La Frano Metabolomics Analysis

Materials & Methods

Metabolomics assays for primary metabolomics, aminomics, and lipidomics were performed on plasma using protein precipitation extraction with ultra-performance liquid chromatography tandem quadrupole mass spectrometry (UPLC-MS), as previously described (1,2). For each sample, 25 µL of plasma was added to a 1.5 mL tube before the addition of 10 µL of 1 µM internal standard solution, followed by 750 µL or 500 uL chilled methanol. Samples were then vortexed 30 seconds prior to being centrifuged at 15,000 x G for 10 min at 4°C. The supernatant was transferred to 1.5 mL high performance liquid chromatography (HPLC) amber glass vials, dried by centrifugal vacuum evaporation, and reconstituted in 100 µL 3:1 acetonitrile:methanol solution with the internal standard 1-cyclohexyl ureido, 3-dodecanoic acid (CUDA) at 100 nM. The reconstituted solution was vortexed 30 seconds and placed on ice for 10 minutes. The solution was then centrifuged at 10,000 x G for 3 minutes at room temperature after being transferred to microfilter tubes. The supernatant was then transferred to a HPLC vial to be analyzed using the UPLC-MS.

All UPLC-MS analyses, including primary metabolomics, aminomics, and lipidomics, were conducted on a Waters Acquity I-Class UPLC (Waters, Milford, MA, USA) coupled with an API 4000 QTrap (Sciex, Framingham, MA) and quantified with AB Sciex MultiQuant version 3.0. Primary metabolomics and aminomics used multiple reaction monitoring (MRM) while the lipidomics assay used full scan MS over m/z 400-1000 using Q1 scans at unit mass resolution and specific lipid species were identified using a range to capture the full width of the monoisotopic ion, as previously described (2). For the primary metabolomics assay, metabolites were separated using a 150 X 2.0 mm Luna NH2 column (Phenomenex, Torrance, CA) and detected by negative ion mode electrospray ionization. For the aminomics assay, metabolites were separated using a 150 × 2.1 mm Atlantis HILIC column (Waters) and detected by positive ion mode electrospray ionization. For the lipidomics assay, metabolites were separated using a 150 × 3.0 mm Prosphere HP C4 column (Grace, Columbia, MD, USA) and detected by positive ion mode electrospray ionization. Gradient, mobile phase, and instrument parameters for the UPLC are listed in Tables 1, 2, and 3. MRMs and other parameters for the MS are listed in Tables 4, 5, and 6.

Primary metabolomics and aminomics assay metabolite identities were confirmed using pure standards in order to establish retention time and MRM, as well as optimize instrument parameters. Standards included those within the Mass Spectrometry Metabolite Library of Standards (MSMLS; Sigma-Aldrich, St. Louis, MO, USA), as well as individually purchased standards from Sigma-Aldrich, Cambridge Isotope Laboratories, Inc (Tewksbury, MA, USA), and Cerilliant Corporation (Round Rock, TX). Some acylcarnitine species were identified based on MRM only, as noted in Table 5. For the lipidomics assay, the SPLASH® LIPIDOMIX® Mass Spec Standards purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) were utilized to identify select lipid species retention times in order to establish retention time indexes that adjust for retention time differences in specific lipid species between our method and those provided by Townsend et al. (2). Purified egg yolk extracts (99-97% purity by TLC; Sigma-Aldrich) of each targeted lipid class, consisting of a variety of species, were analyzed in order to confirm approximate retention time ranges for phosphatidylcholines, phosphatidylethanolamines, lysophosphatidylcholines,

lysophosphatidylethanolamines, and sphingomyelins. Surrogate standards used in the primary metabolomics assay included succinate-$^{13}C_4$, sorbitol-1,1,2,3,4,5,6,6-d8, octanoate-$^{13}C_8$, adenine-2-d1, and histamine-α,α,β,β-d4 while the aminomics assay used L-tryptophan-$^{13}C_{11}$, adenine-2-d1, 2-(3,4-Dihydroxyphenyl)ethyl-1,1,2,2-d4-amine, and histamine-α,α,β,β-d4 were utilized to monitor extraction efficiency and recovery percentage for each sample analyzed. Surrogates were purchased from Santa Cruz Biotechnology, Inc, (Dallas, TX, USA), CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada), and Cambridge Isotope Laboratories, Inc. The internal standard CUDA (Sigma Aldrich), included in the reconstitution solvent that was added post-extraction, was used for controlling instrument and injection parameters. All primary metabolomics and aminomics raw data were normalized to the internal standard CUDA (Sigma Aldrich). The lipidomics assay raw data were normalized to the mTIC.

For quality control purposes, compounds whose background (as determined by method blank response) was greater than 50% of the average sample response or that had more than 1/3 of samples with signal to noise less than 3:1, were excluded from the dataset. To assess reproducibility, five replicates of the current study samples were separately extracted and analyzed. A pooled plasma sample collected from a different study was used as a long-term reference QC sample for an inter-study assessment of data. All samples were run in a single batch.

Reference

1. La Frano MR, Brito A, Johnson C, Wilhelmson B, Gannon B, Fanter R, Pedersen TL, Tanumihardjo SA, Newman JW. Metabolomics Reveals Altered Hepatic Bile Acids, Gut Microbiome Metabolites, and Cell Membrane Lipids Associated with Marginal Vitamin A Deficiency in a Mongolian Gerbil Model. Mol Nutr Food Res. 2020. 64:1901319. doi: 10.1002/mnfr.201901319.

2. Townsend MK, Clish CB, Kraft P, Wu C, Souza AL, Deik AA, Tworoger SS, Wolpin BM. Reproducibility of metabolomic profiles among men and women in 2 large cohort studies. Clin Chem. 2013. Nov;59(11):1657-67. doi: 10.1373/clinchem.2012.199133.

Table 1. Primary Metabolomics Assay UPLC parameters

Time (min)	A%	B%
0	10	90
10	95	5
11	95	5
13	10	90
15	10	90

Solvent A = 20 mM ammonium acetate, 20 mM ammonium hydroxide in water
Solvent B = 10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol

Flow Rate = 0.3 mL/min
Column = 150 x 2.0 mm Luna NH2, 3 um, 100 angstrom (Phenomenex, Torrance, CA)
Column Temp = 30 °C

Table 2. Aminomics Assay UPLC parameters

Time (min)	A%	B%
0	5	95
1	5	95
11	60	40
13	5	95
15	9	95

Solvent A = 10 mM ammonium formate, 0.1% formic acid in water
Solvent B = 0.1% formic acid in acetonitrile
Flow Rate = 0.25 mL/min
Column = 150 x 2.1 mm Atlantis HILIC, 3 um, 100 angstrom (Waters, Milford, MA)
Column Temp = 30°C

Table 3. Lipidomics Assay UPLC parameters

Time (min)	A%	B%
0	80	20
2	80	20
3	20	80
7	0	100
10	0	100
11	80	20
15	80	20

Solvent A = 95:5:0.1 v/v/v 10 mM ammonium acetate in water/methanol/acetic acid
Solvent B = 99.9:0.1 v/v methanol/acetic acid
Flow Rate = 0.35 mL/min
Column = 150 x 3.0 mm Prosphere HP C4, 3 um, 300 angstrom (Grace, Columbia, MD)
Column Temp = 30 °C

Table 4. Primary Metabolomics UPLC/ESI QTRAP Analyte and Instrument-specific Parameters

Metabolite	Parent	Daughter	Declustering Potential	Cell Exit Potential	Collision Energy	Curtain Gas	Dwell Time
2-aminoadipate	160.1	116	-40	-7	-15	-19	5
2-hydroxyglutarate	147.1	57	-40	-9	-25	-9	5
3-methyladipate_pimelate	159.4	115	-60	-15	-20	-7	5
4-pyridoxate	182.1	138	-40	-11	-20	-11	5
5-methyltetrahydrofolic_acid_(5-methyl-THF)	458.2	329	-80	-9	-35	-9	5
6-phosphogluconate	275.4	96.9	-140	-1	-110	-29	5
aconitate	173	129	-20	-5	-20	-21	5
adenine	134.1	107	-80	-15	-25	-9	5
adenosine_diphosphate_(ADP)	426	79	-40	-13	-110	-5	5
adenosine_monophosphate_(AMP)	346.1	79	-40	-11	-90	-13	5
adipate	145.1	101	-60	-9	-15	-17	5
alpha-glycerophosphate	171	79	-60	-15	-65	-1	5
alpha-hydroxybutyrate	103.1	57.2	-20	-7	-20	-3	5
alpha-ketoglutarate	145.1	101	-60	-15	-15	-9	5
beta-hydroxybutyrate	103.4	59.1	0	-3	-15	-13	5
citrate	191	87	-40	-7	-30	-5	5
cystathionine	221.1	134	-40	-11	-20	-7	5
cytidine	242.1	109	-60	-15	-30	-5	5
cytidine_diphosphate_(CDP)	402	158.9	-40	-5	-30	-19	5
cytidine_monophosphate_(CMP)	322.1	79	-40	-3	-65	-11	5
dihydroxyacetone_phosphate_(DHAP)	169	97	-100	-13	-20	-13	5
flavin_mononucleotide_(FMN)	455.7	97.2	-300	-1	-45	-3	5
folate	440.1	311	-60	-7	-15	-5	5
fructose_glucose_galactose	179.1	89	-20	-11	-20	-7	5
fumarate_maleate	115	71	-60	-15	-10	-9	5

gentisate	153	108	-300	-1	-25	0	5
glucuronate	193	113	-80	-11	-25	-5	5
glutathione_oxidized	611.2	306	-80	-11	-40	-19	5
glyceraldehyde-3-phosphate	169.3	97	-360	-7	-45	-35	5
glycocholate	464.3	74	-60	-15	-80	-11	5
SUM_glycodeoxycholate_glycochenodeoxycholate	448.3	74	-60	-9	-65	-5	5
guanine	150	133	-60	-11	-20	-1	5
guanosine	282.1	150.1	-60	-13	-30	-7	5
guanosine_diphosphate_(GDP)	442	79	-40	-9	-60	-1	5
guanosine_monophosphate_(GMP)	362.1	79	-40	-5	-70	-5	5
SUM_hexose_diphosphates_(F16DP_F26DP_G16DP)	339	79	-80	-9	-75	-13	5
SUM_hexose_monophosphates_(F1P_F6P_G1P_G6P)	259	97	-40	-9	-35	-3	5
hippurate	178.1	134	-20	-7	-15	-7	5
homocystine	267.1	132	-40	-1	-55	-29	5
hydroxyphenylacetate	151.1	107	-20	-7	-20	-5	5
hypoxanthine	135	92	-60	-15	-20	-3	5
indole-3-propionate	188.1	116	-60	-13	-20	-23	5
indoleacetate	174.1	130	-40	-13	-20	-7	5
indoxylsulfate	212	80	0	-1	-125	-1	5
inosine	267.1	135	-60	-13	-25	-5	5
inosine_monophosphate_(IMP)	347	79	-60	-13	-80	-5	5
inositol	179.1	81	-40	-5	-25	-3	5
isocitrate	191	73	-20	-7	-30	-11	5
kynurenine	207.1	144	-20	-5	-30	-15	5
lactate	89	43	-40	-7	-20	-5	5
lactose	341.1	161	-80	-15	-20	-9	5
malate	133.1	73.9	-20	-3	-15	-11	5
methylmalonate	117	73	-20	-9	-20	-5	5
nicotinamide_adenine_dinucleotide_phosphate_(NADP)	742.1	620	-400	-1	-35	-15	5
oxalate	89	61	-20	-5	-10	-1	5
pantothenate	218.1	88	-60	-9	-20	0	5

phosphocreatine	210.1	79.1	-180	-7	-10	-27	5
phosphoenolpyruvate_(PEP)	167	79	-200	-1	-30	-3	5
phosphoglycerate	185	97	-280	-7	-60	-11	5
phosphotyrosine	260	79	-40	-13	-45	-5	5
pyruvate	87	43	-80	-9	-10	-7	5
quinolinate	166	122	-40	-13	-15	-9	5
SUM_ribose-5-phosphate_ribulose-5-phosphate	229	97	-60	-13	-20	-7	5
salicylurate	194.1	150	-20	-9	-20	-9	5
sebacate	201.2	157.1	-60	-13	-20	-5	5
sorbitol	181.1	89	-60	-15	-20	-3	5
succinate	117	73	-20	-5	-15	-3	5
sucrose	341.1	179	-80	-13	-25	-17	5
taurocholate	514.3	80	-60	-3	-115	-17	5
SUM_taurodeoxycholate_taurochenodeoxycholate	498.3	80	-60	-11	-60	-1	5
SUM_UDP-galactose_UDP-glucose	565.1	323	-40	-9	-30	-7	5
UDP-glucuronate	579	403	-60	-1	-120	-55	5
urate	167	124	-100	-15	-10	-21	5
uridine	243.1	110	-60	-9	-25	-5	5
uridine_5-monophosphate_(UMP)	323	79	-60	-5	-80	-5	5
uridine-5-diphosphate_(UDP)	403	79	-20	-5	-70	-7	5
SUM_ursodeoxy_chenodeoxy_deoxycholate	391.3	391.3	-100	-9	-15	-5	5
vanillylmandelic_acid_(VMA)	197.4	137	-200	-3	-35	-31	5
xanthine	151	108	-40	-13	-25	-5	5
xanthurenate	204	160	-60	-7	-15	0	5
HAS ISO STD 1 (Histamine-α,α,β,β-d4, surrogate internal standard)	214.4	75.8	-25	-10	-15	12	5
SBL inf 3 (Sorbitol-1,1,2,3,4,5,6,6-d8, surrogate internal standard)	189.2	123	-60	-5	-24	12	5
AND ISO STD 3 (Adenine-2-d1, surrogate internal standard)	116.1	99	-25	-10	-25	12	5
OCT inf (Octanoate-13C8, surrogate internal standard)	134.5	107.1	-15	-9	-10	12	5
SCC inf 2 (Succinate-13C4, surrogate internal standard)	134.5	92	-20	-9	-17	12	5

CUDA (Internal standard)	339.2	214.3	-30	-10	-33	12	5

Note: Additional MRMs were screened but are not listed here because they were either being used as qualifier ions, had unoptimized parameters, or had not been validated using this LC method.

Table 5. Aminomics UPLC/ESI QTRAP Analyte and Instrument-specific Parameters

Metabolite	Parent	Daughter	Declustering Potential	Cell Exit Potential	Collision Energy	Curtain Gas	Dwell Time
2-deoxyadenosine	252.1	136.3	40	10	20	10	5
2-deoxycytidine	228.1	112.1	45	10	15	10	5
3-hydroxyanthranilic_acid	154	136.2	30	10	18	10	5
5-adenosylhomocysteine	385.1	136.3	60	10	32	10	5
5-hydroxyindoleacetic_acid_5-HIAA	192.3	146.2	30	10	18	10	5
5-hydroxytryptophan	221.1	204	40	10	18	10	5
acetylcholine	146.1	87	25	10	21	10	5
adenosine	268.5	136.3	36	10	23	10	5
alanine	90	44	25	10	17	10	5
allantoin	159	116	50	10	11	10	5
alpha-glycerophosphocholine	258.3	104.1	75	10	54	10	5
aminoisobutyric_acid	104.1	86	40	10	16	10	5
anthranilic_acid	138	120	25	10	18	10	5
arginine	175.1	70	25	10	32	10	5
asparagine	133.1	74	30	10	23	10	5
betaine	118.1	58	40	10	41	10	5
carnitine	162.1	85	25	10	29	10	5
choline	104.1	60	50	10	27	10	5
cis trans_hydroxyproline	132.1	86.2	50	10	18	10	5
citrulline	176	113.2	35	10	20	10	5
cotinine	177.1	80	60	10	34	10	5
creatine	132.1	90	50	10	17	10	5
creatinine	114.1	44	25	10	28	10	5
cyclic_AMP_cAMP	330.3	136.2	52	10	30	10	5
cytosine	112	95.1	40	10	26	10	5
dimethylglycine	104.1	58	30	10	20	10	5

dimethyl-L-arginine_ADMA	203.1	70.3	50	10	40	10	5
gamma-aminobutyric_acid_GABA	104.1	87	30	10	17	10	5
glucose	163.1	85	25	10	29	10	5
glutamate	148.1	84	25	10	23	10	5
glutamine	147.1	84	25	10	25	10	5
glycerol	93	57	30	10	12	10	5
glycine	76	48	40	10	10	10	5
histamine	112	95	40	10	26	10	5
histidine	156.1	110	25	10	21	10	5
homocysteine	136	90	50	10	20	10	5
isoleucine	132.1	86.2	50	10	18	10	5
kynurenic_acid	190.2	144	40	10	29	10	5
leucine	132.1	86.2	50	10	18	10	5
lysine	147.1	84	25	10	25	10	5
methionine	150.1	61	40	10	31	10	5
N-carbamoyl-beta-alanine	133.1	115	40	10	12	10	5
niacinamide	123	80	30	10	30	10	5
N-monomethyl-L-arginine_NMMA	189.1	70	60	10	40	10	5
ornithine	133.4	70	40	10	30	10	5
phenylalanine	166.1	120.2	50	10	19	10	5
proline	116.1	70	50	10	20	10	5
serine	106	60	25	10	18	10	5
serotonin	177.1	160	50	10	18	10	5
symmetrical_dimethylarginine_SDMA	203.1	70.3	50	10	40	10	5
taurine	126.2	44.1	65	10	31	10	5
thiamine	265.5	122.2	35	10	22	10	5
threonine	120.1	74	25	10	18	10	5
thyroxine	777.8	732	75	10	35	10	5
triiodothyronine	651.9	606.1	60	10	35	10	5
trimethylamine-N-oxide	76.1	42	20	10	50	10	5
tryptophan	205.5	188.3	25	10	16	10	5

tyrosine	182.5	136.1	25	10	19	10	5
valine	118.1	72	25	10	18	10	5
xanthosine	285.1	153	40	10	18	10	5
Acetyl-carnitine	204.2	85	42	10	26	10	5
Propionyl-carnitine	218.1	85	42	10	27	10	5
Isobutyryl-carnitine	232.2	85	42	10	29	10	5
Butyryl-carnitine	232.2	85	42	10	29	10	5
Isovaleryl-carnitine	246.2	85	48	10	30	10	5
Valeryl-carnitine	246.2	85	48	10	30	10	5
Malonyl-carnitine	248.1	85	48	10	28	10	5
3-Hydroxy-isovaleryl-carnitine	262.2	85	50	10	32	10	5
3-Hydroxy-hexanoyl-carnitine*	276.1	85	50	10	32	10	5
Glutaryl-carnitine	276.1	85	43	10	27	10	5
Octenoyl-carnitine*	286.2	85	50	10	30	10	5
Octanoyl-carnitine	288.2	85	50	10	30	10	5
Decenoyl-carnitine*	314.2	85	55	10	38	10	5
Decanoyl-carnitine	316.2	85	55	10	38	10	5
Dodecenoyl-carnitine*	342.3	85	60	10	39	10	5
Lauroyl-carnitine	344.3	85	60	10	39	10	5
Myristoyl-carnitine	372.3	85	65	10	42	10	5
Palmitoleoyl-carnitine*	398.3	85	63	10	45	10	5
Palmitoyl-carnitine	400.3	85	63	10	45	10	5
3-Hydroxy-palmitoleoyl-carnitine*	414.3	85	63	10	45	10	5
Linoleyl-carnitine*	424.3	85	75	10	49	10	5
Oleyl-carnitine*	426.4	85	70	10	50	10	5
Stearoyl-carnitine	428.4	85	70	10	50	10	5
AND ISO STD 4 (Adenine-2-d1, surrogate internal standard)	135	07	25	10	25	10	5
TRY ISO STD 2 (L-Tryptophan-13C11, surrogate internal standard)	216.1	155.1	25	10	20	10	5

DPA pos inf 2 (2-(3,4-Dihydroxyphenyl)ethyl-1,1,2,2-d4-amine, surrogate internal standard)	158.3	123.2	40	23	28	10	5
HAS pos inf 1 (Histamine- α,α,β,β-d4, surrogate internal standard)	115.7	99.2	30	15	25	10	5
CUDA 1 (Internal Standard)	341.3	216.2	30	10	32	10	5

Note: *Denotes metabolites that were not confirmed with authentic standards. Additional MRMs were screened but are not listed here because they were either being used as qualifier ions, had unoptimized parameters, or had not been validated using this LC method.

Table 6. Lipidomics UPLC/ESI QTRAP Analyte and Instrument-specific Parameters

Name	[M+H]+/[M+NH4]+/[M+Na]+*
C32:2 PC	729.9384 - 731.2884
C32:1 PC	731.9540 - 733.3040
C32:0 PC	733.9696 - 735.3196
C34:4 PC	753.9385 - 755.2885
C34:3 PC	755.9541 - 757.3041
C34:2 PC	757.9697 - 759.3197
C34:1 PC	759.9853 - 761.3353
C36:4 PC-A	781.9699 - 783.3199
C36:4 PC-B	781.9699 - 783.3199
C36:3 PC	783.9855 - 785.3355
C36:2 PC	786.0011 - 787.3511
C36:1 PC	788.0167 - 789.3667
C38:6 PC	805.9700 - 807.3200
C38:5 PC	807.9856 - 809.3356
C38:4 PC	810.0012 - 811.3512
C38:3 PC	812.0168 - 813.3668
C38:2 PC	814.0324 - 815.3824
C40:6 PC	834.0013 - 835.3513
C14:0 LPC	467.7086 - 469.0586
C16:1 LPC	493.7243 - 495.0743
C16:0 LPC	495.7399 - 497.0899
C18:2 LPC	519.7400 - 521.0900
C18:1 LPC	521.7556 - 523.1056
C18:0 LPC	523.7712 - 525.1212
C20:4 LPC	543.7401 - 545.0901
C22:6 LPC	567.7402 - 569.0902
C32:0 PE	691.9228 - 693.2728

C34:1 PE	717.9385 - 719.2885
C36:1 PE	745.9699 - 747.3199
C36:0 PE	747.9855 - 749.3355
C16:0 LPE	453.6931 - 455.0431
C18:2 LPE	477.6932 - 479.0432
C18:1 LPE	479.7088 - 481.0588
C18:0 LPE	481.7244 - 483.0744
C20:4 LPE	501.6933 - 503.0433
C22:6 LPE	525.6934 - 527.0434
C14:0 SM	674.9430 - 676.2930
C15:0 SM	688.9586 - 690.3086
C16:1 SM	700.9587 - 702.3087
C16:0 SM	702.9743 - 704.3243
C18:2 SM	726.9744 - 728.3244
C18:1 SM	728.9900 - 730.3400
C18:0 SM	731.0056 - 732.3556
C20:0 SM	759.0369 - 760.3869
C21:0 SM	773.0526 - 774.4026
C22:1 SM	785.0526 - 786.4026
C22:0 SM	787.0682 - 788.4182
C23:1 SM	799.0683 - 800.4183
C23:0 SM	801.0839 - 802.4339
C24:1 SM	813.0840 - 814.4340
C24:0 SM	815.0996 - 816.4496

Lipidomics assay notes: Lipids were profiled as described in Townsend et al. (2013). doi: 10.1373/clinchem.2012.199133
A full scan MS over m/z 400-1000 using Q1 scans at unit mass resolution was utilized and specific lipid species were identified using a range to capture the full width of the monoisotopic ion.