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A Validated Method for the Quantification of Amino Acids in Mammalian Urine

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APPLICATION BENEFITS

- A simple, robust, LC/MS/MS Method for the absolute quantification of 20 amino acids in rat urine has been developed over the range of 0.2–200.0 µMol.
- Fast analytical throughput: two microtiter plates (192 samples) per day.

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KEY WORDS

TQ-S micro, amino acid, quantification, urine, LC-MS

INTRODUCTION

Amino acids play a critical role in mammalian biochemistry, forming the simple building blocks of proteins, acting as neurotransmitters in biosynthesis and are essential in lipid transports, to name but a few. The rapid and accurate quantification of amino acids is critical to understanding the underlying biochemistry of multiple physiological and disease states. Previous methodologies have employed either ion exchange chromatography followed by derivatization with fluorescence detection or sample derivatization followed by analysis by LC-UV or LC-Fluorescence. However, both of these approaches are time consuming and require complete chromatographic resolution of the amino acids from other amine-containing compounds, so are not always suitable for the analysis of biological fluids. Here we present a bioanalytical method for the rapid, simple, quantification of amino acids by UPLC-MS/MS for research use.

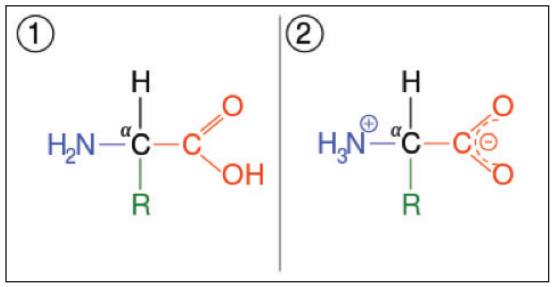

Figure 1. Amino acid structure.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC I-Class
Detector:	Xevo TQ-S micro
Vials:	Maximum Recovery vials
Column:	ACQUITY UPLC HSS T3 1.8 µm, 150 x 2.1 mm
Column temp.:	45 °C
Sample temp.:	Room temperature
Injection vol.:	2-µL
Flow rate:	0.6 mL/min
Mobile phase A:	water + 0.1% formic acid
Mobile phase B:	acetonitrile + 0.1% formic acid
Gradient:	Maintained at 4% B for 0.5 min; increasing to 10% B at 2.5 min; increasing to 28% B at 5 min, increasing to 95% B at 5 .1 min; reverting to 4% B at 6.2 min for a 1.3 min re-equilibration see Table 1

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	Positive
Acquisition range:	MRM mode
Capillary voltage:	1.5 kV
Collision energy:	Compound specific see Table 2
Cone voltage:	Compound specific see Table 2

Sample preparation

The sample preparation procedure employed for the analysis of the amino acids is shown below in Figure 2. A 50-µL aliquot of the samples and standards was vortex mixed with 150-µL of methanol, to precipitate protein. A 10-µL aliquot of the resultant sample was then transferred to a sample tube for derivatization according to the process defined in Figure 2.

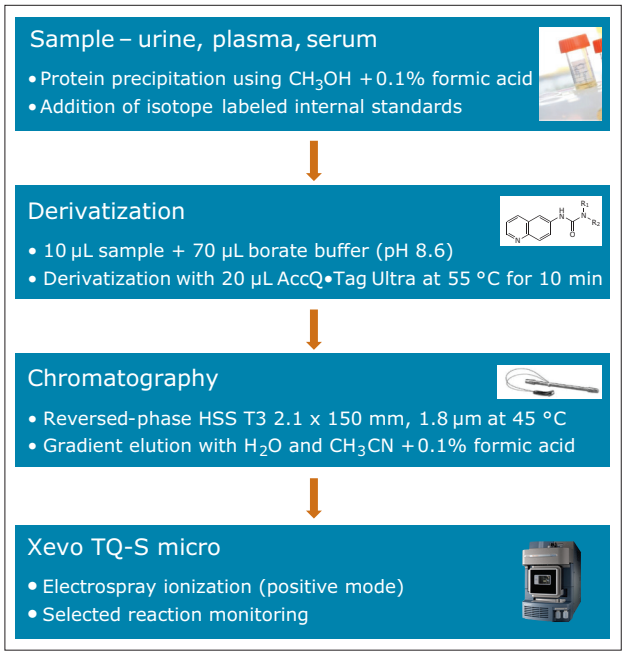

Figure 2. Sample preparation scheme for amino acid analysis.

Time (min)	Flow rate (mL/min)	%B
0.0	0.6	4
0.5	0.6	4
2.5	0.6	10
5.0	0.6	28
5.1	0.6	95
6.1	0.6	95
6.2	0.6	4
7.5	0.6	4

Table 1. Chromatographic gradient table.

Compound	Parent	Fragment	Window (min)	Dwell time (s)	CV (V)	CE (eV)	RT (min)
Histidine-d3	329.1	159.1	0.0–2.0	0.026	20	10	1.51
Histidine	326.1	156.1	0.0–2.0	0.026	20	10	1.52
1-methylhistidine	340.1	170.1	0.0–2.0	0.026	30	18	1.70
4-hydroxyproline	302.0	171.1	1.5–2.0	0.026	10	22	1.78
3-methylhistidine	340.1	124.2	0.0–2.0	0.026	30	28	1.78
Carnosine	397.1	227.2	1.8–2.2	0.026	30	14	1.99
Asparagine	303.1	171.1	1.8–2.1	0.026	30	22	2.00
Arginine	345.1	70.1	1.8–2.2	0.026	30	36	2.01
Taurine	296.1	116.3	2.0–2.5	0.026	30	60	2.22
Glutamine-d5	322.1	171.1	2.0–2.5	0.026	30	24	2.27
Glutamine	317.1	171.1	2.0–2.5	0.026	30	24	2.29
Serine-d3	279.1	171.1	2.0–2.5	0.026	30	20	2.30
Serine	276.1	171.1	2.0–2.5	0.026	30	20	2.32
Homoserine	290.1	171.1	2.2–2.6	0.030	10	18	2.46
Ethanolamine	232.1	171.1	2.2–2.7	0.030	10	20	2.47
Glycine	246.1	116.1	2.2–2.8	0.013	30	14	2.54
Aspartic acid-d3	307.0	171.1	2.5–2.8	0.030	30	20	2.67
Aspartic acid	304.1	171.1	2.5–2.8	0.030	30	22	2.69
Citrulline	346.2	171.1	2.5–2.9	0.030	30	26	2.77
Sarcosine	260.1	116.1	2.6–3.6	0.024	30	44	2.85
Glutamic acid-d3	321.0	171.0	2.5–3.2	0.030	30	20	2.90
Glutamic acid	318.1	171.1	2.5–3.2	0.030	30	22	2.91
β-alanine	260.1	116.1	2.6–3.6	0.024	30	44	3.06
Threonine	290.1	171.1	2.9–3.2	0.062	30	20	3.11
Threonine-13C4 15N	295.1	171.0	2.9–3.2	0.062	30	26	3.11
Alanine-d3	263.1	171.0	3.2–3.6	0.024	30	16	3.45
Alanine	260.1	116.1	2.6–3.6	0.024	30	44	3.47
γ-amino-n-butyric acid	274.1	171.1	3.2–4.2	0.013	10	20	3.51
Hydroxylysine	333.2	171.1	3.4–4.0	0.013	16	16	3.56
Aminoadipic acid	332.1	171.1	3.2–3.8	0.024	30	18	3.61
Proline-d7	293.0	171.1	3.5–4.0	0.013	30	24	3.74
Proline	286.1	116.1	3.5–4.0	0.013	30	50	3.77
β-aminoisobutyric acid	274.1	171.1	3.2–4.2	0.013	10	20	3.78
Ornithine	303.1	171.1	3.6–4.2	0.013	54	22	3.85
Cystathionine	393.2	171.1	3.5–4.1	0.013	66	18	3.86
Phosphoserine	356.1	171.1	3.8–4.2	0.013	20	18	4.05
Lysine 224-d4	246.1	171.1	3.8–4.3	0.013	30	14	4.07
Cystine 291	291.1	171.1	3.8–4.2	0.013	10	12	4.07
Cystine 411	411.0	171.1	3.8–4.2	0.013	60	18	4.07
Cystine 581	581.0	171.1	3.8–4.2	0.013	34	26	4.07
Lysine 487-d4	491.2	171.0	3.8–4.3	0.013	30	32	4.07
Lysine 317-d4	321.1	171.0	3.8–4.3	0.013	50	24	4.07
Lysine 244	244.2	171.1	3.8–4.3	0.030	30	12	4.08
Lysine 317	317.1	171.1	3.8–4.3	0.013	56	16	4.08
Lysine 487	487.1	171.0	3.8–4.3	0.013	18	34	4.08
α-amino-n-butyric acid	274.1	171.1	3.2–4.2	0.013	10	20	4.09
Tyrosine	352.1	171.1	4.2–4.8	0.046	30	24	4.45
Methionine	320.1	171.1	4.3–4.8	0.046	30	22	4.58
Valine-d8	296.1	171.1	4.4–4.8	0.046	30	20	4.61
Valine	288.1	171.1	4.4–4.8	0.046	30	16	4.64
Isoleucine-d10	312.1	171.0	5.0–5.5	0.048	30	20	5.21
Isoleucine	302.1	171.1	5.0–5.5	0.048	30	20	5.24
Leucine-d10	312.1	171.0	5.0–5.5	0.048	30	20	5.27
Leucine	302.1	171.1	5.0–5.5	0.048	30	20	5.31
Phenylalanine-d5	341.1	171.1	5.2–5.7	0.048	30	22	5.43
Phenylalanine	336.1	171.1	5.2–5.7	0.048	30	22	5.45
Tryptophan-d3	378.1	171.1	5.2–5.8	0.048	6	24	5.54
Tryptophan	375.1	171.1	5.2–5.8	0.048	30	26	5.55

Table 2. Mass spectrometric analysis conditions for amino acids.

Data management

- MassLynx Mass Spectrometry Software
- TargetLynx Application Manager

One day validation

The bioanalytical method was subjected to a one day validation according to the FDA guidelines for bioanalytical method validation. The samples employed are shown below:

- Double blank (with no analyte or internal standard)
- Single blank (with analyte but no internal standard)
- Eight point calibration curve (performed at the beginning and end of run)
- Six replicates of:
  - LLOQQC (0.2 µM)
  - LQC (0.6 µM)
  - MQC (30 µM)
  - HQC (160 µM)
  - ULOQQC (20 µM)
- Twenty rat urine samples from a toxicology study ([4-chloro-6-(2, 3-xylidino)-2-pyrimidinylthio]acetic acid) were used to test the robustness of the method.

Amino acids quantified in this method

The amino acids analyzed in this study are listed in Table 3. The twenty proteinogenic amino acids were subjected to validation for absolute quantification using stable isotope labeled internal standards. Those additional eighteen amino acids, for which no stable isotope labeled internal standard was used, were subjected to relative quantification. The stable isotopes employed for each amino acid is listed in Table 4.

Validated For Absolute Quantification	Monitored For Relative Quantification
L-Alanine	3-Methyl-L-histidine
L-Arginine	1-Methyl-L-histidine
L-Asparagine	Cystathionine
L-Aspartic acid	DL-β-Aminoisobutryic acid
L-Cystine	Ethanolamine
L-Glutamic acid	Homoserine
L-Glutamine	Hydroxy-L-proline
Glycine	Hydroxylysine
L-Histidine	L-Carnosine
L-Isoleucine	L-Citrulline
L-Leucine	L-Ornithine
L-Lysine	L-α-aminoadipic acid
L-Methionine	L-α-Amino-n-butyric acid
L-Phenylalanine	Phosphoserine
L-Proline	Sarcosine
L-Serine	Taurine
L-Threonine	β-Alanine
L-Tryptophan	γ-Amino-n-butyric acid
L-Tyrosine	
L-Valine	

Table 3. Amino acids subjected to absolute and relative quantification.

Compound	Labeled internal standard
L-Alanine	Alanine-d3
L-Arginine	Serine-d3
L-Asparagine	Serine-d3
L-Aspartic acid	Aspartic acid-d3
L-Cystine	Proline-d7
L-Glutamic acid	Glutamic acid-d3
L-Glutamine	Glutamine-d5
Glycine	Serine-d3
L-Histidine	Histidine-d3
L-Isoleucine	Isoleucine-d10
L-Leucine	Leucine-d10
L-Lysine	Lysine-d4
L-Methionine	Valine-d8
L-Phenylalanine	Phenylalanine-d5
L-Proline	Proline-d7
L-Serine	Serine-d3
L-Threonine	Threonine-13C4
L-Tryptophan	Tryptophan-d5
L-Tyrosine	Tyrosine-d7
L-Valine	Valine-d8

Table 4. Amino acids quantified using stable isotope labeled internal standards.

Calibration curves

The calibration line was prepared from a Sigma Aldrich physiological, amino acid standard (acidics, basics, neutrals) by spiking into 50/50 CH_3OH/H_2O. The calibration curve was generated over three orders of magnitude, covering the physiological range of 0.2–200.0 µM. The QC samples were prepared from a separate Sigma Aldrich physiological amino acid standard.

RESULTS AND DISCUSSION

A typical separation obtained from the amino acid standard mix is shown in Figure 3. The data displayed illustrates the separation obtained for the amino acids and the throughput of the method. The peak shape obtained from the chromatography was excellent with a peak width at the base of approximately 3 seconds. The method was shown to be reproducible and reliable with no retention time drift. The Xevo TQ-S micro is equipped with a new novel, state of the art, ion transfer optics which allows more ions to be sampled from the source and transferred to the analyzer. The StepWave ion guide in the Xevo TQ-S micro is designed to cope with the challenges of the modern laboratory that are produced by high sample throughput and difficult matrices. Neutrals and gas load are passively removed for enhanced transmission with the ions actively transferred into the mass analyzer, improving sensitivity and robustness.

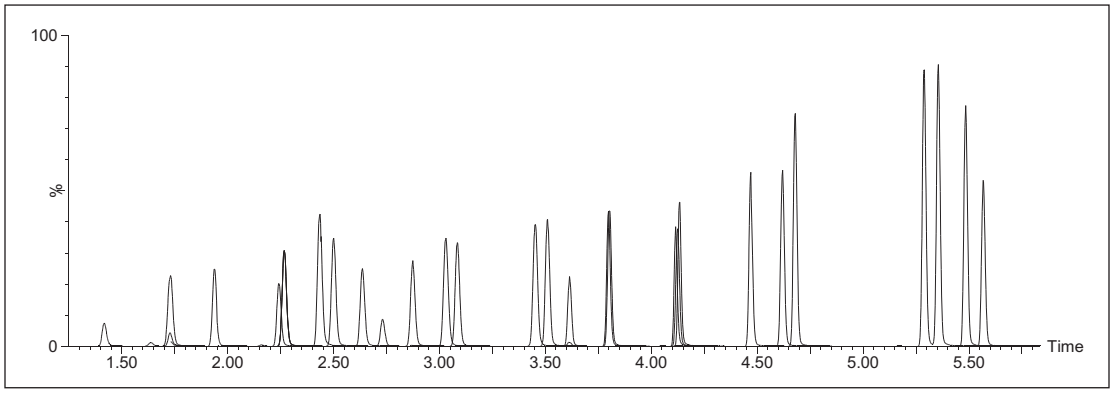

Figure 3. Amino acid LC-MS QC chromatogram 200 µM.

Validation results

The method validation was carried out according to FDA guidelines for bioanalytical methods. The method is not validated for clinical diagnostic use. The resulting amino acid data was processed and quantified using Waters® MassLynx TargetLynx application manager employing internal standard calibration and 1/x weighting. A summary of the QC data for each amino acid is listed below in Table 5. The data obtained for the quantification of each amino acid was acceptable for routine quantification.

Amino acid	0.2 µM	0.6 µM	6 µM	30 µM	160 µM	200 µM
Aspartic acid	7.26	3.40	2.02	0.91	0.80	0.83
Asparagine	6.30	4.13	1.01	1.71	1.74	2.58
Cystine	5.73	3.17	3.00	4.72	2.13	1.70
Glutamic acid	9.81	3.02	1.28	0.88	0.88	3.31
Glutamine	3.23	3.86	0.49	1.18	0.73	1.11
Glycine	17.92	8.48	2.32	2.31	2.27	3.21
Histidine	2.68	5.30	3.26	3.95	2.06	3.37
Isoleucine	5.29	0.57	3.06	1.57	0.63	0.75
Leucine	5.09	1.19	1.09	3.07	0.31	0.38
Lysine	7.46	3.11	2.48	1.10	1.07	1.29
Methionine	0.91	2.25	1.59	1.16	2.12	1.40
Phenylalanine	4.85	1.15	0.95	0.77	5.13	0.83
Proline	8.56	3.31	1.68	1.58	0.95	1.09
Serine	23.57	6.47	2.46	1.85	1.61	2.33
Threonine	4.30	2.45	1.25	1.24	1.06	0.68
Tryptophan	1.88	2.14	1.74	1.00	5.86	1.38
Tyrosine	5.32	2.52	2.96	2.32	4.43	2.79
Valine	3.55	1.12	1.65	0.44	1.01	0.84

Table 5. Coefficient of variation (%) for QCs at various concentrations.

The validation results as well as example chromatograms and calibration lines for the amino acids glutamic acid and aspartic acid are shown in Figures 4–7 and Tables 6 and 7. Here we can see that the methodology demonstrated acceptable peak shape and signal to noise ratio at the lowest level of quantification. The method demonstrated excellent bias and precision for every amino acid.

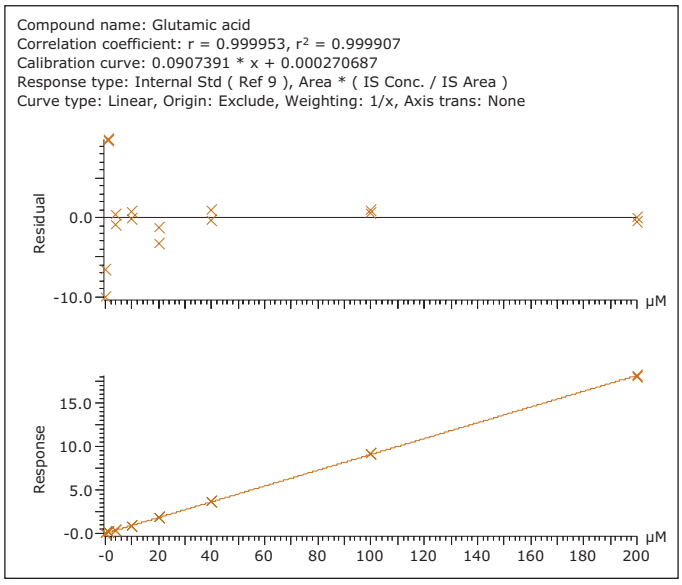

Figure 4. Calibration line for glutamic acid.

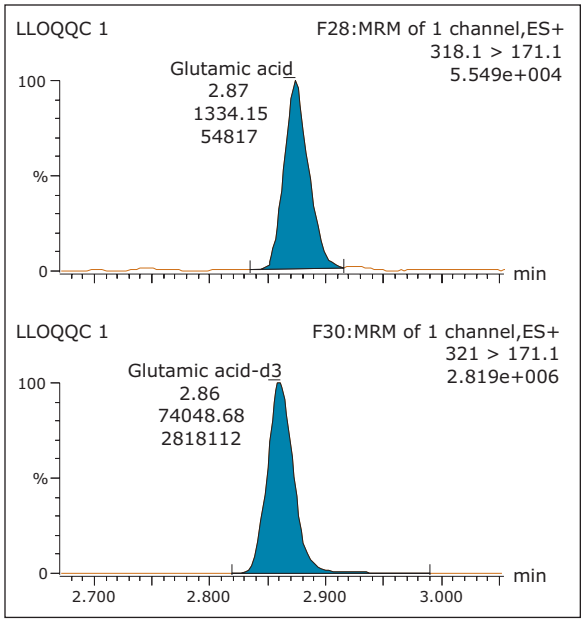

Figure 5. Lower limit of quantification QC (0.2 µM) LC-MS chromatogram for glutamic acid.

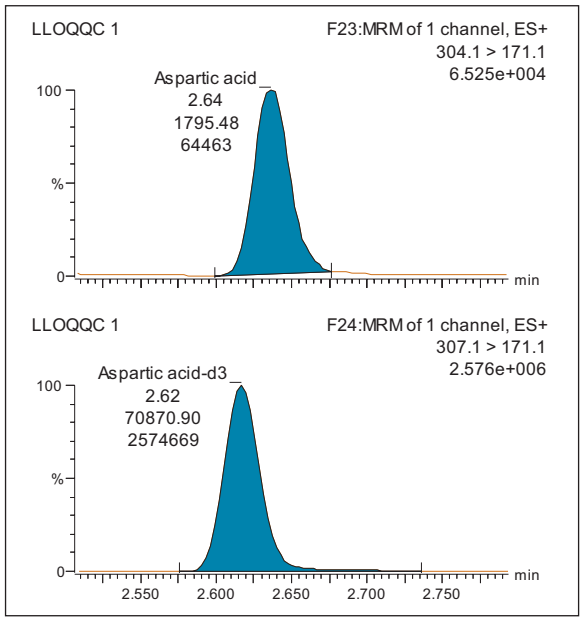

Figure 6. Lower limit of quantification QC (0.2 µM) LC-MS chromatogram for aspartic acid.

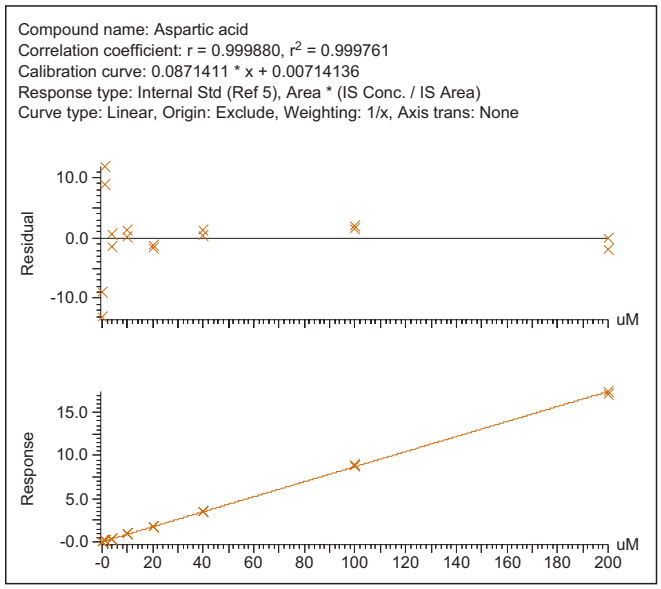

Figure 7. Calibration line for aspartic acid.

Occasion	QC nominal concentration (µM) glutamic acid				
	0.2	0.6	30	160	200
1	0.20	0.58	28.6	154	211
2	0.22	0.58	29.3	158	194
3	0.20	0.58	29.2	155	198
4	0.20	0.58	28.8	156	194
5	0.25	0.62	28.9	156	197
6	0.19	0.57	29.0	158	194
Mean	0.21	0.58	29.0	156	198
STDEV	0.02	0.02	0.25	1.37	6.56
%CV	9.81	3.02	0.88	0.88	3.31
Bias	4.08	-2.56	-3.36	-2.37	-1.05

Table 6. QC validation data for glutamic acid.

Occasion	QC nominal concentration (µM) aspartic acid				
	0.2	0.6	30	160	200
1	0.21	0.58	28.7	154	194
2	0.24	0.60	29.3	157	191
3	0.21	0.62	29.4	157	192
4	0.21	0.56	29.2	155	195
5	0.24	0.59	29.3	155	195
6	0.20	0.60	29.2	158	195
Mean	0.22	0.59	29.2	156	194
STDEV	0.02	0.02	0.27	1.24	1.61
%CV	7.26	3.40	0.91	0.80	0.83
Bias	9.17	-1.22	-2.66	-2.50	-3.19

Table 7. QC validation data for aspartic acid.

CONCLUSION

A robust, reliable bioanalytical method for the absolute quantification of twenty amino acids and the relative quantification of a further eighteen amino acids in mammalian urine has been developed and evaluated. The method had an analysis time of 7.5 minutes per sample. This allows the analysis of two, 96-well, microtitre plates of samples in a 24 hour time period. The method was found to be valid over the physiologically important range of 0.2–200.0 µMol. The chromatography was reproducible and reliable with no retention time drift detected for any of the amino acids. This data demonstrates that LC-MS/MS provides an attractive, viable, and alternative bioanalytical method to traditional modes of amino acid analysis, providing fast and accurate quantification.

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