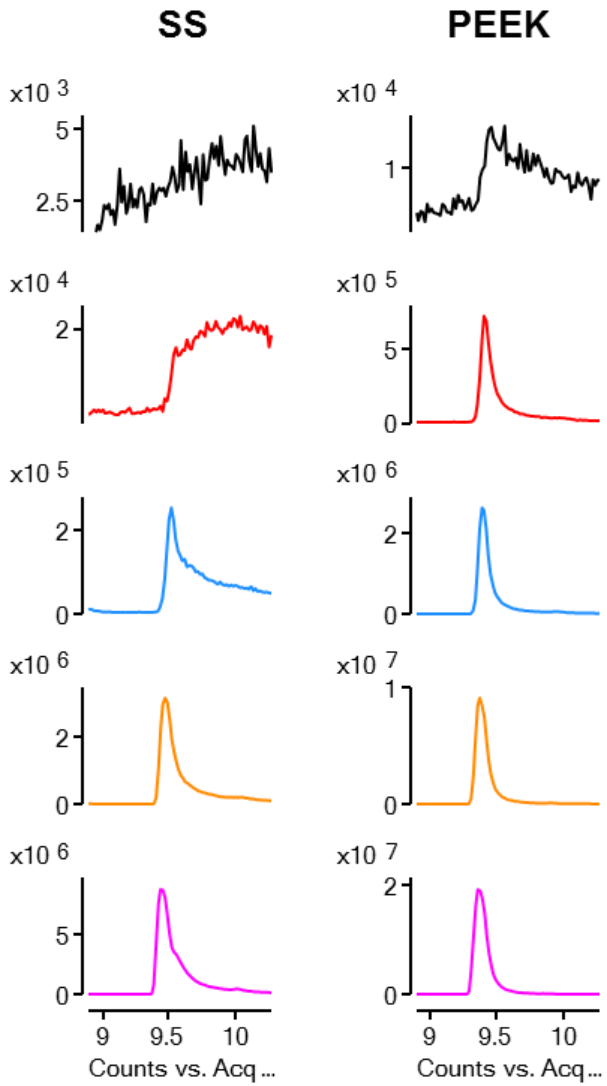
Buffer: pH 6.8 ammonium acetate

ATP



Stainless steel column

PEEK-lined steel column

Optimizing Analysis of Metabolites

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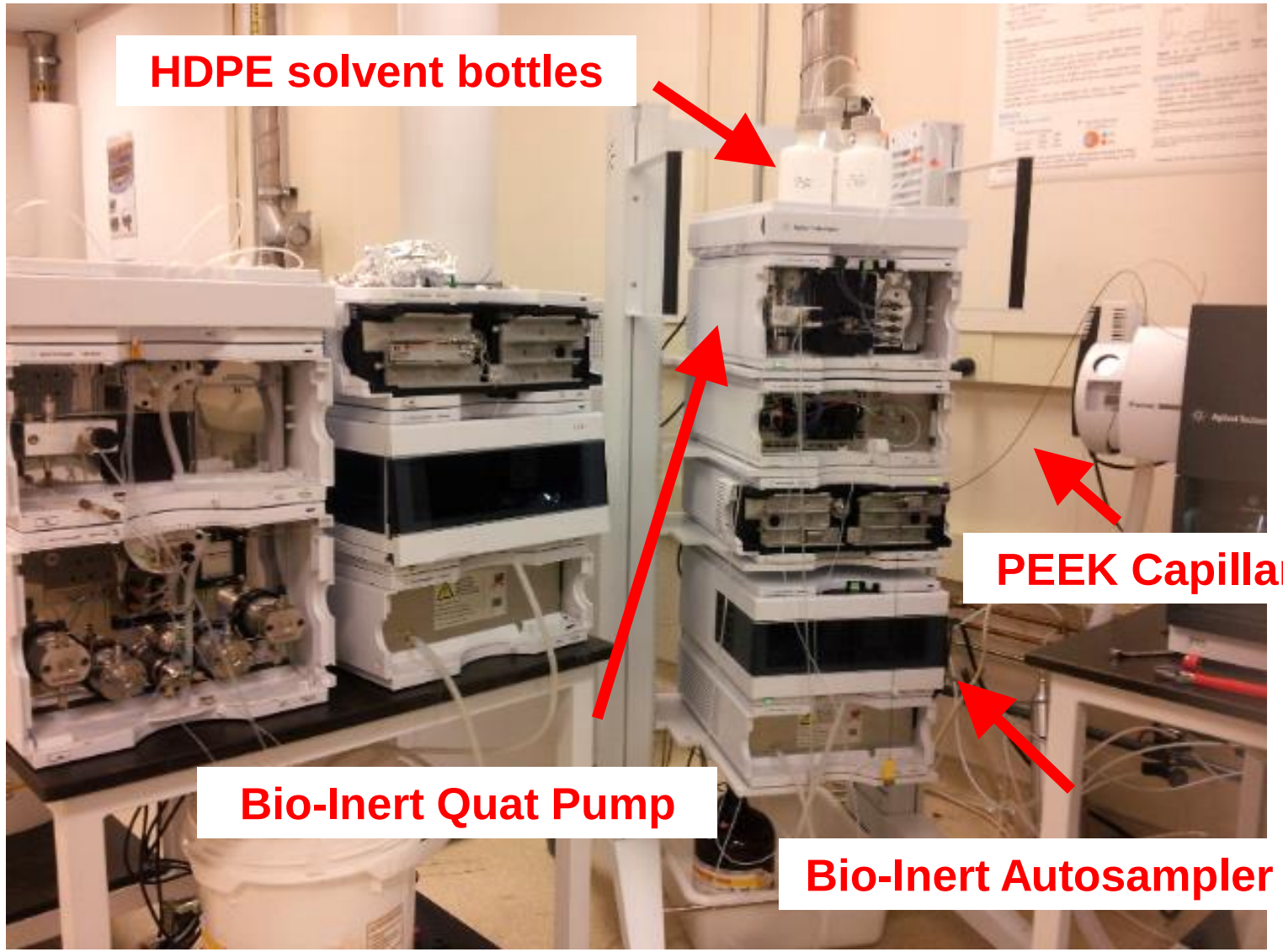

HILIC/MS Sensitivity Can Be Improved with PEEK-lined Capillaries

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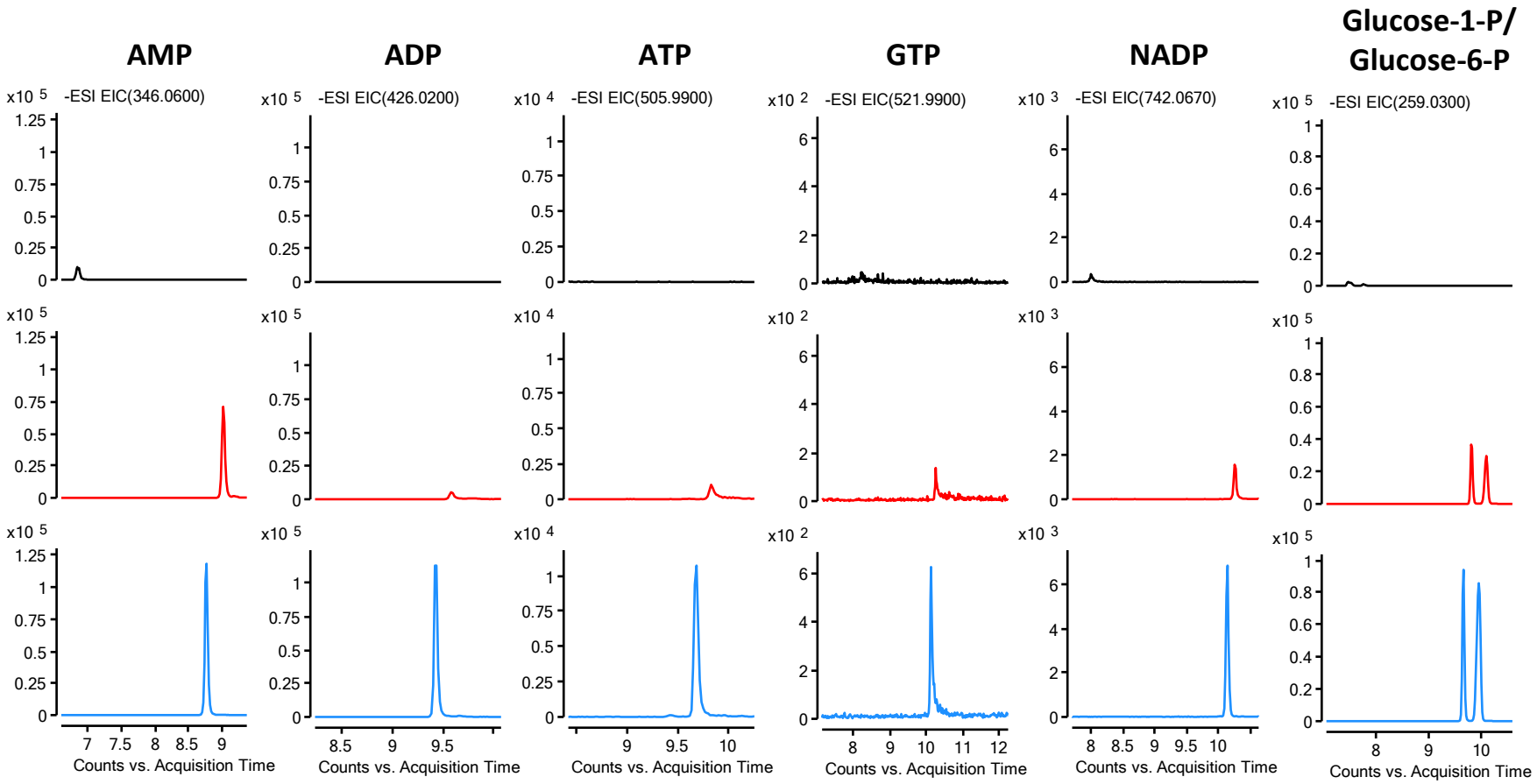

But Not Everyone Has a Bioinert Pump!

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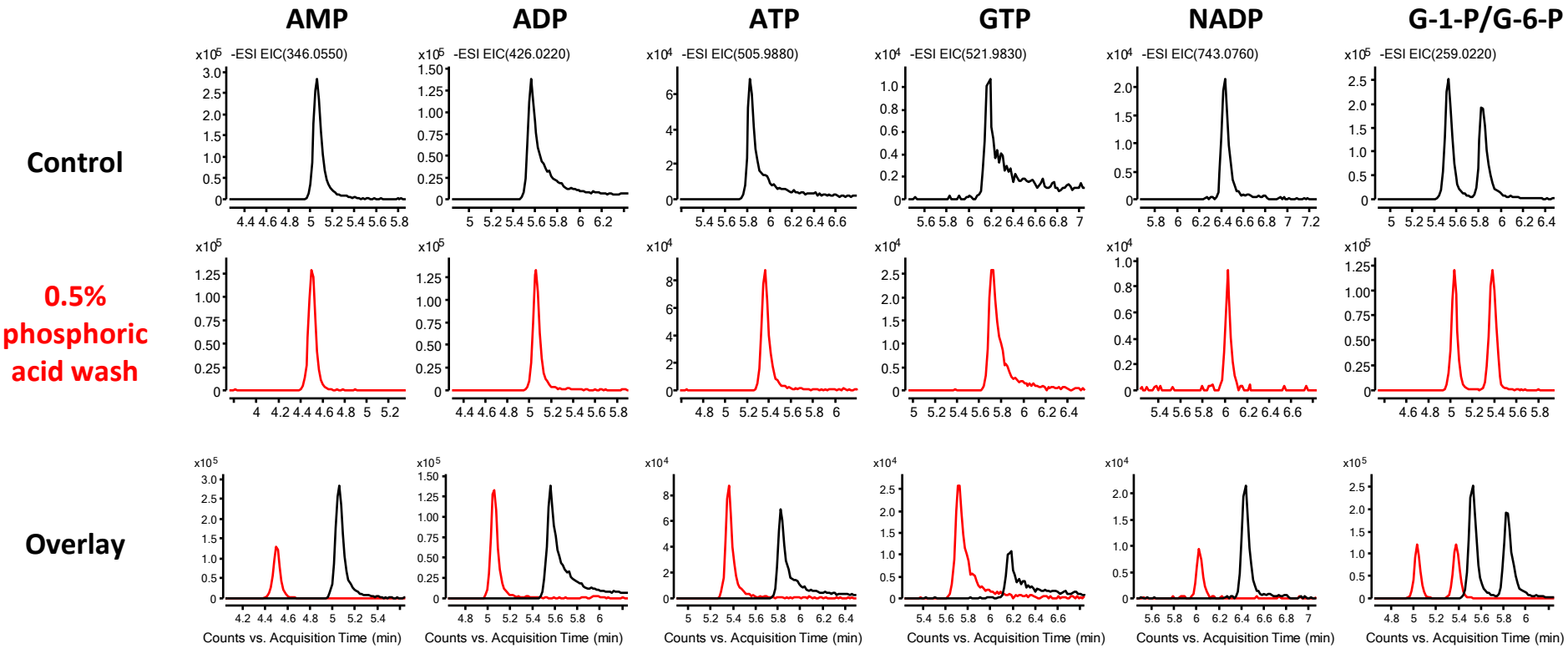
- Using a stainless steel 1290 binary pump
- Buffer[1]: pH 9.0 ammonium acetate



Peak shape and signal improves for phosphorylated analytes

(LC system passivation using 0.5% phosphoric acid wash)

[1] Tuytten R et al, J Chromatogr A, 2006

Effect of Passivation on Chelating Organic Acids

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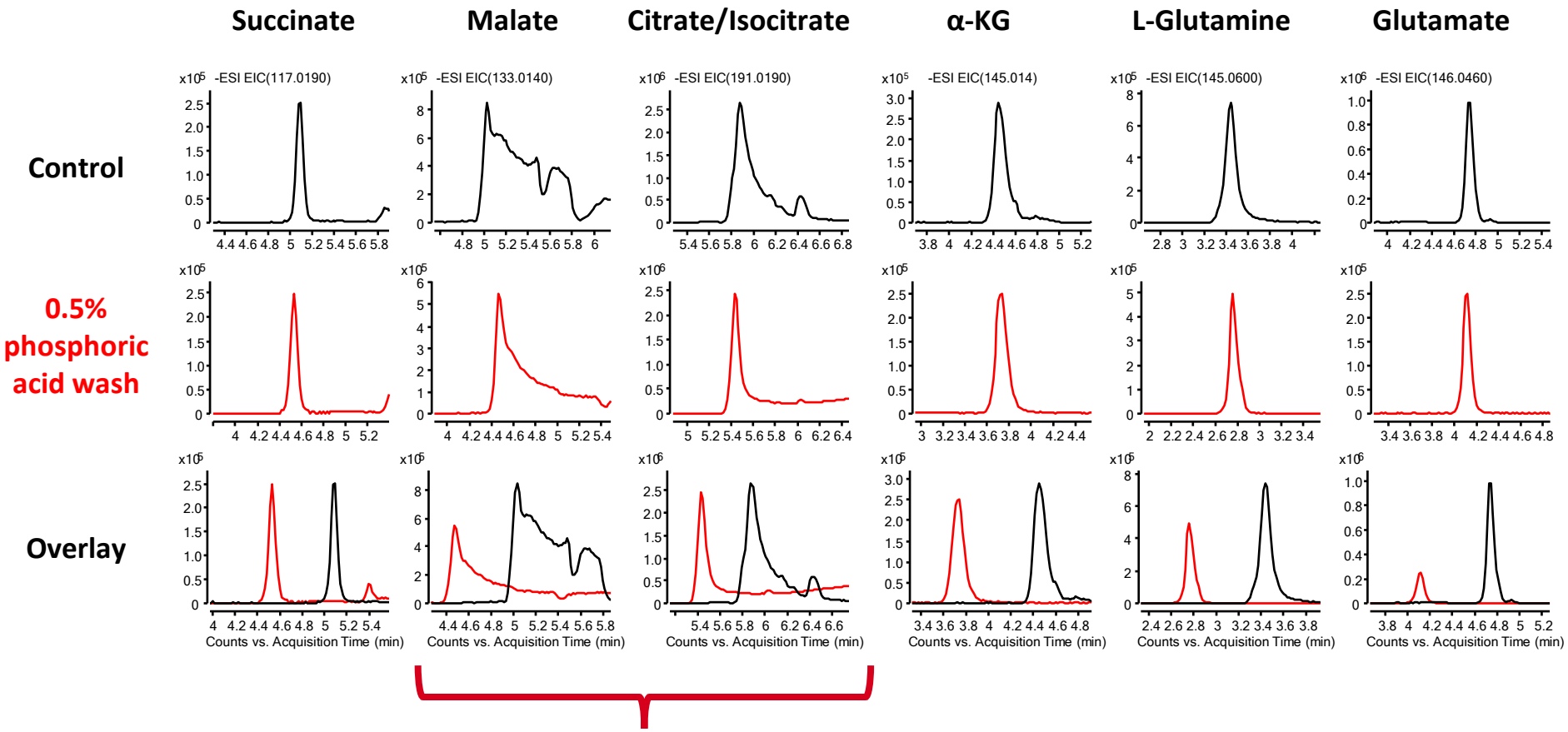


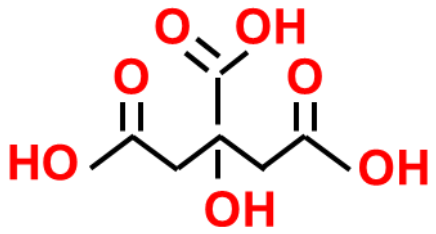
Peak shape is still poor even after wash

What Comes Next?

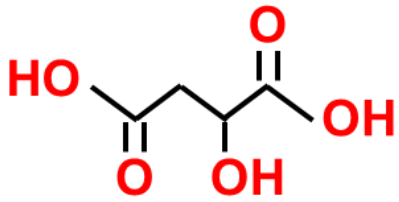
This method has great performance for amino acids, sugars and phosphorylated compounds.

However, you will have to passivate regularly if you are using a steel pump and steel autosampler flow path.

. . . . and we still have problems with strongly metal-chelating organic acids



Citrate



Malate

EDTA in Mobile Phase Solvents

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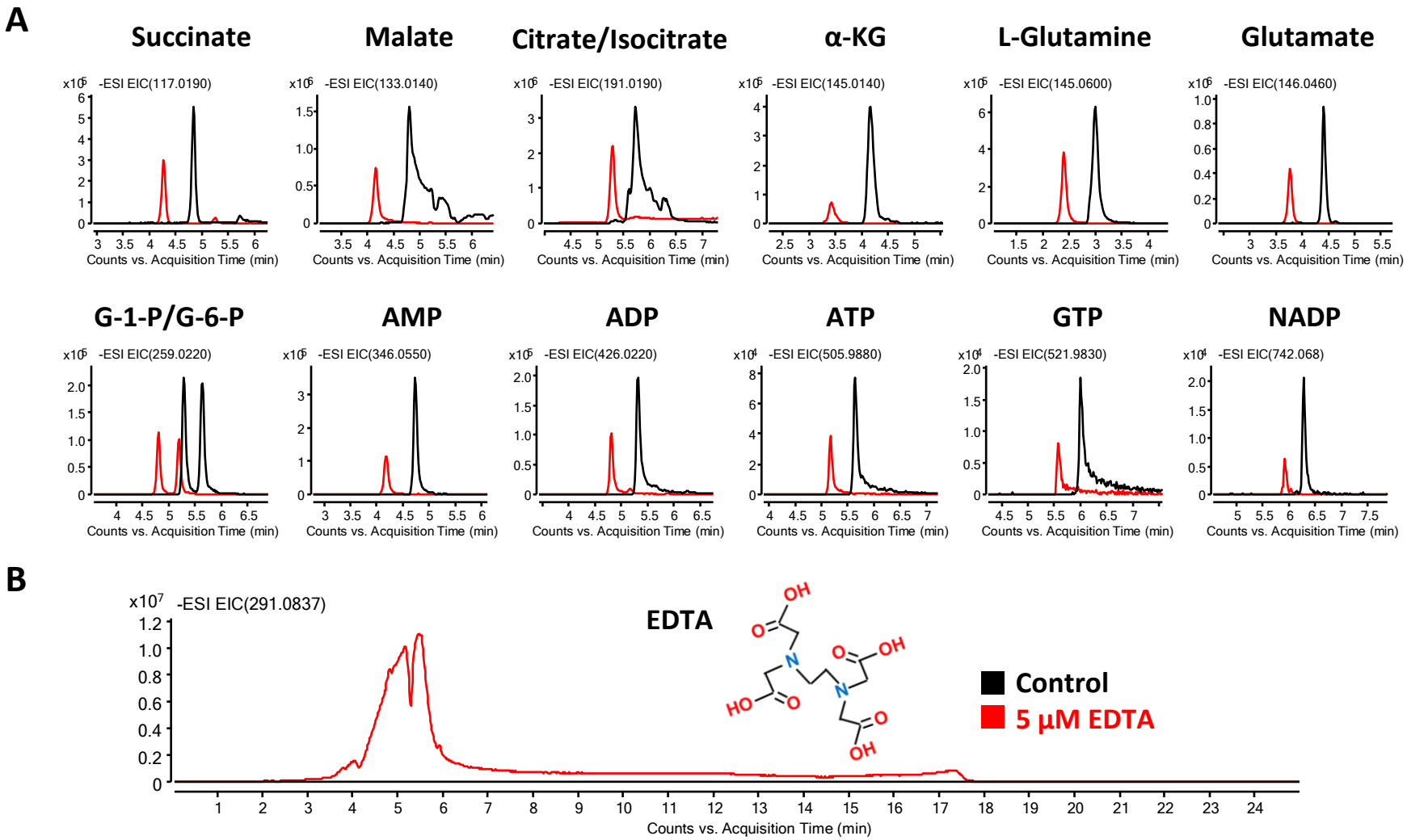

Medronic Acid in Mobile Phase Solvents

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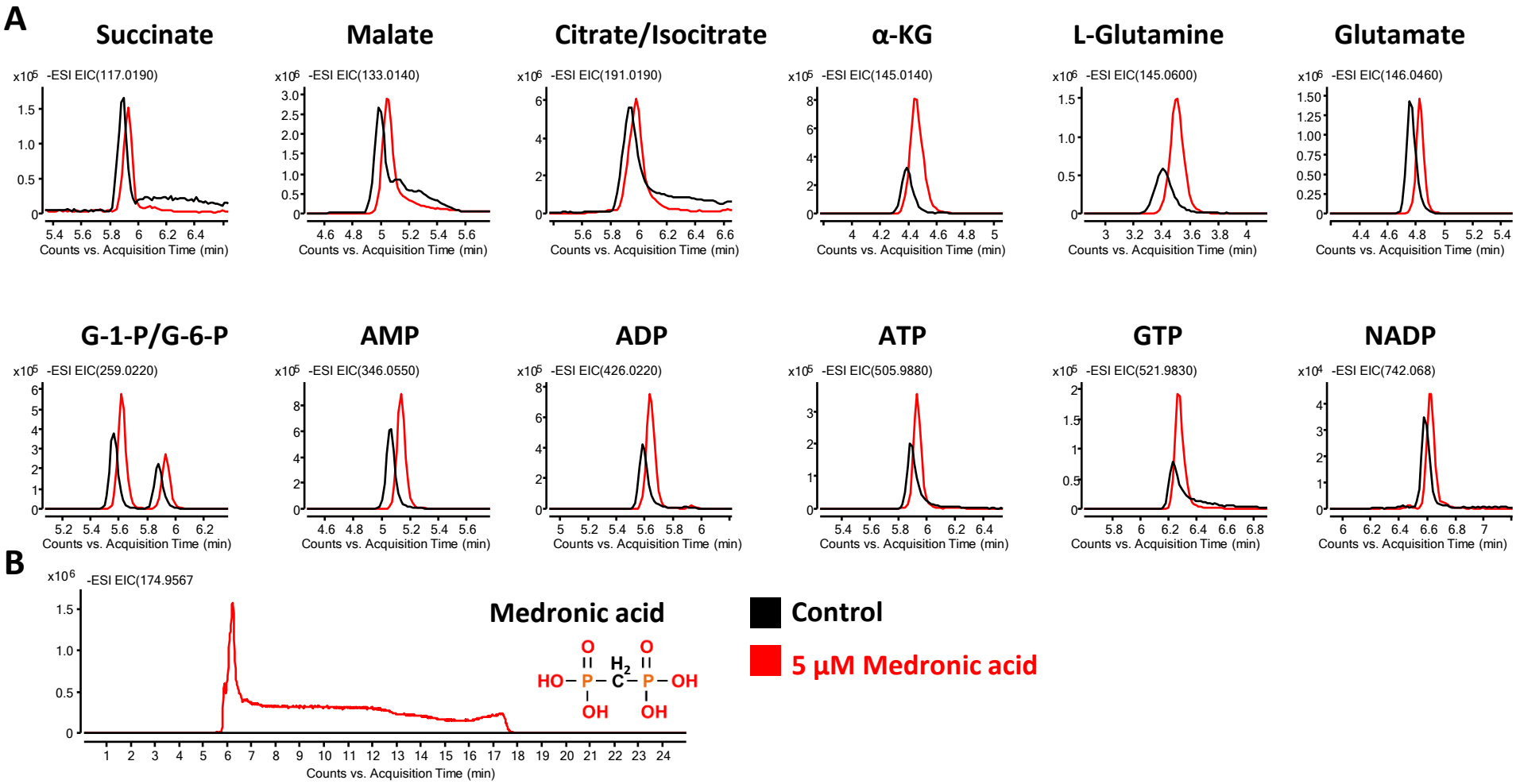

InfinityLab Deactivator Additive is Easily Cleared From the LC/MS System

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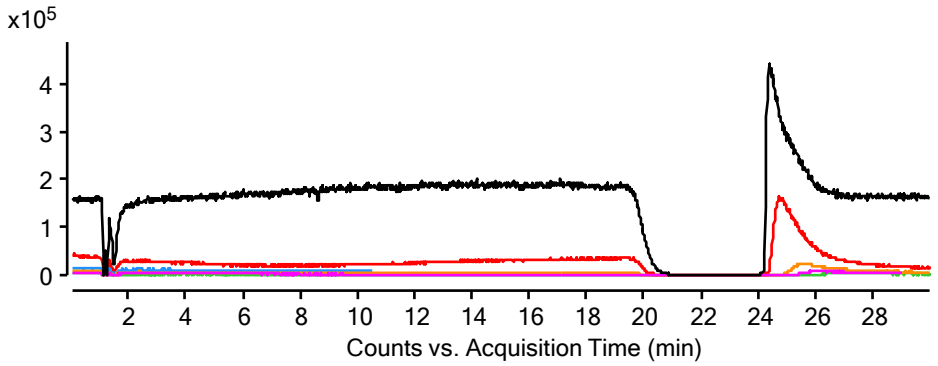
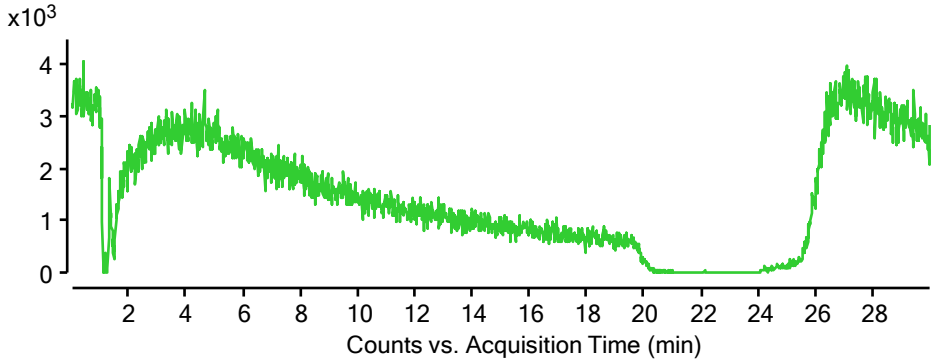


6th run with additive
1st post-additive run (no additive)
2nd post-additive run (no additive)
3rd post-additive run (no additive)
4th post-additive run (no additive)



5th post-additive run (no additive)

InfinityLab
Deactivator Additive
(part no. 5191-4506)

Hsiao JJ et al, Anal Chem, 2018

LOD of SSTL and PEEK-lined Columns on 6490 QQQ

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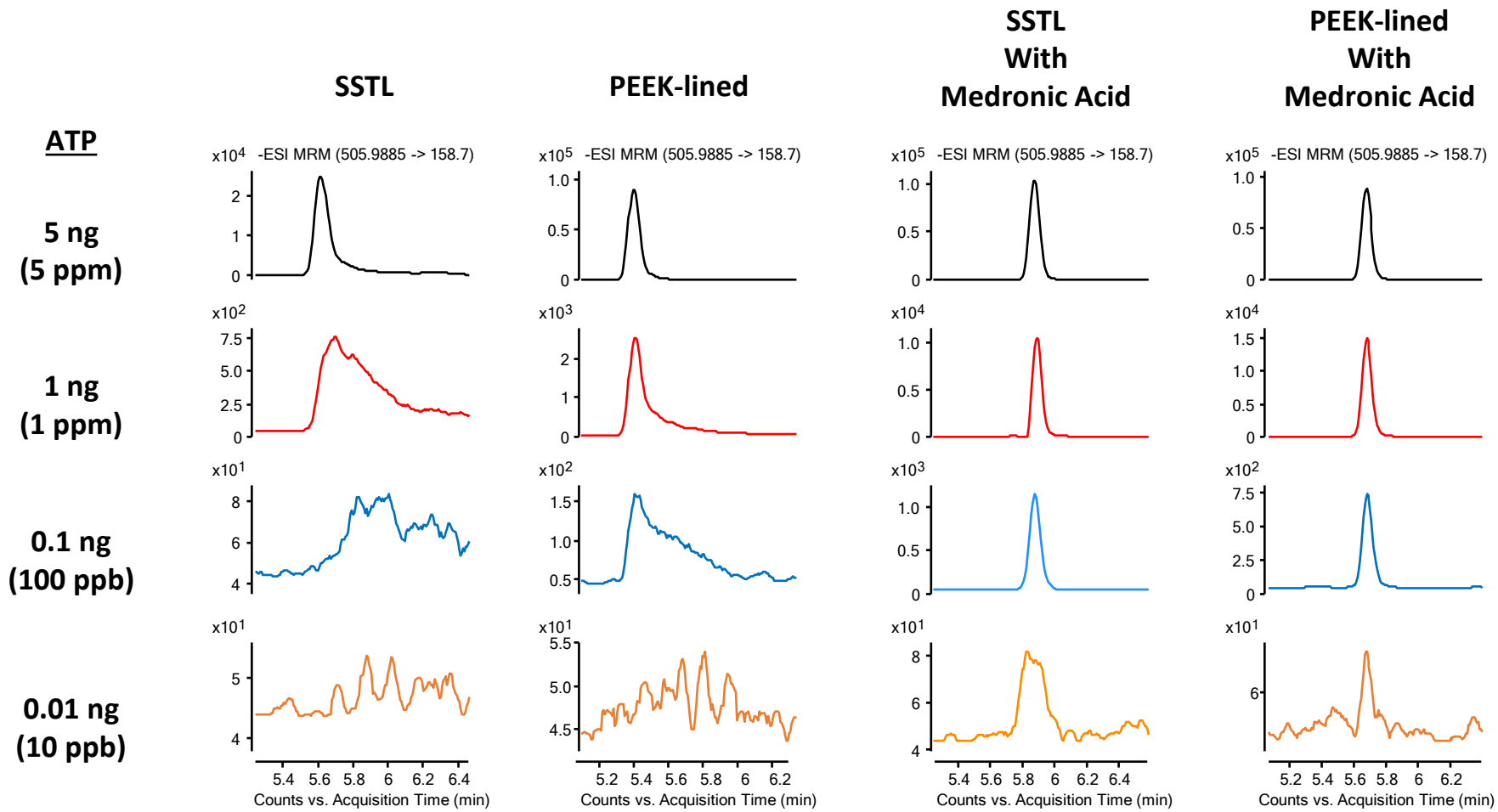

Improved Run to Run Reproducibility with the Additive

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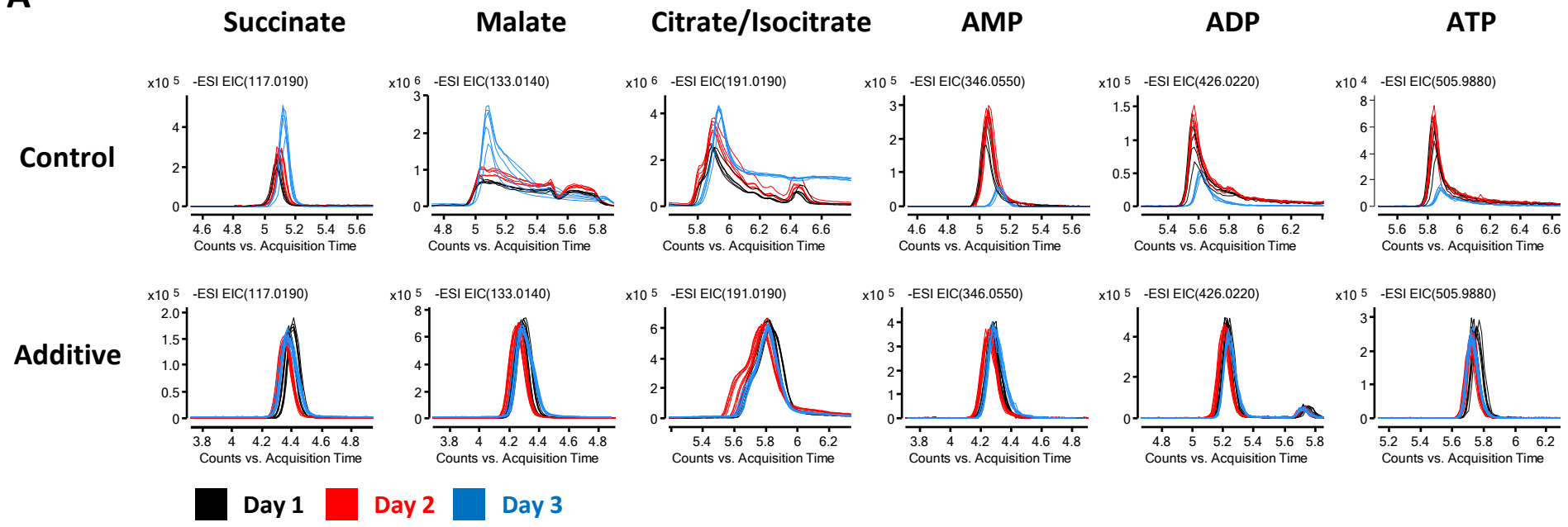
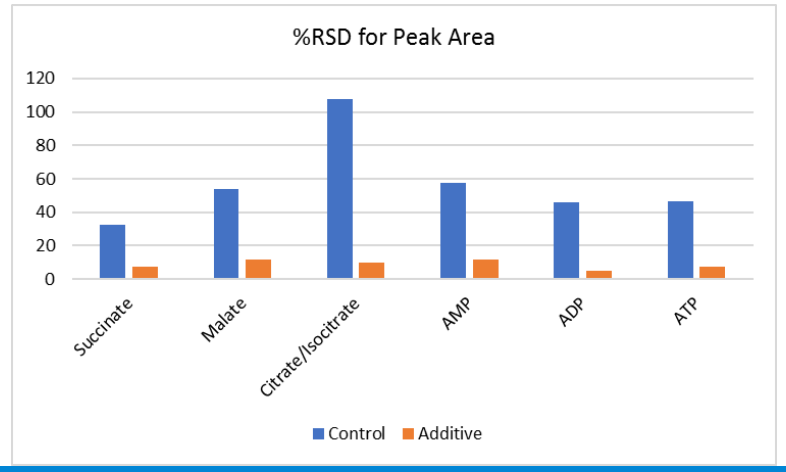
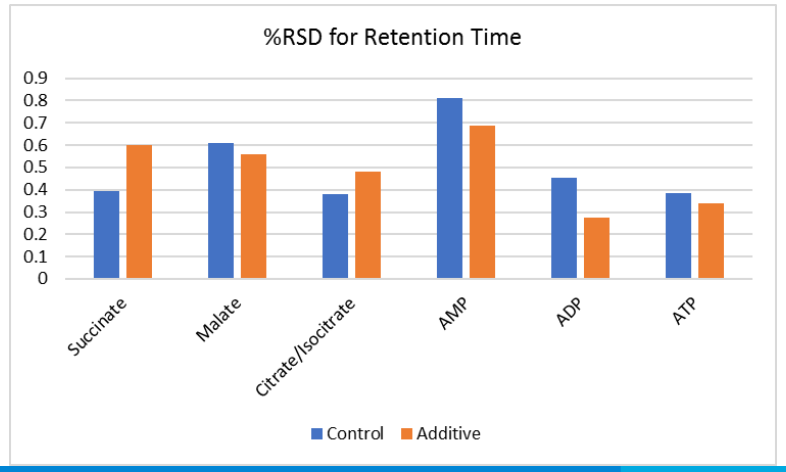
A



B



C



Salt Tolerance Study

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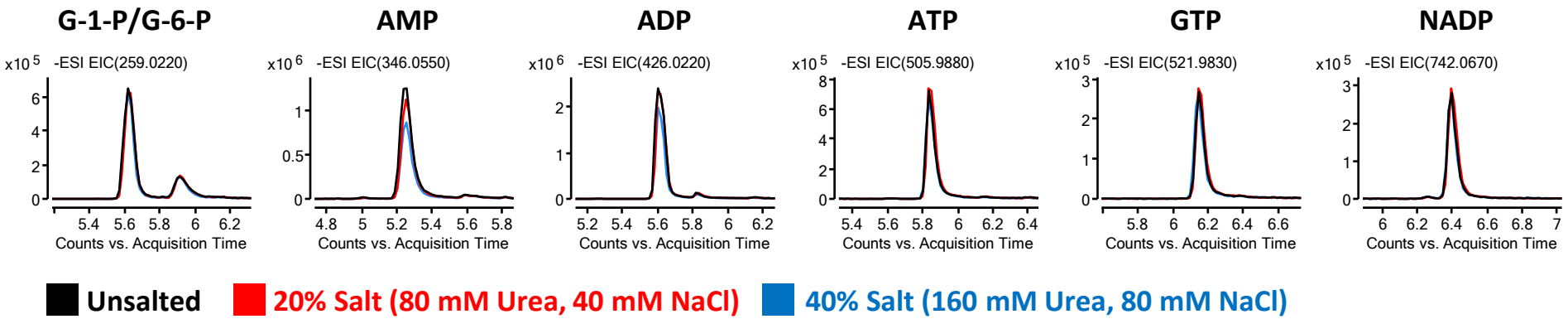
Upper Range of a Human Urine Sample		
Major Urine Components	**[Concentration]**	**[mM]**
Urea	23.3 g/L	390 mM
Chloride	8.4 g/L	236 mM
Sodium	4.4 g/L	191 mM
pH 6.3		

A 4M Urea, 2M NaCl stock solution was made for salt spike-in experiment.



Outline

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Brief Introduction

- Background
- Current methods
- Challenges

Story of the InfinityLab Poroshell 120 HILIC-Z column

- Discovery of a novel mobile phase additive

Applications

- Metabolomics analysis of MTX-treated cells
- Monitoring metabolites from white blood cells (USC collaboration)
- Monitoring metabolites in cell culture media

Metabolomics Analysis of MTX-treated Cancer Cells

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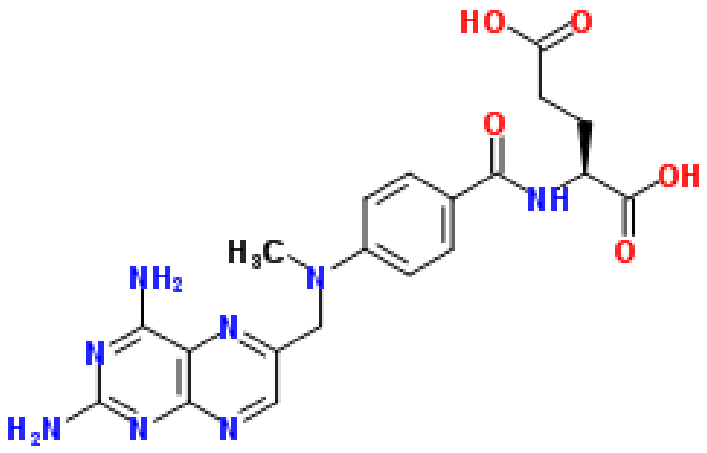


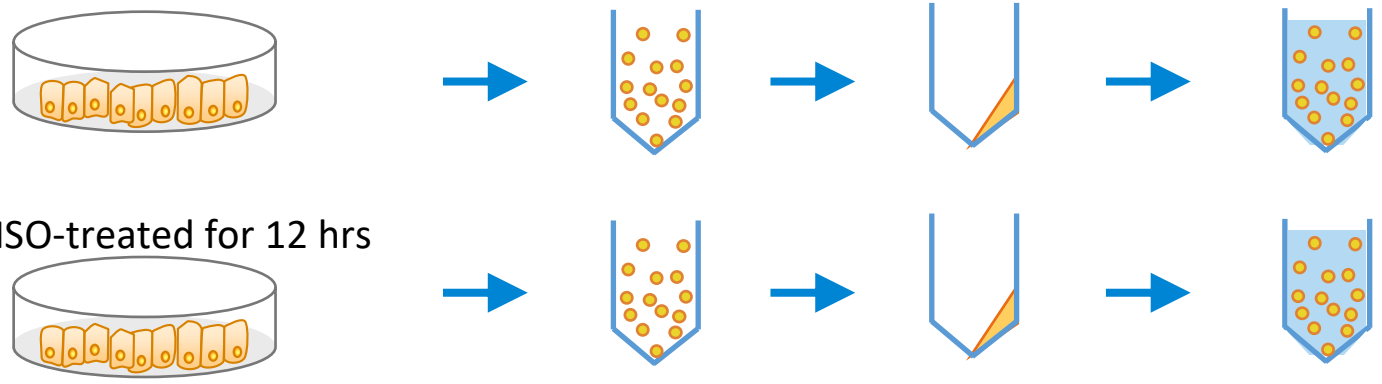
Methotrexate inhibits dihydrofolate reductase (DHFR) and thereby inhibits the synthesis of folate -> DNA, RNA, and proteins

5 µM MTX-treated for 12 hrs

DMSO-treated for 12 hrs

Cells were spun down, washed and collected

Extraction solution
(2:2:1 MeOH:ACN:H_2O with 0.1% formic acid)



MTX Induces Fructose 1,6-bisphosphate and Adenosine Levels

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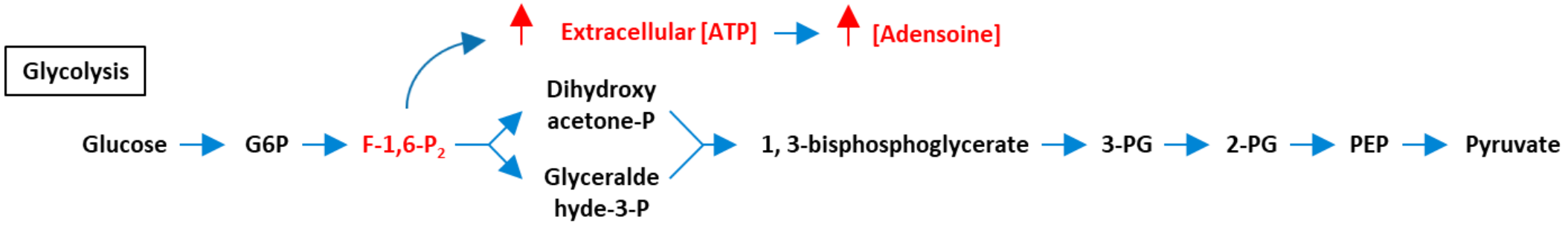

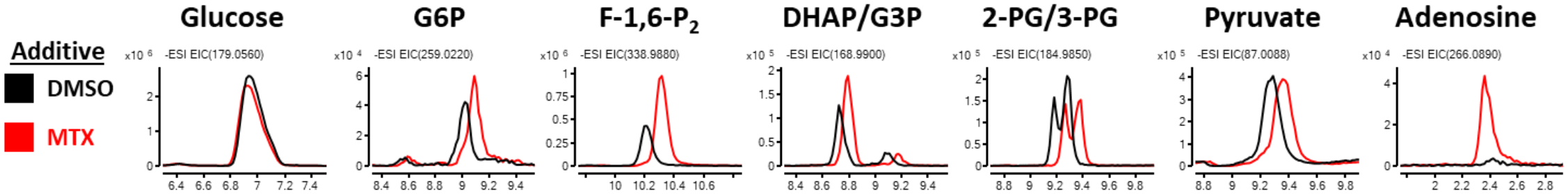

- Exogenous administration of F-1,6-P2 has been shown to sustain glycolysis and increase ATP production[1-3].
- Methotrexate systematically generates extracellular adenosine and subsequent activation of adenosine receptor A2a[4].

[1] Planas ME et al, Eur J Pharmacol, 1993
[2] Alves Filho, JC et al, Pharmacol Res, 2004
[3] Ahn SM et al, Br J Pharmacol, 2002
[4] Veras FP et al, Sci Rep. 2015

Analysis of Polar Metabolites Extracted from White Blood Cells

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Lawrence J. Ellison Institute for Transformative Medicine of USC

University of Southern California
USC Center *for* Applied Molecular Medicine

Drs. Jonathan Katz and Sujatha Chilakala

Sample Information

- WBCs extracted from whole blood of two volunteers using density gradient
- Polar metabolites were extracted using 80:20 methanol: water (chilled)

Analytical Method

Mobile phase:
A: 90/10 Water/ACN, 10 mM Ammonium formate, pH 9.2, 5 µM additive
B: 90/10 ACN/Water, 10 mM Ammonium formate, equal volume of ammonium hydroxide added as to mobile phase A, 5 µM additive

Column: Agilent Poroshell 120 HILIC-Z, P (2.1 X100 mm)
Gradient: 98 % B to 50% B in 10 min

Analysis of Polar Metabolites Extracted from WBCs

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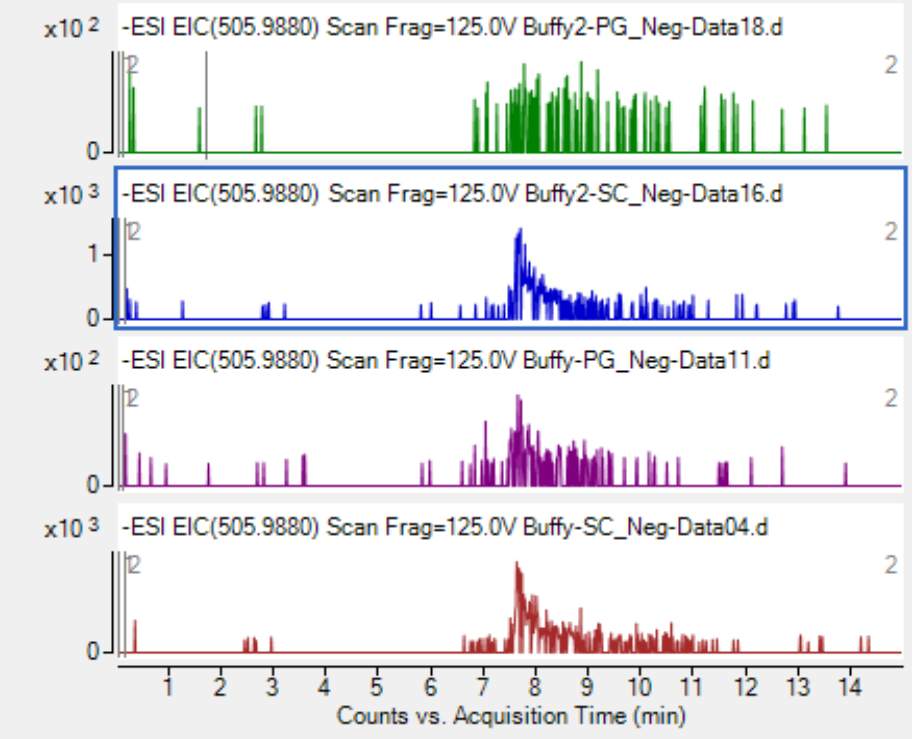
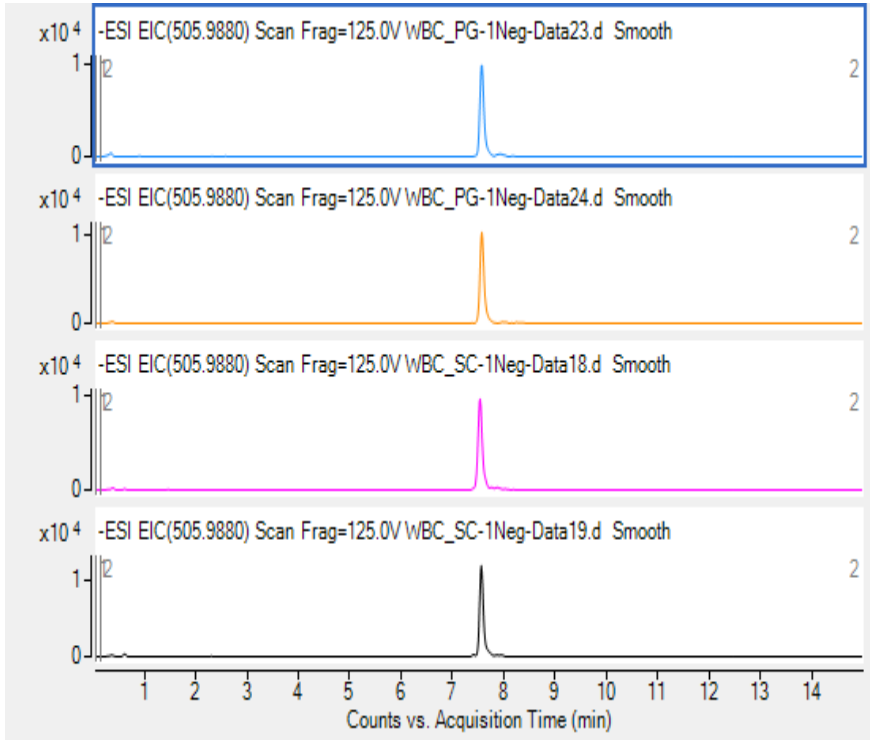
ATP

Control



With Additive



Analysis of Polar Metabolites Extracted from WBCs

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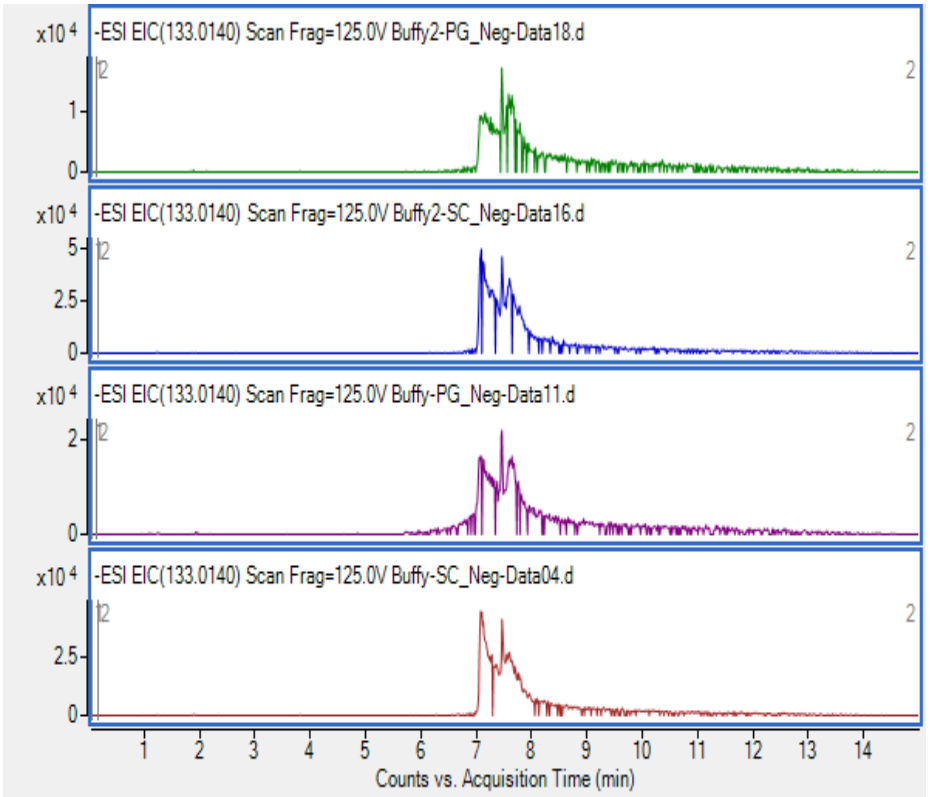
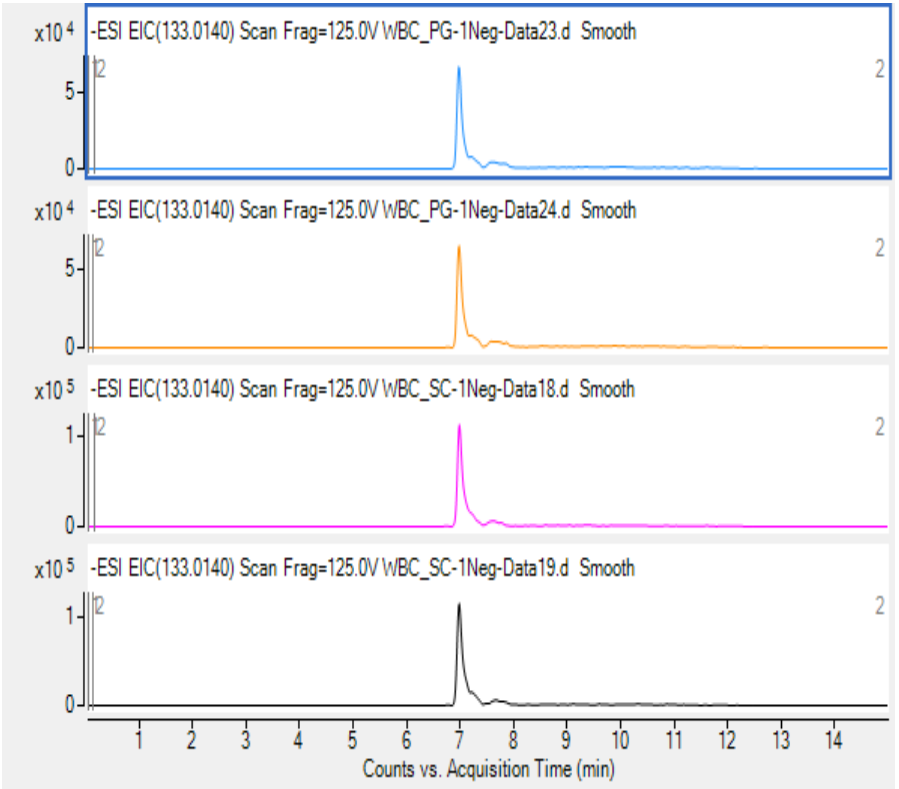
Malate

Control



With Additive



Analysis of Polar Metabolites Extracted from WBCs

Molecular feature extraction

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MFE Score	With additive(1424)	No additive(1417)
100	677	540
99.9-90	264	196
89.9-80	246	163
79.9-70	237	518

Why is Cell Media Analysis Critical?

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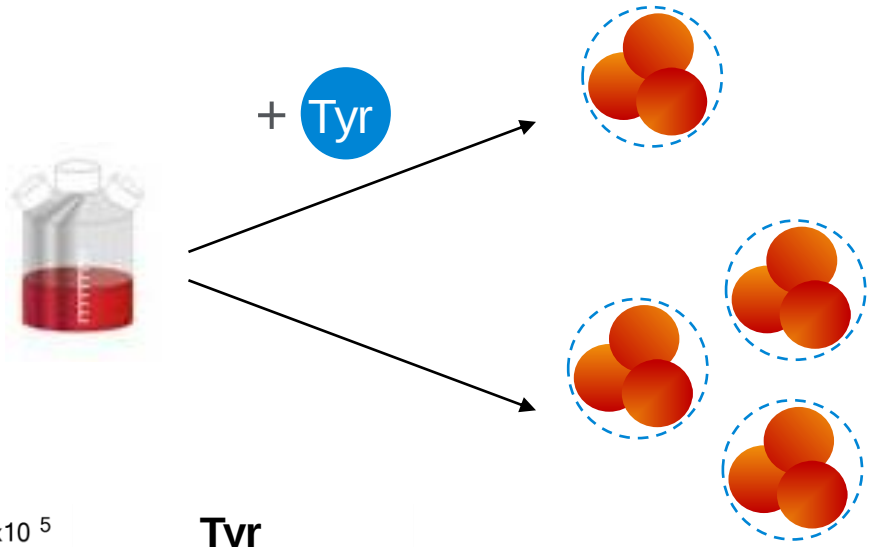
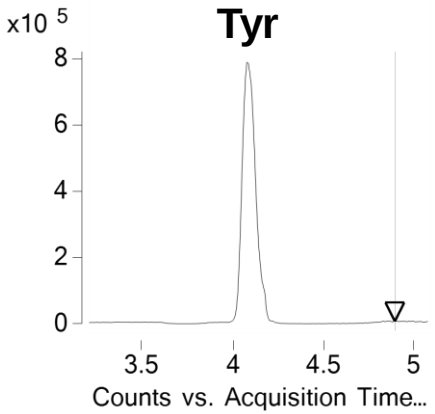
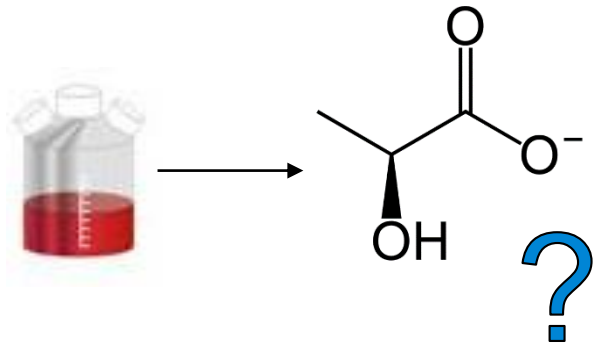
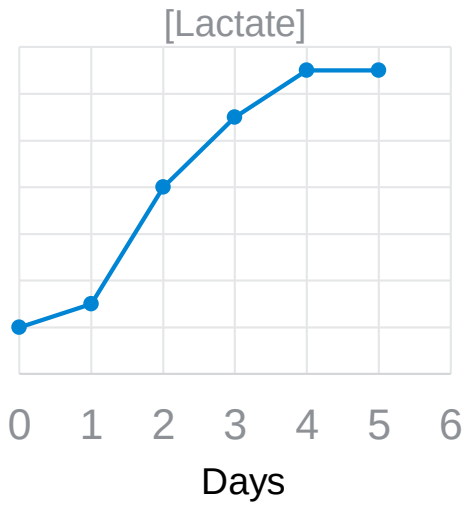
Example: Optimize tyrosine concentration to increases recombinant protein production

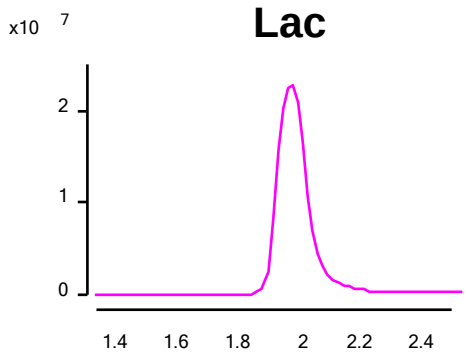




Example: Monitoring excreted lactate concentration and effects on cell growth







Experimental Workflow

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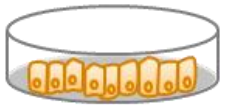
Mammalian Cell Culture



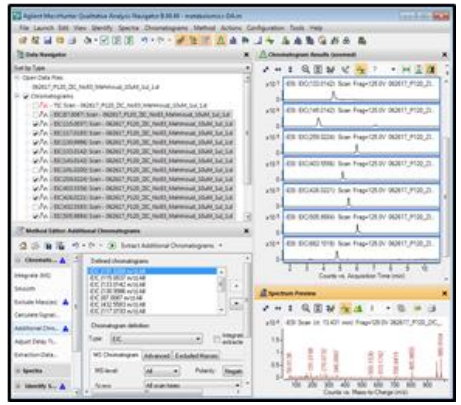
Harvest 100 µL at 0, 1, 2, 3, and 6 days

Add 400 µL of 50% acetonitrile

Analyze 1 µL

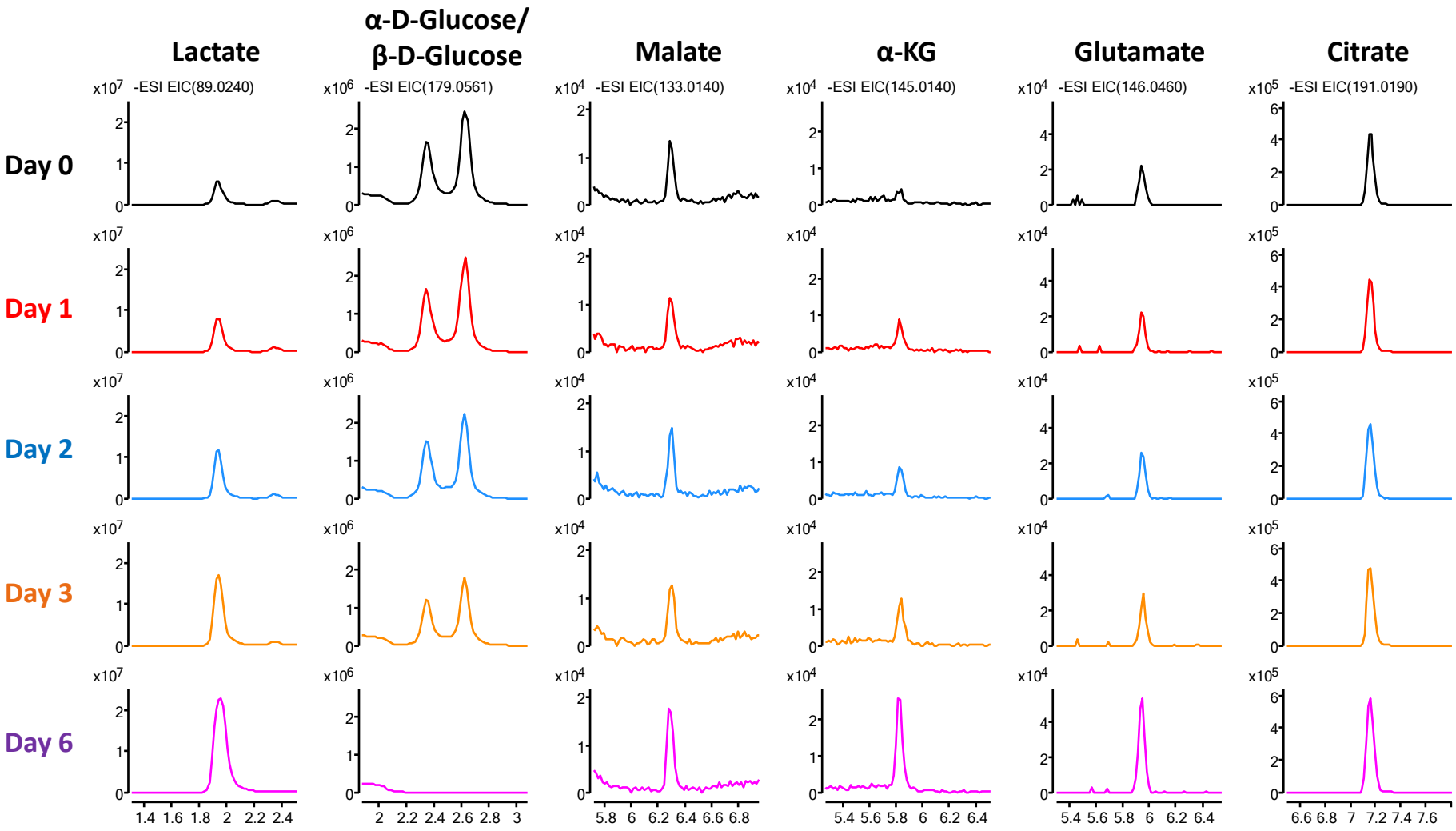
HILIC-LC/MS analysis

Data Analysis



Monitoring Secreted Metabolic Waste in Cell Culture Media

Agilent CrossLab
From Insight to Outcome



Monitoring Consumed Nutrients in Cell Culture Media

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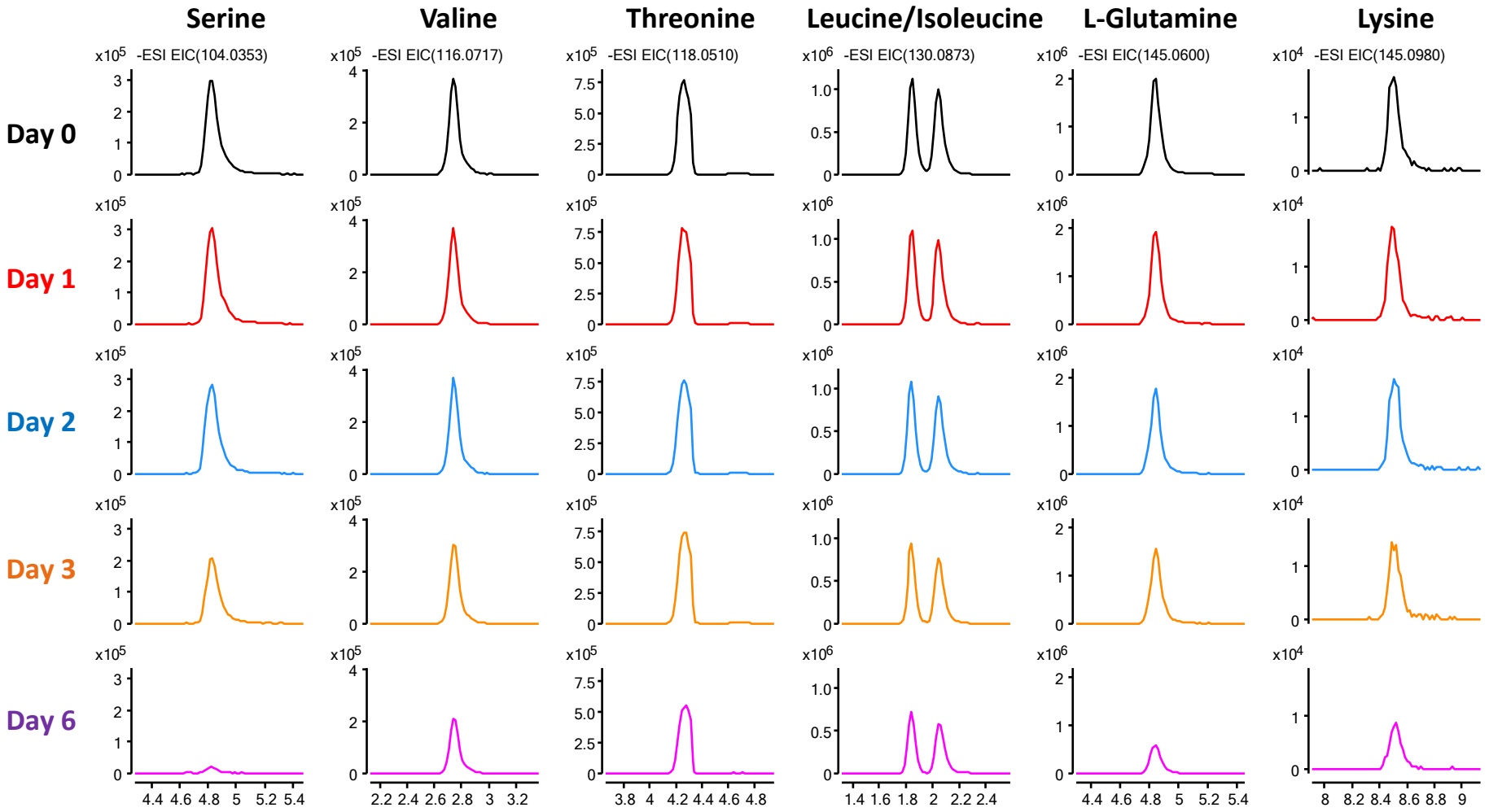

Monitoring Consumed Nutrients in Cell Culture Media

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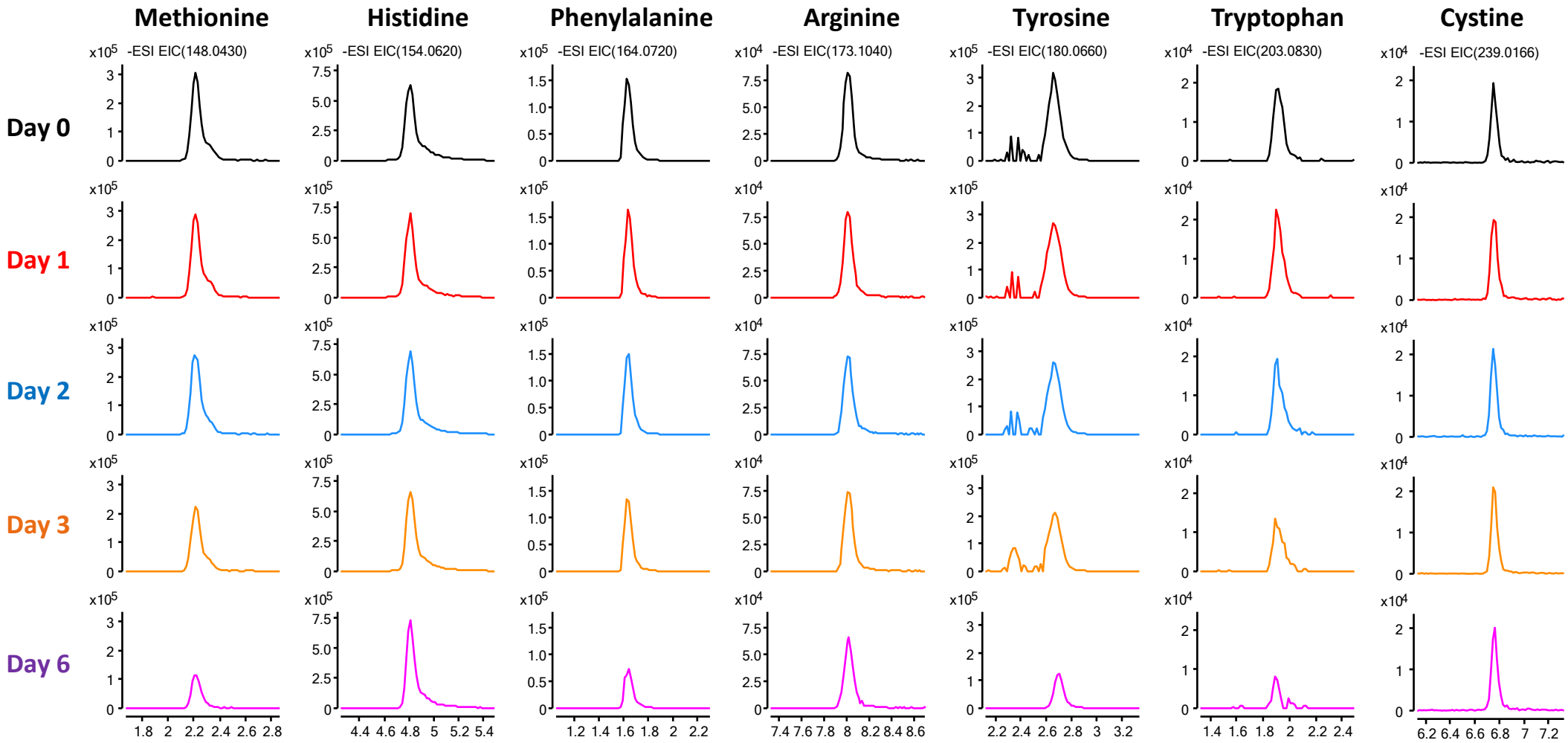
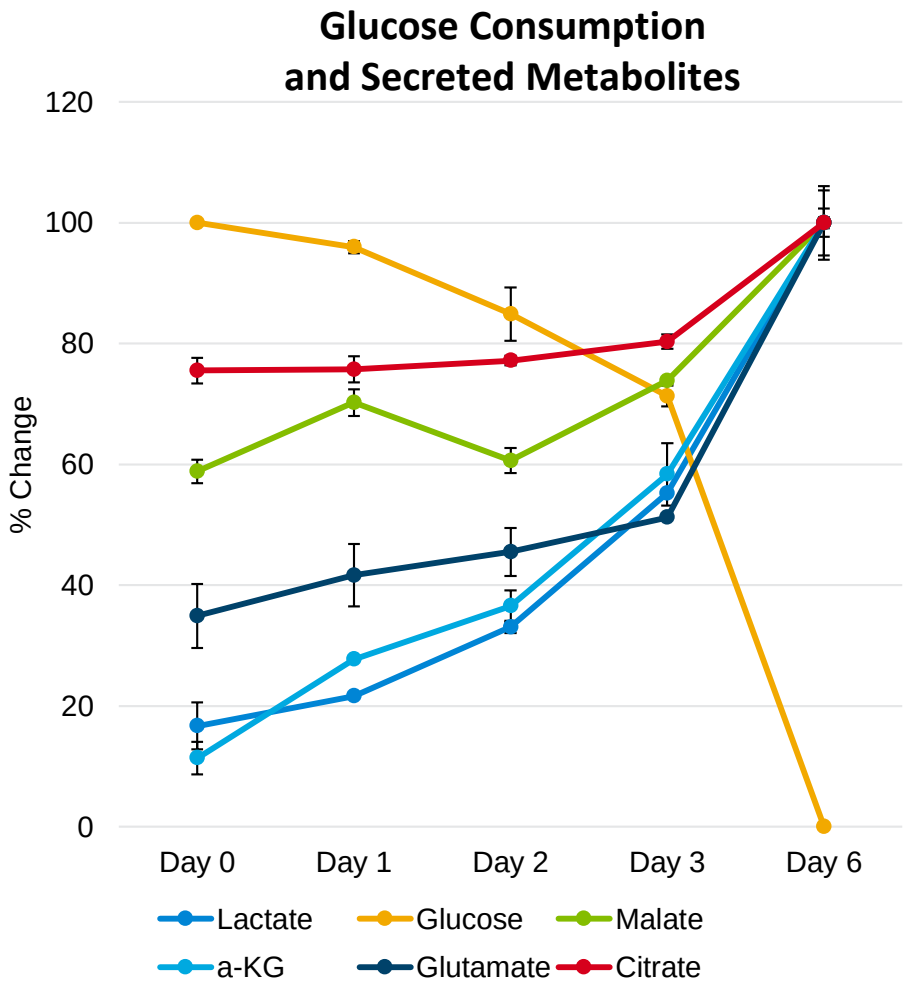
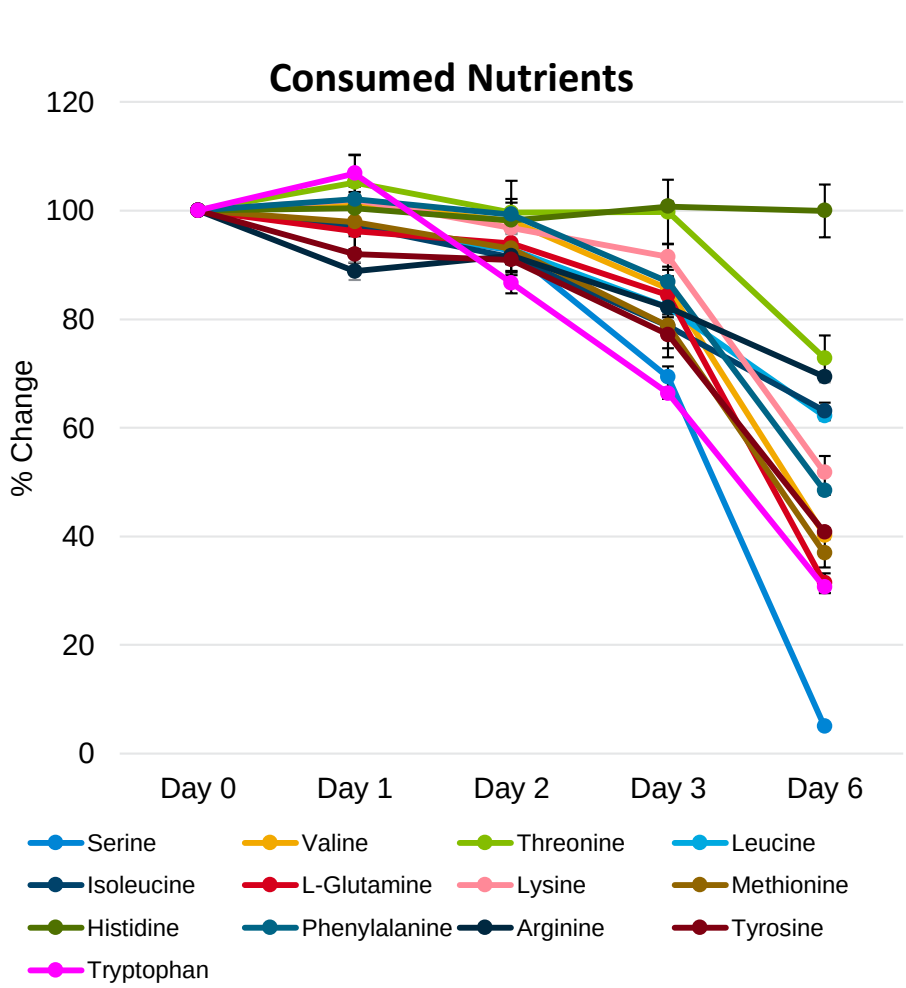


Monitoring Spent Culture Media by HILIC-LC/MS

Agilent CrossLab
From Insight to Outcome





Quantitative values were normalized to the max value of each analyte (n=3).

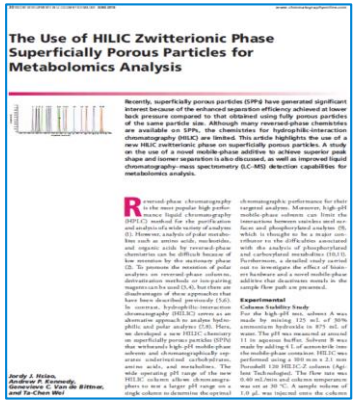
Summary

- A new zwitterionic HILIC column was developed to improve the separation of polar metabolites.
- An optimized method was developed to address **poor peak shapes** and **low sensitivity** for acidic metabolites.

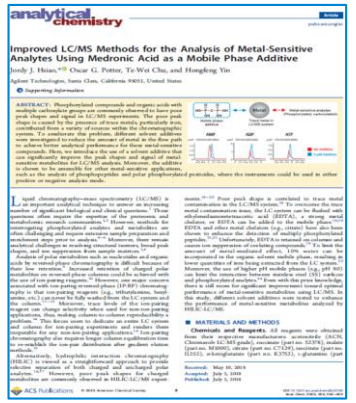
Solutions:

- PEEK-lined stainless steel hardware
- High pH mobile phase solvents
- InfinityLab Deactivator Additive

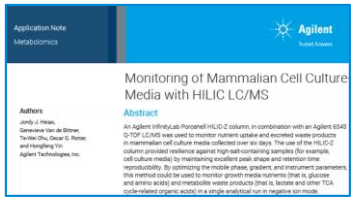
LCGC article (June 2018) – HILIC-Z


The Use of HILIC Zwitterionic Phase Superficially Porous Particles for Metabolomics Analysis

Analytical Chemistry (July 2018) - Additive


Improved LC/MS Methods for the Analysis of Metal-Sensitive Analytes Using Medronic Acid as a Mobile Phase Additive

Application Note 5994-0024EN


Monitoring of Mammalian Cell Culture Media with HILIC LC/MS

Acknowledgements

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Thank You!