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Application News

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Halal Authentication Analysis / LCMS-8060

Detection and Differentiation of Bovine and Porcine Gelatins in Food and Pharmaceutical Products By LC/MS/MS Method

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❑ Introduction

Gelatin is a mixture of polypeptides derived from hydrolysis of collagens of animal origins and has been widely utilized in food, cosmetic, and pharmaceutical industries. Commercial gelatins are often manufactured from bovine and porcine materials (skin, bone and hide etc.) because of the practicability and cost-effectiveness. However, general approval of animal-based gelatin is restricted due to incongruity with several religious customs and individual dietary preference which require the gelatin to be free from porcine, bovine or any animals-by-products [1-2]. Furthermore, there is a concern of animal-based gelatin could be a media for zoonotic disease risks [2]. Accurate food authentication and labelling are requisite for consumers' assurance towards the products. As a part of application news series to support Halal authentication testing method based upon targeted proteomic approach [3], a practical method by using sensitive LC/MS/MS was developed to pinpoint species-specific peptide markers for most widely used gelatins, bovine and porcine, as well as to determine gelatin source in commercial food and pharmaceutical products. The specificity of more than 40 signature peptides as marker candidates was tested using in-house gelatin references due to the similarity in their sequences and properties [4]. The established LC/MS/MS method was proven to be a robust approach for source authentication of gelatin of processed food and pharmaceutical capsules.

❑ Experimental

Preparation of gelatin samples and references

Extraction of gelatins from samples is relatively undemanding due to their high solubility in water, as compared to the extraction of proteins for specific peptide markers [3]. The experimental protocol is comprised of three steps including gelatin extraction, tryptic digestion and LC/MS/MS analysis (**Figure 1**). Additional SPE clean up is not needed. However, before injection to LC/MS/MS, centrifugation of sample is applied to segregate the digested peptides from insoluble solid extract. Because low concentration of ammonium bicarbonate is compatible to LC/MS/MS, the liquid sample (supernatant) is subjected directly to analysis without dilution. The capsule shell of pharmaceutical product is rinsed with milli-Q-water to remove any content. Then, the capsule shell is cut using clean scissor and placed into extraction tube.

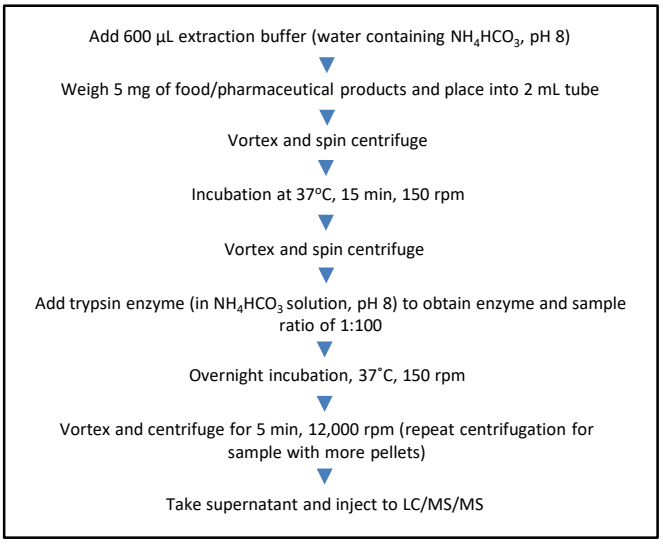

Figure 1. Experimental protocol for gelatin extraction, digestion and LC/MS/MS analysis

Commercial certified reference materials (CRM) of gelatins were obtained and used to confirm detection of peptide markers. However, the stability of those CRM remained unverified as bovine and porcine-specific markers were detected at trace levels in both standards (data not shown). In this report, in-house gelatin references derived from raw materials such as beef tendon and pork skin are used. These gelatin reference materials can last for months at -20°C. **Figure 2** shows the details of the procedure.

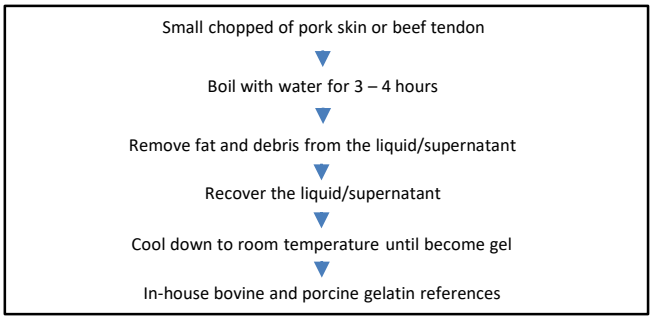

Figure 2. Protocol to obtain gelatin references from raw meat materials

LC/MS/MS analytical conditions

Table 1. Analytical conditions for detection and differentiation of bovine and porcine-specific peptide markers on LCMS-8060

Column	Aeris Peptide 1.7 µm XB-C18 100Å (150 mm x 2.1 mm I.D.)
Mobile Phase	A: Water with 0.1% formic acid B: Acetonitrile with 0.1% formic acid
Elution Program	Gradient elution, 5%B (0 – 2 min) ➔ 25%B (15min) ➔ 50%B (15.21 – 16min) ➔ 5%B (16.01 – 19min)
Flow Rate	0.3 mL/min
Oven Temp.	40 ºC
Injection	1 µL

Table 1. Analytical conditions for detection and differentiation of bovine and porcine-specific peptide markers on LCMS-8060 (continue)

Interface	Heated ESI
MS Mode	MRM, Positive mode
Block Temp.	400 ºC
DL Temp.	250 ºC
Interface Temp.	300 ºC
Nebulizing gas	N_2, 3 L/min
Drying gas	N_2, 10 L/min
Heating Gas	Zero air, 10 L/min

The details of the UHPLC and MS/MS parameters are shown in **Table 1**. A LC/MS/MS system, a Nexera X2 UHPLC connected to LCMS-8060 which is same as that used for Halal testing method [3] was employed to develop MRM based method for detection and differentiation of bovine and porcine-specific peptide markers. Both column and mobile phase are also deliberately set as the same with the Halal testing method.

❑ Results and discussion

Establishment of MRM-based method for detection of peptides of gelatins

It is well known that the high similarity in amino acid sequences between bovine and porcine gelatins is a main challenge for differentiation of their sources. It was reported [4] that it is possible to differentiate bovine and porcine gelatins through analysis of amino acid compositions by using HPLC and PCA methods. A systematic study using LC-MS was carried out by Zhang et al [5] to differentiate gelatins from bovine and porcine by detection of the small differences in amino acid sequences of 39 pairs of polypeptides. Sequence alignment analysis was a key approach. However, unpredictable proline hydroxylation (+16 mass) in the gelatin peptides may cause misleading in identification due to a same mass difference between alanine and serine (+16 mass) if they are present in the same peptide of different species.

In this study, MRM method development was based on over 40 pairs of polypeptides reported in open literatures [1, 5-6]. Each pair of polypeptides has small sequence differences between bovine and porcine species. The Skyline program and the UniProt database are the main tools to predict MRM transitions and respective collision energies (CE) of these targeted peptides, which had expedited greatly the MRM method development process. The predicted sets of MRM transitions with CEs were used to generate initial MRM chromatographic peaks using the in-house gelatin reference materials of bovine and porcine. The MRM parameters were further optimized using the auto-MRM optimisation program on LCMS-8060 for best sensitivity and intensity ratios of MRMs transitions for each targeted peptide.

Validation of peptide markers for differentiation of bovine and porcine gelatins

The specificity of the candidates to be used as specific peptide markers was investigated. It is worth noting that the sequences possess small discrepancy, often in only one amino acid, in each pair of the 39 pairs of reported peptides [5]. Moreover, the frequent and unpredicted occurrence of proline hydroxylation to collagens during hydrolysis may cause bovine and porcine-specific peptides generating exactly same mass and very similar MRM transitions. With Skyline, sequence alignment or verification, i.e., the site and number of proline hydroxylation, can be performed to obtain all MRM transitions and find the differences due to proline hydroxylation. For example, as shown in **Figure 3,** a bovine specific peptide (GPPGSAGSPGK) and the corresponding porcine-specific peptide (GPPGSAGAPGK) have a discrepancy in the 8th amino acid. Theoretically, the 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 *m/z* as bovine, which would result in almost identical product ions (MS/MS fragments) and MRM transition except y9 ion. It is therefore essential to validate experimentally by LC/MS/MS analysis. The MRM peak pattern or MS/MS spectrum of peptides with reliable gelatin reference materials on LC/MS/MS is compulsory to pinpoint unique marker for each species.

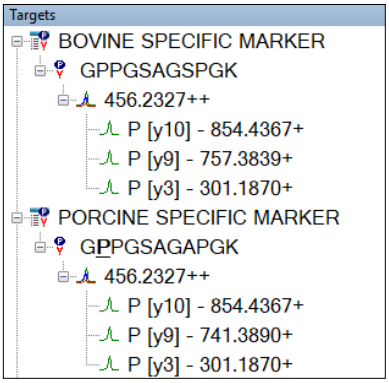

Figure 3. In silico prediction of MRM transitions for bovine and porcine-specific peptides by Skyline. Bold-underlined proline (**P**) indicates proline hydroxylation. The mass-to-charge ratio (*m/z*) of precursor ions and product ions (y3 and y10 ion) between bovine and porcine markers are identical. Product ion (y9) is different for Bovine and porcine.

The results of the validation study are compiled into **Tables 2** and **3**. The corresponding total MRM chromatograms are shown in **Figures 4** and **5**. In summary, nine peptides are found to be specific to bovine gelatins, which are not detected in the porcine gelatin reference materials. Eight different peptides are specific to porcine gelatins, which are not observed in bovine reference materials.

Table 2. Validation of bovine-specific peptides and MRM transition on LCMS-8060.

Protein	UniProt No.	Peptide marker (Abbr.)	Precursor ion & Charges	Number of MRM	RT (min)
Alpha-1 chain of type 1 bovine collagen	P02453	BG1	658.85++	4	9.0
		BG2	780.9++	5	9.9
		BG4	641.3++	5	5.3
		BG5	473.2++	4	1.0
Alpha-2 chain of type 1 bovine collagen	P02465	BG3	644.3++	3	7.9
		BG6	451.75++	3	5.1
		BG7	393.2++	4	2.3
		BG8	357.65++	3	0.9
		BG9	596.85++	5	9.2

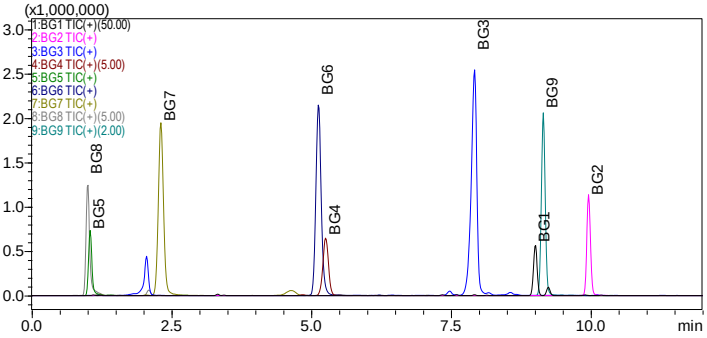

Figure 4. Total MRM chromatograms of nine validated bovine-specific peptide markers detected in trypsin-digested extract of bovine gelatin reference.

Table 3. Validation of porcine-specific peptides and MRM transition on LCMS-8060.

Protein	UniProt No.	Peptide marker (Abbr.)	Precursor ion & charges	Number of MRM	RT (min)
Alpha-1 chain of type 1 porcine collagen	A0A1S7J210	PG2	486.25++	4	1.3
		PG5	656.3++	4	5.4
		PG6	773.9++	5	9.1
Alpha-2 chain of type 1 porcine collagen	A0A1S7J1Y9	PG1	1103.05++	3	8.5
		PG3	921.45++	5	9.5
		PG4	620.8++	5	10.4
		PG7	731.85++	5	8.0
		PG8	590.85++	5	7.9

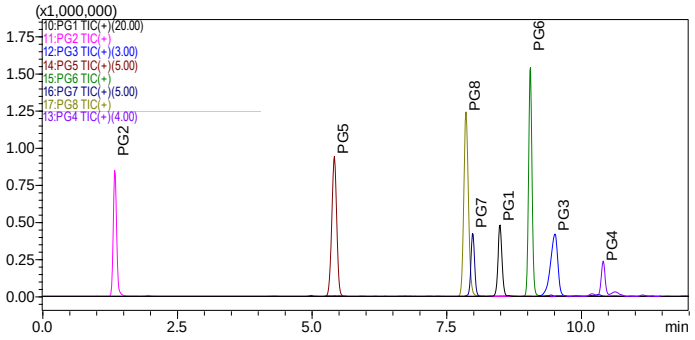

Figure 5. Total MRM chromatograms of eight validated porcine-specific peptide markers detected in trypsin-digested extract of porcine gelatin reference.

Performance of the established MRM method for detection and identification of gelatins

A total of nine bovine and eight porcine-specific markers were validated (**Tables 2** and **3**). The rest of the peptide candidates were found either not specific or not detected by LC/MS/MS. The criteria for peak detection included at least MRM transitions (one quantifier and two confirmation ions) with prominent peak shape and signal-to-noise ratio (S/N)>3. A few peptide markers are hydroxylated at 2-3 proline sites. Each peptide markers exhibit different sensitivities but remain feasible for detection and relative quantitation considering low initial sample amount (5mg) and injection volume (1µL, **Figures 4** and **5**). All the peptide markers display good repeatability (RSD<15%) across six repeated injections (**Table 4**).

Table 4. Repeatability evaluation of peptide markers on LCMS-8060 (*n*=6)

Peptide marker	Area RSD (%) (*n*=6)	Peptide marker	Area RSD (%) (*n*=6)
BG1	12.2*	PG1	10.2
BG2	4.4	PG2	9.2
BG3	1.1	PG3	4.9
BG4	5.5	PG4	7.4
BG5	11.2	PG5	5.9
BG6	2.4	PG6	6.8
BG7	2.1	PG7	9.5
BG8	14.9	PG8	9.4
BG9	2.5		

*four repeated injections (*n*=4)

Detection and differentiation of bovine and porcine gelatins in food and pharmaceutical products

A total of ten commercial processed food samples and two pharma capsules were brought from local shops. These samples were selected randomly, some with gelatin content labelled on the on packings (**Table 5**). The established MRM method was applied for analysis of these samples and the results are shown in **Table 5.** Bovine and porcine-specific markers were successfully detected in samples No. 1-7. Gelatin markers were not seen in samples with undeclared gelatin content (No. 8-12).

Bovine-specific markers demonstrate better sensitivity than porcine as indicated by detection of six or more designated peptide markers and higher abundance in gelatin-containing or presumably containing samples (samples No. 1 and 4-7). The number of detected porcine-specific markers in samples ranged between four to six (samples No. 2, 3, and 7). It is suggested that the most sensitive peptide markers for porcine gelatin are PG2, PG5, PG6 and PG8 as all are observed in the aforementioned samples. Most of the samples contain either bovine or porcine gelatin except for pharma capsule II (sample No. 7).

The analysis results for pharma capsule II shown in **Table 5** indicates that nine bovine-specific and four porcine-specific markers were detected. Three representative individual MRM chromatograms of bovine markers and three of porcine markers are displayed in **Figure 6** for detailed review of the results. Since half of the eight validated porcine markers were detected, it can be concluded that both bovine and porcine gelatins are present in pharmaceutical capsule II. However, the current method could not provide quantitative information. The relative abundance of the peptide markers in the sample was estimated relative to the gelatin reference materials. It was found that the level of bovine gelatin in

Table 5. Results of screening analysis of bovine and porcine gelatin markers in food and pharmaceutical products (ND: not detected)

No	Sample	Gelatin declaration & source	Number of detected peptide markers	
			Bovine	Porcine
1	Gummy bear I	Yes, beef	8	ND
2	Marshmallows I	Yes, pork	ND	5
3	Chewing gum	Yes, undeclared	ND	6
4	Marshmallows II	Yes, undeclared	6	ND
5	Gummy bear II	Yes, undeclared	8	ND
6	Pharma capsule I	Yes, undeclared	9	ND
7	Pharma capsule II	Yes, undeclared	9	4
8	Vanilla cookie	Undeclared	ND	ND
9	Chocolate cookie	Undeclared	ND	ND
10	Chocolate bar I	Undeclared	ND	ND
11	Chocolate bar II	Undeclared	ND	ND
12	Chocolate bar III	Undeclared	ND	ND

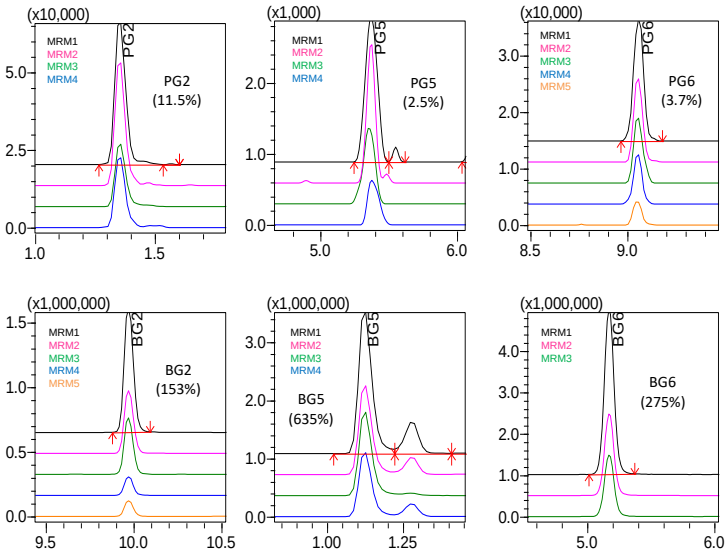

Figure 6. Detection of bovine and porcine-specific peptide markers BG2, BG5, BG6, PG2, PG5, and PG6 in commercial pharmaceutical capsule II (sample No. 7). The value inside bracket indicates % gelatin marker present in sample relative to reference.

pharma capsule II is very high and the level porcine gelatin seems much less. However, as reported by Flaudrops [7], this may not be the case since bovine gelatin signal tended to mask the porcine gelatin when both present or mixed at the same matrix. This will be address in future investigation.

In summary, this LC/MS/MS method presents accurate confirmation of the availability of gelatin in the samples (samples No. 1-2) as well as determination of the source of gelatin (samples No. 3-7). The established method could give substantial support for Halal and kosher testing application since porcine gelatin is forbidden for Muslim and Jew communities. It is also applicable for Hindu religious customs as they strictly require free bovine materials and its-by-products.

❑ Conclusions

Development of practical method for detection and differentiation of bovine and porcine gelatins was established by using LCMS-8060. The current method shaves off several steps and significant amount of time for sample preparation as compared to Halal testing on meat products [3]. Featured with excellent sensitivity and a high number of specific peptide markers, this method offers a reliable approach for Halal testing as well as other religious customs and safety evaluation to authenticate animal of origin based upon detection and differentiation of bovine and porcine gelatins in food and pharmaceutical products.

❑ References

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