Application Note

Food Testing, Metabolomics, Agricultural Chemistry, Environmental

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Quantitative Analysis of Underivatized Amino Acids in Plant Matrix by Hydrophilic Interaction Chromatography (HILIC) with LC/MS Detection

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Abstract

This Application Note presents a method for quantitative analysis of a wide range of underivatized amino acids by triple quadrupole LC/MS. Sensitivity, linearity, and recovery are shown to be excellent, even in the presence of significant amounts of plant matrix (cucumber plant tissue extract).

Hydrophilic interaction chromatography (HILIC) was used to separate the highly polar amino acids. The next generation Agilent InfinityLab Poroshell 120 HILIC-Z phase was used to take advantage of its excellent resolution and peak shape under LC/MS friendly conditions.

Introduction

A wide range of amino acids are present in plant material at parts-per-million (ppm) and parts-per-billion (ppb) levels. As previously found[1], underivatized amino acids can be analyzed with excellent resolution and sensitivity using hydrophilic interaction chromatography (HILIC) mode with low pH solvents and positive mode MS detection. These conditions were further optimized in this Application Note for quantitative analysis of amino acids in plant matter.

Reagents and chemicals

All reagents were HPLC grade or higher. Ultra LC/MS grade acetonitrile was bought from J.T. Baker (Center Valley, PA, U.S.A.). Water was purified using an EMD Millipore Milli-Q Integral System (Darmstadt, Germany). Reagent-grade formic acid (FA, p/n G2453-85060) was from Agilent Technologies. Ammonium formate and amino acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amino acids were stored at –70 °C until day of use.

- Agilent InfinityLab fittings
  - **Column front:** Agilent InfinityLab Quick Connect LC fitting (p/n 5067-5965)
  - **Column back:** Agilent InfinityLab Quick Turn LC fitting (p/n 5067-5966)
- Agilent vial, screw top, amber, write-on spot, certified, 2 mL (p/n 5182-0716)
- Agilent bonded screw cap, PTFE/red silicone septa (p/n 5190-7024)
- Eppendorf pipettes and repeater
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- Agilent InfinityLab solvent bottle, amber, 1,000 mL (p/n 9301-6526)
- Agilent InfinityLab Stay Safe cap, GL45, 3 ports, 1 vent valve (p/n 5043-1219)
- Centrifuge (VWR, Radnor, PA, USA)
- Sonicator (VWR, Radnor, PA, USA)

Instrumentation

- Agilent 1260 Infinity binary pump (G1312B)
- Agilent 1260 Infinity autosampler (G7129A)
- Agilent 1260 Infinity thermostatted column compartment (G1316A)
- Ultralow dispersion kit for Agilent 1290 Infinity LC Series (5067-5189)
- Agilent MassHunter workstation software
- Agilent 6470 triple quadrupole LC/MS
- Agilent Jet Stream Electrospray ionization source

Sample preparation

The amino acid standards were mixed to stated concentrations in water, and analyzed with no further sample preparation.

The following deuterated and ^{15}N-enriched internal standards (ISTD) were added to each sample to a final concentration of 1,500 ng/mL: isoleucine-$^{15}N_1$, methionine-d_8, alanine-d_3, glycine-d2, glutamic acid-$^{15}N_1$, aspartic acid-d_3, and lycine-d_8.

Cucumber plant tissue extracts were prepared from freshly harvested cucumber leaves, which were immediately placed in liquid nitrogen for rapid freezing. The frozen cucumber tissues were homogenized in liquid nitrogen into a fine powder using a mortar and pestle, then stored in a freezer

Instrument conditions

HPLC Conditions	
Column	Agilent InfinityLab Poroshell 120 HILIC-Z 2.1 × 100 mm, 2.7 µm, (p/n 685775-924)
Mobile phase A	20 mM ammonium formate in water at pH = 3
Mobile phase B	20 mM aqueous ammonium formate at pH = 3 in 9:1 acetonitrile/water
Flow rate	0.50 mL/min
Column temperature	25 °C
Injection volume	1 µL
Total run time	15 minutes
Gradient	Time (min) %B 0 100 11.5 70 12 100
MS Conditions	
Ionization mode	ESI Positive
Gas temperature	330 °C
Gas flow	13.0 L/min
Nebulizer	35 psi
Sheath gas temperature	390 °C
Sheath gas flow	12 L/min
Capillary voltage	1,500 V
Nozzle voltage	0 V

at −85 °C. For extraction, 100 mg of frozen cucumber leaf powder was weighed into 2-mL Eppendorf microcentrifuge tubes, and 1 mL of 0.5 M aqueous HCl was added. The tubes were vortexed at 8,000 rpm for 20 minutes, sonicated in a 25 °C water bath for 20 minutes, then centrifuged at 20,000 g for 20 minutes. Finally, 250 µL of the extraction supernatant was transferred into LC vials with ISTD already added, and the mixture was diluted to 1 mL with 20 % water in ACN.

Data collection

Index	Start time (min)	Scan type	Ion mode	Diverter valve	Delta EMV	Store
1	0	dMRM	ESI + Agilent Jet Stream	To waste	0	No
3	2.5	dMRM	ESI + Agilent Jet Stream	To MS	200	Yes

For spike recovery tests, the amino acid standard mix was added to the LC vials along with the extraction supernatant and ISTD mixture before being diluted to 1 mL with 20 % water in ACN. The total increase in amino acid concentration after dilution was 500, 1,000, and 2,000 ng/mL.

Mobile phase preparation

A 200 mM ammonium formate stock solution was prepared in water and adjusted to pH 3 with formic acid. Mobile phase A (aqueous) was prepared by diluting the stock solution 9:1 in water, and mobile phase B (organic) was prepared by diluting the stock solution 9:1 in acetonitrile (final ionic strength of both mobile phases = 20 mM).

MS Parameters

Compound	Retention time (min)	Precursor ion (m/z)	Product ions				
			Quant ion (m/z)	Collision energy (V)	Qual ion (m/z)	Collision energy (V)	Fragmentor (V)
Amino acids							
Phenylalanine	2.95	166.1	120.1	13	103	29	80
Leucine	3.38	132.1	86.1	9	30.2	17	75
Tryptophan	3.41	205.1	188.0	8	146	20	80
Isoleucine	3.75	132.1	86.1	9	44.2	25	75
Methionine	4.22	150.1	104.0	9	56.1	17	75
Valine	4.95	118.1	72.1	9	55.1	25	70
Proline	4.96	116.1	70.1	17	43.2	37	75
Tyrosine	5.01	182.1	136.1	13	91.1	33	85
Cysteine	5.63	122.0	59.1	29	76	13	65
Alanine	6.61	90.1	44.2	9	45.3	40	40
Threonine	6.72	120.1	74.1	9	56.1	17	75
Homoserine	6.91	120.1	74.1	9	56.1	21	70
Glycine	7.00	76.0	30.3	12	NA	NA	35
Glutamine	7.23	147.1	84.1	17	130.1	9	80
Serine	7.26	106.1	88.1	8	42.2	24	67
Asparagine	7.31	133.1	87.1	5	74	17	75
Glutamic acid	7.68	148.1	84.1	17	130	5	75
Citrulline	7.89	176.1	159.1	9	70.1	25	80
Aspartic acid	8.38	134.0	88.1	9	74	13	70
Histidine	9.06	156.1	110.1	13	83.1	29	90
Arginine	9.54	175.1	70.1	24	60.1	12	100
Lysine	10.16	147.1	84.1	17	130.1	9	75
Internal standards							
Isoleucine-$^{15}N_1$	3.75	133.1	87.1	8	NA	NA	75
Methionine-d_8	4.26	158.1	112.1	8	NA	NA	75
Alanine-d_3	6.61	93.1	47.2	12	NA	NA	40
Glycine-d_2	7.00	78.1	32.2	12	NA	NA	40
Glutamic acid-$^{15}N_1$	7.68	149.1	85.1	16	NA	NA	75
Aspartic acid-d_3	8.37	137.1	75.0	16	NA	NA	60
Lysine-d_8	10.16	155.2	92.1	20	NA	NA	80

Results and Discussion

Chromatography

The excellent peak shape and resolving power of the Agilent InfinityLab Poroshell 120 2.7 µm HILIC-Z allowed the complex mixture of amino acids to be separated in under 15 minutes (Figures 1 and 2). Baseline separation was also achieved for both the leucine/isoleucine isobars and threonine/homoserine isobars (Figure 3).

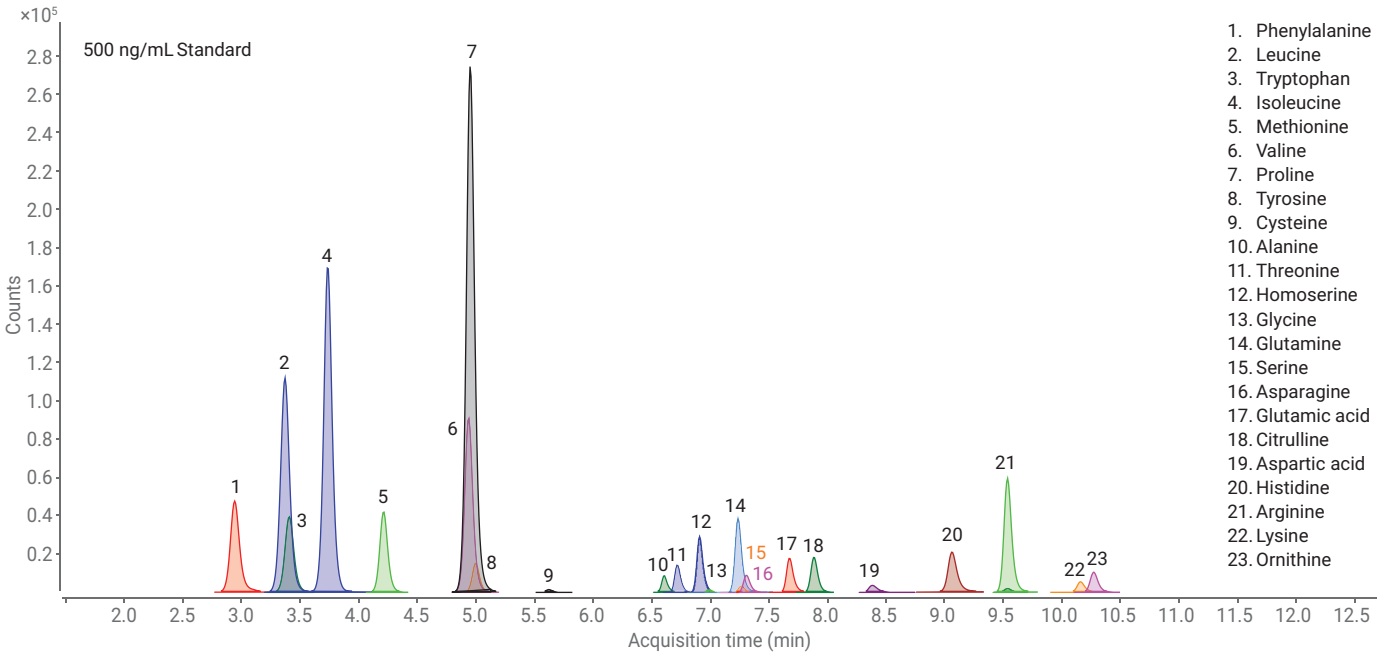

Figure 1. Amino acid standards at a concentration of 500 ng/mL.

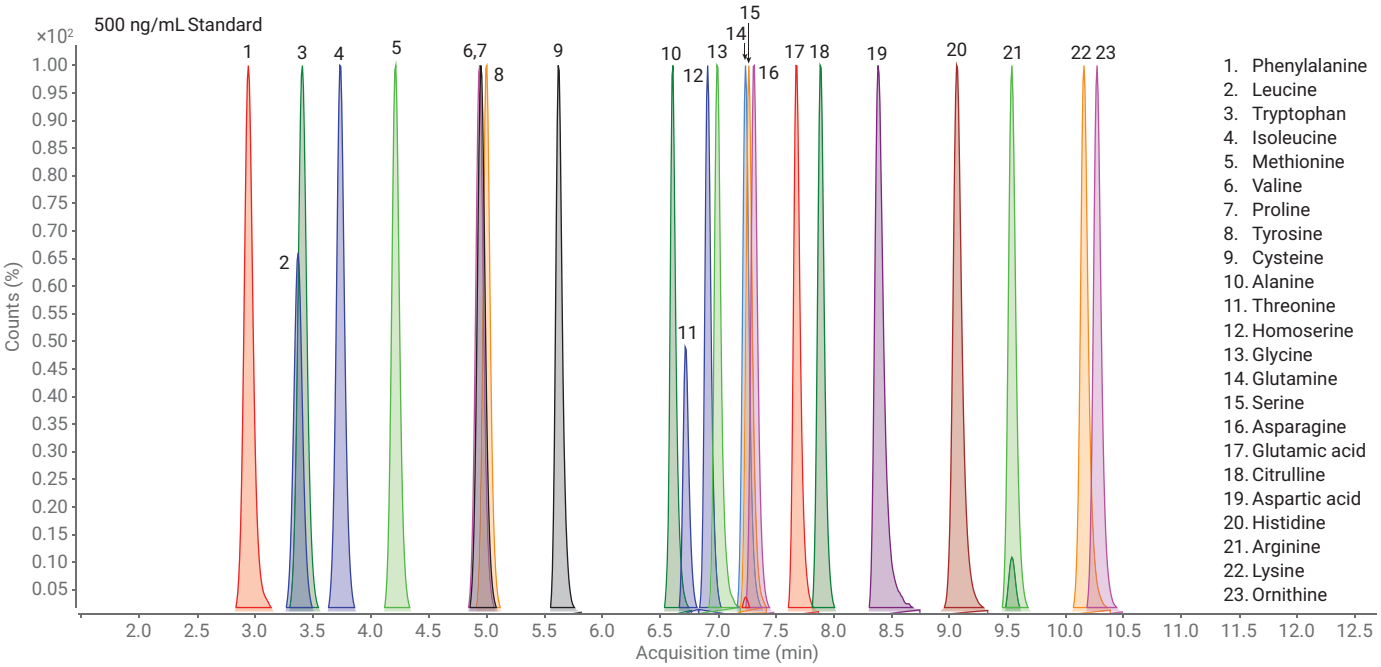

Figure 2. Amino acid standards from Figure 1, with peak heights normalized.

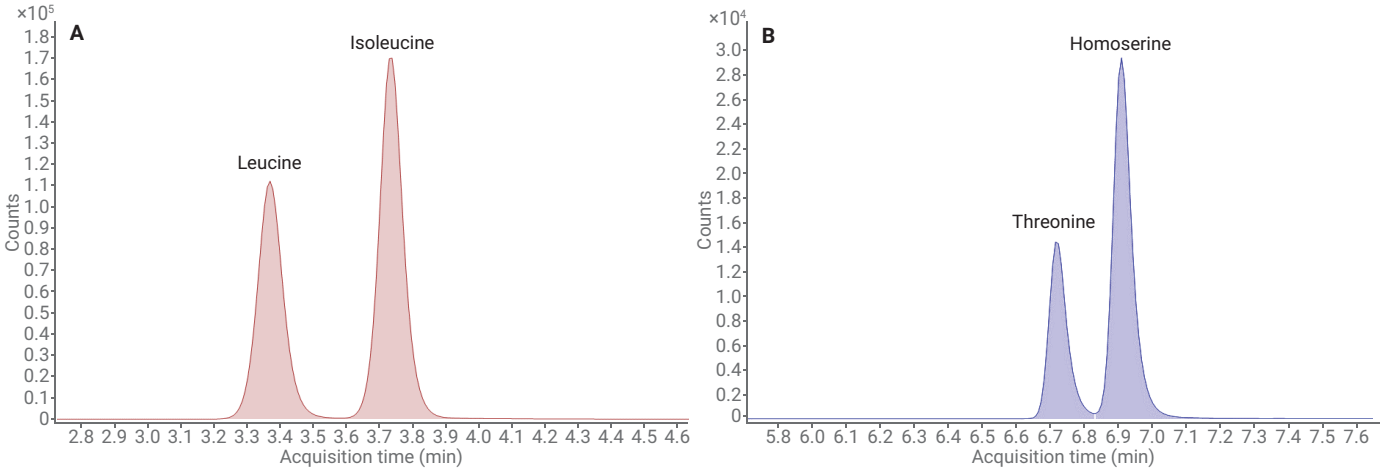

Figure 3. Separation of isobars leucine/isoleucine and threonine/homoserine.

Quantitative analysis

Quantitation of each amino acid was accomplished with a combination of seven-point calibration curves from 50–10,000 ng/mL, and comparison against closely eluting isotope-labeled internal standards (Figure 4). Relative standard deviation (RSD) for response and retention time showed excellent reproducibility from run to run (Table 2).

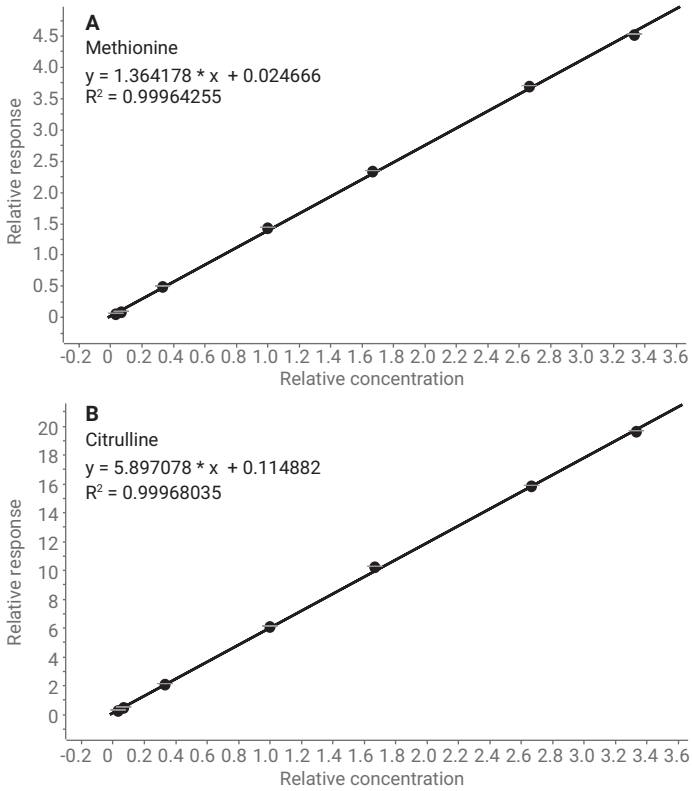

Figure 4. Example calibration curves for methionine and citrulline.

Table 1. Calibration and signal-to-noise (S/N).

Compound	Internal standard	R^2 for calibration curve (50–10,000 ng/mL)	S/N at 50 ng/mL
Phenylalanine	Isoleucine-$^{15}N_1$	0.9999	281.2
Leucine	Isoleucine-$^{15}N_1$	0.9979	244.4
Tryptophan	Isoleucine-$^{15}N_1$	0.9999	748.7
Isoleucine	Isoleucine-$^{15}N_1$	0.9978	379.4
Methionine	Methionine-d_8	0.9996	2315.6
Valine	Alanine-d_3	0.9981	349.2
Proline	Alanine-d_3	0.9983	861.3
Tyrosine	Alanine-d_3	0.9987	161.4
Cysteine	Alanine-d_3	0.9999	121.8
Alanine	Alanine-d_3	0.9994	94.6
Threonine	Alanine-d_3	0.9987	162.0
Homoserine	Alanine-d_3	0.9969	336.6
Glycine	Glycine-d_2	0.9999	10.8
Glutamine	Glycine-d_2	0.9993	30.8
Serine	Glycine-d_2	0.9957	3.4
Asparagine	Glycine-d_2	0.9994	39.7
Glutamic acid	Glutamic acid-$^{15}N_1$	0.9978	53.9
Citrulline	Aspartic acid-d_3	0.9997	106.5
Aspartic acid	Aspartic acid-d_3	0.9999	32.1
Histidine	Aspartic acid-d_3	0.9961	35.6
Arginine	Aspartic acid-d_3	0.9972	184.6
Lysine	Lysine-d_8	0.9992	37.2

A spike recovery test was performed to verify robustness in the presence of matrix. A sample of cucumber tissue was analyzed, followed by the same sample spiked with 500, 1,000, and 2,000 ng/mL of amino acid standard. The recovery at these different levels is shown as the percentage of measured concentration versus the theoretical value (Table 3).

Table 2. Reproducibility of n = 15 samples of cucumber plant tissue extract spiked with 2,500 ng/mL of amino acids.

Compound (2,500 ng/mL)	Response RSD (%)	RT RSD (%)
Phenylalanine	0.99	0.35
Leucine	2.97	0.43
Tryptophan	0.65	0.24
Isoleucine	2.08	0.37
Methionine	0.96	0.35
Valine	1.21	0.73
Proline	1.04	0.58
Tyrosine	8.77	0.69
Cysteine	1.25	0.07
Alanine	3.76	0.00
Threonine	1.51	0.10
Homoserine	1.20	0.00
Glycine	1.82	0.06
Glutamine	0.73	0.06
Serine	1.08	0.08
Asparagine	1.38	0.00
Glutamic acid	1.19	0.00
Citrulline	1.29	0.05
Aspartic acid	2.77	0.13
Histidine	0.70	0.06
Arginine	0.68	0.09
Lysine	1.02	0.06
Ornithine	0.63	0.08

Table 3. Recovery tests for cucumber tissue extract spiked with the amino acid standard mix.

Amino acid	Pre-spike concentration (ng/mL)	% Recovery (500 ng/mL spike)	% Recovery (1,000 ng/mL spike)	% Recovery (2,000 ng/mL spike)
Phenylalanine	1078.36	102	103	102
Leucine	2216.61	119	117	114
Tryptophan	1218.47	125	126	114
Isoleucine	493.07	100	99	96
Methionine	223.24	77	81	77
Valine	1445.24	105	111	93
Proline	524.56	91	102	98
Tyrosine	704.05	101	105	93
Cysteine	336.61	94	109	115
Alanine	5046.64	70	70	89
Threonine	923.93	101	105	96
Homoserine	157.02	92	105	103
Glycine	293.78	101	92	90
Glutamine	1861.98	139	120	124
Serine	1794.39	110	100	93
Asparagine	1145.53	103	92	93
Glutamic acid	7358.15	114	79	92
Citrulline	<MDL	87	86	85
Aspartic acid	6465.33	107	91	92
Histidine	4130.88	118	103	127
Arginine	1083.89	96	91	93
Lysine	2380.70	92	92	94
Ornithine	<MDL	108	138	160

MDL- Method Detection Level

Conclusions

Underivatized amino acids were quantitated in plant tissue to trace levels by combining the excellent resolution and peak shape of the Agilent InfinityLab Poroshell 120 HILIC-Z column with the powerful Agilent 6470 Triple Quadrupole LC/MS. The method is shown to be quantitative, reproducible, and robust even in matrix.

Reference

1. A. Kennedy, A. Bivens. Methods for the Analysis of Underivatized Amino Acids by LC/MS. *Agilent Technologies Application Note*, publication number 5991-8582EN, **2017**.

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This information is subject to change without notice.

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