

Targeted Proteomics Approach for Sensitive Detection of Bovine and Porcine Gelatins in Food, Pharmaceutical Capsules and Personal Care Products

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Introduction

Gelatin has been extensively utilized in food, cosmetic and pharmaceutical industries and is often manufactured from bovine and porcine materials. The use of animal-based gelatin is restricted if it is incompatible with several religious custom and personal dietary preferences which require the gelatin to be free from porcine, bovine or any animal by-products [1-3]. Accurate labelling is requisite for consumers' confidence towards the products. However, high similarity in amino acid sequences between bovine and porcine gelatins is a main challenge for differentiation of the animal sources [4]. A targeted proteomic analysis employing sensitive LC/MS/MS platform was developed to pinpoint species-specific peptide markers for gelatin speciation.

Experimental

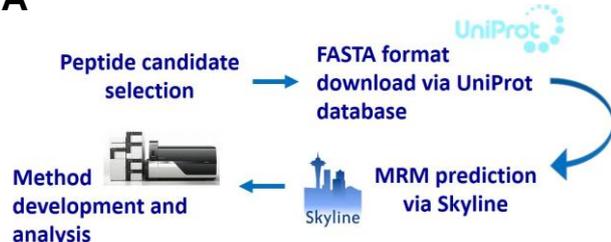
Analytical conditions and sample preparation

A 5 mg of sample was dissolved in 600 μ L extraction buffer (water containing NH_4HCO_3 , pH 8), vortexed and spin centrifuged. Extract was incubated at 37°C with slow shaking (150 rpm) for 15 min. Trypsin enzyme (in NH_4HCO_3 , pH 8) was added to the extract to obtain enzyme and sample ratio of 1:100. The mixture was then incubated for overnight at 37°C, 150 rpm. After incubation, the mixture was vortexed and centrifuged (5 min, 12,000 rpm) to collect supernatant from the pellets. This step needs to be repeated for sample with more pellets. At last, supernatant was injected to LC/MS.

Table 1. LC and MS acquisition parameters

Column	: Aeris peptide XB-C18 100Å (150 x 2.1 mm, 1.7 μ m)
Mobile phase	: A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Flow rate of mobile phase	: 0.3 mL/min
Column temp.	: 40 °C
Elution mode	: Gradient elution, 5%B (0 – 2 min) \rightarrow 25%B (15 min) \rightarrow 50%B (15.21 – 16 min) \rightarrow 5% (16.01 – 19 min)
Interface & temp.	: ESI, 300°C
MS mode	: Positive, MRM
Block temp.	: 400°C
DL temp.	: 250°C
Nebulizing gas flow	: N_2 , 3 L/min
Drying gas flow	: N_2 , 10 L/min
Heating gas flow	: zero air, 10 L/min

A



B

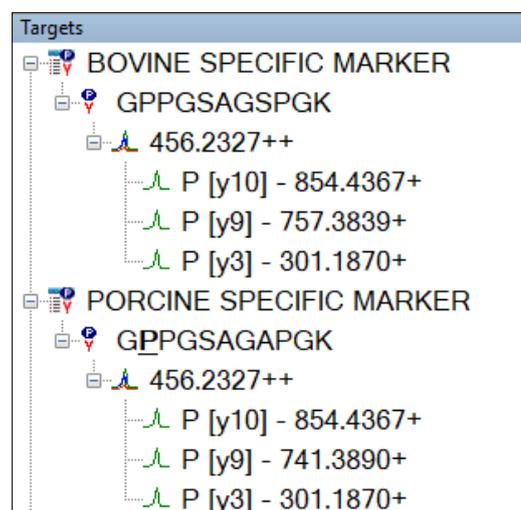


Figure 1. (A) Schematic procedure of MRM method development, (B) In silico prediction of MRM transitions for bovine and porcine peptides by Skyline. The mass-to charge ratio (m/z) of precursor and product ions (y_3 and y_{10}) between two peptides are identical except for y_9 . Bold underlined proline (P) indicates proline hydroxylation.

Results and Discussion

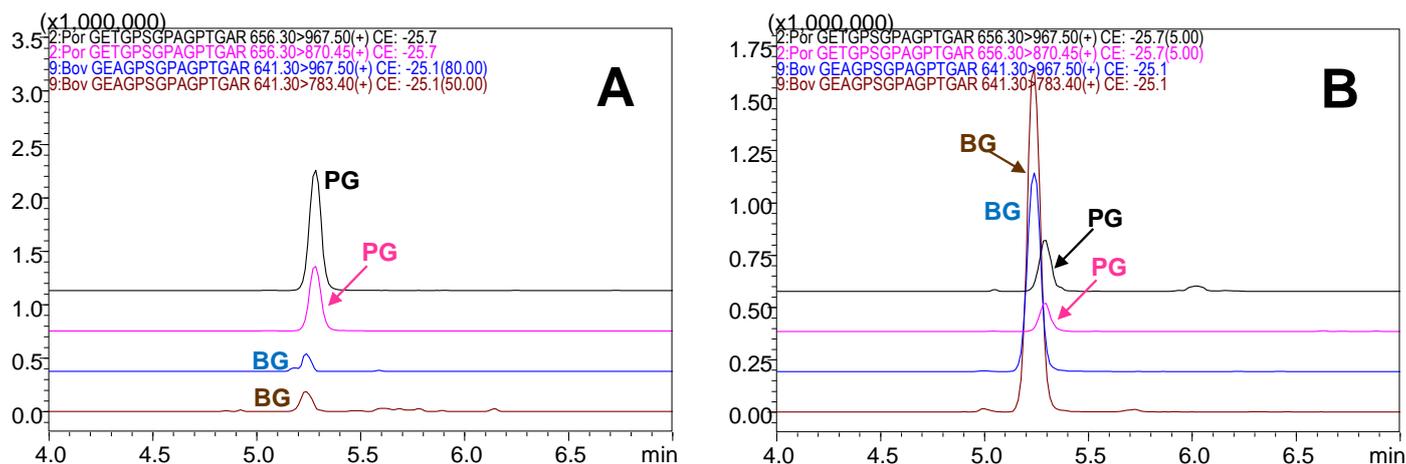


Figure 2. Individual MRM chromatograms show detection of bovine peptide (GEAGPSGPAGPTGAR) in commercial porcine gelatin reference (A) and porcine peptide (GETGPSGPAGPTGAR) in commercial bovine gelatin reference (B). Discrepancy in amino acid composition is shown in bold. BG: beef gelatin, PG: pork gelatin.

Development of MRM-based method for detection of gelatin peptides

Development of MRM method was carried out based on more than 40 pairs of unique peptides [1,5,6]. The process was expedited greatly with the UniProt database and Skyline program (Figure 1A). The later platform is the main tool to predict MRM transitions and collision energies (CE) of targeted peptides. Gelatin references prepared in-house was utilized to generate MRM chromatographic peaks. It was because the stability and purity of commercial gelatin references remained dubious as bovine and porcine-specific markers were detected at trace levels in corresponding references by LC/MS/MS (Figure 2).

It is worth noting that peptide sequences derived from both gelatins possess small discrepancy, often in only one amino acid [5]. Furthermore, the frequent and unpredicted occurrence of proline hydroxylation (+16 Da) to gelatin might cause bovine and porcine-specific peptides generating same masses and very similar MRM transitions. As shown in Figure 2B, bovine specific peptide (GPPGSAG**S**PGK) and porcine specific peptide (GPPGSAG**A**PGK) have a discrepancy in the 8th amino acid. Theoretical mass-to-charge ratio (m/z) of doubly charged precursor ion of the porcine peptide is 448.23. However, the m/z becomes 456.23 if hydroxylation occurs at one proline site, generating same precursor ion as bovine peptide and near-identical product ions (MS/MS spectra). Therefore, sequence verification based on MRM-based fragmentation in LC/MS/MS is necessary.

Table 2. Validation of bovine-specific peptide markers and MRM transitions on LCMS-8060

Protein	UniProt No.	Peptide marker	Precursor ion (m/z)	Number of MRM	RT (min)	Area RSD (%) (n=6)
Alpha-1 chain of type 1 bovine collagen	P02453	BG1	658.85++	4	9.0	12.2
		BG2	780.9++	5	9.9	4.4
		BG4	641.3++	5	5.3	1.1
		BG5	473.2++	4	1.0	5.5
Alpha-2 chain of type 1 bovine collagen	P02465	BG3	644.3++	3	7.9	11.2
		BG6	451.75++	3	5.1	2.4
		BG7	393.2++	4	2.3	2.1
		BG8	357.65++	3	0.9	14.9
		BG9	596.85++	5	9.2	2.5

Table 3. Validation of porcine-specific peptide markers and MRM transitions on LCMS-8060

Protein	UniProt No.	Peptide marker	Precursor ion	Number of MRM	RT (min)	Area RSD (%) (n=6)
Alpha-1 chain of type 1 porcine collagen	A0A1S7J210	PG2	486.25++	4	1.3	10.2
		PG5	656.3++	4	5.4	9.2
		PG6	773.9++	5	9.1	4.9
Alpha-2 chain of type 1 porcine collagen	A0A1S7J1Y9	PG1	1103.05++	3	8.5	7.4
		PG3	921.45++	5	9.5	5.9
		PG4	620.8++	5	10.4	6.8
		PG7	731.85++	5	8.0	9.5
		PG8	590.85++	5	7.9	9.4

Validation, semi-quantitation of peptide markers, and screening of gelatin content in commercial products

Following sequence verification, 9 and 8 peptides were found to be specific for bovine gelatin and porcine gelatin, respectively, which were not detected in the corresponding in-house gelatin references (Table 2-3 and Figure 3). The criteria for peak detection includes confirmation of prominent peak in minimum 3 MRM transitions with signal-to-noise ratio (S/N) > 3. A few peptides are hydroxylated at 2-3 proline sites. Sensitivity of each peptide marker was varied but remained feasible for detection considering low initial sample amount (5 mg) and injection volume (5 µL). All the peptide markers exhibited good repeatability (RSD<15%) across six consecutive injections (Table 2-3). The method is feasible to detect as low as 0.1% adulteration in spiked bovine and porcine gelatin matrices (Figure 4). A total of 19 commercial samples (food, pharmaceutical capsule, and personal care product) was utilized for screening of gelatin content. Bovine or porcine gelatin was detected in 9 commercial samples while 2 samples (pharma capsule II and III) contained both gelatins (Table 4 and Figure 5).

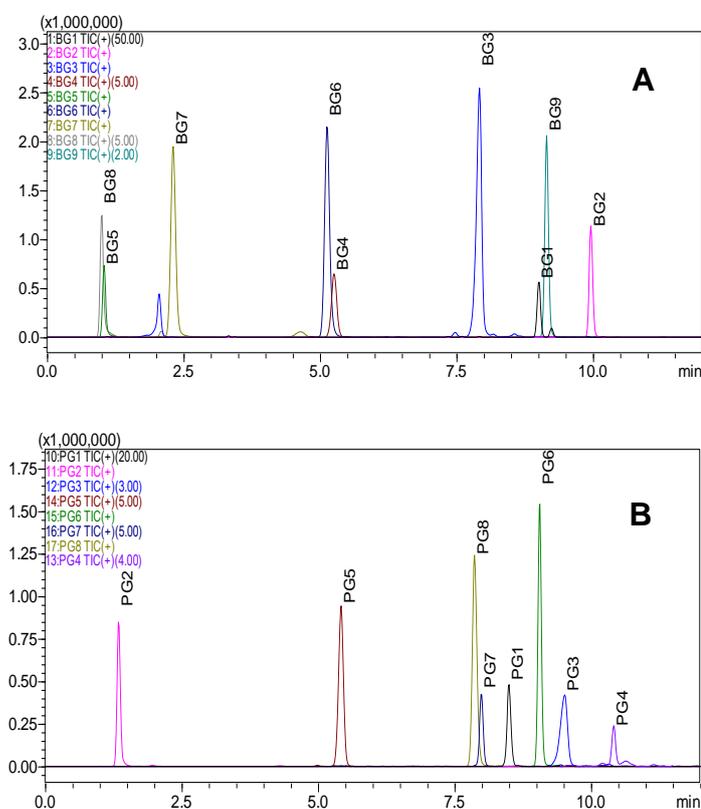


Figure 3. Total MRM chromatograms of nine bovine-specific peptides (A) and eight porcine-specific peptides (B) detected in trypsin-digested extract of in-house gelatin references.

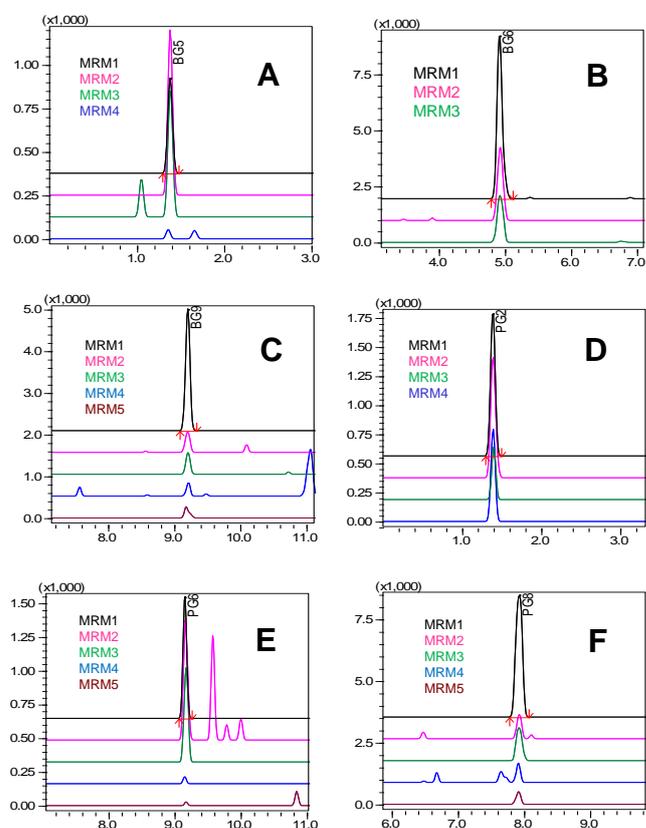


Figure 4. Individual MRM chromatograms of detected peptide markers at 0.1% adulteration. (A, B, C): Bovine gelatin marker BG5, BG6, BG9 detected in porcine gelatin matrix; and (D, E, F): Porcine gelatin markers PG2, PG6, PG8 detected in bovine gelatin matrix.

Table 4. Screening analysis of bovine and porcine gelatins in commercial products
(✓: detected, ND: not detected, *under LOD)

No	Sample (from local supermarket)	Gelatin declaration & source on label	Detection result	
			Bovine	Porcine
1	Gummy bear I	Yes, beef	✓	ND
2	Marshmallows I	Yes, pork	ND	✓
3	Chewing gum	Yes, undeclared	ND	✓
4	Marshmallows II	Yes, undeclared	✓	ND
5	Gummy bear II	Yes, undeclared	✓	ND
6	Vanilla cookie	Undeclared	ND	ND
7	Chocolate cookie	Undeclared	ND	ND
8	Chocolate bar I	Undeclared	ND	ND
9	Chocolate bar II	Undeclared	ND	ND
10	Chocolate bar III	Undeclared	ND	ND
11	Pharma capsule I	Yes, undeclared	✓	ND
12	Pharma capsule II	Yes, undeclared	✓	✓
13	Pharma capsule III	Yes, undeclared	✓	✓
14	Hand cream I	Hydrolysed collagen	ND	✓
15	Hand cream II	Hydrolysed collagen	ND	ND
16	Face cream I	Hydrolysed collagen	ND	✓
17	Face cream II	Hydrolysed collagen	ND	✓
18	Face cream III	Hydrolysed collagen	ND	ND*
19	Hair conditioner	Hydrolysed collagen	ND	ND*

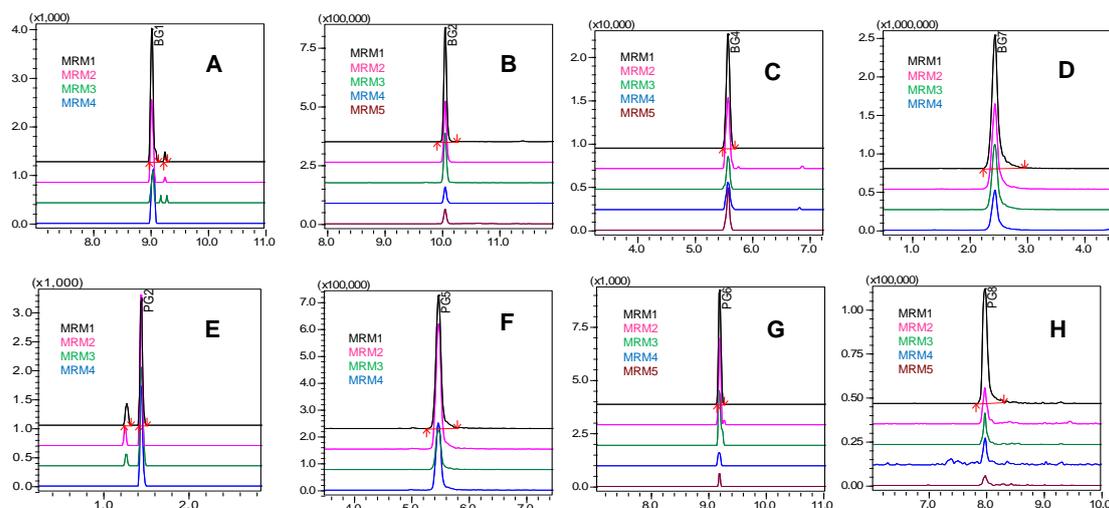


Figure 5. Individual MRM chromatograms of peptide markers in commercial products: (A) BG1 in gummy bear II, (B) BG2 in pharma capsule I, (C) BG4 in gummy bear I, (D) BG7 in pharma capsule II, (E) PG2 in face cream II, (F) PG5 in pharma capsule III, (G) PG6 in hand cream I, (H) PG8 in pharma capsule II.

❑ Conclusion

A MRM based method for screening of bovine and porcine gelatins was established on LCMS-8060. Featured with high sensitivity and a high number of specific peptide markers, this method offers a reliable approach for detection and speciation of animal-based gelatins (bovine and porcine) in commercial products as low as 0.1% adulteration. The method could give substantial support for food labelling approach with the respect of religious customs (Muslim, Jew, Hindu) like Halal testing.

❑ References

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