Supporting Information:

Development of a Universal Second-tier Newborn Screening LC-MS/MS Method for Amino Acids, Lysophosphatidylcholines, and Organic Acids

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TABLE OF CONTENTS

SUPPLEMENTAL MATERIALS:

Standards used in the study and their abbreviations are detailed in Supplemental Table 2.

SUPPLEMENTAL FIGURES:
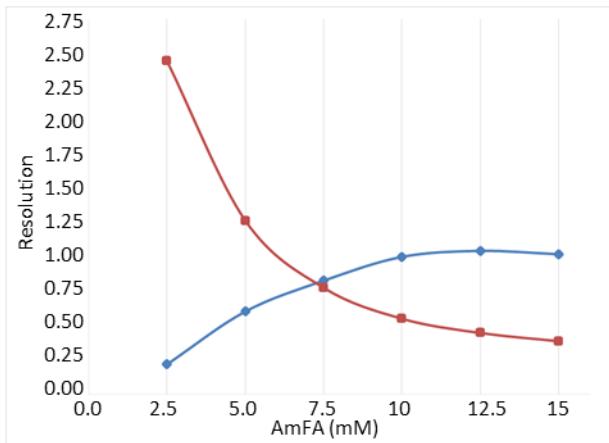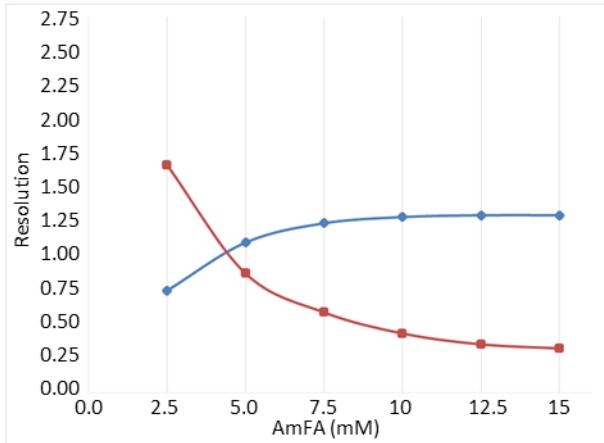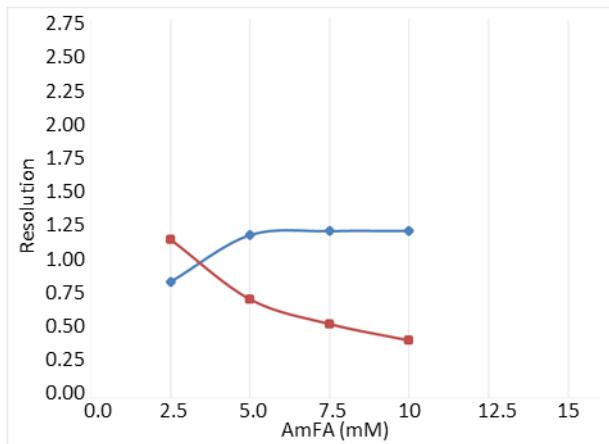
Supplemental Figure 1. Resolution of aIle and Leu (blue) and aIle and Ile (red) with differing concentrations of mobile phase ammonium formate (AmFA) on the x-axis. Each panel shows the results for a different percentage of ACN and water in the mobile phase, all conditions have 0.2% FA A) 80% ACN and 20% water, B) 85% ACN and 15% water, and C) 90% ACN and 10% water. Experiment uses a waters TQD mass spectrometer with an Imtakt Intrada amino acid column (3 μm, 50 x 3 mm) with an isocratic gradient at 0.4 ml/min and a column temperature of 30°C.
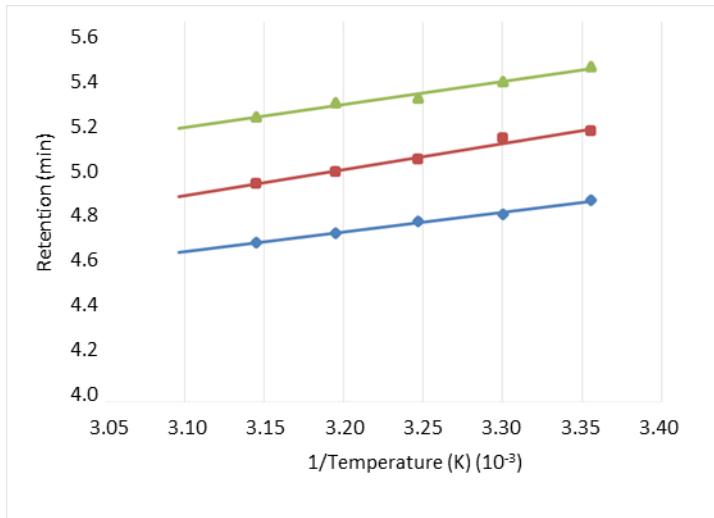
Supplemental Figure 2. Change in the retention time of Ile (green), aIle (red), and Leu (blue) with changing column 1/temperature in Kelvin (K). Method is using a waters TQD mass spectrometer with an Imtakt Intrada amino acid column (3 μm, 50 x 3 mm) with an isocratic gradient 85% ACN, 15% water, 0.2% FA, and 5 mM AmFA at a flow rate of 0.4 ml/min.
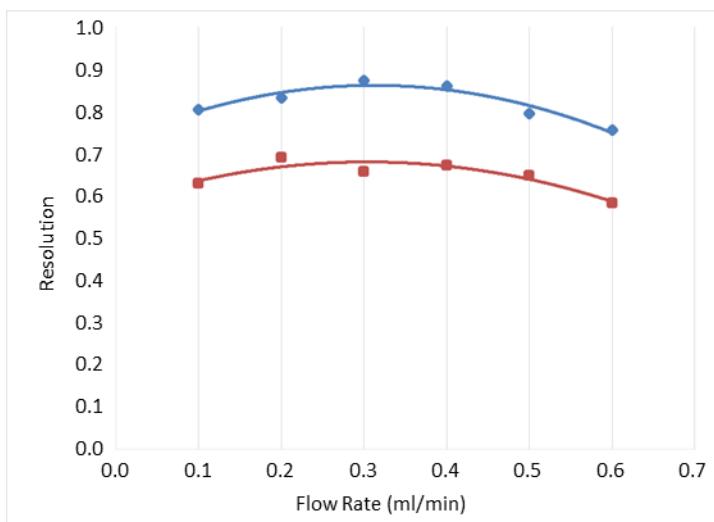
Supplemental Figure 3. Change in the resolution of Ile and aIle (red) and Leu and aIle (blue) with changing flow rate in ml/min. Method is using a waters TQD mass spectrometer with an Imtakt Intrada amino acid column (3 μm, 50 x 3 mm) with an isocratic gradient 85% ACN, 15% water, 0.2% FA, and 5 mM AmFA and a column temperature 30°C.
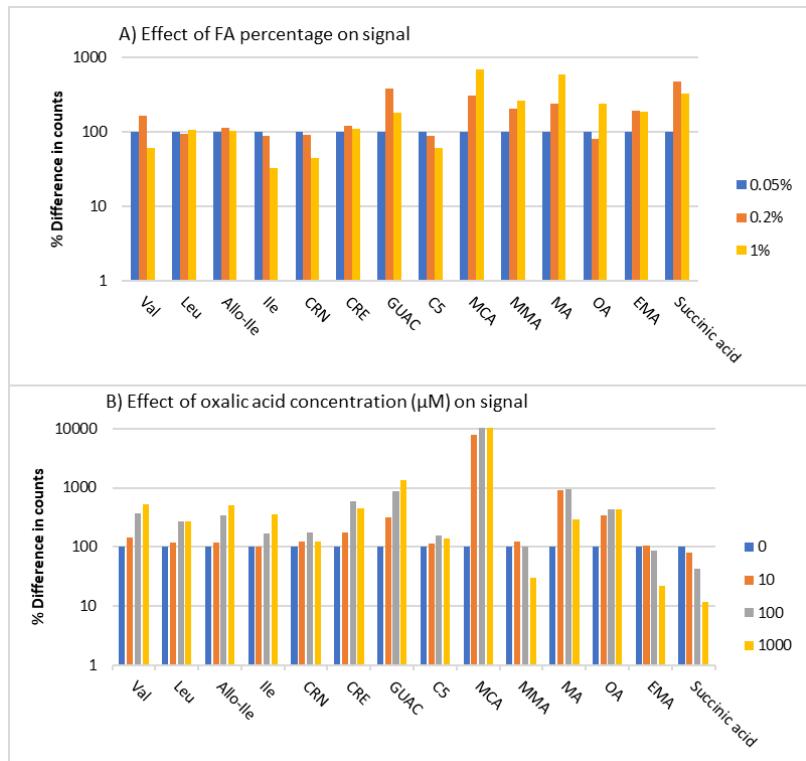
Supplemental Figure 4. Improvement of analyte signal on addition to the mobile phase of A) FA percentage or B) oxalic acid concentration (μM). Using a TQD waters mass spectrometer, the chromatography method differs from the chromatography used for validation in that it uses a different default mobile phase A (85% ACN, 15% water, 6.5 AmFA, and 0.2% FA) and mobile phase B (10% ACN, 90% water, and 140 mM AmFA) with a constant flow rate (0.35 ml/min). The gradient proceeds as follows, with linear gradients between time points mobile phase A is 100% at 0.0 min, 100% at 7.0 min, 0 % at 12 min, 0% at 15 min, 100% at 25 min, and 0% at 30 min.
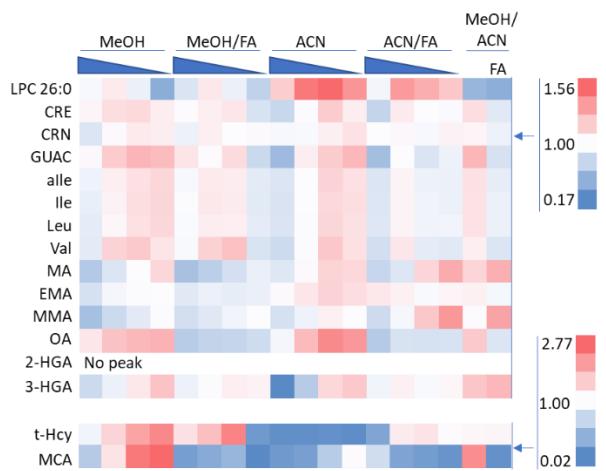
Supplemental Figure 5. A heat map showing the effect of extraction solvent composition on internal standard fold change relative to the mean value of each analyte. Solvents tested include either equal percentages MeOH/ACN/water (MeOH/ACN) or variable percentages of MeOH/water (MeOH) and ACN/water (ACN), 100/0, 90/10, 80/20, and 70/30, with or without 0.1% FA. Above 1 is red, below 1 is blue, and 1 is white. Material extracted was CDC NSQAP QC material lot# D1814.
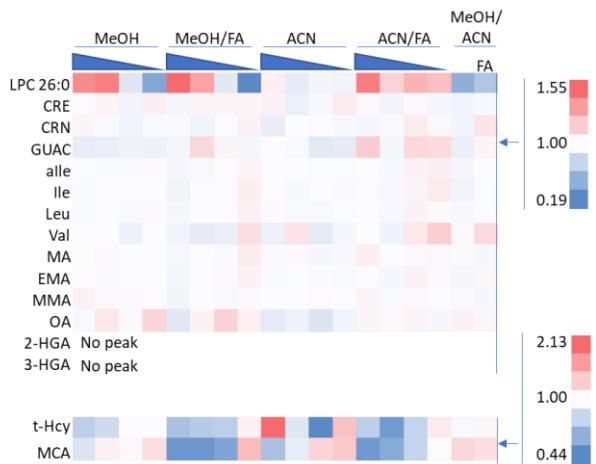
Supplemental Figure 6. A heat map showing the effect of extraction solvent composition on concentration fold change relative to the mean value of each analyte. Solvents tested include either equal percentages MeOH/ACN/water (MeOH/ACN) or variable percentages of MeOH/water (MeOH) and ACN/water (ACN), 100/0, 90/10, 80/20, and 70/30, with or without 0.1% FA. Above 1 is red, below 1 is blue, and 1 is white. Material extracted was CDC NSQAP QC material lot# D1814.
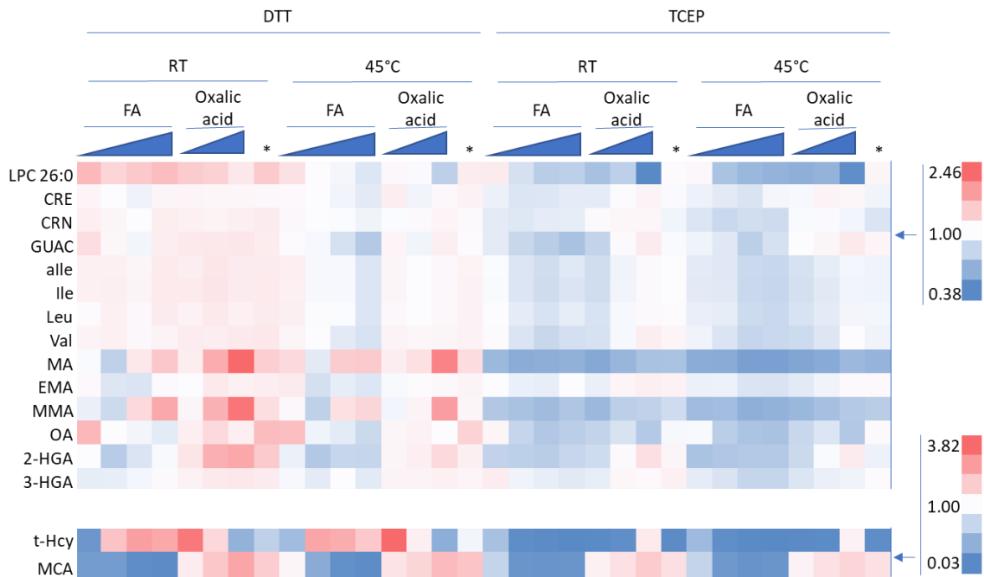
Supplemental Figure 7. A heat map showing the effects of varying extraction conditions on internal standard fold change relative to the mean value of each analyte. Conditions tested include DTT or TCEP reduction, room temperature or 45°C extraction, variable formic acid (FA at 0, 0.05, 0.1, or 0.2%) or variable oxalic acid (1, 3, or 9 mM) using 0.1% FA. In addition, 3 mM oxalic acid with no FA was tested (*). All conditions use 80% acetonitrile with 20% water. Above 1 is red, below 1 is blue, and 1 is white. Material extracted was CDC QC material lot# D1814.
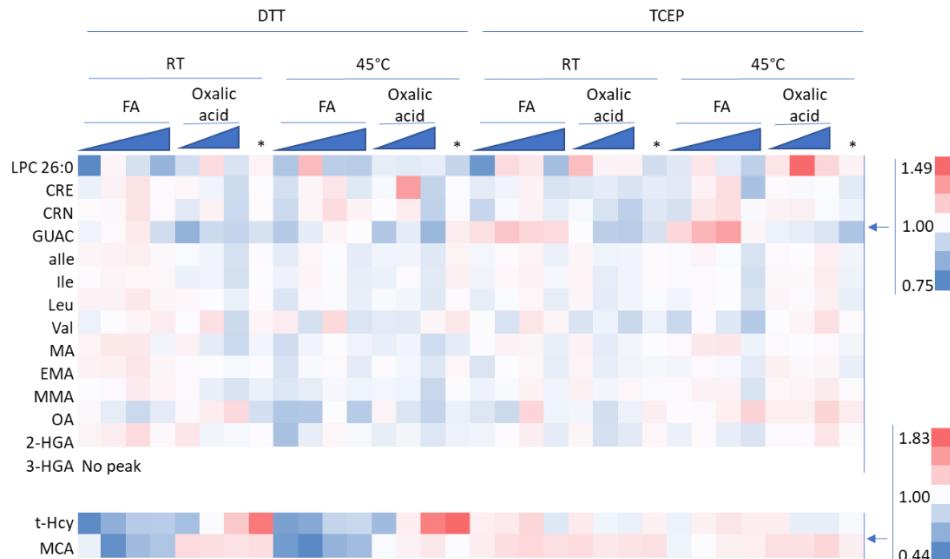
Supplemental Figure 8. A heat map showing the effects of varying extraction conditions on concentration fold change relative to the mean value of each analyte. Conditions tested include DTT or TCEP reduction, room temperature or 45°C extraction, variable formic acid (FA at 0, 0.05, 0.1, or 0.2%) or variable oxalic acid (1, 3, or 9 mM) using 0.1% FA. In addition, 3 mM oxalic acid with no FA was tested (*). All conditions use 80% acetonitrile with 20% water. Above 1 is red, below 1 is blue, and 1 is white. Material extracted was CDC QC material lot# D1814.

SUPPLEMENTAL TABLES:

Supplemental Table 1. Diseases and second-tier biomarkers of interest

Disease	Biomarkers
Maple syrup urine disease[a]	Leu, Ile, aIle, and Val[1]
Homocystinuria[a]	t-Hcy[2]
Methylmalonic academia (Methylmalonyl-CoA mutase)[a]	MMA, t-Hcy, and MCA can be informative[3]
Methylmalonic academia (cobalamin disorders)[a]	MMA, t-Hcy, and MCA can be informative[3]
Propionic acidemia[a]	MCA[3]
Isovaleric acidemia[a]	Isovalerylcarnitine[4]
Glutaric acidemia type 1[a]	3-HGA and GA[5]
Adrenoleukodystrophy[a]	LPC 26:0[6]
Congenital adrenal hyperplasia[a]	17-OHP, 21-deoxycortisol, 11-deoxycortisol, cortisol, and androstenedione[7]
Pompe disease[b]	(CRE/CRN)/(acid alpha-glucosidase) ratio[8]
Malonic Acidemia	MAL[9]
Glutaric acidemia type 2[b]	GA, MSA, EMA, and 2-HGA[10]
Short-chain acyl-CoA dehydrogenase deficiency[b]	EMA and MSA[11]
Guanidinoacetate methyltransferase deficiency	GUAC[12]
Ornithine transcarbamylase deficiency[c]	OA[13]
Krabbe	Psychosine[14]

[a]Part of recommended uniform screening panel primary conditions

[b]Part of recommended uniform screening panel secondary conditions

[c]Usefulness of biomarker questionable in DBS

Superscript numbers are citations where these biomarkers are used or discovered, see references at the end of supplemental material

Supplemental Table 2 Part 1. Manufacturer information for chemicals

Study Analyte Information: Unlabeled and labeled			
Unlabeled Chemical, Manufacturer	Catalog Number: Lot Number	Labeled Chemical, Manufacturer	Catalog Number: Lot Number
Methylmalonic acid (MMA), Sigma	M54058:STBFS304V	MMA-D_3, Cambridge Isotope Laboratories, Inc. (CIL)	DLM-387:I-21371
Ethylmalonic acid (EMA), Sigma	102687:BCCB4912	EMA-D_3, CIL	DLM-6013:PR-28264
Malonic acid (MA), Sigma	M1296:WXBC1802V	MA-$^{13}C_3$, CIL	CLM-6123:PR-31412
Methylcitric acid (MCA), C/D/N Isotopes	X-4176:DE-209	MCA-D_3, C/D/N isotopes	D-4162:DE-268
Alloisoleucine (aIle), Sigma	I8754:BCBX0375	aIle-D_{10}, CIL	DLM-1505:PR-17413B
Isoleucine (Ile), Sigma	I2752:SLCG1332	Ile-D_{10},^{15}N, CIL	DNLM-7325:PR-25770
Leucine (Leu), Sigma	L8000:BCBV3850	Leu-D_3, CIL	DLM-1259:PR-29679B
Valine (Val), Sigma	V0500:SLBT4145	Val-D_8, CIL	DLM-488:PR-22504
Creatine (CRE), Sigma	C0780:BCBZ3033	CRE-D_3, CIL	DLM-1302
DL-Homocystine (Hcy), Sigma	H0501:127K0773V	Hcy-D_8, CIL	DLM-3619:PR-26158
Guanidinoacetic acid (GUAC), Sigma	G11608:MKCJ7552	GUAC-$^{13}C_2$,^{15}N, CIL	CNLM-8300
Creatinine (CRN), Sigma	C4255:SLBW4791	CRN-D_3, CIL	DLM-3653
Orotic acid (OA), Sigma	O2750:MKCK0782	OA-$^{15}N_2$, CIL	NLM-1048:PR-26650
3-Hydroxyglutaric acid (3-HGA), Sigma	04725:BCBW6359	3-HGA-D_5, CIL	D-6391:V-129
2-Hydroxyglutaric acid (2-HGA), Sigma	61313:BCBZ4748	2-HGA-$^{13}C_5$, Sigma	80559:BCCC7143
Lysophosphatidylcholine (LPC) 20:0, Avanti	855777P:200LPC-30	LPC 20:0-D_4, CIL	DLM-10520:PR-29690
LPC 22:0, Avanti	855779P:220LPC-18	LPC 22:0-D_4, CIL	DLM-10500:PR-29619
LPC 24:0, CIL	ULM-10495:PR-29619	LPC 24:0-D_4, CIL	DLM-10497:PR-29620
LPC 26:0, CIL	ULM-9791:PR-27475	LPC 26:0-D_4, CIL	DLM-10501:PR-29618

Supplemental Table 2 Part 2. Manufacturer information for chemicals

Attempted Analyte Information: Unlabeled and labeled			
Unlabeled Chemical, Manufacturer	Catalog Number: Lot Number	Labeled Chemical, Manufacturer	Catalog Number: Lot Number
Glutaric acid (GA), Sigma	G3407:STBF5476V	GA-D$_4$, CIL	DLM-3106:PR-30114
Methylsuccinic acid (MSA), Sigma	M81209:53265	MSA-D$_6$, CIL	DLM-2960:V-610
Isovalerylcarnitine, CIL	ULM-4697:PR-30290	Isovalerylcarnitine-D$_9$, CIL	DLM-3974:PR-27967
21-Deoxycortisol, Sigma	P9521	21-Deoxycortisol-D$_8$, CIL	DLM-8305:PR-25872
11-Deoxycortisol, Sigma	R0500	11-Deoxycortisol-D$_2$, CIL	DLM-7209:I-22071A
Cortisol, Sigma	H0888	Cortisol-D$_4$, CIL	DLM-2218:PR-20446
Androstenedione, CIL	ULM-8472-0	Androstenedione, CIL	DLM-7976:PR-25180
17-α-hydroxyprogesterone (17-OHP), Sigma	H5752	17-OHP-D$_8$, CIL	DLM-6598:I-19002
		Psychosine-D$_5$, Avanti	330714W-1EA: 330714W-1ML-010
Interferents and other chemicals			
Chemical, Manufacturer		Catalog Number: Lot Number	
Succinic acid, Sigma		S3674:SLBX6330	
Trans-hydroxyproline, Sigma		H54409:MKCL1320	
pivaloyl-L-carnitine HCl, Dr. Ten Brick		pivaloyl-L-carnitine.HCl	
(D,L)-2-methylbutyryl-L-carnitine HCl, Dr. Ten Brick*		(D,L)-2-methylbutyryl-L-carnitine.HCl	
Deoxycorticosterone, Sigma		D6875:SLCC6035	
17-Deoxycortisol, Sigma		27840:BCCB3706	
DL-dithiothreitol (DTT), Sigma		43819:BCCB7858	
tris(2-carboxyethyl)phosphine (TCEP), EMD Millipore Corp.		580567:3467213	
Oxalic acid, Sigma		241172	
0.25 N sodium hydroxide, Fisher		SS272-1	
LC/MS grade water, Fisher		W6-4	
LC/MS grade acetonitrile (ACN), Fisher		A955-4	
LC/MS grade formic acid (FA), Fisher		A117-50	
Ammonium formate (AmFA), Sigma		798568:MKCM2018	
Blood bank saline isotonic solution 0.9% w/v (saline), Thermo Scientific		23-062-125	

* Dr. Ten Brick, Amsterdam University Medical Centers, Department of Clinical Chemistry

Supplemental Table 3. Enrichment of every DBS level made for validation

Chemical	Stock solvent	Level 1 (μM)	Level 2 (μM)	Level 3 (μM)	Level 4 (μM)	Level 5 (μM)	Level 6 (μM)	Level 7 (μM)	Level 8 (μM)	Level 9 (μM)	Level 10 (μM)	Level 11 (μM)
CRE	water	0	7	11.9	20.2	34.4	58.5	99.4	169	287	488	830
GUAC	water	0	0.2	0.34	0.58	0.98	1.67	2.84	4.83	8.21	14	23.7
CRN	water	0	2	3.4	5.78	9.83	16.7	28.4	48.3	82.1	140	237
OA	0.1 N NaOH	0	0.5	0.85	1.45	2.46	4.18	7.1	12.1	20.5	34.9	59.3
3-HGA	water	0	0.15	0.26	0.43	0.74	1.25	2.13	3.62	6.16	10.5	17.8
2-HGA	water	0	0.8	1.36	2.31	3.93	6.68	11.3	19.3	32.8	55.8	94.9
LPC 20:0	methanol	0	0.025	0.043	0.072	0.123	0.209	0.355	0.603	1.03	1.74	2.97
LPC 22:0	methanol	0	0.025	0.043	0.072	0.123	0.209	0.355	0.603	1.03	1.74	2.97
LPC 24:0	methanol	0	0.025	0.043	0.072	0.123	0.209	0.355	0.603	1.03	1.74	2.97
LPC 26:0	methanol	0	0.025	0.043	0.072	0.123	0.209	0.355	0.603	1.03	1.74	2.97
MMA	water	0	0.5	0.85	1.45	2.46	4.18	7.1	12.1	20.5	34.9	59.3
EMA	water	0	0.5	0.85	1.45	2.46	4.18	7.1	12.1	20.5	34.9	59.3
MA	water	0	1	1.7	2.89	4.91	8.35	14.2	24.1	41	69.8	119
MCA	water	0	0.25	0.43	0.72	1.23	2.09	3.55	6.03	10.3	17.4	29.7
aIle	water	0	0.11	0.19	0.32	0.54	0.92	1.56	2.66	4.51	7.67	13
Ile	water	0	7	11.9	20.2	34.4	58.5	99.4	169	287	488	830
Leu	water	0	7	11.9	20.2	34.4	58.5	99.4	169	287	488	830
Val	water	0	7	11.9	20.2	34.4	58.5	99.4	169	287	488	830
Hcy*	0.1 N NaOH	0	1	1.7	2.89	4.91	8.35	14.2	24.1	41	69.8	119

*dimer enriched. Values shown are monomer value

Supplemental Table 4. Multiple reaction monitoring table with retention times for final method

Compound	Transition (ion/fragment m/z)	Dwell time (sec)	Ionization mode	Collision energy (eV)	Cone voltage (V)	Retention time (min)
CRN	114/44	0.075	ES+	12	30	10.3
CRN-D_3	117/47	0.075	ES+	12	30	10.3
Val	118/72	0.01	ES+	10	20	8.7
Val-D_8	126/80	0.01	ES+	10	20	8.7
t-Hcy	136/90	0.01	ES+	10	20	8.5
t-Hcy-D_4	140/94	0.01	ES+	10	20	8.5
GUAC	118/76	0.052	ES+	11	10	9.6
GUAC-$^{13}C_2,^{15}N$	121/79	0.052	ES+	11	10	9.6
CRE-$^{13}C_1$[#]	133/91	0.052	ES+	12	20	9.3
CRE-$^{13}C_1,D_3$[#]	136/94	0.052	ES+	12	20	9.3
aIle and Ile	132/69	0.009	ES+	20	25	7.2 and 7.5
aIle-D_{10}	142/78	0.009	ES+	20	25	7.2
Ile-$D_{10},^{15}N$	143/78	0.009	ES+	20	25	7.5
Leu	132/86	0.009	ES+	8	22	6.8
Leu-D_3	135/89	0.009	ES+	8	22	6.8
LPC 20:0	552.7/104.1	0.038	ES+	28	55	3.3
LPC 20:0-D_4	556.7/104.1	0.038	ES+	28	55	3.3
LPC 22:0	580.7/104.1	0.038	ES+	28	55	3.2
LPC 22:0-D_4	584.7/104.1	0.038	ES+	28	55	3.2
LPC 24:0	608.7/104.1	0.038	ES+	28	55	3.1
LPC 24:0-D_4	612.7/104.1	0.038	ES+	28	55	3.1
LPC 26:0	636.7/104.1	0.038	ES+	28	55	3.1
LPC 26:0-D_4	640.7/104.1	0.038	ES+	28	55	3.1
MA	103/59	0.01	ES-	8	15	1.2
MA-$^{13}C_3$	106/61	0.01	ES-	8	15	1.2
MMA	117/73	0.01	ES-	8	15	1.2
MMA-D_3	120/75.8	0.01	ES-	8	15	1.2
EMA	131/87	0.01	ES-	10	20	1.1
EMA-D_3	134/90	0.01	ES-	10	20	1.1
2-HGA	147/85	0.018	ES-	8	25	2.0
2-HGA-$^{13}C_5$	152/89	0.018	ES-	8	25	2.0
3-HGA	147/85	0.018	ES-	8	22	1.9
3-HGA-D_5	152/89	0.018	ES-	8	22	1.9
OA	155/111	0.018	ES-	10	20	2.1
OA-$^{15}N_2$	157/113	0.018	ES-	10	20	2.1
MCA	205/125	0.1	ES-	12	10	1.5* and 2.1
MCA-D_3	208/128	0.1	ES-	12	10	1.5* and 2.1

*time used for quantification of analyte

#unlabeled creatine was so intense that the endogenous $^{13}C_1$ isotope was used for quantification. Internal standard is only labeled for deuterium.

Supplemental Table 5. Multiple reaction monitoring transitions with retention times for attempted biomarkers

Compound	Transition (ion/fragment m/z)	Dwell time (sec)	Ionization mode	Collision energy (eV)	Cone voltage (V)	Retention time (min)
Isovalerylcarnitine	246/85	0.052	ES+	20	10	5.9
Isovalerylcarnitine-D$_9$	246/85	0.052	ES+	20	10	5.9
GA	131/87	0.006	ES-	10	20	1.4
GA-D$_4$	135/91	0.006	ES-	10	20	1.4
MSA	131/87	0.006	ES-	10	20	1.3
MSA-D$_6$	137/93	0.006	ES-	10	20	1.3
Androstenedione	287.3/97.1	0.003	ES+	24	40	1.1
Androstenedione-D$_7$	294.3/100.1	0.003	ES+	24	40	1.1
17-OHP	331.2/97.0	0.003	ES+	45	25	1.2
17-OHP-D$_8$	339.2/100.1	0.003	ES+	45	25	1.2
11-Deoxycortisol	347.2/97.0	0.003	ES+	24	40	1.3
11-Deoxycortisol-D$_2$	349.2/97.0	0.003	ES+	24	40	1.3
21-Deoxycortisol	347.2/121.0	0.003	ES+	22	35	1.2
21-Deoxycortisol-D$_8$	355.2/125.0	0.003	ES+	22	35	1.2
Cortisol	363.2/121.0	0.003	ES+	30	24	1.5
Cortisol-D$_4$	367.2/121.0	0.003	ES+	30	24	1.5
Psychosine transition 1	462.5/282.5	0.003	ES+	20	20	Elutes after run
Psychosine-D$_5$ transition 1	467.5/287.5	0.003	ES+	20	20	Elutes after run
Psychosine transition 2	462.5/264.5	0.003	ES+	18	20	Elutes after run
Psychosine-D$_5$ transition 2	467.5/269.5	0.003	ES+	18	20	Elutes after run

Supplemental Table 6. Concentration of biomarker spikes, relative to DBS volume, for accuracy analysis

Chemical	Zero enrichment (µM)	Low enrichment (µM)	Medium enrichment (µM)	High enrichment (µM)
CRE	0	34.4	99.4	488
GUAC	0	0.58	2.84	14
CRN	0	9.83	28.4	140
OA	0	1.45	7.1	34.9
3-HGA	0	0.43	2.13	10.5
2-HGA	0	2.31	11.4	55.8
LPC 20:0	0	0.07	0.35	1.74
LPC 22:0	0	0.07	0.35	1.74
LPC 24:0	0	0.07	0.35	1.74
LPC 26:0	0	0.07	0.35	1.74
MMA	0	1.45	7.1	34.9
EMA	0	1.45	7.1	34.9
MA	0	2.89	14.2	69.8
MCA	0	0.72	3.55	17.4
aIle	0	0.54	1.56	7.67
Ile	0	34.4	99.4	488
Leu	0	34.4	99.4	488
Val	0	34.4	99.4	488
Hcy*	0	2.89	14.2	69.8

*dimer enriched. Values shown are monomer values

Supplemental Table 7. Separation of biomarker and interferents

Biomarker/Interferent	Separated?
MMA/succinic acid[3]	Yes
LPC 26:0/unknown interferent[6]	Yes
3-HGA/2-HGA[15]	Yes
Leu/Ile/aIle/hydroxyproline[1]	Yes
GA/MSA	No
EMA/(GA and MSA)	Yes
GUAC/unknown interferent[12]	Unknown interferent not observed
Isovalerylcarnitine/pivaloylcarnitine/2-methylbutyrylcarnitine[4]	No
21-Deoxycortisol/11-deoxycortisol[16]/17-deoxycortisol	No
17-OHP/deoxycorticosterone[7]	No

Superscript numbers are references where interferents are documented

Supplemental Table 8 Part 1. Biomarker recovery and accuracy

Compound	Matrix	Enrichment level	Accuracy (%)	Recovery (%)	Accuracy corrected recovery (%)
CRN	Level 1 DBS*	Low	103		
		Medium	104		
		High	100	78	78
	MSUD-PKU DBS QC Lot# A2013	Low	102		
		Medium	104		
		High	101		
Val	Level 1 DBS*	Low	103		
		Medium	92		
		High	93	62	69
	MSUD-PKU DBS QC Lot# A2013	Low	92		
		Medium	92		
		High	91		
t-Hcy	Level 1 DBS*	Low	100		
		Medium	99		
		High	99	26	27
	MSUD-PKU DBS QC Lot# A2013	Low	97		
		Medium	97		
		High	98		
GUAC	Level 1 DBS*	Low	91		
		Medium	93		
		High	93	73	80
	MSUD-PKU DBS QC Lot# A2013	Low	89		
		Medium	94		
		High	93		
Leu	Level 1 DBS*	Low	86		
		Medium	89		
		High	89	64	75
	MSUD-PKU DBS QC Lot# A2013	Low	86		
		Medium	90		
		High	89		
CRE	Level 1 DBS*	Low	101		
		Medium	113		
		High	113	81	68
	MSUD-PKU DBS QC Lot# A2013	Low	110		
		Medium	118		
		High	115		

*DBS made in this study, unenriched

Supplemental Table 8 Part 2. Biomarker recovery and accuracy

Compound	Matrix	Enrichment level	Accuracy (%)	Recovery (%)	Accuracy corrected recovery (%)
MA	Level 1 DBS*	Low	86		
		Medium	85		
		High	84	58	74
	MSUD-PKU DBS QC Lot# A2013	Low	95		
		Medium	90		
		High	85		
MMA	Level 1 DBS*	Low	109		
		Medium	108		
		High	104	71	67
	MSUD-PKU DBS QC Lot# A2013	Low	103		
		Medium	107		
		High	104		
EMA	Level 1 DBS*	Low	87		
		Medium	91		
		High	90	62	72
	MSUD-PKU DBS QC Lot# A2013	Low	89		
		Medium	92		
		High	91		
3-HGA	Level 1 DBS*	Low	77		
		Medium	78		
		High	79	52	73
	MSUD-PKU DBS QC Lot# A2013	Low	80		
		Medium	78		
		High	78		
2-HGA	Level 1 DBS*	Low	80		
		Medium	88		
		High	88	60	72
	MSUD-PKU DBS QC Lot# A2013	Low	86		
		Medium	90		
		High	88		
OA	Level 1 DBS*	Low	93		
		Medium	94		
		High	93	70	77
	MSUD-PKU DBS QC Lot# A2013	Low	95		
		Medium	94		
		High	93		

*DBS made in this study, unenriched

Supplemental Table 8 Part 3. Biomarker recovery and accuracy

Compound	Matrix	Enrichment level	Accuracy (%)	Recovery (%)	Accuracy corrected recovery (%)
MCA	Level 1 DBS*	Low	89		
		Medium	91		
		High	92	60	68
	MSUD-PKU DBS QC Lot# A2013	Low	90		
		Medium	91		
		High	92		
LPC 20:0	Level 1 DBS*	Low	103		
		Medium	105		
		High	106	92	86
	MSUD-PKU DBS QC Lot# A2013	Low	103		
		Medium	105		
		High	106		
LPC 22:0	Level 1 DBS*	Low	91		
		Medium	106		
		High	101	105	104
	MSUD-PKU DBS QC Lot# A2013	Low	93		
		Medium	108		
		High	102		
LPC 24:0	Level 1 DBS*	Low	86		
		Medium	105		
		High	104	110	106
	MSUD-PKU DBS QC Lot# A2013	Low	88		
		Medium	105		
		High	104		
LPC 26:0	Level 1 DBS*	Low	86		
		Medium	110		
		High	104	106	102
	MSUD-PKU DBS QC Lot# A2013	Low	87		
		Medium	112		
		High	105		
Ile	Level 1 DBS*	Low	97		
		Medium	100		
		High	98	75	77
	MSUD-PKU DBS QC Lot# A2013	Low	98		
		Medium	101		
		High	99		
aIle	Level 1 DBS*	Low	105		
		Medium	101		
		High	107	66	59
	MSUD-PKU DBS QC Lot# A2013	Low	102		
		Medium	100		

		High	103		

*DBS made in this study, unenriched

Supplemental Table 9 Part 1. Biomarker imprecision and
reproducibility results broken down by enrichment level

Compound	Level	Total Relative Standard Deviation (%)	Reproducibility (%)
CRN	4	8	7
	7	9	9
	10	10	10
Val	4	16	9
	7	15	11
	10	15	13
t-Hcy	4	19	12
	7	20	18
	10	22	18
GUAC	4	6	0
	7	6	3
	10	7	4
Leu	4	6	2
	7	5	2
	10	6	3
CRE	4	6	3
	7	6	5
	10	7	7
MA	4	10	6
	7	5	1
	10	6	3
MMA	4	10	2
	7	8	2
	10	8	5
EMA	4	7	0
	7	5	2
	10	6	2
3-HGA	4	15	1
	7	7	0
	10	7	4
2-HGA	4	5	2
	7	5	2
	10	6	3
OA	4	6	1
	7	5	3
	10	6	3
MCA	4	6	0
	7	5	3

	10	6	5
LPC 20:0	4	9	2
	7	7	0
	10	9	4

Supplemental Table 9 Part 2. Biomarker imprecision and
reproducibility results broken down by enrichment level

Compound	Level	Total Relative Standard Deviation (%)	Reproducibility (%)
LPC 22:0	4	8	1
	7	6	3
	10	8	6
LPC 24:0	4	8	1
	7	9	6
	10	10	6
LPC 26:0	4	16	15
	7	13	6
	10	12	1
Ile	4	11	7
	7	11	3
	10	10	2
aIle	4	12	9
	7	11	7
	10	11	4

Supplemental Table 10 Part 1. Ruggedness with biomarker concentrations over different treatments and limits

Condition	Level (DBS)	CRE (μM)	GUAC (μM)	CRN (μM)	OA (μM)	3-HGA (μM)	2-HGA (μM)	LPC 20:0 (μM)	LPC 22:0 (μM)	LPC 24:0 (μM)	LPC 26:0 (μM)
FA +10%	4	315	1.03	22.4	0.859	0.268	14.8	0.113	0.0986	0.117	0.08
FA +10%	10	669	10.3	127	23.3	5.23	44.9	1.33	1.58	1.72	1.48
FA -10%	4	300	0.99	18.8	0.849	0.404	14.1	0.106	0.0888	0.112	0.08
FA -10%	10	732	11.1	114	25.3	6.2	48.5	1.41	1.7	1.86	1.55
AmFA +10%	4	252	0.894	19	0.765	0.458	12.6	0.0907	0.082	0.091	0.0733
AmFA +10%	10	617	9.95	112	23.4	5.64	43.7	1.32	1.64	1.71	1.61
AmFA -10%	4	292	0.994	18.5	0.882	0.193	14.2	0.102	0.0907	0.104	0.082
AmFA -10%	10	607	9.87	104	23.3	4.91	43.7	1.25	1.55	1.59	1.54
DTT -10%	4	289	0.995	21.4	0.839	0.285	13.6	0.105	0.0813	0.108	0.0783
DTT -10%	10	644	10.1	125	22.6	5.41	43.2	1.44	1.66	1.88	1.67
DTT +10%	4	268	0.902	19.8	0.772	0.259	12.4	0.1	0.078	0.102	0.0723
DTT +10%	10	672	10.6	131	23.8	5.74	46.1	1.4	1.65	1.88	1.58
Extraction Temperature 30°C	4	295	0.998	24.5	0.844	0.283	13.8	0.118	0.088	0.123	0.083
Extraction Temperature 30°C	10	670	10.6	133	24.1	5.63	45	1.47	1.63	1.78	1.6
Column Temperature -10%	4	296	0.979	20.6	0.797	0.332	13.8	0.104	0.089	0.109	0.0836
Column Temperature -10%	10	685	10.7	127	24.5	5.93	45.9	1.46	1.77	1.92	1.61
Column Temperature +10%	4	287	0.949	18.1	0.791	0.339	13.4	0.103	0.0895	0.106	0.08
Column Temperature +10%	10	668	10.1	112	23.4	5.6	44.6	1.33	1.59	1.7	1.46
Lower limit*	4	234	0.787	16.1	0.676	0.181	11.2	0.0786	0.0625	0.0763	0.0403
Upper limit^	4	326	1.13	26.3	0.966	0.465	15.5	0.135	0.0997	0.127	0.11
Lower limit*	10	549	8.84	93.2	20.4	4.69	38.8	1.08	1.26	1.22	0.973
Upper limit^	10	816	13.1	174	29.6	7.04	55.3	1.87	2.1	2.3	2.13

*(Imprecision mean - 3*SD)

^(Imprecision mean + 3*SD)

Items in **bold** are outside the specified limits.

Supplemental Table 10 Part 2. Ruggedness with biomarker concentrations over different treatments and limits

Condition	Level (DBS)	MMA (μM)	EMA (μM)	MA (μM)	MCA (μM)	aIle (μM)	Ile (μM)	Leu (μM)	Val (μM)	t-Hcy (μM)
FA +10%	4	**1.93**	1.34	6.25	0.537	**1.12**	**43.1**	61	70.4	3.31
FA +10%	10	26	21.4	44.9	10.5	7.35	439	357	346	17.9
FA -10%	4	1.53	1.17	6.12	0.531	0.856	35.5	58.1	67.4	3.34
FA -10%	10	28	23.1	49.1	11.6	6.69	420	385	371	20
AmFA +10%	4	1.33	1.05	4.52	0.477	0.911	35.2	53.1	74.3	3.4
AmFA +10%	10	26	21.1	42.3	10.4	6.42	401	350	407	18.6
AmFA -10%	4	1.57	1.24	4.56	0.499	**1.24**	41.1	60	67.4	4.11
AmFA -10%	10	25.8	21	41.1	9.92	**8.05**	417	352	325	21.5
DTT -10%	4	1.47	1.15	4.48	0.506	0.699	32.9	56	90	3.3
DTT -10%	10	24.5	21.1	42.8	10.5	5.28	357	344	466	17.9
DTT +10%	4	1.38	1.07	4.29	0.468	0.662	30.4	52	77.2	3.46
DTT +10%	10	26.2	21.8	45.4	10.7	5.26	376	359	482	19.7
Extraction Temperature 30°C	4	1.42	1.16	4.81	0.506	0.773	29.9	56.4	85.6	4.01
Extraction Temperature 30°C	10	24.7	21	43.3	10.6	4.87	334	350	475	21.2
Column Temperature -10%	4	1.53	1.13	5.54	0.525	0.91	33.8	57.1	61.4	3.61
Column Temperature -10%	10	26	21.8	46.4	11	6.84	389	365	318	21.5
Column Temperature +10%	4	1.78	1.19	5.61	0.49	0.934	35.9	55.4	80	3.21
Column Temperature +10%	10	25.2	21.2	44.6	10.5	6.49	406	355	424	19.6
Lower limit*	4	0.99	0.895	3.86	0.404	0.489	21.2	45.4	36.4	1.61
Upper limit^	4	1.83	1.38	7.33	0.581	1.03	42.6	63.7	102	5.84
Lower limit*	10	19.7	18.4	37.6	8.89	3.88	262	303	216	7.66
Upper limit^	10	32.8	26.3	54.3	13.1	7.52	503	429	565	35.7

*(Imprecision mean - 3*SD)

^(Imprecision mean + 3*SD)

Items in **bold with a gray background** are outside the specified limits.

S21

Supplemental Table 11 Part 1. Stability DBS concentration after different treatments with upper and lower limits

Treatment	Compound	Level (DBS)	Mean (μM)	SD (μM)	Upper limit (imprecision mean + 3 x SD) (μM)	Lower limit (imprecision mean - 3 x SD) (μM)
	CRN	4	24.4	0.293	26.3	16.1
		10	138	1.99	174	93.2
	Val	4	67.6	0.114	102	36.4
		10	331	10.6	565	216
	t-Hcy	4	4.25	0.0928	5.84	1.61
		10	22.8	0.809	35.7	7.66
	GUAC	4	1.03	0.0216	1.13	0.787
		10	10.9	0.442	13.1	8.84
	Leu	4	57.6	0.350	63.7	45.4
		10	354	6.08	429	303
	CRE	4	312	4.89	326	234
		10	668	12.6	816	549
	MA	4	5.69	0.101	7.33	3.86
		10	43.5	1.18	54.3	37.6
	MMA	4	1.38	0.0452	1.83	0.99
		10	24.2	0.648	32.8	19.7
	EMA	4	1.18	0.0173	1.38	0.895
		10	21.3	0.455	26.3	18.4
Extract stored for 24 hr at 4°C	3-HGA	4	0.368	0.0386	0.465	0.181
		10	5.72	0.127	7.04	4.69
	2-HGA	4	14.3	0.200	15.5	11.2
		10	45.1	1.48	55.3	38.8
	OA	4	0.918	0.0214	0.966	0.676
		10	24.8	0.759	29.6	20.4
	MCA	4	0.531	0.0116	0.581	0.404
		10	10.6	0.321	13.1	8.89
	LPC C20:0	4	0.124	0.00638	0.135	0.0786
		10	1.56	0.0463	1.87	1.08
	LPC C22:0	4	0.0917	0.0048	0.0997	0.0625
		10	1.73	0.0709	2.1	1.26
	LPC C24:0	4	0.113	0.00616	0.127	0.0763
		10	1.74	0.0930	2.3	1.22
	LPC C26:0	4	0.0913	0.00967	0.11	0.0403
		10	1.53	0.0939	2.13	0.973
	Ile	4	29.9	0.453	42.6	21.2
		10	326	8.96	503	262
	aIle	4	0.715	0.0204	1.03	0.489
		10	4.81	0.176	7.52	3.88

Items in **bold with a gray background** are outside the specified limits.

Supplemental Table 11 Part 2. Stability DBS concentration after different treatments with upper and lower limits

Treatment	Compound	Level (DBS)	Mean (µM)	SD (µM)	Upper limit (imprecision mean + 3 x SD) (µM)	Lower limit (imprecision mean – 3 x SD) (µM)
Extract stored for 24 hr at RT	CRN	4	**30.7**	0.628	26.3	16.1
		10	157	1.95	174	93.2
	Val	4	64.6	2.52	102	36.4
		10	360	8.82	565	216
	t-Hcy	4	4.93	0.0763	5.84	1.61
		10	25.8	0.293	35.7	7.66
	GUAC	4	1.06	0.0276	1.13	0.787
		10	11.2	0.246	13.1	8.84
	Leu	4	58.3	1.30	63.7	45.4
		10	367	5.17	429	303
	CRE	4	312	4.34	326	234
		10	706	9.45	816	549
	MA	4	5.76	0.135	7.33	3.86
		10	45.2	0.540	54.3	37.6
	MMA	4	1.34	0.0164	1.83	0.99
		10	25.1	0.600	32.8	19.7
	EMA	4	1.19	0.0430	1.38	0.895
		10	22	0.311	26.3	18.4
	3-HGA	4	0.363	0.0118	0.465	0.181
		10	5.94	0.214	7.04	4.69
	2-HGA	4	14.6	0.198	15.5	11.2
		10	47.3	1.23	55.3	38.8
	OA	4	0.892	0.0299	0.966	0.676
		10	25.7	0.705	29.6	20.4
	MCA	4	0.524	0.0147	0.581	0.404
		10	11	0.172	13.1	8.89
	LPC C20:0	4	0.127	0.00471	0.135	0.0786
		10	1.65	0.0609	1.87	1.08
	LPC C22:0	4	0.0943	0.00263	0.0997	0.0625
		10	1.84	0.0981	2.1	1.26
	LPC C24:0	4	**0.128**	0.0151	0.127	0.0763
		10	1.95	0.0860	2.3	1.22
	LPC C26:0	4	0.0977	0.0193	0.11	0.0403
		10	1.71	0.0439	2.13	0.973
	Ile	4	30.1	0.332	42.6	21.2
		10	341	6.01	503	262
	aIle	4	0.71	0.0188	1.03	0.489
		10	4.89	0.144	7.52	3.88

Items in **bold with a gray background** are outside the specified limits.

Supplemental Table 11 Part 3. Stability DBS concentration after different treatments with upper and lower limits

Treatment	Compound	Level (DBS)	Mean (μM)	SD (μM)	Upper limit (imprecision mean + 3 x SD) (μM)	Lower limit (imprecision mean – 3 x SD) (μM)
Spot stored for 24 hr at RT	CRN	4	24.2	0.129	26.3	16.1
		10	143	2.25	174	93.2
	Val	4	67	2.90	102	36.4
		10	335	6.11	565	216
	t-Hcy	4	3.9	0.0822	5.84	1.61
		10	21.8	0.290	35.7	7.66
	GUAC	4	1.05	0.0196	1.13	0.787
		10	11	0.133	13.1	8.84
	Leu	4	58.4	0.677	63.7	45.4
		10	361	6.76	429	303
	CRE	4	312	4.98	326	234
		10	696	11.2	816	549
	MA	4	5.88	0.113	7.33	3.86
		10	45.9	0.642	54.3	37.6
	MMA	4	1.36	0.0301	1.83	0.99
		10	24.7	0.616	32.8	19.7
	EMA	4	1.22	0.0209	1.38	0.895
		10	22.1	0.602	26.3	18.4
	3-HGA	4	0.341	0.00497	0.465	0.181
		10	5.86	0.171	7.04	4.69
	2-HGA	4	14.6	0.267	15.5	11.2
		10	47	1.15	55.3	38.8
	OA	4	0.907	0.0281	0.966	0.676
		10	25.1	0.456	29.6	20.4
	MCA	4	0.533	0.0110	0.581	0.404
		10	10.7	0.264	13.1	8.89
	LPC 20:0	4	0.122	0.00236	0.135	0.0786
		10	1.57	0.0186	1.87	1.08
	LPC 22:0	4	0.0947	0.00330	0.0997	0.0625
		10	1.77	0.0503	2.1	1.26
	LPC 24:0	4	0.115	0.00492	0.127	0.0763
		10	1.86	0.0565	2.3	1.22
	LPC 26:0	4	0.081	0.00356	0.11	0.0403
		10	1.61	0.0120	2.13	0.973
	Ile	4	29.1	0.612	42.6	21.2
		10	320	7.62	503	262
	aIle	4	0.681	0.0271	1.03	0.489

		10	5.02	0.162	7.52	3.88

Items in **bold with a gray background** are outside the specified limits.

Supplemental Table 11 Part 4. Stability DBS concentration after different treatments with upper and lower limits

Treatment	Compound	Level (DBS)	Mean (μM)	SD (μM)	Upper limit (imprecision mean + 3 x SD) (μM)	Lower limit (imprecision mean – 3 x SD) (μM)
DBS exposed to 3x freeze thaw cycles	CRN	4	22.6	0.726	26.3	16.1
		10	139	5.81	174	93.2
	Val	4	65.1	4.88	102	36.4
		10	330	4.74	565	216
	t-Hcy	4	4.05	0.0977	5.84	1.61
		10	22	0.293	35.7	7.66
	GUAC	4	1.01	0.0199	1.13	0.787
		10	10.6	0.373	13.1	8.84
	Leu	4	55.8	2.62	63.7	45.4
		10	352	10.1	429	303
	CRE	4	296	10.6	326	234
		10	681	24.4	816	549
	MA	4	5.6	0.220	7.33	3.86
		10	44.9	1.43	54.3	37.6
	MMA	4	1.28	0.0800	1.83	0.99
		10	24.3	0.980	32.8	19.7
	EMA	4	1.14	0.0289	1.38	0.895
		10	21.7	0.719	26.3	18.4
	3-HGA	4	0.32	0.0236	0.465	0.181
		10	5.78	0.167	7.04	4.69
	2-HGA	4	13.9	0.681	15.5	11.2
		10	45.9	1.60	55.3	38.8
	OA	4	0.884	0.0371	0.966	0.676
		10	24.7	0.691	29.6	20.4
	MCA	4	0.51	0.0200	0.581	0.404
		10	10.6	0.302	13.1	8.89
	LPC 20:0	4	0.118	0.00283	0.135	0.0786
		10	1.5	0.0421	1.87	1.08
	LPC 22:0	4	0.0953	0.00419	0.0997	0.0625
		10	1.67	0.0515	2.1	1.26
	LPC 24:0	4	0.108	0.00309	0.127	0.0763
		10	1.72	0.0875	2.3	1.22
	LPC 26:0	4	0.078	0.00327	0.11	0.0403
		10	1.56	0.152	2.13	0.973
	Ile	4	28.1	1.68	42.6	21.2
		10	314	11.4	503	262

		4	0.695	0.0189	1.03	0.489
	aIle	10	4.96	0.245	7.52	3.88

Items in **bold with a gray background** are outside the specified limits.

REFERENCES

1.   Oglesbee, D.; Sanders, K. A.; Lacey, J. M.; Magera, M. J.; Casetta, B.; Strauss, K. A.; Tortorelli, S.; Rinaldo, P.; Matern, D., Second-tier test for quantification of alloisoleucine and branched-chain amino acids in dried blood spots to improve newborn screening for maple syrup urine disease (MSUD). *Clin Chem* **2008,** *54* (3), 542-9.

2.   Okun, J. G.; Gan-Schreier, H.; Ben-Omran, T.; Schmidt, K. V.; Fang-Hoffmann, J.; Gramer, G.; Abdoh, G.; Shahbeck, N.; Al Rifai, H.; Al Khal, A. L.; Haege, G.; Chiang, C.-C.; Kasper, D. C.; Wilcken, B.; Burgard, P.; Hoffmann, G. F., Newborn Screening for Vitamin B(6) Non-responsive Classical Homocystinuria: Systematical Evaluation of a Two-Tier Strategy. *JIMD Rep* **2017,** *32,* 87-94.

3.   Turgeon, C. T.; Magera, M. J.; Cuthbert, C. D.; Loken, P. R.; Gavrilov, D. K.; Tortorelli, S.; Raymond, K. M.; Oglesbee, D.; Rinaldo, P.; Matern, D., Determination of Total Homocysteine, Methylmalonic Acid, and 2-Methylcitric Acid in Dried Blood Spots by Tandem Mass Spectrometry. *Clinical Chemistry* **2010,** *56* (11), 1686-1695.

4.   Janzen, N.; Steuerwald, U.; Sander, S.; Terhardt, M.; Peter, M.; Sander, J., UPLC-MS/MS analysis of C5-acylcarnitines in dried blood spots. *Clinica chimica acta; international journal of clinical chemistry* **2013,** *421,* 41-5.

5.   Al-Dirbashi, O. Y.; Kölker, S.; Ng, D.; Fisher, L.; Rupar, T.; Lepage, N.; Rashed, M. S.; Santa, T.; Goodman, S. I.; Geraghty, M. T.; Zschocke, J.; Christensen, E.; Hoffmann, G. F.; Chakraborty, P., Diagnosis of glutaric aciduria type 1 by measuring 3-hydroxyglutaric acid in dried urine spots by liquid chromatography tandem mass spectrometry. *J Inherit Metab Dis* **2011,** *34* (1), 173-80.

6.   Haynes, C. A.; De Jesús, V. R., Improved analysis of C26:0-lysophosphatidylcholine in dried-blood spots via negative ion mode HPLC-ESI-MS/MS for X-linked adrenoleukodystrophy newborn screening. *Clinica Chimica Acta* **2012,** *413* (15), 1217-1221.

7.   Janzen, N.; Peter, M.; Sander, S.; Steuerwald, U.; Terhardt, M.; Holtkamp, U.; Sander, J., Newborn Screening for Congenital Adrenal Hyperplasia: Additional Steroid Profile using Liquid Chromatography-Tandem Mass Spectrometry. *The Journal of Clinical Endocrinology & Metabolism* **2007,** *92* (7), 2581-2589.

8.   Tortorelli, S.; Eckerman, J. S.; Orsini, J. J.; Stevens, C.; Hart, J.; Hall, P. L.; Alexander, J. J.; Gavrilov, D.; Oglesbee, D.; Raymond, K.; Matern, D.; Rinaldo, P., Moonlighting newborn screening markers: the incidental discovery of a second-tier test for Pompe disease. *Genetics in Medicine* **2018,** *20* (8), 840-846.

9.   Ramaswamy, M.; Skrinska, V. A.; Abdoh, G.; Mahmoud Ahmed, L.; Mitri, R. F.; Joshi, R., A Rare Case of Malonic Aciduria Diagnosed by Newborn Screening in Qatar. *Int. J. Neonatal Screen.* **2017,** *3* (1), 5.

10.   Test ID: HGEM Hydroxyglutaric Acids, Glutaric Acid, Ethylmalonic Acid, and Methylsuccinic Acid, Blood Spot. https://www.mayocliniclabs.com/test-catalog/Clinical+and+Interpretive/62230 (accessed 4/23/2020).

11.   Gallant, N. M.; Leydiker, K.; Tang, H.; Feuchtbaum, L.; Lorey, F.; Puckett, R.; Deignan, J. L.; Neidich, J.; Dorrani, N.; Chang, E.; Barshop, B. A.; Cederbaum, S. D.; Abdenur, J. E.; Wang, R. Y., Biochemical, molecular, and clinical characteristics of children with short chain acyl-CoA dehydrogenase deficiency detected by newborn screening in California. *Molecular Genetics and Metabolism* **2012,** *106* (1), 55-61.

12.   Sinclair, G. B.; van Karnebeek, C. D. M.; Ester, M.; Boyd, F.; Nelson, T.; Stockler-Ipsiroglu, S.; Vallance, H., A three-tier algorithm for guanidinoacetate methyltransferase (GAMT) deficiency newborn screening. *Molecular Genetics and Metabolism* **2016,** *118* (3), 173-177.

13.   D'Apolito, O.; Garofalo, D.; Paglia, G.; Zuppaldi, A.; Corso, G., Orotic acid quantification in dried blood spots and biological fluids by hydrophilic interaction liquid chromatography tandem mass spectrometry. *Journal of separation science* **2010,** *33* (6-7), 966-73.

14.   Chuang, W. L.; Pacheco, J.; Zhang, X. K.; Martin, M. M.; Biski, C. K.; Keutzer, J. M.; Wenger, D. A.; Caggana, M.; Orsini, J. J., Jr., Determination of psychosine concentration in dried blood spots from newborns that were identified via newborn screening to be at risk for Krabbe disease. *Clinica chimica acta; international journal of clinical chemistry* **2013,** *419,* 73-6.

15.   Baric, I.; Wagner, L.; Feyh, P.; Liesert, M.; Buckel, W.; Hoffmann, G. F., Sensitivity and specificity of free and total glutaric acid and 3-hydroxyglutaric acid measurements by stable-isotope dilution assays for the diagnosis of glutaric aciduria type I. *J Inherit Metab Dis* **1999,** *22* (8), 867-81.

16.   Janzen, N.; Sander, S.; Terhardt, M.; Steuerwald, U.; Peter, M.; Das, A. M.; Sander, J., Rapid steroid hormone quantification for congenital adrenal hyperplasia (CAH) in dried blood spots using UPLC liquid chromatography–tandem mass spectrometry. *Steroids* **2011,** *76* (13), 1437-1442.