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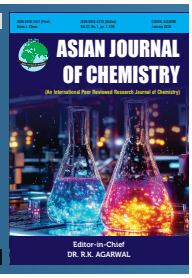

Quantitative LC-MS/MS Determination of Source-Specific Variations of Underivatized Free Amino Acids in Protein Powders

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This study reports a novel, validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to comprehensively profile underivatized free amino acids (FAAs) in diverse whey and plant-based protein powders from global sources, which are commonly consumed for their nutritional value. Though their overall protein composition is frequently promoted, the accurate profiles and patterns of unmodified FAAs, whose functions are significant in metabolism, taste and product quality, are less well described. The validated LC-MS/MS technique showed a broad linearity range, high precision, accuracy and recoveries of 75-121%, with low detection and quantification limits. The analysis and separation time of less than 9 min increases its efficiency. Substantial differences in the FAAs present and their levels, both in kind and quantity, were observed between whey and plant-based proteins from various geographical region. The amino acids like glutamine and L-tryptophan were consistently high-ranking in most species of whey and plant-based proteins investigated. Notably, amino acids linked to sweet taste such as glycine, alanine, serine, proline and threonine were significantly greater in the majority of whey proteins that directly causes acute disorders in healthy individuals as per the International Council on Amino Acid Science (ICAAS). Principal component analysis (PCA) was capable of separating the protein samples that were tested which accounted for 86.88% of the total variance, thus verifying different clustering by FAA profiles. These observations reinforce the significance of source diversity and geographical origin in characterizing the nutritional profile of protein powders beyond bulk protein content. The large-scale profiling of underivatized FAAs furnishes rich information useful for consumers, manufacturers and researchers interested in deciphering and optimizing the nutritional, functional properties of these ubiquitous dietary supplements.

Keywords: Nutritional profiling, LC-MS/MS, Free amino acids, Principal component analysis, Ubiquitous dietary supplements.

INTRODUCTION

Protein supplements are increasingly popular for enhancing health, fitness and overall well-being [1,2]. Animal (whey) and varied plant-based protein powders have become integral parts of contemporary diets and are consumed extensively due to their multifaceted nutritional utility, from muscle development and recuperation to body weight regulation and general well-being [3,4]. Though their most direct appeal is as a high total protein source, the specific profiles and ranges of their individual free amino acids (FAAs) are now understood to be essential determinants of their functional attributes, metabolic effect and organoleptic properties, such as taste and mouthfeel [5].

However, concerns have arisen regarding their quality, purity and potential contamination, which may include adulterants or inaccurate labeling. To address these concerns, a comprehensive analytical approach is essential for evaluating the safety and efficacy of these products [6,7]. Free amino acids are not just building blocks; they play active roles in key physiological processes as signaling compounds, neurotransmitter precursors and gene expression modulators [8].

High concentrations of specific free amino acids, while generally well-tolerated from dietary protein sources, can be associated with adverse health outcomes when present pathologically due to metabolic dysregulation or excessive supplementation [9]. Elevated L-tryptophan, for instance, has been historically linked to Eosinophilia-Myalgia Syndrome (EMS), a severe multi-system disorder caused due to metabolites of tryptophan mainly 1′-ethylene*bis*[tryptophan] (EBT), L-5-hydroxytryptophan (5-HTP), serotonin (5-hydroxytryptamine,

5-HT), 5-hydroxyindoleacetic acid (5-HIAA). Indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), although this outbreak was primarily attributed to contaminated supplements rather than pure L-tryptophan itself [10]. Furthermore, supraphysiological levels of L-tryptophan, particularly when combined with serotonergic medications, can precipitate Serotonin Syndrome, a potentially life-threatening condition characterized by neurological and autonomic hyperactivity [11].

Beyond acute toxicity, dysregulation of tryptophan metabolism, especially shifts towards neurotoxic kynurenine pathway metabolites, is implicated in the pathophysiology of various neurological and psychiatric conditions, including depression and schizophrenia [12,13]. Similarly, pathologically increased concentrations of "sweet-tasting" amino acids like alanine, glycine, serine, proline, glutamine and threonine are predominantly observed in rare inborn errors of metabolism. For example, persistently high glycine levels are characteristic of severe disorders such as nonketotic hyperglycinemia (NKHG) due to deficiencies in the glycine cleavage system [14].

Elevated proline can indicate Hyperprolinemia Type I or II, stemming from defects in proline catabolism, with Type II sometimes associated with neurological symptoms [15]. While glutamine is generally safe, extremely high supplemental doses or accumulation in individuals with compromised liver or kidney function may exacerbate issues related to ammonia or glutamate toxicity [16,17]. These examples underscore that while dietary amino acids are vital, extreme or uncontrolled elevations, often due to underlying metabolic conditions or inappropriate supplementation, can disrupt physiological homeostasis and lead to significant health impairments.

The precise and complete characterization of FAAs in intricate food matrices such as protein powders poses severe analytical challenges. Traditional reverse-phase liquid chromatography (RP-LC), the most commonly employed methodology, tends to have poor resolution properties for underivatized amino acids because of their very polar nature and absence of significant hydrophobic side-chains required for robust retention on conventional C_{18} columns [18-23]. While hydrophilic interaction liquid chromatography (HILC) in conjunction with UHPLC-MS/MS has proven to be a sensitive and effective alternative method for the analysis of underivatized FAAs [24-27], the intricacy of its mobile phase demands and optimization can remain as constraints for routine high-throughput analysis of a wide range of samples. In addition, the determination of FAAs from intricate protein matrices requires an efficient cleanup procedure to eliminate interfering chemicals that may degrade LC-MS/MS sensitivity and precision. Although ion exchange techniques are known to be selective in amino acid extraction, their use is regularly limited due to time demands and their expense, precluding their utility for large sample sets [28-35]. Thus, the fundamental analytical deficiency remains for a fast, inexpensive and rugged method that can extract and isolate underivatized FAAs from a variety of protein powder samples with high sensitivity and reproducibility.

Responding to these issues, this article presents a new LC-MS/MS approach for the overall analysis of underivatized free amino acids in protein powders. The approach uniquely combines a simplified but efficient liquid-liquid extraction with filtration and evaporation sample preparation, avoiding the drawbacks of more laborious cleanup methods. Key to the technique is the chromatographic separation on a size-exclusion stationary phase with tandem mass spectrometry, a different approach that exploits the subtle size discrepancies and hydrodynamic volumes of underivatized amino acids to provide superior separation. This new methodological combination provides a durable, efficient and fast solution, with fast separation and analysis time of under 9 min.

This proven technique has been used to routinely analyze the differences in FAA amounts in a wide variety of commercial whey and plant-based protein powders. Through presenting a comprehensive picture of the non-derivatized FAA scene, this research seeks to provide insightful information on the nutritional, functional and sensory variations inherent in protein powders of various origins, according to FSSAI, EU and the International Council on Amino Acid Science (ICAAS), ultimately benefiting researchers, manufacturers and consumers alike in the quest for optimized protein supplementing schemes.

EXPERIMENTAL

Eighteen dietary protein supplements [whey protein and plant based protein powders], sourced from a India, China and Japan nutraceutical export exhibition, were utilized in this study. These supplements, available in powder sachet form, contained a daily recommended dose of 500 mg essential amino acids, including various amino acids along with additional nutrients like vitamins and minerals. A comprehensive set of standard L-amino acids was employed, encompassing lysine, serine, histidine, glutamic acid, alanine, tryptophan, methionine, leucine, phenylalanine, aspartic acid, threonine, arginine, glycine, isoleucine, cysteine, tyrosine, proline, asparagine, valine and glutamine. To ensure analytical precision, ultra LC-MS grade solvents and formic acid were procured from Biosolve (Hyderabad, India), pipecolic acid, nitric acid, hydrochloric acid were obtained from E-Merck (Bengaluru, India) and ultrapure water was sourced from a Direct Q (Millipore) water purification system (Millipore, UK).

Preparation of standard amino acid solution: Individual amino acid standard stock solutions were prepared by dissolving 10 mg of each amino acid in a mixed acid solvent comprising 1 mL of 0.05 M HNO_3 and 1 mL of 0.05 M HCl. Subsequent working concentrations were achieved by diluting these stock solutions with deionized water as required by maintaining suitable pH. All the prepared standard solutions were stored at -20 ºC to maintain their stability until use.

Preparation of Sample protein powder solution: Eighteen sample solutions for analysis were prepared through an acid digestion method to liberate free amino acids. Briefly, 10 mg of each protein powder was accurately weighed and dissolved in a solvent mixture of 1 mL of 0.05 M HCl and 1 mL of 0.05 M diluted HNO_3. Samples were then diluted with deionized water to reach their target working concentrations.

To ensure thorough protein breakdown and complete amino acid liberation, the solutions underwent a sequential mechanical treatment by vortexing for 5 min, followed by sonication for

10 min at room temperature. After sonication, samples were shaken on a mechanical shaker for 20 min at 300 strokes per min to ensure complete mixing. Insoluble material was subsequently removed by centrifugation for 15 min at 10,000 rpm yielding a clear supernatant containing the liberated amino acids. Finally, this supernatant was passed through a 0.45 µm nylon syringe filter to eliminate any residual particulates that could interfere with downstream LC-MS/MS analysis.

LC-MS/MS analysis: Liquid chromatography-tandem mass spectrometry (LC-MS/MS) anaysis was performed using an Agilent 6420A triple quadrupole mass spectrometer coupled with an Agilent 1290 Quaternary pump and a 1260 series autosampler (Agilent, USA). For the chromatographic separation of underivatized amino acids, a Phenomenex Yarra size exclusion chromatography column (300 mm length, 4.6 mm internal diameter, 3 µm particle thickness, Phenomenex, USA) was used. The mobile phase, an isocratic mixture of 60:40 acetonitrile: water containing 0.4% formic acid, was delivered at a flow rate of 600 mL/min. The mass spectrometer operated in positive ion electrospray ionization (ESI) mode. Key source parameters included a capillary voltage of 3850 V, nebulizing gas flow rate of 12 L/min, source temperature of 250 ºC, nebulizer gas pressure of 50 psi and a capillary current of 4400 nA (Table-1). Amino acids were quantified using optimized multiple reaction monitoring (MRM) conditions (Table-2 and Fig. 1).

Sample preparation for method validation: For method validation, a stock solution of the amino acid mixture was serially diluted with the mobile phase to prepare 11 calibration standards. These standards covered a wide concentration range: 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 µg/mL. Calibration

TABLE-1
LC-MS/MS ANALYTICAL METHOD PARAMETERS

Parameter category	Specific parameter	Value/Description
Instrumentation	Mass spectrometer	Agilent 6420A Triple Quadrupole
	LC pump	Agilent 1290 Quaternary Pump
	Autosampler	Agilent 1260 Series Autosampler
	Manufacturer	Agilent, USA
Chromatography	Column	Phenomenex yarra size exclusion chromatography
	Column dimensions	300 mm (length) × 4.6 mm (ID) × 3 µm (particle thickness)
	Column manufacturer	Phenomenex, USA
	Mobile phase	60% Acetonitrile : 40% water with 0.4% formic acid
	Elution mode	Isocratic
	Flow rate	600 mL/min
Mass spectrometry	Ionization mode	Positive electrospray ionization (ESI)
	Capillary voltage	3850 V
	Nebulizing gas flow rate	12 L/min
	Source temperature	250 ºC
	Nebulizer gas pressure	50 psi
	Capillary current	4400 nA
	Acquisition mode	Optimized multiple reaction monitoring (MRM)
	Quantification data	Refer to Table-2 (MRM transitions for amino acids)

TABLE-2
AMINO ACIDS VALIDATION PARAMETERS IN MRM USING LC-MS/MS ANALYSIS

Compound name	Retention time (min)	(M+H)	Q1	LOD (ng/g)	LOQ (ng/g)	Recovery (n = 5)		
						100 ng/g	500 ng/g	5000 ng/g
Lys	3.68	147	84.2	6.89	20.89	121.25	119.25	79.81
Arg	3.69	175	70	4.43	13.42	121.82	87.66	103.10
His	3.79	156	110	4.19	12.71	118.84	94.43	80.85
Val	3.89	118	72	8.24	24.99	90.38	109.32	94.45
Ala	4.02	90	44	10.35	31.37	89.63	103.58	97.16
Thr	4.52	120	56	8.65	26.22	105.93	114.17	97.27
Ser	4.61	106	60	7.54	22.86	115.06	105.08	95.77
Trp	4.95	205	188	6.79	20.60	63.66	94.59	81.20
Tyr	5.15	182	91	7.85	23.81	81.66	106.68	91.89
Phe	5.17	166	120	9.38	28.42	89.05	100.18	89.17
Met	5.23	150	56	16.52	50.06	87.28	89.65	94.26
Glu	5.53	148	84	8.87	26.88	90.62	100.17	90.87
Gln	5.57	147	84	2.50	7.60	92.24	121.13	114.92
Asp	5.70	134	75	12.67	38.40	Not detected	66.19	98.73
Ile	5.726	132	86	12.34	37.41	90.78	114.65	88.94
Leu	5.728	132	86	7.94	24.07	96.26	118.89	90.88
Pro	5.83	116	70	9.50	28.78	92.22	105.91	93.60
Gly	6.26	76	30	7.97	24.15	107.78	115.06	103.79
Asn	7.69	133	75	7.04	21.32	120.32	119.17	113.32

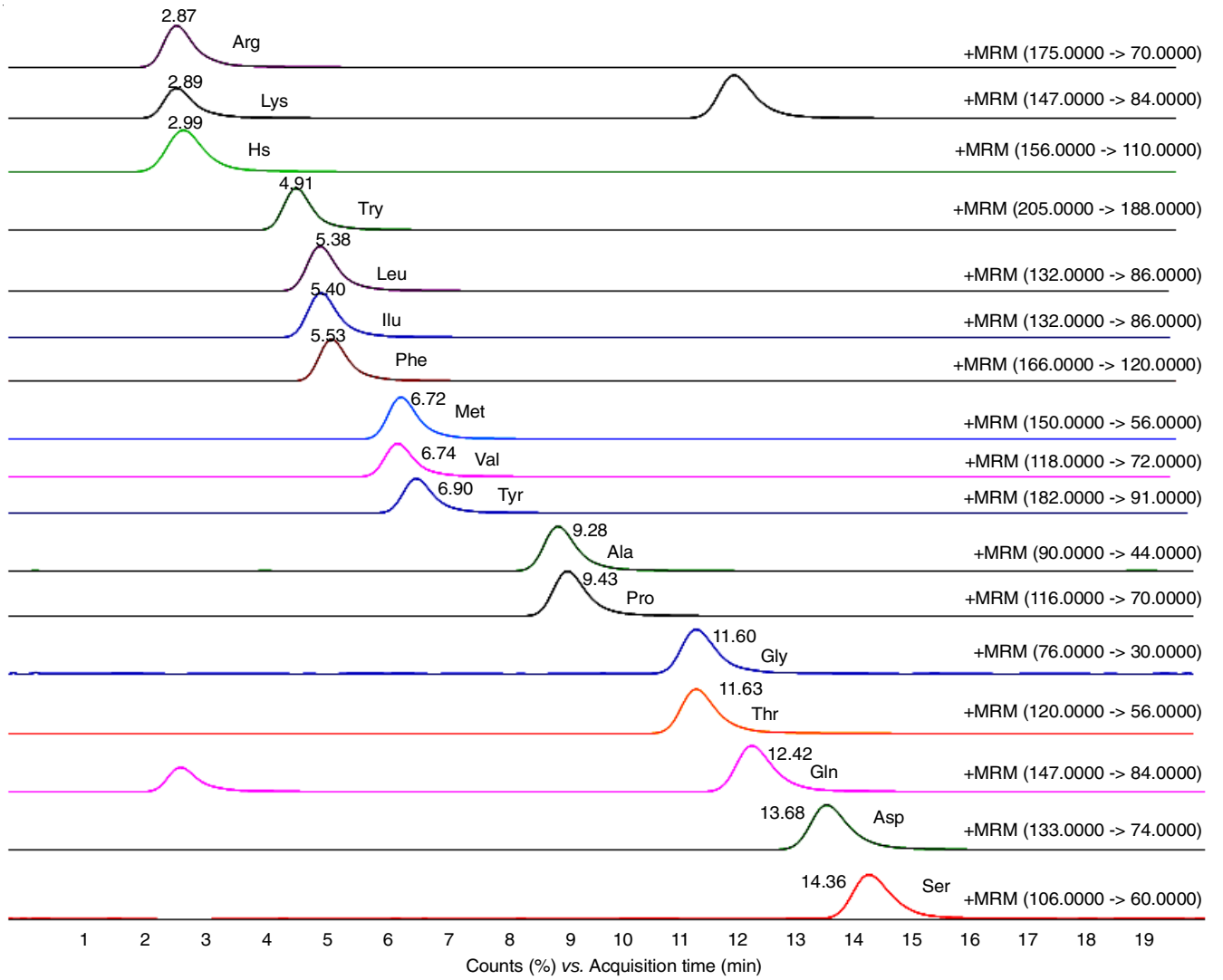

Fig. 1. MRM transitions of total amino acids

curves were then constructed by plotting the peak areas obtained from the standards against their corresponding concentrations. The limits of detection (LOD) and limits of quantification (LOQ) for each amino acid were determined based on signal-to-noise (S/N) ratios of approximately 3:1 and 10:1, respectively.

To assess matrix effects, known concentrations of the standard solution were spiked into the samples. Given the challenge of obtaining a truly blank protein matrix devoid of endogenous amino acids, each protein sample underwent a rigorous washing procedure. Specifically, 100 mg of each protein sample was washed three times with 100 mL of 0.05 M HCl solution to minimize background interference. For recovery studies, appropriate amounts of standard amino acid solutions were spiked into these 100 mg protein samples. Calibration curves were then generated from these spiked samples and their slopes were compared to the slopes obtained from aqueous standards at the identical concentration levels to determine recovery.

RESULTS AND DISCUSSION

Free amino acids (FAAs), the fundamental building blocks of proteins, are crucial for human health, with their metabolism implicated in various disorders, including eosinophilia-myalgia syndrome (EMS), Alzheimer's disease, serotonin syndrome, nonketotic hyperglycinemia (NKHG), hyperprolinemia (Type I or II) and periodontal disease [12,13]. The specific amino acid profiles within protein powders can significantly influence metabolic regulation and overall health outcomes [16].

Previous reports indicate that glutamine, tryptophan and asparagine are often prominent amino acids in protein samples. Our current study confirms the significant presence of essential amino acids such as valine, leucine, threonine and phenylalanine along with a slight increase in the concentrations of alanine, tryptophan, glutamine and methionine (Table-3). However, a more indepth analysis using principal component analysis (PCA) revealed that alanine, valine, asparagine, aspartic acid, tryptophan, glutamine and methionine were the key discriminators, highlighting critical quality differences among the samples. Notably, the whey and vegan protein samples originating from Japan exhibited distinct variations in their amino acid profiles compared to those from India and China. This underscores the impact of geographical source on amino acid composition.

TABLE-3
AMINO ACID CONCENTRATION PRESENT IN THE SELECTED SAMPLES OF PROTEIN POWDERS (VALUES ARE MEAN ± SD)

Compound name	Met	Trp	Gly	Asp	Asn	Val	His
WPC-1 (µg/g)	8.50 ± 1.62	0.14 ± 0.06	1.21 ± 0.15	1.18 ± 0.24	145.60 ± 63.14	152.18 ± 64.38	39.01 ± 11.16
WPC-2 (µg/g)	9.83 ± 0.71	0.52 ± 0.26	1.91 ± 0.19	2.45 ± 0.54	79.91 ± 1.99	105.15 ± 51.89	19.63 ± 1.13
WPC-3 (µg/g)	0.19 ± 0.12	1.53 ± 0.15	1.80 ± 0.34	147.96 ± 62.98	154.40 ± 70.69	16.56 ± 2.26	177.70 ± 11.07
WPJ-1 (µg/g)	0.13 ± 0.05	1.36 ± 0.09	1.63 ± 0.38	116.99 ± 52.48	122.53 ± 56.15	24.20 ± 8.90	169.39 ± 10.75
WPJ-2 (µg/g)	0.19 ± 0.12	.253 ± 0.15	1.80 ± 0.34	147.96 ± 62.98	154.40 ± 70.69	16.56 ± 2.26	177.70 ± 11.07
WPJ-3 (µg/g)	2.14 ± 0.06	2.21 ± 0.15	1.18 ± 0.24	145.60 ± 63.14	152.18 ± 64.38	39.01 ± 11.16	145.61 ± 40.74
WPI-1 (µg/g)	0.52 ± 0.26	3.91 ± 0.19	2.45 ± 0.54	79.91 ± 1.99	105.15 ± 51.89	19.63 ± 1.13	201.55 ± 21.81
WPI-2 (µg/g)	0.19 ± 0.12	1.53 ± 0.15	1.80 ± 0.34	147.96 ± 62.98	154.40 ± 70.69	16.56 ± 2.26	177.70 ± 11.07
WPI-3 (µg/g)	0.13 ± 0.05	1.36 ± 0.09	1.63 ± 0.38	116.99 ± 52.48	122.53 ± 56.15	24.20 ± 8.90	169.39 ± 10.75
VPC-1 (µg/g)	0.19 ± 0.12	1.53 ± 0.15	1.80 ± 0.34	147.96 ± 62.98	154.40 ± 70.69	16.56 ± 2.26	177.70 ± 11.07
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VPI-3 (µg/g)	0.13 ± 0.05	1.36 ± 0.09	1.63 ± 0.38	116.99 ± 52.48	122.53 ± 56.15	24.20 ± 8.90	169.39 ± 10.75

Compound name	Phe	Gln	Ile	Leu	Tyr	Glu	Ser
WPC-1 (µg/g)	145.61 ± 40.74	3.23 ± 1.46	201.89 ± 93.85	22.98 ± 13.36	26.07 ± 14.12	4.08 ± 1.54	3.52 ± 0.56
WPC-2 (µg/g)	201.55 ± 21.81	9.42 ± 2.52	294.36 ± 22.59	40.85 ± 2.11	41.04 ± 2.29	8.70 ± 1.89	3.92 ± 1.49
WPC-3 (µg/g)	2.34 ± 1.43	283.73 ± 21.23	31.61 ± 9.19	34.61 ± 2.45	6.31 ± 1.05	5.24 ± 0.77	51.56 ± 3.70
WPJ-1 (µg/g)	4.99 ± 0.36	263.49 ± 23.77	16.26 ± 8.89	18.11 ± 11.05	36.00 ± 0.06	12.43 ± 2.04	47.88 ± 3.10
WPJ-2 (µg/g)	2.34 ± 1.43	283.73 ± 21.23	31.61 ± 9.19	34.61 ± 2.45	4.31 ± 2.05	5.24 ± 0.77	51.56 ± 3.70
WPJ-3 (µg/g)	3.23 ± 1.46	201.89 ± 93.85	22.98 ± 13.36	26.07 ± 14.12	25.08 ± 1.54	3.52 ± 0.56	17.76 ± 4.90
WPI-1 (µg/g)	9.42 ± 2.52	294.36 ± 22.59	40.85 ± 2.11	41.04 ± 2.29	8.70 ± 1.89	3.92 ± 1.49	48.01 ± 5.09
WPI-2 (µg/g)	2.34 ± 1.43	283.73 ± 21.23	31.61 ± 9.19	34.61 ± 2.45	4.31 ± 1.05	5.24 ± 0.77	51.56 ± 3.70
WPI-3 (µg/g)	4.99 ± 0.36	263.49 ± 23.77	16.26 ± 8.89	18.11 ± 11.05	36.00 ± 0.06	12.43 ± 2.04	47.88 ± 3.10
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VPJ-1 (µg/g)	4.99 ± 0.36	263.49 ± 23.77	16.26 ± 8.89	18.11 ± 11.05	36.00 ± 0.06	12.43 ± 2.04	47.88 ± 3.10
VPJ-2 (µg/g)	2.34 ± 1.43	283.73 ± 21.23	31.61 ± 9.19	34.61 ± 2.45	4.31 ± 2.05	5.24 ± 0.77	51.56 ± 3.70
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VPI-1 (µg/g)	9.42 ± 2.52	294.36 ± 22.59	40.85 ± 2.11	41.04 ± 2.29	8.70 ± 1.89	3.92 ± 1.49	48.01 ± 5.09
VPI-2 (µg/g)	2.34 ± 1.43	283.73 ± 21.23	31.61 ± 9.19	34.61 ± 2.45	4.31 ± 1.05	5.24 ± 0.77	51.56 ± 3.70
VPI-3 (µg/g)	4.99 ± 0.36	263.49 ± 23.77	16.26 ± 8.89	18.11 ± 11.05	36.00 ± 0.06	12.43 ± 2.04	47.88 ± 3.10

Compound name	Arg	Pro	Ala	Thr	Lys
WPC-1 (µg/g)	17.76 ± 4.90	51.71 ± 8.61	5.49 ± 0.63	2.23 ± 0.71	7.56 ±1.57
WPC-2 (µg/g)	48.01 ± 5.09	58.32 ± 4.75	8.01 ± 0.8	10.82 ± 4.88	9.59 ± 1.07
WPC-3 (µg/g)	53.47 ± 4.87	8.23 ± 1.06	8.29 ± 6.08	8.37 ± 0.90	9.70 ± 0.76
WPJ-1 (µg/g)	42.07 ± 4.48	8.12 ± 0.95	4.44 ± 0.76	7.38 ± 0.93	7.96 ± 0.65
WPJ-2 (µg/g)	53.47 ± 4.87	8.23 ± 1.06	8.29 ± 6.08	8.37 ± 0.90	9.70 ± 0.76
WPJ-3 (µg/g)	51.71 ± 8.61	5.49 ± 0.63	2.23 ± 0.71	7.56 ±1.57	8.50 ± 1.62
WPI-1 (µg/g)	58.32 ± 4.75	8.01 ± 0.8	10.82 ± 4.88	9.59 ± 1.07	9.83 ± 0.71
WPI-2 (µg/g)	53.47 ± 4.87	8.23 ± 1.06	8.29 ± 6.08	8.37 ± 0.90	9.70 ± 0.76
WPI-3 (µg/g)	42.07 ± 4.48	8.12 ± 0.95	4.44 ± 0.76	7.38 ± 0.93	7.96 ± 0.65
VPC-1 (µg/g)	53.47 ± 4.87	8.23 ± 1.06	8.29 ± 6.08	8.37 ± 0.90	9.70 ± 0.76
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VPI-2 (µg/g)	53.47 ± 4.87	8.23 ± 1.06	8.29 ± 6.08	8.37 ± 0.90	9.70 ± 0.76
VPI-3 (µg/g)	42.07 ± 4.48	8.12 ± 0.95	4.44 ± 0.76	7.38 ± 0.93	7.96 ± 0.65

WPC = Whey protein China samples; WPJ = Whey protein Japan samples; WPI = Whey protein Indian samples; VPC = Vegan protein China samples; VPJ = Vegan protein Japan samples; VPI = Vegan protein Indian samples.

To facilitate this detailed analysis, we developed a simple and rapid LC-MS/MS method utilizing a size exclusion chromatography (SEC) column for effective separation, identification and quantification of FAAs. The extraction of FAAs from protein samples was achieved *via* a liquid-liquid extraction procedure using acetonitrile and water (60:40) with 0.4% formic acid. Chromatographic elution was performed in an isocratic mode with a mobile phase of 60% acetonitrile and 40% water containing 0.4% formic acid. This optimized LC-MS/MS method achieved the separation and elution of all target amino acids within a remarkable 9 min.

The linearity of the method was validated across a broad concentration range from 0.05 to 100 µg/mL, accommodating the high concentrations of amino acids in most of the samples. The limit of detection (LOD) ranged from 4 to 16 ng/g and the limit of quantification (LOQ) ranged from 7 to 50 ng/g. Notably, glutamine exhibited the lowest LOQ, while tryptophan and methionine had the highest. Further details on the LOD and LOQ values for individual analytes are presented in Table-2. Method precision was assessed through intra- and inter-day variations by analyzing five replicate samples of a total of 18 protein powders at three concentration levels (100, 500 and 5000 ng). The calculated relative standard deviation (RSD) values were consistently less than 18%, demonstrating the robust precision and reliability of the method.

Recovery of amino acids: To assess the recovery of amino acids, we conducted spiking experiments by adding known concentrations of a standard amino acid mixture to protein samples. Recognizing the inherent challenge in obtaining a truly analyte-free protein matrix (due to the high natural abundance of amino acids), the protein samples were pre-treated. Each protein sample (100 mg) was washed three times with 100 mL of 0.05 M HCl to minimize the native amino acid background.

After this washing step, the treated protein samples were spiked with the standard amino acid mixture at three concentration levels *viz*. 100 ng, 500 ng and 5000 ng. These spiked samples were then extracted with the mobile phase, followed by centrifugation at 10,000 rpm for 5 min to separate solids. The resulting supernatant was collected and evaporated to dryness under a gentle stream of nitrogen. The dried residues were then reconstituted with 0.5 mL of the mobile phase and subjected to LC-MS/MS analysis.

The percentage recovery for each analyte was determined by comparing the peak areas obtained from the spiked samples to those of standard analytes prepared at equivalent concentrations in the mobile phase. Overall, the recovery for most amino acids was observed to be within the range of 75-121%. Specifically, the majority of amino acids showed recoveries between 87-121%. Tyrosine, tryptophan, aspartic acid and glutamic acid exhibited slightly lower, yet acceptable, recoveries ranging from 70-106%. These recovery rates demonstrate the robustness and effectiveness of the sample preparation and analytical method.

Conclusion

This study successfully developed and validated a rapid precise and accurate LC-MS/MS method for the quantitative determination of underivatized free amino acids (FAAs) in diverse whey and vegan protein powders. The method demonstrated excellent linearity and recoveries (75-121%). The comprehensive analysis revealed significant variations in FAA profiles, not only between whey and plant-based protein sources but also based on their geographical origin. Tyrosine, tryptophan and glutamine were consistently observed to be major amino acids present at high concentrations in many samples, aligning with essential nutritional requirements and considerations from regulatory standards such as FSSAI, EU and the International Council on Amino Acid Science (ICAAS). Furthermore, amino acids associated with a sweet taste (glycine, alanine, serine, proline, glutamine, tryptophan and threonine) were found in significantly higher concentrations in the most whey and vegan protein samples especially samples from the Japan. Principal component analysis (PCA) effectively distinguished the protein samples based on their unique FAA fingerprints, with 86.88% of the total variance explained by the principal components, confirming distinct clustering patterns. These findings underscore the critical importance of both source diversity and geographical origin in shaping the unique nutritional and functional characteristics of protein powders, extending beyond their bulk protein content. The detailed profiling of underivatized FAAs provides valuable insights for consumers, manufacturers and researchers aiming to optimize the nutritional value and functional properties of these widely consumed dietary supplements, which directly causes acute disorders in healthy individuals.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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