Materials and Instrumentation:

- AccQ·Tag 2 x 100 mm, 1.7 µm column, Waters Corporation, Cat. No. 186003837
- AccQ·Tag Eluent A Concentrate, Waters Corporation, Cat. No. 186003838
- AccQ·Tag Eluent B, Waters Corporation, Cat. No. 186003839
- MassTrak Amino Acid Derivatization Kit, Waters Corporation, Cat. No. 186004095
- Amino acid standards and heavy isotope-labeled internal standards
- Agilent 1290 Infinity HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer
    - Software: MassHunter Acquisition (B.04.01); MassHunter Quantitative Analysis (B.05.00)

Assay Background/Protocol:

The Waters AccQ·Tag Amino Acids Assay relies on derivatization of each amino acid with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate ("AQC").

Sample prep protocol:

- Aliquot 100 µL of either biological fluid or tissue homogenate (at a concentration of 100 mg tissue per mL of 50/50 ACN/0.3% formic acid) into an Eppendorf tube.
- Add 10 µL of internal standard mix to the tube (more info on internal standards below)
- Add 800 µL of ice-cold MeOH to the tube and vortex thoroughly to mix
- Centrifuge the tube at 18,000 x g for five minutes at 4°C; protein precipitate will form a tight pellet at the bottom of the tube
- After centrifugation, transfer 100 µL of supernatant methanolic extract to a 96-well plate
- Dry the contents of the plate under nitrogen at 45°C until all wells are completely dry
- Meanwhile, prepare the derivatization reagent by adding 2 mL of MassTrak AAA Reagent Diluent (2B) to the MassTrak AAA Reagent Powder (2A). Vortex the solution and heat at 55°C for 10 minutes, vortexing occasionally
    - Note: Solubility of the reagent is limited; precipitate will likely remain in the vial, even after heating
- Once the wells are dry, reconstitute the samples in 80 µL of Borate Buffer (provided in the derivatization kit) and 20 µL of the MassTrak AAA Reagent. Seal the plate, vortex on a plate mixer for 30 seconds, and incubate for 10 minutes at 55°C
- After incubation, transfer the plate to the autosampler of the LC-MS

Calibrator curve prep protocol:

- To nine Eppendorf tubes, add either 90 µL of either H_2O (for fluid analysis) or 50/50 ACN/0.3% formic acid (for tissue analysis)
- Add 10 µL of the appropriate calibrator mix to the tube (more info on calibrators below)
- Add 10 µL of internal standard mix to the tube (more info on internal standards below)
- All remaining steps, beginning with the addition of MeOH, follow the steps used in study sample prep

Standards/Internal Standards Information:

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

Amino Acid	Carrier	Cat. No.	Stock Conc'n (mM)	Diluent, if not H_2O
Glycine	Sigma-Aldrich	G7126	500	
L-Alanine	Sigma-Aldrich	44526	500	
L-Serine	Sigma-Aldrich	S4500	500	
L-Proline	Sigma-Aldrich	P0380	500	
DL-Valine	Sigma-Aldrich	94640	200	
L-Leucine	Sigma-Aldrich	L8000	100	0.1 M KOH
L-Isoleucine	Sigma-Aldrich	I2752	150	0.1 M KOH
L-Methionine	Sigma-Aldrich	M9625	200	
L-Histidine	Sigma-Aldrich	H8000	100	
L-Phenylalanine	Sigma-Aldrich	40541	100	
L-Tyrosine	Sigma-Aldrich	93829	50	0.1 M KOH
L-Asparagine	Sigma-Aldrich	A0884	150	0.1 M KOH
L-Aspartic Acid	Sigma-Aldrich	A8949	75	0.1 M KOH
L-Glutamine	Sigma-Aldrich	G3126	100	
L-Glutamic Acid	Sigma-Aldrich	49449	100	0.1 M KOH
L-Ornithine·HCl	Sigma-Aldrich	75469	200	
L-Arginine	Sigma-Aldrich	11009	200	
L-Threonine	Sigma-Aldrich	T8625	500	
L-Lysine	Sigma-Aldrich	L5501	500	
L-Tryptophan	Sigma-Aldrich	T0254	100	0.1 M KOH
L-Citrulline	Sigma-Aldrich	C7629	250	

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:

Amino Acid	Initial Individual Stock Conc'n (mM)	Final Combined Stock Conc'n (mM)
Glycine	500	10
L-Alanine	500	10
L-Serine	500	5
L-Proline	500	10
DL-Valine	200	10
L-Leucine	100	5
L-Isoleucine	150	5
L-Methionine	200	5
L-Histidine	100	5
L-Phenylalanine	100	5
L-Tyrosine	50	5
L-Asparagine	150	5
L-Aspartic Acid	75	5
L-Glutamine	100	10
L-Glutamic Acid	100	5
L-Ornithine·HCl	200	5
L-Arginine	200	10
L-Threonine	500	10
L-Lysine	500	10
L-Tryptophan	100	2.5
L-Citrulline	250	5

This is the stock solution for the highest calibrator, C_9. Serial dilution of C_9 yields the stock solutions for all nine calibrator samples, C_1-C_9. For simplicity, the table below only shows the serial dilution scheme in terms of Glycine's concentration:

Calibrator Stock Sol'n	Conc'n of Gly in Solution (mM)

C_9	10
C_8	5
C_7	2.5
C_6	1
C_5	0.5
C_4	0.25
C_3	0.1
C_2	0.05
C_1	0.01

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

Calibrator	Conc'n of "A" AAs present (uM)	Conc'n of "B" AAs present (uM)	Conc'n of "C" AAs present (uM)
C_9	1000	500	250
C_8	500	250	125
C_7	250	175	87.5
C_6	100	50	25
C_5	50	25	12.5
C_4	25	12.5	6.25
C_3	10	5	2.5
C_2	5	2.5	1.25
C_1	1	0.5	0.25

"A" Amino Acids: Gly, Ala, Pro, Val, Arg, Thr, Lys, Gln

"B" Amino Acids: Ser, Leu, Ile, Met, His, Phe, Tyr, Asn, Asp, Glu, Orn, Cit

"C" Amino Acid: Trp

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:

Amino Acid-Int. Std.	Carrier	Cat. No.	Stock Conc'n	Diluent, if not H_2O

			(mM)	
Glycine-D5	Isotec	175838	20	
DL-Alanine-D4	Isotec	488917	20	
L-Serine-D3	Cambridge Isotopes	DLM-582	20	
L-Proline-D7	Cambridge Isotopes	DLM-487	20	
L-Valine-D8	CDN Isotopes	D-1076	20	
L-Leucine-D3	Cambridge Isotopes	DLM-1259	20	0.1 M KOH
L-Isoleucine-13C6	Cambridge Isotopes	CLM-2248	20	0.1 M KOH
L-Methionine-D3	CDN Isotopes	D-2230	200	
L-Histidine-13C6	Isotec	722871	20	
L-Phenylalanine-D8	Cambridge Isotopes	DLM-372	2	0.1 M KOH
L-Tyrosine-D4	Cambridge Isotopes	DLM-451	2	0.1 M KOH
L-Asparagine-13C4	Isotec	588695	20	0.1 M KOH
L-Aspartic Acid-D3	CDN Isotopes	D-898	20	0.1 M KOH
L-Glutamine-D7	CDN Isotopes	D-2532	20	
DL-Glutamic Acid-D3	CDN Isotopes	D-1196	20	0.1 M KOH
L-Ornithine-D6	Isotec	749443	5	
L-Arginine-13C6	Isotec	643440	20	
L-Threonine-13C4	Cambridge Isotopes	CLM-2261	20	
DL-Lysine-D4	Isotec	489034	20	
L-Tryptophan-D8	Cambridge Isotopes	DLM-6903	2	0.1 M KOH
L-Citrulline-D4	Isotec	578886	5	

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

Amino Acid-Int. Std.	Initial Individual	Final Combined

	Stock Conc'n (mM)	Stock Conc'n (mM)
Glycine-D5	20	0.2
DL-Alanine-D4	20	0.2
L-Serine-D3	20	0.2
L-Proline-D7	20	0.2
L-Valine-D8	20	0.2
L-Leucine-D3	20	0.2
L-Isoleucine-13C6	20	0.2
L-Methionine-D3	200	5
L-Histidine-13C6	20	0.2
L-Phenylalanine-D8	2	0.02
L-Tyrosine-D4	2	0.02
L-Asparagine-13C4	20	0.2
L-Aspartic Acid-D3	20	0.2
L-Glutamine-D7	20	0.2
DL-Glutamic Acid-D3	20	0.2
L-Ornithine-D6	5	0.05
L-Arginine-13C6	20	0.2
L-Threonine-13C4	20	0.2
DL-Lysine-D4	20	0.2
L-Tryptophan-D8	2	0.02
L-Citrulline-D4	5	0.05

The resulting mixture is then diluted 1:1 in H_2O to generate the "working" internal standard (wIS) mix. The concentration of Glycine in the wIS is 0.1 mM.

Based on the protocol above, the final concentration of the internal standards will be as follows:

Sample	Conc'n of "A" ISs present	Conc'n of "B" ISs present	Conc'n of "C"	Conc'n of "D" ISs present

	(uM)	(uM)	ISs present (uM)	(uM)
C_1-C_9	250	10	2.5	1
Tissue/Fluid	250	10	2.5	1

"A" Internal Standard: Met

"B" Internal Standards: Gly, Ala, Ser, Pro, Val, Leu, Ile, His, Asn, Asp, Gln, Lys, Glu, Thr, Arg

"C" Internal Standards: Orn, Cit

"D" Internal Standards: Phe, Tyr, Trp

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

Assay Conditions:

- Autosampler
  - Temperature: 4°C
  - Injection Volume: 0.5 µL
  - Needle Wash Solution: 80/20 Methanol/Water
- Column
  - Temperature: 55°C
  - Maximum Pressure: 900 bar
- Binary Pump
  - Flow Rate: 0.7 mL/min
  - Solvent A: AccQ·Tag Eluent A (diluted from the concentrate provided @ 1:10 in water)
  - Solvent B: AccQ·Tag Eluent B (use as received)
  - Gradient Conditions:

Segment	Time (min)	% B	Flow Rate (mL/min)
0 (Start)	0.00	0.0	0.7
1	0.54	0.0	0.7
2	5.74	9.1	0.7
3	8.24	21.2	0.7
4	9.54	50.0	0.7
5	9.55	90.0	0.7
6	10.14	90.0	0.7
7	10.15	0.0	0.7
Re-equil.	16.15	0.0	0.7

- Mass Spectrometer
  - Gas Temperature: 325˚ C
  - Gas Flow: 11 L/min.
  - Nebulizer: 50 psi

- Sheath Gas Temperature: 325˚ C
- Sheath Gas Flow: 10 L/min.
- Capillary Voltage: 3500 V
- Nozzle Voltage: 500 V
- Electrospray ionization: Positive
- See Excel spreadsheet for MRM transition information, collision energies, etc.

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Approved By:	Christopher Petucci	Date: June 05,2013

Revision Number	Name	Reason for Revision	Effective Date
01			
02			