Food Control 168 (2025) 110909

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Authentication of porcine, bovine, and fish gelatins based on quantitative profile of amino acid and chemometric analysis

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A R T I C L E I N F O

Keywords:
Amino acids
Hydrophilic interaction chromatography
Mass spectrometry
gelatin
Principle component analysis

A B S T R A C T

Gelatin is a biological macromolecule derived from the partial hydrolysis of collagen protein. This paper describes a sensitive and rapid method for the detection of gelatin sources based on the composition of amino acids. Hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry (HILIC-MS/MS) and chemometric tools such as principle component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used for the analysis and spectral classification, respectively. Twenty amino acids are identified and quantified with the limit-of-detection (LOD) of 0.03–2.62 μg/mL and limit-of-quantification (LOQ) of 0.10–7.93 μg/mL, a precision of 0.19–11.21% and 0.03–13.62% (%RSD) for intra and inter-day, respectively, and a total recovery of 85.1–107.6%. Porcine gelatin showed correlation with glycine, proline, hydroxyproline, tyrosine glutamine, and glutamic acid; Bovine gelatin was correlated with lysine, leucine, and isoleucine histidine, phenylalanine, and alanine, and fish gelatin showed correlation with methionine, threonine, serine arginine and cysteine in PCA analysis. Verification of the developed method was confirmed by using different commercial and laboratory prepared gelatin products containing gelatin and the samples were successfully categorized into their respective sources.

1. Introduction

Gelatin, a water-soluble and flavorless protein, is obtained when collagen protein undergoes considerable processing. Collagen proteins are extracted from various parts of animals, including the skins, bones, and cartilage (Ranasinghe et al., 2022). When collagen is partially hydrolyzed, it converts into a mixture of polypeptide chains resulting in gelatin formation (Noor et al., 2021). The gelatin can be in the form of granules, powder, or sheets. The gelatins obtained from acid hydrolysis are categorized as type A and basic hydrolysis is categorized as type B (Uddin et al., 2021).

Gelatin is used as gelling, binding, glazing, and coating materials in different cosmetics, food, and pharmaceutical products due to its appealing properties (Usman et al., 2023). Studies reveal that gelatin has a market size of USD 3.6 billion approximately and the global gelatin production is estimated to be 326,000 tons (Hassan et al., 2023). The gelatin market is expected to reach USD 6.7 billion by the end of 2027. Mammalian species are the primary source of commercially accessible gelatins that are mainly obtained from the skins of porcine (46%) and bovine (29.4%), bones of both (23.1%), and other animals' raw materials (1.5%) (donkey and horsehide, fish skin, etc.) (Tukiran, 2019). Gelatin obtained from marine species and poultry feet and skin is another alternative source of gelatin. However, both gelatins exhibit low yields in the global market (Gaspar-Pintiliescu et al., 2019).

Furthermore, the acceptability of gelatin relies on the origin of collagen from which it is derived. The religious beliefs of Muslims, Jews, and some communities of Christians avoid any pork components while the Hindu community's religious convictions demand gelatin products to be free from bovine sources (Zhu et al., 2023). Therefore, religious considerations have shaped the demand for alternative sources like fish

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https://doi.org/10.1016/j.foodcont.2024.110909
Received 17 May 2024; Received in revised form 26 August 2024; Accepted 21 September 2024
Available online 23 September 2024
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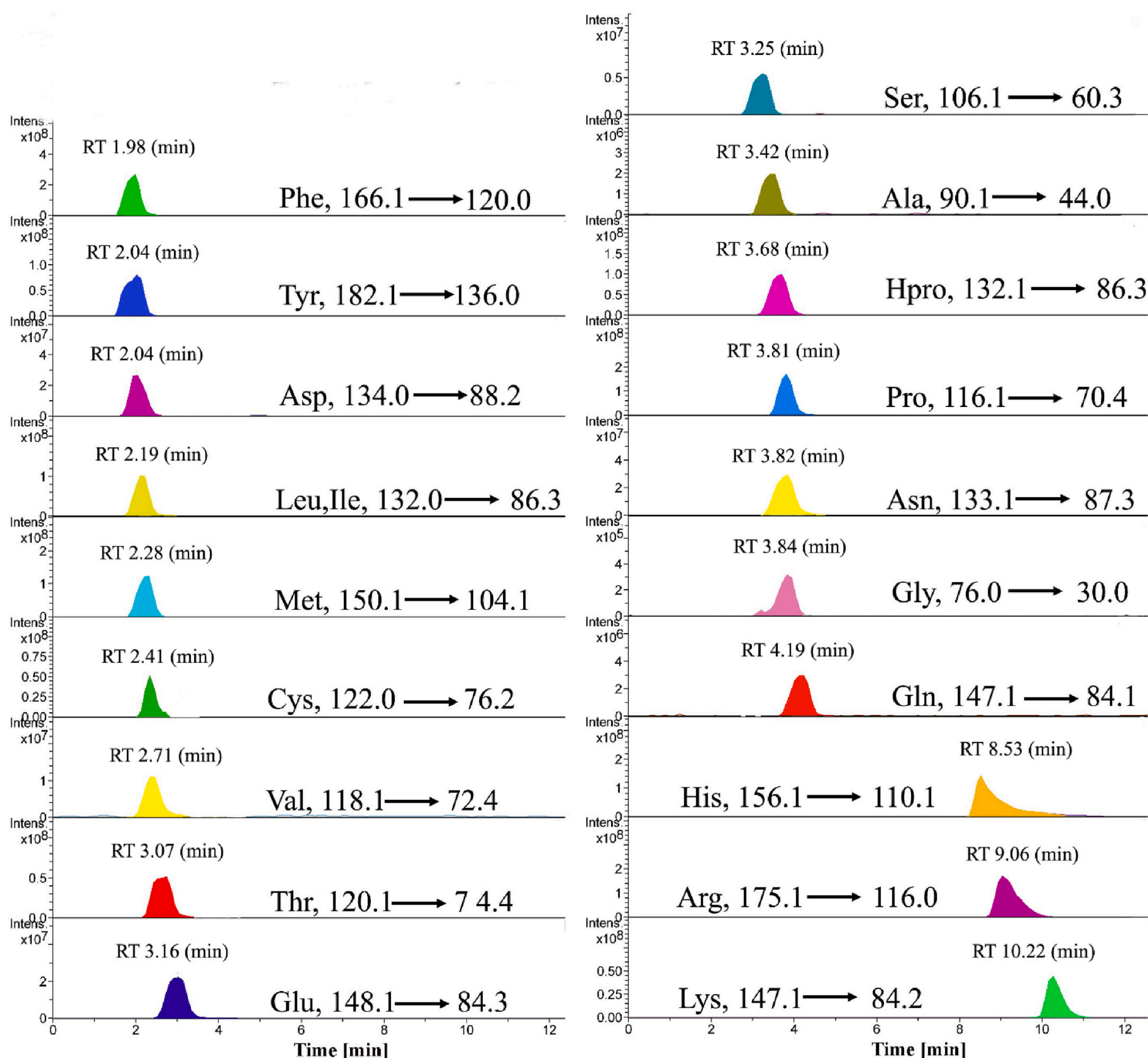

Fig. 1. Typical HILIC chromatogram of mixed standards with MRM method.

and poultry to cater to the requirements of halal and Kosher markets (Tukiran et al., 2023).

The extensive production of porcine gelatin globally has raised uncertainties among consumers regarding the products containing gelatin. Other major issues are the adulteration of different animals' gelatin and fraud of false labeling on packages to increase profits (Han et al., 2022). These matters have stimulated a wider interest among researchers and academic communities to develop accurate, precise, and reliable analytical methods for authentication of gelatin sources and adulteration in gelatin-based products (Girish et al., 2020).

Therefore, various methods have been developed based on different working principles and approaches to differentiate gelatin origin (Ishaq et al., 2020) such as enzyme-linked immune sorbent assay (ELISA) (Tukiran et al., 2016; Venien & Levieux, 2005), gel electrophoresis (Aina et al., 2013; Yap & Gam, 2019), polymerase chain reaction (PCR) (Sultana, Hossain, Zaidul, & Ali, 2018; Zhang, Chan, et al., 2019a,b), liquid chromatography combined with PCA (Raraswati et al., 2013; Widyaninggar et al., 2012) and mass spectrometry (Cai et al., 2021; Sha et al., 2020), and spectroscopic (Jariyah et al., 2021; Zhang et al., 2018). However, the manufacturing of gelatin involves the utilization of high temperatures due to which species-specific DNA is degraded and denatured making PCR methods challenging or inappropriate for species detection in highly processed gelatin samples (Mortas et al., 2022). ELISA being a relatively cost-effective and simple method for routine analysis exhibits several limitations such as it can only detect a limited sequence of epitopes, and repeated analysis is not feasible due to the denaturation of biomarkers epitopes which compromises the specificity of antibodies. This is particularly challenging because various gelatin sources exhibit similarities in the sequences of collagen (Kuramata et al., 2022). In the spectroscopic method, the sensitivity of Fourier transform infrared (FTIR) is low and the requirement of highly pure samples makes it unsuitable for the analysis of processed food samples (Hassan et al., 2023). Distinguishing gelatin sources through liquid chromatography-tandem mass spectrometry (LC-MS/MS) based on

Table 1
Retention time and precursor/product ion pairs of respective amino acids.

Amino acids	Retention time (min)	$[M+H]^+$ (*m/z*)	Transition (precursor → product)		Relative Collision Energy (%)
			Identification (q)	Quantification (Q)	
L-Phenylalanine (Phe)	1.98	166.08	166.1 → 103.0	166.1 → 120.0	16
L-Tyrosine (Tyr)	2.04	182.08	182.1 → 91.2	182.1 → 136.0	12
L-Aspartic acid (Asp)	2.04	134.00	134.0 → 74.0	134.0 → 88.2	15
L-Leucine (Leu), L-Iso-leucine (Ile)	2.19	132.10	132.1 → 69.1	132.1 → 86.3	10
L-Methionine (Met)	2.28	150.05	150.1 → 56.0	150.1 → 104.1	18
L-Cysteine (Cys)	2.41	122.02	122.0 → 59.3	122.0 → 76.2	35
L-Valine (Val)	2.71	118.08	118.1 → 55.0	118.1 → 72.4	20
L-Threonine (Thr)	3.07	120.06	120.1 → 102.0	120.1 → 74.4	14
L-Glutamic acid (Glu)	3.16	148.06	148.1 → 130.1	148.1 → 84.3	32
L-Serine (Ser)	3.25	106.04	106.0 → 70.0	106.1 → 60.3	22
L-Alanine (Ala)	3.42	90.05	90.1 → 62.1	90.1 → 44.0	25
L-Hydroxyl-proline (Hpro)	3.68	132.06	132.1 → 70.0	132.1 → 86.3	37
L-Proline (Pro)	3.81	116.07	116.1 → 43.1	116.1 → 70.4	27
L-Asparagine (Asn)	3.82	133.06	133.1 → 74.2	133.1 → 87.3	24
Glycine (Gly)	3.84	76.03	76.0 → 45.0	76.0 → 30.0	29
L-Glutamine (Gln)	4.19	147.07	147.1 → 130.0	147.1 → 84.1	16
L-Histidine (His)	8.53	156.07	156.1 → 93.1	156.1 → 110.1	19
L-Arginine (Arg)	9.06	175.11	175.1 → 157.0	175.1 → 116.0	36
L-Lysine (Lys)	10.22	147.11	147.1 → 130	147.1 → 84.2	34

Table 2
Regression equation, determination coefficient, and limits of detection (LODs) and quantitation (LOQs) of respective amino acids (n = 4).

Amino acids	Linearity range (10–200 μg mL^{-1})		LOD (μg mL^{-1})	LOQ (μg mL^{-1})
	Calibration linear equation	Determination coefficient (R^2)		
L-Phenylalanine (Phe)	y = 0.0446x + 2.6238	0.9931	2.48	7.52
L-Tyrosine (Tyr)	y = 0.0206x + 1.3802	0.9947	0.89	2.71
L-Aspartic acid (Asp)	y = 0.0027x + 0.1413	0.9953	0.19	0.58
L-Leucine (Leu), L-Iso-leucine (Ile)	y = 0.028x + 1.9628	0.9934	2.14	6.50
L-Methionine (Met)	y = 0.0164x + 1.2215	0.9944	1.79	5.44
L-Cysteine (Cys)	y = 0.0025x + 0.1454	0.9956	0.26	0.79
L-Valine (Val)	y = 0.008x + 0.7578	0.9963	0.40	1.23
L-Threonine (Thr)	y = 0.0304x + 1.2712	0.9946	1.57	4.76
L-Glutamic acid (Glu)	y = 0.0069x + 0.4218	0.9948	0.12	0.38
L-Serine (Ser)	y = 0.001x + 0.0858	0.9936	0.17	0.52
L-Alanine (Ala)	y = 0.0021x + 0.1629	0.9934	0.24	0.71
L-Hydroxyl-proline (Hpro)	y = 0.0295x + 1.1947	0.9941	1.36	4.12
L-Proline (Pro)	y = 0.0424x + 1.6834	0.9955	1.71	5.17
L-Asparagine (Asn)	y = 0.0095x + 0.5142	0.9952	0.37	1.12
Glycine (Gly)	y = 0.0001x + 0.0054	0.9936	0.03	0.10
L-Glutamine (Gln)	y = 0.007x + 0.4343	0.9961	0.64	1.94
L-Histidine (His)	y = 0.0476x + 0.8852	0.9972	1.17	3.56
L-Arginine (Arg)	y = 0.0588x + 1.7285	0.9955	2.21	6.71
L-Lysine (Lys)	y = 0.0132x + 0.4784	0.9969	0.42	1.28

biomarker peptides could lead to false positive peptide matches. This is attributed to the presence of highly repetitive motifs within collagen and gelatin molecules, introduced through hydroxylation sites, along with variations in their relative abundance (Nurilmala, Hizbullah, Karnia, Kusumaningtyas, & Ochiai, 2020).

Various methods have been reported to differentiate the species origin of gelatin based on the composition of amino acid and profiling using reverse-phase high-performance liquid chromatography (RP-HPLC) methods (Azilawati et al., 2015; Sani et al., 2021; Widyaninggar et al., 2012). These methods have used a derivatization approach to detect amino acids since most of the amino acids have little ultraviolet absorbance. Unfortunately, the derivatization-based approaches possess some limitations, including unstable derivatives, interference of reagents, and inadequate reproducibility of derivative yield (Kipuraet al., 2024). To overcome these limitations, a method free of derivatization needs to be established. In this regard, mass spectrometry is one of the promising candidates with high sensitivity, versatility, and vast attractive features. Similarly, the detection and accurate quantitation of amino acids using RP-HPLC with ion-pair reagents is challenging due to insufficient separation and weak retention. Alternatively, using HILIC, amino acids have good separation and retention time (Guo et al., 2013). Several methods have been reported for the detection and quantification of underivatized amino acids using HILIC-tandem mass spectrometry. For instance, Virgiliou et al. (2021) accurately quantified endogenous amino acids in urine by HILIC-tandem MS method. Wen Ma et al., (2022) also determined 20 amino acids in honey through a rapid underivatized method using HILIC-MS/MS. Wudy et al. (2023) recently studied different underivatized amino acids in infant serum by HILIC-ESI-MS/MS.

In this study, we report the establishment, validation, and utilization of a sensitive and rapid HILIC method coupled with tandem mass spectrometry with the incorporation of PCA for underivatized amino acids detection and quantification to distinguish porcine, bovine, and fish gelatin sources and to the best of authors' knowledge, this has not been previously reported. The effectiveness of the developed method is determined by analyzing a range of real samples, including both gelatin-based food and non-food products. Additionally, multivariate statistical analysis was conducted to explore variations in amino acid profiles among these gelatin-based products with reference to gelatin standards. This approach addresses the demands of consumers for authenticating gelatin sources, particularly concerning religious restrictions and health safety. It also proves valuable as a screening method for identifying gelatin sources in various commercial products.

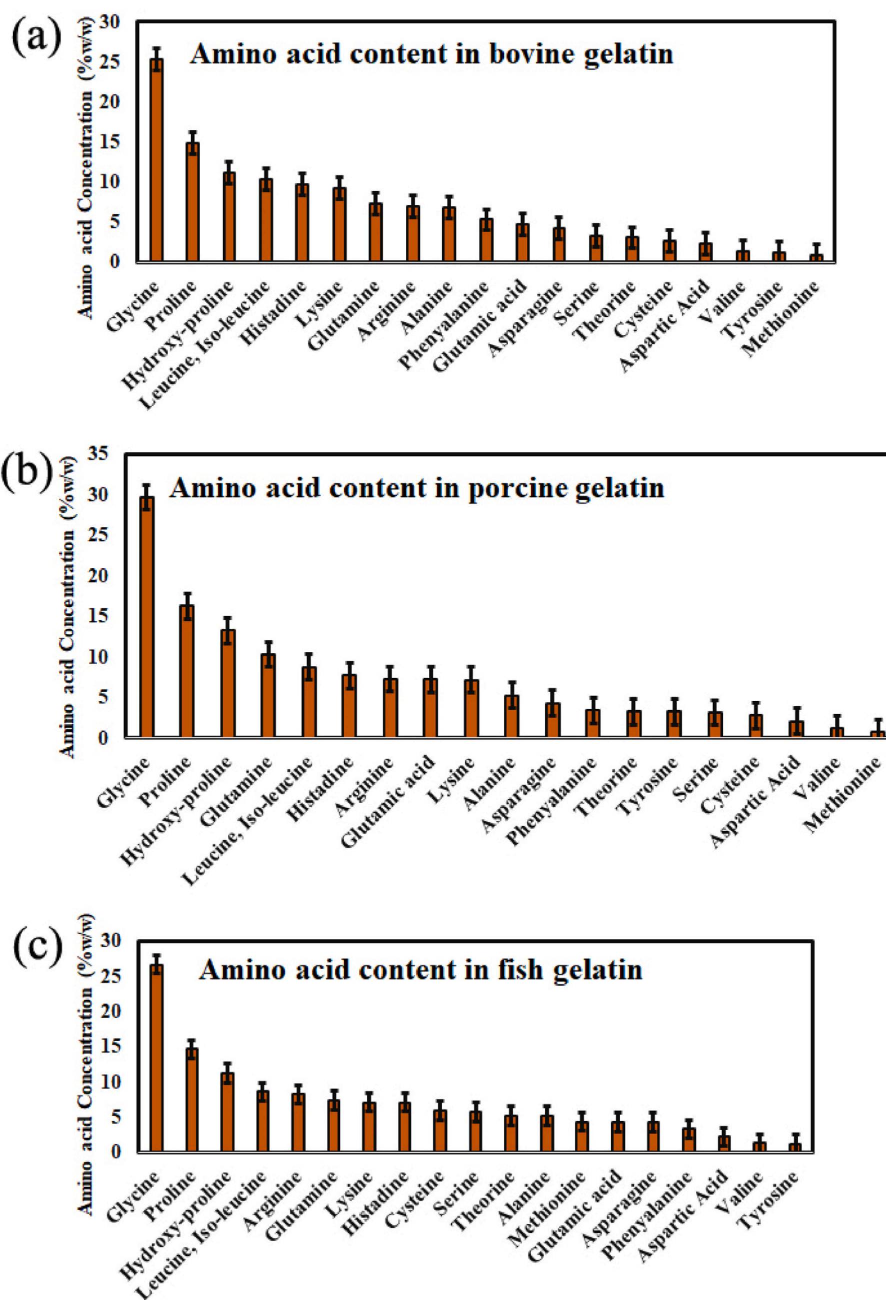

Fig. 2. Concentration of amino acids in (a) bovine, (b) porcine, and (c) fish gelatins.

2. Methodology

2.1. Chemical and reagents

The gelatin standards of porcine (40–50 KDa), fish (60 KDa), and bovine (50–100 KDa) were provided by Carl Roth (Karlsruhe, Germany), Sigma Aldrich (Schnelldorf, Germany), and Leap Chem (Hong Kong, China), respectively. All the standards of L-amino acids (>99%) used in this study were purchased from Sigma Aldrich (St. Louis, USA). Acetonitrile and formic acid were HPLC grade and supplied by Sigma Aldrich (St. Louis, USA). Ultrapure water was taken in the laboratory using a Milli-Q water purification system (Millipore, Billerica, MA, USA). Formic acid (100%) and ammonium formate (>98%) were purchased from Sigma Aldrich (St. Louis, USA)

The solution of hydrochloric acid (6 N) purchased from Sigma Aldrich (St. Louis, USA) was prepared by mixing concentrated hydrochloric acid (37%) with distilled water. All real samples containing gelatin were purchased from different local markets and some of the details are given in Table S1. However, due to fishy odour and dark colour of fish gelatin, it has not been used in commercialized samples (Al-Nimry et al., 2021; Usman et al., 2022). Therefore, we have purchased different brands fish gelatin powder and their samples (jellies, marshmallows, and capsule shells) were prepared according to Sudjadi et al. (2016) and Sani et al. (2021) to generate data for chemeometric analysis. The information of fish-derived gelatin samples is listed in Table S2.

2.2. Preparation of calibration standard solutions

A mixture of 20 L-amino acids standard stock solutions including glutamine (Gln), arginine (Arg), aspartic acid (Asp), hydroxy-proline (Hpro), tyrosine (Tyr), methionine (Met), asparagine (Asn), histidine

Table 3
Composition of amino acids in different gelatin standards (expressed in %W/W).

Amino acids	Bovine Gelatin	Porcine Gelatin	Fish Gelatin
L-Phenylalanine (Phe)	5.3 ± 0.1	3.39 ± 0.01	3.3 ± 0.1
L-Tyrosine (Tyr)	1.2 ± 0.2	3.23 ± 0.04	1.2 ± 0.1
L-Aspartic acid (Asp)	2.3 ± 0.2	2.1 ± 0.1	2.19 ± 0.09
L-Leucine (Leu), L-Iso-leucine (Ile)	10.31 ± 0.09	8.7 ± 0.2	8.61 ± 0.04
L-Methionine (Met)	0.89 ± 0.07	0.8 ± 0.1	4.4 ± 0.1
L-Cysteine (Cys)	2.69 ± 0.05	2.76 ± 0.04	5.91 ± 0.01
L-Valine (Val)	1.29 ± 0.03	1.2 ± 0.2	1.3 ± 0.1
L-Threonine (Thr)	3.04 ± 0.01	3.27 ± 0.02	5.23 ± 0.08
L-Glutamic acid (Glu)	4.7 ± 0.2	7.2 ± 0.2	4.32 ± 0.08
L-Serine (Ser)	3.2 ± 0.2	3.2 ± 0.2	5.79 ± 0.04
L-Alanine (Ala)	6.9 ± 0.1	5.32 ± 0.09	5.2 ± 0.2
L-Hydroxyl-proline (Hpro)	11.1 ± 0.2	13.3 ± 0.2	11.3 ± 0.1
L-Proline (Pro)	14.9 ± 0.1	16.3 ± 0.2	14.7 ± 0.2
L-Asparagine (Asn)	4.19 ± 0.2	4.3 ± 0.2	4.2 ± 0.1
Glycine (Gly)	25.34 ± 0.09	29.7 ± 0.2	26.66 ± 0.09
L-Glutamine (Gln)	7.2 ± 0.2	10.3 ± 0.3	7.35 ± 0.06
L-Histidine (His)	9.7 ± 0.3	7.7 ± 0.2	7.08 ± 0.09
L-Arginine (Arg)	6.94 ± 0.07	7.3 ± 0.2	8.1 ± 0.2
L-Lysine (Lys)	9.2 ± 0.2	7.2 ± 0.2	7.2 ± 0.1

Values are mean ± standard deviation (n = 3).

(His), proline (Pro), leucine (Leu), serine (Ser), lysine (Lys), phenylalanine (Phe), threonine (Thr), isoleucine (Ile), alanine (Ala), valine (Val), glutamic acid (Glu), glycine (Gly), and cysteine (Cys) were prepared by dissolving them in 60:40 v/v of 10- and 2-mM ammonium formate buffer in water and acetonitrile, respectively having pH 3. Each amino acid has a concentration of 250 μg/mL. Aminobutyric acid (AABA) used as an internal standard was prepared at 50 μg/mL in the same ratio of buffer as amino acid standards.

The stock solutions of calibration standard were prepared from the mixed standard stock solutions through serial dilutions with fixed buffer ratios to form different concentrations of 10, 20, 50, 75, 100, 150, and 200 μg/mL. AABA has a similar concentration (50 μg/mL) at each calibration point.

2.3. Preparation of sample solutions

2.3.1. Extraction of gelatin from real samples

The extraction of gelatin was performed using the previously reported method with some modifications (Zhu et al., 2023). The jellies, marshmallows, and soft and hard capsule shells were cut into small pieces. The pieces were transferred into a conical flask and were dissolved in deionized water through heating at 60 °C for 30 min. The mixture was cooled down at room temperature and vortexed. The centrifugation was performed at 5000 rpm (3070×*g*) for 5 min. Subsequently, the supernatants were collected into a 50 mL falcon tube and mixed with 4 vol of cold acetone having a temperature of −20 °C. The acetone-containing mixture was incubated for 12 h at −20 °C and followed by centrifugation at 14,000 rpm (16435×*g*) for 20 min. Then protein pellets were collected and air-dried.

2.3.2. Acid hydrolysis of gelatin samples

Acid hydrolysis was followed by a previously reported method with some changes (Niquet-Léridon & Tessier, 2011). To hydrolyze gelatin protein into respective amino acids, an amount of 0.2 g of gelatin standard samples from porcine, bovine, and fish and gelatin pellets of real samples were dissolved in HCl (5 mL, 6 N). The mixture was subjected to incubation at 110 °C for 18 h. The mixture was then cooled down at room temperature. Each residue was reconstituted in 100 μL of internal standard (AABA, 50 μg/mL) and filtered through a 0.45 μm filter before chromatography.

2.3.3. Sample clean-up

2 mL of each hydrolysate residue was adapted to the Empore™ C18-SD solid phase extraction (SPE) cartridge (7 mm/3 mL). The activation of cartridges was carried out using 6 mL of acetonitrile and 6 mL of deionized water. Subsequently, the samples were filtered through the cartridges, followed by elution with 6 mL of water/acetonitrile (20:80 v/v). Finally, the eluates were evaporated to dryness. The residues were redissolved in 900 μL 60:40 v/v of 10- and 2-mM ammonium formate buffer in water and acetonitrile, respectively having pH 3, and subjected to chromatographic separation.

2.4. Separation and quantification

Both, separation and quantification of standard amino acids and hydrolysates were performed on ultra-performance liquid chromatography (UHPLC) (Thermo Scientific 3000) equipped with a binary solvent system and an autosampler and coupled with Bruker Amazon ion trap mass spectrometer. Separation on HILIC was performed on ZORBAX RRHD HILIC Plus (2.1 mm × 100 mm, 1.8 μm) from Agilent. The composition of mobile phase A was 10 mM ammonium formate buffer in water and 0.15% formic acid, pH 3.0, and mobile phase B was 2 mM ammonium formate buffer in acetonitrile and 0.15% formic acid, pH 3.0. The gradient elution was as follows; 0–6 min, 85-80% B; 6–10 min, 80-70% B; 10–12 min, 70-60% B. Subsequently, the column was re-equilibrated for 6 min under initial conditions. The column temperature was maintained at room temperature (25 °C) followed by the flow rate of 0.4 mL/min 10 μL of the samples were injected in triplicate in the complete study. The eluent was then directly subjected to the mass spectrometer. Each sample was operated using Chromeleon software.

The mass spectrometer was operated in the electrospray ionization (ESI) positive mode in the scan range of 50–1000 *m/z*. The source parameters were established with a capillary voltage of 4500, a source temperature of 180 °C, a nebulizer gas flow of 4.0 L/min, gas pressure of 15.0 psi. Quantitative analysis of amino acids was monitored in multiple reaction monitoring (MRM) mode. The relative collision energy for each analyte was optimized and the isolation width was fixed at 4.0. The maximum accumulation time was 200 ms and the time allowed for fragmentation in collision-induced dissociation (CID) was set as 40 ms.

2.5. Validation of method

The validation of the method was performed in terms of LODs and LOQs, linearity, precision (interday and intraday), and recovery studies. Calibration curves were constructed from the ratio of the peak area of standard amino acids and internal standard (AABA) versus the concentration of amino acids. Each amino acid calibration curve was constructed with seven concentration points (10, 20, 50, 75, 100, 150, and 200 μg/mL) in four replicates. The limits of detection (LOD) and quantification (LOQ) for each amino acid were determined based on signal-to-noise ratios of approximately 3 and 10, respectively.

To assess the precision of the developed method, intra and interday variations were investigated. In the case of intraday variability, triplicate analyses of mixed solutions containing standards at three different concentrations (20, 75, 150 μg mL^{-1}) were conducted. For interday variability, triplicate analyses of standard solutions were performed over four consecutive days. An accuracy assessment was carried out through a recovery test. This involved adding corresponding amino acids at high, middle, and low levels (20, 75, 150 μg mL^{-1}) to 0.2 g hydrolyzed of three distinct gelatin sources (porcine, bovine, and fish). Each experiment was conducted in triplicate at each level. The percent recovery was calculated using the formula: recovery (%) = [(amount found - original amount)/amount added] × 100.

2.6. Data processing and statistical analysis

Data analysis software (Bruker, 4.4) was used for chromatographic

Table 4
Amino acids composition in commercialized real samples purchased in market (expressed in %W/W).

Amino acids	B-220	B-250	B-280	ED-G	G-C-S	K-J	K-MAR	PUR-GE	R-C-S	S-2	GEL-A	J-P	M-P	S-1	S-3	S-C-S	SOF-G	W-C-S
L-Phenylalanine (Phe)	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.8	5.1 ± 0.1	5.46 ± 0.04	5.2 ± 0.2	5.21 ± 0.06	5.2 ± 0.1	5.1 ± 0.1	3.06 ± 0.04	3.1 ± 0.2	3.42 ± 0.04	3.2 ± 0.1	3.2 ± 0.1	3.06 ± 0.04	3.3 ± 0.3	3.3 ± 0.2
L-Tyrosine (Tyr)	1.36 ± 0.08	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.1	3.2 ± 0.2	3.1 ± 0.1	3.32 ± 0.04	3.2 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	3.2 ± 0.1	3.3 ± 0.3
L-Aspartic acid (Asp)	2.4 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	2.15 ± 0.2	2.3 ± 0.1	2.38 ± 0.05	2.11 ± 0.01	2.2 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.3 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	2.2 ± 0.3	2.2 ± 0.1
L-Leucine (Leu), L-Iso-leucine (Ile)	10.2 ± 0.2	10.2 ± 0.2	10.2 ± 0.1	10.2 ± 0.1	10.2 ± 0.2	10.1 ± 0.2	10.14 ± 0.08	10.25 ± 0.08	10.1 ± 0.1	10.2 ± 0.2	8.8 ± 0.2	8.7 ± 0.1	8.7 ± 0.1	8.7 ± 0.2	8.7 ± 0.2	8.8 ± 0.2	8.7 ± 0.3	8.7 ± 0.1
L-Methionine (Met)	0.82 ± 0.02	0.8 ± 0.2	0.79 ± 0.04	0.83 ± 0.09	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.79 ± 0.07	0.8 ± 0.1	0.82 ± 0.07	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.83 ± 0.07	0.8 ± 0.04	0.8 ± 0.2
L-Cysteine (Cys)	2.3 ± 0.1	2.31 ± 0.03	2.24 ± 0.02	2.66 ± 0.06	2.75 ± 0.02	2.8 ± 0.2	2.8 ± 0.2	2.6 ± 0.1	2.8 ± 0.1	2.75 ± 0.02	2.8 ± 0.2	2.72 ± 0.05	2.89 ± 0.05	2.7 ± 0.2	2.7 ± 0.2	2.8 ± 0.2	2.72 ± 0.08	2.72 ± 0.05
L-Valine (Val)	1.24 ± 0.04	1.24 ± 0.08	1.2 ± 0.2	1.26 ± 0.07	1.2 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.12 ± 0.05	1.21 ± 0.06	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.3	1.1 ± 0.1	1.4 ± 0.3	1.4 ± 0.1
L-Threonine (Thr)	3.25 ± 0.09	3.2 ± 0.1	3.1 ± 0.1	3.3 ± 0.1	3.29 ± 0.04	3.1 ± 0.1	3.3 ± 0.1	3.17 ± 0.04	3.34 ± 0.03	3.29 ± 0.04	3.2 ± 0.2	3.2 ± 0.2	3.3 ± 0.2	3.2 ± 0.3	3.2 ± 0.2	3.2 ± 0.2	3.2 ± 0.1	3.2 ± 0.2
L-Glutamic acid (Glu)	4.3 ± 0.1	4.3 ± 0.2	4.41 ± 0.08	4.79 ± 0.03	4.7 ± 0.1	4.8 ± 0.2	4.67 ± 0.09	4.7 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	7.3 ± 0.2	7.2 ± 0.2	7.41 ± 0.06	7.2 ± 0.2	7.2 ± 0.05	7.3 ± 0.2	7.2 ± 0.1	7.2 ± 0.2
L-Serine (Ser)	3.8 ± 0.1	3 ± 1	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.97 ± 0.08	3.2 ± 0.2	3.98 ± 0.07	3.8 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	3.95 ± 0.04	3.8 ± 0.1	3.8 ± 0.3	3.4 ± 0.1	4.1 ± 0.2	3.15 ± 0.05	3.15 ± 0.04
L-Alanine (Ala)	6.2 ± 0.1	6.4 ± 0.1	6.6 ± 0.1	6.7 ± 0.2	6.74 ± 0.04	6.8 ± 0.1	6.7 ± 0.2	6.62 ± 0.04	6.8 ± 0.1	6.74 ± 0.05	5.3 ± 0.1	5.1 ± 0.2	5.2 ± 0.2	5.2 ± 0.2	5.2 ± 0.1	5.2 ± 0.1	5.1 ± 0.1	5.1 ± 0.2
L-Hydroxyl-proline (Hpro)	11.2 ± 0.1	11.2 ± 0.2	11.2 ± 0.1	11.23 ± 0.06	11.3 ± 0.1	11.28 ± 0.03	11.4 ± 0.2	11.23 ± 0.09	11.45 ± 0.05	11.36 ± 0.09	13.3 ± 0.2	13.2 ± 0.1	13.3 ± 0.2	13.38 ± 0.08	13.48 ± 0.08	13.2 ± 0.2	13.3 ± 0.2	13.2 ± 0.1
L-Proline (Pro)	14.3 ± 0.2	14.7 ± 0.2	14.15 ± 0.07	14.8 ± 0.2	14.6 ± 0.1	14.52 ± 0.09	14.46 ± 0.04	14.8 ± 0.1	14.61 ± 0.04	14.6 ± 0.1	16.3 ± 0.2	16.2 ± 0.2	16.32 ± 0.02	16.2 ± 0.1	16.2 ± 0.2	16.3 ± 0.2	16.2 ± 0.2	16.2 ± 0.2
L-Asparagine (Asn)	4.11 ± 0.09	4.36 ± 0.08	4.2 ± 0.1	4.2 ± 0.1	4.42 ± 0.05	4.2 ± 0.1	4.3 ± 0.2	4.2 ± 0.2	4.31 ± 0.05	4.42 ± 0.06	4.1 ± 0.1	4.3 ± 0.2	4.21 ± 0.05	4.2 ± 0.1	4.2 ± 0.2	4.1 ± 0.1	4.3 ± 0.2	4.2 ± 0.2
Glycine (Gly)	25.2 ± 0.2	25.4 ± 0.1	25.2 ± 0.1	25.47 ± 0.01	25.38 ± 0.09	25.24 ± 0.02	25.12 ± 0.06	25 ± 1	25.13 ± 0.05	25.38 ± 0.06	29.4 ± 0.5	29.16 ± 0.09	29.3 ± 0.2	29.2 ± 0.1	29.3 ± 0.1	29.3 ± 0.5	29.16 ± 0.09	29.16 ± 0.09
L-Glutamine (Gln)	7.36 ± 0.05	7.3 ± 0.2	7.1 ± 0.2	7.445 ± 0.009	7.1 ± 0.1	7.1 ± 0.2	7.21 ± 0.09	7.3 ± 0.2	7.3 ± 0.1	7.18 ± 0.09	10.3 ± 0.2	10.2 ± 0.2	10.1 ± 0.1	10.2 ± 0.2	10.2 ± 0.2	10.3 ± 0.2	10.2 ± 0.2	10.2 ± 0.2
L-Histidine (His)	9.2 ± 0.1	9.3 ± 0.2	9.6 ± 0.1	9.47 ± 0.03	9.7 ± 0.1	9.72 ± 0.03	9.8 ± 0.2	9.7 ± 0.2	9.8 ± 0.1	9.7 ± 0.2	7.7 ± 01	7.8 ± 0.3	7.97 ± 0.04	7.7 ± 0.2	7.7 ± 0.2	7.6 ± 0.1	7.8 ± 0.3	7.8 ± 0.2
L-Arginine (Arg)	6.491 ± 0.009	6.3 ± 0.2	6 ± 1	6.63 ± 0.05	6.7 ± 0.1	6.69 ± 0.03	6.57 ± 0.03	6.58 ± 0.01	6.8 ± 0.1	6.7 ± 0.2	7.2 ± 0.1	7.2 ± 0.1	17.21 ± 0.02	7.15 ± 0.02	7.15 ± 0.02	7.1 ± 0.1	7.2 ± 0.1	7.1 ± 0.1
L-Lysine (Lys)	9.11 ± 0.08	9.12 ± 0.04	9.15 ± 0.05	9.38 ± 0.05	9.08 ± 0.09	9.19 ± 0.01	9.1 ± 0.5	9.2 ± 0.2	9.3 ± 0.2	9.17 ± 0.09	7.11 ± 0.05	7.16 ± 0.08	7.11 ± 0.03	7.2 ± 0.1	7.2 ± 0.1	7.1 ± 0.05	7.16 ± 0.08	7.16 ± 0.08

Values are mean ± standard deviation (n = 3).

Table 5
Amino acids composition in fish-derived gelatin samples prepared in laboratory (expressed in %W/W).

Amino acids	FF-101	FF-102	FF-103	FF-104	FF-105	FP-201	FP-202	FP-203	FP-204	FP-205
L-Phenylalanine (Phe)	3.3 ± 0.2	3.5 ± 0.3	3.1 ± 0.1	3.08 ± 0.05	3.6 ± 0.2	3.06 ± 0.3	2.9 ± 0.2	3.4 ± 0.2	3.4 ± 0.1	2.8 ± 0.3
L-Tyrosine (Tyr)	0.9 ± 0.2	1.2 ± 0.1	0.8 ± 0.2	0.9 ± 0.3	1.2 ± 0.3	1.0 ± 0.5	1.1 ± 0.1	1.3 ± 0.5	1.0 ± 0.3	1.3 ± 0.3
L-Aspartic acid (Asp)	1.9 ± 0.1	2.0 ± 0.1	2.3 ± 0.2	2.05 ± 0.05	2.1 ± 0.1	2.3 ± 0.3	2.06 ± 0.02	2.4 ± 0.1	1.9 ± 0.5	1.8 ± 0.5
L-Leucine (Leu), L-Isoleucine (Ile)	8.6 ± 0.5	8.5 ± 0.2	8.6 ± 0.5	8.8 ± 0.5	8.9 ± 0.2	8.7 ± 0.1	8.6 ± 0.5	8.5 ± 0.2	8.8 ± 0.6	8.7 ± 0.8
L-Methionine (Met)	4.4 ± 0.9	4.3 ± 0.3	4.5 ± 0.3	4.09 ± 0.12	4.1 ± 0.6	4.5 ± 0.6	4.2 ± 0.2	4.2 ± 0.6	4.3 ± 0.2	4.5 ± 0.2
L-Cysteine (Cys)	5.91 ± 0.09	5.8 ± 0.1	5.6 ± 0.6	5.5 ± 0.1	5.9 ± 0.3	5.7 ± 0.2	5.8 ± 0.1	5.91 ± 0.81	5.93 ± 0.9	5.7 ± 0.2
L-Valine (Val)	1.3 ± 0.2	1.5 ± 0.2	1.05 ± 0.09	1.3 ± 0.3	1.2 ± 0.2	1.1 ± 0.2	1.51 ± 0.3	1.4 ± 0.2	1.21 ± 0.15	1.34 ± 0.6
L-Threonine (Thr)	5.23 ± 0.6	5.3 ± 0.5	5.09 ± 0.01	5.3 ± 0.9	5.5 ± 0.2	5.6 ± 0.5	5.1 ± 0.6	5.08 ± 0.08	5.1 ± 0.2	5.33 ± 0.5
L-Glutamic acid (Glu)	4.32 ± 0.25	4.09 ± 0.05	4.15 ± 0.02	4.5 ± 0.2	4.2 ± 0.1	4.1 ± 0.1	4.3 ± 0.3	4.7 ± 0.5	4.2 ± 0.2	4.13 ± 0.21
L-Serine (Ser)	5.79 ± 0.01	5.8 ± 0.2	5.7 ± 0.6	5.6 ± 0.2	5.7 ± 0.2	5.9 ± 0.1	5.5 ± 0.8	5.32 ± 0.6	5.54 ± 0.03	5.7 ± 0.3
L-Alanine (Ala)	5.2 ± 0.1	5.09 ± 0.3	5.2 ± 0.8	5.1 ± 0.5	5.3 ± 0.2	5.4 ± 0.2	5.09 ± 0.05	5.5 ± 0.6	5.15 ± 0.06	5.3 ± 0.2
L-Hydroxyl-proline (Hpro)	11.3 ± 0.1	11.3 ± 0.2	11.5 ± 0.2	11.4 ± 0.3	11.6 ± 0.1	11.59 ± 0.03	11.58 ± 0.02	11.65 ± 0.05	11.3 ± 0.2	11.2 ± 0.5
L-Proline (Pro)	14.7 ± 0.1	14.9 ± 0.2	14.8 ± 0.9	14.76 ± 0.12	14.61 ± 0.06	14.5 ± 0.2	13.98 ± 0.09	14.6 ± 0.2	14.9 ± 0.2	14.8 ± 0.3
L-Asparagine (Asn)	4.2 ± 0.2	4.1 ± 0.6	4.12 ± 0.01	4.2 ± 0.2	4.3 ± 0.1	4.4 ± 0.6	4.44 ± 0.04	4.35 ± 0.35	4.2 ± 0.2	4.1 ± 0.5
Glycine (Gly)	26.66 ± 0.05	26.59 ± 0.05	26.9 ± 0.01	26.7 ± 0.3	26.7 ± 0.2	26.6 ± 0.5	26.7 ± 0.1	26.6 ± 0.3	26.59 ± 0.05	26.86 ± 0.05
L-Glutamine (Gln)	7.35 ± 0.06	7.1 ± 0.2	7.02 ± 0.05	7.5 ± 0.2	7.3 ± 0.2	7.2 ± 0.2	7.1 ± 0.2	7.3 ± 0.2	7.6 ± 0.5	7.25 ± 0.02
L-Histidine (His)	7.08 ± 0.08	7.1 ± 0.1	7.2 ± 0.1	7.05 ± 0.02	7.1 ± 0.1	7.2 ± 0.3	7.12 ± 0.2	7.3 ± 0.1	7.6 ± 0.1	7.09 ± 0.03
L-Arginine (Arg)	8.1 ± 0.2	8.0 ± 0.5	8.1 ± 0.5	8.2 ± 0.1	8.09 ± 0.32	8.1 ± 0.2	8.08 ± 0.05	8.1 ± 0.1	8.09 ± 0.15	8.2 ± 0.3
L-Lysine (Lys)	7.2 ± 0.3	7.1 ± 0.1	7.2 ± 0.2	7.16 ± 0.3	7.17 ± 0.12	7.1 ± 0.1	7.3 ± 0.5	7.1 ± 0.6	7.3 ± 0.2	7.1 ± 0.1

Values are mean ± standard deviation (n = 3).

and mass spectrometer data acquisition and processing. Metaboanalysts 5.0 software was used to perform principle component analysis (PCA).

3. Results and discussion

3.1. Optimization of chromatographic conditions

The chromatographic parameters were investigated and optimized to achieve a satisfactory chromatographic profile with good retention and peak shape. Firstly, ZORBAX RRHD HILIC Plus (2.1 mm × 100 mm, 1.8 μm) from Agilent having non-bonded silica as stationary phase was tested for the separation of amino acids with various conditions. Different additives were used in the mobile phase with different concentrations like formic acid, acetic acid, and ammonium formate. The findings manifest that incorporating ammonium formate salts into the mobile phase leads to significantly enhanced peak shapes for all amino acids as compared to other additives. Moreover, the best peak shapes were obtained at the concentration of 10- and 2-mM ammonium formate in water and acetonitrile as eluents A and B, respectively. The adjustment of buffer pH at 3.0 with 0.15% formic acid improved the shape of peaks and decreased the retention time for most of the amino acids except histidine, lysine, and arginine as basic amino acids. Different gradient elution procedures with flow rates were optimized. However, the developed method could not separate the peaks of leucine and isoleucine in mixtures. The results demonstrated that the optimized HILIC method discussed above could successfully separate the 20 amino acids within 12 min in all gelatin sources (bovine, porcine, and fish) except leucine and isoleucine (Figs. S1, S2, and S3). No such difference in chromatographic spectra was observed in three sources of gelatins as reported previously by Azilawati et al. (2015). Furthermore, in the case of dissolving amino acid, different solvent ratios were used such as water and acetonitrile and mobile phases A and B. From the results for pure water and water, acetonitrile ratios, the peaks were broad with tailing. Therefore, different ratios of the mobile phase were studied and the best peaks were obtained with the ratio 60:40 v/v of mobile phase A and B, respectively. Fig. 1 shows the typical chromatogram of 20 amino acids under optimized conditions.

3.2. Optimization of MS/MS conditions

Additionally, it is well known that amino acids possess both, amino and carboxylic groups which can easily form stable positive and negative ions, respectively. Therefore, all the amino acids were first scanned in both, positive and negative ionization modes (Liyanaarachchi et al., 2018). The results revealed that the intensity of signals and sensitivity of amino acids were higher in positive ion mode than in negative ion mode. Subsequently, ESI with positive ion mode was selected in this study.

All amino acids were investigated separately to select a proper transition for the MS/MS detection in quantification. The precursor ion of each analyte obtained from full scan spectra was stored in the trap and further fragmented with different collision energies (between 10 and 40 %). The most abundant fragment ions were selected for quantification while the other was selected for identification (Fig. S4).

All the analytes were detected as $[M + H]^+$ with abundant intensities and selected as precursor ions. The majority of amino acids showed an abundant product ion at $[M + H - 46]^+$, which is due to the neutral loss of formic acid through a rearrangement as reported in previous studies (Gargano et al., 2021; Németh et al., 2023; Zhang, Chan, et al., 2019a,b).

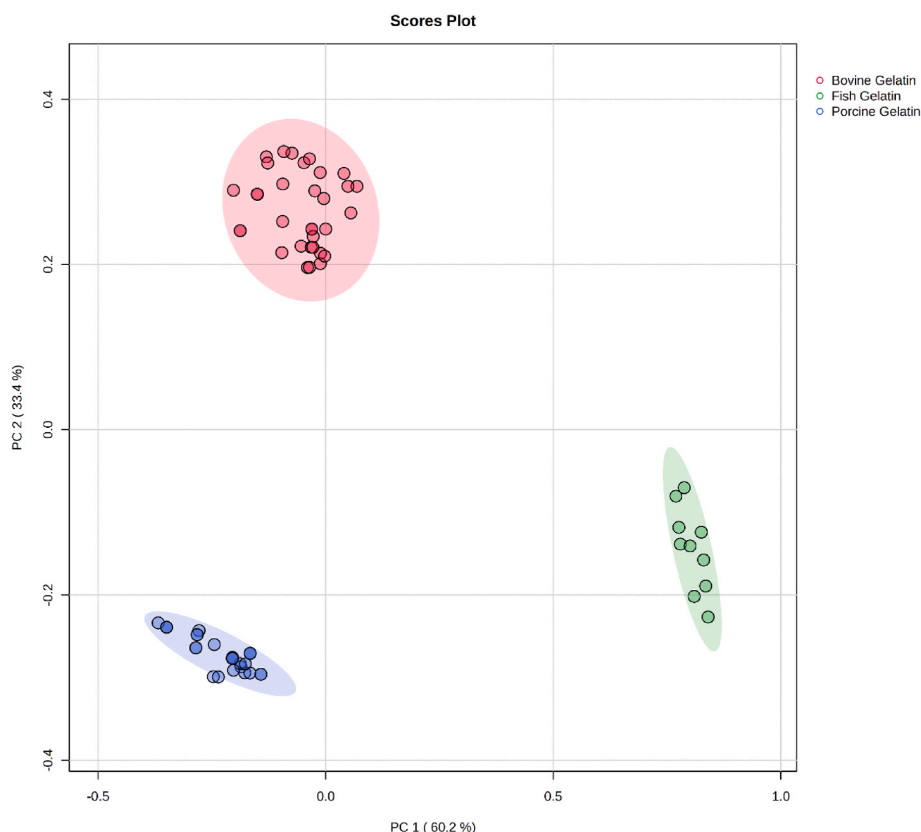

Fig. 3. Scores plot of standards and real samples of porcine, bovine, and fish gelatins.

This transition is selected for most of the amino acids in this study. The amide side chain containing amino acids such as glutamine provides the fragment ions at *m/z* 130 and 84 which is due to the loss of NH_3 (17) and CH_2O_2 (46) simultaneously, $[M + H- 46 -17]^+$. Glutamic acid contains a negatively charged acidic side chain and the first fragmentation of protonated glutamic acid started with the loss of water molecule to form a fragment ion at *m/z* 130.1. The second fragment ion at *m/z* 84.4 was produced due to the subsequent elimination of H_2O + CO (Wang et al., 2019). Moreover, in the case of most basic amino acids such as arginine and lysine, the loss of formic acid was very weak or not detected which can be due to extra amino groups which have stronger basicity than α-amino and causing easily ionized fragments (Mamani-Huanca, Gradillas, Gil de la Fuente, López-Gonzálvez, & Barbas, 2020). The quantitative (Q) and confirmative (q) transitions and optimized collision energy selected for the fragmentation of each analyte in ESI-positive mode are illustrated in Table 1.

3.3. Optimization of acid hydrolysis and solid phase extraction (SPE) conditions

To achieve the optimal temperature and time for the acid hydrolysis of different gelatin sources, the gelatin standards were hydrolyzed at different temperatures (110 and 150 °C) with various time durations (18 and 24 h). The satisfactory results for all amino acids were obtained at 110 °C for 18 h. Above this temperature and time, the peak shapes of some of the amino acids such as serine, cystine, and methionine were destroyed (Fig. S5)

For the SPE, Empore™ C18-SD SPE cartridges were chosen for their unique sorbent properties that make them well-suited for a broad spectrum of analytes, encompassing polar, nonpolar, and neutral compounds. The sorbent can selectively retain the target analytes (amino acids) through polar, hydrophobic, and ion exchange interactions. Two different elution systems with the proportions of methanol and 0.1% formic acid (10: 90 v/v) and water and acetonitrile (20:80 v/v) were tested. The 20 amino acids were eluted successfully when the elution system of water and acetonitrile (20:80 v/v) was used. However, for the former elution system, 15 amino acids were eluted and detected as shown in Table S3. Therefore, this ratio of water and acetonitrile was used as an optimal elution process.

3.4. Validation of optimized method

The validation of the proposed method was determined in terms of linearity, LOD, LOQ, intraday and interday precisions, and accuracy. The determination coefficient values (r^2) were >0.9920 for all amino acids. The LODs and LOQs were in the ranges from 0.03 to 2.62 μg/mL and from 0.10 to 7.93 μg/mL which denotes the acceptable method linearity as shown in Table 2. Intra- and interday variations (%RSD) of the amino acids were in the ranges of 0.19–11.21% and 0.03–13.62%, respectively (Table S4). The total recoveries of amino acids lie between 85.1 and 107.6% while spiked gelatins of bovine, fish, and porcine with 20 μg/mL, 75 μg/mL, and 150 μg/mL amino acids standards made 85–102.1%, 87–104.5%, and 87–107.6% recoveries, respectively (Table S5). The 20 μg/mL cysteine had the lowest recovery at 85.1%, while 20 μg/mL glycine had the highest recovery at 107.6%. All the values are in an acceptable range. These findings suggest that the established HILIC-ESI-MS/MS method is characterized by sensitivity, reproducibility, and accuracy in the quantitative analysis of these amino acids.

3.5. Content of amino acids in bovine, porcine, and fish gelatins

The outcome of the analysis showed that the content of amino acid in terms of concentrations (%w/w) in the bovine gelatin follows the order; Gly > Pro > Hpro > Leu, Ile > His > Lys > Gln > Ala > Arg > Phe > Glu > Asn > Ser > Thr > Cys > Asp > Tyr > Val > Met as shown in Fig. 2 (a),

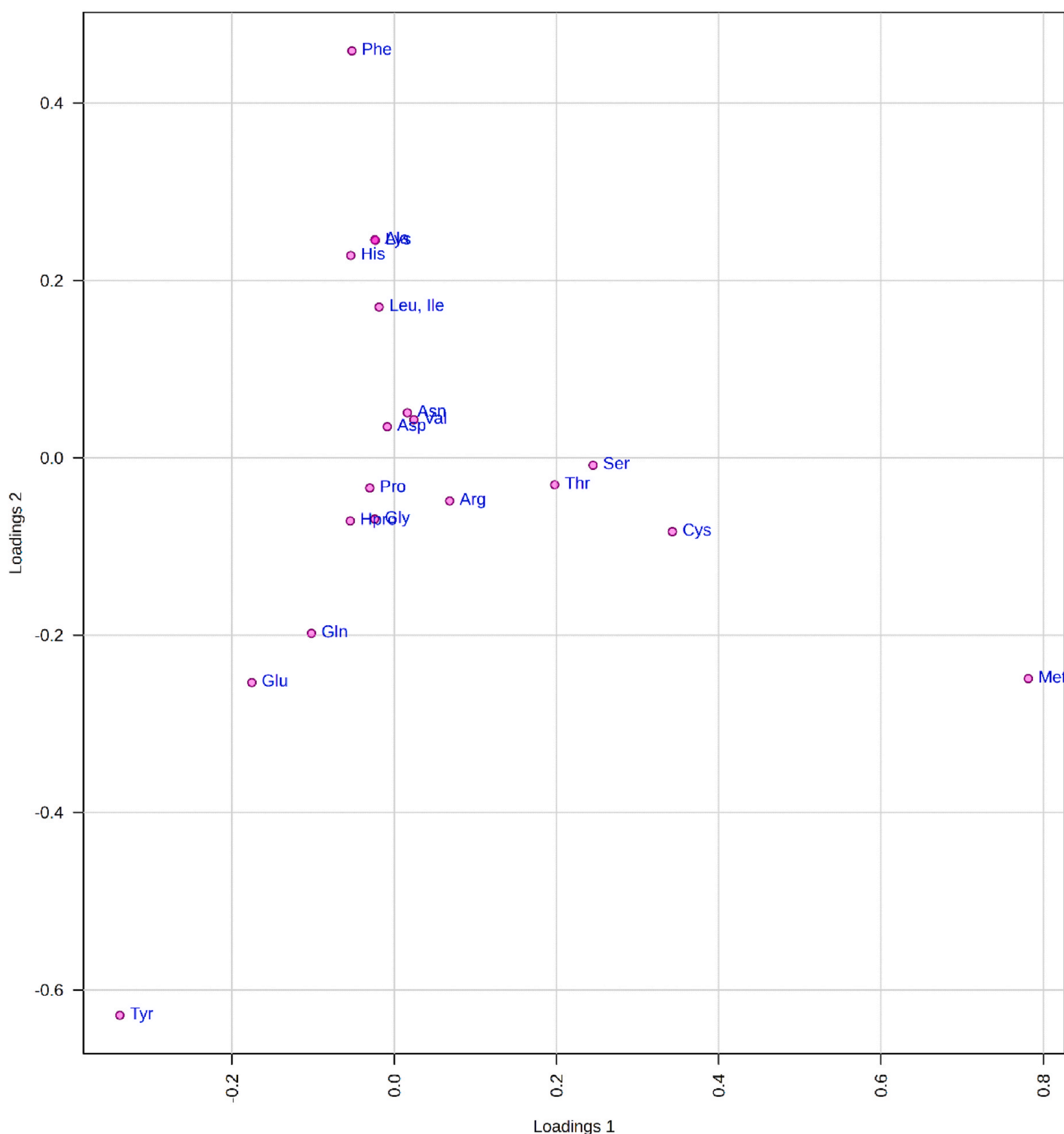

Fig. 4. Correlation loadings plot of respective amino acids.

which has similar distribution order for Gly > Pro > Hpro as Azilawati et al. (2015) and Sani et al. (2021), however, had slight contradictory findings. Both of the studies analyzed gelatin sources without glutamine, cysteine, and asparagine. The former study also excluded histidine. The studies of Widyaninggar et al. (2012) had a similar distribution in amino acid profile for Ala > Arg, and Asp > Tyr only with the exclusion of proline and hydroxyproline, but our study analyzed all 20 important amino acids responsible for distinguishing gelatin sources.

Moreover, in this study concentrations of the amino acid were quantified in porcine gelatin which had the following distribution profile; Gly > Pro > Hpro > Gln > Lue, Ile > His > Arg > Glu = Lys > Ala > Asn > Phe > Thr > Tyr > Ser > Cys > Asp > Val > Met as depicted in Fig. 2 (b). The obtained results are supported by Azilawati et al. (2015) and Sani et al. (2021) study for Gly > Pro > Hpro. However, asparagine, cysteine, glutamine, and histidine are not included in the former study. Widyaninggar et al. (2012) quantified glycine and threonine, serine and cysteine in combined form.

Similarly, the results of fish gelatin revealed that glycine (26.66 %w/w) had the highest and tyrosine (1.2%w/w) had the lowest concentrations as demonstrated in Fig. 2 (c). A similar amino acid distribution is reported by Azilawati et al. (2015) and Sani et al. (2021). However, reported studies manifest that different species and parts of fish exhibit different amino acid distributions. As compared to fish gelatin, porcine and bovine gelatin possess close similarities in amino acid distribution profile which can be due to being mammalians. The other factors that may influence the amino acid distribution in gelatin protein is food intake and habitat of animals.

Table 3 represents the concentrations (%w/w) of individual amino acids in the standards of three gelatin sources (bovine, porcine, and fish). The comparative study between three sources of gelatin showed that fish gelatin has the highest concentration of serine, threonine, methionine, cysteine, and arginine than porcine and bovine gelatin. Despite the fact that amino acids distribution in porcine and bovine gelatin has close similarities, results show that porcine gelatin has higher concentrations of glycine, proline, hydroxyproline, glutamine, lysine, glutamic acid, and tyrosine, while bovine gelatin has the highest

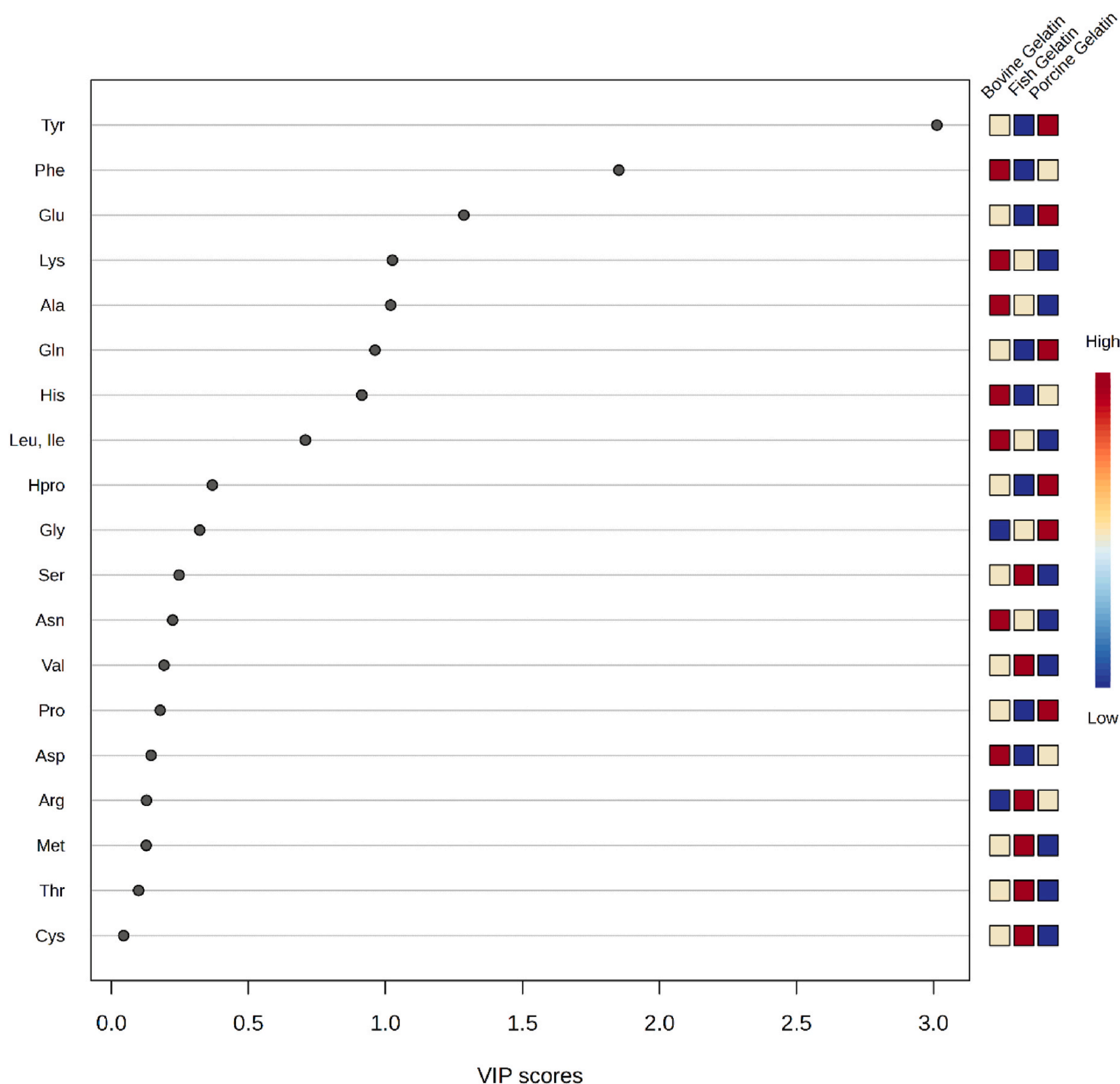

Fig. 5. PLS-DA plot of 20 amino acids responsible for differentiation of three gelatin sources.

concentration of histidine, leucine and isoleucine phenylalanine, and alanine. However, the distribution profile of aspartic acid, valine, and asparagine showed negligible differences in the three types of gelatins.

3.6. Application to real samples

To validate the efficacy of the amino acid profiling method, an experimental application was conducted using commercialized real samples and laboratory prepared fish-derived gelatin samples. The established HILIC-MS/MS method was employed to analyze a total of 18 batches of commercialized real samples and 10 fish-derived gelatin samples. These samples included a diverse range of items, both food and non-food, such as jellies, marshmallows, gelatin powder, and gelatin samples of various applications (gelatin sheets, and soft and hard capsule shells). The aim was to assess variations in the amino acid profiles among these samples and detect the gelatin species.

All amino acid content in different types of commercialized real samples is summarized in Table 4. The amino acid content of fish-derived gelatin samples prepared at laboratory scale is provided in Table 5. The concentration of amino acids obtained from these real samples was compared with the standard gelatins of three sources. Among 18 commercial samples, 10 of the samples showed very close amino acid distribution as bovine gelatin. Each of these 10 samples exhibited a richness in specific amino acids comparable to the bovine gelatin standard such as phenylalanine, leucine and isoleucine, alanine, histidine, and lysine while the remaining 8 samples had a higher concentration of glycine, tyrosine, proline, hydroxyproline, glutamic acid, and glutamine as porcine gelatin. Therefore, the amino acid concentrations of porcine and bovine gelatin in this study could potentially discriminate bovine and porcine gelatin and bovine sources in processed food and non-food samples. The samples from fish gelatin possessed high amount of methionine, serine, threonine, cysteine, and arginine as fish gelatin standard.

3.7. Multivariant analysis of the samples

To assess the differences among three distinct gelatin sources, PCA was conducted using data from the analysis of 20 amino acid contents.

PCA is a mathematical approach that is capable of transforming high-dimensional multivariate data into a lower number of dimensions. It was applied to visualize the general potential to differentiate between three different gelatin sources using amino acid profiles. The scores plot indicates that the first two principal components effectively distinguished the gelatin samples based on their origin. The results demonstrate that

Table 6
Comparison of developed method based on amino acids distribution for differentiation of gelatin sources with other reported methods.

Gelatin sources	Technique	Derivatizing reagents	Total run time (min)	LOD/LOQ (μg mL^{-1})	Linear range	Recovery%	No of amino acids studied	Real samples	Chemometric tools	Ref
Porcine and bovine	RP-HPLC coupled with fluorescence detector	Orthophtaldialdehye (OPA) and 4-chloro7-nitro benzofurazane (NBD-Cl)	33	Not reported	Not reported	Not reported	18	Gelatin powder	PCA	Nemati et al. (2004)
Porcine and bovine	RP-HPLC coupled with fluorescence detector	orthophtalaldehyde (OPA), 3-mercaptoethanol (MCE)	25	Not reported	Not reported	Not reported	18	Commercial and laboratory prepared Capsule shells	PCA	Widyaninggar et al. (2012)
Porcine and bovine	RP-HPLC coupled with fluorescence detector	ortho-phtalaldehyde 2-mercaptoethanol (OPA/2-MCE)	25	Not reported	Not reported	Not reported	19	Commercial and laboratory prepared soft candy	PCA	Raraswati et al. (2013)
Porcine, bovine, and fish	RP-HPLC coupled with fluorescence detector	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)	35	Not reported	37.5–1000 pmol/μL	Not reported	16	Gelatin powder	PCA	Azilawati et al. (2015)
Porcine, bovine, and fish	RP-UHPLC-DAD	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)	9.5	Not reported	37.5–1000 pmol/μL	85–111	17	Gelatin powder and laboratory prepared marshmallows	PCA and DA	Sani et al. (2021)
Porcine, bovine, and fish	HILIC- ion trap-MS/MS	Derivatization free	12	0.03-2.62/ 0.10-7.93	10–200 μg/mL	85.1–107.6	20	Commercial gelatin powder and sheets, soft and hard capsule shells, marshmallows, jelly, soft candy	PCA and PLS-DA	This work

samples from the same group were aggregated. Each source of gelatin shows a clear separation trend in the scores plot as represented in Fig. 3. The differences between the three groups of gelatins confirm that amino acid ratios vary between different sources of gelatin which is helpful to distinguish the gelatin sources. Additionally, PC1 exhibits a linear combination that aligns with the positive side of its score vector, particularly representing fish gelatin. In contrast, a set of porcine gelatins displayed no correlation with fish gelatin and demonstrated a linear function on the negative side of PC2. Simultaneously, a cluster of bovine gelatin samples was positioned on the positive side of PC2. This set of databases also includes various real samples which have known and unknown gelatin samples. In this PCA analysis, 28 gelatin sample mixtures sourced from various commercial products and laboratory prepared samples, comprising porcine, fish, and bovine origins, were examined to validate the database's effectiveness in distinguishing gelatin origin. Among these 18 commercial samples, 10 were classified within the bovine gelatin group, while the remaining 8 were situated in the porcine gelatin group. The remaining 10 samples prepared from different brands of fish gelatin showed exact correlation with fish gelatin group. Therefore, the developed database is beneficial for screening methods to differentiate the sources of gelatin.

The correlation loadings plot depicted in Fig. 4 illustrates the interrelationships among amino acids that played a significant role in shaping the effects observed in the score plot. Aspartic acid, valine, and asparagine contributions to the total explained variance in the data were less than 50%, and were positioned at the center. Consequently, it is insignificant in the differentiation of gelatin. The contribution of methionine, serine, threonine, cysteine, and arginine to the variance in the data was significant to correlate with fish gelatin. Phenylalanine, histidine, leucine, isoleucine, and lysine exhibited a correlation with bovine gelatin as they are given their placement on the positive side of PC1. The linear combination of glutamic acid, glutamine, proline, hydroxyproline, tyrosine, and glycine to the data variance contributes to porcine gelatin.

Following PCA, an additional method, partial least-squares discriminant analysis (PLS-DA) was employed to further validate and authenticate gelatin sources. PLS-DA proved successful in differentiating three gelatin sources based on amino acid distributions. In PLS-DA, the variable importance in the projection (VIP) score of a variable reflects its discriminating power which is considered as a potential key marker for classification of groups. Based on VIP scores, tyrosine, phenylalanine, glutamic acid, and lysine exhibit high VIP scores as shown in Fig. 5, and were identified as variables that contributed largely to the model. The results of PLS-DA were consistent with PCA results for all 18 gelatin standards and commercial gelatin samples. Thus, both PCA and PLS-DA were able to differentiate all gelatin-containing samples from various gelatin sources using the amino acids profile.

3.8. Method comparison

The efficiency and validation of the developed method are also compared with previously reported methods. The comparison results are presented in Table 6 for ease of analysis and interpretation. The developed method shows great efficiency in terms of linearity, LOD, LOQ, and recovery. The current method escapes the step of derivatization with the greater number of amino acids and method validation was studied entirely with all complex commercial samples as compared to the previous method.

4. Conclusion

In a nutshell, gelatin authentication by testing laboratories has become indispensable due to the exponential growth in demand for gelatins used in food applications. The detection of gelatin sources via amino acid distribution through advanced analytical tools incorporated with multivariate tools is essential to address manufacturers' inaccurate

claims and address concerns related to food integrity. Here, using HILIC-MS/MS, a robust, uncomplicated, and sensitive method was established for the quantification of all protein amino acids directly in different gelatin sources, eliminating the need for derivatization. This method successfully distinguished gelatin from porcine, bovine, and fish sources. The application of this method was extended to the analysis of 20 amino acids in 28 commercially available and laboratory prepared gelatin-based products. Our method for detecting gelatin, based on amino acid profiles, differs from existing approaches by initiating sample solution preparation right after a straightforward extraction step. This eliminates the need for the labor-intensive and time-consuming derivatization procedures often required in other developed methods. Using the HILIC mode of separation enables the effective separation of more polar compounds within a short 12-min window, without relying on ion-pairing reagents, as these are not recommended for an ESI source. The analysis of gelatin samples demonstrated distinctive variations in amino acid concentrations across different gelatin sources. The outcomes of PCA and PLS-DA successfully separated porcine, bovine, and fish gelatin into three distinct, unrelated groups. The consistency between amino acid correlations and the grouping in the score plot was evident. The findings from our current study indicate that the combination of the HILIC-MS/MS method with multivariate analysis can serve as a valuable tool for identifying gelatin sources in food and pharmaceutical products based on specific amino acids such as glycine, proline, hydroxyproline, lysine, histidine, phenylalanine threonine, methionine, and serine as the markers. Regulatory bodies at the government level may consider adopting this approach as a standard for authenticating gelatin products.

Funding

This work was supported by the Higher Education Commission, Pakistan, under theCenter of Excellence (CoE-75) funding program.

CRediT authorship contribution statement

Mahjabeen Hassan: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Tehreem Kanwal:** Visualization, Methodology, Data curation. **Amna Jabbar Siddiqui:** Visualization, Validation, Methodology, Investigation. **Arslan Ali:** Validation, Investigation, Data curation, Conceptualization. **Dilshad Hussain:** Writing – review & editing, Visualization, Validation. **Syed Ghulam Musharraf:** Writing – review & editing, Visualization, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

The authors acknowledge the Higher Education Commission (HEC) of Pakistan for this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110909.

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