Ion Pairing Free Mixed-Mode Chromatography for the Separation and Measurement of TCA Cycle Metabolites in Urine Samples of Breast Cancer Patients

Kerri M Smith, Giorgis Isaac, Suraj Dhungana and Paul D. Rainville
Waters Corporation, Milford MA

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INTRODUCTION

The Tricarboxylic acid (TCA) cycle is the ultimate fate of metabolism where Acetyl-CoA or other molecules are formed by the breakdown of carbohydrates, protein, and fats. These molecules are then enzymatically oxidized to produce molecules such as adenosine triphosphate (ATP) to fuel cellular growth and function, as well as to reduce important cofactors that enables other metabolic processes. In addition, the TCA cycle produces precursors for amino acids, proteins, fatty acids, cholesterols, and nucleotide synthesis for cell growth and division.

Due to the essential metabolic processes and pathways where the TCA cycle is involved, there is a need to study the changes along this pathway to understand disease states and cellular processes. Components of the TCA cycle, small polar organic carboxylic acids, are difficult to retain and adequately separate under traditional reversed-phase LC conditions. Current LC methods used to analyze these compounds include: HILIC, ion-pairing, anion exchange, as well as derivatization followed by either gas or liquid chromatography. Each methodology has unique challenges. These challenges can be complicated further by the presence of metal surfaces. Compounds with electron-rich moieties, such as carboxylates and phosphates, are known to chelate metals, notably iron [1]. The use of a hybrid organic-inorganic surface technology, termed MaxPeak High Performance Surfaces (HPS), with the CSH Phenyl-Hexyl column mitigates analyte interactions with metal surfaces (Figure 1).

Additional analytical method for quantification of TCA cycle analytes in human plasma, see our application note: "Improved analytical sensitivity and chromatographic peak shape for the quantification of TCA cycle analytes in human plasma using the ACQUITY PREMIER System Solution."

Here we present an MS compatible, mixed-mode anion exchange LC method for the analysis of TCA cycle analytes, and other related compounds without the use of sample derivatization or ion-pairing reagents using the ACQUITY PREMIER CSH Phenyl-Hexyl column.

Analytical separation

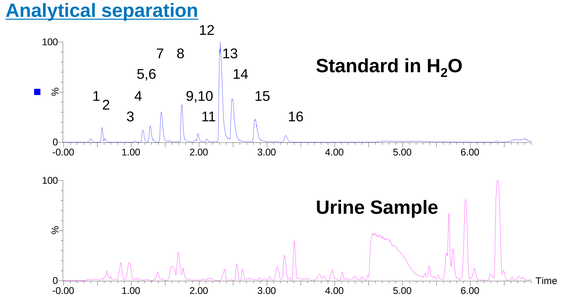

Figure 1. *Representative chromatographic separation of standards in solution (top) and a urine sample (below). 1) glutamine, 2) glutamic acid, 3) lactic acid, 4) malic acid, 5) 2-hydroxyglutaric acid (2HG), 6) succinic acid, 7) isocitric acid, 8) citric acid, 9) fumaric acid, 10) itaconic acid, 11) pyruvic acid, 12) 6-phosphogluconic acid, 13) α-ketoglutaric acid, 14) 3-phosphoglyceric acid, 15) phosphoenolpyruvic acid, 16) cis-aconitic acid*

RESULTS and DISCUSSION

Separation of critical pairs

The two major identifying ions for itaconic acid, $[M-H]^{-1}$ of 129.019 m/z and the fragment at 85.029 m/z, are also present in other analytes such as cis-aconitic acid, 2-hydroxyglutaric acid (2HG) and isocitric acid (as well as citric acid itself). These fragments are used for library matching, therefore chromatographic resolution is essential. Additionally, Isocitric and citric acids are isobaric at 191.019 *m/z*. Although they have unique fragments, the fragments are minor, and a large concentration disparity would challenge the identification. Malic acid and fumaric acid have different structures, however, it has been shown that malic acid undergoes in-source fragmentation and collision cell decomposition to contain the same fragments as fumaric acid [2].

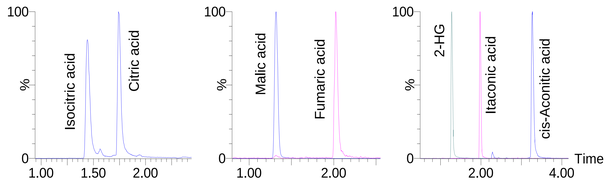

Figure 2*. Separation of critical pairs: Citric and isocitric acid; Malic and fumaric acid; 2HG, Itaconic and cis-aconitic acid (minor peak at 2.15 min is trans-aconitic acid)*

Peak recovery of analytes on column

Analyte loss to metal surfaces is problematic for compounds containing electron rich moieties such as carboxylic and phosphoric acids and is especially troublesome for compounds present at low concentrations. The addition of MaxPeak HPS improves peak area recovery of these compounds.

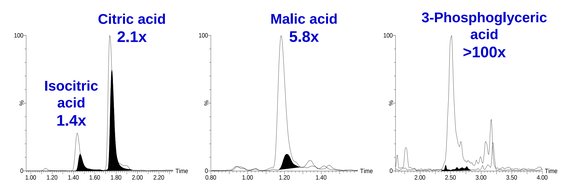

Figure 3*. Peak recoveries from a urine sample for isocitric acid and citric acid respectively, malic acid, and the glycolysis intermediate 3-phosphoglyceric acid for PREMIER CSH Phenyl Hexyl column and a standard CSH Phenyl Hexyl column (filled trace). Numbers show the improvement of peak area on the PREMIER column*

References
.[1] Abrahamson, H.; Rezvani, A.; Brushmiller, J.,*Inorganica chimica acta* 1994, *226*, 117-127
[2] Luo, B.; Groenke, K.; Takors, R.; Wandrey, C.; Oldiges, M., *J Chromatogr A* 2007, *1147* (2), 153-64.

Analytical reproducibility

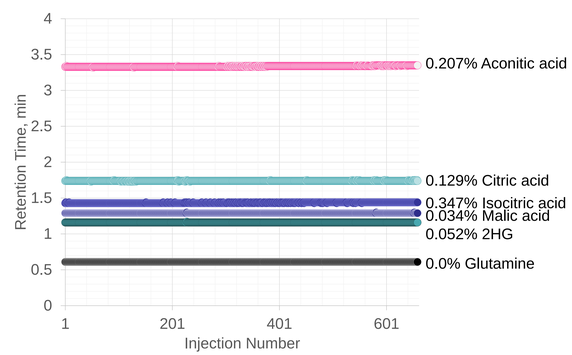

Figure 4*. Injection reproducibility of a pooled urine sample over 462 injections*

Abundance plots of selected analytes

Figure 6*. Abundance plots of succinic acid (A), aconitic acid (B), isocitric acid (C), citric acid (D), 3-phosphoglyceric acid (E), and 2-hydroxyglutaric acid (F) in each of the breast cancer positive as well as the control and pooled urine (QC) injections of this small cohort study example.*

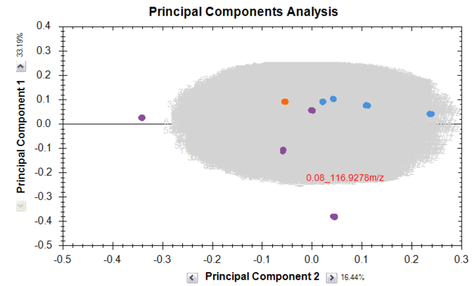

Figure 5*. PCA scores plot of 5 replicate injections for . Blue – healthy control, Purple – breast cancer positive, Orange – QC (Pool) showing tight clustering of the pooled QC sample as well as individual samples*

METHODS

LC Conditions:

LC System – ACQUITY I-Class PLUS
Vials – Waters Total Recovery Silanized [p/n 186000385DV]
Column – ACQUITY PREMIER CSH Phenyl-Hexyl, 1.7µm 2.1x100mm column [p/n 186009475]
Flow Rate – 0.4mL/min
Mobile Phase A – 0.1% formic acid in H_2O
Mobile Phase B – 0.1% formic acid in ACN
Gradient 0% to 25% B over 4 minutes, 25%-95%B for 3 minutes followed by wash and re-equilibration

MS Conditions:

MS System – Xevo G2-XS QTOF, negative ionization mode
Software – MassLynx and Progenesis QI

Sample Preparation:

Urine samples were thawed on ice
– 100 µL of urine was pipetted into an Eppendorf tube, followed by 300 µL of H_2O
– Vortex mix 30sec
– Centrifuge at 21,130 rcf for 10 minutes at 4C
– Pipette into silanized vials

CONCLUSION

- **Here we describe a method for the analysis of organic acids, including those of the TCA cycle, by mixed-mode reversed phase chromatography without the need for ion-pairing reagents**
- **The incorporation of MaxPeak HPS with the analytical column improves recovery of analytes which are sensitive to metal interactions**
- **The method was applied to the analysis of healthy control and breast cancer positive urine samples with unsupervised PCA analysis showing distinct grouping of healthy and disease samples and excellent reproducibility of respective injection clusters**
- **Abundance plots of select analytes show their differences in healthy versus disease samples**

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