Supporting Information

Improving the Speed and Selectivity of Newborn Screening using Ion Mobility Spectrometry – Mass Spectrometry (IMS-MS)

James N. Dodds and Erin S. Baker

Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States.

* Corresponding Author Email: ebaker@ncsu.edu

Comments on IM-MS Data presented in this Work

In this supporting information we detail instrument settings used to collect IMS-MS data in this manuscript. Extended information for analyte drift times, and CCS reproducibility is included in the supplemental Excel Sheet ES1. We also include a list of NBS targets with molecular formula and CCS readily for import into Skyline should other researchers choose to utilize these values in their own experiments. Supplemental isomer separations are also included for steroids and method development information pertaining to the Agilent RapidFire SPE workflow.

Table of Contents

Supporting Information Table S1: Instrumental parameters flow injection analysis (FIA), automated SPE using the Agilent RapidFire 365, and IMS-MS settings the Agilent 6560.

Abbreviations: FA – Formic Acid, AF – Ammonium Formate

Flow Injection Analysis (FIA)

Parameter	Value	Descriptor	Time (min)	% B
Temperature	30	Celsius	0 – 1.5 min	50
Injection Volume	2	uL		
Flow Rate	0.2	mL/min		
Mobile Phase (A)	H_20	0.1% FA		
Mobile Phase (B)	ACN	– both A/B		

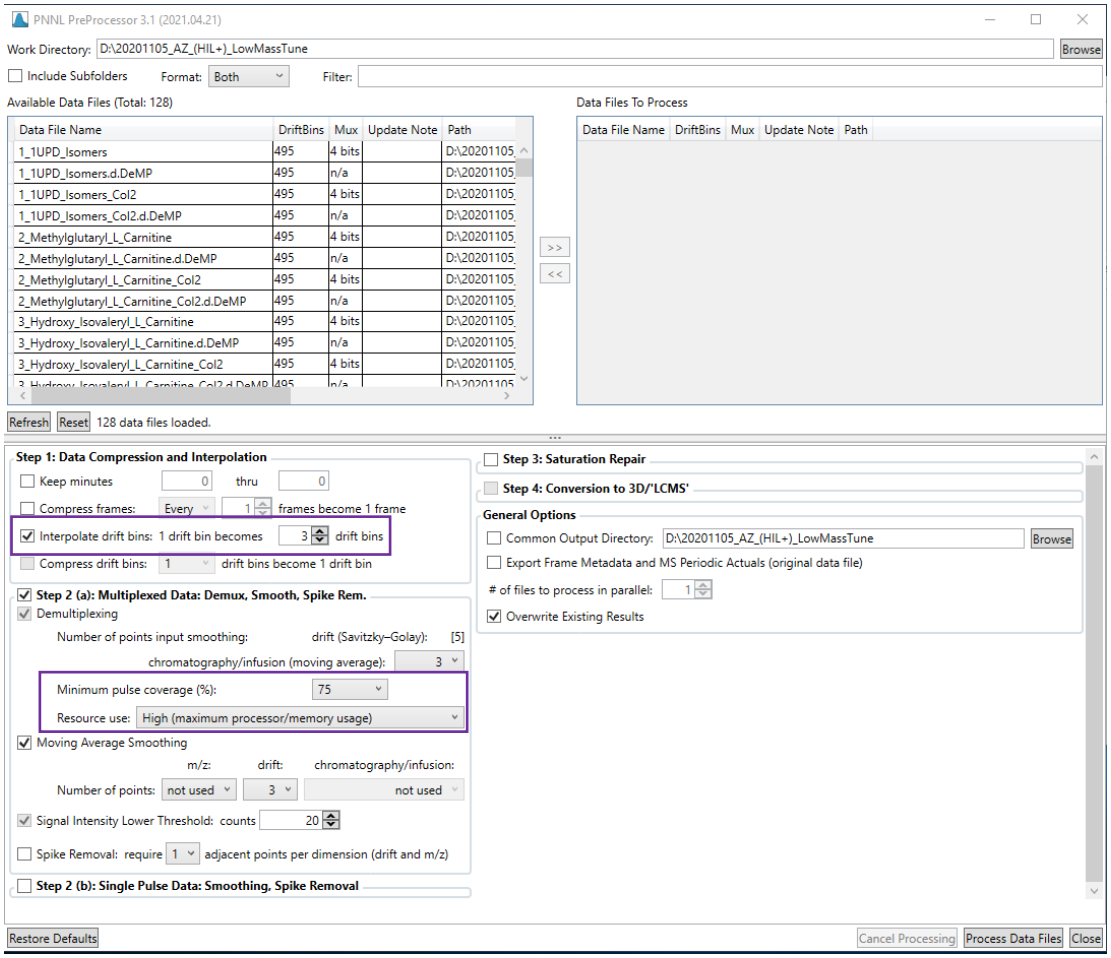
RapidFire 365 (SPE)

Parameter	Value	Descriptor
State (Sequence Timings)		**Cartridge:** C18/C4/C8/Cyano/Hypercarb/Phenyl
Aspirate	600 ms	Pump 1: H_2O (100%)
Load/Wash	3000 ms	Pump 2: H_2O + 0.1% FA (100%)
Extra Wash	0 ms	Pump 3: ACN + 0.1% FA (100%)
Elute	6000 ms	**Cartridge:** HILIC
Requilibrate	1000 ms	Pump 1: 9:1 ACN/H2O - 20 mM FA, pH3 (100%)
Flow Rate to MS	0.6 mL/min	Pump 2: 9:1 ACN/H2O - 20 mM FA, pH3 (100%)
96 Deep Well Plate (Axygen)		Pump 3: 9:1 ACN/H2O - 20 mM AF, pH3 (25%), H2O – 20 mM AF, pH3 (75%)

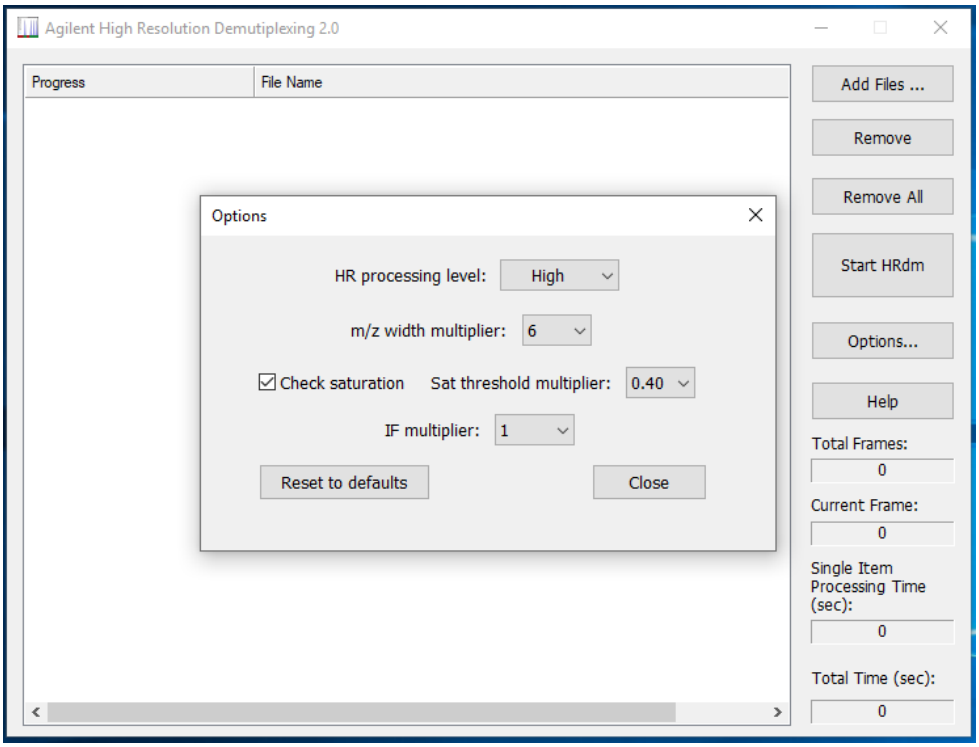
Agilent 6560 Settings

Parameter	Value	Descriptor
Source Settings		
Gas Temperature	325	Celsius
Drying Gas	11	L/min
Nebulizer	15/45	Flow Injection/RapidFire
Sheath Gas Temp	350	Celsius
Sheath Gas Flow	12	L/min
Vcap	4000	V
Nozzle	2000	V
IMS-MS Settings		
Mass Range	50-1700	m/z
Trap Fill Time	3900	µs
Trap Release Time	100	µs
Frame Rate	0.9	Frames/sec
IM Transient Rate	18	IM Transients/Frame
Max Drift Time	60	ms
TOF Transient Rate	600	Transients/IM Transients
Drift Tube Entrance	1574/1174	V (Standard Multiplex/HRdm)
Drift Tube Exit	224	V
Rear Funnel Entrance	217.5	V
Rear Funnel Exit	45	V

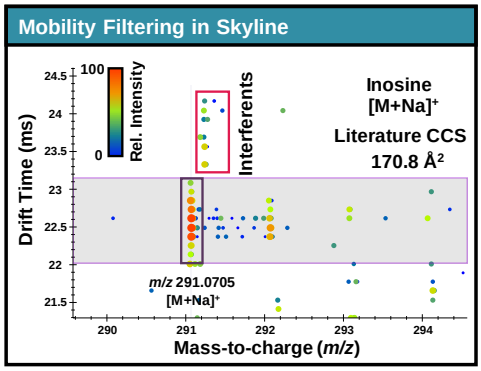
Supporting Information Figure S1: Highlighted Settings utilized for demultiplexing IMS-MS data and data interpolation via the PNNL Preprocessor tool (v.2021.04.21), publicly available for download at https://omics.pnl.gov/software/pnnl-preprocessor. Settings utilized other than default (interpolation of 3 drift bins and minimal pulse coverage of 75%) are highlighted in purple.



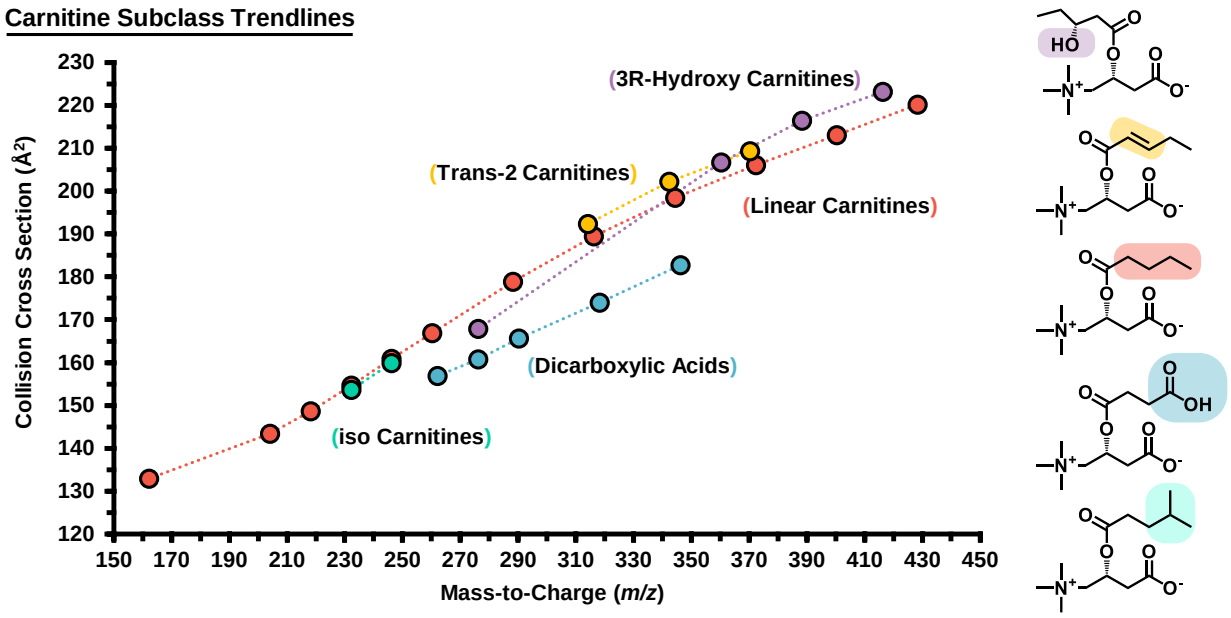
Supporting Information Figure S2: Settings utilized for the High Resolution Demultiplexing (HRdm, v.2_0_B45E) process after feature extraction from IM-MS Browser, v. 10.0.



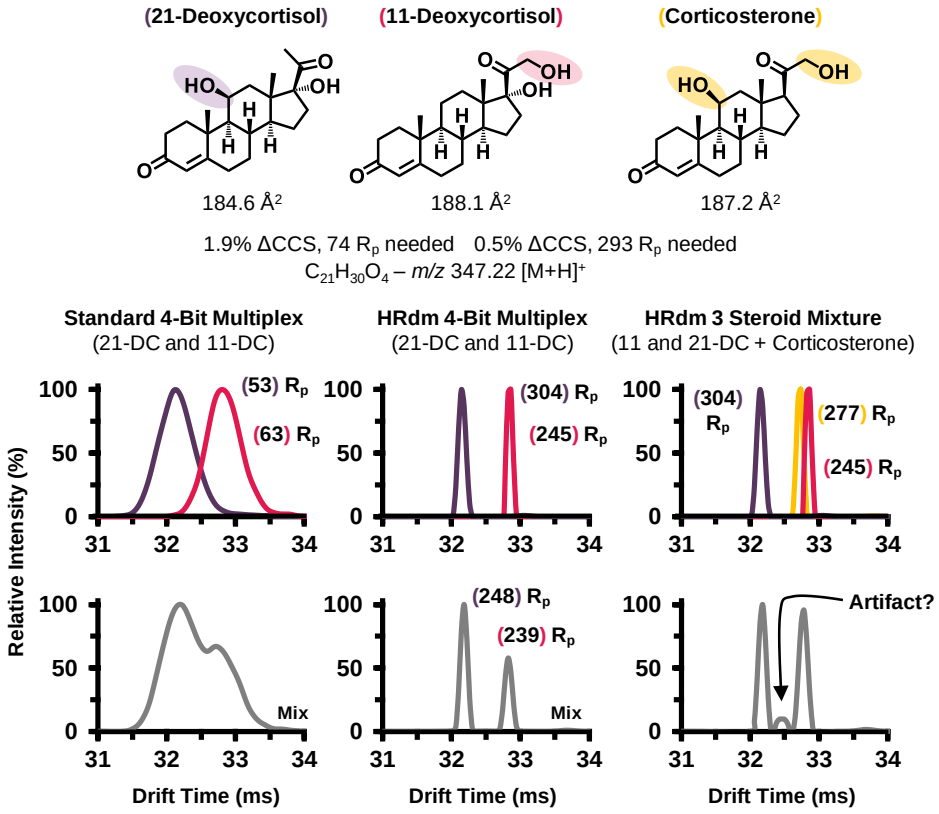
Supporting Information Figure S3: Representative illustration of Skyline mobility filtering of IMS-MS data. Here the sodiated adduct of inosine is spatially separated in *m/z* and drift time space from nearby chemical interferents based on the chemical formula and CCS value of the target ion. The literature CCS value is referenced from the work of Nichols *et. al.* Anal. Chem. **2018**, *24*, 14484-14492. doi: 10.1021/acs.analchem.8b04322 using the same IMS-MS platform.



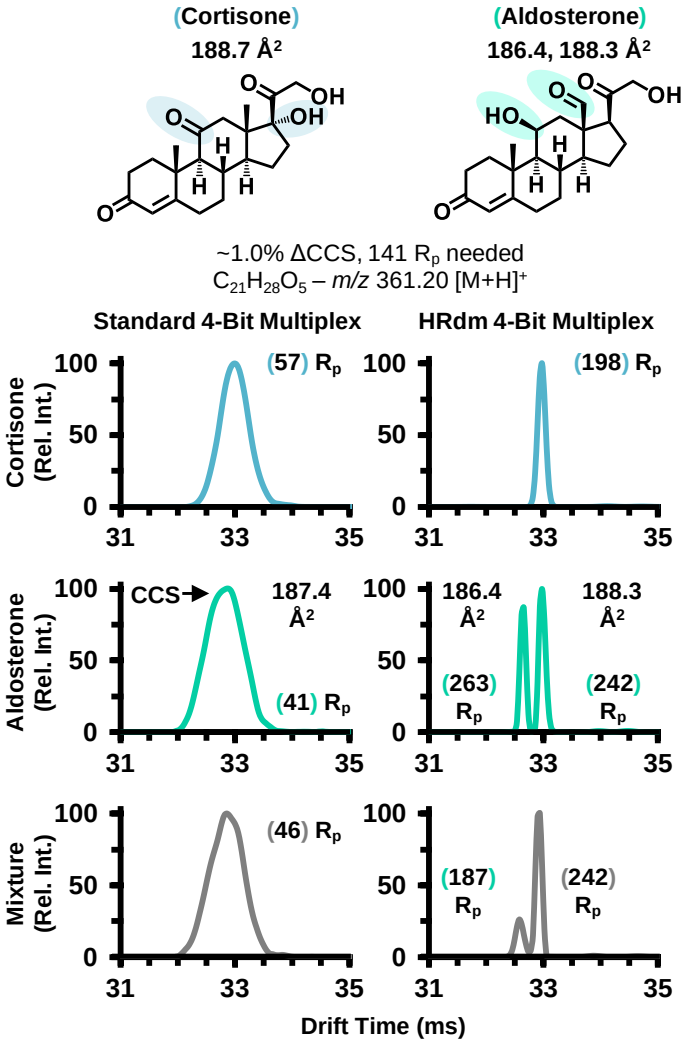
Supplemental Figure S4. Carnitine subclass trendlines observed for the standards utilized in this work. Hydroxylated and Trans-2 carnitines seem to be larger in CCS per m/z unit in comparison to the linear carnitine form. Iso-carnitines are typically smaller than their linear forms, likely a result of a more compact gas phase structure rather than an extended form. The dicarboxylic acid carnitines are by far the smallest in CCS per *m/z*, where the extra carboxylate group adds more mass, yet less length, than a linear CH_2 addition as in the linear base carnitines. Similar trendlines have been noted with other chemical classes as discussed in the main text.



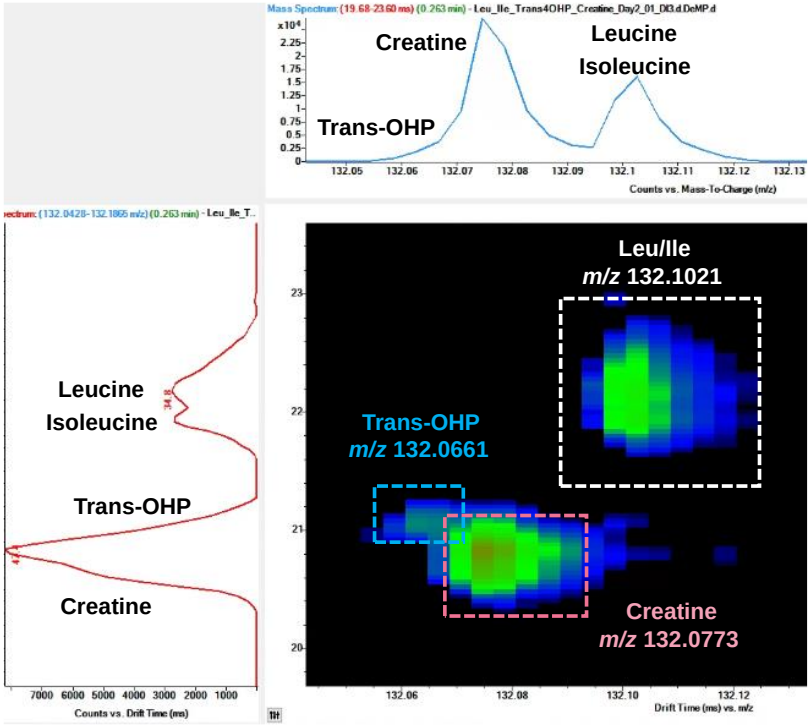
Supplemental Figure S5. Steroid isomers of $C_{21}H_{30}O_4$- Corticosterone added here, where a post processing artifact is noted in the middle pane for the three-component mixture. As the drift time does not correspond to the corticosterone standard (likely unresolved with 11-deoxycortisol) the current thought is that this peak is an artifact of the beta HRdm process and still being investigated.



Supplemental Figure S6. Steroid isomers cortisone and aldosterone. While both analytes are resolvable by the standard 60 Rp using normal demultiplexing, HRdm provides interesting resolution of these isomers. Of note, aldosterone seems to possess two conformations as an individual standard (middle pane, green), which also suggested by the decreased resolving power (41) in "normal" mode. Though the right conformer has a very similar calculated CCS as cortisone (188.3 and 188.7 $Å^2$, respectively), it would be theoretically possible to calculate the relative abundance of each isomer provide that the relative abundance of each aldosterone "conformer" is conserved in subsequent analyses.



Supplemental Figure S7. Small molecule amino acids separations by HRdm. Our preliminary results working with the beta HRdm software suggest there are additional challenges for resolution enhancement when working with particularly small analytes (<150 Da). These ions possess high mobilities (low drift times), and hence do not possess as many points across the peak for the HRdm enhancement process compared to larger molecular ions, and hence the observed Rp from small ions is typically ~80 or so compared to ~200 for larger analytes.



$C_6H_{13}NO_2$ – *m/z* 132.1021

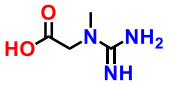
Isoleucine **Leucine**

132.9 Å^2 134.6 Å^2

~1.3% ΔCCS, 110 R_p needed

Creatine

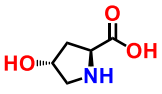
$C_4H_9N_3O_2$ – *m/z* 132.0773



126.0 Å^2

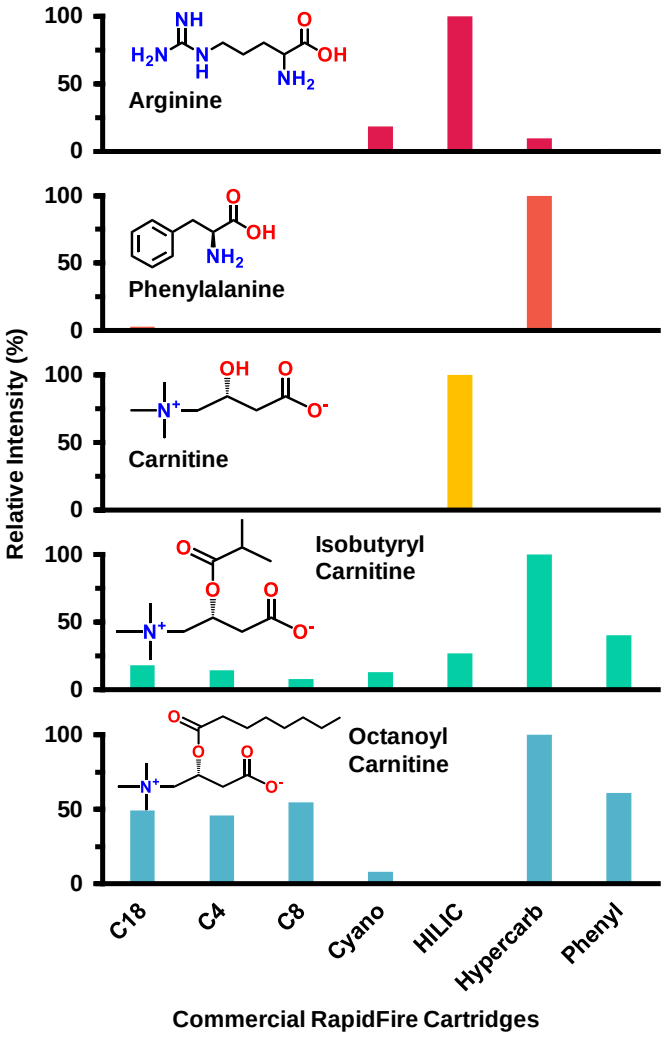
Trans-4-hydroxy-Proline

$C_5H_9NO_3$ – *m/z* 132.0661

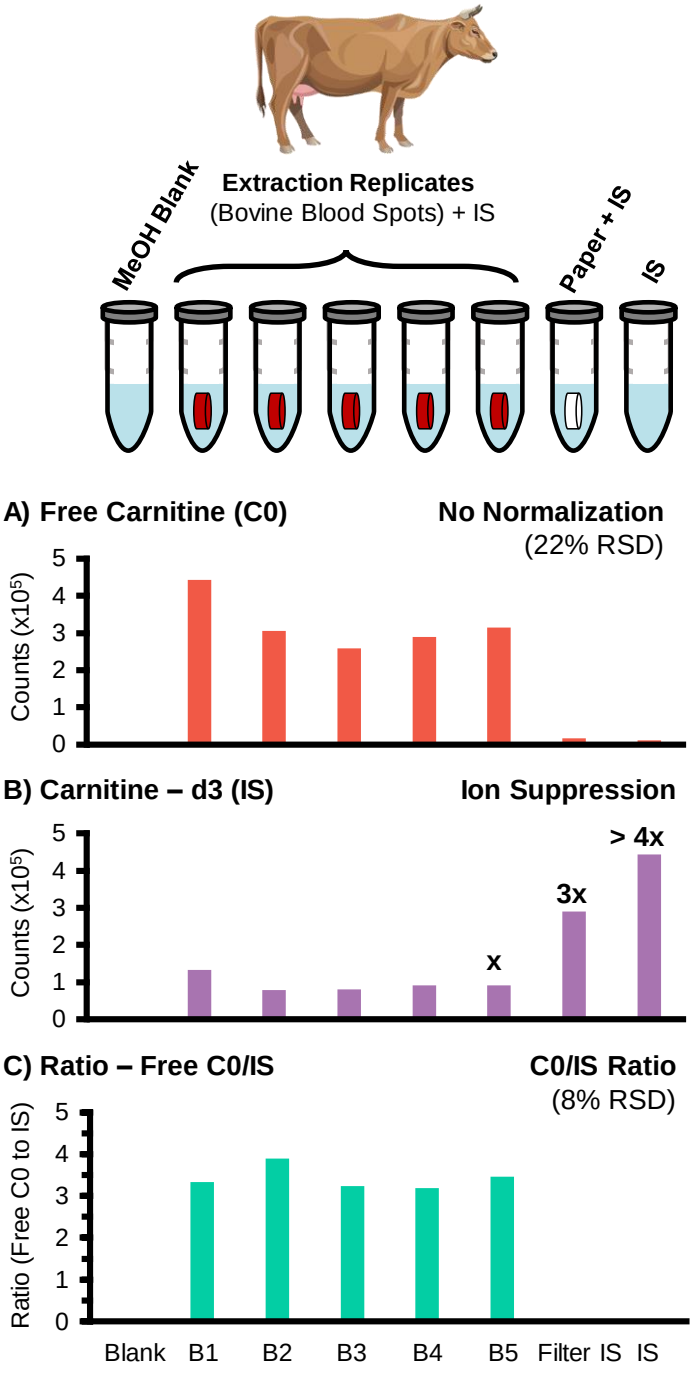


127.8 Å^2

Supplemental Figure S8. SPE cartridge selectivity based on analyte polarity. A standard mixture comprised of both amino acids and carnitines of various chain lengths were assessed by each SPE cartridge. Results suggest that sequential sampling using both the HILIC and Hypercarb cartridges (polar and nonpolar analytes, respectively) should provide sufficient coverage for assessment of most analytes.



Supplemental Figure S9. Extraction reproducibility using sequential bovine blood spots made from the same pooled sample. While run-to-run variation could exceed 20% RSD, normalization with isotopically labeled standards (IS, here carnitine-*d*3) helped correct for ion suppression effects (from blood and filter paper) and variations in sample aspiration (similar to injection volume) from the RapidFire. Corrected peak areas typically were around 10% RSD for the preliminary tests demonstrated in this work.



Supplemental Figure S10. Extraction replicates of simulated NBS samples using bovine and sheep blood. Relative abundance for each NBS target is provided in a bar graph along with the preferred SPE cartridge.