Application Note

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Quantitation of Amino Acids in Soy Flour, Dried Cow's Milk Powder, and Corn Silage by Triple Quadrupole LC/MS/MS

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Abstract

This application note describes a method for the detection, identification and quantitation of underivatized amino acids in ruminant feed ingredients and dried cow milk powder using an Agilent 1290 Infinity II LC coupled to an Agilent 6470 triple quadrupole LC/MS. The sample preparation procedure involves three specific protein/peptide hydrolysis methods. Released amino acids are separated using hydrophilic interaction chromatography (HILIC). The method was applied to soy flour, dried cow's milk powder, and corn silage, achieving great sensitivity, linearity, and accuracy.

Introduction

Amino acids are the organic structural units which form proteins and are often called the building blocks of life. The general structure of amino acids contains a basic amino group (–NH_2) and carboxyl group (–COOH). It is challenging to accurately separate, identify, and quantify amino acids in food and feed products due to interference from endogenous components in the sample. An important sample preparation step for analysis of bound amino acids is protein/peptide hydrolysis, which commonly involves hydrolytic digestion in 6 N hydrochloric acid at approximately 110 °C for 24 hours. Methionine and cystine are present in low concentrations and undergo oxidation to various oxidized derivatives during acid hydrolysis. Controlled oxidation of methionine to methionine sulfone and cystine to cysteic acid are required using performic acid prior to acid hydrolysis. Tryptophan is also present in low concentrations but is extensively degraded during acid hydrolysis, so it is normal to use alkaline hydrolysis.[1,2]

Released amino acids are traditionally separated using ion-exchange or reversed-phase chromatography with ion-pairing reagents, then analyzed using diode array detection or fluorescence detection after derivatization. LC/MS/MS has become increasingly popular for amino acid detection due to greater sensitivity, high selectivity, quantitative accuracy and high throughput. As a consequence of the selectivity and specificity of the mass spectrometer, the need for derivatization during sample preparation and need for ion-pairing reagents in LC detection are eliminated, which also increases reproducibility and robustness in the analysis.

In this study, a fast and sensitive UHPLC-MS/MS method was evaluated to provide identification and accurate quantification of amino acids in complex food and feed matrices. The postextraction matrix-matched standard was included to evaluate any effect of the matrix on recoveries and accuracy of detection. Method criteria for data acceptance were established.

Experimental

Equipment

The LC/MS analysis was performed using an Agilent 1290 Infinity II LC consisting of an Agilent 1290 Infinity II multisampler (G7167B), an Agilent 1290 Infinity II high-speed pump (G7120A), and an Agilent 1290 Infinity II multicolumn thermostat (G7116B) coupled to a 6470 triple quadrupole LC/MS (G6470A). The system was controlled by Agilent MassHunter Acquisition software version 10.1. Data processing was performed with Agilent MassHunter quantitative analysis software version 10.1 and Agilent MassHunter qualitative analysis software version 10.0.

Samples and standards

The sample matrices in this study included soy flour (NIST Standard Reference Material 3234), dried cow's

Chromatographic conditions

Parameter	Setting
Analytical Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.7 µm, 2.1 × 150 mm, PEEK-lined, (p/n 673775-924)
Column Oven	25 ±2 °C
Injection Volume	1 µL
Run Time	14 min
Autosampler	15 ±2 °C
Mobile Phase A	10 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	10 mM ammonium formate + 0.1% formic acid in 90% acetonitrile
Seal Wash	90/10 Water/IPA
Needle Wash	50/50 MeOH/H_2O
Gradient	Time (min) / Flow (mL/min) / %A / %B: 0 / 0.4 / 0 / 100 5.0 / 0.4 / 20 / 80 6.0 / 0.4 / 30 / 70 7.0 / 0.4 / 50 / 50 9.0 / 0.4 / 80 / 20 10.0 / 0.4 / 80 / 20 10.5 / 0.4 / 0 / 100 14.0 / 0.4 / 0 / 100

MS parameters

Parameter	Setting
MS Acquisition	dMRM
Ion Source Type	Agilent Jet Stream Electrospray ionization (AJS ESI ±)
Drying Gas Temperature	330 °C
Drying Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Heater	390 °C
Sheath Gas Flow	12 L/min
Capillary	2,000 V (ESI ±)
Nozzle Voltage	0 V (ESI ±)
Precursor Ion and Production Ion Resolution	Unit
Compound-Specific Conditions	See Table 1

milk powder, and dried, ground corn silage. An amino acid standard mix (part number 5061-3330) including alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine was obtained from Agilent Technologies Inc. (Santa Clara, CA, USA); methionine sulfone, cysteic acid and tryptophan were obtained from Millipore Sigma, Inc. (St. Louis, MO, USA). The samples and standards were stored at 5 °C.

Method

Description

The following steps are a detailed description of sample preparation. Table 1 gives the analyte-specific LC/MS conditions.

1. Weigh 0.3 ±0.015 g sample into a 40 mL glass tube with PTFE cap. Add the spiking solution (an amino acid composite standard solution) for spiking samples.

For the detection of cystine and methionine, follow steps 2 through 5:

2. Keep the samples and performic acid on ice for ~30 minutes. Add 5 mL of performic acid into sample. Swirl gently.
3. Store samples for at least 16 hours in an ice bath in refrigerator.
4. Under a fume hood, with samples on ice, add ~0.8 to 1 g of sodium metabisulfite to sample and swirl gently for at least 15 minutes.
5. Add 5 mL of 12 N HCl. Cap and vortex.

For the detection of tryptophan, follow step 6:

6. Add 15 mL 4 N NaOH to sample. Cap and vortex.

Table 1. Analyte-specific LC/MS conditions: precursor to product ion transitions, fragmentor, collision energies (CE), and retention times (RT).

Compound Name	Precursor Ion	Product Ion	RT (min)	Delta RT (min)	Fragmentor	Collision Energy
Alanine	90.1	44.1	6.0	1.2	40	9
Alanine	90.1	45.1	6.0	1.2	40	40
Arginine	175.1	116.1	7.7	1.5	105	2
Arginine	175.1	70.1	7.7	1.5	105	8
Arginine	175.1	60.1	7.7	1.5	105	4
Aspartic Acid	134.1	88.0	7.2	1.2	75	0
Aspartic Acid	134.1	74.0	7.2	1.2	75	4
Aspartic Acid	134.1	70.0	7.2	1.2	75	6
Cystine	241.0	152.0	7.7	1.5	105	0
Cystine	241.0	120.0	7.7	1.5	105	0
Cystine	241.0	74.1	7.7	1.5	105	25
Glutamic Acid	148.1	84.0	6.7	1.2	85	6
Glutamic Acid	148.1	56.1	6.7	1.2	85	22
Glutamic Acid	148.1	41.0	6.7	1.2	85	18
Glycine	76.0	48.0	6.4	1.2	40	0
Glycine	76.0	30.0	6.4	1.2	40	12
Histidine	156.1	110.1	7.6	1.2	95	4
Histidine	156.1	95.1	7.6	1.2	95	6
Isoleucine	132.1	86.1	4.6	1.2	85	0
Isoleucine	132.1	44.1	4.6	1.2	85	16
Isoleucine	132.1	41.0	4.6	1.2	85	18
Isoleucine	132.1	30.0	4.6	1.2	85	6
Leucine	132.1	86.1	4.4	1.2	85	0
Leucine	132.1	44.1	4.4	1.2	85	14
Leucine	132.1	41.0	4.4	1.2	85	25
Leucine	132.1	30.0	4.4	1.2	85	4
Lysine	147.1	130.1	7.9	1.5	85	0
Lysine	147.1	84.1	7.9	1.5	85	6
Methionine	150.1	104.1	4.7	1.2	75	0
Methionine	150.1	61.0	4.7	1.2	75	14
Methionine	150.1	56.1	4.7	1.2	75	6
Methionine	150.1	28.0	4.7	1.2	75	26
Phenylalanine	166.1	120.1	4.1	1.2	85	4
Phenylalanine	166.1	103.1	4.1	1.2	85	22
Phenylalanine	166.1	91.1	4.1	1.2	85	32
Phenylalanine	166.1	77.0	4.1	1.2	85	36
Proline	116.1	70.1	5.3	1.2	85	6
Proline	116.1	43.1	5.3	1.2	85	25
Serine	106.1	88.1	6.5	1.2	65	8
Serine	106.1	42.1	6.5	1.2	65	24
Threonine	120.0	74.1	6.0	1.2	75	0
Threonine	120.0	56.1	6.0	1.2	75	6

For the detection of the remaining amino acids, follow step 7:

7. Add 5 mL of 6 N HCl. Cap and vortex.

Perform the following steps for all above samples:

8. Place samples in a 110 °C heat block for at least 24 hours.
9. Allow samples to cool to room temperature.
10. Quantitatively transfer the samples to a 50 mL centrifuge tube with Milli-Q water. Bring to the maximum volume mark. Mix well.
11. Filter ~1 mL of the sample through a 0.2 µm nylon syringe filter into a 1.8 mL microcentrifuge tube.
12. Dilute further as needed with 0.1 N HCl.
13. Prepare the postspiked sample (post spike an amino acid composite standard solution to the diluted sample extract before injection to evaluate the matrix effect) along with the diluted sample.
14. The samples are now ready for LC/MS/MS injection using both positive/negative ESI modes.

Evaluation procedure

The method performance was evaluated by analyzing a NIST soy flour (standard reference material 3234) sample. The reference mass fraction values for amino acids are listed in Table 2. The reagent blanks (0.1% HCl solution) were spiked with amino acids at 100 to 200 µg and went through the hydrolysis/dilution to confirm the method extraction efficiency without matrix. The quantitation was performed using an external calibration curve with 1/x weight and single point postmatrix spike correction.

Compound Name	Precursor Ion	Product Ion	RT (min)	Delta RT (min)	Fragmentor	Collision Energy
Tryptophan	205.1	187.9	4.2	1.2	50	16
Tryptophan	205.1	146.0	4.2	1.2	50	23
Tryptophan	205.1	117.9	4.2	1.2	50	10
Tyrosine	182.1	136.1	5.0	1.2	95	0
Tyrosine	182.1	119.1	5.0	1.2	95	10
Tyrosine	182.1	91.1	5.0	1.2	95	22
Tyrosine	182.1	77.0	5.0	1.2	95	34
Valine	118.1	72.1	5.2	1.2	75	0
Valine	118.1	55.1	5.2	1.2	75	14
Cysteic Acid*	168.0	150.9	6.6	1.2	90	12
Cysteic Acid*	168.0	80.9	6.6	1.2	90	20
Methionine* Sulfone	180.0	79.1	5.4	1.2	70	30
Methionine* Sulfone	180.0	64.0	5.4	1.2	70	45

* In ESI negative mode

Evaluation criteria

Specificity:

- The relative error (RE %) of retention time of each analyte peak to the average of standard peaks is less than 5%.
- The ion ratio is within the tolerance of 30%.

Linearity and range:

- Calibration curve has R^2 >0.99.
- Calculated working standard values should be within ±30% of the theoretical value.
- The calibration standards should bracket the analyte concentration level.

Accuracy:

- The test result for each amino acid in soy flour is within ±30% deviation of the reference mass fraction value (see Table 2), which is considered the true value.
- The recovery for prespike of amino acid contents in the reagent blank is within 80 to 120%.
- The postspike matrix recovery is within 50 to 150% for result correction (due to the variations in sample preparation, analyte detection, and instrument performance, the criteria should be set by each individual lab).

Table 2. Mass fraction values for amino acids in NIST soy flour standard reference material 3234.

Compound Name	Mass Fraction g/100 g
Alanine	2.28 ±0.16
Arginine	3.72 ±0.31
Aspartic Acid	6.0 ±1.2
Cystine	0.74 ±0.15
Glutamic Acid	10.2 ±1.4
Glycine	2.22 ±0.15
Histidine	1.22 ±0.089
Isoleucine	2.31 ±0.23
Leucine	4.03 ±0.42
Lysine	3.20 ±0.25
Methionine	0.69 ±0.13
Phenylalanine	2.54 ±0.13
Proline	2.71 ±0.23
Serine	2.69 ±0.32
Threonine	2.02 ±0.11
Tryptophan	0.66 ±0.14
Tyrosine	1.76 ±0.43
Valine	2.45 ±0.41

Results and discussion

Column selection

In this study, an Agilent InfinityLab Poroshell HILIC-Z column was used for underivatized amino acids separation.[3] Excellent chromatographic performance in terms of resolution, peak shape and sensitivity were achieved using HILIC column with low pH mobile phase additives. See Figure 1 for the elution profile of amino acids. Baseline separation of leucine and isoleucine isomers was also achieved.

Specificity

A dynamic multiple reaction monitoring (dMRM) acquisition method was used for amino acid identification and quantitation. Monitoring MS/MS transitions with evaluation of the ratio for their relative product ion intensities and RT of analyte peaks enables the target analyte to be distinguished from potential interferences in quantitative analysis. Figure 1 shows an example of an extracted ion chromatogram of a 100 ng/mL working standard in 0.1% HCl. Figure 2 shows that no amino acids are present in the reagent blank at a level greater than 30% of the lowest calibration standard.

Range and linearity

The method was evaluated over the concentration range of 1 to 2,500 ng/mL. To evaluate the linearity of the method, nine working standard (WS) solutions of amino acids were made at 1, 5, 10, 20, 50, 100, 500, 1,000, and 2,500 ng/mL. The calibration curve residuals were ≤30% for WS1 to WS9. Figure 3 demonstrates the statistical data of the calibration curve residuals. The linearity was determined by using a linear calibration with a 1/x weighting factor. The coefficients of determination (R^2) value were >0.99. Table 3 lists the data of the linear concentration range and coefficients of determination.

Approaches for accurate quantitation

Interfering substances in the matrix can be observed and may affect the electrospray ionization process, causing suppression or enhancement of the analyte signal. While good sample preparation and cleanup can mitigate many of these interferences, some may still remain. Currently, there are no guidelines for dealing with matrix effects due to variations in method and instrument performance. However, matrix effects need to be compensated. A postspike matrix-matched standard can address the matrix effect and any other matrix interactions for quantitation purposes when an internal standard is not available or not easy to obtain.[4] Postspike recovery was determined by fortifying samples after extraction with the analyte composite standard solution. The results were corrected using postspike recovery if it was within 50 to 150%.

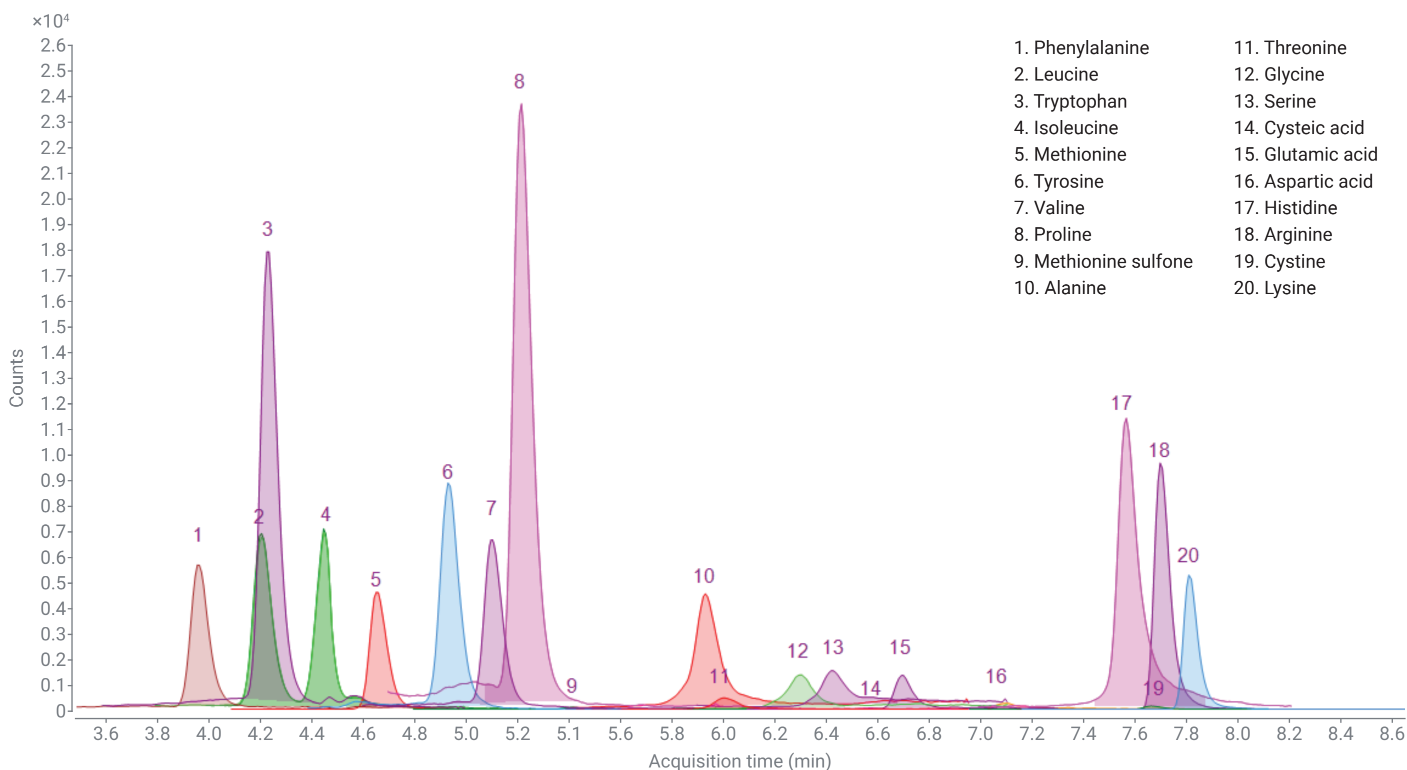

Figure 1. HILIC elution profile of amino acids in a 100 ng/mL working standard mix in 0.1 N HCl, 1 µL injection volume.

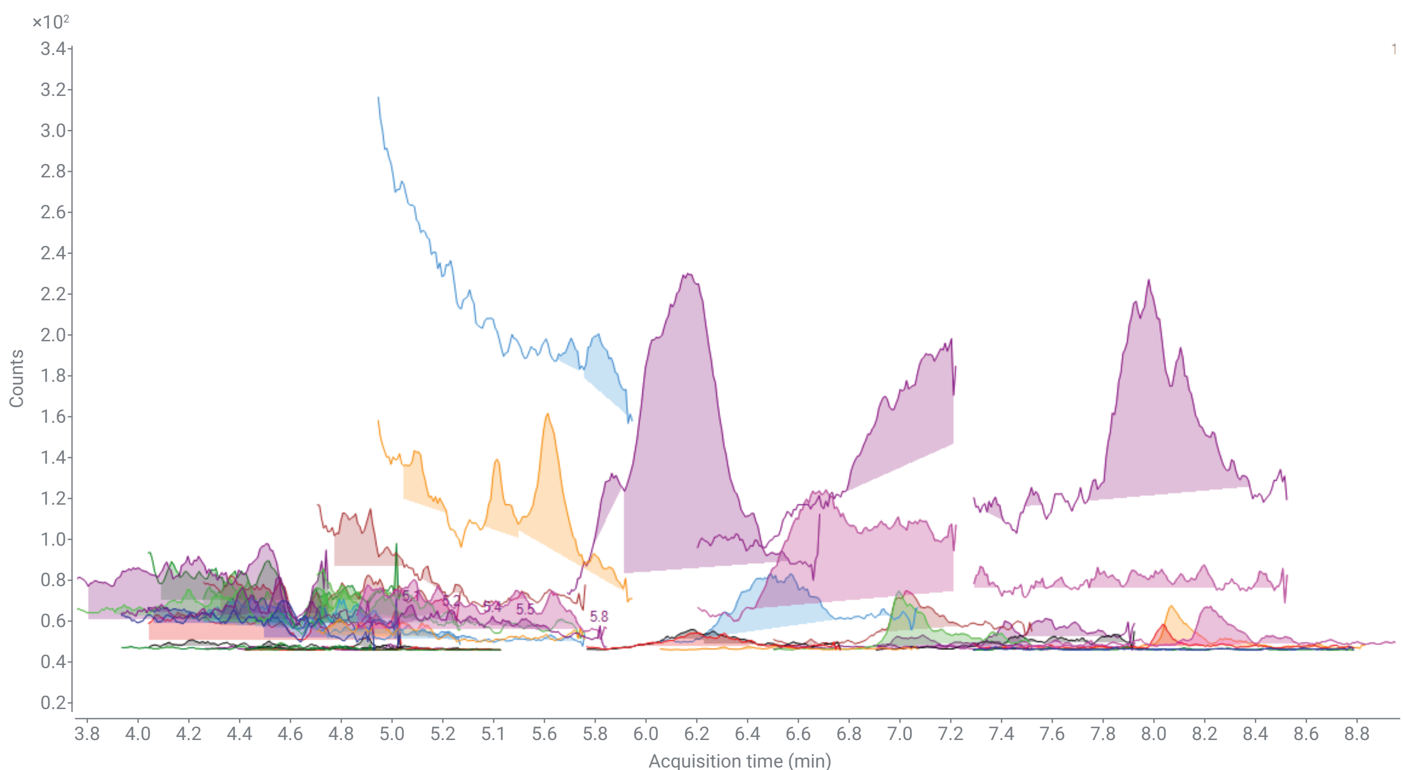

Figure 2. Extracted ion chromatogram of a solvent blank, 0.1% HCl.

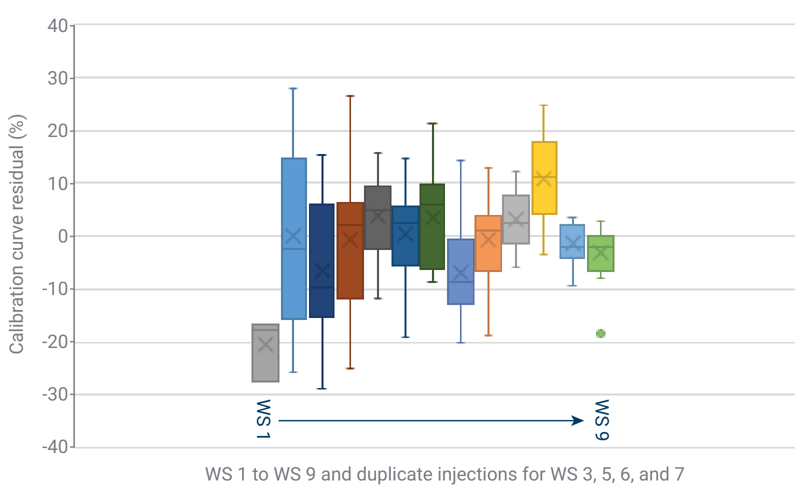

Figure 3. Calibration curve residual for working standards.

Table 3. The linear concentration range and coefficients of determination R^2.

Compound Name	Range (ng/mL)	R^2
Alanine	10 to 1,000	0.9980
Arginine	5 to 1,000	0.9973
Aspartic Acid	20 to 2,500	0.9972
Cysteic Acid	20 to 2,500	0.9988
Cystine	1 to 2,500	0.9917
Glutamic Acid	5 to 2,500	0.9977
Glycine	20 to 1,000	0.9947
Histidine	10 to 2,500	0.9989
Isoleucine	5 to 1,000	0.9949
Leucine	5 to 1,000	0.9906
Lysine	5 to 1,000	0.9988
Methionine sulfone	5 to 1,000	0.9964
Methionine	1 to 1,000	0.9913
Phenylalanine	1 to 1,000	0.9916
Proline	5 to 1,000	0.9992
Serine	10 to 1,000	0.9912
Threonine	10 to 1,000	0.9969
Tryptophan	5 to 1,000	0.9978
Tyrosine	5 to 1,000	0.9939
Valine	5 to 1,000	0.9980

Sample tests

Prespike recoveries were obtained for all amino acids (within 90 to 115% except 81.5% for methionine) in reagent blank samples, indicating that significant losses in analyte amounts are not observed during hydrolysis and extraction. Table 4 shows the recovery for each amino acid.

The evaluated method was applied to a variety of general agricultural products, including soy flour, dried cow's milk powder, and corn silage. The high sensitivity of LC/MS/MS allows a large dilution after sample extraction. The postspike recoveries for all amino acids fell into the accepted range (93% of postspike recoveries were within 70 to 130%) and the results were corrected. The corrected results using matrix-matched standards for all amino acids were in great agreement with the NIST mass fraction values (deviation between –2.4 to 18.3%) with the exception of cystine and methionine, which were at the low-end. Sample results and postspike recoveries for each analyte in each matrix are shown in Table 5.

Table 4. Spike recovery for reagent blank.

Compound Name	Reagent Blank Prespike Recovery (%)
Alanine	90.9
Arginine	104
Aspartic Acid	103
Cystine*	111
Glutamic Acid	104
Glycine	91.6
Histidine	107
Isoleucine	92.0
Leucine	106
Lysine	92.9

Compound Name	Reagent Blank Prespike Recovery (%)
Methionine*	81.5
Phenylalanine	108
Proline	102
Serine	100
Threonine	90.6
Tryptophan	110
Tyrosine	95.0
Valine	95.1

* Cystine and methionine were spiked into the reagent blank, converted to, and calculated from cysteic acid and methionine sulfone.

Table 5. Sample results for soy flour, dried cow's milk powder, and corn silage.

	Soy Flour				Dried Cow's Milk Powder		Corn Silage	
Compound Name	Mass fraction from NIST (g/100 g)	Results on DM* (g/100 g)	Postspike Recovery (%)	Deviation from NIST Value (%)	Results on DM (g/100 g)	Postspike Recovery (%)	Results on DM (g/100 g)	Postspike Recovery (%)
Alanine	2.28	2.12	115	−7.0	0.77	102	0.51	120
Arginine	3.72	3.54	103	−4.7	0.83	97.2	0.10	107
Aspartic Acid	6.00	5.33	127	−11.2	1.60	116	0.39	105
Glutamic Acid	10.20	9.95	122	−2.4	5.04	111	0.73	132
Glycine	2.22	2.10	131	−5.5	0.42	111	0.27	104
Histidine	1.22	1.45	110	18.3	0.92	101	0.12	111
Isoleucine	2.31	2.17	113	−6.3	1.12	106	0.14	148
Leucine	4.03	4.45	116	10.4	2.97	88.7	0.78	91.1
Lysine	3.20	3.56	113	11.2	2.58	102	0.16	108
Phenylalanine	2.54	2.79	115	9.8	1.27	98.2	0.25	107
Proline	2.71	2.62	109	−3.4	2.47	95.4	0.39	112
Serine	2.69	2.53	142	−6.0	1.28	106	0.23	110
Threonine	2.02	1.83	124	−9.6	1.04	101	0.23	98.9
Tryptophan**	0.66	0.64	110	−2.9	0.28	112	0.025	116
Tyrosine	1.76	1.51	120	−14.1	0.97	106	0.10	112
Valine	2.45	2.16	115	−11.8	1.51	96.8	0.31	105
Cysteic Acid	–	1.58	88.6	–	0.28	116	0.090	104
Cystine***	0.74	1.12	–	51.4	0.20	–	0.064	–
Methionine Sulfone	–	1.16	82.1	–	0.65	109	0.12	89.6
Methionine***	0.69	0.95	–	38.0	0.53	–	0.10	–

* DM: Dry matter: 93.24% for soy flour; 95.85% for dried cow's milk powder; 91.45% for corn silage.

** Tryptophan was analyzed after a sample was hydrolyzed using 4 N NaOH.

*** Cystine and methionine were analyzed after a sample was oxidized using performic acid and then hydrolyzed using 6 N HCl. Cystine and methionine results were calculated from cysteic acid (note: cystine in the sample was converted to two cysteic acids) and methionine sulfone, respectively.

Conclusion

A rapid, sensitive, and accurate UHPLC-MS/MS method for the identification and quantitation of underivatized amino acids in complex agricultural products was presented. The method used an Agilent 1290 Infinity II LC stack coupled to an Agilent 6470A triple quadrupole LC/MS with Agilent MassHunter workstation software. The need for a derivatization step in sample preparation and the use of ion-pairing reagents in LC detection are eliminated. The evaluation demonstrated that the method can achieve excellent specificity, linearity, and accuracy.

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DE44209.6102199074

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Printed in the USA, February 15, 2021
5994-3044EN

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