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vMethod™ Application for Food Testing

Screening and Speciation of Raw and Processed Meat Products

A Selective and Robust LC-MS/MS Method for Multiple Meat Speciation and Authentication on the QTRAP® 4500 System

Rapid and Reliable Detection of Multiple Meat Species in Food Products in a Single Injection

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Introduction

In early 2013, horse and pig DNA were identified in beef products sold in several supermarket chains. Further testing across Europe and beyond had revealed widespread incidences of such contamination.[1] This type of contamination not only misleads the consumers, but also has health, religious, and ethical implications. In response to this, the Food Safety Authority (FSA) and Department for Environment Food & Rural Affairs (Defra) have set the threshold for undeclared meat species in meat products to 1% (w/w).[2] Therefore, it is imperative that analytical methods are sensitive and accurate enough to screen for the presence of meat adulteration in food products.

Traditionally, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISA) are used for meat speciation. PCR amplifies fragments of DNA extracted from food samples and demonstrates good sensitivity in unprocessed products. However, DNA can be easily disrupted or removed during food processing and manufacturing, thus limiting the use of PCR for processed or cooked meat products. ELISA is relatively quick and simple to perform, but has poor selectivity and is susceptible to cross-species reactivity which can lead to false positive or false negative results. Moreover, most ELISA tests lack multiplexing capabilities. Hence, LC-MS/MS provides an excellent alternative to these methodologies to identify and confirm different meat species with more accuracy and reliability.

Herein, we present a robust and sensitive LC-MS/MS method using the QTRAP® 4500 LC-MS/MS system that detects and screens pork, beef, lamb, chicken, duck and horse simultaneously in a single injection. The optimized sample preparation procedure is easy to follow and can be used for analyzing raw, cooked and processed meat products. Signature marker peptides unique to each species were identified and verified to ensure that they do not present any cross-species reactivity. Presently, this method can detect peptides from each meat species at a threshold detection limit of 1% w/w (10 mg/g) in a variety of food products.

Experimental

Sample Preparation

Meats or meat products (10 g) were frozen for 1 hour and grounded using a food processor or a coffee grinder. As an optional step, each grounded meat (1 g) was defatted with hexane and dried under a gentle flow of nitrogen. Extraction buffer was added to each defatted meat sample and the mixture was homogenized at high speed using a probe homogenizer to extract the proteins. Standard samples were prepared by combining different amounts of pork, beef, lamb, chicken, duck and horse homogenates to final concentrations of 0% and 1% (w/w) for each meat species (single-point calibration). The mixed meat homogenates (2 mL) were centrifuged and 0.4 mL of supernatant was diluted with ammonium bicarbonate buffer. Reducing reagent was added and the samples were incubated at 60°C for 1 hour. After cooling to room temperature, samples were alkylated using a cysteine blocking reagent. The modified proteins were digested with trypsin (4 to 12 hours). After which, the enzymatic activity was quenched with formic acid. Digested samples were desalted and concentrated using Agela

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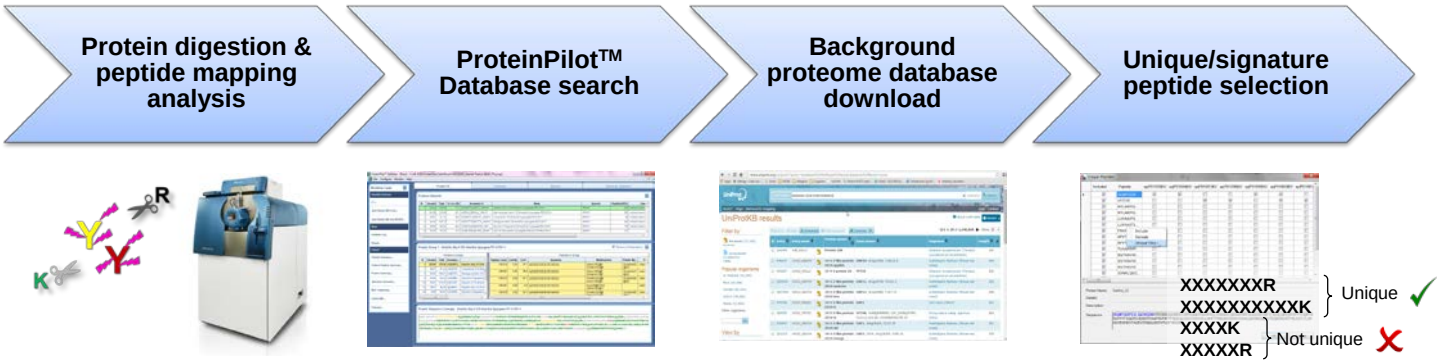

Figure 1. Signature peptide selection workflow using the SCIEX TripleTOF® 6600 system and ProteinPilot™ software

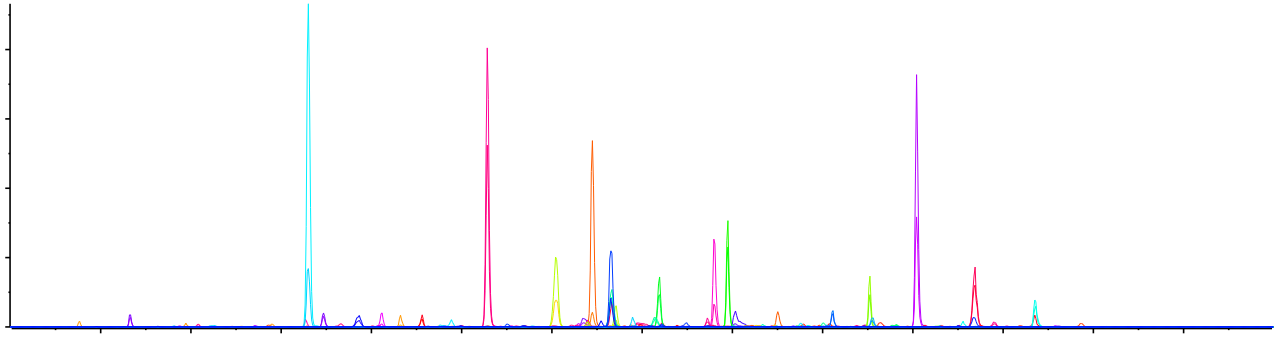

Figure 2. Extracted ion chromatograms from the LC-MS/MS analysis of raw meat mixture containing pork, beef, lamb, chicken, duck and horse (10, 20, 20, 20, 20 and 10% w/w, respectively). Multiple peaks corresponding to tryptic marker peptides are displayed.

Technologies Cleanert PEP SPE cartridges (60 mg/3 mL). The SPE eluents containing the peptides were dried and reconstituted for LC-MS/MS analysis.

LC Separation

Analytes (10 µL injection volume) were chromatographically separated using a ExionLC™ AC system equipped with a Phenomenex Kinetex C18 column (2.6 µm, 100 x 4.6 mm i.d.). A linear gradient was employed over 15 min at a flow rate of 500 µL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

MS/MS Detection

Ion-dependent acquisitions (IDA) on a TripleTOF® 6600 LC-MS/MS System were performed to identify the proteins and peptides representative of pork, beef, lamb, chicken, duck and horse meats (Figure 1). The strategy for the selection of signature peptides can be found in more detail in the Results and Discussion.

Meat speciation and screening analysis was performed on a SCIEX QTRAP® 4500 system with Turbo V™ source in positive ESI mode using an ion source temperature of 650 °C. The Scheduled MRM™ algorithm was used to analyze food samples for 6 meats in a single injection by multiplexing the detection of multiple MRM transitions for unique signature peptides.

Results and Discussion

Comprehensive information of protein/peptide IDs was generated using the ProteinPilot™ software's protein database search features after LC-MS/MS analysis of digested meat samples on a TripleTOF® 6600 System (Figure 1). Selections of signature peptides for each meat species were performed using the Skyline software and NCBI Protein BLAST to ensure that the shortlisted peptides were unique and not found in other common livestock.

Signature peptides were finalized for each meat based on their: 1) specificity for each meat species; 2) uniqueness compared to the cross-species background; 3) sensitivity of detection; and 4) ability to be detected in both raw and cooked or processed meat samples.

For each meat species, two unique proteins, two unique peptides per protein, and two unique MRM transitions per peptide were chosen to ensure confidence in positive identification (Table 1). This corresponds to 24 marker peptides or a total of 48 MRM transitions representing pork, beef, lamb, chicken, duck and horse, for the simultaneous identification of multiple meat species in the same food sample (Figure 2). To monitor many MRM transitions during a single injection, the Scheduled MRM™ algorithm was employed, where each MRM transition was monitored for a short period during its expected retention time, decreasing the total number of concurrent MRM experiments during a cycle and allowing cycle time and dwell time to be maintained. This approach maximized the sensitivity for signature peptide detection and allows the method to be expanded as markers from other meats are identified.

LC-MS/MS analyses of raw and cooked (pan-fried) meat mixtures were performed to evaluate the thermal stability of the marker peptides. As shown in Figure 3, each meat marker peptide was detected without significant changes in sensitivity before (raw) and after cooking.

To demonstrate that signature peptide signals were linear in response to increasing meat concentrations, calibration curves for each peptide were generated over a wide dynamic range (0 to 100% w/w) with good reproducibility in combined meat matrix. For all meat species tested (pork, beef, lamb, chicken, duck and horse), MRM transitions were linear over a broad dynamic range with correlation coefficient values of over 0.99 for both MRM transitions. Figures 4 and 5 show examples of pork and beef with good linear response in meat matrix.

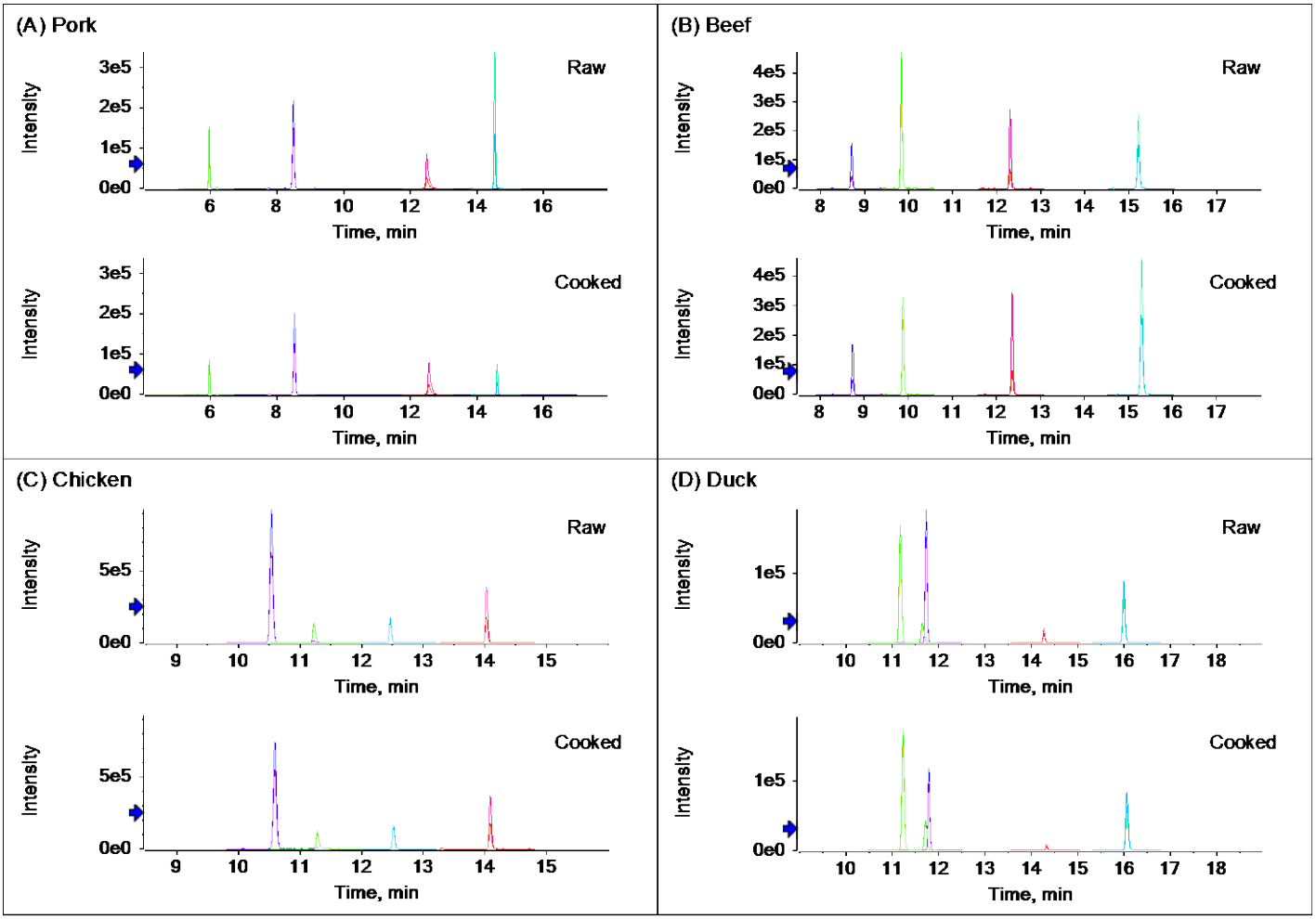

Figure 3. Extracted ion chromatograms (XIC) from the LC-MS/MS analysis of raw (top) and cooked (bottom) meat mixture containing pork, beef, chicken, duck and lamb (data not shown).

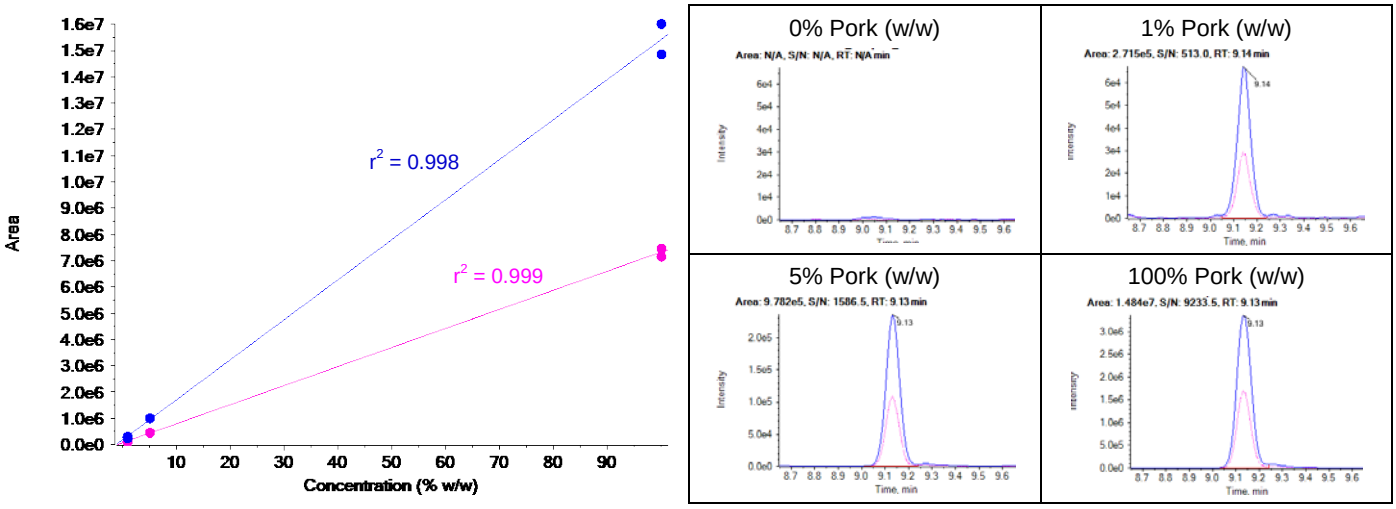

Figure 4. Calibration curves and XICs of Protein_1.Peptide_A from 0 to 100% raw pork (w/w). Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored.

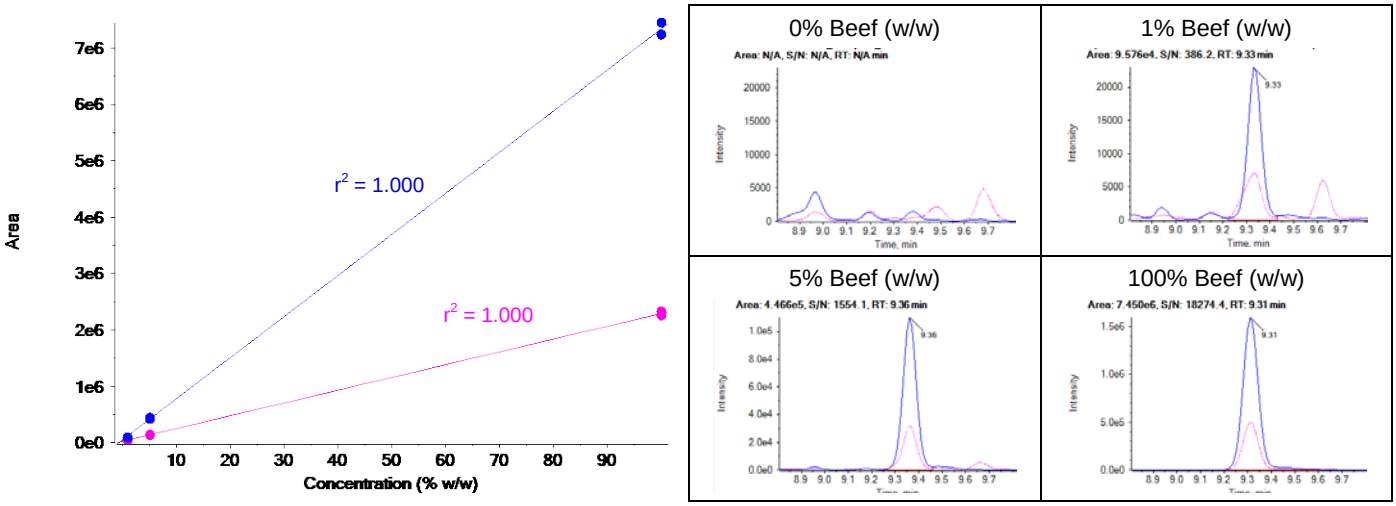

Figure 5. Calibration curves and XICs of Protein_1.Peptide_A from 0 to 100% raw beef (w/w). Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored.

The 1% (w/w) detection threshold limit of meat species in the combined meat matrix was verified on a SCIEX QTRAP® 4500 system by analyzing the 0% and spiked 1% (w/w) meat species in meat matrix. All marker peptides for each meat species were reliably detected at 1% spiked and no interference signals were observed in the background matrix (0%). Figures 5 and 6 show example XICs of quantifier ion (Protein_1.Peptide_A1) for each meat in 0% and 1% (w/w) samples, demonstrating high sensitivity and reliability of detection. It's worth noting that 0.1% (w/w) detection threshold limit of meat can also be achieved with a SCIEX QTRAP® 6500+ system (data not shown).

To verify the effectiveness of the method for detecting meat contamination or adulteration, various raw and processed food products purchased from supermarkets were screened. As an

example in Figure 7, no significant pork marker peptides were detected in the halal certified products. Pork was tested positive only in products that had this meat labeled as one of the ingredients.

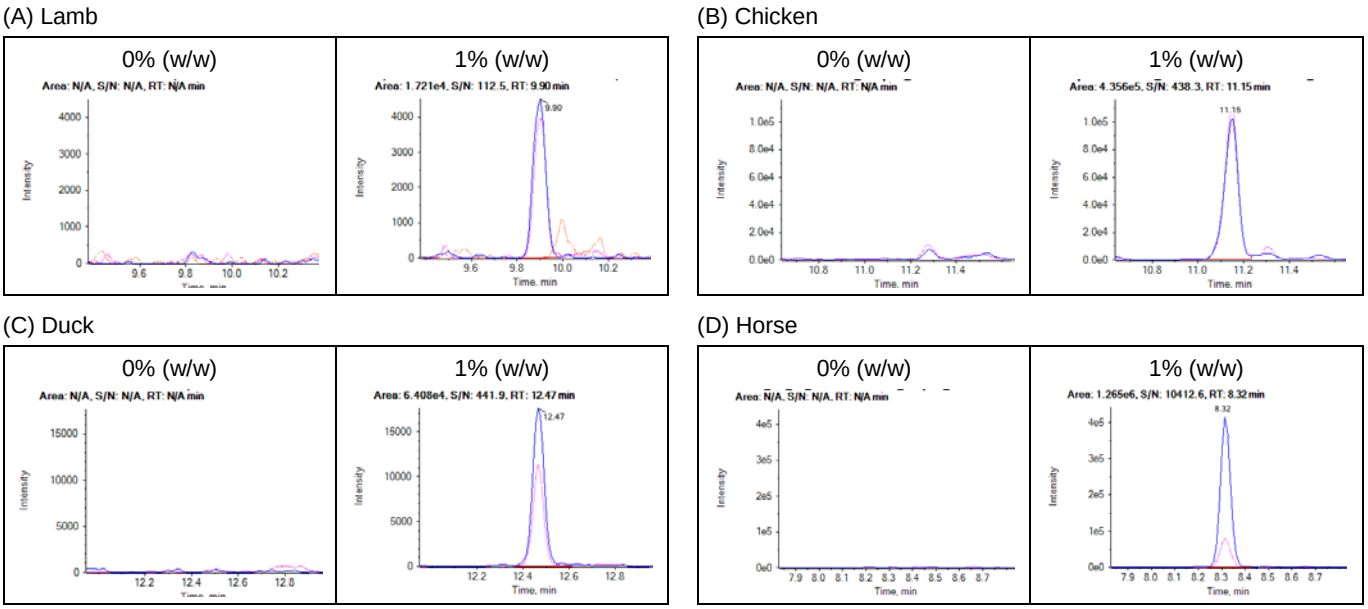

Figure 6. XICs of Protein_1.Peptide_A for 0 and 1% (w/w) of lamb, chicken, duck and horse in combined meat matrix (refer to Figure 5 for detection of pork and beef at 0 and 1% w/w). Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored for each marker peptide.

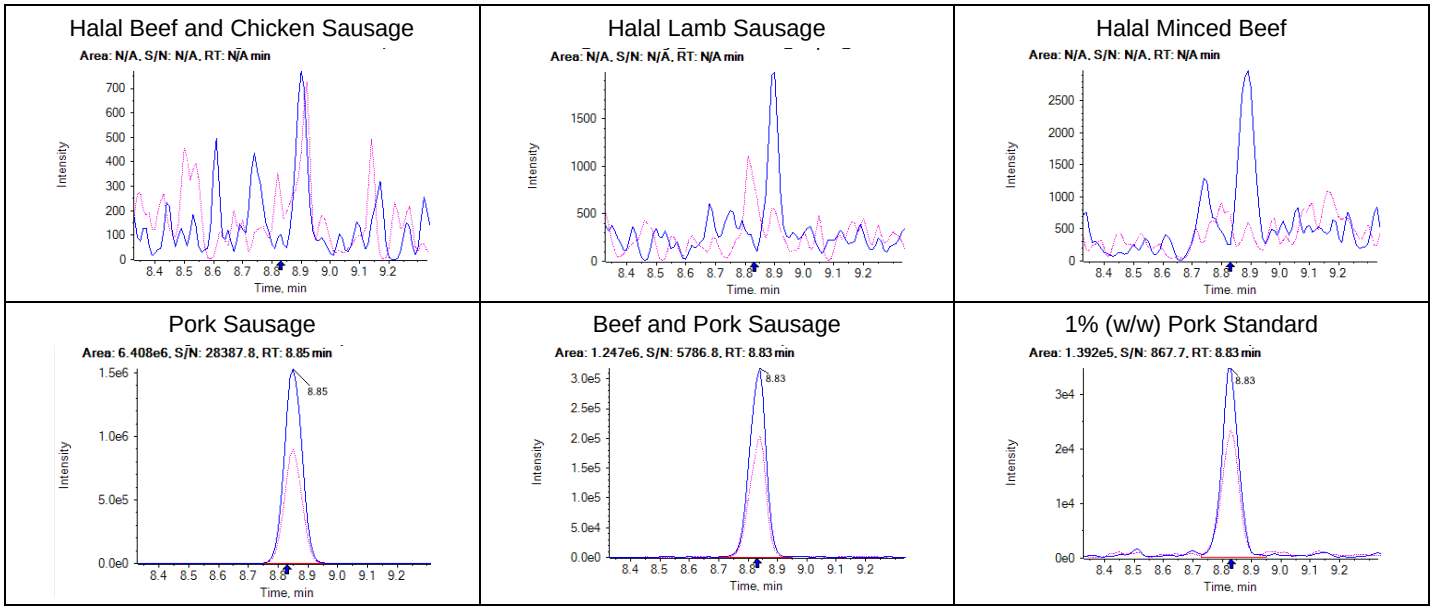

Figure 7. XIC of Pork.Protein_1.Peptide_A in commercial sausage products. Two MRM transitions, fragment 1 (blue) and fragment 2 (pink), were monitored for the marker peptide.

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Summary

We have developed an LC-MS/MS-based meat speciation method for screening meat adulteration at 1% (w/w) for pork, beef, lamb, chicken, duck and horse. This method identifies MRM transitions corresponding to unique peptides for each meat species, and multiplexes their detection into a single injection. Unlike PCR and ELISA, the method is applicable to both unprocessed and processed meat matrices, providing high specificity and sensitivity in a single analysis. In addition to 1% meat adulteration screening on a SCIEX QTRAP® 4500 system, the method also demonstrates good linear responses at different meat concentrations in meat matrix, indicating its potential capability for relative quantitation. The vMethod package includes an easy-to-follow and robust sample preparation procedure, an optimized LC-MS/MS acquisition method, established templates for data processing and reporting to facilitate the rapid detection and identification of meat adulteration or contamination in food products.

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Determination of Animal Species Origin from Gelatin in Food and Pharmaceutical Products by LC-MS/MS

For Research Use Only. Not for use in diagnostic procedures.

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ABSTRACT

Gelatin has many applications in the food, pharmaceutical and nutritional industries supported by its excellent properties and functionalities. Pig skins and bovine hide and bones are the largest commercial sources of gelatin. For religious reasons, Muslims, Jews, Hindus etc. in the world would need to ensure that their gelatin based food products do not contain pork or beef hence the development a method for determination of animal species origin from gelatin products is necessary. In this paper we present a fast, robust, and reliable method, which has been validated for determination of animal species origin from gelatin products. The LC-MS/MS method using the Multiple Reaction Monitoring (MRM) detects gelatin with Limits of Quantitation (LOQ) of 1% w/w in gelatin mixture.

INTRODUCTION

Gelatin is a protein based product derived from the fibrous protein collagen and produced by partial denaturation of native collagen extracted from skins, bones and connective tissues of animals like bovine and porcine.[1] In its production, dilute acid or alkali is used to treat raw animal material to achieve partial cleavage of crosslink and break the structure, resulting in formation of ''warm-water-soluble collagen'', namely gelatin.[2] It is widely used as a gelling and thickening agent in a variety of foodstuffs such confectionary products, water-based desserts and in the pharmaceutical industry e.g. in gel capsules for medicines. Pig skin was the largest commercial source of gelatin, followed by bovine hide and bones as sources of gelatin.

In the mid-1980s, the world was shaken by the emergence of bovine spongiform encephalopathy (“mad cow disease”) epidemic that swept the European countries. Since then, there has been much concern about using the gelatin from the infected animals. In addition, religious and socio-cultural factors have influenced the need for a method to identify the species origin of gelatin to fulfil the halal and kosher markets.

Under the conditions of gelatin production, species-specific DNA present from the original animal is often denatured or removed making the use of the polymerase chain reaction (PCR), often used in species identification, difficult[3-5] or impossible6. ELISA (enzyme-linked immunosorbent assay) has been used for speciation[7] but this approach has limitations due to it’s risk of false negatives and positives. Some nano UHPLC-ESI-Q-TOF based methods have also been reported for speciation of gelatin[8], but this approach has issues due to the inherent complexities of nano UHPLC as well as long run times. Triple Quad/TRAP systems have excellent sensitivity, and the combination of UHPLC can quickly and accurately identify markers in a short time, making it more suitable for deployment in a routine setting.

MATERIALS AND METHODS

Sample Preparation:

5 mg of gelatin or gelatin production sample was dissolved using 50 mM ammonium bicarbonate solution at 37˚C to the final concentration of 1mg/ml. Digestion took place either overnight at 37˚C (10-15 hours) or using a microwave burst technique.

HPLC Conditions:

An EXIONLC™ LC system with an Phenomenex Kinetex® C18 , 100 Å, 50x3 mm, 2.6 µm column at 40˚C with a gradient of eluent A 0.1% formic acid in water and eluent B 0.1% formic acid in acetonitrile was used at a flow rate of 250 µL/min. The injection volume was set to 20 µL. The LC gradient conditions are shown in Table 1.

MS/MS Conditions:

A SCIEX QTRAP™ 4500 LC-MS/MS system with Turbo V™ source and Electrospray Ionization (ESI) probe was used. Porcine and bovine gelatin were detected using 3 MRM transitions per species to allow identification based on the number of detected markers.

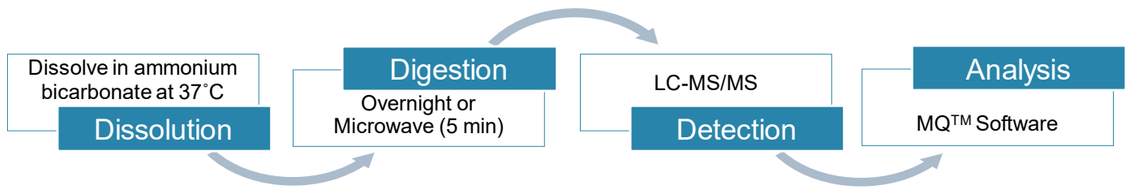

Figure 1. Work flow of the method to determine the animal species origin from gelatin by LC-MS/MS.

Step	Total Time (min)	Module	Event	Parameter (%)
1	2	Pumps	Pump B Conc.*	5
2	12	Pumps	Pump B Conc.*	40
3	12.5	Pumps	Pump B Conc.*	90
4	13.5	Pumps	Pump B Conc.*	90
5	14	Pumps	Pump B Conc.*	5
6	19	Controller	Stop*	-

Table 1. LC gradient conditions used for separation at a flow rate of 250 µL/min

RESULTS

A quick, simple and effective method for identification of porcine and bovine gelatin was developed (Figure 1). Three markers for each species of gelatin are using for identification (Figure 2). As shown in Figure 3 and 4, 1% contamination of bovine gelatin with porcine gelatin or porcine gelatin with bovine gelatin could be easily identified by microwave digestion, and the overnight digestion shows the same result.

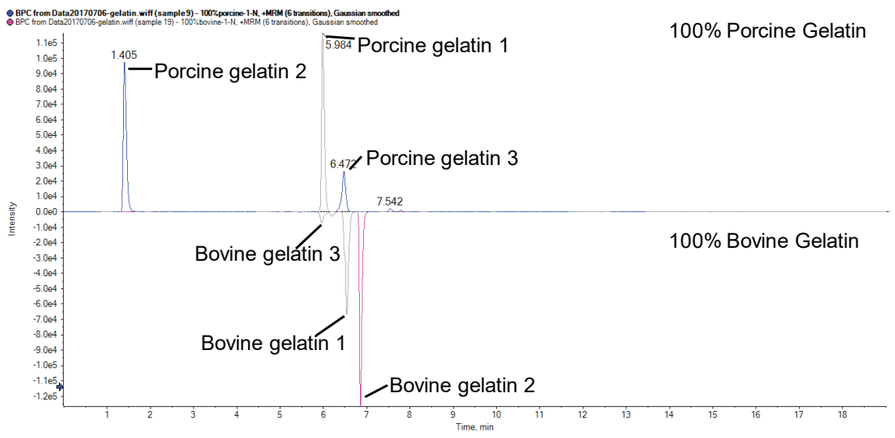

Figure 2. Porcine gelatin markers and bovine gelatin markers for identification the animal species origin from gelatin.

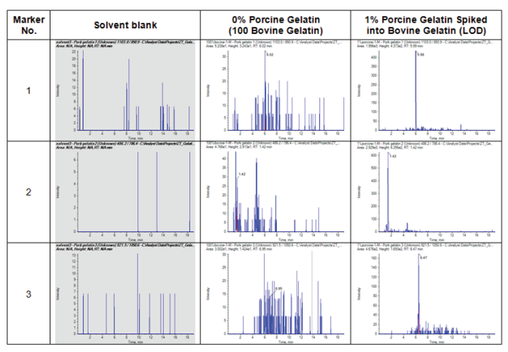

Figure 3. 1% porcine gelatin could be easily identified by microwave digestion

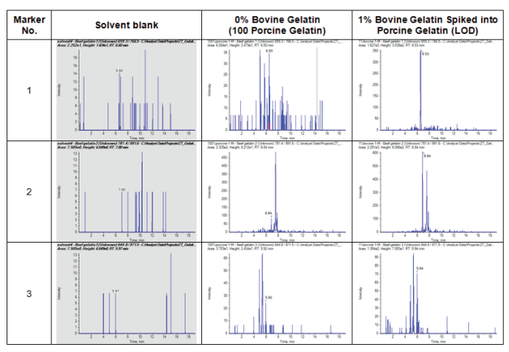

Figure 4. 1% bovine gelatin could be easily identified by microwave digestion

System stability was evaluated using the 10% added gelatin samples. As shown in Figure 3, the method has good system stability. A total of 14 commercial gelatin products (raw gelatin, dairy products, candies, sausages and capsules) were used as unknown samples to assess the feasibility of this method for screening the species of gelatin in food or pharmaceutical products. As shown in Table 2 and Figure 5, the method was able to accurately screen for the species of gelatin both in raw and processed gelatin products using both digestion methods.

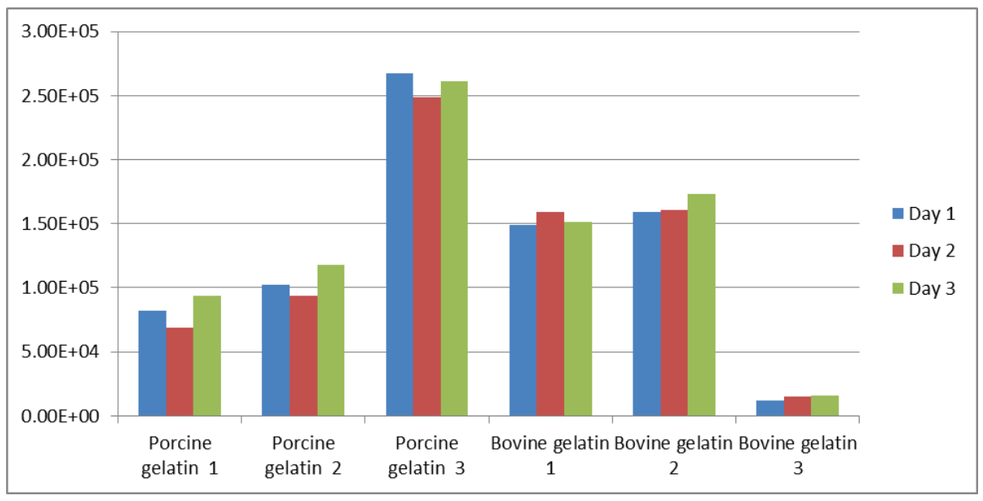

Figure 5. System stability test result. all the markers were found to be stable up to 3 days at 10°C in the autosampler.

ID	Description	Raw or Processed	Number of gelatin marker with positive identification (n of 3)			
			Overnight Digestion		Microwave Digestion	
			Porcine	Bovine	Porcine	Bovine
1	Raw Gelatin 1	Raw	3 of 3	3 of 3	3 of 3	3 of 3
2	Raw Gelatin 2	Raw	3 of 3	3 of 3	3 of 3	3 of 3
3	Raw Gelatin 3	Raw	3 of 3	3 of 3	3 of 3	3 of 3
4	Yoghourt Brand 1 (Halal)	Processed	N/A	N/A	0 of 3	0 of 3
5	Yoghourt Brand 2	Processed	3 of 3	3 of 3	3 of 3	3 of 3
6	Yoghourt Brand 3	Processed	3 of 3	3 of 3		
7	Dairy Product Brand 1 (Halal)	Processed			0 of 3	0 of 3
8	Beef Sausage Brand 1 (Halal)	Processed			0 of 3	3 of 3
9	Capsules 1	Processed	3 of 3	3 of 3	3 of 3	3 of 3
10	Capsules 2	Processed	3 of 3	3 of 3	3 of 3	3 of 3
11	Candy Brand 1	Processed	3 of 3	3 of 3	N/A	N/A
12	Candy Brand 2	Processed	3 of 3	1 of 3	N/A	N/A
13	Candy Brand 3	Processed	1 of 3	2 of 3	N/A	N/A
14	Candy Brand 4	Processed	3 of 3	1 of 3	N/A	N/A

Table 2. Screening of commercial gelatin products. For gelatin to be identified as present, 2 out of 3 markers will have to be positively identified. It did not demonstrate false positive where gelatin is not present, such as the absence of gelatin in Halal certified products (sample 4 and 7, sample 8 also absence of gelatin but it is a kind of beef product so bovine gelatin marker can be detected in it).

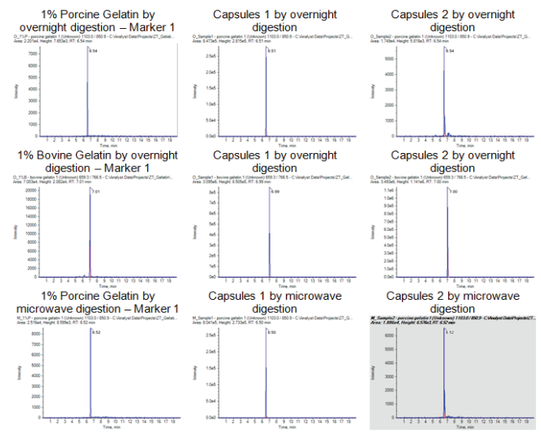

Figure 6i. A part of example XICs for gelatin markers in commercial gelatin products..

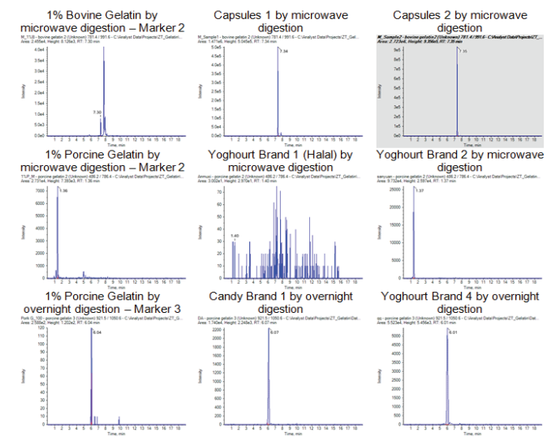

Figure 6ii. A part of example XICs for gelatin markers in commercial gelatin products..

CONCLUSIONS

A fast, robust, and reliable method, for the determination of animal species origin from gelatin in food and pharmaceutical products by LC-MS/MS was developed and validated. This method has the advantages of simple operation, quick analysis and accurate identification. High resolution LC using a small particle size column was combined with high sensitivity detection using an SCIEX Triple le Quad/QTRAP™ 4500 LC-MS/MS system. Multiple Reaction Monitoring (MRM) was used because of its high selectivity and sensitivity.

By using this method, 1% contamination of gelatin could be easily identified. The method was validated in 14 commercial gelatin products include raw gelatin, dairy products, candies, sausages and capsules. It can accurately identify the animal species origin of gelatin in the gelatin product and avoid false positives.

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TRADEMARKS/LICENSING

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