Are Pork Extracts Present in My Gummy Bears? Gelatin Speciation by LC-MS/MS

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Overview

The accidental or fraudulent blending of meat and animal products from different species is highly relevant for consumers with ethical concerns against eating species such as horse or pork in particular the Jewish and Muslim communities. In this work, we present the results from the initial development of an LC-MS/MS method utilizing AB SCIEX TripleTOF® 5600 and 4000 QTRAP® LC/MS/MS systems for the determination of the origin of gelatin used in food products and also pharmaceutical capsules.

Introduction

Following the Food Standards Agency (FSA)'s announcement in January that horse and pig DNA had been identified in beef products sold by several supermarket chains, further testing across Europe and beyond has revealed widespread incidences of such food contamination. This intended adulteration for financial gain or careless false declaration of meat products is a severe problem for consumers who have ethical or religious concerns about the consumption of pork or horse, more specifically the Muslim or Jewish communities who represent about 23 % of the worldwide population. As the tolerance level for porcine and equine content in foods is 0 %, for religious reasons, the limit of detection (LOD) needs to be as low as possible and so the continued development of more sensitive methods is necessary.

However, pork based products are not only used as the meat but can also be found in gelling agents in food (for example in candy, ice cream, and marshmallows) as well as in the cosmetic and pharmaceutical industry in the form of gelatin. Gelatin is made from collagen, a protein, which has been extracted from the skin, bones, and connective tissues of animals such as cows, chicken, pigs, and fish. After extraction the collagen is partially hydrolyzed to form the gelatin which is a mixture of peptides and proteins and is used in the form of sheets, granules or powder.

In the production of gelatin the protein hydrolysis normally occurs with hot water or under acidic conditions. The gelatin so produced is purified and used in food manufacturing and this process again may involve elevated temperatures. Under these conditions 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[1-3] or impossible.[4]

An alternative protein-based method, ELISA (enzyme-linked immunosorbent assay), has also been used for speciation[5] but this approach has limitations, including that it detects only one part of the protein and not multiple protein markers and so can pose a risk of producing false negatives and positives.

So an LC-MS/MS approach, detecting multiple tryptic peptides as markers for confirmation offers a more accurate and reliable approach to gelatin speciation than PCR or ELISA-based techniques. Initial identification of markers was by a shotgun proteomics approach using a high-resolution mass spectrometer, AB SCIEX TripleTOF® 5600 system, coupled to an Eksigent LC system. The method developed in this work uses the AB SCIEX 4000 QTRAP® system where multiple reaction monitoring (MRM) was used to detect markers which then automatically trigger the acquisition of enhanced product ion (EPI) scan to provide additional sequence information to further identify the peptides and proteins and therefore the gelatin species.

Experimental

Sample Preparation

Each sample (5mg) was dissolved in 600 µL of 50 mM ammonium bicarbonate buffer (37°C for 10-15 minutes). This extract was digested using trypsin (50 mM in ammonium bicarbonate buffer) in a trypsin to sample ratio of 1:100. Digestion took place either overnight at 37˚C (10-15 hours) or using a microwave burst technique where the samples are placed in ice and subject to 5 x 30 sec of microwave digestion, between each microwave burst the sample was shaken.

Once digested the samples were spun (12,000 rpm for 5 minutes). The top supernatant layer (500 µL) was removed carefully, not to disturb the bottom sediment, and centrifuged again (12,000 rpm for 5 minutes). The top portion of the supernatant (200 µL) was used for analysis.

LC-MS/MS

Initial identification of species specific peptides, from tryptic digests of porcine and bovine gelatin, was done by a shotgun proteomics approach using the high resolution and accurate mass AB SCIEX TripleTOF® 5600 system coupled to an Eksigent ekspert™ ultraLC 100-XL system. In these survey experiments a Phenomenex Aeris wide pore column was used for separation of the peptides using a 45 minutes gradient at a flow rate of 250 µL/min. An information dependent acquisition (IDA) method was used to automatically trigger 30 TOF-MS/MS spectra from the information in the TOF-MS survey scan. Principle components analysis within the MarkerView™ software was then used to identify species specific markers (Figures 1 and 2).

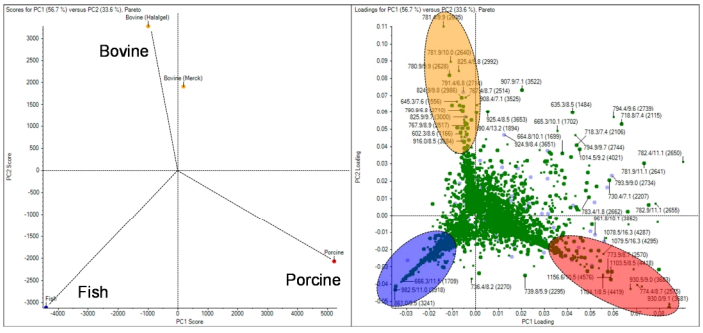

Figure 1. MarkerView™ software was used to identify characteristic markers for gelatin speciation, PCA Scores plot for bovine, porcine and fish (left) and PCA Loadings showing characteristic markers (right)

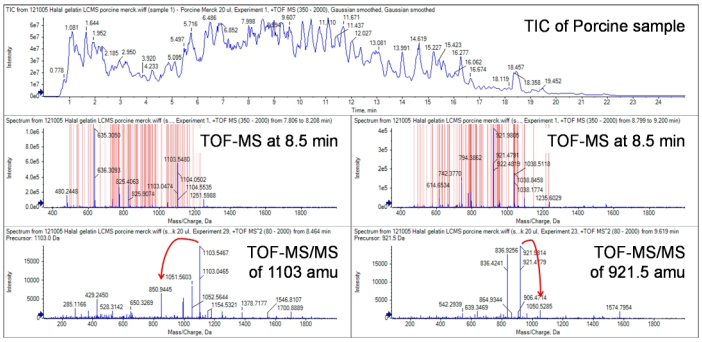

Figure 2. TOF-MS and TOF-MS/MS data of two selected marker compounds for porcine, TripleTOF® data was used to develop the MRM method

From this information seven markers were identified and the method was transferred to an AB SCIEX 4000 QTRAP® system where MRM transitions for each marker were optimized. In this final screening method samples were separated on a C18 column using the gradient shown in Table 1 where eluent A was water and eluent B was acetonitrile with both mobile phases containing 0.1% formic acid. The flow rate was set to 250 µL/min, column oven temperature to 40°C, and 20 µL of the sample volume was injected.

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

Step	Time	A (%)	B (%)
0	0.0	95.0	5.0
1	2.0	95.0	5.0
2	12.0	60.0	40.0
3	12.5	10.0	90.0
4	13.0	95.0	5.0
5	19.0	95.0	5.0

In the optimized method the Turbo V™ source conditions used were gas 1, gas 2 set at 30 psi and the curtain gas set to 25 psi, the temperature of the source was set at 450°C and IS voltage was 5500V.

MRM conditions for the most intense marker transitions are given in Table 2. In addition 3 qualifier transitions were monitored for each peptide marker. MRM transitions were acquired at a dwell time of 20 msec and were used as IDA triggers to automatically acquire full scan EPI spectra for identification of the gelatin marker.

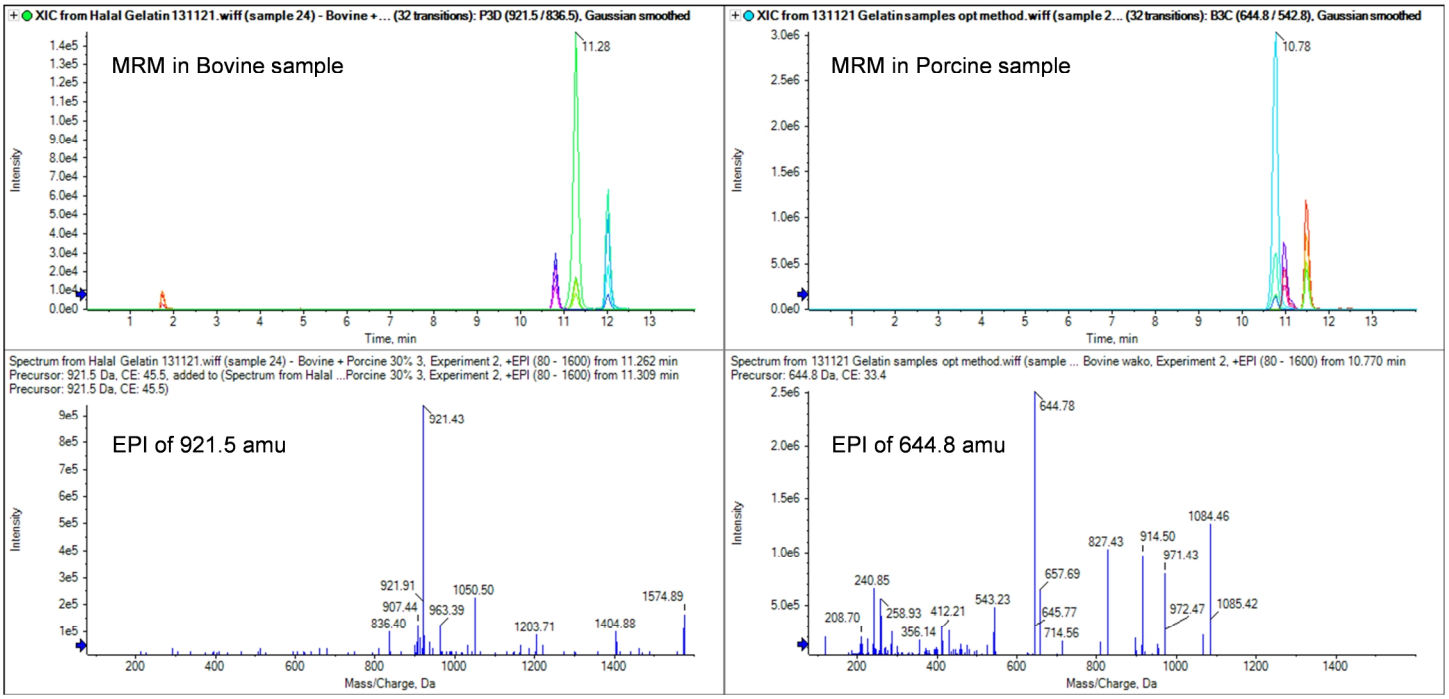

Figure 3. Comparison of a tryptic digest of a porcine (left) and a bovine (right) gelatin standard, MRM transitions were used to automatically acquire full scan EPI spectra for identification of the gelatin marker

Table 2. MRM transitions for the marker peptides of bovine (beef) and porcine (pork) gelatin, DP was set to 100 V for all transitions

Gelatin Marker	Q1 (amu)	Q3 (amu)	CE (V)
Pork gelatin 1	1103	850.9	57.5
Pork gelatin 2	486.2	786.4	26.4
Pork gelatin 3	921.5	1050.6	49.4
Pork gelatin 4	620.8	618.3	28.3
Beef gelatin 1	659.3	766.5	34
Beef gelatin 2	781.4	991.6	42.3
Beef gelatin 3	644.8	971.5	37.3

Results and Discussion

When burst microwave digestion was compared to the traditional overnight trypsin digestion results were identical, as this method was quicker this approach was used for this study. Alkylation and reduction of the proteins was also not necessary as the disulfide bridges and the secondary structure of the collagen had already been broken during extraction and purification of the gelatin. Trypsin extracts produced from beef and pork samples using this method were compared (Figure 3) and marker peptides which have different sequences and generated different MRM and fragmentation patterns could be produced.

Beef gelatin was then spiked with pork gelatin so that levels of contamination of beef with pork gelatin could be determined and a 1% contamination of bovine gelatin with porcine gelatin could be easily identified (Figure 4).

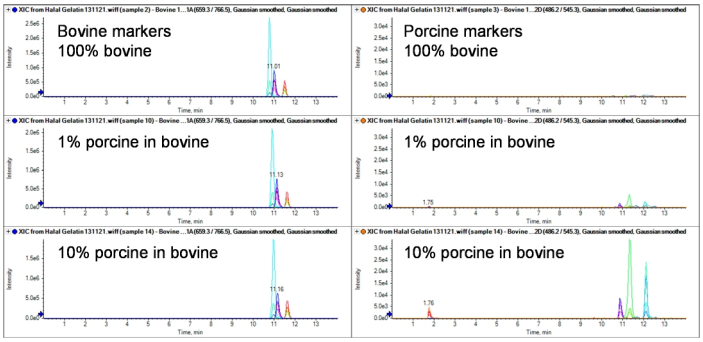

Figure 4. Results of spiking pork gelatin into beef gelatin, porcine markers were easily detected at 1% contamination

The method was then tested on extracts of gummy bears, fruit and chocolate candies as well as pharmaceutical capsules used for drug delivery and examples are shown in Figure 5. Here pork gelatin was detected but with no trace of bovine gelatin seen in the sweets and in the capsules only bovine gelatin was detected.

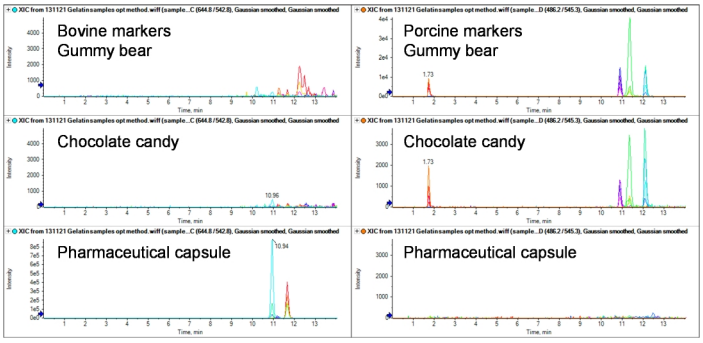

Figure 5. Results from the analysis of gummy bears, candy and pharmaceutical capsule for the presence of bovine (left) and porcine (right) markers. These examples show the presence of pork gelatin in gummy bear and chocolate candy. In the pharmaceutical capsule only bovine gelatin was detected.

Summary

In this study, we have identified 7 markers which are either specific for pork or beef gelatin and highlight the use of LC-MS/MS for gelatin speciation. These first results have shown that the gelatin ingredient can be extracted and analyzed in less than 1 hour and a 1% impurity of pork in beef gelatin can be detected. Further to this, this method can be used to detect the presence of pork gelatin in processed food such as sweets and also the animal source gelatin used in pharmaceutical capsules and offers multiple points of identification previously not available by ELISA analysis

In the future lower detection limits will be possible with the use of microLC[6] and more sensitive LC-MS/MS systems which mean that gelatin speciation at even lower levels is possible. This will help alleviate ethical concerns of the source of gelatin used in food manufacturing and pharmaceutical capsules used to deliver drugs.

References

1 P. J. O'Mahony: 'Finding horse meat in beef products - a global problem' QJM 106 (2013) 595-597

2 S. Ghovvati, M. R. Nassiri, S. Z. Mirhoseini, A. H. Moussavi, A. Javadmanesh: 'Fraud identification in industrial meat products by multiplex PCR assay' Food Control 20 (2009) 696-699

3 S. Soares, J. S. Amaral, M. B. Oliveira, I. Mafra: 'A SYBR Green real-time PCR assay to detect and quantify pork meat in processed poultry meat products' Meat Science 94 (2013) 115-120

4. S. R. Murray, R. C. Butler, A. K. Hardcare, G. M. Timmerman-Vaughan: 'Use of quantitative real-time PCR to estimate maize endogenous DNA degradation after cooking and extrusion or in food products' J Agric Food Chem 55 (2007) 2231-2239

5. F.-C. Chen, Y. H. P. Hsieh: 'Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA' Journal of AOAC International 83 (2000) 79-85

6 Stephen Lock: 'Can LC-MS/MS Be Used in Horse Meat Detection?' Application Note AB SCIEX (2013) # 7580213-01

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