

Comparative Proteomic Analysis of Three Gelatinous Chinese Medicines and Their Authentications by Tryptic-digested Peptides Profiling using Matrix-assisted Laser Desorption/Ionization-time of Flight/Time of Flight Mass Spectrometry

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ABSTRACT

Background: Gelatinous Chinese medicines (GCMs) including Asini Corii Colla, Testudinis Carapacis ET Plastris Colla, and Cervi Cornus Colla, were made from reptile shell or mammalian skin or deer horn, and consumed as a popular tonic, as well as hemopoietic and hemostatic agents. Misuse of them would not exert their functions, and fake or adulterate products have caused drug market disorder and affected food and drug safety. GCMs are rich in denatured proteins, but insufficient in available DNA fragments, hence commonly used cytochrome c oxidase I barcoding was not successful for their authentication. **Objective:** In this study, we performed comparative proteomic analysis of them and their animal origins to identify the composition of intrinsic proteins for the first time. **Materials and Methods:** A reliable and convenient approach was proposed for their authentication, by the incorporation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two-dimensional electrophoresis, and matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF-MS).

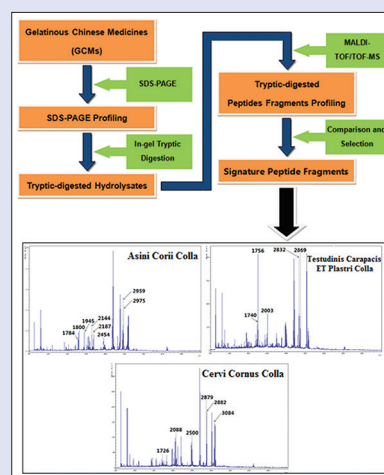
Results: A total of 26 proteins were identified from medicinal parts of original animals, and GCMs proteins presented in a dispersive manner in electrophoresis analyses due to complicated changes in the structure of original proteins caused by long-term decoction and the addition of ingredients during their manufacturing. In addition, by comparison of MALDI-TOF/TOF-MS profiling, 19 signature peptide fragments originated from the protein of GCM products were selected according to criteria.

Conclusion: These could assist in the discrimination and identification of adulterates of GCMs and other ACMs for their form of raw medicinal material, the pulverized, and even the complex.

Key words: Authentication, gelatinous Chinese medicines, matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry, signature peptide fragments

SUMMARY

- Comparative proteomic analysis of three gelatinous Chinese medicines was conducted, and their authentications were based on tryptic-digested peptides profiling using matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry.



Abbreviations used: GCMs: Gelatinous Chinese medicines, COI: Cytochrome c oxidase I, SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis, 2-DE: Two-dimensional electrophoresis, MALDI-TOF/TOF-MS: Matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry, LC: Liquid chromatography, ChP: Chinese Pharmacopoeia, HPLC: High performance liquid chromatography, LC-ESI⁺-MS: Liquid chromatography-electro spray ionizing-mass spectrometry, IEF: isoelectric focusing, HCCA: α -Cyano-4-hydroxycinnamic acid.

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INTRODUCTION

Gelatinous Chinese medicine (GCM) was made from reptile shell or mammalian skin or deer horn, and consumed as a popular tonic or nutritional supplement for sickly or weak people, such as postpartum women, cancer patients undergoing radiotherapy or chemotherapy, the elderly, postoperative patients,^[1] and also often used as hemopoietic agents.^[2] There are three frequently-used GCMs including Asini Corii Colla (ACC), Testudinis Carapacis ET Plastris Colla (TCPC), and Cervi Cornus Colla (CCC) listed in the Chinese Pharmacopoeia. Moreover, the application of GCM has already been diversified since pre-Qin

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Dynasty (BC2000~BC221) due to the difference in their functions.^[3] Misuse of them would not exert their functions, and fake or adulterate products have exposed consumers to a high risk and hence, it is an important task to construct an appropriate approach for their quality assessment.

GCM is much different from herbs containing abundant secondary metabolites such as flavonoids, steroids that can be conveniently profiled by liquid chromatography (LC)-based technologies.^[4-6] It is rich in denatured proteins but insufficient in available DNA fragment due to long-term decoction and addition of extra ingredients in the course of manufacturing. Moreover, their quality control scheme is based on an amino acid determination by high-performance LC and identification of a certain oligopeptide by LC-electro spray ionization-mass spectrometry. However, the amino acids are of no specificity, and assay of the oligopeptide after enzymatic hydrolysis is also not reliable because the artificial addition of short synthesized peptide into GCMs is quite readily nowadays. Moreover, it is difficult to compare intrinsic proteins for their discriminations, so few investigations were reported due to the lack of specifically designed strategies.^[7]

In this paper, our attempts were made to compare these GCMs in terms of their proteins for the first time. Moreover, a novel approach was proposed for their authentication [Figure 1], where some of powerful technologies employed in proteomics research, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis (2-DE), matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF-MS),^[8-10] were incorporated. In particular, the changes in proteins (10–250 kDa) of GCM products after manufacturing from medicinal parts of original animals were profiled by SDS-PAGE and 2-DE. Moreover, the enzymatic-digested peptides from proteins existing in the medicinal parts as well as GCMs were analyzed using MALDI-TOF/TOF-MS and MASCOT search. Finally, signature peptide fragments were selected, which would assist in their discrimination and identification of their adulterate of raw medicinal material, the pulverized and even the complex.

MATERIALS AND METHODS

Materials

Three GCM references including ACC (B/N: 121274-200301, code: E1; B/N: 121274-201202, code: E2), TCPC (B/N: 121693-201301, code: G) and CCC (B/N: 121694-201301, code: L) were all purchased from the National Institutes for Food and Drug Control, China.

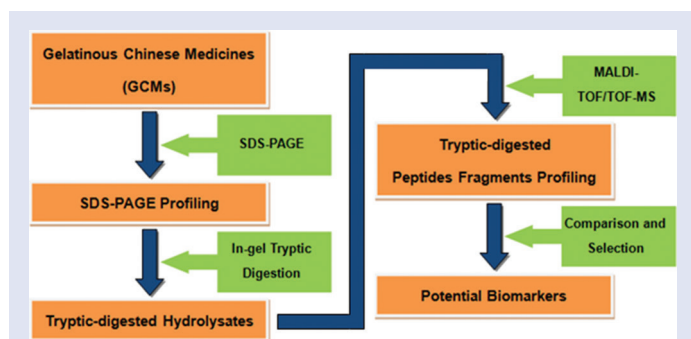


Figure 1: Proposed strategy for the authentication of three gelatinous Chinese medicine products

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional electrophoresis analysis of gelatinous Chinese medicines and their animal origins

Preparation of sample solutions

About 20 mg of GCM powder was dissolved in 200 μ L of rehydration solution. And, 40 mg of their animal origins were precisely weighed and macerated into 200 μ L of rehydration solution, cell lysis buffer or H₂O, respectively. Then, protein in GCMs and their animal origins were extracted at 4°C for 4 h, followed by centrifugation at 12,000 rpm for 30 min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis

The sample solution (20 μ L) and protein ladder (5 μ L) were separated by 12% resolving gel, which was run under 80 V for 30 min and then 110 V for another 1 h.

Two-dimensional electrophoresis analysis

The protein supernatant (1.5 mg/mL) previously extracted by rehydration solution was analyzed. In the analysis, the separation was performed by isoelectric focusing with an 11 cm immobilized pH gradient strip (pH 3-10, linear), followed by protein transfer to a 12% SDS-PAGE gel.

Matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry analysis and identification of proteins

In-gel digestion of proteins

The digestion steps were made with slight modifications according to the Zhao's method.^[6] Each coomassie-stained gel area and spot of interest was manually cut into 1 mm \times 1 mm pieces and then subjected to in-gel tryptic digestion. The tryptic-digested peptides mixture was extracted from the gel pieces with 20 μ L of 5%TFA in 50% ACN. The pooled extracts were evaporated to *ca.* 10 μ L. 1 μ L of concentrated solution mixed with 1 μ L of 5 mg/mL α -Cyano-4-hydroxycinnamic acid matrix was subject to subsequent MS analysis.

Matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry analysis

MALDI-TOF/TOF-MS analysis was performed on an Ultraflex Extreme mass spectrometer. Raw spectra were recorded in the positive linear mode (shots, 500; laser frequency, 1000 Hz; ion source 1 voltage, 25.01 kV; ion source 2 voltage, 22.51 kV; lens voltage, 7.29 kV; pulsed ion extraction time, 150 ns; matrix suppression, deflection; suppress up to, 600 Da; mass range, 500–5000 Da).

Identification of proteins

The resulting files from MALDI-TOF/TOF-MS was subjected to the MASCOT search engine based on NCBI nr and SWISS-PROT database with the following parameter settings: trypsin-cleavage one missing cleavage site allowed, cysteine (carbamidomethyl) set as static modification, methionine (oxidation) was allowed as dynamic modification, and mass tolerances of precursor was 0.2 Da.

Selection of signature peptides for gelatinous Chinese medicines' authentication

After MALDI-TOF/TOF-MS profiling of tryptic-digested hydrolysates of GCM samples, their peptide fragments were compared in view of their *m/z* value to find out the difference. Moreover, signature peptides

for GCMs' authentication purpose were then selected according to the following criteria:

- The detection of m/z of the peptide fragments should be repeatable
- The m/z of the peptide fragments should be different among three GCMs
- The peptide fragments should be the largest peak among isotope peaks cluster in MALDI-TOF/TOF-MS profile
- The signal of the peptide fragments should be from the mono-charged precursor
- m/z value of the peptide fragments was set at above 1500 for a high specificity.

RESULTS AND DISCUSSION

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional electrophoresis profiles

Before the electrophoresis analysis of three GCMs, initial protein expression patterns of their corresponding animal origins were investigated for comparison purpose. It was shown that visual protein bands in SDS-PAGE profiles were plentiful and their molecular weights were ranged from 10 kDa to above 250 kDa, demonstrating that donkey skin, deer antler, and turtle shell have abundant and various proteins. And, extracting solvent should be carefully considered the profiles of proteins extracted by rehydration solution, cell lysis buffer or H_2O , differed to certain extent [Figure 2]. In addition, a protein band at ca. 60 kDa, another band at ca. 37 kDa, and two bands at ca. 24 kDa and 55 kDa were identical to the profile of donkey skin, turtle shell, and deer antler, respectively. These intrinsic differences in their proteins have been disclosed, which allowed those medicinal species to be differentiated by SDS-PAGE profiles quickly. Subsequently, protein sample solution extracted by a proper extracting solvent was analyzed to achieve individual profile of the protein spots by more effective 2-DE technology. As shown in Figure 3, protein spots of three animal origins were mainly distributed in five areas including I-III (donkey skin), IV (turtle shell), and V (deer antler). It can be also seen that proteins in III and V areas were largely expressed and have not been well separated by 2-DE analysis of high resolution. In addition, the positions of protein spots in 2-DE profiles matched with that of protein bands in their corresponding SDS-PAGE profiles, further suggesting the high reliability of these electrophoresis analyses. However, protein bands in the SDS-PAGE profiles of original animal's medicinal parts were not observed in any of three GCMs' profiles; although, the loaded protein has been much reduced. Instead, E1 and E2 lanes were wholly stained in a smear manner all the way down, whereas proteins in G and L lanes were mainly presented in the area below 50 kDa. These were probably due to the high temperature in the course of decoction of animal's medicinal parts for a long time, and the intermolecular hydrogen bonds maintaining the stability of the collagen structure were greatly destroyed. Consequently, some collagen molecules were randomly hydrolyzed into numbers of polypeptide chains of various relative molecular weights. The regions of diffusion distribution in GCMs also differed, implying that those original proteins of high molecular weight in deer antler and turtle shell were unstable to heating and easily degraded into proteins of small molecule weight. In addition, deer antler and turtle shell contained a lower protein content of high and medium molecular weight according to their SDS-PAGE profiles. The major proteins in GCMs were widely distributed between pI 3 and 7 and the rest of proteins was scattered in the area <25 kDa between pI 8 and 10, presenting dispersion state as well. There were obvious differences in 2-DE profiles among GCMs, and the molecular weight of ACC protein was ranged from 15 kDa to 250 kDa or above, whereas those of TCPC and CCC were both below 50 kDa.

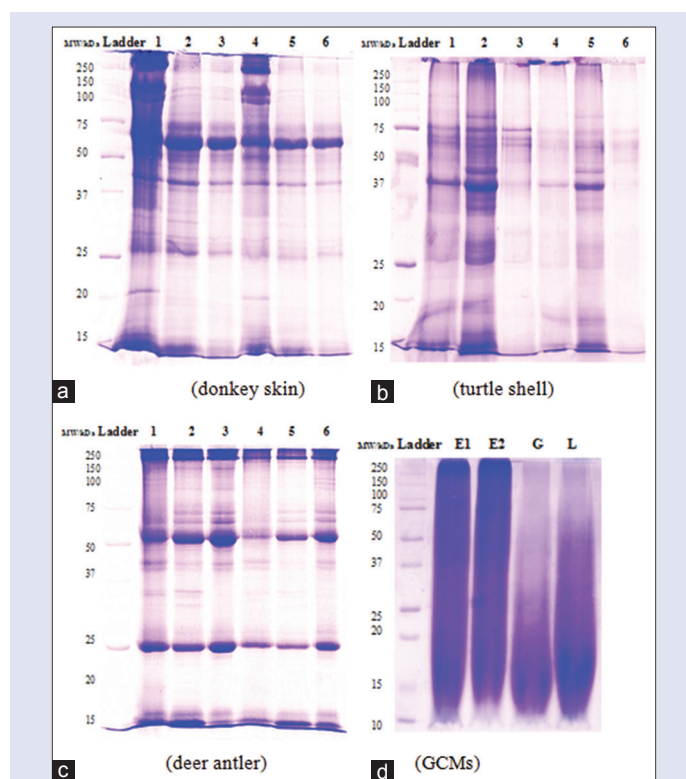


Figure 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of animal origins and gelatinous Chinese medicines (a) donkey skin, (b) turtle shell, (c) deer antler, (d) GCMs. (1: Protein extracted by rehydration solution; 2: Protein extracted by cell lysis buffer; 3: Protein extracted by ultrapure water; 4-6: 2 times dilution of 1~3 solutions)

A comparison of proteome patterns of GCMs was also made to that of their original animal parts. During the GCMs' production, long-term decoction was involved for the extraction from an animal's medicinal part, and in subsequent refinement and concentration, they were dealt with the addition of rice wine, soya bean oil, and rock sugar as ingredients. These have led to complicated changes in the structure of original proteins. For instance, this result could be caused by Maillard reaction, an important chemical reaction between the nucleophilic amino group of amino acids in the protein and the reactive carbonyl group of reducing sugars. Therefore, GCMs were much more complex than respective medicinal parts, and even 2-DE of the high resolution did not manage to effectively analyze specific proteins of these tonics.

Identification of proteins

Identified proteins from medicinal parts of original animals were summarized in Table 1. Among 26 proteins, 12 unique proteins belonged to 9 protein spots of donkey skin after 2-DE analysis, 6 unique proteins were identified from 4 protein spots of tortoise shell after 2-DE profiling, and other 8 unique proteins were from 4 protein bands after SDS-PAGE analysis of three medicinal parts. Many of them were close to the species of our concern and interest.

Matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry profiling and signature peptide fragments for gelatinous Chinese medicines' authentication

The areas of 50–75 kDa and nearly 25 kDa from SDS-PAGE profile of three GCMs were selected since there were relatively more proteins in these

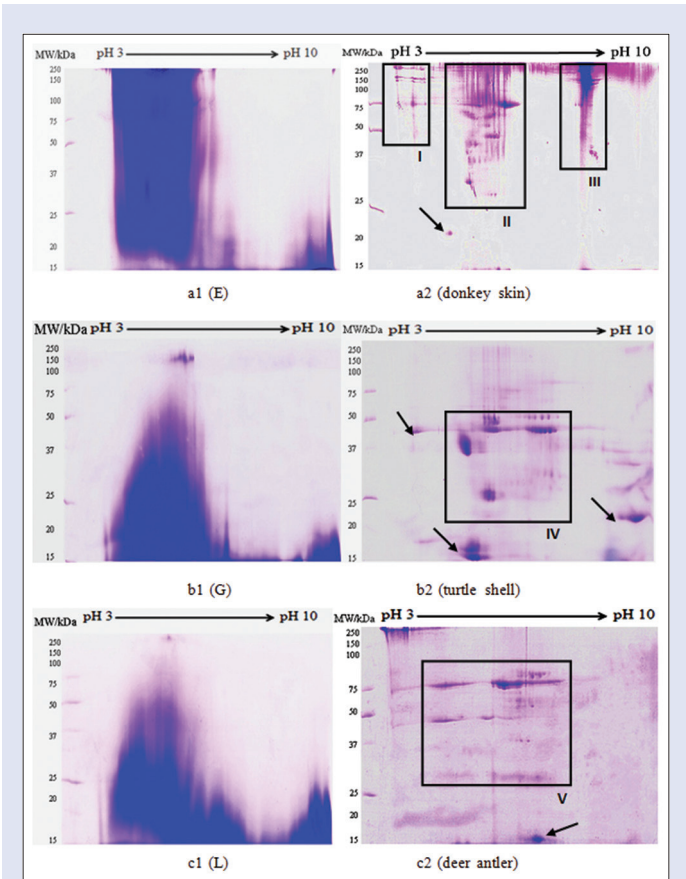


Figure 3: Two-dimensional electrophoresis profiles of three gelatinous Chinese medicines and their animal origins

areas in terms of SDS-PAGE profile of their original medicinal parts. To find out signature peptide fragments, *m/z* values of enzymatic-digested peptides originated from each GCM in their MALDI-TOF/TOF-MS profiles [Figure 4] were compared and selected according to the criteria. And as summarized in Table 2, the peptide fragments could be used as their signature peptides to discriminate three GCM products and identify their adulterates.

CONCLUSION

In this study, we compared three GCMs including ACC, TCPC and CCC in terms of their proteins. The changes in proteins after manufacturing from original animal parts to GCM products were profiled using SDS-PAGE and 2-DE. Then, the peptides originated from the proteins were analyzed using MALDI-TOF/TOF-MS. It was found that original animal parts have abundant and various proteins and the intrinsic differences were shown in their proteins, allowing these medicinal animal species to be differentiated by their electrophoresis profiles readily. In both SDS-PAGE and 2-DE, GCMs proteins presented in a dispersive manner, these could be caused by complicated changes in the structure of original proteins after long-term decoction and the addition of ingredients during their manufacturing. Moreover, 19 selected signature peptide fragments from the protein of GCM products could be employed for the authentication of their form of raw medicinal material, the pulverized and even the complex. And, the proposed strategy would be a promising tool for the discrimination and identification of adulterates of GCMs, and we would analyze commercial GCM products collected from the market in further work. It is also worth to conducting more investigation for the authentication of other animal-derived Chinese medicines by use of this proposed strategy.

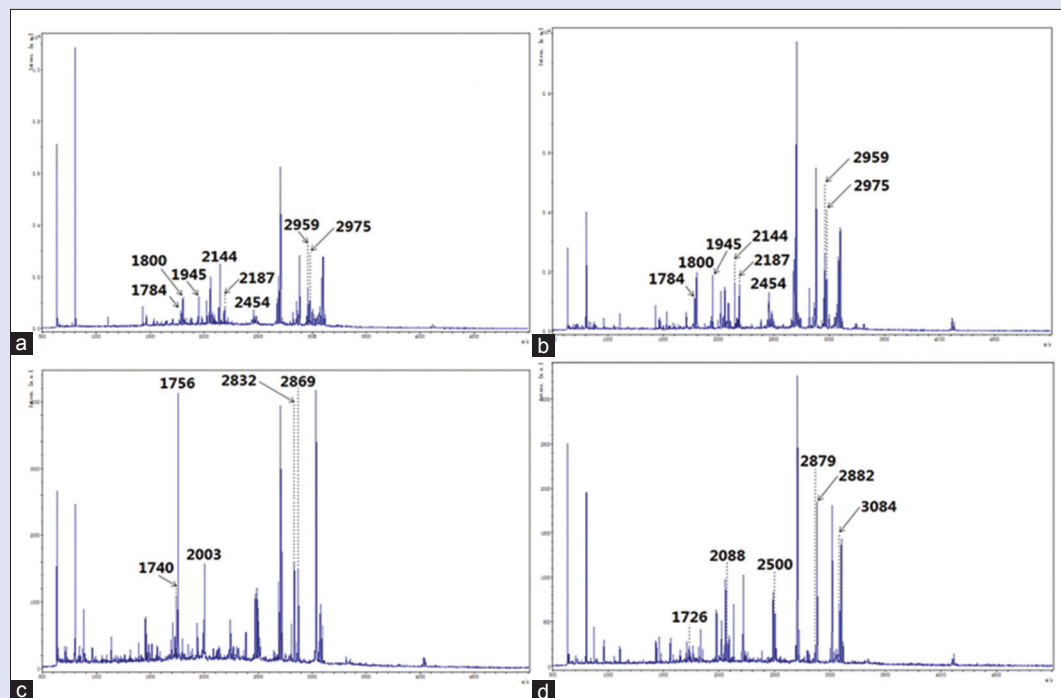


Figure 4: Matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry profiles of gelatinous Chinese medicines after sodium dodecyl sulfate polyacrylamide gel electrophoresis (a: E1; b: E2; c: G; d: L)

Table 1: Identified proteins from animal origins

n	Accessions	Protein name	Taxonomy	Scores
1	gi 126352407	Fatty acid-binding protein, intestinal	<i>E. caballus</i>	64
2	gi 958672048	Protein MON2 homolog	<i>E. asinus</i>	78
3	gi 545177509	cAMP-dependent protein kinase inhibitor beta isoform X1	<i>E. caballus</i>	75
4	gi 150456439	Fin bud initiation factor homolog	<i>E. caballus</i>	72
5	gi 958785268	Basic salivary proline-rich protein 1-like	<i>E. asinus</i>	82
6	gi 190701011	Uterine serpin, partial	<i>E. caballus</i>	68
7	gi 664779357	Nonreceptor tyrosine-protein kinase TYK2-like	<i>E. przewalskii</i>	55
8	gi 664752763	NACHT, LRR and PYD domains-contain g protein 1 isoform X2	<i>E. przewalskii</i>	61
9	gi 124271129	Hexokinase-2	<i>E. grevyi</i>	84
10	gi 124271127	Hexokinase-2	<i>E. zebra</i>	63
11	gi 126352418	Hexokinase-2	<i>E. caballus</i>	75
12	gi 664741951	ADAMTS-like protein 1	<i>E. przewalskii</i>	60
13	gi 946645187	Uncharacterized protein LOC106731913 isoform X1	<i>P. sinensis</i>	52
14	gi 946645191	Uncharacterized protein LOC106731913 isoform X2	<i>P. sinensis</i>	61
15	gi 946645194	Uncharacterized protein LOC106731913 isoform X3	<i>P. sinensis</i>	57
16	gi 946677156	Myosin light polypeptide 6	<i>P. sinensis</i>	43
17	gi 530574731	Coiled-coil domain-containing protein 23	<i>C. picta bellii</i>	67
18	gi 465971403	Small nuclear ribonucleoprotein F, partial	<i>C. mydas</i>	46
19	gi 189176141	Interferon alpha-1	<i>E. caballus</i>	66
20	gi 1567962	Interferon alpha-2	<i>E. caballus</i>	73
21	gi 1567972	Interferon alpha-3	<i>E. caballus</i>	51
22	gi 56237697	Testis-specific histone, H1t	<i>E. caballus</i>	58
23	gi 953873276	Ubiquitin-like protein ATG12 isoform X2	<i>E. caballus</i>	74
24	gi 2506853	Metallothionein-1A	<i>E. caballus</i>	67
25	gi 896269	Unknown protein, partial (mitochondrion)	<i>E. burchellii quagga</i>	45
26	gi 545682	Thyroxine (T4)-binding protein=Vitamin D-binding protein homolog	<i>Testudines</i>	62

E. caballus: *Equus caballus*; *E. asinus*: *Equus asinus*; *E. przewalskii*: *Equus przewalskii*; *E. grevyi*: *Equus grevyi*; *E. zebra*: *Equus zebra*; *P. sinensis*: *Pelodiscus sinensis*; *C. picta bellii*: *Chrysemys picta bellii*; *C. mydas*: *Chelonia mydas*; *E. burchellii quagga*: *Equus burchellii quagga*

Table 2: Signature peptide fragments from three gelatinous Chinese medicine products

Asini Corii Colla (m/z)	Testudinis Carapacis ET Plastris Colla (m/z)	Cervi Cornus Colla (m/z)
1784	1740	1726
1800	1756	2088
1945	2003	2500
2144	2832	2879
2187	2869	2882
2454		3084
2959		
2975		

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Conflicts of interest

There are no conflicts of interest.

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