Proceeding Paper

Nutritional Protein Value of Flours via LC-MS/MS Analysis †

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Abstract: The growth of the world's population and the reduction in the average annual global individual carbon footprint are current issues. With the aim of assessing nutritional protein values, we developed a sensitive analytical methodology for the identification and quantification of amino acids. Strategies have been developed to reduce sample complexity and improve detection for analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The method is suitable for the purpose and is a useful tool for protein value assessment, according to the Food and Agriculture Organization of the United Nations.

Keywords: nutritional protein value; amino acids; protein hydrolysis; LC-MS/MS

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1. Introduction

Innovative processed foods that not only satiate nutritional needs but also contribute to improved health, increased longevity, protection from nutrition-related diseases, and enhanced physical and mental well-being, need to be developed. In this context, we are involved in the InsectERA PRR Project dedicated to facilitating the industrialization and commercialization of groundbreaking products based on insect flours [1]. To accurately assess the protein content, we have developed a highly sensitive analytical methodology for the identification and quantification of amino acids (AAs). This quantification is pivotal because protein value assessments, as per the Food and Agriculture Organization of the United Nations tables [2], hinge on the summation of the masses of the 20 essential AAs.

One of the most critical aspects of protein analysis by mass spectrometry is sample preparation, a step that is both variable and time-consuming. The quality and reproducibility of sample extraction and preparation significantly influence the accuracy of the results. Over time, strategies have evolved to streamline sample complexity and enhance detection [3–5]. Our research work sought to develop a rapid, precise, and dependable LC-MS/MS method for the simultaneous and targeted analysis of 20 underivatized AAs. Notably, this method encompasses the sample preparation, chromatographic separation, and mass spectrometric detection of AAs without the need for chemical derivatization reagents.

The initial step consists in analyzing the AA composition of proteins and involves the hydrolysis of proteins, breaking them down into their constituent AA building blocks. The separation of these 20 AAs was achieved using hydrophilic interaction chromatography, which offered superior retention and peak symmetry for all analytes. Importantly, we quantified all 20 AAs, employing commercially available isotope-labeled internal standards and ensuring a linear range that effectively accounts for signal intensity losses, interference from the biological matrix, and degradation, which are otherwise problematic.

The method validation demonstrated that our LC-MS/MS approach is not only specific but also highly accurate and reliable. It exhibits minimal matrix effects and yields excellent extraction efficiency, falling within the range of 81% to 104%.

2. Material and Methods

2.1. Chemicals

Analytical standard chemicals of LC-MS grade were acquired. The analytical solvents formic acid and acetonitrile were purchased from Carlo Erba® (Emmendingen, Germany) Reagents S.A.S. The ammonium formate buffer was supplied by Sigma-Aldrich® (St. Louis, MO, USA). A Milli-Q® ultrapure water system, equipped at the end of an assembly line with a Milli-Q® Reference and a Q-POD® element, was used to obtain ultrapure water.

The hydrolysis reagent, namely a methane sulfonic acid solution (4 M) with 0.2% tryptamine (*w*/*v*), was purchased from Sigma-Aldrich® and HCl (6 M) was acquired from Thermo Scientific (Waltham, MA, USA).

AA analytical standards and stable isotope standards for MS were acquired from Sigma-Aldrich®. Certified Reference Material BSA was purchased from Sigma-Aldrich® and the 3234 standard reference material was acquired from NIST® (Gaithersburg, MD, USA).

2.2. Sample Preparation for Amino Acid Profile Analysis

Briefly, for the AA profile of flour, a sample of 10.0 ± 0.5 mg was placed in a hydrolysis tube along with methane sulfonic acid solution with tryptamine as the agent for the antioxidant reduction. Oxygen-free conditions were attained by flushing the tube with N_2 gas. This tube was sealed and heated at 110 °C for 22 h. Afterwards, the mixture was moved to a centrifuge tube with HCl (0.1 M) and centrifuged for 5 min at 4200× *g*. The supernatant was filtered using a polytetrafluoroethylene syringe filter (0.22 µm, PTFE). Then, 0.5 mL of the extract was diluted with 5 mL acetonitrile/water 95:5 *v*/*v* and analyzed using the UHPLC-HILIC-ESI-MS/MS system.

2.3. Amino Acid Profile Analysis

2.3.1. Instrumental

A Dionex® Ultimate 3000 System UHPLC+ focused and a TSQ QuantisTM triple-stage quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA) were used to the LC-MS/MS analysis.

2.3.2. Conditions of Chromatography and Mass Spectrometry

A total of 10 µl of the sample was injected. The separation of AAs was attained with an AccuroreTM HILIC Column (2.6 µm, 150 × 2.1 mm, Thermo Fisher Scientific, Waltham, MA, USA). The mobile phase gradient was composed of ammonium formate (10 mM) with 0.1% formic acid (A) and acetonitrile (B). The flow rate was 0.3 mL/min.

A triple-stage quadrupole mass spectrometer was used to carry out the Mass Spectrometry (MS) analysis. The injection of the samples was in select reaction monitoring (SRM) mode and in positive polarity mode, with the spray voltage set at 3.5 kV, vaporization temperature at 370 °C and capillary temperature at 270 °C. The SRM results are presented in Table 1.

Table 1. Performance validation parameters of 20 AAs for quantitative analysis.

AA	RT	Precursor (*m*/*z*)	Product (*m*/*z*)	Range [a] (μmol.L^{-1})	R^2	Weighting, Type [b]
Alanine	2.03	90.274	44.44	20–500	0.9968	1/X, L
Arginine	12.12	175.12	70.26	5–200	0.9910	1/X, L
Asparagine	1.9	133.2	87.15	20–500	0.9977	1/X, L
Aspartic acid	2.03	134.11	74.12	10–500	0.9959	1/X, L
Cystine	8.71	241.1	152	5–500	0.9979	1/X, L
Glutamine	1.98	147.12	130.04	5–500	0.9936	1/X, L
Glutamic Acid	2.11	148.1	84.15	5–500	0.9978	1/X, L
Glycine	2.16	76.3	76.3	50–500	0.9992	1/X, L
Histidine	10.46	156.13	110.1	5–100	0.9694	1/X, L
Isoleucine	3.52	132.18	69.26	5–500	0.9992	1/X, L

Table 1. Performance validation parameters of 20 AAs for quantitative analysis.

AA	RT	Precursor (*m*/*z*)	Product (*m*/*z*)	Range [a] (μmol.L^{-1})	R^2	Weighting, Type [b]
Leucine	3.74	132.18	86.22	20–200	0.9937	1/X, L
Lysine	10.49	147.17	84.22	5–500	0.9915	1/X, L
Methionine	3.08	150.098	133.07	5–500	0.9991	1/X, L
Phenylalanine	6.85	166.12	103.7	2–500	0.9885	1/X^2, L
Proline	2.13	116.243	70.26	5–500	0.9942	1/X, Q
Serine	1.88	106.191	60.33	20–500	0.9982	1/X, L
Threonine	1.89	120.16	74.28	10–500	0.9985	1/X, L
Tryptophan	9.64	205.078	187.99	5–500	0.9993	1/X, L
Tyrosine	5.95	182.078	136.04	5–500	0.9991	1/X, L
Valine	2.62	118.17	72.26	20–500	0.9973	1/X, L

[a] Range of concentrations examined; [b] calibration weighting and type of calibration (L-linear, Q quadratic).

2.4. Method Validation

Method validation encompassed assessments of linearity, precision, and trueness, as well as the determination of the limit of detection (LOD) and limit of quantification (LOQ) [6]. The method's linearity was meticulously examined by analyzing standard solution mixtures at six distinct concentration levels, which covered the entire working concentration range for all amino acids. Calibration curves were carefully constructed by plotting the peak areas of the respective AAs, followed by linear regression analysis (R^2), employing the standard addition method. LODs and LOQs were determined using the signal-to-noise ratio (S/N) and the slope (S) in the equations LOD = 3 SD/S and LOQ = 10 SD/S, with SD representing the standard deviation of peak areas at the least detectable concentration.

The assessment of precision and trueness was conducted with the NIST Soy flour standard reference material ® 3234 and the BSA standard as reference. The results were obtained using the concentration values expressed in grams per 100 g for each individual AA. Additionally, we calculated the relative standard deviation (RSD) as a percentage and determined the recoveries as part of our analysis.

3. Results

The performance validation parameters for LC-MS/MS conditions are presented in Table 1. The recoveries for the soy flour NIST reference material fell within the range of 81% to 104%, as represented in Figure 1.

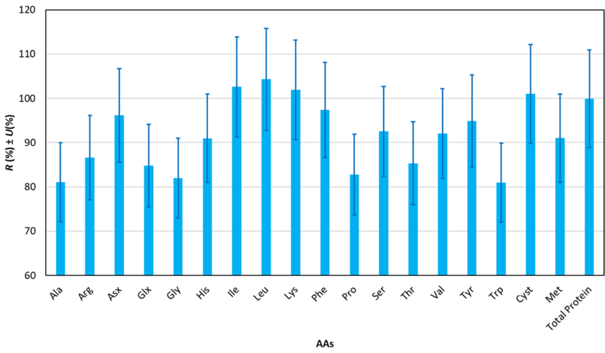

Figure 1. AAs recovery (R (%)) and uncertainty (%) for soy flour NIST SRM 3234.

4. Discussion and Conclusions

For almost a century, amino acids detection has relied heavily on the use of ninhydrin with quantification through spectrophotometry. The current widely accepted methods in research laboratories often involve multiple intricate steps, making them notably cum-

bersome and time-consuming. In contrast, the method presented offers a streamlined, straightforward, and highly reproducible approach.

Our innovative approach seamlessly integrates amino acid compositional analysis and protein concentration determination through HILIC LC-MS/MS, leveraging isotope dilution-based quantitation. This synergy empowers the accurate resolution and quantification of all twenty amino acids. This precision is made possible by the utilization of a readily available blend of labeled amino acids, ensuring meticulous protein level determination. This method not only exemplifies robustness and efficiency but also boasts adaptability in detecting non-proteinogenic and modified amino acids. Furthermore, it lends itself to semi-high throughput protein analysis without the need for derivatization, simplifying sample preparation, slashing overall preparation and analysis time, and substantially curbing reagent costs, signifying a remarkable advancement over conventional techniques.

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