Separation and Analysis of Low Molecular Weight Organic Acid Metabolites by Mixed-Mode Chromatography Coupled to Mass Spectrometry

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INTRODUCTION

The components of the Tricarboxylic acid (TCA) cycle are small and very polar organic carboxylic acids. Traditional methods of reversed phase chromatography do not always yield enough retention or selectivity to confidently measure these analytes. Current separations include HILIC, ion-pairing, anion exchange, and derivitization followed by gas or liquid chromatography separation with each technique presenting its own unique challenges. Here we present a new analytical method for the analysis of the TCA cycle metabolites as well as other related compounds without sample derivitization or ion-pairing reagents in the mobile phase. We applied the analytical method to a breast cancer urine samples and used statistical software for feature analysis.

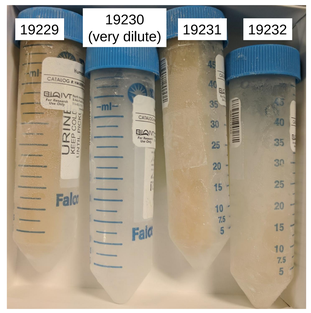


Figure 1. Four breast cancer positive urine samples (female)

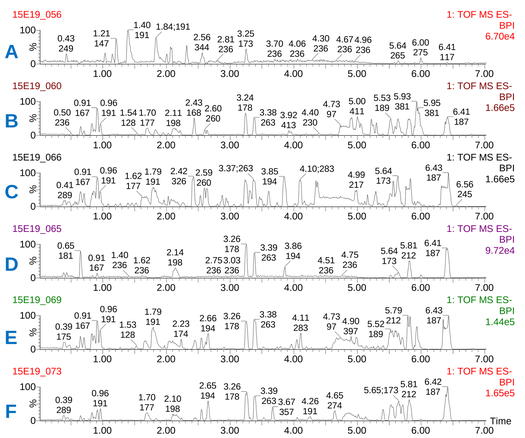
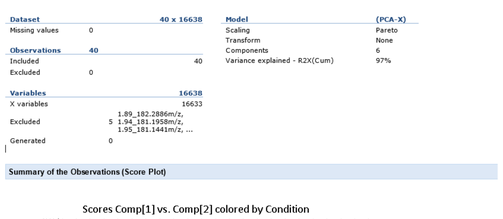


Figure 2. Separation of urinary metabolites, underivatized, on an ACQUITY CSH Phenyl-Hexyl Column; A) Standard Mix in H2O, B) Non-Disease female urine sample, C) 19929, D) 19930, E) 19931, F) 19932
Retention times of standards: lactate, 1.0; malate, 1.15; succinate, 1.2; 2-hydroxyglutarate, 1.2; isocitrate, 1.4; citrate, 1.8; fumarate, 2.0; pyruvate, 2.3; α-ketoglutarate, 2.3; phosphoenolpyruvate, 2.9; cis-aconitate, 3.3;

METHODS

Human breast cancer positive urine samples (figure 1) were diluted 10x with MQ H2O, centrifuged 10 minutes at 4˚ C and 21130 rcf. The supernatant was transferred to a silanized total recovery vial for analysis. The samples were separated on an ACQUITY I-Class LC with an ACQUITY UPLC CSH Phenyl-Hexyl 2.1 x 100mm 1.7um column using 0.1% formic acid in water and ACN for mobile phases A and B. The gradient was from 0 - 25%B over 4 minutes with a flow rate of 0.4 mL/min and a column temperature of 60˚ C. The LC was connected to a Xevo G2-XS time of flight mass spectrometer which was operated in negative ionization ionization. The data was acquired in continuum format using MSᵉ acquisition mode of MassLynx 4.1 and further processed using Progenesis QI as well as EZinfo software.



Dataset	**40 x 16638**	**Model**	**(PCA-X)**
Missing values	0	Scaling	Pareto
		Transform	None
Observations	**40**	Components	6
Included	40	Variance explained - R2X(Cum)	97%
Excluded	0		
Variables	**16638**		
X variables	16633		
Excluded	5 1.89_182.2886m/z, 1.94_181.1958m/z, 1.95_181.1441m/z, ...		
Generated	0		

Summary of the Observations (Score Plot)

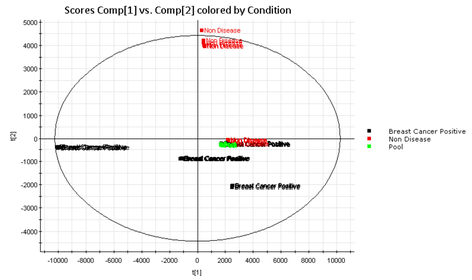


Figure 3. PCA plot of replicate injections of breast cancer positive, non-disease, and pooled urine samples

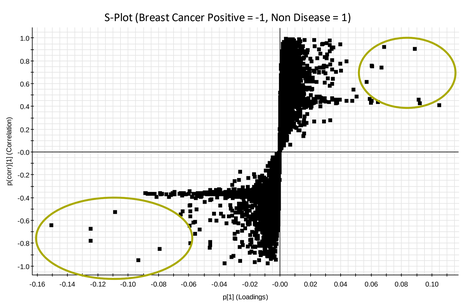


Figure 4. S-Plot of the major differences between the breast cancer positive and non-disease urine samples

WORKFLOW AND PRELIMINARY RESULTS

The components of the TCA cycle are small and very polar organic carboxylic acids. Traditional methods of reversed phase chromatography do not always yield enough retention or selectivity to confidently measure these analytes. Citric acid and isocitric acid, for example, are isobaric at 191 m/z and require chromatographic resolution for accurate determination. To address the separation and retention of the critical pairs and polar species of the TCA metabolites and other biologically relevant compounds, a mixed-mode chromatography method was developed. Here the ACQUITY UPLC CSH Phenyl-Hexyl column was employed for separation and analysis of organic acid metabolites in urine. Figure 2 gives examples of the separation in standard as wells as non-disease and breast cancer positive urine.
Breast cancer positive urine as well as non-disease urine samples (female) were injected with 5 replicates. A pooled sample of equal volumes of each disease and non-disease sample was also acquired (labeled pool). The injections were imported into and processed by Progenesis QI with further statistical modeling performed by EZinfo (figures 3 and 4). Markers were selected and transferred back to Progenesis QI for investigation and identification by library searches: HMDB and KEGG as well as METLIN MS/MS (figures 5 and 6). Compound abundance for the top markers were reviewed (figure 7) and XIC extracted from original data (figure 8). Proposed identifications for the compounds were listed and will be confirmed using commercial standards.

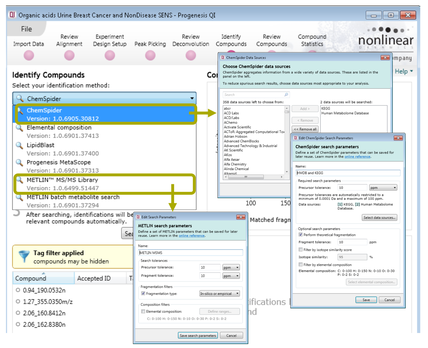


Figure 5. Major differences in ions for breast cancer positive and non-disease samples were subjected to library searching for identification

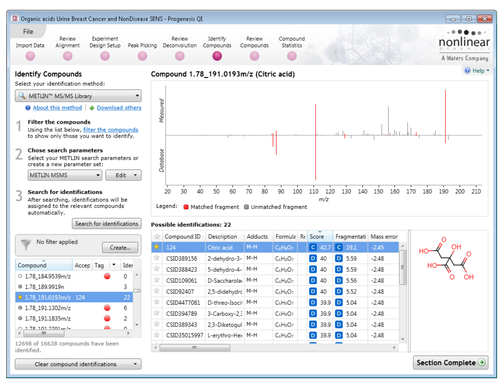


Figure 6. Identification of known compound, citric acid

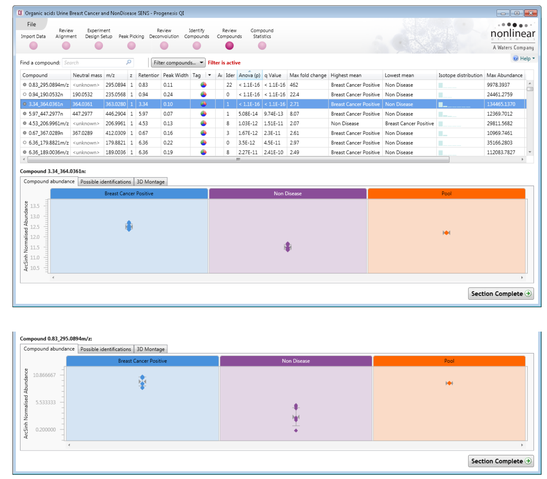


Figure 7. Ion abundance of 363.0280 at 3.34 min and 295.0894 m/z at 0.83 min

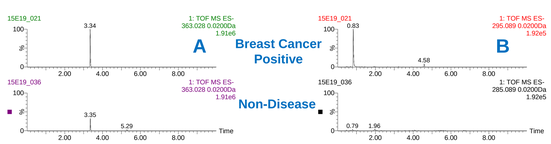


Figure 8. A) XIC 363.0280 m/z and B XIC 295.0894 m/z for breast cancer positive (top) and non disease (bottom) urine samples

Top proposed IDs from ChemSpider and METLIN search:
A) xanosine monophosphate
B) aspartate-tyrosine dipeptide,

CONCLUSION AND FUTURE WORK

Breast cancer positive and non-disease female urine samples were separated and analyzed using an ACQUITY CSH Phenyl-Hexyl column and simple mobile phase of 0.1% formic acid in water and ACN. The analysis was interrogated by Progenesis QI and EZinfo software to determine the most prominent differences between the samples. Library searching revealed potential identifications of features. These identifications will be tested using commercial standards

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