Sensitive and Reproducible Mass Spectrometry-Compatible RP-UHPLC Analysis of Tricarboxylic Acid Cycle and Related Metabolites in Biological Fluids: Application to Human Urine

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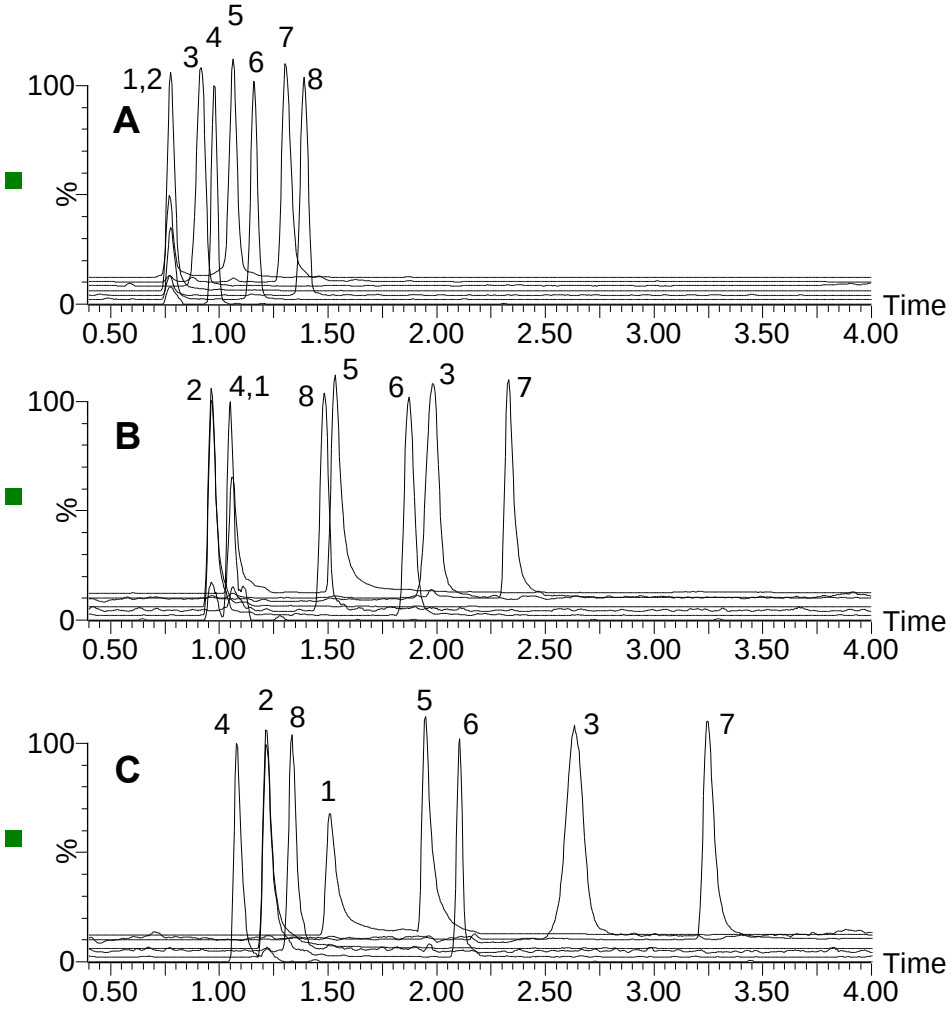

Figure S1. Separation of Isocitric acid (1), malic acid (2), α-ketoglutaric acid (3), lactic acid (4), citric acid (5), fumaric acid (6), cis-aconitic acid (7), succinic acid (8) 100 µm standards spiked into 1:3 diluted urine by 0.1% formic acid in H2O as well as ACN on ACQUITY UPLC BEH C18 (p/n 186002352) (A), ACQUITY UPLC CSH C18 (p/n 186005297) (B), ACQUITY UPLC CSH Phenyl-Hexyl (p/n 186005407) (C). All columns were standard, 2.1x100 mm dimensions and 1.7 µm particle size. This preliminary data was acquired on a Xevo TQ-S tandem mass spectrometer in MRM mode.

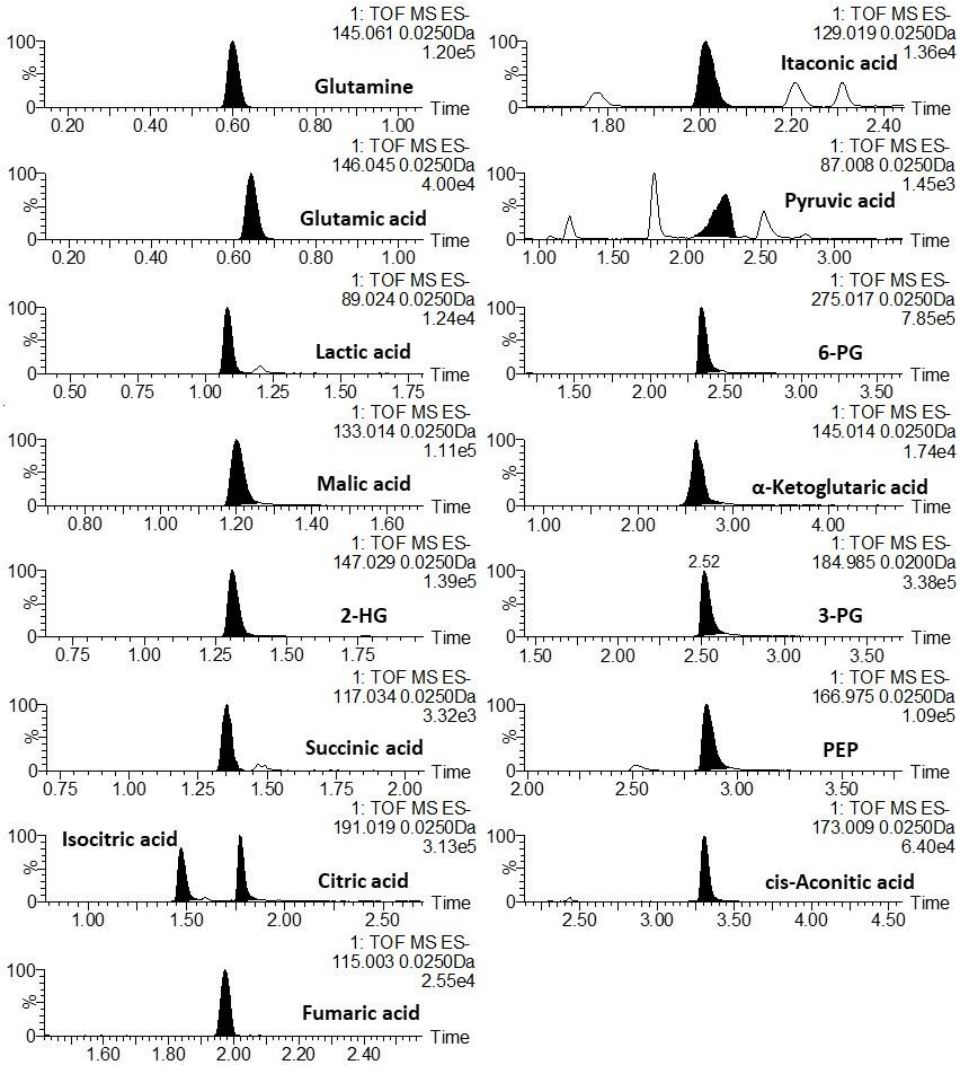

Figure S2. XIC of the analytes in standard solution. All analytes were at 100μM, except α-Ketoglutaric acid which was 32μM.

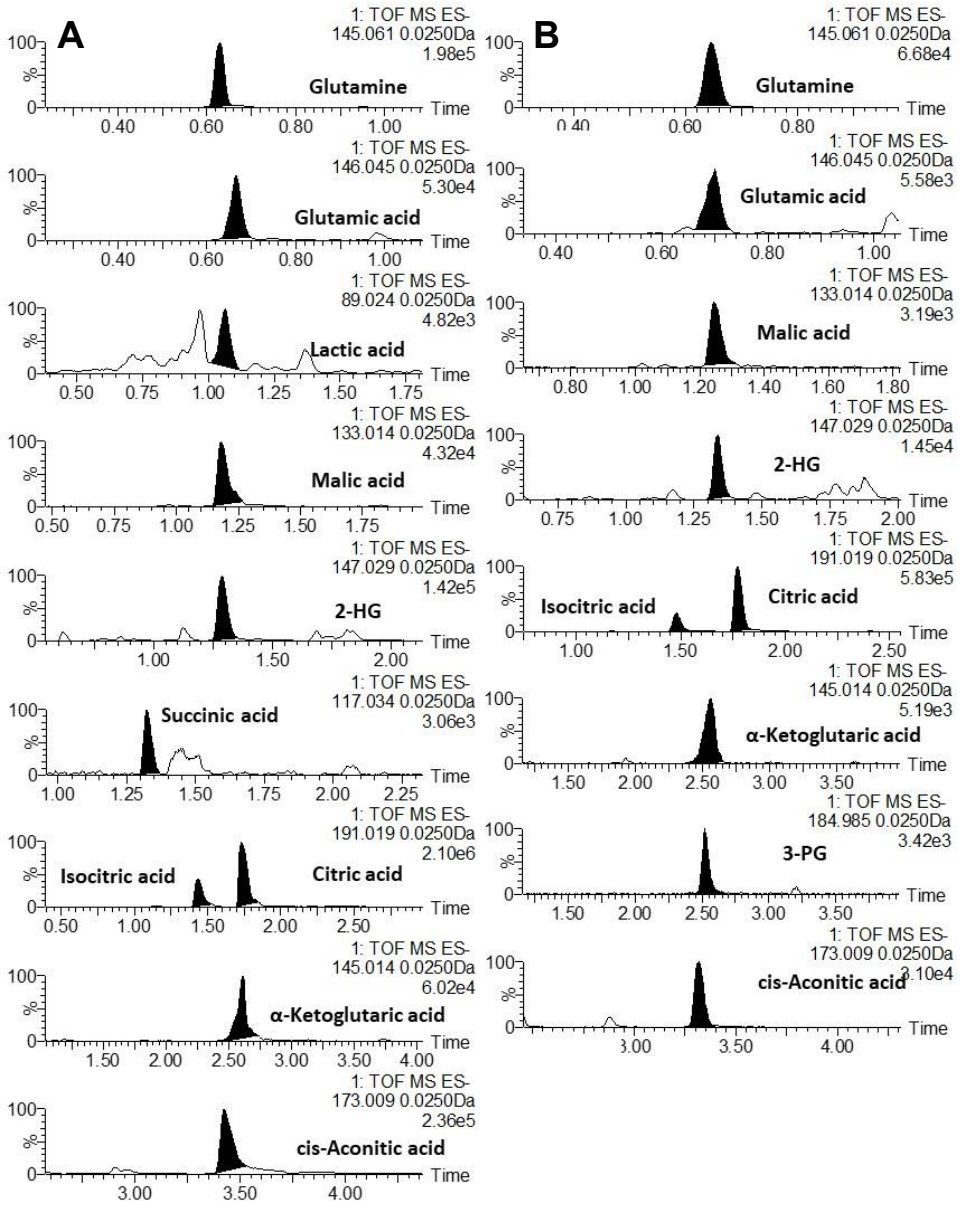

Figure S3. XIC of identified analytes in a control urine (A) and breast cancer positive urine (B) sample.

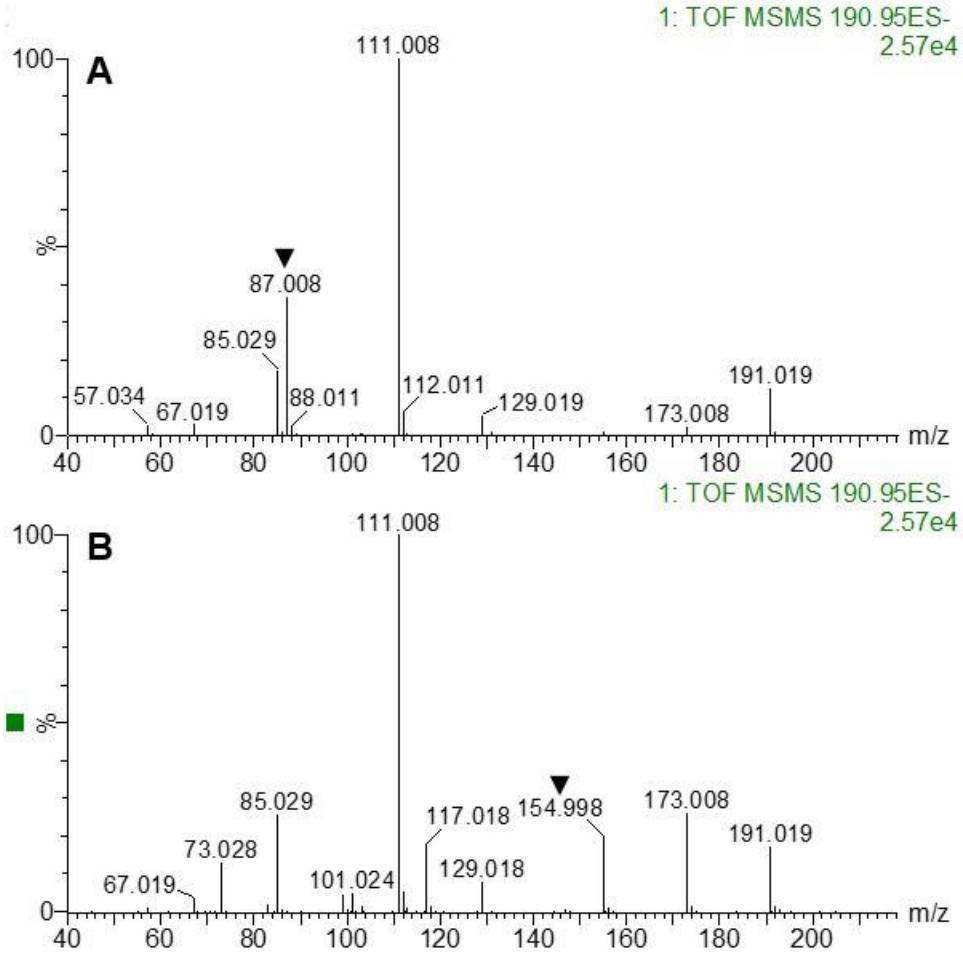

Figure S4. Fragmentation of citric acid (A) and isocitric acid (B) showing unique fragments.

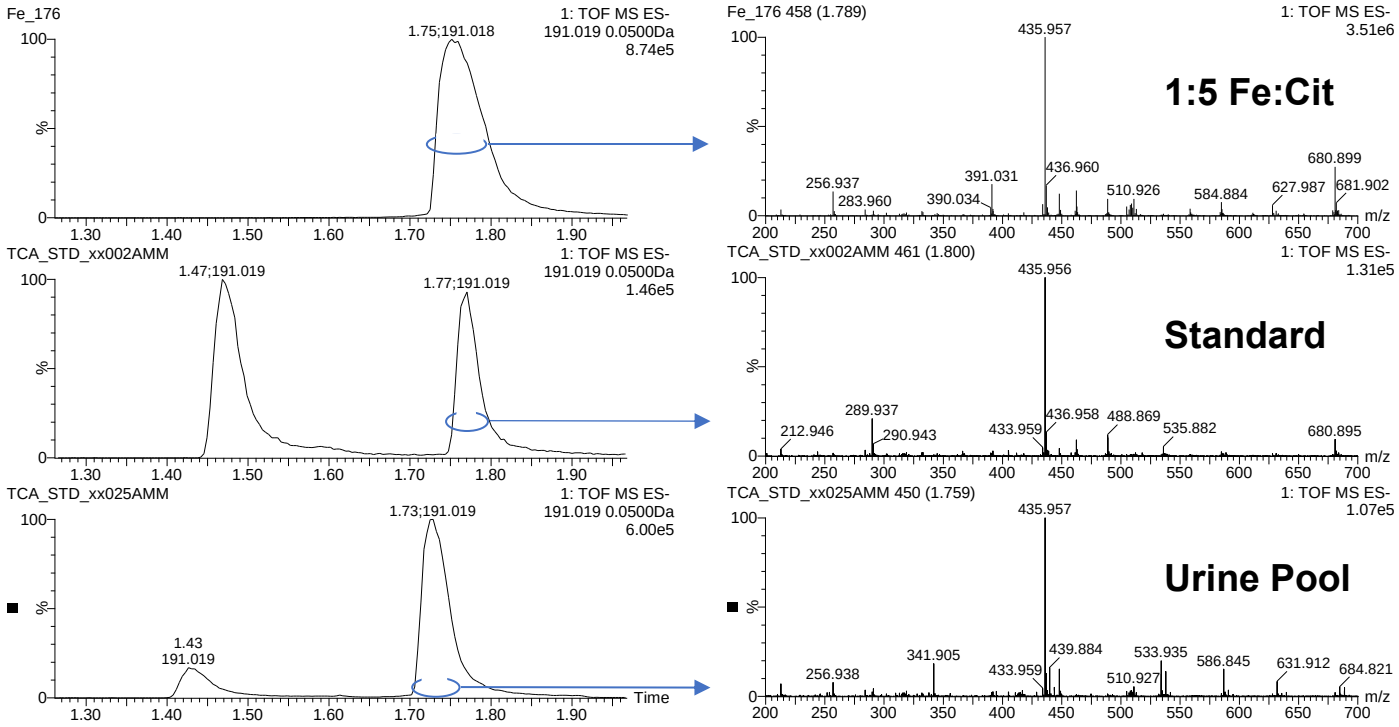

Figure S5. To confirm the presence of Fe-Citrate complexes present, a 1 mM $Fe_2(SO)_3$ solution was incubated with a 10 mM citric acid solution resulting in a 1:5 molar ratio of Fe:Citrate. The solution was diluted 10x with water and injected onto the CSH Phenyl-Hexyl column (p/n 186005407), using the same methodology as the current assay. Chromatograms are shown on the left for the Fe:Citrate solution, 100 µM citrate and isocitrate standard solution and the pooled urine sample. Summed mass spectra for the citric acid peaks are shown on the right. It is noted that the major ion at 435.957 m/z is that of the complex of $Fe(Citrate)_2$.

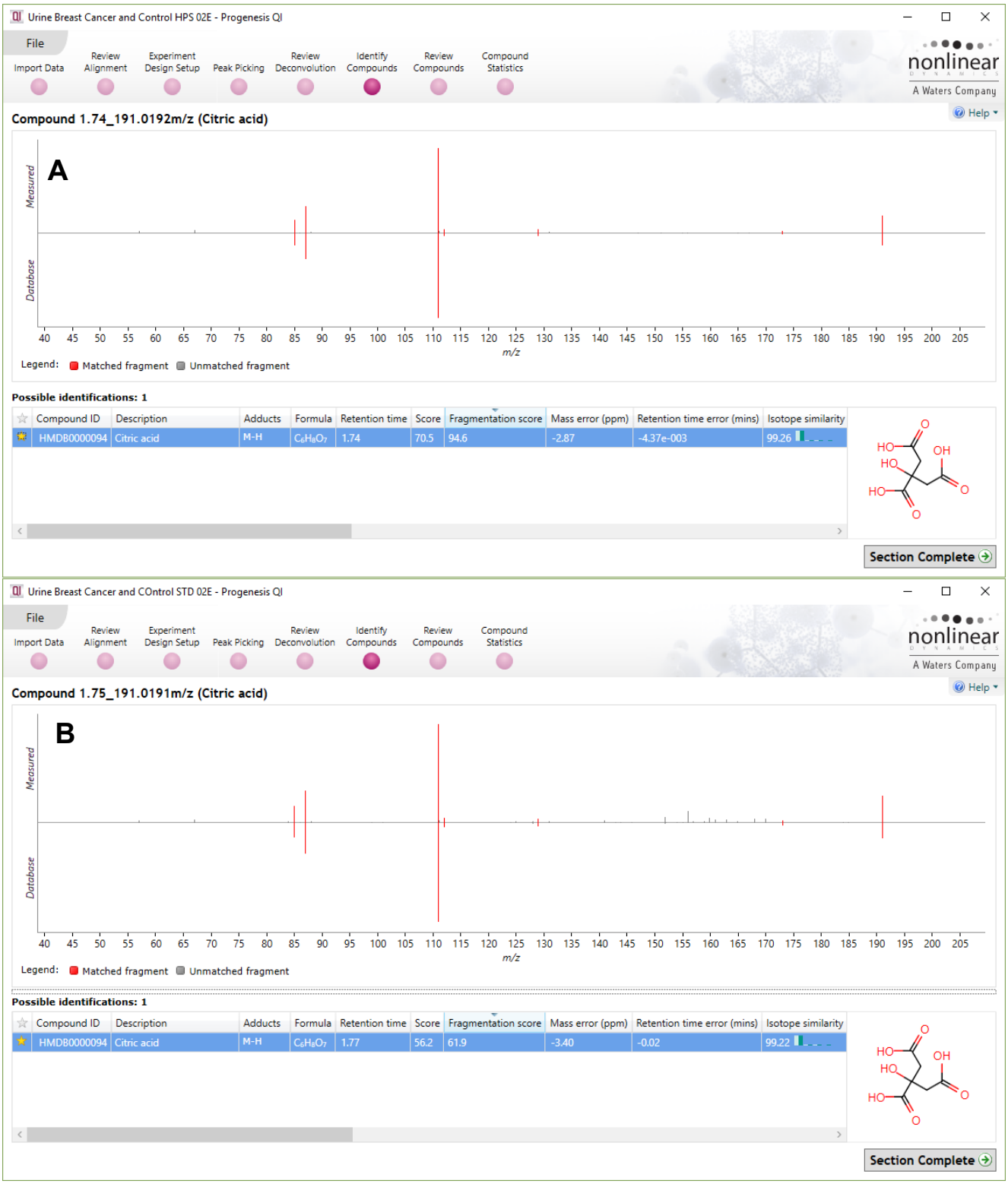

Figure S6. Database matching for citric acid for HST column (A) and Standard column (B). Fragmentation scores were higher at 94.6 for HST column compared to 61.9 for standard column on the same analysis.

File Name	*Sample Text*	***Citric acid***		***Isocitric acid***		***cis-Aconitic acid***		***Glutamine***		***2-Hydroxyglutaric acid***	
		RT, min	Response	RT, min	Response	RT, min	Response	RT, min	Response	RT, min	Response
TCA_HPS_xx019	Pool	1.7433	39452	1.4336	10185	3.2909	1958	0.6075	1707	1.2917	905
TCA_HPS_xx025	Pool	1.7394	37133	1.4336	10566	3.2908	1926	0.6075	1693	1.2916	867
TCA_HPS_xx026	Pool	1.7395	36306	1.4336	9742	3.2908	1899	0.6075	1689	1.2916	876
TCA_HPS_xx044	Pool	1.7433	38214	1.4336	9669	3.2945	1937	0.6075	1615	1.2917	891
TCA_HPS_xx048	Pool	1.7395	38601	1.4336	9710	3.2908	1922	0.6075	1629	1.2917	885
	Average	***1.74***	***37941***	***1.43***	***9975***	***3.29***	***1928***	***0.61***	***1667***	***1.29***	***885***
	RSD	***0.12%***	***3.26%***	***0.00%***	***3.92%***	***0.05%***	***1.11%***	***0.00%***	***2.50%***	***0.00%***	***1.61%***

File Name	Sample Text	***Glutamic acid***		***Malic acid***		***α-Ketoglutaric acid***		***Succinic acid***		***3-Phosphoglyceric acid***	
		RT, min	Response	RT, min	Response	RT, min	Response	RT, min	Response	RT, min	Response
TCA_HPS_xx019	Pool	0.6571	313	1.1811	708	2.5342	347	1.3412	451	2.5152	373
TCA_HPS_xx025	Pool	0.6571	302	1.1811	703	2.5303	338	1.3412	445	2.5151	354
TCA_HPS_xx026	Pool	0.6571	301	1.1811	705	2.5303	324	1.3412	464	2.5036	369
TCA_HPS_xx044	Pool	0.6571	297	1.1811	726	2.5265	335	1.3412	436	2.5074	389
TCA_HPS_xx048	Pool	0.6571	293	1.1811	714	2.5303	326	1.3412	436	2.5074	395
	Average	***0.66***	***301***	***1.18***	***711***	***2.53***	***334***	***1.34***	***446***	***2.51***	***376***
	RSD	***0.00%***	***2.50%***	***0.00%***	***1.31%***	***0.11%***	***2.78%***	***0.00%***	***2.60%***	***0.21%***	***4.42%***

Table S1. Retention time and MS response for analytes in the pooled urine sample.