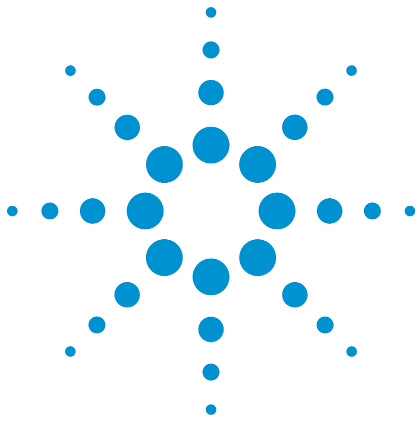

Quantitative Analysis of Underivatized Glutamine, Glutamic Acid, Asparagine, and Aspartic Acid in Cell Media using Agilent 6460 Triple Quadrupole LC/MS

Application Note

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Abstract

A robust and sensitive method was demonstrated for rapid quantitation of underivatized amino acids in complex biological matrices using ion pair chromatographic separation and triple quadrupole LC/MS detection. Excellent quantitation performance, measured by linearity, accuracy, and reproducibility, was achieved in neat standards and biological samples.

Agilent Technologies

Introduction

Glutamine (Gln), glutamic acid (Glu), asparagine (Asn), and aspartic acid (Asp) (Figure 1 and Table 1) are important amino acids in the fields of medicine, food industry, and metabolomics and clinical research.[1,2] Quantitation of amino acids in a complex biological matrix without derivatization[3,4] is advantageous as it eliminates laborious sample preparation procedures and reduces potential experimental errors. However, due to the highly hydrophilic nature of these compounds, LC/MS analysis is challenging because they have poor chromatographic separation and LC retention, especially in complex biological matrices. In this application note, a rapid and sensitive LC/MS/MS method is presented for the separation and quantitation of Gln, Glu, Asn, and Asp in cell media using an Agilent 1290 Infinity UHPLC System coupled to an Agilent 6460 Triple Quadrupole LC/MS with Jet Stream technology. The method uses an ion pairing reagent, heptafluorobutyric acid (HFBA), to achieve baseline chromatographic separation. Therefore, it eliminates amino acid derivatization and prevents signal interference between amino acid pairs (for example Gln and Glu, and Asn and Asp). The method demonstrated excellent sensitivity, linearity, dynamic range, accuracy, reproducibility, and precision. Ion pairing liquid chromatography in combination with triple quadrupole MRM detection provides a valuable approach for quantitation of underivatized amino acids in the pharmaceutical industry and clinical laboratories.

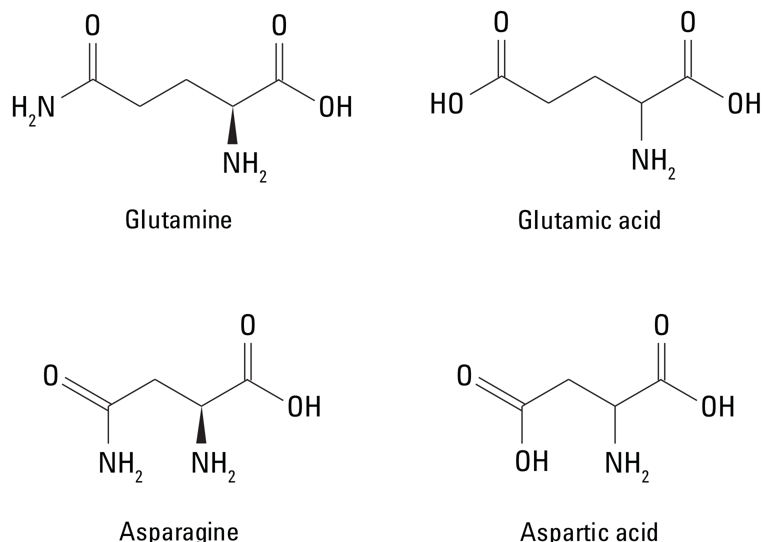


Figure 1. Underivatized glutamine, glutamic acid, asparagine, and aspartic acid.

Table 1. Four amino acid test compounds.

Amino acid name	Formula	Mass
Asparagine	$C_4H_8N_2O_3$	132.12
Aspartic acid	$C_4H_7NO_4$	133.10
Glutamine	$C_5H_{10}N_2O_3$	146.14
Glutamic acid	$C_5H_9NO_4$	147.13

Experimental

Sample Preparation

Neat calibration standards containing a mixture of the four amino acids were prepared in water from 5 to 100,000 nM (Table 2). Cell media calibration standards were prepared by spiking the four amino acids at varied concentrations (0.5 – 10,000 µM). Media A is RPMI 1640 without Gln and media B is media A + 5 % fetal bovine serum. Unknown samples 1 – 6 were prepared by spiking different levels of Gln in media A, while unknown samples 7 and 8 were prepared by spiking Gln in media B. The cell media calibration standards and the eight unknown cell media samples (100 µL) were extracted using ice cold acetonitrile (200 µL). After centrifugation, the supernatant (100 µL) was diluted 100 times in water before LC/MS/MS analysis.

Instrumentation

Liquid chromatography was performed on a 1290 Infinity UHPLC System consisting of a binary pump, vacuum degasser, high performance thermostatted autosampler, and a thermostatted column compartment. LC/MS/MS analysis was performed on a 6460 Triple Quadrupole LC/MS equipped with an Agilent Jet Stream source in positive ionization mode. Source conditions were optimized for quantitative analysis of amino acids (Table 3). The specific MRM transitions used for quantitation of Gln, Glu, Asn, and Asp and the optimized compound dependent MRM parameters, such as fragmentor voltage and collision energy, are summarized in Table 4.

Table 2. Calibration standard solutions of the four amino acids in water.

Level	Injection volumn (µL)	Concentration (nM)	Concentration (fmol on-column)
1	1	5	5
2	1	10	10
3	1	50	50
4	1	100	100
5	1	500	500
6	1	1,000	1,000
7	1	5,000	5,000
8	1	10,000	10,000
9	1	50,000	50,000
10	1	100,000	100,000

Table 3. Liquid chromatography and triple quadrupole MS source conditions.

LC Conditions			
Column	Agilent ZORBAX SB-C18 Rapid Resolution HB column, 3.0 × 50 mm, 1.8 µm (p/n: 829975-302)		
Column temperature	25 °C		
Injection volume	1 µL		
Autosampler temperature	4 °C		
Needle wash	10 seconds in wash port		
Mobile phase	A = 0.5 % formic acid and 0.3 % HFBA in water B = 0.5 % formic acid and 0.3 % HFBA in acetonitrile		
Flow rate	0.4 mL/min		
Gradient program	Time (min)	A (%)	B (%)
	Initial	100	0
	5.00	95	5
	5.01	10	90
	6.00	10	90
	6.01	100	0
Post time	1 min		
Triple quadrupole MS source conditions			
Ion mode	Positive		
Drying gas temperature	275 °C		
Drying gas flow	9 L/min		
Sheath gas temperature	325 °C		
Sheath gas flow	12 L/min		
Nebulizer pressure	40 psi		
Capillary voltage	3750 V		
Nozzle voltage	0 V		
Delta EMV	0 V		

Data acquisition and analysis

A MassHunter Workstation (version B.03.01) was used for data acquisition. MassHunter Qualitative Analysis (version B.03.01) and Quantitative Analysis Software (version B.04.00) were used for data processing. The two most abundant MRM transitions were selected for each analyte as quantifier and qualifier ions, the ratio of which was used as confirmatory evidence for the analyte of interest in biological matrices.

Results and Discussion

As demonstrated in Figure 2, the four amino acids were well separated with retention times of 3.00, 3.48, 3.89, and 4.61 minutes for Asn, Asp, Gln, and Glu, respectively. 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) from Asp (*m/z* 134.1 > 74.1). This significantly improved the quantitation performance, for example sensitivity, linearity, and accuracy.

Table 4. Agilent triple quadrupole MRM acquisition method parameters.

Compound name	Precursor ion	MS1 resolution	Product ion	MS2 resolution	Fragmentor (V)	CE (V)
Asparagine	133.1	Unit	74.1	Unit	74	10
Asparagine	133.1	Unit	87.1	Unit	74	4
Aspartic acid	134.0	Unit	74.0	Unit	80	10
Aspartic acid	134.0	Unit	88.0	Unit	80	5
Glutamine	147.1	Unit	84.1	Unit	80	15
Glutamine	147.1	Unit	130.1	Unit	80	5
Glutamic acid	148.1	Unit	84.1	Unit	80	14
Glutamic acid	148.1	Unit	130.1	Unit	80	4

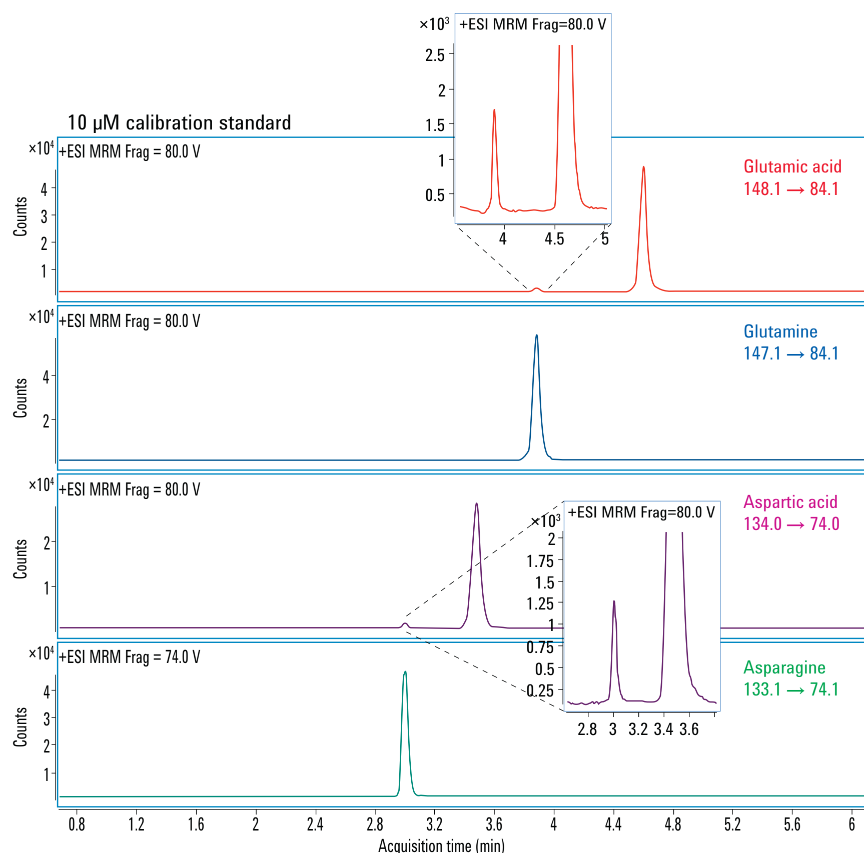


Figure 2. MRM chromatograms of the four amino acids.

Sensitivity

The limits of detection (LOD) are 5, 10, 5, and 10 nM, or 5, 10, 5, and 10 fmol on-column for Asn, Asp, Gln, and Glu, respectively, with a signal-to-noise ratio of > 3:1 (Figure 3). The limits of quantitation (LOQ) are 5, 10, 5, and 50 nM, or 5, 10, 5, and 50 fmol on-column for Asn, Asp, Gln, and Glu, respectively, with a signal-to-noise ratio of > 5:1 (Figure 3). As illustrated by Figure 3, excellent reproducibility (% RSD < 5 from triplicate results) of both retention time and peak area response was obtained at the LOQ levels. Since the four amino acids have their endogenous concentrations in cell media, LOD and LOQ levels are not evaluated in cell media.

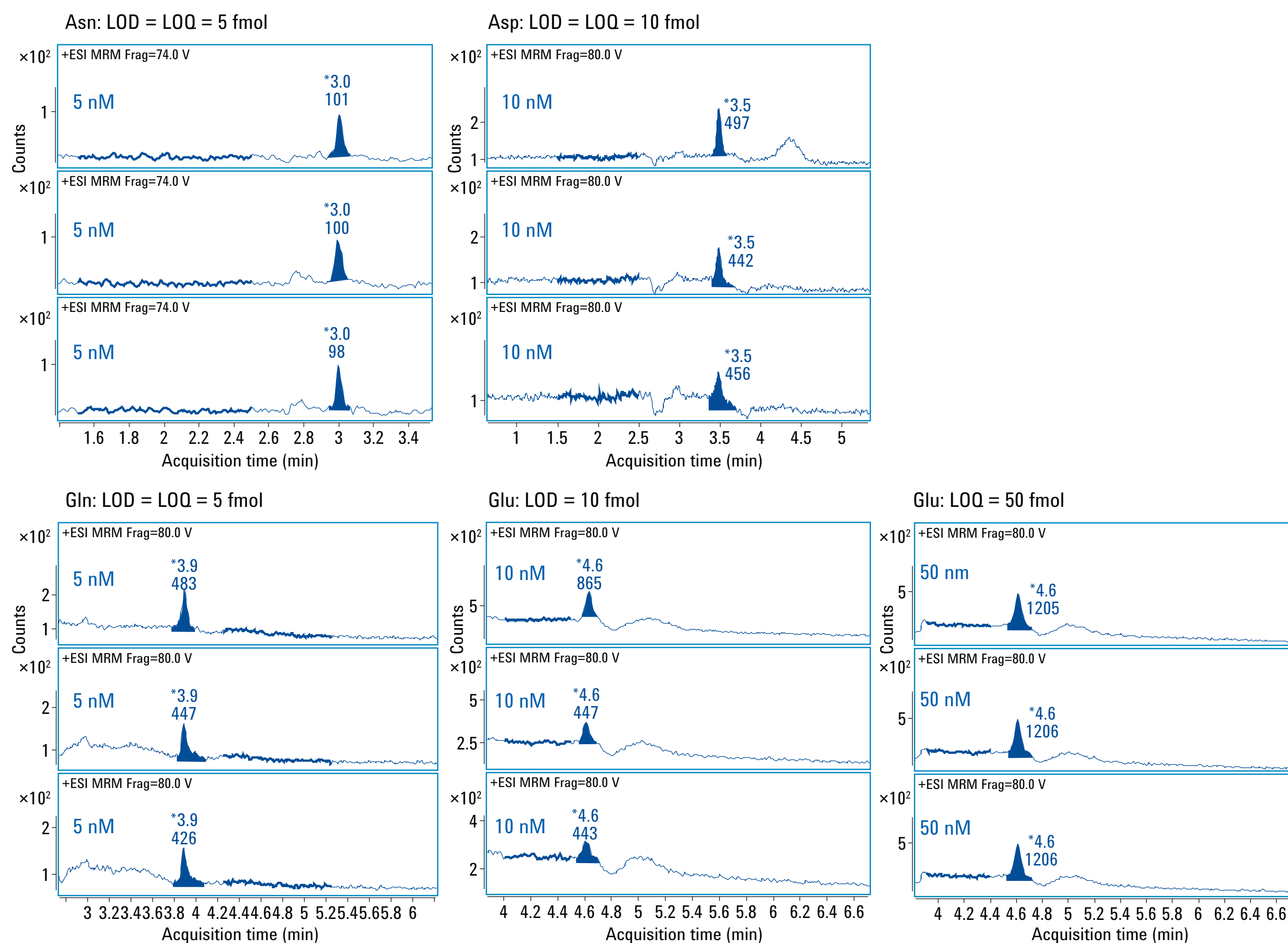
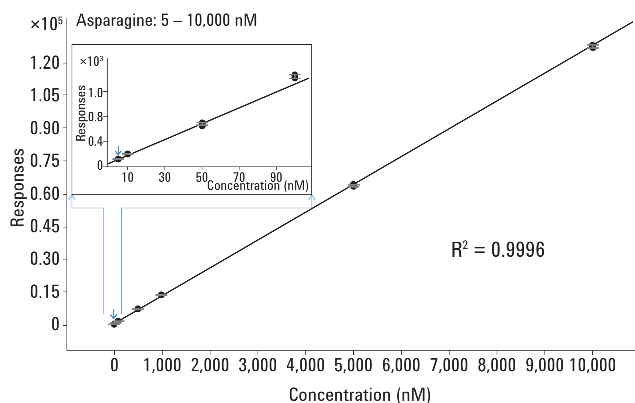
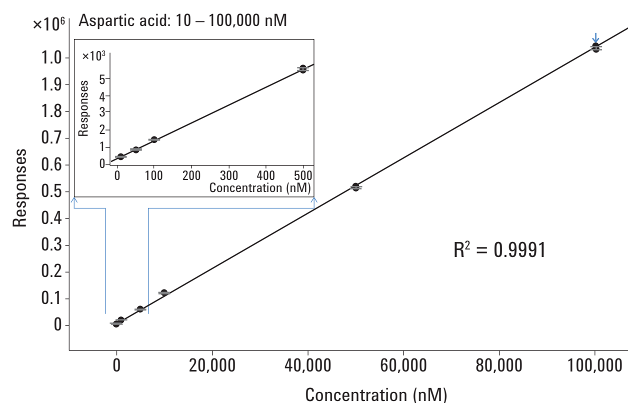


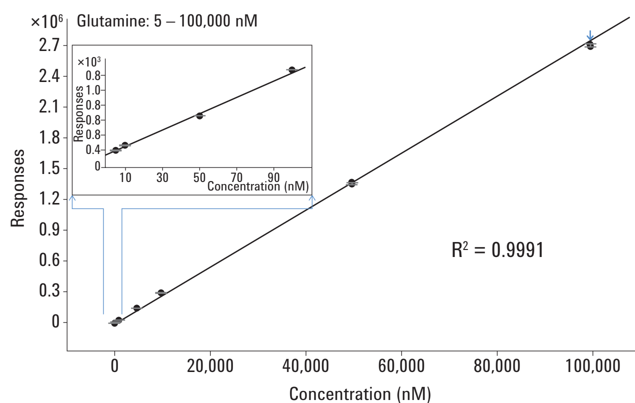
Figure 3. MRM chromatograms of Asn, Asp, Gln, and Glu at their LOD and LOQ levels (3 replicate injections).

Calibration curve linearity and range

The calibration curves for the four amino acids in water (Figure 4) and in cell media (Figure 5) show excellent linearity ($R^2 > 0.999$) and wide dynamic range (≥ 3 orders). Notably, the dynamic range for Gln in water is greater than 4 orders of magnitude (5 – 100,000 nM). The inserts in Figure 4 demonstrate the excellent detection accuracy and reproducibility even at low concentration levels (that is LOQ levels indicated in the previous section).







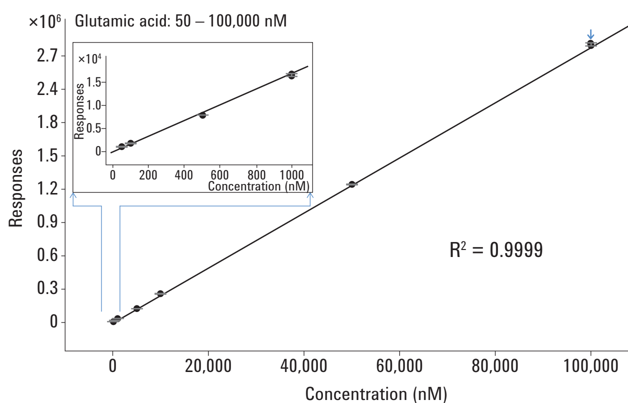
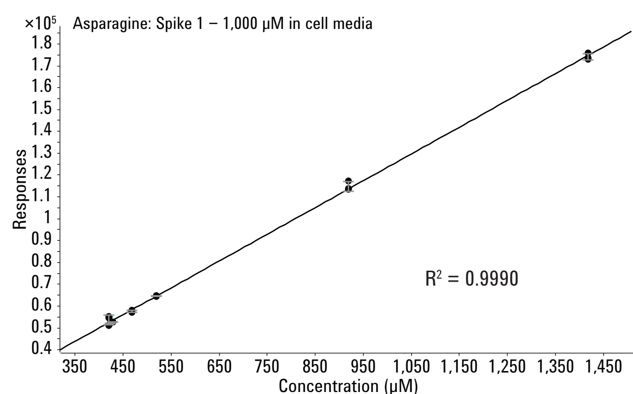
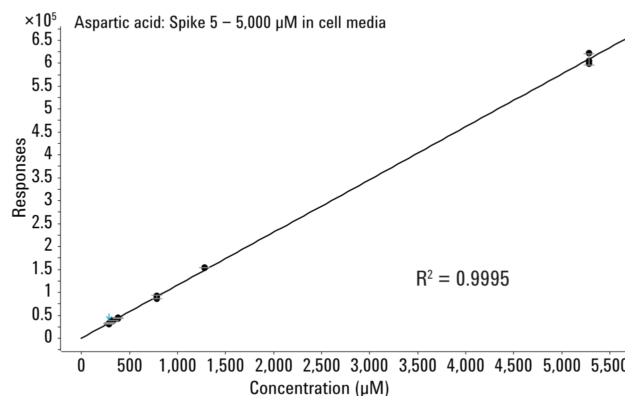


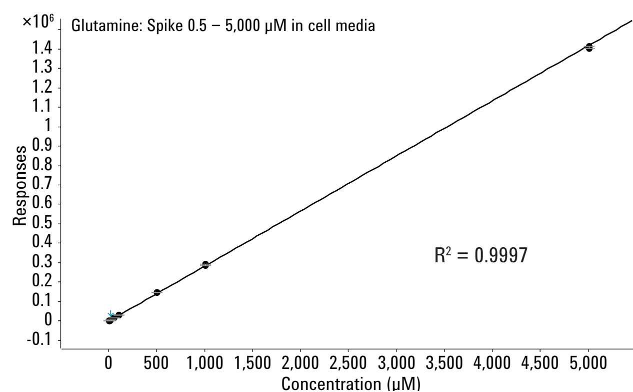
Figure 4. Calibration curves of Asn, Asp, Gln, and Glu in water. Inserts demonstrate low concentration range.

Accuracy, reproducibility, and precision

The accuracy, reproducibility, and precision were evaluated for each amino acid at nine to ten standard concentrations. The results obtained from analytes in water and in cell media are summarized in Table 5. Comparable accuracy, reproducibility, and precision were achieved for both neat and cell media standards, demonstrating excellent quantitation performance of the LC/MS/MS method in biological matrices.







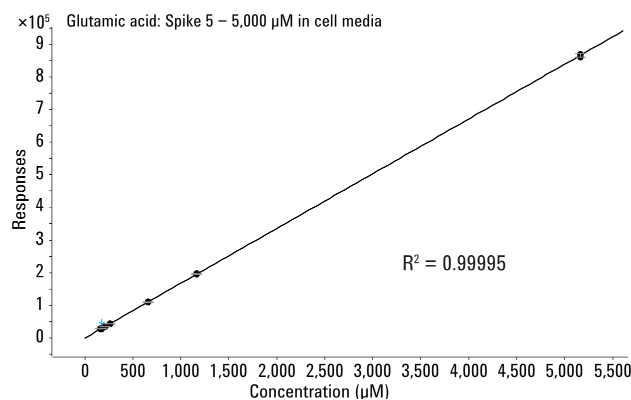


Figure 5. Calibration curves of Asn, Asp, Gln, and Glu in cell media (RPMI 1640 without Gln).

Table 5. Accuracy, reproducibility, and precision results in water and in cell media.

	Water			Cell media		
Amino acid name	Accuracy (%)	Reproducibility (% RSD, n = 3)	Precision (%RSD, n =10)	Accuracy (%)	Reproducibility (% RSD, n = 3)	Precision (% RSD, n =9)
Asparagine	84.4 – 107.0	0.55 – 4.27	7.23	98.6 – 102.6	0.03 – 4.07	1.53
Aspartic acid	92.4 – 112.8	0.48 – 1.98	8.63	94.1 – 103.6	0.09 – 3.90	2.82
Glutamine	86.7 – 109.7	0.14 – 1.09	8.03	94.7 – 105.2	0.23 – 5.42	4.59
Glutamic acid	82.0 – 109.6	0.22 – 3.54	5.61	98.3 – 101.1	0.13 – 0.75	1.08

Quantitation of amino acids in cell media samples

The LC/MS/MS method was successfully implemented to measure the concentrations of the four amino acids in media A, media B, and the eight unknown cell media samples (Figures 6 & 7). The measured amino acid concentrations in the unknown samples were very consistent with previously spiked values. The results in Table 6 demonstrate excellent reproducibility (% RSD < 5 %).

Table 6. Measured concentration in cell media samples.

	Asparagine		Aspartic acid		Glutamine		Glutamic acid	
Media A and unknowns	Conc. (µM)	%RSD (n = 3)	Conc. (µM)	%RSD (n = 3)	Conc. (µM)	%RSD (n = 3)	Conc. (µM)	%RSD (n = 3)
Media A	397.8	0.9	270.3	3.0	7.3	0.9	174.0	0.3
1	393.3	0.5	260.8	0.7	13.0	0.3	167.0	0.1
2	395.4	4.1	254.6	1.2	7.0	0.4	164.5	1.4
3	390.3	2.9	264.7	0.5	1071.3	1.4	173.0	1.4
4	391.2	2.7	264.2	0.9	6.8	0.1	168.8	0.6
5	397.9	1.9	267.5	1.0	6.9	0.6	170.7	0.3
6	407.7	4.6	257.2	4.3	271.3	2.4	171.7	0.6
	Asparagine		Aspartic acid		Glutamine		Glutamic acid	
Media B and unknowns	Conc. (µM)	%RSD (n = 3)	Conc. (µM)	%RSD (n = 3)	Conc. (µM)	%RSD (n = 3)	Conc. (µM)	%RSD (n = 3)
Media B	326.8	1.8	241.8	1.1	26.3	1.8	179.5	1.6
7	334.0	1.0	244.5	1.1	3930.8	1.0	199.0	1.9
8	347.6	0.5	256.6	1.2	29.9	1.6	199.1	1.2

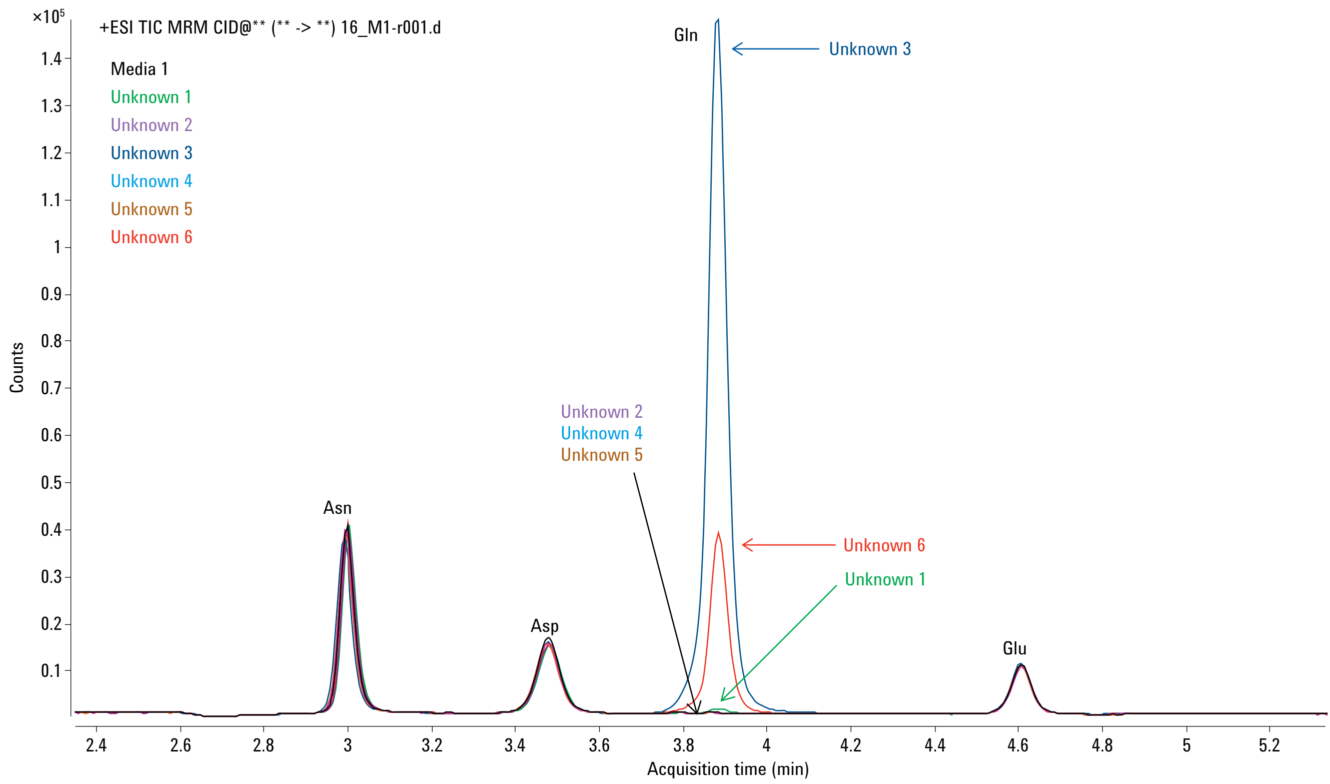


Figure 6. Overlaid LC/MS/MS chromatograms of media A and unknown 1 – 6.

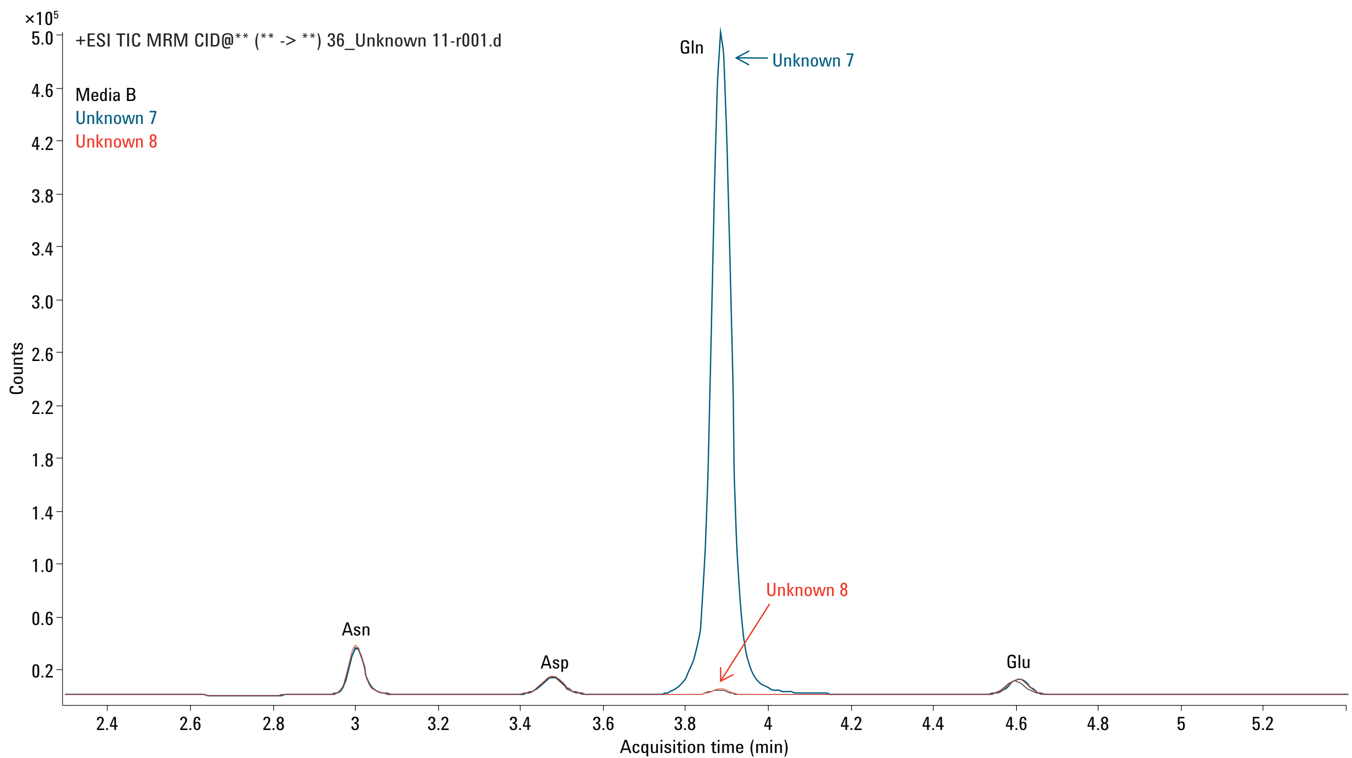


Figure 7. Overlaid LC/MS/MS chromatograms of media B and unknown 7 and 8.

Conclusions

This application note describes a rapid and sensitive LC/MS/MS method for the quantitation of amino acids in complex biological samples without derivatization. Baseline separation of Asn, Asp, Gln, and Glu was achieved using ion paring chromatography. The LC/MS/MS method demonstrates excellent sensitivity with an LOQ of low-fmol level on column. Great linearity (> 0.999), dynamic range (≥ 3 orders), accuracy (82 – 113 %), precision (< 6 %), and reproducibility (< 9 %) were observed for all four amino acids.

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