Journal of Chromatography A 1705 (2023) 464153

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Discovery of species-specific peptide markers and development of quality-evaluation strategies for deer horn gelatin using liquid chromatography-tandem mass spectrometry and a label-free methodology

Fei Xue[a], Bing Wang[a], Dong-xiao Guo[a], Yang Jiao[a], Wei-liang Cui[a], Xian-long Cheng[b], Zhi-bin Wang[c], Xue Yin[a], Shuang-cheng Ma[b,*], Yong-qiang Lin[a,*]

[a] Shandong Engineering Laboratory for Standard Innovation and Quality Evaluation of TCM, Shandong Engineering Research Center for Generic Technologies of Traditional Chinese Medicine Formula Granules, NMPA Key Laboratory for Quality Evaluation of Gelatin Products, Shandong Institute for Food and Drug Control, Jinan 250101, PR China
[b] National Institutes for Food and Drug Control, Beijing 100000, PR China
[c] Scientific Research Institute of Beijing Tongrentang Corporation, Beijing 100000, PR China

ARTICLE INFO

Article history:
Received 7 April 2023
Revised 1 June 2023
Accepted 12 June 2023
Available online 13 June 2023

Keywords:
Deer horn gelatin
Peptide markers
LC-MS/MS

ABSTRACT

Deer horn gelatin (DHG) is a valuable nutritional dietary supplement. Due to the significant variation in the price of DHG from different sources, it is important to assess its quality and to clarify the species of its raw material. However, due to the similarity in appearance and physicochemical properties, as well as the destruction of genetic material during the manufacturing process, it is difficult to distinguish DHG from gelatin derived from other sources. Furthermore, current methods are unable to evaluate the overall quality of DHG. Using Nano LC-Orbitrap MS and data analysis software, DHG samples from five deer species were analyzed to identify peptide markers specific to alpha-2-HS-glycoprotein (AHSG) and collagen. The peptide markers were validated using HPLC-Triple Quadrupole MS, and strategies for assessing the quality of DHG were developed. Eighteen peptide markers were discovered, comprising peptides with differing specificities. Three strategies for the identification, characteristic mapping, and content determination of DHG were developed. These strategies can be used to assess the quality of deer gelatin.

1. Introduction

Deer horn gelatin (DHG) is widely used as a health care product and traditional Chinese medicine in Asia [1]. It is a solid gelatin obtained by boiling the ossified horns of deer with water and then concentrating the resulting solution. DHG has been used in medicine for over 2000 years [2]. In traditional Chinese medicine, it is commonly used to treat vertigo, palpitations, hematuria, and insomnia, and it is a common tonic for elderly or postpartum women and other debilitated individuals. It is also further processed into health products. In recent years, it has been used to treat persistent anemia [3], femoral head necrosis [4], and osteoporosis [5]. It is also an effective anti-oxidant, anti-inflammatory agent [6], and immunomodulator [7]. Recent studies have demonstrated that DHG can prevent Alzheimer's disease [8] and aid in the regeneration of skin equivalents by promoting stem cell activity and epidermal differentiation [9]. The peptides in DHG have also been demonstrated to be effective against lung injury [10] and liver injury [4]. We believe that DHG is gaining global recognition as a healthy supplement and medicine and that market regulation is essential for its global expansion. The raw material that is used to make DHG are antlers that have already been calcified. Sika deer (*Cervus nippon*) and red deer (*Cervus elaphus*) are widely distributed in China. Since the sika deer is a nationally protected species in China, and the antler velvet of sika deer and red deer is extremely valuable and is frequently harvested when unossified, their ossified horns are relatively uncommon [11]. On the contrary, both male and female reindeer (*Rangifer tarandus*) are capable of producing horns, and the Eurasian elk (*Alces alces*), the largest deer in the world, has a pair of palm-like horns that spread flat on both sides and can be as wide as two meters [12]. Moreover, white-tailed deer (*Odocoileus virginianus*) are widespread in North America [13]. The people in the region where reindeer, Eurasian elk, and white-tailed deer are found do not practice the cutting of antlers (the unossified horns), and so obtaining a large quantity

* Correspondong authors.
E-mail addresses: masc@nifdc.org.cn (S.-c. Ma), 13864067104@163.com (Y.-q. Lin).

https://doi.org/10.1016/j.chroma.2023.464153
0021-9673/

of horns is relatively simple. The price of reindeer horn gelatin (RDHG), Eurasian elk horn gelatin (EDHG), and white-tailed deer horn gelatin (WDHG) are therefore significantly lower than sika deer horn gelatin (SDHG) and red deer horn gelatin (REDHG). The price of SDHG and REDHG has increased to approximately US$530 per kilogram [14], which is more than 10 times the price of other deer sources. No studies, however, have distinguished DHG produced from closely related species of the deer family.

One constraint on the production of deer horn gelatin (DHG) is its limited capacity. The drive for profit often leads manufacturers to substitute cheaper and more abundant raw materials, such as pig, horse, cattle, and donkey hides, for deer antlers. Using hides can produce more gelatin, and because deer antlers and hides come from the same species, it can be challenging to tell them apart. Some manufacturers have even used deer hides instead of more easily identifiable cattle and pig hides to boost profits. To solve this problem, a study reported that peptides with significantly different expressions in horn and hide could be used as peptide markers to differentiate horn gelatin from hide gelatin based on the difference in glycosylation sites [15]. However, the experimental samples in that study were homemade SDHG, and the peptide markers did not demonstrate species specificity. Given the large variation in DHG prices across species, we aim to develop a strategy that considers species specificity and can differentiate between hide and horn origins. This is the challenge we seek to overcome in this research.

Since the preparation of gelatin from deer horn necessitates repeated decoction, which destroys the DNA in the raw material, it is difficult to use nucleic acid-based testing for identification. The resolution of this problem requires a strategy with high specificity and detection efficiency. In a previous study, we identified peptide markers capable of distinguishing antlers of closely related deer species and developed strategies for antler identification [16]. However, the strategy encountered some difficulties when applied to DHG, as these peptide markers were difficult to detect. We suspect that the protein to which the markers belonged was not heat-resistant and was destroyed during the gelatin production process. Determining the peptide markers applicable to DHG and establishing quality-evaluation strategies are the objectives of the present study.

Nano LC-Orbitrap MS is widely used in the exploration of peptidomics and proteomics [17]. Combined with data analysis software, it can analyze the amino acid sequence of proteins and is a method of protein sequencing. In this study, using Nano LC-Orbitrap MS and data analysis software, peptide markers of DHG in alpha-2-HS-glycoprotein (AHSG) and collagen were identified. The strategies of identification, characteristic mapping, and content determination were established by HPLC-Triple Quadrupole MS. The three strategies can be used to evaluate all aspects of the quality of DHG, not just source differentiation, but also whether the gelatin is produced in sufficient quantity from raw materials. Fig. 1 shows a flowchart of our experimental design.

2. Materials and methods

2.1. Materials and reagents

Trypsin (proteomics grade) and ammonium bicarbonate were obtained from Sigma-Aldrich (St. Louis, MO, USA), formic acid (optima LCMS) from Thermo Fisher Scientific (Waltham, MA, USA), and acetonitrile (gradient grade) and methanol (gradient grade) from Merck KGa (Darmstadt, Germany). Millipore's Milli-Q Gradient A10 system was used to prepare water (Schwalbach, Germany).

In this study, twenty batches of deer horns were collected from various regional markets across China for the purpose of producing homemade gelatin: three batches of sika deer horns, four batches of red deer horns, four batches of reindeer horns, five batches of Eurasian elk horns, and four batches of white-tailed deer horns (Table S1). The species were identified by the National Institutes for Food and Drug Control (Beijing, China). For comparison, one batch of donkey hide gelatin, one batch of pig hide gelatin, and one batch of cattle hide gelatin were acquired from control herbs, which were calibrated and made publicly available by the National Institutes for Food and Drug Control (Beijing, China). Additionally, one batch of deer hide gelatin was sourced from Guangjitang Pharmaceutical Co., Ltd (Guiyang, Guizhou Province, China), while one batch of horse hide gelatin was procured from Hongjitang Pharmaceutical Co., Ltd (Jinan, Shandong Province, China). In this study, all 18 peptide markers and one peptide internal standard were synthesized by China Peptides Co., Ltd (Shanghai, China) and Yuanpeptide Co., Ltd (Nanjing, China).

2.2. Sample preparation

The preparation of DHG was conducted as follows. The raw materials were first crushed into 3–5 cm segments or blocks, and 250 g of the crushed material were soaked in 1000 ml of ultrapure water for 48 h. This process required four changes of ultrapure water. After soaking, the samples were placed in a pot and boiled with 1000 ml of water at 110 °C, under atmospheric pressure, while ensuring that the water volume remained constant. The liquid was collected every 8 h and continued to be boiled until the sample softened. The resulting gelatin liquid was then combined, filtered, concentrated, and dried.

For mass spectrometry analysis, sample preparation was carried out as follows. A 20 mg portion of crushed gelatin was taken in powder form and combined with 5 ml of a 25 mM ammonium bicarbonate solution. The mixture was then sonicated for 30 min. Subsequently, the gelatin solution was filtered through a microporous membrane. To 95 µl of filtrate, 5 µl of a 10 mg/ml bovine trypsin solution was added, and the mixture was digested at 37 °C for four hours. Finally, the temperature of the gelatin solution was reduced to room temperature.

2.3. Nano LC-MS/MS analysis

The prepared samples were analyzed by nano-flow liquid chromatograph (EASY-nLC 1000, Thermo Scientific, San Jose, CA, USA) coupled with high-resolution mass spectrometry (Orbitrap-Fusion, Thermo Scientific, San Jose, CA, USA) under the following conditions: desalination and enrichment on a Thermo Acclaim PepMap C18 column (75 µm × 2 cm, 3 µm, Thermo Scientific, San Jose, CA, USA), separation on a Thermo Acclaim PepMap C18 column (75 µm × 15 cm, 3 µm, Thermo Scientific, San Jose, CA, USA) at a flow rate of 300 nl/min; mobile phase A was aqueous 0.1% formic acid solution with 2% acetonitrile, and mobile phase B was aqueous 0.1% formic acid solution containing 98% acetonitrile, with gradient elution (0–1 min, 1% B→6% B; 1–96 min, 6% B→22% B; 96–113 min, 22% B→30% B; 113–117 min, 30% B→95% B; 117–120 min, 95% B); and the injection volume was 1 µl.

The conditions for the high-resolution mass spectrometry were as follows. The ion source was Nanospray Flex; analysis was conducted in positive ion mode with a spray voltage of 1800 V; ion transport capillary temperature was 275 °C; and the S-Lens transport efficiency was set to 60%. The primary mass spectrometry was performed on an Orbitrap mass analyzer with a resolution of 60,000 and an acquisition range of 350–1550 (m/z), and the secondary mass spectrometry was performed on an Orbitrap mass analyzer with Rapid Scan mode for scanning, Top speed data-dependent mode for precursor ion selection, HCD mode for fragmentation, and fragmentation energy NCE set to 40%.

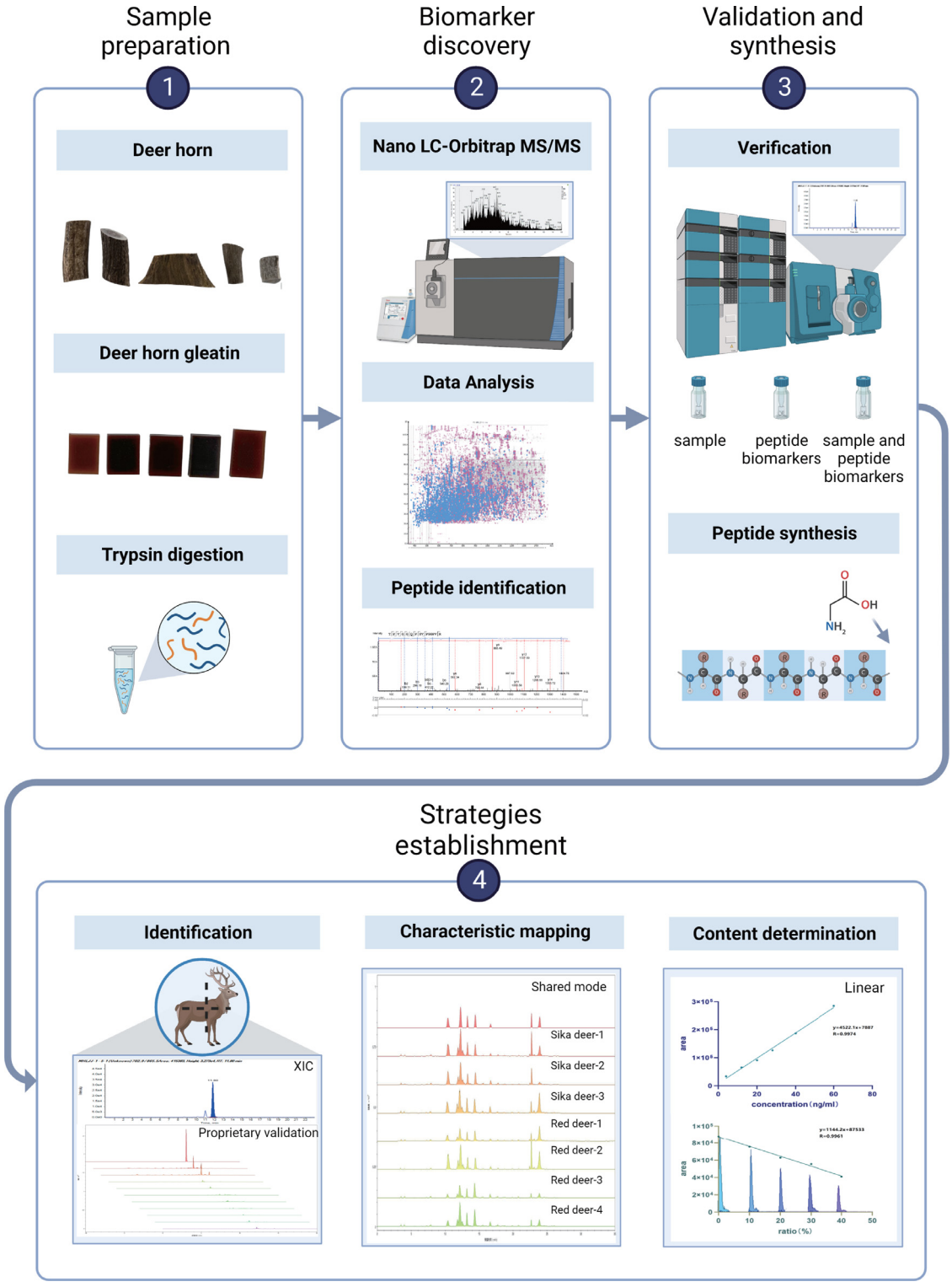

Fig. 1. Analytical scheme for the discovery of peptide markers in DHG and establishment of quality evaluation strategies.

2.4. Mass spectrometry data analysis and discovery of peptide markers

The mass spectrometry data were imported into PEAKS Studio software (8.5 Edition, Bioinformatics Solutions Inc., Waterloo, Canada) using the following parameters: parent mass error tolerance was 15.0 ppm, fragment mass error tolerance was 0.02 Da, max missed cleavages was set to 3, De novo score threshold was set to 15, and filter charge was set to 2–8. A number of even-toed ungulate "collagen and AHSG" protein databases downloaded from Uniport (containing a total of 19 proteins, https://www.uniprot.org/, accessed May 2, 2022; for more detailed counts, see Table S2) were used and all peptide sequencing and peptide matching was performed from scratch.

The SPIDER mode in PEAKS Studio can predict amino acid sequence mutations in peptides. Further filtering of the analy-

sis results was performed, and the rules were as follows: peptides with high specificity, lengths between 7 and 25 amino acids, and few post-translational modifications were chosen for further validation. Peptide markers were identified for which sequences have not been reported. The mass spectrometry proteomics data for these markers were uploaded to PRIDE. The data were also deposited to the ProteomeXchange Consortium *via* the PRIDE [18] partner repository with the dataset identifier PXD036244 and 10.6019/PXD036244.

2.5. Verification of the specificity of peptide markers by HPLC-MS/MS

HPLC-Triple Quadrupole MS (QTRAP6500 LC/MS, AB SCIEX, Foster City, CA, USA) was used to verify the specificity of the peptide markers and to establish the MRM method. To verify the specificity of the peptide markers, we used 20 batches of DHG from five deer species, one batch of deer hide gelatin (red deer), one batch of horse hide gelatin, one batch of donkey hide gelatin, one batch of pig hide gelatin, and one batch of cattle hide gelatin. The peptides identified in PEAKS were selected, and the product ions with the highest response intensity were analyzed using HPLC-Triple Quadrupole MS and chosen as the quantitative ions and qualitative ions. The HPLC parameters were set as follows: the column was C18 (2.1 mm × 100 mm, 1.8 μm, ZORBAX SB RRHD, Agilent Technologies, Santa Clara, CA, USA; 2.1 mm × 100 mm, 1.7 μm, ACQUITY UPLC BEH, Waters Corporation, Milford, MA, USA; 2.1 mm × 100 mm, 1.7 μm, ACQUITY UPLC HSS, Waters Corporation, Milford, MA, USA; 2.1 mm × 100 mm, 3 μm, Acclaim RSLC, Thermo Scientific, San Jose, CA, USA), the column temperature was 43 °C, the flow rate was 0.3 ml/min, mobile phase A was an aqueous solution containing 0.1% formic acid, B was a 1:1 methanolic acetonitrile solution, and gradient elution was performed (0–12 min, 10% B →20% B; 12–21 min, 20% B→97% B; 21–25 min, 97% B; 25–25.5 min, 97% B→10% B; 25.5–30 min, 10% B) with an injection volume of 5 μl, and the solvent delay was 0–4 min and 26–30 min.

The mass spectrometry parameters were set as follows: a mass spectrometry detector with electrospray ionization (ESI) in positive ion mode for multiple reaction monitoring, sheath gas flow rate of 46 l/h, auxiliary gas flow rate of 850 l/h, spray voltage of 5.5 kV, ion source temperature of 550 °C, auxiliary gas temperature of 400 °C, cone hole voltage of 30 V, and collision voltage of 35 V.

2.6. Synthesis and verification of peptide markers

The peptide markers were artificially synthesized and made into solutions using ultrapure water. HPLC-Triple Quadrupole MS was employed to determine whether the retention times of the synthesized peptides and gelatin samples were consistent. The retention times of both the qualitative and quantitative ions were utilized to simultaneously confirm the synthesized peptide markers. The synthesized peptide was added to the prepared gelatin sample, reaching a final concentration of 50 ng/ml, and their peak numbers were verified using HPLC-Triple Quadrupole MS. If the qualitative and quantitative ions in the peptide markers displayed peaks within the designated time (±0.2 min for both qualitative and quantitative ions in the sample), and the spiked sample exhibited a single peak, it could be inferred that the sequence of the peptide marker corresponded to that of the gelatin samples.

2.7. Establishment of quality-evaluation strategies for DHG

With the newly discovered peptide markers, we developed three quality-evaluation strategies for DHG, each focusing on a different aspect of the product's overall quality.

2.7.1. Establishment of identification strategy for DHG

To develop an identification strategy for DHG, a peptide marker unique to DHG and undetectable in gelatin from other sources was selected. Subsequently, specificity experiments were conducted. Since the prices of SDHGand REDHG are comparable, these two types of horn gelatin were assessed together. The parameters of the HPLC-Triple Quadrupole MS were consistent with those described in Section 2.5.

Utilizing the strategy building method outlined in the previous paragraph, identification strategies for SDHG, REDHG, RDHG, EDHG, and WDHG were established to identify the species of authentic DHG. Based on this foundation, identification strategies were devised for donkey hide and horse hide gelatin, cattle hide gelatin, pig hide gelatin, sika deer and red deer hide gelatin, reindeer hide gelatin, Eurasian elk hide gelatin, and white-tailed deer hide gelatin to determine the species of counterfeit DHG.

2.7.2. Establishment of characteristic mapping strategy for DHG

To construct the characteristic mapping, we selected peptides present in DHG but absent in gelatin from several other species and compared the response intensities of the peptides using the MRM method. The parameters of the HPLC-Triple Quadrupole MS were identical to those described in Section 2.5. Furthermore, experiments were conducted using four distinct C18 columns to determine the relative retention time (RRT). The mass spectrometry data from 20 batches of DHG were employed as a standard reference and imported into ChemPattern Studio software (2020 Edition, Chemmind Technologies Inc., Beijing, China) to fit a shared mass spectrum and establish the characteristic mapping strategy.

2.7.3. Establishment of content determination strategy for DHG

A strategy for the determination of DHG content was established using a label-free quantitative method. The following HPLC parameters were modified: (0–5 min, 3% B →7.5% B; 5–9 min, 7.5% B→25% B; 9–13 min, 25% B→90% B; 13–16 min, 90% B; 16–16.5 min, 90% B→3% B; 16.5–20 min, 3% B) with an injection volume of 5 μl; the solvent delay was 0–4 min and 16–20 min. The rest of the setting parameters were the same as those described in Section 2.5. We used Pep12, Pep11, Pep13, and Pep14 as reference standard (RS) of five species sources of deer horn gelatin. SDHG and REDHG both used Pep12. For each RS, the two most stable and highest response product ions were selected as quantitative and qualitative ions, respectively. The content was determined by the value of the peak area of the quantitative ion in the sample in the linear regression equation of the RS.

The total runtime for a single sample, according to the liquid chromatography method, was 23 min, including pre-equilibration and post-equilibration. The sample tray was set to 37 °C, and RS and DHG samples, which had just been treated with trypsin, were placed on it. The samples were then alternately injected for a period of 24 h to assess stability. Subsequently, linearity was evaluated using synthetic peptide markers as RSs. Furthermore, methodological experiments were conducted using samples labeled as SDHG-2, RDHG-1, EDHG-1, and WDHG-3, specifically for repeatability testing and sample addition recovery testing. Lastly, an internal standard control with the amino acid sequence TPV(13C5, 15N)GGQPSVPGGPVR was synthesized. The content of four parallel samples from the same batch of red deer horn gelatin (REDHG) was determined using both the internal standard method and the established label-free strategy to compare the differences between the two methods.

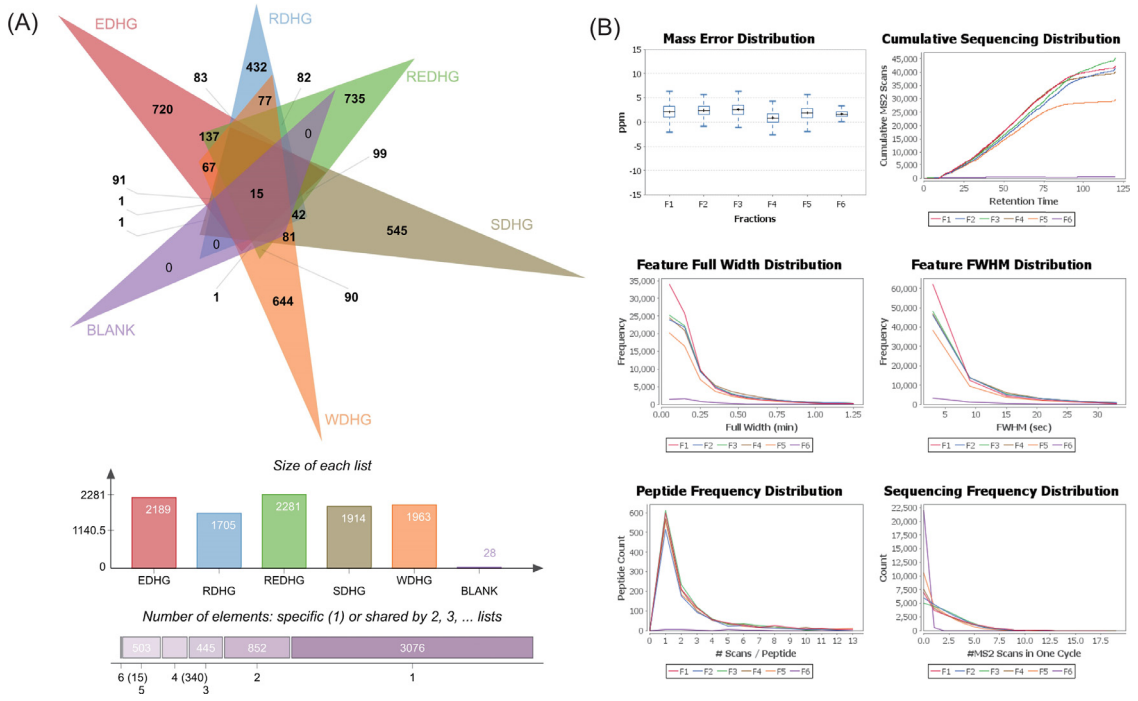

Fig. 2. (A) Venn diagram of peptide identification results. (B) Mass spectrometry data analysis quality control results.

3. Results

3.1. Results of nano LC-MS/MS data analysis

In previous experiments, we discovered a high concentration of collagen type I alpha 1 (COL1A1), collagen type II alpha 1 (COL2A1), and AHSG proteins in DHG. Collagen, the fundamental structural component of connective tissue, is abundant in skin and bone [19], can be soluble in hot water, and dissociates into protein gelatin [20]. It is also the primary ingredient in the renowned Chinese traditional medicine E Jiao (donkey hide gelatin). However, our attention was drawn to AHSG protein, as one of the main components of the non-collagenous bone matrix protein, which is abundant in horns [21,22]. Consequently, we hypothesized that peptide markers in AHSG would be crucial for evaluating the quality of DHG. The peptide mixture of DHG digested by trypsin was injected into Nano LC-Orbitrap MS, resulting in 5232 matched peptides (Table S3). The specificity of the identified peptides was statistically analyzed (Fig. 2), and peptides with high specificity, lengths between 7 and 25 amino acids, and minimal post-translational modifications were selected for further validation.

3.2. Determination and specificity verification of peptide markers

Multiple reaction monitoring (MRM) is a mass spectrometry technique that selectively obtains quantitative mass spectrometry information through statistical analysis of the data. Using this technique to build the method, it is only necessary to identify the parent ion and the corresponding quantitative and qualitative ions to capture information about the target peptide [23]. As a result, 18 peptide markers with varying specificities were identified and numbered according to the retention time of the mass spectral peaks' appearance, as acquiring method described in Section 2.5. Among these markers, Pep6 has been reported as a marker for adulterated reindeer antlers in sika deer antlers or red deer antlers [24], and Pep4 has been reported as a peptide marker in deer hide gelatin and DHG, distinguishing it from pig, cattle, sheep, horse, and donkey hide gelatins [2–25]. Notably, we identified several peptide markers witch previously unreported sequences. To enhance the study's reliability, all the 18 peptide markers were artificially synthesized and analyzed by HPLC-Triple Quadrupole MS. The peak times of the quantifier and qualifier ions of the synthesized peptides matched those of the peptide markers in the samples. When the synthesized peptide was added to each sample, the peak times of the quantifier and qualifier ions in both spiked and unspiked samples were identical, and there was only a single peak at the peptide markers. Consequently, we believe that our predicted sequence aligns with the sequences of the peptide biomarkers in the samples. The MRM parameters and specificity are displayed in Table 1 and Fig. 3, respectively. Corresponding sequence analysis maps of peptide biomarkers are shown in Figs. S1–S3.

3.3. Establishment of the quality evaluation strategies for deer horn gelatin

HPLC-Triple Quadrupole MS was chosen to establish the MRM quality-evaluation strategies for DHG, due to its shorter analysis time, ease of operation, less expensive instrumentation, and ability to focus on target peptides.

3.3.1. Identification strategy for DHG

The identification strategy is a method for evaluating the authenticity of DHG samples. Using the strategy, it is possible to assess whether the samples were produced using materials of authentic origin in sufficient quantities. Pep12, Pep11, Pep13, and Pep14 were chosen as the primary subjects for the identification of DHG. These peptides are present in the AHSG protein of deer, located near the C-terminus, and are readily detectable following enzymatic digestion (Fig. 4A). AHSG is scarce in the hide but abundant in the horn and is greatly enriched during the intricate preparation of horn gelatin. Pep12 was not detected in hide gelatin (including deer hide gelatins) in our experiments, confirming the high specificity and suggesting that Pep12 can be used as a marker for the exclusive identification of SDHG and REDHG.

Table 1
Sequences of peptide biomarkers and their specificity and MRM parameters in positive mode (p is hydroxyproline).

No	Sequence	Charge	m/z	Quantifier	Qualifier	Accession No
1	GETGPAGRpGEVGPpGPpGPAGEK	3	739.36	938.46	1035.52	A0A6J0Z691
2	GERGPpGESGAAGPAGPIGSR	3	631.65	754.42	586.33	OWK06316.1
3	GERGPpGESGAAGPTGPIGSR	3	641.65	841.46	940.42	A0A6J0Z5J9
4	SGETGASGPpGFAGEK	3	732.84	875.43	1090.51	A0A6J0Z5J9
5	TGETGASGPpGFAGEK	2	739.85	962.46	818.41	A0A1U7R801
6	AGQpGAVGPAGIR	2	583.82	257.12	353.14	PXD036244
7	GPpGESGAAGPAGPIGSR	2	775.89	541.26	965.44	OWK06316.1
8	GPpGESGAAGPTGPIGSR	2	790.89	319.17	454.19	A0A6J0Z5J9
9	TGQPGAVGPAGIR	2	590.83	797.47	287.14	OWK06316.1
10	GEpGPAGAVGSAGAVGPR	2	761.89	771.41	998.54	PXD036244
11	TPVGGQPGVPGGPVR	2	687.88	835.48	1177.58	PXD036244
12	TPVGGQPSVPGGPVR	2	702.88	865.49	1107.59	OWK05631.1
13	TPVEGQPSVPGGPVR	2	738.89	865.50	582.34	PXD036244
14	TPVGGLPGVPGGPVR	2	680.39	835.48	1062.61	A0A6J0WBI5
15	LGApGFLGLpGSR	3	637.35	602.33	715.41	OWK06316.1
16	LGPpGFLGLpGSR	2	650.36	1032.55	602.33	A0A6J0Z5J9
17	DLGFLPQPPQEK	2	684.86	598.32	823.42	PXD036244
18	DLSFLPQPPQEK	2	699.87	598.32	501.24	A0A6J0Z691
19	TPV(13C5 15N)GGQPSVPGGPVR	2	705.88	865.49	1107.59	Stable isotope internal standard

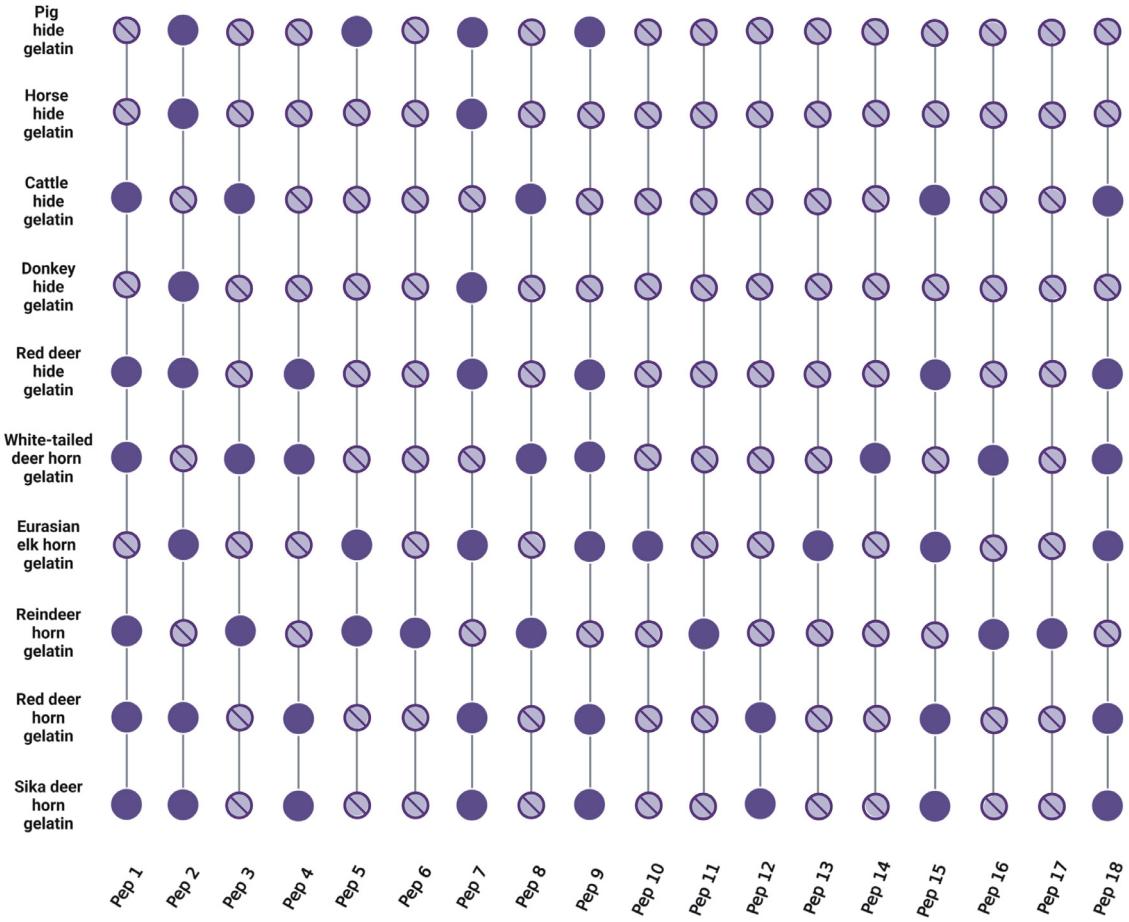

Fig. 3. The specificity of peptide markers. A large purple circle indicates that the peptide marker was detected in the sample, while a purple prohibition sign signifies that the peptide marker was not detected.

Based on the aforementioned experimental findings, we hypothesized that Pep11, Pep13, Pep14, which are the peptides at the same position of AHSG proteins in different species of deer families could also serve as markers for horn gelatin in the respective deer families. On the basis of the experimental results, the identification strategy for DHG was developed, which can be used to distinguish horn gelatin from different species of the deer family, to distinguish DHG from its homologous deer hide gelatin, and to distinguish DHG from pig, cattle, donkey, and horse hide gelatins (Fig. 4B). We tested 25 batches of gelatin samples using the established identification strategy, and the results were consistent with the identification strategy.

3.3.2. Characteristic mapping strategy of DHG

The strategy based on the characteristic mapping identifies the authenticity of DHG and can be used to ascertain its quality. Char-

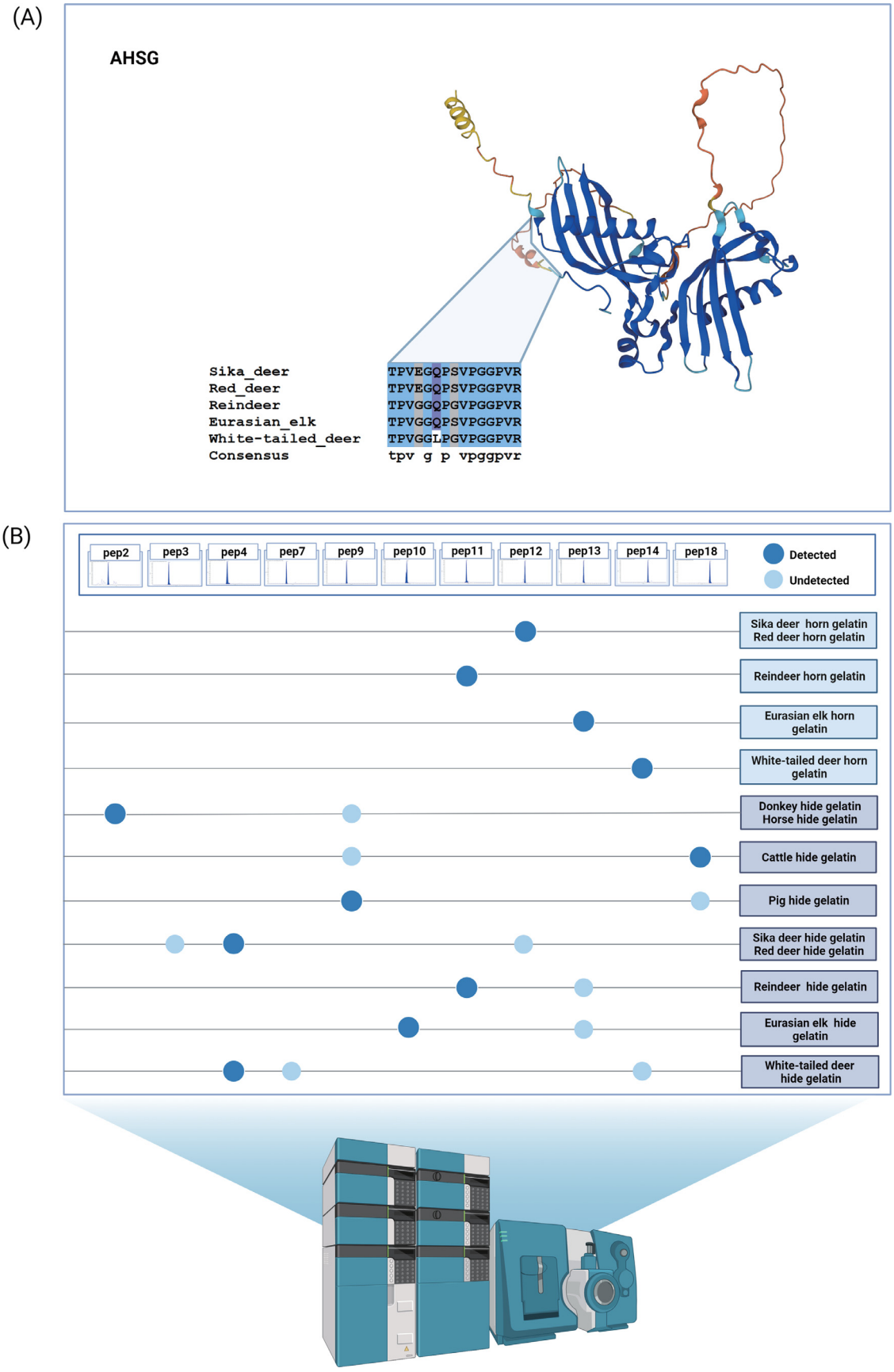

Fig. 4. Establishment of a strategy for identifying DHG. (A) Peptide markers in the AHSG of various species of deer. Structure of AHSG downloaded from AlphaFold Protein Structure Database, Alpha-2-HS-glycoprotein, Source organism: Bos taurus, https://alphafold.ebi.ac.uk/entry/B0JYN6 [26,27] (accessed on 23 September 2022). (B) Strategy for identifying DHG using peptide markers. Method in Section 2.2 was applied to DHG, which was then analyzed using HPLC-Triple Quadrupole MS. Large blue circles represent detections and small light blue circles represent non-detections.

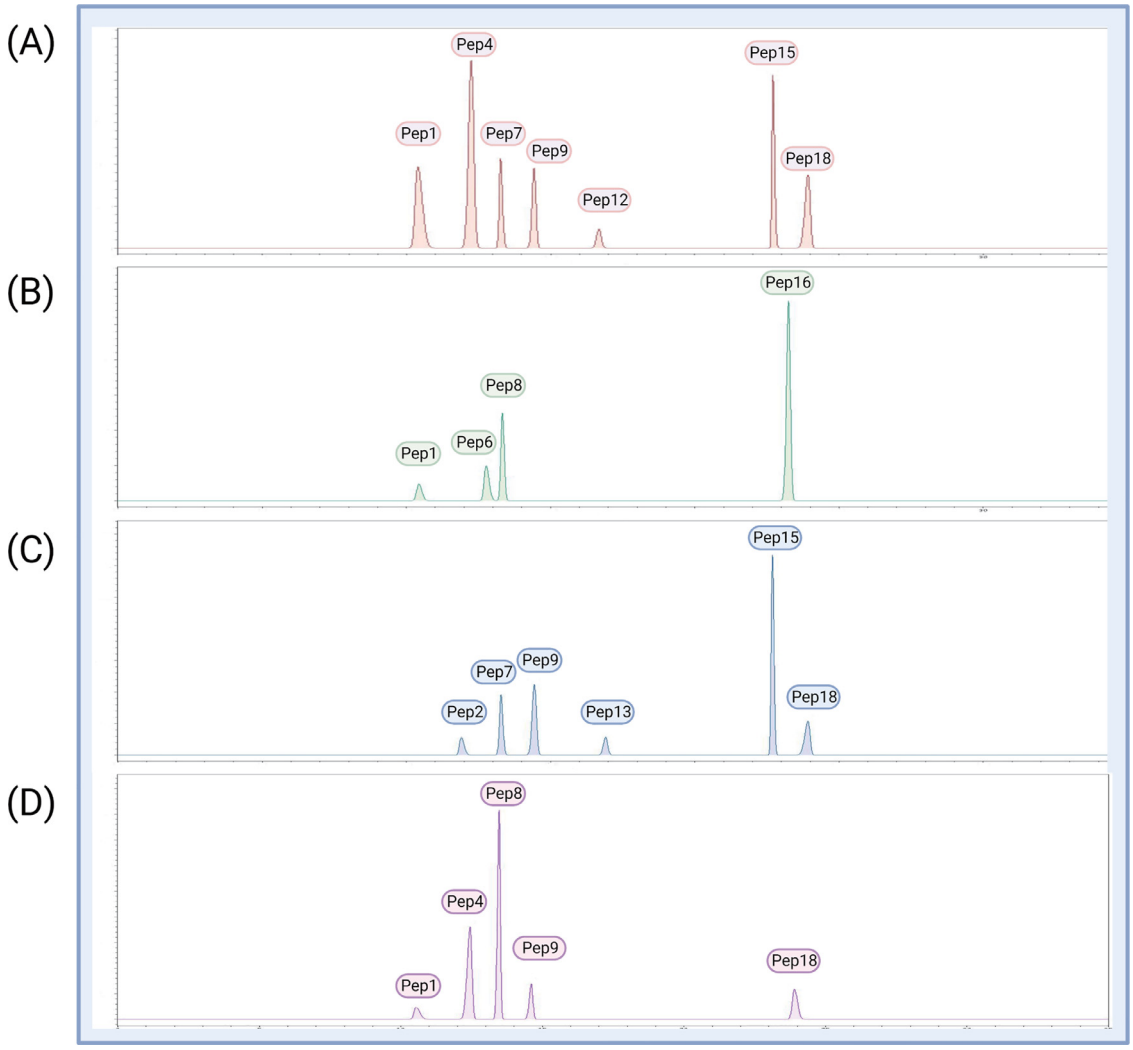

Fig. 5. A strategy for building a DHG characteristic mapping based on peptide markers. (A) Characteristic mapping of SDHG and REDHG. (B) Characteristic mapping of RDHG. (C) Characteristic mapping of EDHG. (D) Characteristic mapping of WDHG.

acteristic mapping is currently one of the internationally accepted methods for evaluating the quality of natural products with complex composition. This method is based on the separation of peptide markers in samples with different partition coefficients between the mobile and stationary phases in HPLC. The MRM mass spectra of the peptide markers shared by the same species of DHG should be similar after the separation by liquid chromatography. The peaks of the mass spectra were the shared peaks, the peak with high stability was selected as the S-peak, and the peptide marker represented by it was the RS. The ratio of retention time of the shared peak to the S-peak is stable under the same liquid chromatographic elution parameters. The characteristic mapping method prioritizes accurate identification over precise calculation and uses shared peaks to indicate the characteristics of different component groups. It can be used to evaluate the sample's quality based on the RRT of the shared peak in the spectrum, and it requires the synthesis of only the peptide marker represented by the S-peak, which significantly reduces the cost and duration of the experiment.

The RRT of markers were obtained using four different C18 columns and averaged. The results were as follows: for SDHG and REDHG, seven shared peaks were revealed, with the corresponding peak of Pep12 serving as the S-peak. The RRT of each shared peak and S-peak were calculated (RT of shared peaks/RT of peak S): 0.47 (Pep1), 0.56 (Pep4), 0.63 (Pep7), 0.75 (Pep9), 1.36 (Pep15), and 1.40 (Pep18). RDHG revealed four shared peaks, with the corresponding peak of Pep8 serving as the S-peak. The RRT of each shared peak and S-peak were calculated as follows: 0.75 (Pep1), 0.97 (Pep6), and 2.24 (Pep16). EDHG revealed six shared peaks, with the corresponding peak of Pep13 serving as the S-peak. The RRT of each shared peak and S-peak were calculated as follows: 0.49 (Pep2), 0.60 (Pep7), 0.72 (Pep9), 1.35 (Pep15), and 1.38 (Pep18). WDHG revealed five shared peaks, with the corresponding peak of Pep8 serving as the S-peak. The RRT of each shared peak and S-peak were calculated as follows: 0.76 (Pep1), 0.89 (Pep4), 1.21 (Pep9), and 2.26 (Pep18).

Finally, we conducted experiments on 20 batches of DHG using three different columns, and determined that the RRT were within 10% of the specified values and conformed to the characteristic mapping. The fitted characteristic mapping of DHG is shown in Fig. 5, calculation procedures are detailed in Table S4 and the characteristic mapping of DHG are shown in Fig. S4.

The characteristic mapping strategy can prevent the detection of false positives to a greater extent. Taking a batch of samples marked SDHG as an example, the strategy of characteristic mapping is adopted, if any of Pep1, Pep4, Pep7, Pep9, Pep12, Pep15, or Pep18 did not appear in the mass spectra of this sample, it was

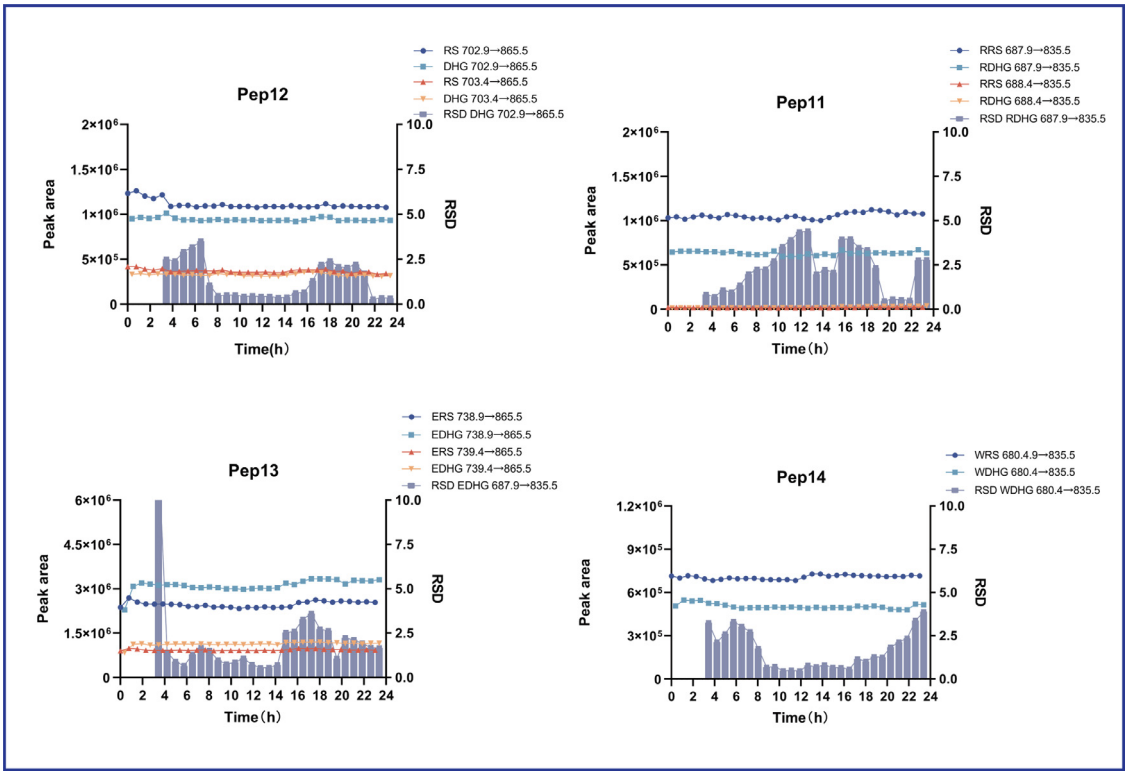

Fig. 6. Sample stability study for label-free content determination strategy (n=31). The peptide quantified ion's peak area is shown on the ordinate. The dark blue line represents the quantitative ion of the RS, while the red line represents the quantitative ion of the oxidized peptide of the RS. The light blue line indicates the quantitative ion of the DHG, while the yellow line represents the quantitative ion of the oxidized peptide in the DHG. The RSD value of five consecutive samples is illustrated in the purple histogram.

considered that the raw material source of the sample was not sika deer horn.

3.3.3. Content determination strategy for DHG

The content determination strategy can detect the number of deer horns added as raw material, and it can detect whether manufacturers add resin, industrial synthetic glue, and other non-animal sources of gelatin, which is crucial for evaluating the quality of DHG. Peptide marker on the AHSG protein were selected to serve as the RS for the development of content determination strategy. Pep12 for SDHG and REDHG, Pep11 for RDHG, Pep13 for EDHG, and Pep14 for WDHG.

From the experiments to investigate the stability of the digestion samples, it was revealed that the RSD values of the peak areas for five consecutive DHG samples after 4 h were less than 5%, so the digestion time was set to 4 h. Moreover, since Pep12, Pep11, and Pep13 contain glutamine, which is easily oxidized to glutamate, we also carried out the determination of the oxidized peptide content and found that the content of oxidized peptides in the samples exhibited no significant changes within 24 h (Fig. 6). In addition, the correlation coefficients of the linear regressions for four linearity experiments with the RS were greater than 0.99. Notably, the ideal recovery was not consistently achieved during the experiment due to the severe matrix effect. This issue was resolved by replacing the blank matrix with RDHG solution (4 mg/ml) for Pep12 and SDHG solution (4 mg/ml) for Pep11, Pep13, and Pep14. The recoveries for the six concentrations ranged between 80% and 120% (refer to Table 2). To ensure the accuracy of the content determination, three samples were repeatedly made for each of the four DHGs, resulting in an RSD of 4.09%, 3.94%, 2.80%, and 1.32%. To assess the feasibility of the label-free content determination strategy, we compared it with a stable isotope internal standard method. The results showed no significant difference in the content of a batch of REDHG using either the label-free or stable isotope internal standard method. Considering that the label-free strategy involves the synthesis of only one peptide marker as a RS, and has relatively low experimental cost, we believe that it is worthy of further development for the determination of DHG. The amounts of peptide markers in each species are presented in Table 3, while the raw data and calculation procedures are detailed in Table S4 and can be used as a reference for the quality of DHG.

Table 2
Results of sample adding recovery test.

Percentage of RS	SDHG-2	RDHG-1	EDHG-1	WDHG-3
15	107.42	99.10	100.69	109.23
25	106.25	99.74	104.62	97.87
35	106.20	115.72	95.02	88.46
50	109.42	108.47	105.22	95.13
75	108.74	112.96	116.41	111.49
85	109.17	111.63	98.75	115.98

Table 3
Results of DHG's content-determination.

RS	Quantifier ion	Qualifier ion	Species	No.	Calculated content (μg/g)
TPVGGQPSVPGGPVR	702.88→865.49	702.88→1107.59	SDHG	1	7.99
				2	11.03
				3	3.22
			REDHG	1	9.13
				2	3.42
				3	8.02
				4	13.31
TPVGGQPGVPGGPVR	687.88→835.48	687.88→1177.58	RDHG	1	3.62
				2	7.66
				3	11.14
				4	8.29
TPVEGQPSVPGGPVR	738.89→865.49	738.89→582.34	EDHG	1	15.92
				2	12.88
				3	13.24
				4	11.14
				5	9.41
TPVGGLPGVPGGPVR	680.39→835.48	680.39→1062.61	WDHG	1	1.22
				2	1.47
				3	1.98
				4	1.23

4. Discussion and conclusion

In this study, we utilized Nano LC-Orbitrap MS data analysis to identify AHSG and collagen as the key factors in differentiating DHG species, resulting in the identification of 18 peptide markers with varying specificities. The HPLC-Triple Quadrupole MS technique was utilized to confirm the specificity of the peptide markers and establish quality-evaluation strategies. Our analysis resulted in the identification of 18 peptide markers with distinct specificities from the matched 5232 peptides, and three different strategies were developed to evaluate the quality of DHG.

Four peptide markers unique to DHG were identified, enabling the development of a highly specific identification strategy for DHG, and its implementation in the quality-evaluation study of Chinese patent medicines containing DHG [28]. However, the distinction between horn gelatin and hide gelatin from reindeer, Eurasian elk, or white-tailed deer requires further investigation as we have not yet collected the hides of these species. Additionally, a characteristic mapping strategy for DHG was developed, which provides a simple, low-cost, and convenient approach to assess quality. Finally, we established a strategy for content-determination of DHG based on label-free methods, which has high specificity and detection efficiency compared to traditional methods for determining amino acid content and total nitrogen amount. This strategy can provide a reference for raw material quality control and production process enhancement of DHG. By integrating the three established strategies, we developed a comprehensive quality-evaluation tool for gelatin. This tool overcomes the limitations of a single evaluation method and can guide quality-control strategies for other animal-derived gelatin medicines.

While our study primarily focused on the identification of peptide markers and the establishment of quality-evaluation strategies for DHG, we recognize the importance of investigating the active sources within DHG for a comprehensive understanding of its medicinal properties. We hope that our research can serve as a valuable starting point for future studies aimed at uncovering the active compounds present in DHG and elucidating their potential therapeutic benefits.

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.

CRediT authorship contribution statement

Fei Xue: Writing – original draft, Methodology, Investigation. **Bing Wang:** Investigation, Data curation. **Dong-xiao Guo:** Writing – review & editing. **Yang Jiao:** Investigation, Data curation. **Wei-liang Cui:** Investigation, Data curation. **Xian-long Cheng:** Investigation, Resources. **Zhi-bin Wang:** Investigation, Resources. **Xue Yin:** Investigation, Data curation. **Shuang-cheng Ma:** Conceptualization, Writing – review & editing. **Yong-qiang Lin:** Conceptualization, Writing – review & editing, Supervision.

Acknowledgments

This research was funded by the Major Scientific and Technological Innovation Projects of Shan-dong Key R & D Plan, grant number [2021CXGC010511], and the Leading Talent Program of Quancheng.

Funding

This research was funded by the Major Scientific and Technological Innovation Projects of Shan-dong Key R & D Plan, grant number [2021CXGC010511], and the Leading Talent Program of Quancheng.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464153.

References

[1] Chinese Pharmacopoeia CommissionChinese Pharmacopoeia 2020 Editioned, The Medicine Science and Technology Press of China, Beijing, 2020.
[2] J. Chen, X.L. Cheng, F. Wei, Q.Q. Zhang, M.H. Li, S.C. Ma, Detection of gelatin adulteration in traditional Chinese medicine: analysis of deer-horn glue by rapid-resolution liquid chromatography-triple quadrupole mass spectrometry, J. Anal. Methods Chem. 2015 (2015) 259757, doi:10.1155/2015/259757.
[3] Y. Hijikata, T. Kano, L. Xi, Treatment for intractable anemia with the traditional Chinese medicines Hominis Placenta and Cervi Cornus Colla (deer antler glue), Int. J. Gen. Med. 2 (2009) 83–90, doi:10.2147/ijgm.s5253.
[4] C.J. Yun, W.J. Qian, J.Y. Wu, C.X. Yuan, S.Z. Jiang, J.P. Lv, Pilose antler peptide promotes osteoblast proliferation, differentiation and mineralization *via* the insulin signaling pathway, Exp. Ther. Med. 19 (2) (2020) 923–930, doi:10.3892/etm.2019.8286.
[5] J.C. Li, X.Z. Liang, D. Luo, B.Z. Yan, J.B. Liu, G. Li, Study on the molecular mechanism of BuShenHuoXue capsule in treatment of steroid-induced osteonecrosis of the femoral head, Ann. Transl. Med. 8 (24) (2020) 1680, doi:10.21037/atm-20-7040.

[6] B. Yao, Z. Zhou, M. Zhang, X. Leng, D. Zhao, Investigating the molecular control of deer antler extract on articular cartilage, J. Orthop. Surg. Res. 16 (1) (2021) 8, doi:10.1186/s13018-020-02148-w.
[7] B. Yao, M. Zhang, X. Leng, M. Liu, Y. Liu, Y. Hu, D. Zhao, Y. Zhao, Antler extracts stimulate chondrocyte proliferation and possess potent anti-oxidative, anti-inflammatory, and immune-modulatory properties, *In Vitro* Cell Dev. Biol. Anim. 54 (2018) 439–448, doi:10.1007/s11626-018-0266-2.
[8] D. Cheng, X.J. Yang, L. Zhang, Z.S. Qin, W.Q. Li, H.C. Xu, Z.J. Zhang, Tortoise plastron and deer antler gelatin prevents against neuronal mitochondrial dysfunction *in vitro*: implication for a potential therapy of Alzheimer's disease, Front Pharmacol. 12 (2021) 690256, doi:10.3389/fphar.2021.690256.
[9] H.R. Choi, K.M. Nam, D.S. Kim, C.H. Huh, J.I. Na, K.C. Park, Cervi cornus Colla (deer antler glue) induce epidermal differentiation in the reconstruction of skin equivalents, Int. J. Cosmet. Sci. 35 (2013) 281–285, doi:10.1111/ics.12045.
[10] C. Ma, H. Long, C. Yang, W. Cai, T. Zhang, W. Zhao, Anti-inflammatory role of pilose antler peptide in LPS-induced lung injury, Inflammation 40 (2017) 904–912, doi:10.1007/s10753-017-0535-3.
[11] R. Zhang, Y. Li, X. Xing, Comparative antler proteome of sika deer from different developmental stages, Sci. Rep. 11 (2021) 10484, doi:10.1038/s41598-021-89829-6.
[12] Edinburgh Zoo and Highland Wildlife Park, Alces alces alces (Eurasian elk). https://www.highlandwildlifepark.org.uk/animals-attractions/animals/eurasian-elk/, 2022 (accessed 31 May 2023).
[13] V.L. Hale, P.M. Dennis, D.S. McBride, J.M. Nolting, C. Madden, D. Huey, M. Ehrlich, J. Grieser, J. Winston, D. Lombardi, S. Gibson, L. Saif, M.L. Killian, K. Lantz, R.M. Tell, M. Torchetti, S. Robbe-Austerman, M.I. Nelson, S.A. Faith, A.S. Bowman, SARS-CoV-2 infection in free-ranging white-tailed deer, Nature 60 (2022) 481–486, doi:10.1038/s41586-021-04353-x.
[14] Plum Dragon HERBS, Chinese HERB: lu Jiao Jiao (deer antler gelatin). https://plumdragonherbs.com/products/lu-jiao-jiao, 2021 (accessed 31 May 2023).
[15] S. Han, K. Zhao, S. Cai, M. Jiang, X. Huang, S. Chen, S. Li, M. Zhao, J.A. Duan, R. Liu, Discovery of peptide biomarkers by label-free peptidomics for discrimination of horn gelatin and hide gelatin from Cervus nippon Temminck, Food Chem. 363 (2021) 130347, doi:10.1016/j.foodchem.2021.130347.
[16] F. Xue, B. Wang, D.X. Guo, Y. Jiao, X. Yin, W.L. Cui, Q.Q. Zhou, F.R. Yu, Y.Q. Lin, Peptide biomarkers discovery for seven species of deer antler using LC-MS/MS and label-free approach, Molecules 27 (2022), doi:10.3390/molecules27154756.
[17] X.K. Yang, H.C. Bai, L.J. Yin, J. Wang, W.T. Xue, X. Jia, Evaluation of allergenic protein profiles in three Chinese high-oleic acid peanut cultivars using NanoLC-Orbitrap mass spectrometry, Food Sci. Hum. Well. 12 (2023) 851–860, doi:10.1016/j.fshw.2022.09.026.
[18] Y. Perez-Riverol, J. Bai, C. Bandla, D. García-Seisdedos, S. Hewapathirana, S. Kamatchinathan, D.J. Kundu, A. Prakash, A. Frericks-Zipper, M. Eisenacher, M. Walzer, S. Wang, A. Brazma, J.A. Vizcaíno, The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences, Nucleic Acids Res. 50 (2022) D543-d552, doi:10.1093/nar/gkab1038.
[19] A.M. Holwerda, L.J.C. van Loon, The impact of collagen protein ingestion on musculoskeletal connective tissue remodeling: a narrative review, Nutr. Rev. 80 (2022) 1497–1514, doi:10.1093/nutrit/nuab083.
[20] F.M. Sinex, B. Faris, Isolation of gelatin from ancient bones, Science 129 (1959) 969, doi:10.1126/science.129.3354.969.
[21] L. Kalabay, K. Cseh, A. Pajor, E. Baranyi, G.M. Csákány, Z. Melczer, G. Speer, M. Kovács, G. Siller, I. Karádi, G. Winkler, Correlation of maternal serum fetuin/alpha2-HS-glycoprotein concentration with maternal insulin resistance and anthropometric parameters of neonates in normal pregnancy and gestational diabetes, Eur. J. Endocrinol. 147 (2002) 243–248, doi:10.1530/eje.0.1470243.
[22] J.T. Triffitt, U. Gebauer, B.A. Ashton, M.E. Owen, J.J. Reynolds, Origin of plasma alpha2HS-glycoprotein and its accumulation in bone, Nature 262 (1976) 226–227, doi:10.1038/262226a0.
[23] S. Cai, M.T. Jiang, K.X. Zhao, X.Z. Huang, F. Fei, B. Cao, X.B. Cui, J.A. Duan, M. Zhao, S.Y. Han, A quantitative strategy of ultrasound-assisted digestion combined UPLC-MS/MS for rapid identifying species-specific peptide markers in the application of food gelatin authentication, LWT 147 (2021) 111590, doi:10.1016/j.lwt.2021.111590.
[24] X.H. Guo, X.L. Cheng, W.X. Liu, M.H. Li, F. Wei, S.C. Ma, Identification of velvet antler and its mixed varieties by UPLC-QTOF-MS combined with principal component analysis, J. Pharm. Biomed. Anal. 165 (2019) 18–23, doi:10.1016/j.jpba.2018.10.009.
[25] R. Liu, Y. Huang, H. Xu, Y. Zheng, Y. Liu, S. Han, M. Yang, Y. Xie, K. Wang, J.A. Duan, L. Li, A strategy for identifying species-specific peptide biomarkers in deer-hide gelatin using untargeted and targeted mass spectrometry approaches, Anal. Chim. Acta 1092 (2019) 32–41, doi:10.1016/j.aca.2019.09.064.
[26] J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Zidek, A. Potapenko, A. Bridgland, C. Meyer, S.A.A. Kohl, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, Highly accurate protein structure prediction with AlphaFold, Nature 596 (2021) 583–589, doi:10.1038/s41586-021-03819-2.
[27] M. Varadi, S. Anyango, M. Deshpande, S. Nair, C. Natassia, G. Yordanova, D. Yuan, O. Stroe, G. Wood, A. Laydon, A. Zidek, T. Green, K. Tunyasuvunakool, S. Petersen, J. Jumper, E. Clancy, R. Green, A. Vora, M. Lutfi, M. Figurnov, A. Cowie, N. Hobbs, P. Kohli, G. Kleywegt, E. Birney, D. Hassabis, S. Velankar, AlphaFold protein structure database: massively expanding the structural coverage of protein-sequence space with high-accuracy models, Nucleic Acids Res. 50 (2022) D439–D444, doi:10.1093/nar/gkab1061.
[28] L. Xu, Y. Jiao, W. Cui, B. Wang, D. Guo, F. Xue, X. Mu, H. Li, Y. Lin, H. Lin, Quality evaluation of traditional chinese medicine prescription in naolingsu capsule based on combinative method of fingerprint, quantitative determination, and chemometrics, J. Anal. Methods Chem. 2022 (2022) 1429074, doi:10.1155/2022/1429074.