Materials and Instrumentation:

- Acquity UPLC BEH C18 2.1 x 100 mm, 1.7 µm column, Waters Corporation, Cat. No. 186002352
- O-Benzylhydroxylamine, Sigma-Aldrich Cat. No. B22984
- *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, Sigma-Aldrich Cat. No. E1769
- Organic acid standards and heavy isotope-labeled internal standards
- Dionex UltiMate 3000 HPLC coupled to a Thermo Quantiva triple quadrupole mass spectrometer
    - Software: Xcalibur (v. 3.0), Dionex (v. 2.14), TraceFinder (v. 3.2), Quantiva (v. 1.1)

Sample Preparation:

1. Retrieve study samples (-80°C freezer) and calibrator/internal standard solutions (-20°C freezer) and allow them to thaw on ice.
    - If not done previously, aliquot bulk study samples at 50 µL into fresh tubes

2. Obtain ten 1.7-mL Eppendorf tubes and place in a plastic tube rack on ice; label them for the calibration samples, C_1-C_{10}; note that C_{10} will be your high calibrator. Transfer 40 µL of either 50% ACN/0.3% Formic Acid (if assaying tissue) or dH_2O (if assaying fluid) to each tube.
    - If tissue was homogenized in a different solvent, use that solvent

3. Once thawed, vortex the calibrator and IS solutions retrieved from the freezer. Stock calibrator solutions are labeled as "Cals" from Cal 1 to Cal 10, with each Cal corresponding to a single calibration sample (See table below). Transfer 10 µL of a Cal solution to the corresponding calibration tube, and tap lightly to mix.

The stock calibrator "Cals" are concentrated combinations of all organic acids in water. Once diluted as prescribed below, organic acids are present at the following concentrations:

Cal	Volume Spiked into Cal Tubes (uL)	Calibrator	Conc'n of "A" OAs present (uM)	Conc'n of "B" OAs present (uM)	Conc'n of "C" OAs present (uM)	Conc'n of "D" OAs present (uM)
10	10	C_{10}	5000	1000	250	100
9	10	C_9	2000	400	100	40
8	10	C_8	1000	200	50	20
7	10	C_7	500	100	25	10
6	10	C_6	200	40	10	4
5	10	C_5	50	10	2.5	1
4	10	C_4	10	2	0.5	0.2

3	10	C_3	5	1	0.25	0.1
2	10	C_2	2	0.4	0.1	0.04
1	10	C_1	1	0.2	0.05	0.02

"A" Organic Acid: Lactate

"B" Organic Acids: 2-hydrxoybutyrate, 3-hydroxybutyrate

"C" Organic Acids: Pyruvate, Succinate, Fumarate, Malate, Citrate

"D" Organic Acid: α-ketoglutarate

4. All tubes (both calibrator and study sample) should now contain 50 µL. Vortex the thawed internal standard solution (labeled IS Mix #2) and transfer 10 µL of IS Mix #2 to all tubes.

   The stock Internal Standard Mix #2 (IS Mix #2) solution is a concentrated mix of all "heavy" organic acids in water. Once diluted as prescribed below, organic acid ISs are present at the following concentrations:

Solution	Volume Spiked into All Tubes (uL)	Conc'n of "A" ISs present (uM)	Conc'n of "B" ISs present (uM)	Conc'n of "C" ISs present (uM)	Conc'n of "D" ISs present (uM)	Conc'n of "E" ISs present (uM)
IS Mix #2	10	12.5	62.5	12.5	0.5	25

"A" Internal Standard: Lactate

"B" Internal Standards: Succinate, Fumarate, Malate, Citrate

"C" Internal Standard: 3-hydroxybutyrate*

"D" Internal Standard: Pyruvate

"E" Internal Standard: α-ketoglutarate

*The 3-hydroxybutyrate internal standard will govern the calibrator curve for 2-hydroxybutyrate

5. To all tubes, add 50 µL of 0.4M OBA, followed by 10 µL of 2M EDC. Close the tubes, vortex, and allow the mixture to incubate at room temperature for 10 minutes.
   - OBA = o-Benzylhydroxylamine hydrochloride (Sigma Cat. No. B22984). Prepare the OBA solution in 50/50 MeOH/200 mM Ammonium formate, pH 5. The solution must be prepared daily.
   - EDC = *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (Sigma Cat. No. E1769). Prepare the solution in dH_2O. The solution must be prepared daily.

6. After incubation, add 100 µL of dH_2O to all tubes, followed by 600 µL of ethyl acetate. Close the tubes and vortex thoroughly (at least 5 seconds). Centrifuge all tubes at 18,000 x g/5 min/10°C. A bilayer will form.

7. To a 96-well plate (Corning 1-mL), transfer 100 µL of each the ethyl acetate extract (top layer) according to the following scheme:

	1	2	3	4	5	6	7	8	9	10	11	12
A	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}		
B	1	2	3	4	5	6	7	8	9	10	11	12
C	13	14	15	16	17	18	19	20	21	22	23	24
D	25	26	27	28	29	30	31	32	33	34	35	36
E	37	38	39	40	41	42	43	44	45	46	47	48
F	49	50	51	52	53	54	55	56	57	58	59	60
G	61	62	63	64	65	66	67	68	69	70	71	72
H	73	74	75	76	77	78	79	80	81	82	83	84

*Note: Calibrators should progress from C_1 (low cal) to C_{10} (high cal)

8. Dry the extract under N_2. Once dry, reconstitute each well in 1 mL of 50/50 MeOH/dH_2O. Seal the plate with a 96-well plate seal and place in the autosampler.

Sample Preparation:

Below is a list of organic acid standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Organic Acid	Carrier	Cat. No.	Stock Conc'n (mM)	Diluent
Lactate	Sigma	71720	250	Water
Pyruvate	Sigma	P2256	50	Water
3-hydroxybutyrate	Sigma	54965	50	Water
Succinate	Sigma	S3674	25	Water
Fumarate	Sigma	204745	50	Water
Malate	Sigma	204176	50	Water

α-ketoglutarate	Sigma	75890	50	Water
Citrate	Sigma	251275	50	Water

Individual stock solutions are then combined to prepare the highest calibrator standard stock solution. The millimolar concentration of each amino acid in the combined mixture is given below:

Organic Acid	Initial Individual Stock Conc'n (mM)	Final Combined Stock Conc'n (mM)
Lactate	250	25
Pyruvate	50	1.25
3-hydroxybutyrate	50	5
Succinate	25	1.25
Fumarate	50	1.25
Malate	50	1.25
α-ketoglutarate	50	0.5
Citrate	50	1.25

This is the stock solution for the highest calibrator, C_{10}. Serial dilution of C_{10} yields the stock solutions for all ten calibrator samples, C_1-C_{10}. For simplicity, the table below only shows the serial dilution scheme in terms of Lactate's concentration:

Calibrator Stock Sol'n	Conc'n of Lac in Solution (mM)
C_{10}	25
C_9	10
C_8	5
C_7	2.5
C_6	1
C_5	0.25
C_4	0.05
C_3	0.025
C_2	0.01
C_1	0.005

Based on the protocol above, the calibrator curve ultimately generated during prep will have the following range. Note that there are four "groups" of organic acids, each with its own range in the curve:

	Conc'n of "A" OAs present	Conc'n of "B" OAs present	Conc'n of "C"	Conc'n of "D" OAs present

Calibrator	(uM)	(uM)	OAs present (uM)	(uM)
C_{10}	5000	1000	250	100
C_9	2000	400	100	40
C_8	1000	200	50	20
C_7	500	100	25	10
C_6	200	40	10	4
C_5	50	10	2.5	1
C_4	10	2	0.5	0.2
C_3	5	1	0.25	0.1
C_2	2	0.4	0.1	0.04
C_1	1	0.2	0.05	0.02

"A" Organic Acid: Lactate

"B" Organic Acid: 3-hydroxybutyrate

"C" Organic Acids: Pyruvate, Succinate, Fumarate, Malate, Citrate

"D" Organic Acids: α-ketoglutarate

Below is a list of heavy-isotope-labeled amino acid internal standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Organic Acid- Int. Std.	Carrier	Cat. No.	Stock Conc'n (mM)	Diluent
Lactate-d3	CDN Isotopes	D-2646	50	Water
Pyruvate-13C3	Aldrich	490717	25	Water
3-hydroxybutyrate-13C4	Aldrich	606030	50	Water
Succinate-13C4	Isotec	491985	25	Water
Fumarate-13C4	Cambridge Isotopes	CLM-1529	25	Water
Malate-13C4	Aldrich	750484	25	Water
α-ketoglutarate-13C4	Cambridge isotopes	CLM-4442	50	Water
Citrate-d4	Aldrich	485438	25	Water

Below is a list of heavy-isotope-labeled amino acid internal standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Organic Acid- Int. Std.	Initial Individual Stock Conc'n (mM)	Final Combined Stock Conc'n (mM)
Lactate-d3	50	0.5
Pyruvate-13C3	25	0.02
3-hydroxybutyrate-13C4	50	0.5
Succinate-13C4	25	2.5
Fumarate-13C4	25	2.5
Malate-13C4	25	2.5
α-ketoglutarate-13C4	50	1
Citrate-d4	25	2.5

The above combined mix is then diluted 4-fold in water to generate the working internal standard solution.

Our limits of quantitation are set by the high and low points of our calibrator curves. Some calibrator curves display quadratic character under our instrument conditions.

Assay Conditions:

- Autosampler
  - Temperature: 10°C
  - Injection Volume: 5 µL
  - Needle Wash Solution: 80/20 Methanol/Water
- Column
  - Temperature: 45°C
  - Maximum Pressure: 900 bar
- Binary Pump
  - Flow Rate: 0.3 mL/min
  - Solvent A: 0.1% Formic acid in water
  - Solvent B: 0.1% Formic acid in acetonitrile
  - Gradient Conditions:

Segment	Time (min)	% B	Flow Rate (mL/min)
0 (Start)	0.00	5.0	0.3
1	3.00	20.0	0.3
2	5.00	60.0	0.3
3	7.50	85.0	0.3
4	7.60	95.0	0.6
5	9.00	95.0	0.6
6	9.10	5.0	0.6
7	10.50	5.0	0.6

8	10.60	5.0	0.3

- Mass Spectrometer
  - Positive Ion Spray Voltage: 3500 V
  - Sheath Gas: 40
  - Aux Gas: 10
  - Sweep Gas: 1
  - Ion Transfer Tube: 350°C
  - Vaporizer: 300°C
  - Q1 Resolution: 0.7
  - Q3 Resolution: 0.7
  - CID Gas: 1.5 mTorr

- MRM Transitions

Organic Acid	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	RF Lens (V)
Lactate	196.1	91.062	13	30
Lactate-d3	199.1	91.062	13	30
3-hydroxybutyrate	210.1	91.153	16	41
3-hydroxybutyrate-13C4	214.1	91.153	16	41
Pyruvate	299.1	91.186	15	51
Pyruvate-d3	302.1	91.186	15	51
Fumarate	327.1	91.192	15	48
Succinate	329.1	206.233	9	44
Fumarate-13C4	331.1	91.192	15	48
Succinate-13C4	333.1	210.233	9	44
Malate	345.1	91.18	20	50
Malate-13C4	349.1	91.18	20	50
α-ketoglutarate	462.2	91.2	23	70
α-ketoglutarate-13C4	466.2	91.2	23	70
Citrate	508.25	385.0	9	51
Citrate-d4	512.2	389.0	9	51

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Reviewed By:	Christopher Petucci	Date: August 14, 2014
Approved By:	Christopher Petucci	Date: August 14, 2014

Revision Number	Name	Reason for Revision	Effective Date
01			
02			