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Original Article

Collagen derived species-specific peptides for distinguishing donkey-hide gelatin (*Asini Corii Colla*)

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ABSTRACT

Objective: As an important food therapy product with traditional Chinese medicine (TCM) applications, donkey-hide gelatin (*Asini Corii Colla*, ACC) has been used for thousands of years. However, till now few effective strategy had been proposed to distinguish ACC from other animal hide gelatins, especially closely related horse- and mule-hide gelatins, which was an embarrassment of ACC quality control.
Methods: Combined mass spectrometry and bioinformatic methods have been applied to identify and verify two ACC-specific peptides (Pep-1 and Pep-2) capable of distinguishing ACC from other closely related animal gelatins with high selectivity.
Results: It confirmed that these two peptides could be not only used for distinguishing ACC from highly homologous horse-hide and mule-hide gelatins as well as other animal hide gelatins.
Conclusion: The present study provides a simple method for species-specific peptides discovery, which can be used for assessing the quality of animal gelatin products, and ensure they are authenticable and traceable.

1. Introduction

Donkey-hide gelatin (*Asini Corii Colla*, ACC), a gelatinous Chinese medicine known as Ejiao in Chinese, is obtained from donkey hide after a complex extraction procedure. ACC has been applied as an important food therapy product to optimize immune response, improve metabolic balance and treat gynecological diseases for centuries since it was first documented in *Shennong Bencao Jing* (from 200 BCE to 200 CE) and then included in the *Compendium of Materia Medica* (Ming Dynasty, 1593 CE) (Li et al., 2016; Wang et al., 2014; Wu et al., 2016; Yang et al., 2015).

As an important food product with TCM applications, the price of donkey hide continues to increase, which forces up the price of ACC in recent years. For this reason, some dishonest businesses produce ACC using other animal skins from horse, cattle, and pig in pursuit of excessive profits, and/or adulterate other animal skins to prepare ACC. Unfortunately, genuine ACC cannot be distinguished from adulterated materials and products made from other animal skins by visual observation or chemical identification approaches after the complex heating and high-pressure processing (Li et al., 2017). The recently popular DNA barcoding-based analytical methods for identifying species are also facing the problem of DNA degradation under high temperature and pressure, which may lead to the difficulty in extracting and amplifying DNA from gelatin products.

Lately it has been reported that proteins and peptides are more stable than DNA under the severe processing conditions. Integrated nano-LC-MS/MS and triple-quadrupole MS method with high throughput, sensitivity, and selectivity has been reported to discover species-specific peptides efficiently (Liu et al., 2019). Consequently, species-specific peptides generated from proteins after enzyme treatment have been applied for authentication purposes (Carrera et al., 2011; Fornal & Montowska, 2019; Montowska, Alexander, Tucker, & Barrett, 2014; Şakalar, Abasiyanik, Bektik, & Tayyrov, 2012). However, collagen proteins, the main ingredients of ACC, are highly conserved between species, and almost identical

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in donkey, horse and mule. That is, ACC, horse-hide gelatin (HHG) and mule-hide gelatin (MHG) are hard to be distinguished by the existing methods. Thus, a more reliable strategy is urgently needed to solve the current dilemma of ACC adulteration.

In the present study, collagens in ACC were identified using a comprehensive proteomics analysis method. In order to obtain peptides of appropriate length and specificity, Lys-C was employed to digest collagens instead of commonly used trypsin in the present work. Alignment was then used to compare sequences of collagens in donkey and horse, with which the specific peptides that can distinguish ACC and HHG were found for the first time. Finally, the discovered peptide biomarkers were verified by successfully distinguishing ACC from HHG, MHG, and some other products from closely related species. The findings may prove particularly helpful for animal-hide gelatin product quality control.

2. Materials and methods

2.1. Chemicals and materials

Lys-C (sequencing grade) and trypsin (sequencing grade) were purchased from Promega (Fitchburg, WI, USA). HPLC-grade formic acid (FA), acetonitrile, and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals and reagents were of the highest grade available.

Three batches of ACC samples were collected from Dong'e Co., Ltd. (Shandong, China) and Fu Co., Ltd. (Ji'nan, China), three horse skins and three mule skins were collected and authenticated. HHG and MHG were prepared in-house following the protocol as: Skin pieces were soaked in 1% Na_2CO_3 solution for 30 min at 70 °C, and were decocted in water for 4 h at 0.8 MPa and 120 °C. Then gelatinous samples were concentrated and dried.

Sample preparation was performed according to the method in the Chinese Pharmacopeia with slight modification. Dried and powdered gelatin sample (50 mg) was weighed into a 5 mL centrifuge tube, and 4 mL 1% (volume percentage) NH_4HCO_3 solution was added. The tube was ultrasonicated for 30 min. The sample solution was centrifuged at 16 000×*g* at 4 °C for 15 min, and the supernatant was collected and the protein concentration was determined using bicinchoninic acid method. Lys-C or trypsin was added to 1% (mass ratio), samples were incubated at 37 °C overnight. The digestion products were desalted with SepPak C_{18} cartridge (Waters) and then dried.

2.2. LC–MS/MS method

Samples were analyzed using a Dionex Ultimate 3000 nanoLC system (Thermo Scientific) coupled to an LTQ-Orbitrap hybrid mass spectrometer (ThermoFisher Scientific, Bremen, Germany). Chromatographic separations were performed on a reversed-phase capillary column (75 μm × 15 cm, particle size 1.7 μm, pore size 15 nm). Then the peptides in 0.1% FA were separated using a solvent gradient of increasing from 3% to 30% solvent B (0.1% FA in 98% acetonitrile) over 100 min at a flow rate of 300 nL/min.

Data-dependent acquisition in positive mode recorded MS scans in profile mode from *m*/*z* 300–2000. The 20 most intense precursor ions were selected for MS2 collision induced dissociation fragmentation with an isolation window of 2.0 Da and dynamic exclusion set at 10.0 s. Automatic gain control targets of 5E4 were accumulated for MS/MS spectra generation.

2.3. Protein identification

All MS/MS spectra were analyzed using PEAKS Software (8.5 Edition, Bioinformatics Solutions Inc., Waterloo, Canada). It was set up to search the *Laurasiatheria* UniProt database (downloaded on October 6th, 2019). The cutoff of false discovery rate (FDR) for identification was set as FDR ≤ 1%. Trypsin or Lys-C was chosen as the enzyme and two missed cleavages allowed. Variable modifications consisted of oxidation (+15.99), hydroxylation (+15.99), deamidation (+0.98), and acetylation of the protein *N*-terminus (+42.01). Maximum precursor ion tolerance of 10 ppm and fragment ion tolerance of 0.02 Da were set as well.

2.4. Sequence alignment analysis for selecting specific peptide biomarkers in ACC

Collagen α-1 I (COL1A1) and collagen α-2 I (COL1A2) were identified as the main collagen components in ACC, and COL1A1 and COL1A2 sequences from donkey and horse were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) software to identify amino acid residues of collagens that differ between these two species.

2.5. Analysis of specificity of each corresponding specie-specific peptide biomarker

ACC, HHG, MHG, cattle-hide gelatin (CHG), pig-hide gelatin (PHG) and deer-hide gelatin (DHG) were newly acquired. HHG and ACC were mixed at 1:99, 20:80 and 50:50 ratios by weight. ACC, HHG, MHG, DHG, CHG and PHG were mixed at a 1:1:1:1:1:1 ratio by weight. All samples were prepared and digested by Lys-C using the protocol in section 2.1. Then peptides were identified using the protocol in section 2.2. In order to search precisely, a home-made database contained only 10 homologous species-specific peptides from gelatin samples were established (Supplemental Data 1). Some specific PEAKS parameters were set as follow: Lys-C enzyme, modifications of hydroxylation (+15.99) and deamidation (+0.98). Species-specific peptides were all detected, and *m*/*z* values for each homologous targeted precursor are shown in Table 1.

2.6. Verification by multiple-reaction monitoring analysis

Shimadzu Nexera UPLC LC-20A system (Shimadzu, Kyoto, Japan) and a QTRAP 5500 mass spectrometer (Applied Biosystems, Foster City, CA, USA) were used for peptide biomarkers verifications. Chromatographic separation of samples was performed on an Acquity UPLC T3 column (2.1 mm × 50 mm, 1.7 μm), and separated using a solvent gradient of holding 2% B (0.1% FA in ACN) for 0–4 min, increasing from 2% to 40% B, and holding 40% A for 5 min at a flow rate of 0.4 mL/min. Electrospray ionization source was set in positive mode. Ion source temperature was set at 450 °C; Ionizing voltage was set to 5000 V; Desolvation temperature was set to 500 °C, and data was collected in multiple-reaction monitoring (MRM) mode.

3. Results and discussion

3.1. Collagens identification

Collagens including COL1A1, COL1A2, Collagen α-1 II, Collagen α-1 III, Collagen α-1 IV, Collagen α-4 IV, Collagen α-5 IV, Collagen α-1X, Collagen α-1 XI, Collagen α-1 XVII, and Collagen α-2 XI were identified in ACC. COL1A1 and COL1A2 displayed the high coverage, with 91 and 108 unique peptides, respectively (Table S1). COL1A1 and COL1A2 were the main constituents of ACC, and collagens are known to be highly conserved between species. Even though it is difficult to identify ACC-specific peptides from COL1A1 and/or COL1A2, it is important to develop peptide biomarkers for

Table 1
Homologous peptides in other samples.

Species	Peptides	Mass	*m/z*	z	RT	Transitions	Protein
Homologous peptides of Pep-1							
Donkey	HGN(+0.98)RGEP(+15.99)GPVGSVGPVGAVGPRGP(+15.99)SGPQGVRGDK	3220.6084	806.1582	4+	43.34	645.530 → 659.810	COL1A2
Horse	HGH RGEP(+15.99)GPVGSVGPVGAVGPRGP(+15.99)SGPQGVRGDK	3242.6404	811.6687	4+	37.44	649.740 → 670.830	
Deer	HGN(+0.98)RGEP(+15.99)GPAGAVGPAGAVGPRGPSGP(+15.99)QGIRGDK	3162.5667	791.6497	4+	40.85	791.900 → 974.510	
Cattle	HGN(+0.98)RGEP(+15.99)GPAGAVGPAGAVGPRGPSGP(+15.99)QGIRGDK	3162.5667	791.6511	4+	38.23	791.900 → 974.510	
Pig	HGN(+0.98)RGEP(+15.99)GPAGSVGPAGAVGPRGPSGP(+15.99)QGIRGEK	3192.5771	799.1520	4+	44.47	639.720 → 567.750	
Homologous peptides of Pep-2							
Donkey	GPTGEPGK	741.3657	371.6900	2+	11.06	371.690 → 487.250	COL1A2
Horse	GPSGEPGK	727.3500	364.6820	2+	11.04	364.682 → 487.250	
Deer	GPTGDPGK	727.3500	364.6823	2+	13.10	364.682 → 574.280	
Cattle	GPSGDPGK	713.3344	357.6746	2+	11.87	357.675 → 301.190	
Pig	GPTGDPGK	727.3500	364.6822	2+	13.29	364.682 → 574.280	

distinguishing products from donkey and closely related species such as horse and mule. Therefore, sequence alignment was performed to compare differences between donkey collagens (COL1A1 and COL1A2) and horse collagens.

3.2. Selecting specific peptide biomarkers in ACC

After sequence alignment, we noticed that only one amino acid residue differed among the 1463 amino acids of COL1A1 in donkey and horse (red box in Fig. S1). For COL1A2, four amino acid residues differ among the 1364 amino acids (red box in Fig. S2). After trypsin or Lys-C digestion, four peptides with different amino acid residues were predicted for COL1A1 and COL1A2 (Table S2), that could serve as potential specific peptide biomarkers for distinguishing ACC and HHG. Trypsin and Lys-C are commonly used enzymes for hydrolyzing proteins into peptides at specific cleavage sites. To obtain good peptide biomarkers, species specificity and an appropriate number of amino acids are key factors. Peptide TGDAGPVGPPGPPGPPGPPSAGFDFSFLPQPPQEK is derived from COL1A1 by trypsin digestion, and it possesses theoretical specificity for distinguishing ACC and HHG. However, we did not detect this sequence in the trypsin-treated ACC sample, possibly because the theoretical molecular weight (MW) of 3646.78 Da for this peptide is high, and may be outside the MS detection range.

There are four amino acid differences in COL1A2 (Fig. S2), but only three theoretical specific peptides could be generated. Peptides GHKGLDGLK and GHNGLDGLK contain amino acid 181 from COL1A2, but peptide GHKGLDGLK was difficult to obtain because Lys_{181} in the donkey protein would likely be cleaved by trypsin or Lys-C.

Amino acid residue 424 in COL1A2 is Thr and Ser in donkey and horse, respectively, which would generate peptides GATGPAGVR and GASGPAGVR. Horse-specific peptide GASGPAGVR has been reported by Gong et al. and Jiao et al. as peptide biomarker for finding horse-adulterants in ACC (Gong, Hang, Chi, & Li, 2018; Jiao et al., 2019), however, peptide GATGPAGVR is not a donkey specific peptide and cannot distinguish ACC from other animal gelatins. Therefore, this pair of peptides only can be applied to detect HHG in ACC, but not identify the authenticity of ACC.

Trypsin and Lys-C are commonly used enzymes in proteomic analysis. Trypsin cleaves peptide bonds after Arg and Lys amino acid residues, while Lys-C only cleaves bonds after Lys (Greer, Parker, & Brodbelt, 2015). Peptides cleaved by Lys-C may display higher specificity than those resulting from trypsin treatment because Lys-C generates longer peptides (i.e. collagen is cut into shorter peptides when using trypsin). Shorter peptides are associated with lower specificity, and specific peptides must be of an appropriate length. Thus, to identify species-specific peptides, trypsin is typically employed to cleave proteins. Trypsin-digested peptides can be used for authentication of highly treated products such as DHG and pork cuts (Liu et al., 2019; Nalazek-Rudnicka, Klosowska-Chomiczewska, Wasik, & Macierzanka, 2019).

However, in order to generate peptides of appropriate length that display good specificity and MS detectability, Lys-C might be more suitable for cleaving highly conserved collagens into specific peptides. Treatment with Lys-C generated peptide pair Pep-1 HGNRGEPGPVGSVGPVGAVGPRGPSGPQGVRGDK/HGHRGEPGPVGS VGPVGAVGPRGPSGPQGVRGDK and Pep-2 GPTGEPGK/GPSGEPGK containing sites 499 and 975 different amino acid residues, respectively. GPTGEPGK and HGNRGEPGPVGSVGPVGAVG PRGPSGPQGVRGDK were identified as potential species-specific peptides for distinguishing donkey and horse, and MS/MS spectra and sequences of Pep-1 and Pep-2 were shown in Fig. 1A and 1B. Peptide GPTGEPGKPGDK containing sequence of GPTGEPGK has been reported by Shi et al. for identifying ACC (Shi et al., 2017), however, theoretically, GPTGEPGKPGDK can be further cleaved to GPTGEPGK under trypsin or Lys-C digestion. Thus, GPTGEPGK might be more stable under long time digestion treatment compared to GPTGEPGKPGDK.

Furthermore, we noticed that Pep-1 HGNRGEPGPVGSVGPVGAVGPRGPSGPQGVRGDK and its corresponding homologous Pep-1 showed good specificity by Basic Local Alignment Search Tool (BLAST) search against the NCBI non-redundant protein sequences database, suggesting that Pep-1 and its homologous peptides may serve as species-specific peptide biomarkers. Alignment of partial COL1A2 sequences from donkey, horse, deer, cattle, sheep, and pig were displayed in Fig. 1C and 1D. The MEGA results showed that Pep-1 and Pep-2 included some unique amino acids in different species. However, Pep-1 exhibited better specificity than Pep-2.

3.3. Peptide biomarkers verification

MS/MS spectra for each sample were searched against an in-house peptide database to verify if peptides GPTGEPGK and HGNRGEPGPVGSVGPVGAVGPRGPSGPQGVRGDK were species-specific. The results showed that Pep-1 and its homologous peptides could be exclusively detected in each corresponding animal sample (Table S3). Interestingly, in the MHG sample, both ACC- and HHG-specific Pep-1 were identified, which suggests that two collagen species might exist simultaneously.

Furthermore, to demonstrate the ability of Pep-1 to distinguish adulterated ACC products from horse and other animal species, ACC mixed with HHG at ratios of 1%, 20% and 50% were analyzed, and ACC, HHG, CHG, DHG, MHG and PHG were mixed (1:1:1:1:1:1 weight ratio) and analyzed. Using full-scan MS mode combined with in-house peptide database searching, all Pep-1 homologous peptides could be detected in each corresponding mixed sample. HHG could be easily detected even HHG added into ACC with an amount of 1%.

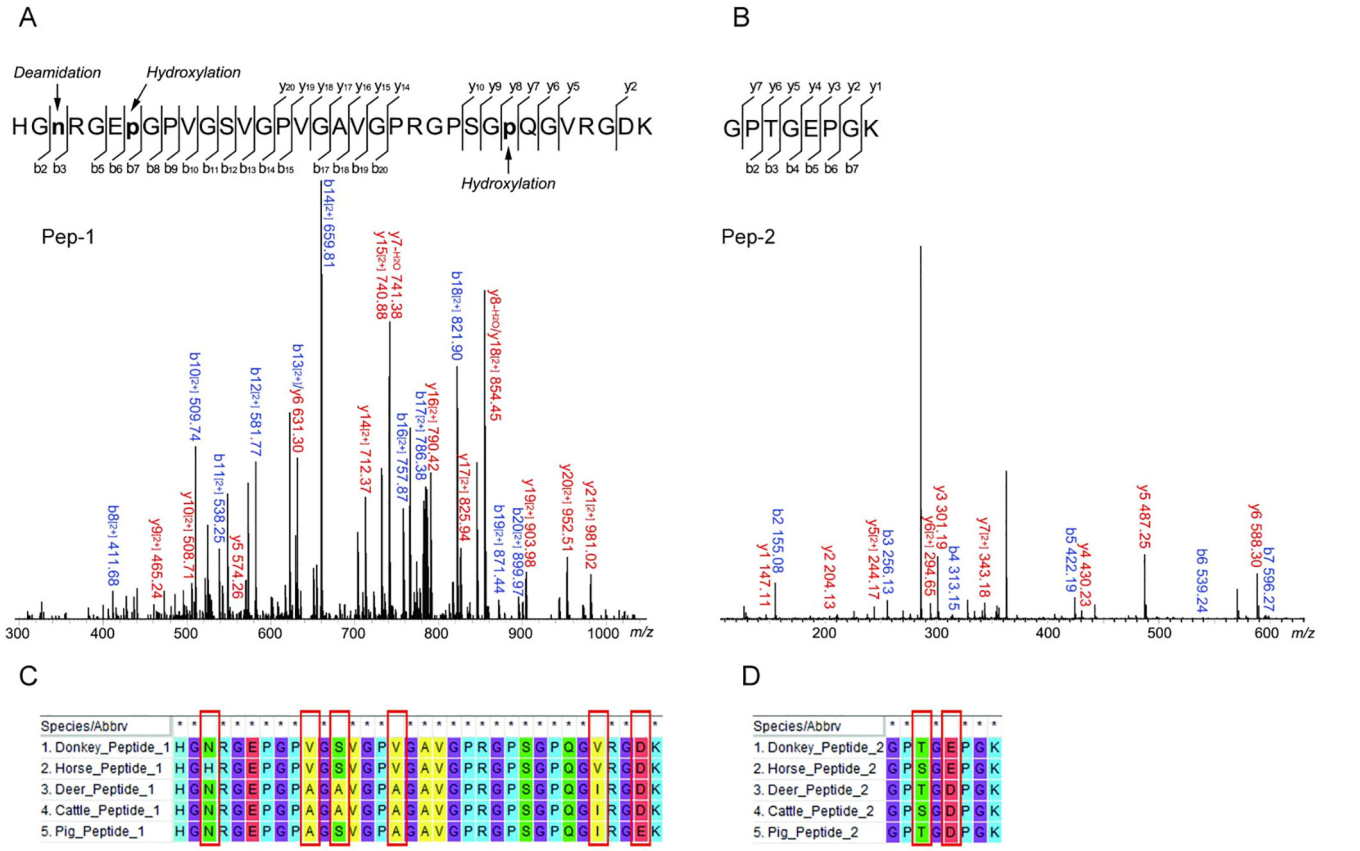

Fig. 1. MS/MS spectra of ACC-specific Pep-1 and Pep-2 (A, B). Sequence alignment of homologous peptides from different gelatin samples (C, D). The red box showed the different amino acid residues in different homologous peptides.

Pep-1 showed good species specificity, and ACC Pep-1 optimized transitions m/z 645.530 (5+) → m/z 659.810 (2+) were chosen for distinguishing ACC from other gelatin samples. The other optimized transitions of other gelatin samples were shown in Table 1, and MRM chromatograms of gelatin samples were shown in Fig. 2. Specific peptides of Pep-1 homologs could be detected exclusively in each corresponding gelatin sample, and chromatographic peak area values in MRM mode are shown in Table S4.

In addition, there were only four Pep-1 homologous peptides identified in the sample containing gelatin samples from six different species because DHG and CHG share the same Pep-1, and MHG includes both ACC- and HHG-specific Pep-1. Although the coexistence of ACC- and HHG-specific peptides in MHG makes it hard to distinguish MHG from other gelatins, it is obvious that ACC was adulterated with MHG or HHG if HHG-specific Pep-1 is detected. Pep-1 and its homologous peptides show good specificity due to the amino acid sequence length, and also show good stability under long time enzymatic digestion treatment. Therefore, it is feasible to use Pep-1 and its homologous peptides as biomarkers for distinguishing adulterated HHG in ACC products, even for contamination from closely related species. In addition to Lys-C, we believe that trypsin and Glu-C can also be used as proteases to obtain specific peptides, peptides GPTGEPGKGDK and GASGPAGVR cleaved by trypsin have been reported as ACC specific peptide. Furthermore, ACC specific peptide from COL1A2 under Glu-C cleavage can also be obtained, such as RGYPGNAGPVGAVGAPGPHGPV GPTGKHGNRGE, and this hypothesis needs to be further verified.

3.4. Hydroxylation and deamidation of peptide biomarkers

Repeating Gly-Xaa-Yaa triplets are basic motif of collagen, and Pro hydroxylation is one of the most abundant post-translational modifications (PTMs) (Ma et al., 2018; Pawelec, Best, & Cameron, 2016). It has been reported that up to 40% of Pro residues in collagen may be hydroxyproline. While deamidation may also occur during the production of gelatin (Silva, Kirkpatrick, Brodsky, & Ramshaw, 2005). In total, 24 peptides derived from ACC-specific Pep-1 were searched against the in-house database for possible hydroxylation and deamidation sites (Table S2). As shown in Table S2, taking ACC-specific Pep-1 as an example, five peptides with different PTMs were detected from one sample; there was a single deamidation on Asn in each Pep-1, while hydroxylation occurred at various positions in each Pep-1. The theoretical ACC-specific Pep-1 HGNRGEPGPVGSVGPVGAVGPRGPSGPQGVRGDK was selected, but all five peptides listed in Table S2 with various PTMs could also be selected as ACC-specific peptides for distinguishing adulterated ACC. In the present study, ACC-specific Pep-1 HGN(+0.98)RGEP(+15.99)GPVGSVGPVGAVGPRGP(+15.99)SGPQG VRGDK with one deamidation and two hydroxylation was selected due to its high intensity. Our previous results showed that the number of deamidation and hydroxylation modification increased significantly during the heating and high-pressure processing (Liu et al., 2020). Therefore, it is rational to analyze potential PTMs in these species-specific peptides, and modified peptides could be utilized as species-specific peptide biomarkers.

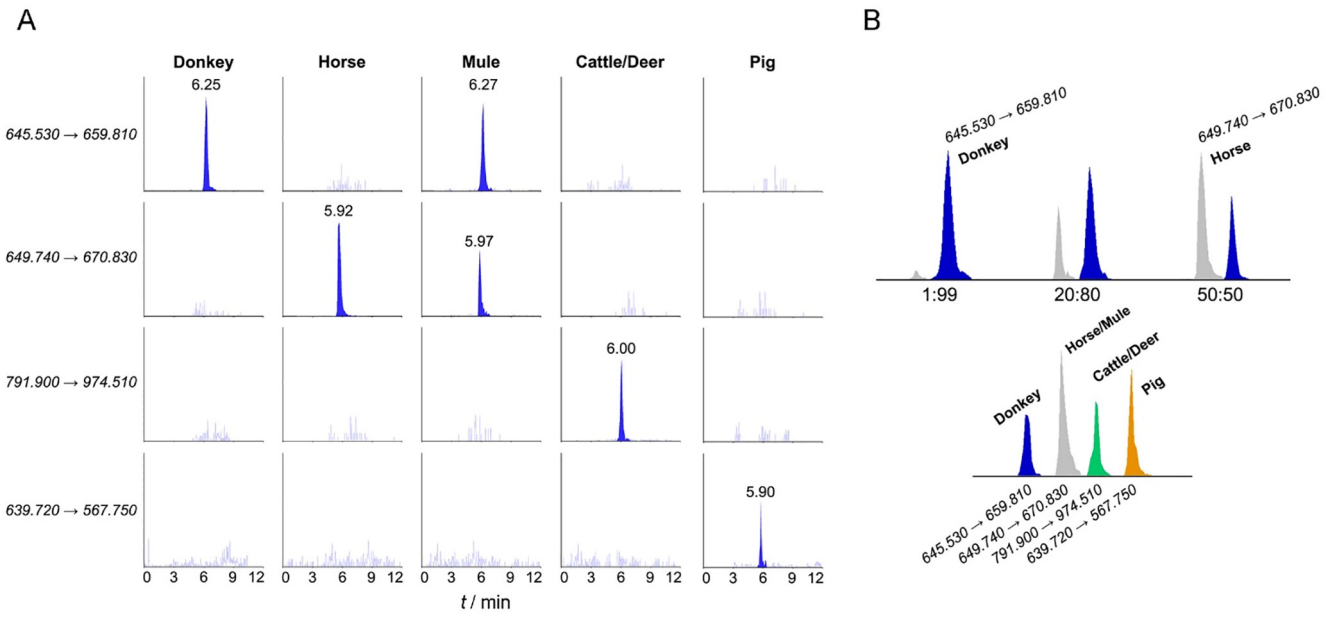

Fig. 2. (A) Chromatograms of different gelatin samples in MRM mode. Transitions are m/z 645.530 → m/z 659.810 for ACC-specific Pep-1, m/z 649.740 → m/z 670.830 for HHG-specific Pep-1, m/z 791.900 → m/z 974.510 for CHG/DHG-specific Pep-1, and m/z 639.720 → m/z 567.750 for PHG-specific Pep-1. (B) MRM chromatograms of animal-hide gelatin mixtures. Peak areas of transitions m/z 645.530 → m/z 659.810 and m/z 649.740 → m/z 670.830 in ACC/HHG mixtures with different ratios, and peak areas of transitions in ACC/HHG/MHG/CHG/DHG/PHG mixtures are shown.

4. Conclusions

In summary, we discovered collagen-derived species-specific peptides that can be employed for distinguishing ACC from products derived from closely related species. In the present study, two ACC-specific peptide biomarkers digested from COL1A2 by Lys-C were identified. It is feasible to distinguish ACC from other gelatin products and adulterations, especially HHG and MHG products by using ACC-specific peptide. The developed method appears to be effective and competitive for ACC authentication and traceability, and could help to improve the quality standards of ACC and ACC-containing products.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2020.12.006.

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