Application Note

Food Testing and Agriculture

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Trusted Answers

Routine Quantitation of 17 Underivatized Amino Acids by LC/MS

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Abstract

The global nutraceuticals market was valued at 493.1 billion US dollars as of 2022. This market is expected to grow at a rate of 9.1%, reaching a market value of 991 billion by 2030. Amino acids are the essential ingredients of many nutraceuticals. Verification of the amino acid composition on the product label is crucial in this industry. Historic methods using only HPLC required a laborious derivatization step. The method presented here provides for the cost-effective and rapid quantitative analysis of 17 underivatized amino acids in one injection using an Agilent InfinityLab LC/MSD iQ. The working ranges were selected to be those most useful to the industry and all were linear. Good accuracy and precision were observed, both in the solvent and in a matrix.

Introduction

The valuation of the amino acid supplement market is expected to be 8.1 billion US dollars in 2023 and is anticipated to reach a high of 19.6 billion by the year 2033. Key factors that are anticipated to increase the sales of supplemental amino acids include an aging population in advanced nations, rising health consciousness, and consumer expenditure on dietary supplements, which has been rising quickly.[1]

There are over 700 types of amino acids known in nature. Almost all of these types are alpha-amino acids and have been found in bacteria, fungi, plants, and animals.[2]

The 20 amino acids that comprise the building blocks of proteins are necessary for life. Of these 20, nine are considered essential amino acids (EAA) since they cannot be produced in the human body and must be obtained from food sources. The group of essential amino acids known as branched chain amino acids (BCAAs) consist of L-leucine, L-isoleucine, and L-valine and all have been shown to have an anabolic effect on muscle protein synthesis. These three essential amino acids constitute nearly 40% of the daily requirement for all essential amino acids.[3]

L-leucine helps to regulate blood sugar levels and promotes growth and recovery of muscle and bone tissue. L-leucine is obtained by the consumption of a protein rich diet (fish, chicken, beef, dairy, and eggs). L-isoleucine, an isomer of L-leucine, acts in much the same way as leucine and also participates in hemoglobin synthesis. Isoleucine is also thought to increase endurance and aid in the repair of tissues after injury or surgery. Isoleucine must also be obtained through a diet of protein rich foods.

L-valine is important for the nervous system and cognitive function. Valine inhibits the transport of tryptophan across the blood-brain barrier, which has been shown to alleviate insomnia and nervousness. L-valine is important in the alleviation of muscle disorders and the regulation of the immune system. L-valine, unlike some other amino acids, is not processed by the liver. It can be obtained in a diet of kidney beans, leafy vegetables, poultry, and milk.

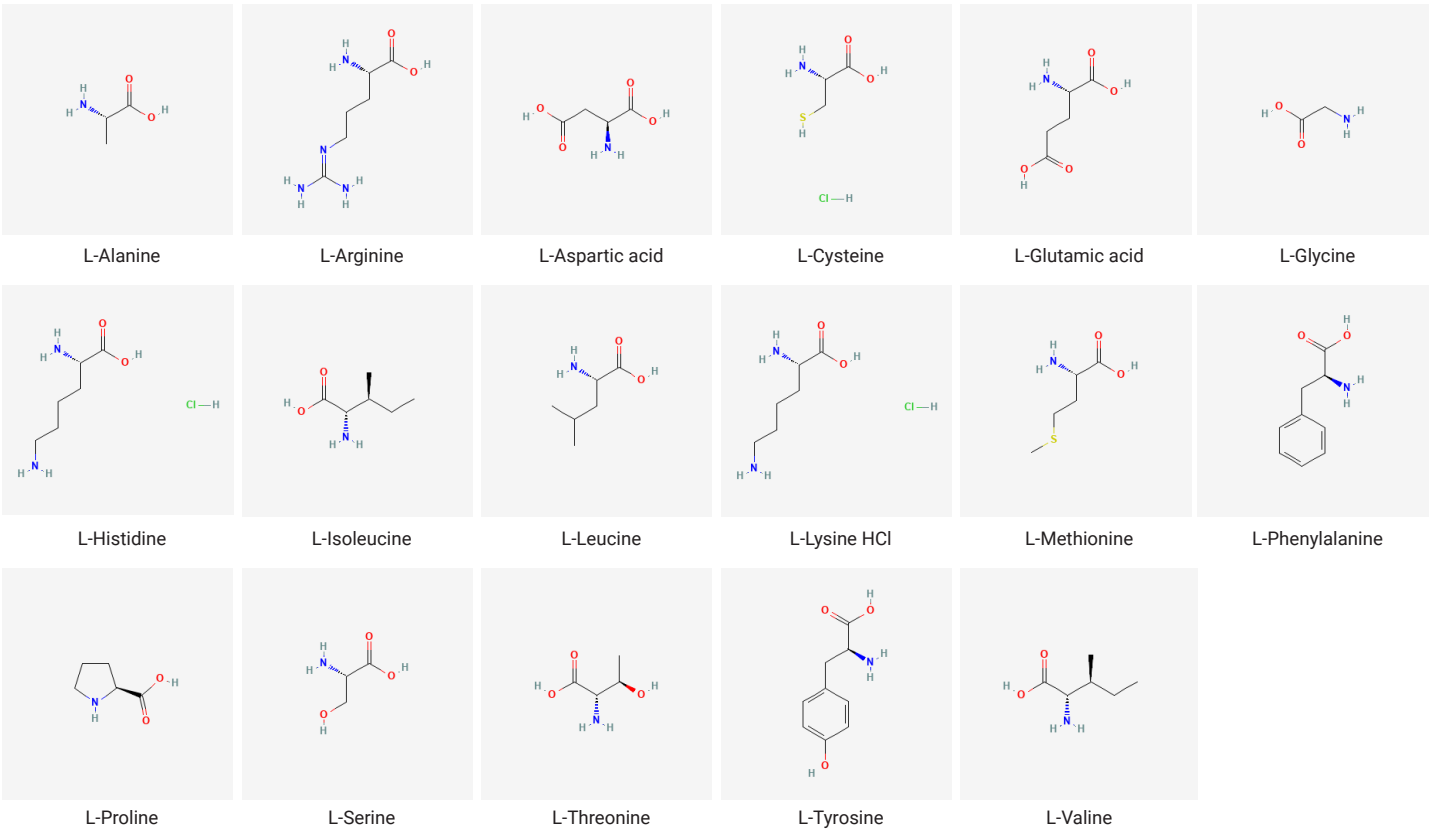


Figure 1. Structures for the 17 amino acids used in this study.

Experimental

Equipment

All experiments in this study were performed using an Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II multisampler (G7104A), an Agilent 1290 Infinity flexible pump (G7120A), and an Agilent 1290 Infinity II multicolumn thermostat column compartment (G7116B) coupled to an Agilent InfinityLab LC/MSD iQ (G6160A) mass spectrometer. The system was controlled by Agilent OpenLab CDS software, version 2.6. Data processing was performed using the same OpenLab CDS software.

Samples, standards, and consumables

- Agilent HILIC-Z column, 3.0 × 150 mm, 2.7 µm
- Agilent amino acid standards, part numbers 5061-3331, 5061-3332, 5061-3333, and 5061-3334
- HPLC grade acetonitrile
- Formic acid
- Ammonium formate

Method

Table 1. Chromatographic conditions.

Parameter	Setting
Analytical Column	Agilent HILIC-Z, 3 × 150 mm, 2.7 µm (p/n 683975-324)
Column Oven	25.0 °C
Injection Volume	1 µL
Run Time	14.00 min
Postrun Time	8.00 min
Mobile Phase Flow Rate	0.6 mL/min

Table 2. Solvent composition and gradient.

Channel	Solvent
A	0.1% Aqueous formic acid
B	Acetonitrile
C	Not used
D	10 mM Aqueous ammonium formate

Table 3. HPLC gradient timetable.

Timetable						
Time (min)	%A	%B	%C	%D	Flow	Pressure
0	5	90	0	5	0.600 mL/min	1,300 bar
5	15	70	0	15		
6	20	60	0	20		
7	40	30	0	30		
9	45	10	0	45		
10	45	10	0	45		

Table 4. Mass spectrometer source parameters.

Source Parameter		
Parameter Id	Positive Value	Negative Value
Gas Temperature (°C)	325	325
Gas Flow (L/min)	11	11
Nebulizer (psi)	50	50
Capillary Voltage (V)	3,500	3,500

Table 5. Single ion monitoring (SIM) parameters.

Compound Name	Internal Standard (ISTD)	Mass (*m/z*)	Quadrupole Resolution	Dwell (MS)	Fragmentor (V)	Polarity	Detector Gain Factor	Ion Mode
L-Alanine	No	90.1	Unit	55	90	+	1	ESI
L-Arginine	No	175.1	Unit	55	105	+	1	ESI
L-Aspartic Acid	No	134.1	Unit	55	75	+	1	ESI
L-Cysteine	No	241	Unit	55	105	+	1	ESI
L-Glutamic Acid	No	148.1	Unit	55	85	+	1	ESI
L-Glycine	No	76	Unit	55	120	+	1	ESI
L-Histidine $HCl \cdot H_2O$	No	210	Unit	55	95	+	1	ESI
L-Isoleucine	No	132.1	Unit	55	85	+	1	ESI
L-Leucine	No	132.1	Unit	55	85	+	1	ESI
L-Lysine HCl	No	183.1	Unit	55	95	+	1	ESI
L-Methionine	No	150	Unit	55	75	+	1	ESI
L-Phenylalanine	No	166.1	Unit	55	85	+	1	ESI
L-Proline	No	116.1	Unit	55	85	+	1	ESI
L-Serine	No	106.1	Unit	55	65	+	1	ESI
L-Threonine	No	120	Unit	55	75	+	1	ESI
L-Tyrosine	No	182.1	Unit	55	95	+	1	ESI
L-Valine	No	118	Unit	55	75	+	1	ESI

Table 6. Amino acids standards preparation. Amino acids certified reference materials at 10, 100, 250, and 1,000 pmol/µL were used as-is. The load-on-column method was used (Table 7) to generate a calibration curve for each of the 17 amino acids studied.

Standard Concentration (pmol/µL)	Injection Volume (µL)	Equivalent Concentration (pmol/µL)
10	0.5	5
	1.0	10
100	0.5	50
	1.0	100
250	0.5	125
	1.0	250
1,000	0.5	500
	1.0	1,000

Results and discussion

Amino acids in water – method evaluation

Table 7. Linearity and range of amino acids in water. The instrumental portion of this method was evaluated using standard solutions of 17 amino acids in acidified water. The calibration curves were generated using the load-on-column method to minimize propagation of pipetting errors. The calibration range was chosen to be 10 to 1,000 pmol/µL. The calibration curves of all 17 amino acids were linear.

Amino Acid	Retention Time (min)	Fragmentor Voltage	*m/z*	r	R^2	Range (pmol/µL)	Range (ppm)
L-Alanine	6.117	90	90	0.99949	0.99899	10 to 1,000	0.891 to 89.1
L-Arginine	7.988	105	175	0.99745	0.99490	10 to 1,000	1.742 to 174
L-Aspartic Acid	7.845	75	134	0.99904	0.99807	10 to 1,000	1.33 to 133.1
L-Cysteine	8.179	105	241	0.99997	0.99994	10 to 1,000	1.2 to 121
L-Glutamic Acid	7.214	85	148	0.99932	0.99864	10 to 1,000	1.47 to 147.1
L-Glycine	6.515	120	76	0.99979	0.99958	10 to 1,000	0.75 to 75
L-Histidine HCL·H_2O	4.377	95	210	0.99614	0.99229	10 to 1,000	2.10 to 210
L-Isoleucine	4.652	85	132.1	0.99912	0.99824	10 to 1,000	1.31 to 131
L-Leucine	4.489	85	132.1	0.99888	0.99775	10 to 1,000	1.31 to 131
L-Lysine HCl	4.365	85	183	0.99691	0.99224	10 to 1,000	1.83 to 183
L-Methionine	4.868	75	150	0.99970	0.99840	10 to 1,000	1.49 to 149.2
L-Phenylalanine	4.371	85	166	0.99990	0.99980	10 to 1,000	1.65 to 165.2
L-Proline	5.349	85	116.1	0.99922	0.99844	10 to 1,000	1.15 to 115
L-Serine	6.618	65	106	0.99972	0.99945	10 to 1,000	1.05 to 105
L-Threonine	4.371	75	120	0.99986	0.99982	10 to 1,000	1.19 to 119
L-Tyrosine	5.267	95	182	0.99553	0.99109	10 to 1,000	1.81 to 181.2
L-Valine	5.280	75	118	0.99688	0.99378	10 to 1,000	1.17 to 117.2

Table 8. Precision data for amino acids in water.

Amino Acid	Average Retention Time (min)	Retention Time RSD%	Concentration RSD%
L-Alanine	6.279	0.293	2.029
L-Arginine	8.130	0.102	1.5636
L-Aspartic Acid	8.090	0.089	1.299
L-Cysteine	8.261	0.083	1.863
L-Glutamic Acid	7.590	0.147	1.998
L-Glycine	6.657	0.260	1.593
L-Histidine HCL·H_2O	4.537	0.018	1.172
L-Isoleucine	4.844	0.378	1.428
L-Leucine	4.672	0.398	1.4378
L-Lysine HCl	5.389	0.326	1.218
L-Methionine	5.045	0.348	1.3132
L-Phenylalanine	4.539	0.404	1.6932
L-Proline	5.440	0.335	1.573
L-Serine	6.754	0.243	1.522
L-Threonine	6.313	0.280	1.704
L-Tyrosine	5.387	0.330	1.1617
L-Valine	5.367	0.346	2.988

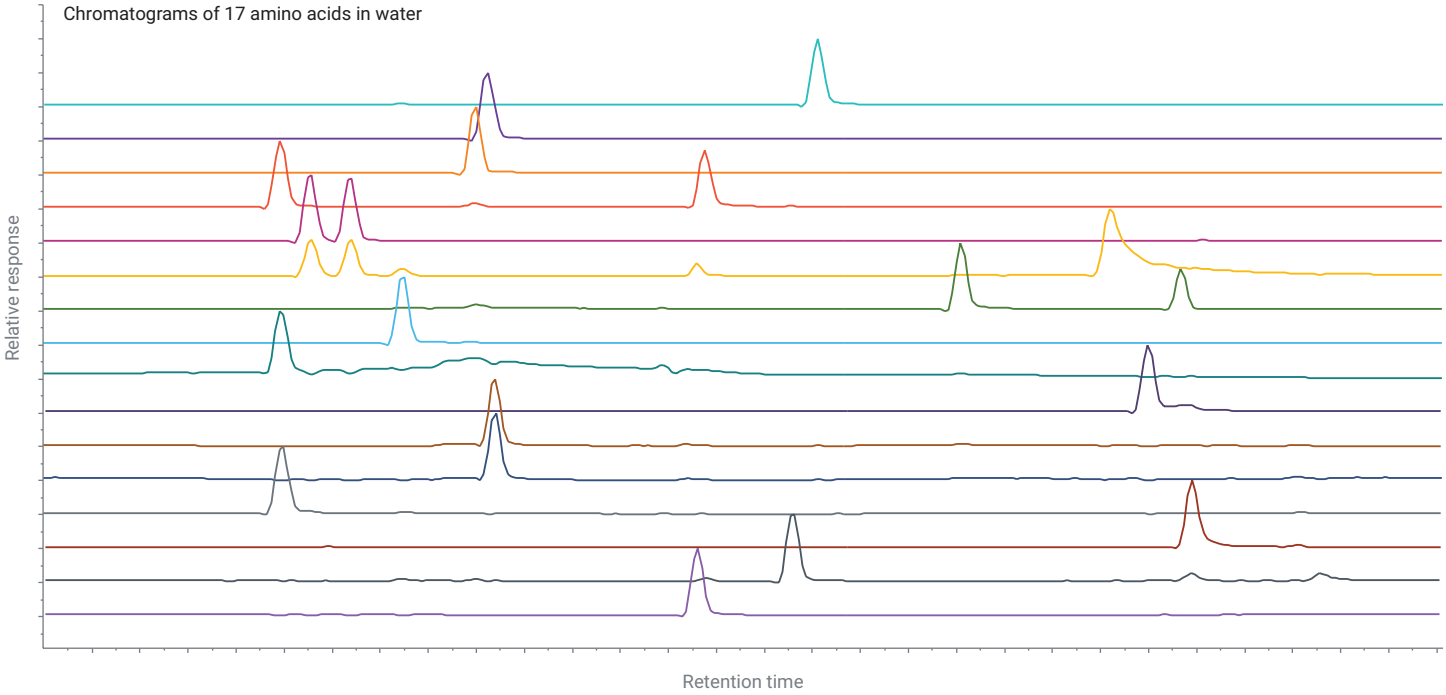


Figure 2. SIM chromatograms of 17 amino acids in water.

A L-Leucine

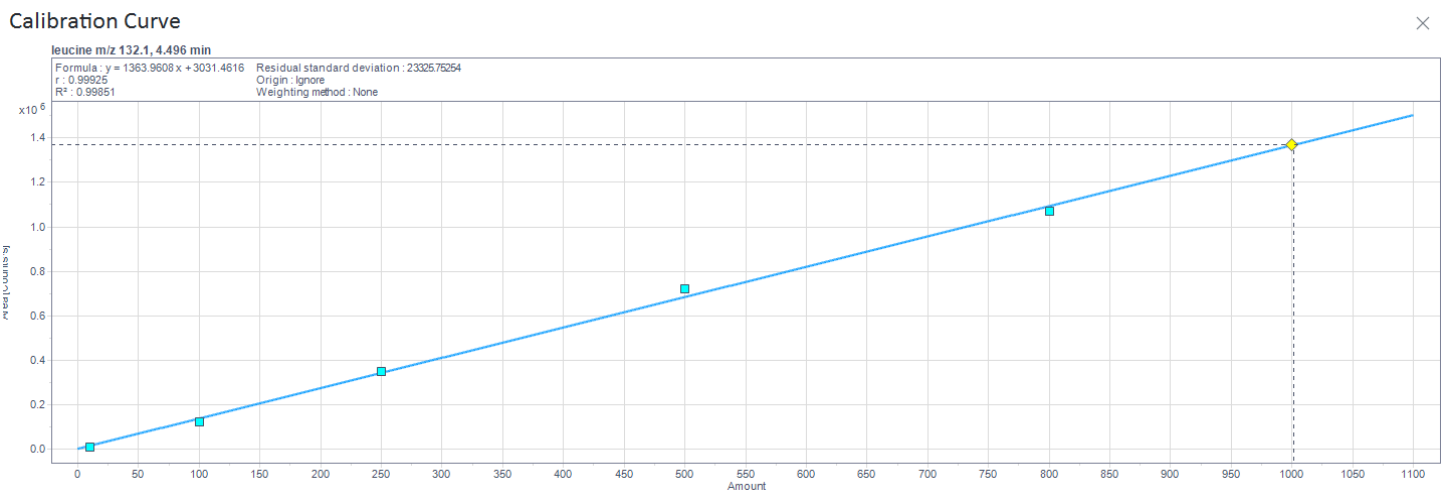
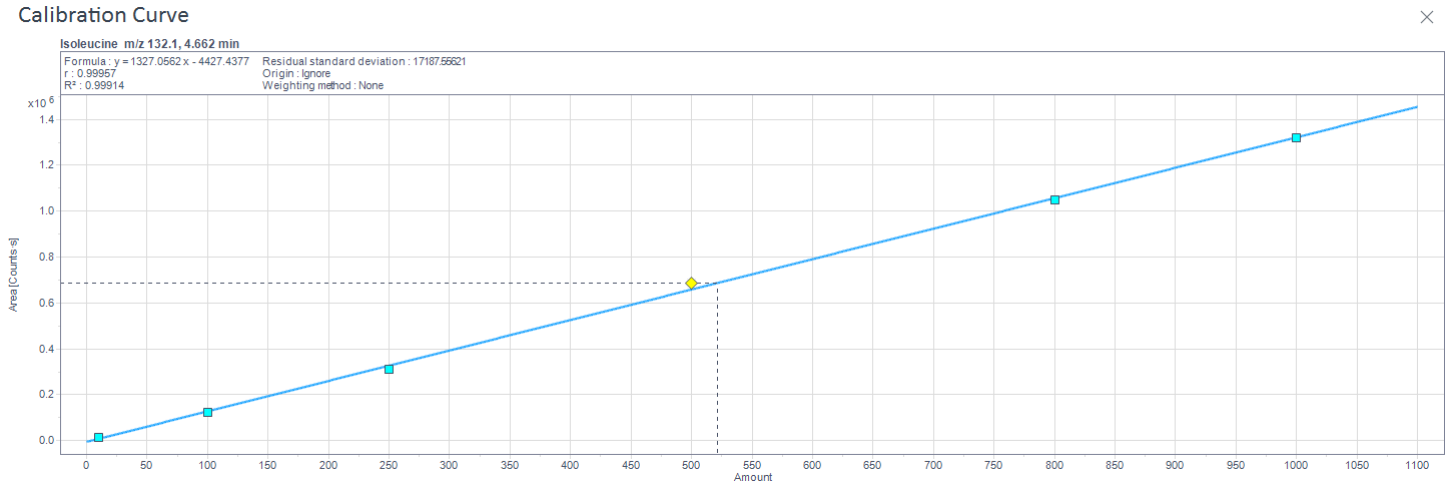
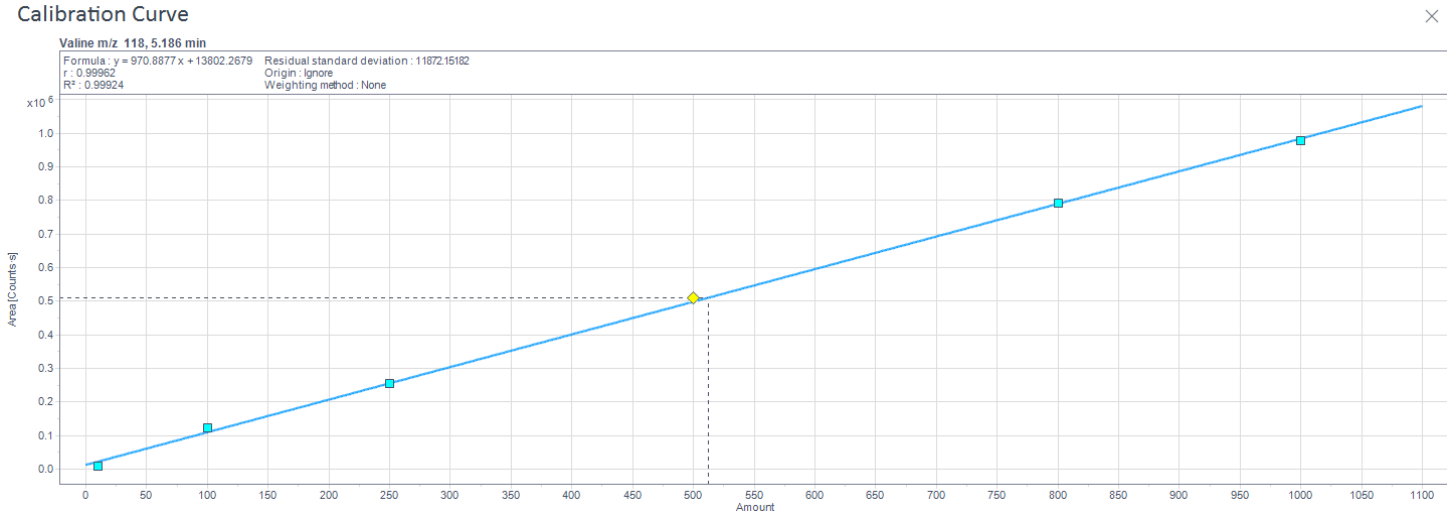


Figure 3. Calibration curves of leucine, isoleucine, valine, phenylalanine, methionine, and lysine (continued on the next 2 pages).

B L-Isoleucine



C L-Valine



D L-Phenylalanine

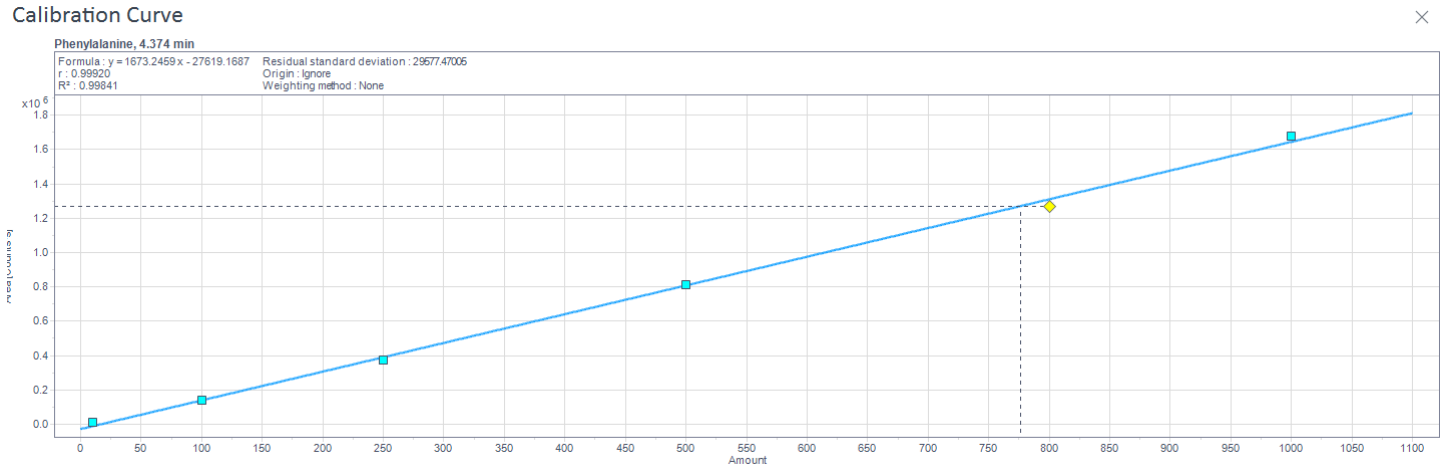
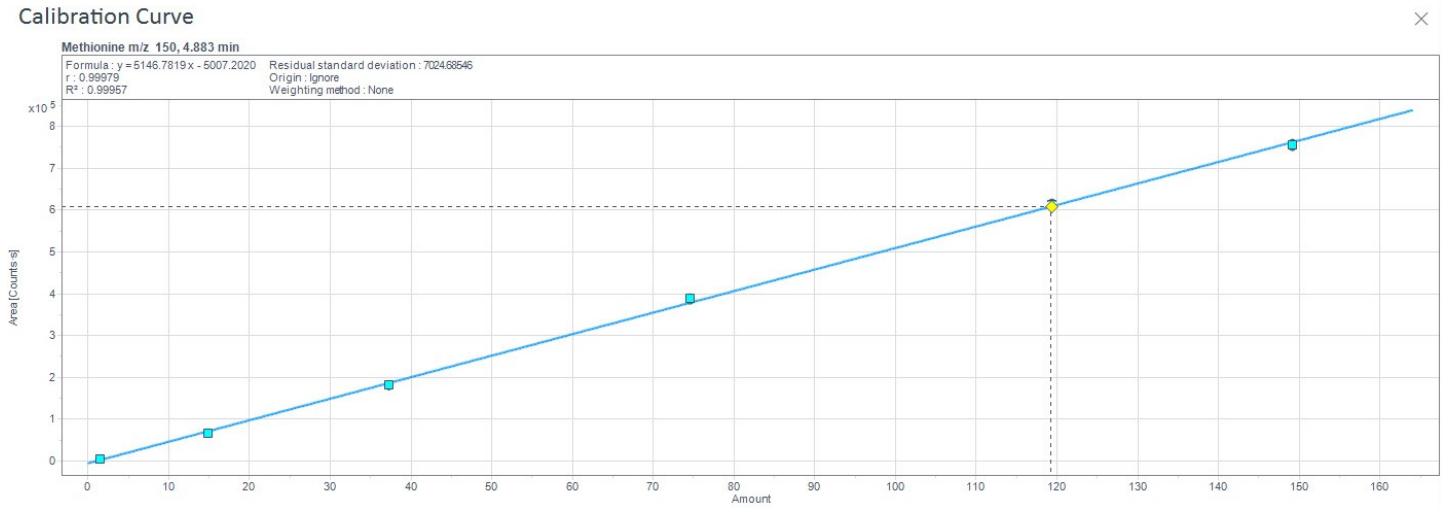


Figure 3. Calibration curves of leucine, isoleucine, valine, phenylalanine, methionine, and lysine (continued).

E L-Methionine



F L-Lysine HCl

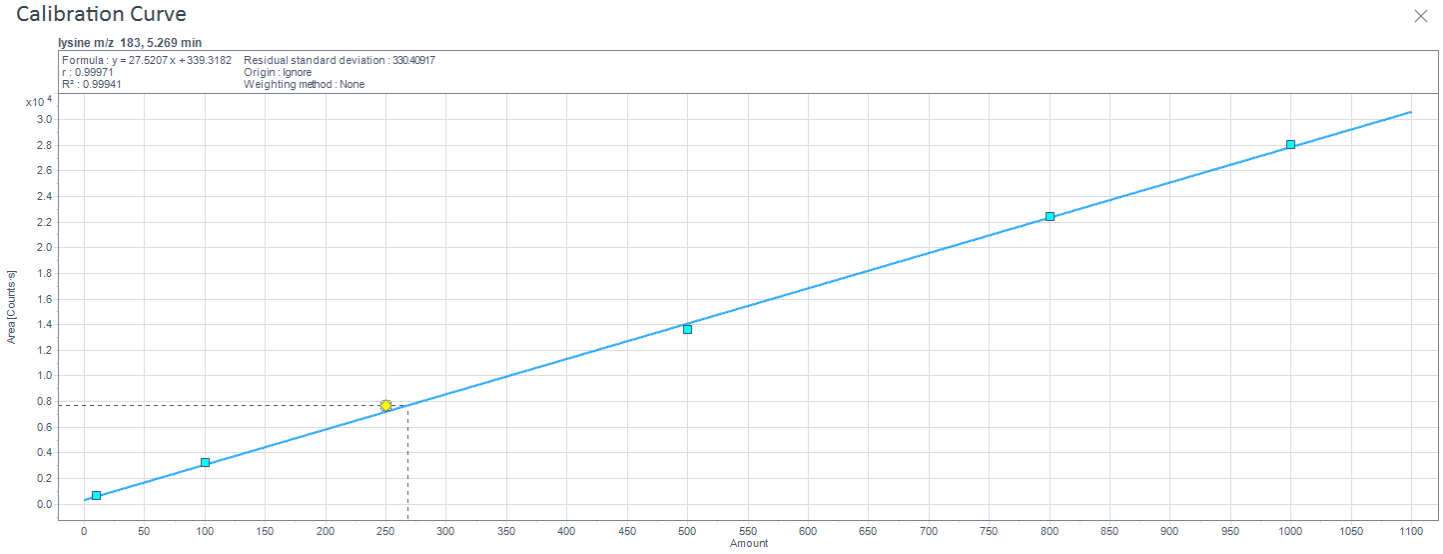


Figure 3. Calibration curves of leucine, isoleucine, valine, phenylalanine, methionine, and lysine (continued).

Accuracy data for amino acids in water

Table 9. Summary of the average accuracies for all amino acids measured in this study.

Amino Acid	Average Accuracy
L-Alanine	101.49
L-Arginine	101.76
L-Aspartic Acid	102.32
L-Cysteine	101.07
L-Glutamic Acid	100.42
L-Glycine	101.36
L-Histidine HCl·H_2O	100.89
L-Isoleucine	100.93
L-Leucine	101.49
L-Lysine·HCl	101.93
L-Methionine	101.98
L-Phenylalanine	102.03
L-Proline	102.14
L-Serine	102.17
L-Threonine	102.47
L-Tyrosine	100.69
L-Valine	100.31

Amino acids in supplement 1 – method evaluation

Supplement 1 was analyzed in this study and consisted of a capsule containing a powdered mix of L-leucine, L-isoleucine, and L-valine only. Four capsules (one serving) were dissolved in one liter of water and the resulting solution was analyzed. It is noted that exact concentrations were not known. Recoveries indicate no matrix effects.

Table 10. Amino acid recovery data for supplement 1.

Amino Acid	Calculated* (pmol/L)	Unspiked Quantitated (pmol/L)	Calculated Spiked from Unspiked Quantitated** (pmol/L)	Quantitated Spike (Spiked with 100 pmol) (pmol/L)	% Recovery (pmol/L)
L-Leucine	590	631	667	685	103
L-Isoleucine	387	340	406	406	100
L-Valine	331	394	455	455	100

* Amino acid amounts are given as a range by the manufacturer.
Actual exact amounts are not known.
** 900 µL unspiked amino acid solution + 100 µL of 1,000 pmol spike
(0.9(unspiked quant) + 100 pmol = calculated spike)

Table 11 . Amino acids precision data for supplement 1.

	Retention Time Mean (min)	Retention Time RSD%	Concentration RSD%
L-Leucine	4.495	0.444	3.097
L-Isoleucine	4.670	0.438	3.165
L-Valine	5.203	0.345	5.200

Amino acids in supplement 2 – method evaluation

Supplement 2 was analyzed in this study and consisted of capsules containing a powdered mix of L-leucine, L-lysine, L-threonine, L-isoleucine, L-valine, L-phenylalanine, L-histidine, L-tryptophan, and L-methionine. Three capsules (one serving) were dissolved in one liter of water and the resulting solution was analyzed for L-phenylalanine, L-leucine, L-isoleucine, L-methionine, and L-valine. No matrix effects were observed.

Table 12 . Amino acid recovery for supplement 2.

Amino Acid	Quantitated Unspiked Concentration (pmol/µL)	Spiked Concentration Calculated (pmol/µL)	Spiked Concentration Quantitated (pmol/µL)	% Recovery (pmol/µL)
L-Phenylalanine	284.3	355.9	399.9	112
L-Leucine	914.3	922.9	960.4	104
L-Isoleucine	509.9	558.9	575.6	103
L-Methionine	123.2	210.9	226.1	107
L-Valine	690.6	721.5	728.6	100

Table 13 . Amino acid precision data for supplement 2.

Amino Acid	Retention Time Mean (min)	Retention Time RSD%	Concentration RSD%
L-Phenylalanine	4.244	0.058	3.037
L-Leucine	4.394	0.071	2.911
L-Isoleucine	4.572	0.067	2.967
L-Methionine	4.761	0.058	2.357
L-Valine	5.109	0.073	1.783

Conclusion

This method, using the Agilent 1290 Infinity II LC coupled with an Agilent MSD iQ mass spectrometer, has been shown to be effective, sensitive, accurate, and robust for the screening and analysis of amino acids in nutritional supplements. The ability to analyze 17 underivatized amino acids in one injection, as well as the ability to resolve the L-leucine and L-isoleucine peaks, make this method a time-saving and cost-effective tool for commercial and industrial label verification.

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