Quantitative Analysis of Underivatized Glutamine, Glutamic Acid, Asparagine, and Aspartic Acid in Cell Media using LC/MS/MS

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Introduction

Glutamine (Gln), glutamic acid (Glu), Asparagine (Asn), and Aspartic acid (Asp) are important amino acids in the fields of medicine, food industry, and metabolomics research. Amino acid determination in a complex matrix without derivatization is advantageous as it eliminates laborious sample preparation procedures and reduces experimental errors. However, difficulties are found in the separation and detection of those highly hydrophilic compounds, especially in complex biological matrices. Herein, a rapid and sensitive LC/MS/MS method is presented for the separation and quantitation of Gln, Glu, Asn, and Asp in cell media. The method utilizes an ion pairing reagent to achieve baseline HPLC separation thus eliminating amino acid derivatization and preventing interference between related amino acids (e.g. Gln/Glu, and Asn/Asp). Excellent sensitivity, linearity, range, accuracy, reproducibility and precision were demonstrated using this LC/MS/MS method.

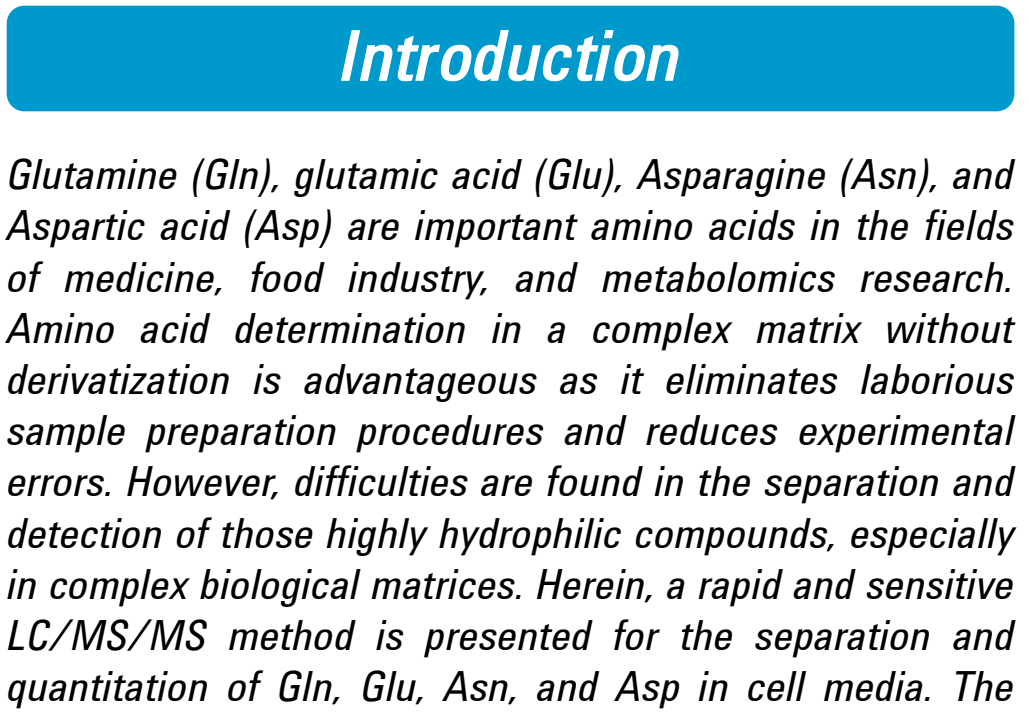

Figure 1: Underivatized Glutamine, Glutamic acid, Asparagine, and Aspartic acid.

Experimental

Sample Preparation

Calibration standard solutions containing the four amino acids at varied concentrations (10 nM – 10 mM in water and 50 nM – 5 mM spiked in cell media) were prepared in water and in cell media. Calibration standard solutions and eight unknown cell media samples (100 µL) were deproteinized using ice-cold acetonitrile (200 µL). After centrifugation, the supernatant (100 µL) was diluted 100 times in water before LC/MS/MS analysis.

Experimental

LC Method

Liquid chromatography was performed on an Agilent 1290 infinity UHPLC system. The four amino acids were physically separated using ion pairing chromatography with heptafluorobutyric acid (HFBA) on a Zorbax SB-C18 Rapid Resolution HT column (3.0 x 50 mm, 1.8 µm). The column temperature was 25 °C. Mobile phases were 0.5% formic acid and 0.3% HFBA in water (A) and in acetonitrile (B). Autosampler was kept at 4 °C and samples (1 µL) were injected with an LC flow rate of 0.4 mL/min. The gradient was from 0% to 5% mobile phase B in 5 min, kept at 90% B for 1 min followed by 3 min equilibration at 0% B. As demonstrated by Figure 2, the four amino acids were well separated with retention time of 3.00, 3.48, 3.89, and 4.61 min for Asn, Asp, Gln, and Glu, respectively.

Mass Spectrometry Method

LC/MS/MS analysis was performed on an Agilent 6460 triple quadrupole mass spectrometer equipped with Agilent Jet Stream source in positive ionization mode. Source conditions were optimized for amino acid analysis. Drying gas temperature and flow were kept at 275 °C and 9L/min. Nebulizer gas was 40 psi. Sheath gas temperature and flow were 325 °C and 12 L/min. Capillary voltage was 3750 V. The specific MRM transitions employed for quantitation of Gln, Glu, Asn, and Asp were m/z 147.1 > 84.1, 148.1 > 84.1, 133.1 > 74.1, and 134.1 > 74.0, respectively, as illustrated by Figure 2. Fragmentor voltage and collision energy were optimized for each amino acid analyte.

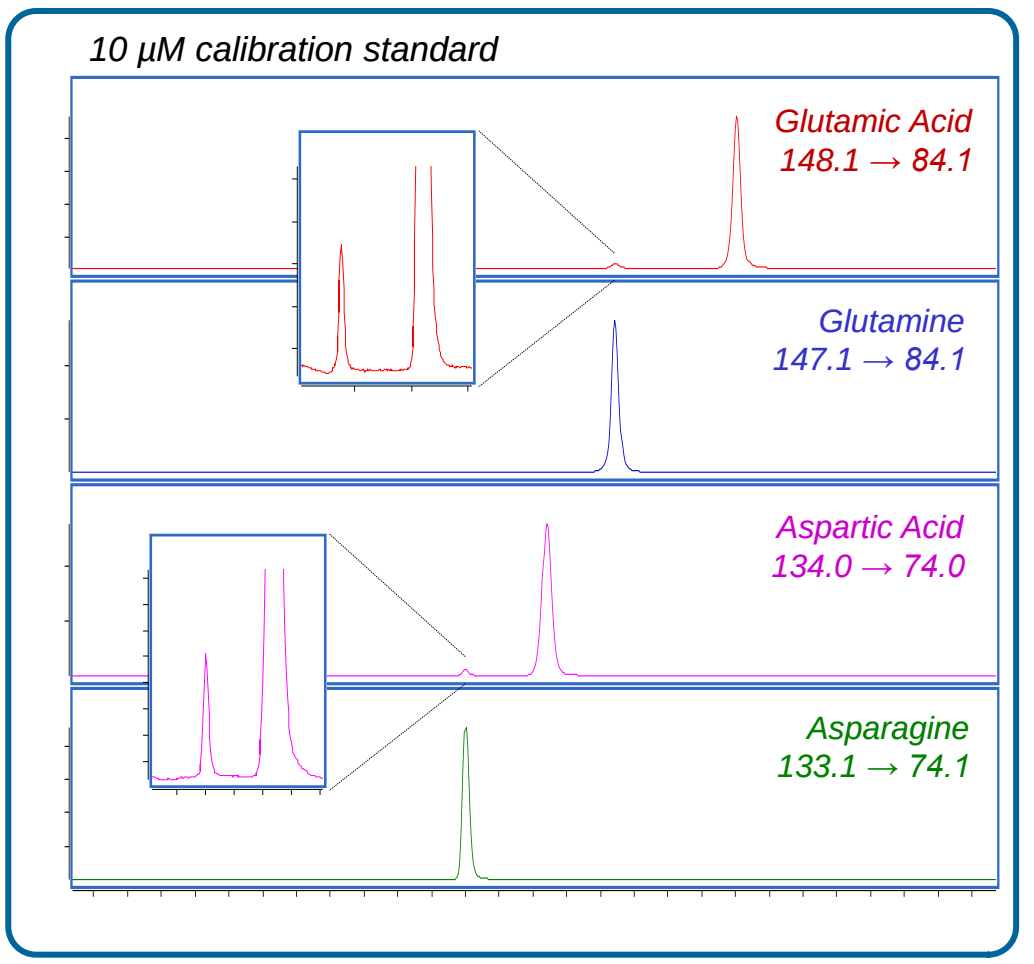

Figure 2: MRM chromatograms of the four amino acids.

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Results and Discussion

Method Validation

The baseline separation of the four amino acids prevented MRM signal interference of Gln (m/z 147.1 > 84.1) to Glu (m/z 148.1 > 84.1), as well as Asn (m/z 133.1 > 74.1) to Asp (m/z 134.1 > 74.1). As demonstrated by the expanded views in Figure 2, the interference peak was separated from the analyte peak. This significantly improved the sensitivity, linearity and accuracy of the LC/MS/MS analysis.

Sensitivity (LOD and LOQ)

The limit of detection (LOD) is 5, 10, 5, and 10 nM, and 5, 10, 5, and 10 fmol on column for Asn, Asp, Gln, and Glu, respectively, with signal to noise ratio of > 5:1. The limit of quantitation (LOQ) is 5, 10, 5, and 50 nM, and 5, 10, 5, and 50 fmol on column for Asn, Asp, Gln, and Glu, respectively. As illustrated by Figure 3, excellent reproducibility (%RSD < 5%) were obtained at the LOQ levels.

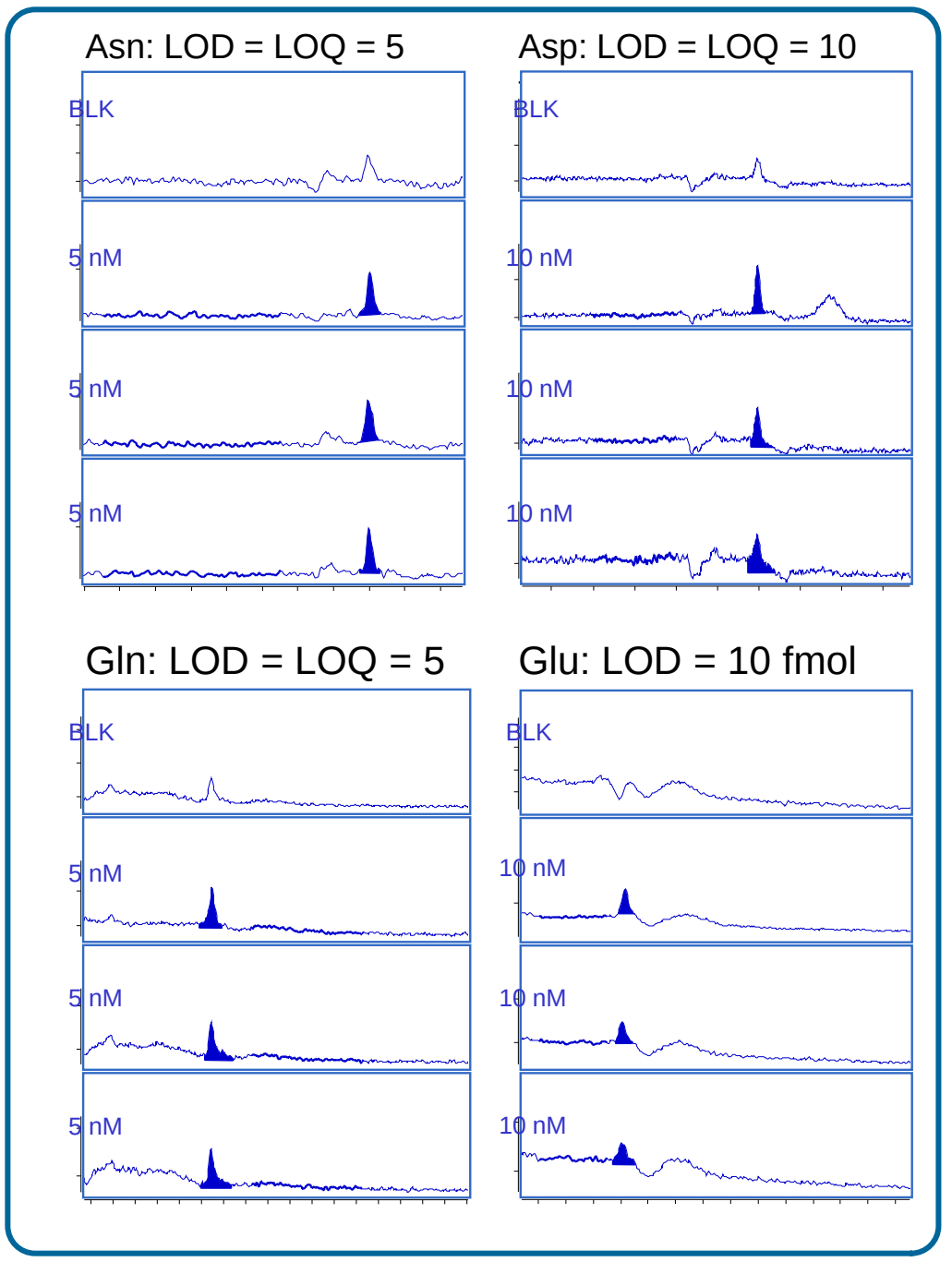

Figure 3: MRM chromatograms of Asn, Asp, Gln, and Glu at their LOD and LOQ levels (3 replicate injections).

Calibration curve linearity and range

The calibration curves for the four amino acids in water (Figure 4) show excellent linearity (R^2 > 0.999) and wide dynamic range (> 3 orders). Notably, the dynamic range for Gln is greater than 4 orders in magnitude (5 – 100000 nM). The inserts of Figure 4 demonstrate the great detection accuracy (80 – 120%) and reproducibility (%RSD < 5%) at low concentration levels, particularly at the LOQ levels.

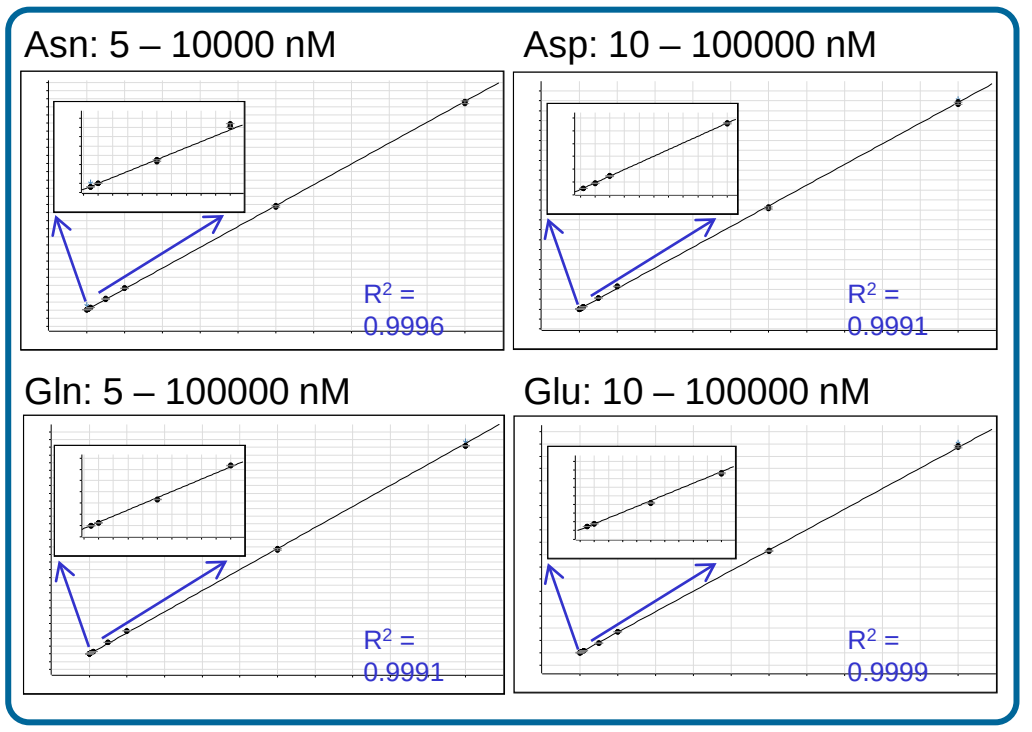

Figure 4: Calibration curves of Asn, Asp, Gln, and Glu in water. Inserts demonstrate low concentration range.

Cell media calibration curves were obtained by spiking varied concentrations of Asn, Asp, Gln, and Glu in cell media. As demonstrated in by Figure 5, excellent linearity (R^2 > 0.999) and dynamic range (≥ 3 orders) were obtained for the four amino acids in cell media.

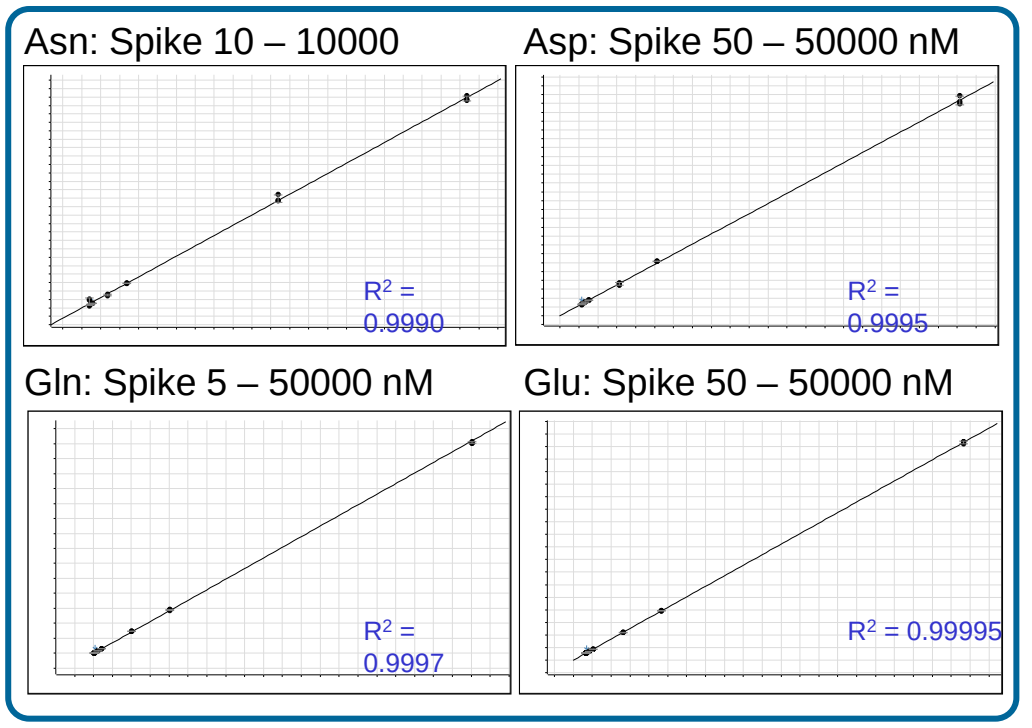

Figure 5: Calibration curves of Asn, Asp, Gln, and Glu in cell media (RPMI 1640 without Gln).

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Results and Discussion

Accuracy, reproducibility and precision

The method accuracy, reproducibility, and precision were evaluated at nine or ten standard concentrations for each amino acid. The results in water and in cell media are summarized in Table 1.

In Water

Compounds	Accuracy (%)	Reproducibility (%RSD, n = 3)	Precision (%RSD, n = 10)
Asparagine	84.4 – 107.0	0.55 – 4.27	7.23
Aspartic Acid	92.4 – 112.8	0.48 – 1.98	8.63
Glutamine	86.7 – 109.7	0.14 – 1.09	8.03
Glutamic Acid	82.0 – 109.6	0.22 – 3.54	5.61

In Cell Media

Compounds	Accuracy (%)	Reproducibility (%RSD, n = 3)	Precision (%RSD, n = 9)
Asparagine	98.6 – 102.6	0.03 – 4.07	1.53
Aspartic Acid	94.1 – 103.6	0.09 – 3.90	2.82
Glutamine	94.7 – 105.2	0.23 – 5.42	4.59
Glutamic Acid	98.3 – 101.1	0.13 – 0.75	1.08

Table 1: Accuracy, reproducibility and precision of the LC/MS/MS method in water and in cell media.

Quantitation of amino acids in cell media samples

The LC/MS/MS method was applied to measure the concentrations of the four amino acids in media A (RPMI 1640 without Gln) and media B (media A + 5% fetal bovine serum), and eight unknown cell media samples. Unknown 4 – 9 were prepared by spiking different levels of Gln in media A, while unknown 10 and 11 were prepared by spiking Gln in media B. .

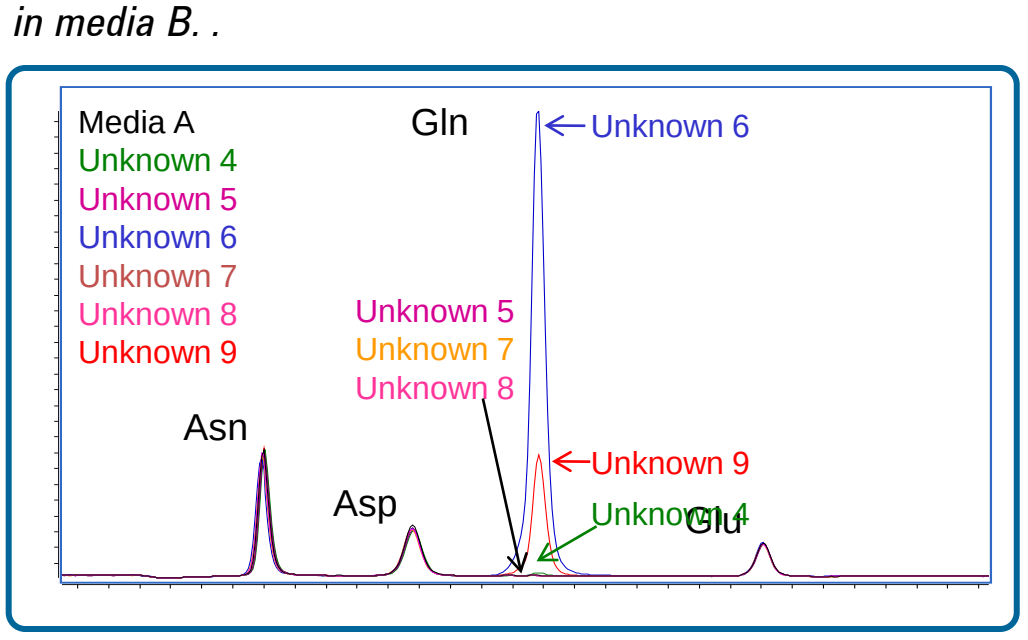

Figure 6: Overlaid LC/MS/MS chromatograms of media A and unknown 4 – 9.

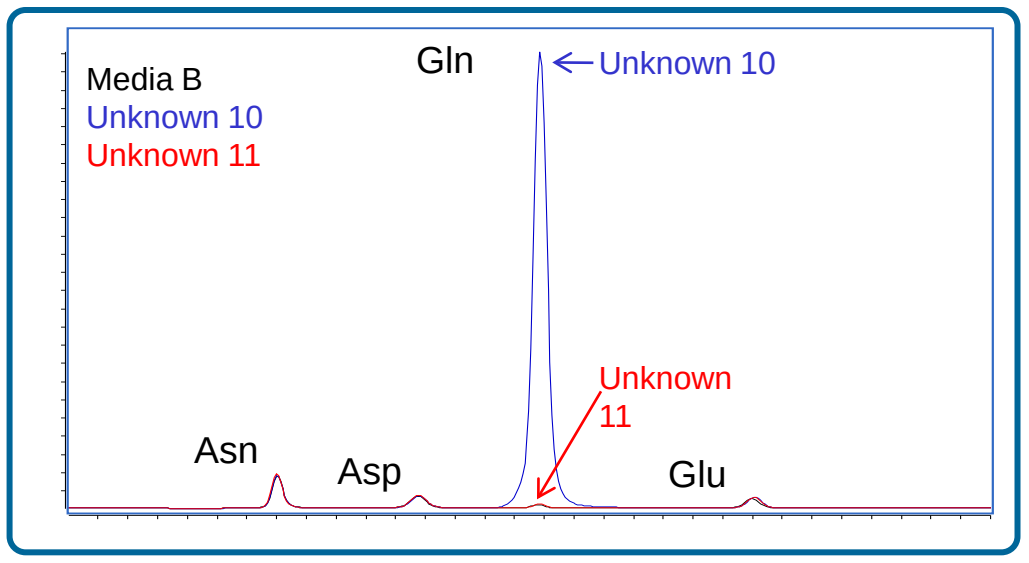

Figure 7: Overlaid LC/MS/MS chromatograms of media B and unknown 10 and 11.

The measured concentrations of Asn, Asp, Gln, and Glu in media A , media B, and the unknown samples were very consistent with previously spiked values. The results in Table 2 demonstrate excellent reproducibility (%RSD < 5%).

Media A and Unknown 4 - 9	Asparagine		Aspartic Acid		Glutamine		Glutamic Acid	
	Conc. (µM)	%RSD (n=3)	Conc. (µM)	%RSD (n=3)	**Conc. (µM)**	**%RSD (n=3)**	Conc. (µM)	%RSD (n=3)
Media 1	397.76	0.92	270.31	2.97	**7.26**	**0.87**	174.01	0.26
4	393.30	0.45	260.82	0.70	**13.03**	**0.33**	166.96	0.04
5	395.38	4.11	254.58	1.16	**7.00**	**0.43**	164.47	1.36
6	390.32	2.85	264.67	0.51	**1071.33**	**1.36**	172.95	1.43
7	391.17	2.67	264.20	0.91	**6.83**	**0.06**	168.75	0.61
8	397.94	1.90	267.48	1.00	**6.88**	**0.58**	170.73	0.33
9	407.66	4.58	257.22	4.26	**271.29**	**2.38**	171.70	0.58

Media B and Unknown 10 and 11	Asparagine		Aspartic Acid		Glutamine		Glutamic Acid	
	Conc. (µM)	%RSD (n=3)	Conc. (µM)	%RSD (n=3)	**Conc. (µM)**	**%RSD (n=3)**	Conc. (µM)	%RSD (n=3)
Media 2	326.76	1.81	241.80	1.12	**26.28**	**1.82**	179.50	1.57
10	334.02	1.04	244.53	1.11	**3930.81**	**0.95**	198.96	1.93
11	347.61	0.52	256.58	1.16	**29.92**	**1.59**	199.14	1.22

Table 2: Measured concentrations in cell media samples.

Conclusions

- *Underivatized Asn, Asp, Gln, and Glu were baseline separated using ion pairing chromatography.*
- *The LC/MS/MS method demonstrates excellent sensitivity with LOD/LOQ of sub-fmol level on column.*
- *Great linearity (> 0.999), range (> 4 orders), accuracy (82 – 113%), precision (< 6%), and reproducibility (< 9%) were observed for all four amino acids.*
- *Accurate quantitation results of the four amino acids were achieved in unknown cell media samples.*
- *This rapid and sensitive LC/MS/MS method can be conveniently used to measure amino acids in complex biological samples without derivatization.*