Defining the Healthy Infant Metabolome: Liquid Chromatography Tandem-Mass Spectrometry Analysis of Dried Blood Spot Extracts from the Prospective Research on Early Determinants of Illness and Children's Health Trajectories Birth Cohort Study

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Newborn screening using dried plasma spots offers preanalytical advantages over conventional cards for plasma-associated targets of interest. Herein we present dried plasma spot–based methods for measuring metabolites using a 250+ compound liquid chromatography tandem mass spectrometry library. Quality assurance reduced this library to 134, and from these, 30 compounds determined the normal newborn reference ranges. *(J Pediatr 2021;■:1-6).*

The dried blood spot (DBS) sampling matrix is a ubiquitous staple of state-to-state newborn health screening programs as it is widely available, cheap, and readily amenable to quality assurance programs, such as the Centers for Disease Control and Prevention's Newborn Screening Quality Assurance Program. Newborn screening is widely regarded as one of the most successful public health programs in the US, with approximately 4 million infants tested annually for inborn errors in metabolism (IEMs).[1] Screening programs using DBS have evolved over 5 decades of use, beginning with manual bacterial inhibition assays for early identification of phenylketonuria and other IEMs,[2] to more sophisticated radioimmunoassay testing platforms.[3] Contemporary advances using genomic sequencing technologies[4] and tandem mass spectrometry[5] now enable DBS-based screening for other rare diseases, where early diagnosis, often before the onset of symptoms, provides considerable benefits to newborns if intervention is provided as close to birth as possible.

Conventional newborn screening with DBS begins with the addition of capillary blood droplets, derived either from heel- or finger-stick within 24-48 hours after birth, onto a solid, cellulose-based filter card, which is then passively dried in ambient conditions. In this context, preanalytical issues emerge, as blood in a nonuniform manner dries in the presence of cells, and differential blood cells lyse and introduce contaminants.[6] Hematocrit variation,[7] as well as the inconsistency between successive blood drops from finger-sticks often used to increase total spotting volumes,[8] add to preanalytical variation. Cells present in spotted blood may continue to undergo metabolism and respond to clotting factors.[9] These preanalytical variables are difficult to measure and pose challenges to the utility of conventional DBS cards in ultrasensitive testing platforms, such as therapeutic drug monitoring using mass spectrometry (MS) and other discovery applications.[10] A commercially available DBS matrix (Noviplex; Novilytic) separates volumetric plasma samples from blood drops at the point of DBS collection using the top layer of the card to trap cells, wherein plasma travels by capillary action to a separate disc beneath it. Plasma is thus isolated without the presence of whole or lysed cells, potentially mitigating the preanalytical challenges described previously in the use of highly sensitive testing in DBS, herein referred to as dried plasma spots (DPS).

In a comprehensive review of the literature in 2017, we identified more than 2000 analytes potentially detectable in DBS using a variety of different analytical methods.[11] With the development of highly sensitive large multiplex liquid chromatography tandem mass spectrometry (LC-MS/MS) assays, the diversity of compounds found in whole

DBS	Dried blood spot
DPS	Dried plasma spot
HPLC	High-performance liquid chromatography
IEM	Inborn errors of metabolism
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MS	Mass spectrometry
QC	Quality control
RUSP	Recommended universal screening panel

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blood spots on Whatman Filter cards, the traditional card type in most IEMs screening, using a single method has surpassed 250 compounds.[12] Herein, we describe a workflow that combines sample collection on Noviplex Duo Cards, extraction of plasma from the lower discs, and subsequent analysis of the extracts using LC-MS/MS with a pentafluorophenylpropyl stationary phase with a 250 compound multiplex assay developed with authentic chemical standards. This workflow was applied to the Johns Hopkins All Children's Prospective Research on Early Determinants of Illness and Children's Health Trajectories birth cohort study.[13]

The objectives of the present work were to develop and describe a standardized procedure for infant DBS collection, storage, and metabolomics analysis suitable for broad implementation, and, using this approach, to derive normative values for a diverse array of analytes within the healthy infant metabolome.

Methods

A detailed description of all methods is provided online. De-identified samples from the Prospective Research on Early Determinants of Illness and Children's Health Trajectories birth cohort study[13] were used in this study. Our study did not include any exclusion criteria related to premature birth, as mothers were recruited early in their pregnancy. If mother delivered at <32 weeks, they were removed from the study. Median gestational age at birth of the infants in the study population was 39 weeks, with an observed range (36-41 weeks).

Sample collection kits were distributed to enrolled maternal participants containing a 3" by 6" aluminum canister sealed with a 3" aluminum lid with external threading and a polyvinyl chloride coating for a hermetic seal (Elemental Container). This canister held a Noviplex DUO Plasma Prep Card (Novilytic) in its original packaging, along with a 5.0-g molecular sieve desiccant packet (Impak) to preserve the cards for later use. This kit was used for collection and transport of DBS specimens from the point of care at the Johns Hopkins All Children's Hospital newborn nursery to (and transient storage in) the Johns Hopkins All Children's Pediatric Biorepository, located adjacent to the hospital.

At the time of heel stick performed for routine clinical care (ie, state-mandated DBS newborn screening) by trained nurses in the newborn nursery nurses ≥24 hours after birth, 2 additional drops of fresh blood (estimated by the manufacturer to be approximately 60 ≥μL) were collected for the present research during the period of January 23, 2019, to September 20, 2019, via application to the plasma separation DPS card in accordance with manufacturer specifications. Following a 3-minute ambient incubation, the top layer of the plasma separation DPS card was removed by the collection team, allowing the plasma to physically separate from blood cells by capillary action onto dual discs directly underneath the top layer. The plasma-separated DPS discs were subsequently placed back into the sealed transport/transient storage canister, and transported via pneumatic tube system from the newborn nursery to the Johns Hopkins All Children's Pediatric Biorepository.

Upon receipt in the Biorepository, plasma spots were removed and separated from the DPS card as distinct discs by trained biorepository technicians using a clean wooden stick. Isolated discs were then placed inside an internally-threaded 1.4-mL polypropylene cryovial (Micronic), the cryovial was immediately purged with a 3-second nitrogen gas flush while held upright, and then sealed with the accompanying screw cap, which bears a silicon gasket. Cryovials were immediately accessioned into a −80°C semi-automated freezer (SAM, Hamilton Storage) with NIST-certified continuous electronic temperature monitoring.

For analysis, cryovials containing plasma discs were removed from long-term storage and transferred to the Johns Hopkins All Children's Molecular Determinants Core. Each disc was extracted in 500 μL of 50/50 methanol (Optima LC/MS Ref# A456, Fisher Chemical)/water (Optima LC/MS Ref# W6, Fisher Chemical) mix and sonicated for 5 minutes before aliquoting. Each extract was split into 2 analytical aliquots of 200 μL each. The remaining 100 μL of extract was pooled to create two 200 μL quality control (QC) aliquots. Aliquots were dried in a vacuum concentrator (SpeedVac Vacuum Concentrator, Savant; ThermoFisher Scientific) for interim storage at −80°C. Before analysis, one analytical and one QC aliquot for each disc were reconstituted in 50 μL of water with heavy internal standards (**Table I**; available at www.jpeds.com) for normalization. Osmolality for each sample was measured on an osmometer (OsmoPRO Multi-Sample Micro-Osmometer; Advanced Instruments) in accordance with manufacturer instructions, using 20 μL from each aliquot.

High-performance liquid chromatography (HPLC) was performed using a Shimadzu HPLC composed of an SIL-30ACMP 6-MTP Autosampler and Nexera LC-30AD HPLC Pumps (Shimadzu). Mass spectrometry targeted assays employed a Shimadzu 8060 triple quadrupole mass spectrometer equipped with an electrospray Ionization source used in both positive and negative mode. Instrument settings are provided in **Table II** (available at www.jpeds.com). Pentafluorophenylpropyl chromatography was used to resolve small molecule metabolites, using a 150-mm × 2.1-mm internal diameter column from Suppleco, with a particle size of 3 μM. **Tables III** and **IV** (available at www.jpeds.com) provide details on the LC method, gradient concentrations, and durations.

Each batch was run with a system suitability QC, which was created from commercially available pooled human plasma (Innovative Research Inc), to validate instrument performance. Asparagine, methionine, and tryptophan (representative metabolites spread in time through the chromatogram) were evaluated for retention time, raw signal, and signal to noise to ensure QC suitability criteria were met. Data analysis was performed with MetaboAnalyst Software 5.0. The data were reduced and centered by Pareto Scaling (mean-centered and divided by the square root of the standard deviation of

each variable) for multivariate analysis. Missing values were replaced by a small value (one-fifth of minimum positive value in the original data, per metabolite). Regression analysis was performed by analysis of calibrators from 460 000 fmol to 8 fmol on column using a linear regression. Noviplex Duo cards have an indicator of when sufficient blood volume is placed; however, upon separation of the top and bottom layer of the cards, there is no visual indicator demonstrating sufficient plasma reached the residual bottom disc. As an additional quality control check, we performed an analysis of osmolality on the specimens (**Table V**; available at www.jpeds.com). Values were divided by mOsm to normalize for amount of material spotted to the DPS disc. For hypothesis testing related to specific analytes, our general approach is to use standard curves consisting of at least external authentic chemical standards for the analytes targeted in our hypothesis to obtain absolute quantitation relative to a reference standard. Targeted results were generated as femtomoles for each sample, and median and IQR values were reported for each analyte of interest specified (**Table VI**). These values were converted to a μmol/L unit for more direct comparisons with published clinical reference ranges by dividing by the volume of plasma (3.8 μL) deposited per disc as a value established by the manufacturer. Data were analyzed using a nonparametric approach to construct reference values (2.5th-97.5th percentiles). Outliers were not excluded, unless due to previously defined analytical or biological reasons.

Noviplex Duo manufacturer's instructions suggest that most analytes are stable for up to 7 days at room temperature storage. As the stability of analytes may vary depending on many factors, we assessed the impact of room temperature storage time on analytes measured by LC-MS/MS before freezing. Following peak selection criterion that were applied equally to all samples regardless of the duration of storage temperature, samples were divided into 2 groups in silico: Group 1: Less than 7 days of room temperature storage (n = 20); and, Group 2 (n = 10): More than 7 days of room temperature storage. Given the rather small sample size and possibility of nonparametric distribution of data, intergroup differences were tested via both the Student *t* test and a Mann–Whitney *U* test. As the goal of this study was to assess our workflows and sample stability at room temperature before low temperature storage, we chose 32 representative small molecules out of the 134 reported from our panel, representing ~20% of our analytical platform.

Results

Thirty samples were collected from healthy normal infant patients during the study period for the purpose of establishing normative ranges of analytes determined using our multiplex LC-MS/MS assay from Noviplex Duo cards. Of the 30 samples, the time from collection and storage at room temperature to the transfer of the plasma containing discs to ultra-low temperature storage ranged from less than 1 day to 32.9 days with a mean of 7.9 days with an SD of 7.12 days.

Table VI. Normal research reference ranges

Compounds	Median plasma, μmol/L	IQR	Normative range (2.5th-97.5th percentile)	
Amino acids				
Alanine	2.76	1.89	0.51	8.90
Asparagine	5.67	2.01	3.26	9.19
Creatine	21.42	5.27	10.67	26.33
Creatinine	10.17	2.25	5.91	13.56
Glutamic acid	6.91	2.28	4.62	10.98
Glutamine	10.62	3.59	5.28	18.55
Glycine	23.11	7.63	14.57	35.32
Histidine	6.78	3.92	2.86	11.47
Leucine	6.16	3.20	3.63	10.42
Methionine	6.24	2.93	2.76	9.41
Methionine sulfoxide*	11.14	4.32	5.20	38.35
Proline	16.24	5.33	8.01	24.11
Serine	19.99	10.66	12.40	38.96
Taurine	16.31	7.26	9.61	24.13
Threonine	1.07	0.67	0.39	2.24
Tryptophan	2.18	0.92	1.13	3.50
Tyrosine	11.94	5.03	5.95	24.38
Valine	12.13	5.07	6.49	19.46
Carboxylic acids				
Orotic acid*	27.85	3.57	21.92	33.71
Dihydroorotic acid*	5.17	0.69	4.35	6.45
Kynurenine*	0.46	0.07	0.27	0.84
Carnitines				
Acetylcarnitine	1.05	0.32	0.55	1.68
Carnitine	1.76	1.18	0.90	2.85
Bile acids				
Glycocholic acid*	0.05	0.05	0.02	0.23
B vitamins				
Nicotinamide	5.12	2.43	2.91	12.17
Pantothenic acid	1.60	1.64	0.71	5.37
Nucleic acid				
Methyladenosine*	0.34	0.09	0.16	0.39
Oligopeptide				
Ophthalmic acid*	1.36	0.65	0.41	2.85
Phospholipid				
Sn-Glycero-3-phosphocholine*	2.19	0.65	1.21	3.09
Stimulants				
Caffeine	15.41	27.40	0.76	61.79
Paraxanthine*	2.62	3.12	0.32	7.97

*Denotes compounds for which targeted metabolic testing is not currently available.

Samples were extracted as described in the Methods and run in a randomized single batch of 30 samples using our LC-MS/MS panel. After data processing and adjustments, we found that 134 analytes passed our initial QC criterion. For the purposes of testing the effect of duration of room temperature storage before freezing, we divided our samples in silico into 2 groups. Group 1 contained samples stored for less than 7 days (in accordance with manufacturer's recommendations) at room temperature before low-temperature storage (n = 20) and group 2 consisted of samples stored for greater than 7 days at room temperature before low-temperature storage (n = 10). Analysis was performed on raw signal intensity as described previously. Our results showed that 10 of the 134 analytes measured were impacted by storage time at room temperature before cold temperature storage (**Table VII**; available at www.jpeds.com).

Osmolality ranged from 40 mOsm/kg H_2O to 88 mOsm kg H_2O with a median value of 60 mOsm/kg H_2O, a mean value

of 62.6 mOsm/kg H_2O, standard deviation of 13.3 mOsm/kg H_2O (Table V). Our osmolality results showed that no significant differences (mean difference of 0.81 mOsm) were associated with time (P = .71 Mann–Whitney), suggesting that any differences in osmolality were due to actual physiological differences in subjects or variability in blood volume collected.

We performed unsupervised machine learning on the remaining 20 specimens stored for less than 7 days at room temperature in comparison with (a) blank injections (n = 16); (b) sample independent quality control specimens (n = 8); (c) pooled specimen quality control specimens (n = 8); and (d) individual specimens (n = 20) to obtain a qualitative overview of differences between these groups. As expected due to a priori established criteria for variability, we obtained tight clustering of our quality control indicators (groups a, b, and c). Group d exhibited spread in Euclidian space as compared with groups a and b, whereas group c was located within group d (Figure 1; available at www.jpeds.com). Note that group c is a pool of all 30 specimens run with 8 technical replicates, whereas group d represents the remaining 20 individual samples stored <7 days. Interestingly, a full separation of groups c and d were obtained by unsupervised analysis, indicating that the differences contributing to group separation survived a one-third dilution factor (10 > seven days room temperature–stored samples of 30 total samples) (Figure 2; available at www.jpeds.com). In fact, the analytes that were significantly different between group c and d were completely overlapping with the compounds listed in Table VII, with the exceptions of carnitine, phosphoethanolamine, isoleucine, serine, valine, and taurine, indicating the effect of temperature was a profound contributor to variability.

Table VI shows the median plasma values, IQRs, and normative values for the compounds of interest in our random sample of 20 clinically normal pediatric patients. Figure 3 demonstrates the same data, graphed as box and whisker plots, depending on scale. Table VIII (available at www.jpeds.com) shows the remaining 102 reported compounds; instead of concentration, raw instrument intensity values are reported.

Discussion

In this study, we identified and defined normative values among 32 analytes in the healthy newborn metabolome, via tandem LC-MS/MS analysis of capillary venous blood collected via heel-stick onto plasma-separation DBS cards. Our findings are generalizable to a methodology that employs room-temperature storage less than 7 days before extended storage at −80°C, osmolality as a surrogate normalizing measure for volumetric inconsistencies in blood spots, and highly sensitive MS in combination with the use of heavy internal standards. Our findings define the healthy newborn metabolome in a pragmatic and scalable DPS approach at a time in which newborn screening programs look to evolve capabilities and improve laboratory quality, particularly with tandem MS.[14,15] These values are not validated for clinical use; however, they provide a useful reference range for research studies where cross-platform validation can be performed.

A key feature of our study includes the timing of our research sample collection with the state mandated newborn screening DBS collection (>24 hours but no later than 48 hours after birth) as metabolic profiles shift dramatically if the sample collection occurs outside of this window. Furthermore, a previous study demonstrated potential differences in amino acid stabilities between cotton and cellulose filter cards when exposed to varying environmental conditions, particularly in metabolites known to be sensitive to humidity and temperature, such as histidine, tryptophan, arginine, lysine, citrulline, ornithine, asparagine, and glutamine.[16] We anticipate that similar stability differences exist in DPS cards and hence our collection and storage procedure (low oxygen and ultra-cold storage using barcoded cryovials) serves to mitigate variations in measured values of these and other analytes stemming from preanalytical conditions while also streamlining disc management practices for alignment with automated storage and analytics. For example, oxidized glutathione stability has is very prone to variation associated with handling of samples (see Enns and Cowan[17] and Enomoto et al[18]). We observed a high degree of variability in oxidized glutathione, indicating that there is still much room for improvement in methodologies as they relate to analytes highly susceptible to oxidation (Table IX; available at www.jpeds.com).

One study also used LC-MS/MS to establish reference intervals for 25 amino acids measurable in DBS cards collected from the Neonatal Cohort (aged 0-4 days) of the National Children's Study stored desiccated at −80°C.[19] We observe distinctive differences between panels, particularly in overlapping amino acids wherein our reported median values are lower and the normative ranges much narrower. We hypothesize these differences suggest the National Children's Study DBS collection has a wider variation in blood spot volumes or other attributive preanalytical variation, as the authors did not attempt to remove such outliers from their data set. We further note the detection of caffeine and related metabolite paraxanthine in our panel, as a potential surrogate marker for caffeine-based therapeutics provided to mothers after delivery and likely shared via breast-milk given the short half-life of caffeine. Our study has several limitations. Although our study was carried out under Biorepository and Clinical protocols, it is possible that there was some variability in the amount of time before the top layer was removed from the bottom spots. We did perform studies on the amount of blood spotted in an experiment where 10 to 80 μL of blood was spotted in 10-μL increments. We found that the most reproducible range was withing the 30- to 60-μL range. We found significant differences in analytes above or 60 μL and below 30 μL of blood spotted (data not shown, - 3 replicates of each duo card, so 6 DPS spots total).

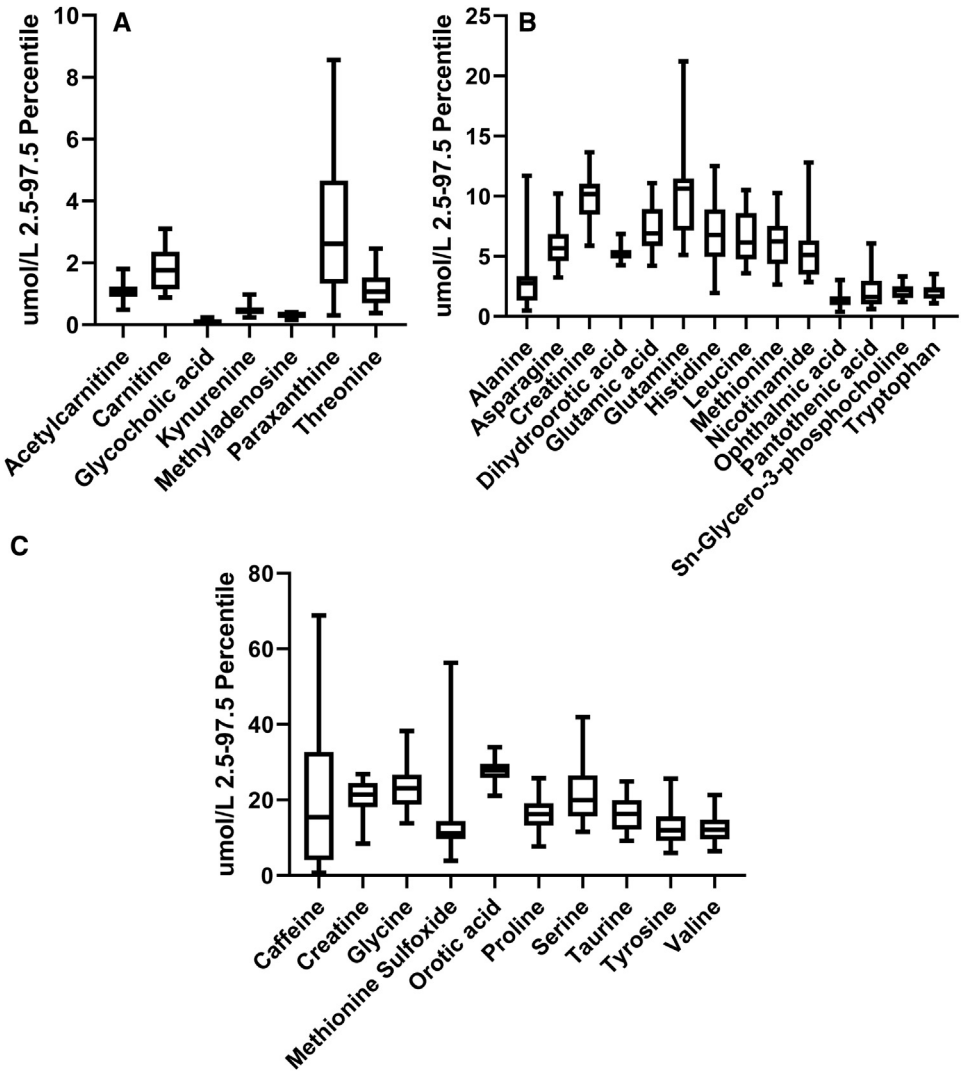

Figure 3. Box and whisker plots demonstrating **A**, 7 analytes with IQRs between 0 and 10 μmol/L; **B**, 14 analytes with IQR range of 0-25 μmol/L; and **C,** 10 analytes with IQR range of 0-80 μmol/L.

We observed significant variability in our analytes, likely the relatively small sample size (n = 20) is in part responsible for the rather wide ranges in the normative values we report; however, physiological variation in some of the measured analytes likely also contributes. It is clear that this methodology is different than venous or DBS blood draws. In fact, our data show considerably lower levels of analytes in comparison with DBS studies for the analytes that overlap,[20] a finding that is consistent with DBS spots as they relate to plasma.[21] Further research must be performed before DPS cards can be considered for clinical use.

The sample size of this initial study particularly limited our power to detect differences in normative metabolomic profiles based on sex or other demographic factors.[22] Another potential limitation was the permissible duration of ambient storage before storage at −80°C; however, we sought to evaluate a pragmatic collection and storage workflow suitable for potential application in less resource-rich environments. Future larger studies will permit sensitivity analyses of the potential influence of duration of ambient storage from 0 to <7 days; however, in preliminary analyses in the present study, we did not find this to be significantly associated with levels of measured analytes.

The focus of this study was not on newborn screening or analytes on the recommended universal screening panel (RUSP) for IEMs. The results are based upon a large mass spectrometry research assay that the laboratory has developed to assess overall metabolism and provide insights into targets not generally previously studied. Unfortunately many of the targets that are indeed of importance for the RUSP IEMs are not included in this assay due to the biochemical limitations of the methods. Given the positive data associated with DPS, the translation of the DPS findings to the RUSP IEMs are an important next step in the line of

investigation. Our panel does involve important markers that could potentially be used to detect synthesis defects in serine and creatinine biosynthesis.

Despite these limitations, this study establishes feasibility of a DPS collection and storage methodology, in combination with LC-MS/MS, for determination of a broad array of analytes within the healthy newborn metabolome. We report normative values for 32 metabolites with suitable stability for targeted comparative studies in larger cohort studies, wherein diagnostic and prognostic utility may be shown valuable for identifying metabolic disorders and molecular early determinants of child health and disease. ■

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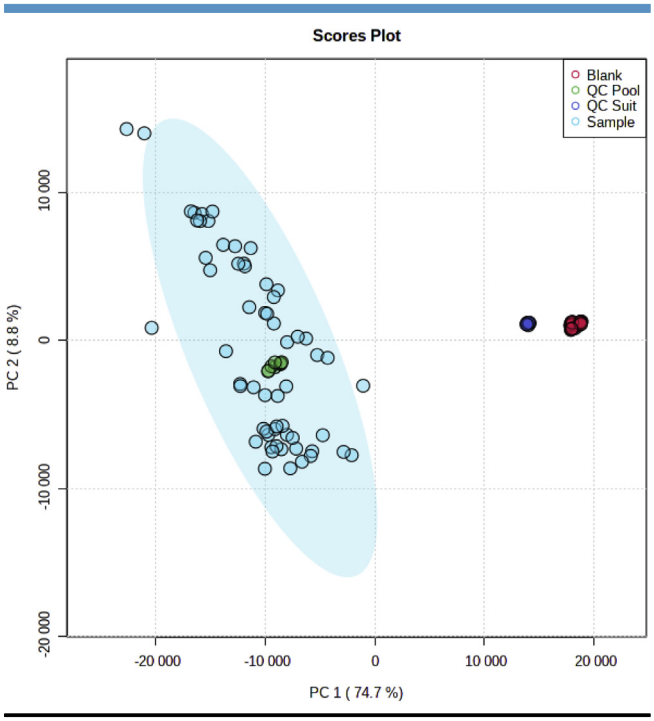

Figure 1. Principal component analysis of procedural blanks, QC pool, QC system suitability standards, and participant samples showing contribution of each principal component (PC).

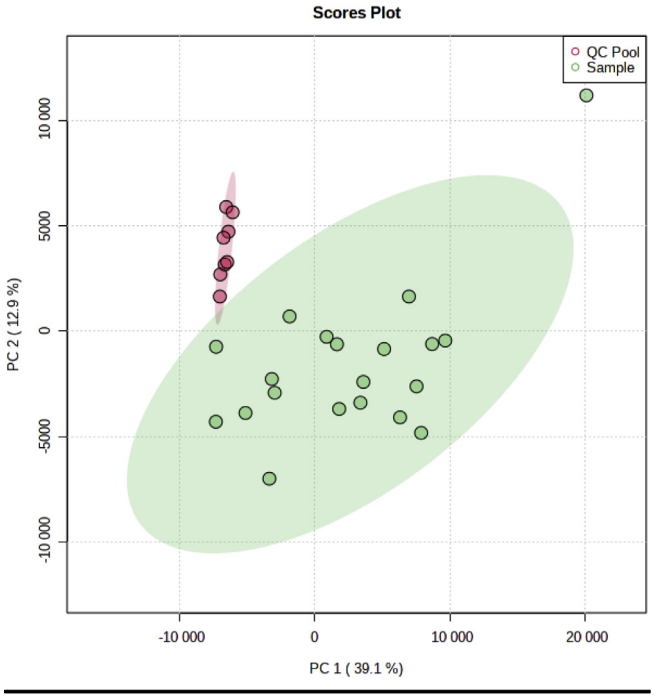

Figure 2. Principal component analysis of QC pool (all 30 samples) vs samples stored for <7 days at room temperature.

Table I. Heavy internal standards and sources

Standards	Vendor	Part number
L-Glutamic acid-13C5,15N	Sigma-Aldrich	607851
L-Isoleucine-13C6,15N	Sigma-Aldrich	608092
L-Proline-13C5,15N	Sigma-Aldrich	608114
L-Tryptophan-13C11,15N2	Sigma-Aldrich	574597
L-Tyrosine-(phenyl-13C6)	Sigma-Aldrich	489794
3-Nitro-L-tyrosine-13C9	Sigma-Aldrich	652296
L-Serine-13C3,15N	Sigma-Aldrich	608130
L-Methionine-13C5,15N	Sigma-Aldrich	608106
Glycine-1-13C,15N	Sigma-Aldrich	299340

Table II. MS variables

Variables	Value
Resolution	1 Dalton
Nebulizing gas flow	3 L/min
Heating gas flow	10 L/min
Interface temperature	300°C
Desolvation line temperature	250°C
Heat block temperature	400°C
Drying gas flow	10 L/min

Table III. Liquid chromatography variables

Variables	Value
Column	Pentafluorophenylpropyl, 150 mm × 2.1 mm ID, particle size 3 μM
Mobile A	Water and 0.1% formic acid
Mobile B	Acetonitrile with 0.1% formic acid
Temperature	40°C

ID, inner diameter.

Table IV. Liquid chromatography gradient

Time, min	Ramp/step	% Mobile B
0-2	Ramp	0
2-5	Ramp	24
5-11	Ramp	34
11-15	Ramp	76
15-17	Step	0

Table V. Osmolality measures of individual samples

Participant_ID	mOsm/kg H_2O
001-0324D	59
001-0444C	75
001-0448C	46
001-0478C	40
001-0479C	49
001-0484C	53
001-0488C	52
001-0489C	83
001-0492C	75
001-0493C	60
001-0494C	59
001-0495C	62
001-0496C	86
001-0497C	73
001-0498C	79
001-0500C	56
001-0501C	76
001-0502C	64
001-0503C	68
001-0507C	56
001-0510C	45
001-0511C	51
001-0515C	67
001-0516C	81
001-0517C	54
001-0521C	45
001-0522C	60
001-0524C	51
001-0533C	88
001-0534C	65

Table VII. Effect of room temperature storage before cold temperature storage (<7 days vs >7 days)

	Parametric (*t* test)		Nonparametric (Mann–Whitney)	
Compounds	*P* value	FDR	*P* value	FDR
Creatine	.002753	0.046463	.000789	0.029014
Taurine	.002753	0.046463	.000823	0.029014
Histidine	.002317	0.046463	.000899	0.029014
Valine	.003258	0.047162	.001004	0.029014
Serine	.001349	0.046463	.001075	0.029014
Isoleucine	.003843	0.047162	.00158	0.030716
Carnitine	.001622	0.046463	.001627	0.030716
Leucine	.002753	0.046463	.00184	0.030716
Glutamine	.003843	0.047162	.002048	0.030716
Phosphoethanolamine	.000616	0.046463	.003629	0.04083

Table VIII. Normal research reference ranges, raw signal intensity

Compounds	Median plasma	IQR	Normative range (2.5th-97.5th percentile)	
Inosinic acid	11 631	9298	7125	20 199
Phosphoethanolamine	662 350	358 297	396 697	1 535 650
Glucosaminic acid	34 126	30 111	19 276	74 206
Ciliatine	11 785	6563	5083	13 472
Citicoline	9891	12 331	5426	50 135
N-methylaspartic acid	26 615	17 584	6801	50 810
Thiourea	19 785	17 948	5333	41 046
N-alpha-acetylasparagine	1 389 785	984 949	556 521	2 517 101
Cystathionine	76 474	45 417	23 391	162 009
Nicotinamide mononucleotide	147 862	49 926	62 697	291 012
S-Carboxymethylcysteine	103 919	25 334	70 279	143 057
Aspartic acid	251 725	175 219	146 595	838 207
Cystine	1 791 118	2 483 750	524 966	5 992 630
4-Hydroxyproline	115 567	56 256	61 995	164 122
N-Acetylserine	48 749	19 050	30 985	74 588
Diaminopimelic acid	10 495	5821	6229	25 385
Homoserine	93 949	41 650	65 963	232 736
Betaine	21 398 166	10 129 769	11 697 196	34 832 042
Citrulline	875 589	651 662	315 007	1 849 058
Mannosamine	1 135 831	264 840	827 266	1 704 202
Trigonelline	164 862	127 483	47 740	596 065
Picolinic acid	11 432	8069	5327	28 959
2,3-Diaminopropionic acid	107 882 798	7 353 332	98 146 826	117 642 726
N-methylglutamic Acid	475 664	227 280	232 196	893 397
Uracil	73 930	20 229	52 811	109 960
N-Acetylaspartic acid	13 181	7228	5466	17 193
5-Oxoproline	171 803	112 520	34 777	319 315
3-Ureidopropionic acid	456 095	115 622	256 748	634 631
Lysine	16 445	15 256	5627	56 073
Pipecolic acid	21 933	8798	8436	47 156
3-Methylhistidine	234 072	92 964	57 248	355 519
3-Aminoisobutanoic acid	223 791	200 245	57 624	909 755
Arginine	290 525	117 362	75 758	542 178
Trimethylamine	2 795 308	3 267 436	568 764	11 288 029
Nicotinic acid	931 131	456 421	366 118	1 844 995
Trimethylamine N-oxide	192 939	114 338	122 999	466 705
Pyridoxamine	2 441 261	2 385 986	511 653	6 282 717
4-Imidazoleacetic acid	16 690	15 812	8193	44 871
2-Hydroxypyridine	8284	4231	5122	13 058
1-Methylhistidine	16 107	15 290	5688	55 132
Inosine	3 950 129	4 511 731	338 082	17 712 505
Glutathione	756 406	502 253	298 911	1 768 606
Cytidine	2 518 073	870 449	1 643 182	3 463 552
3,5-cyclic AMP	317 195	59 031	246 069	385 544
3,5-cyclic GMP	772 567	71 023	648 298	869 180
2,3-cyclic AMP	425 807	56 465	354 442	559 585
Guanosine	4 997 927	410 821	4 192 178	5 291 566
N-Alpha-acetyl lysine	337 645	123 099	246 100	579 101
Adenine	106 625	34 897	16 178	394 665
Thymidine	550 298	111 052	358 451	717 043
Thymine	168 331	32 953	113 410	204 896
Quinolinic acid	741 250	295 642	486 648	1 302 564
O-Succinylhomoserine	7975	5052	5224	19 424
5-Aminopentanoic acid	7167	1530	5422	12 952
4-Pyridoxic acid	163 512	87 214	103 289	524 000
6-Hydroxynicotinic acid	292 485	83 147	214 910	408 292
Pterin	331 671	47 274	260 990	390 106
2-Deoxycytidine	1 974 304	3 854 626	6244	4 515 135
Adenosine	19 121 482	3 016 978	13 665 794	32 997 273
5-Methylcytosine	35 047	23 384	8368	55 828
2-Deoxyguanosine	8 598 424	855 459	7 299 279	11 002 284
1-Methyladenosine	3 413 334	536 860	1 768 481	4 290 438
N-Acetylputrescine	195 448	73 115	96 951	368 323
Pyridoxal	69 646	54 725	35 010	145 011
Selenomethionine	75 230	33 206	44 188	612 131
4-Quinolinecarboxylic acid	19 578	8696	7279	34 182
NG-Methylarginine	629 301	150 607	458 496	825 833
3-Hydroxyanthranilic acid	686 113	245 829	454 557	1 071 428
Theobromine	46 933	78 387	13 787	303 804
5-Hydroxyindoleacetic acid	109 578	85 490	63 293	206 474

(*Continued*)

Table VIII. Continued

Compounds	Median plasma	IQR	Normative range (2.5th-97.5th percentile)	
Pimelic acid	571 145	232 539	411 758	964 764
Epinephrine	40 722	23 133	23 628	73 545
Isoleucine	3 733 875	2 060 186	2 027 950	6 571 492
Urocanic acid	4 176 757	2 851 856	1 789 407	11 477 081
5-Deoxyadenosine	7 094 959	2 190 721	5 346 171	9 681 417
Hippuric acid	65 676	56 506	33 215	1 214 416
3-Hydroxykynurenine	143 610	54 185	76 313	209 946
N-Formylkynurenine	282 426	153 923	123 055	668 286
3-Methyladenine	15 405	7853	6314	36 403
Suberic acid	264 573	95 768	198 220	502 927
3-Methoxytyrosine	22 033	17 236	6355	46 362
Symmetric dimethylarginine	13 485	6448	5621	26 847
Asymmetric dimethylarginine	21 337	11 380	7748	28 207
Normetanephrine	55 018	46 514	13 452	119 233
N-Acetylleucine	851 786	379 247	464 076	1 282 986
Kynurenic acid	8001	3950	5298	16 037
2-Aminophenol	18 744	8354	6321	32 758
Melilotic acid	24 891	14 326	11 117	45 243
Azelaic acid	563 568	124 147	396 033	716 783
Ferulic acid	9130	9384	5470	36 177
Lumichrome	42 301	10 653	34 572	68 208
N-acetyltryptophan	517 352	545 012	152 557	2 172 530
Tyramine	7209	2821	5748	16 080
Indoleacetic acid	652 149	319 154	335 942	987 556
Cortisol	13 665	11 342	5938	24 463
10-Hydroxydecanoic acid	72 968	24 025	56 888	105 935
Gluconic acid	6403	1360	5495	11 872
Succinic acid	31 875	19 377	9094	65 164
Itaconic acid	833 843	216 477	587 983	1 096 668
Ethylmalonic acid	19 865	15 927	7494	32 104
Hydroxyphenyllactic acid	53 428	25 334	12 550	96 789
Cholic acid	50 243	95 925	28 640	359 978

Table IX. Normal range of glutathione oxidized

Compound	Median plasma, fmol	IQR	Normative range (2.5th-97.5th percentile)
Glutathione oxidized	228 970	175 647	62 568-507 856