Can LC-MS/MS Be Used in Horse Meat Detection?

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Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of horse meat at low % levels in beef and the banned substance phenylbutazone (BUTE) using peptides markers for horse proteins and specific MRM transitions for BUTE.

Introduction

Following the Food Standards Agency's (FSA) 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 contamination.[1] However, most testing methods are based on detection of species-specific DNA in meat, using the polymerase chain reaction (PCR) – which does not detect or identify proteins. This is a concern because DNA can be easily disrupted or removed during standard meat processing and food manufacturing. As a result, horse tissue or other contaminants remain undetected in food samples, despite strong presence of the contaminating proteins. An alternative protein-based method, ELISA (enzyme-linked immunosorbent assay), can be used to complement DNA testing, but this method has limitations, including that it detects only one part of the protein and not multiple protein markers.

The LC-MS/MS-based method presented offers a more accurate and reliable approach to meat speciation than PCR or ELISA-based techniques or other indirect methods, and also allows for the detection of veterinary drug residues in the same analysis, which is not possible by ELISA or PCR.

The method was developed using an Eksigent ekspert™ microLC 200 UHPLC system coupled with an AB SCIEX QTRAP® 5500 LC/MS/MS system. The method uses multiple reaction monitoring (MRM) to detect peptide markers for horse and is capable of providing sequence information by acquiring an enhanced product ion (EPI) scan for each triggering MRM which can be used to further confirm the peptide's / proteins and therefore the species identity. This gives greater confidence for food testing when distinguishing between species; for example horse and beef proteins may differ by as little as one or two amino acids.

At the same time it is also possible to detect and quantify veterinary drug residues using the same extraction method and LC conditions by simply adding additional MRM transitions to the method. Here the nonsteroidal anti-inflammatory drug (NSAID) BUTE was detected in meat samples.

Method Details

Standards

For the initial development work some of the target proteins were commercially available and therefore purchased as well as commercially available reference materials of pork, beef, and horse meat and beef reference material which had been spiked at different levels with horse meat. A sample of lamb meat was obtained from a local supermarket.

A sigma standard of BUTE was not available at the time of this work so BUTE had to be extracted from a sample of horse medicine.

Sample Preparation

The meat sample was homogenized using a food processor and mixed (2 g) with an extraction buffer containing tris (2-amino-2-hydroxymethyl-propane-1,3-diol), urea and acetonitrile (10 mL). The meat was broken up by shaking, ultra sonication (15 min) and agitated further using a roller mixer (45 min). This mixture

was centrifuged and the top liquid layer (0.5 mL) was transferred to a 2mL Eppendorf tube. The protein markers were reduced in a thermal mixer with a solution of tris (2-carboxyethyl) phosphine (TCEP, 60 min, 60°C), alkylated by adding methyl methanethiosulfonate (MMTS, 30 min, room temperature in the dark) and digested in a thermal mixer by addition of a digestion buffer containing ammonium bicarbonate, calcium chloride and trypsin (60 min, 40°C).

The filtrate was purified using a conventional conditioned polymeric SPE cartridge from Phenomenex. The peptides were extracted from the cartridge using acetonitrile and the extract was evaporated to dryness and reconstituted in acidified aqueous acetonitrile.

LC Separation

All method development and analysis was done using an Eksigent ekspert™ microLC 200 UHPLC system. Final extracted samples (10 µL) were separated over a 11 minute gradient (Table 1) where A = water and B = acetonitrile both containing 0.1 % formic acid. Peptides were separated on a reversed-phase Halo C18 2.7 µm 90Å 50 x 0.5mm (Eksigent) column at 20 µL/min and at a temperature of 40ºC.

Table 1. Gradient conditions used for separation

Time (min)	A (%)	B (%)
0	98	2
2	98	2
6	60	40
7	2	98
8.5	2	98
8.7	98	2
11	98	2

MS/MS Detection

All analyses were performed on an AB SCIEX 5500 QTRAP® LC/MS/MS system using electrospray ionization (ESI).

Initial method development was carried out using the MIDAS™ workflow (MRM-initiated detection and sequencing, Figure 1) where the electrode was changed to a microLC hybrid electrode (50 µm ID) designed for MicroLC.[2] For MIDAS a set of predicted MRM transitions from the known protein sequence were used as a survey scan to trigger the acquisition of EPI spectra (Figure 2).

This data was then submitted to a database search engine for confirmation of peptide identification and of the feasibility of the MRM transition for meat speciation. With this workflow MRM transitions were designed without the need for synthetic peptides.
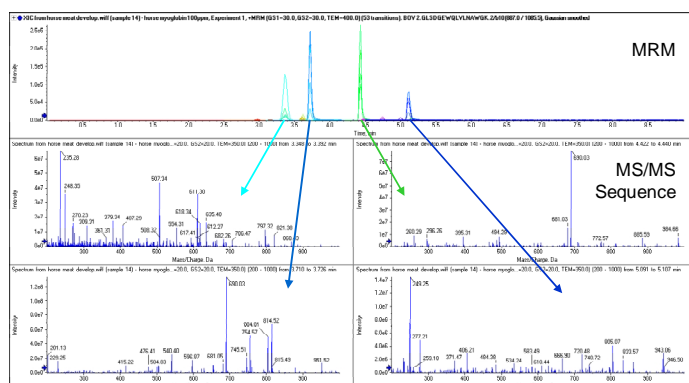

Figure 2. MRM initiated acquisition of MS/MS spectra to sequence characteristic proteins for horse meat

In the final method the Turbo V™ source conditions used were gas 1, gas 2 and the curtain gas set to 30 psi, the temperature of the source was set at 350°C and the IS voltage was 5500 V. The peptides and BUTE were analyzed using the *Scheduled* MRM™ algorithm with an MRM detection window of 50 s and a target scan time of 0.40 s. Q1 resolution was set to low and Q3 resolution was set to unit. A total of 56 MRM transitions were used over the 11 minute run time with 3 dedicated to BUTE, 12
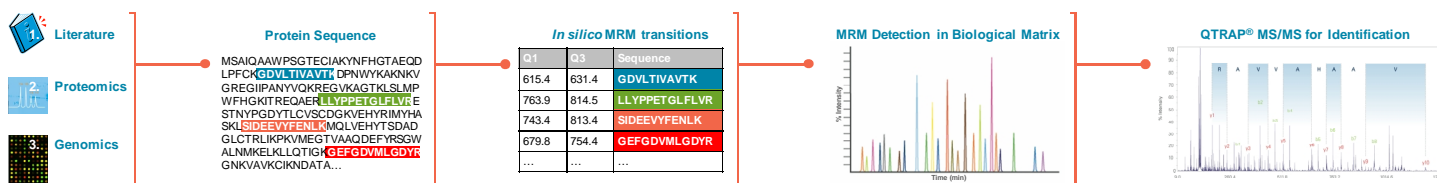

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

for horse meat (4 peptides with 3 MRM transitions each) and the rest for other meat species peptides currently under evaluation.

The MRM conditions for the detection of BUTE were taken from the MRM catalogue of the iMethod™ application for Veterinary Antibiotic Screening 1.1 (Table 2).[3]

Table 2. MRM transitions for the detection of BUTE, taken from the iMethod™ application for Antibiotic Screening

MRM transition	DP (V)	CE (V)
309/160	120	28
309/120	120	32
309/188	120	22

Results and Discussion

In the method development care was taken to make sure that peptides chosen were unique to the meat species. The list was further consolidated by removing peptides that could be susceptible to modification during food processing, e.g. undergo post translational modification or the Maillard reaction (for future application to processed meat samples). This reduced the number of peptides used as triggers for detection and generation of peptide finger prints of species.

Figure 3 shows a comparison of horse, beef, pork and lamb extracts where 4 unique peptides for horse are shown from a method which contains additional markers for other species which are currently under evaluation. This confirmed the BLAST search results for the specific peptides chosen for horse meat were specific to horse and were not seen in beef, pork and lamb.
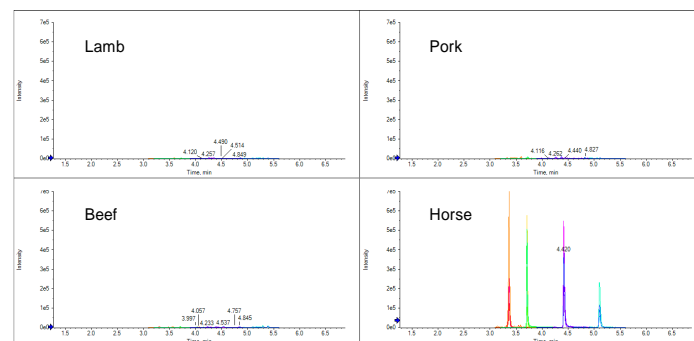

Figure 3. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

Figure 4 shows the comparison of beef and beef reference material which had been spiked at 10% and at 1% horse (current detection limit for PCR analysis).

In this figure the MRM transitions for 3 of the 4 peptides have been extracted and it shows clearly that horse meat can be detected at a 1% spike level. The fourth peptide was detected at 10% level it was below the LOD limit at 1% horse meat in beef. In order to confirm these results extraction of samples were performed multiple times and in each batch 1% horse meat could be detected in beef.
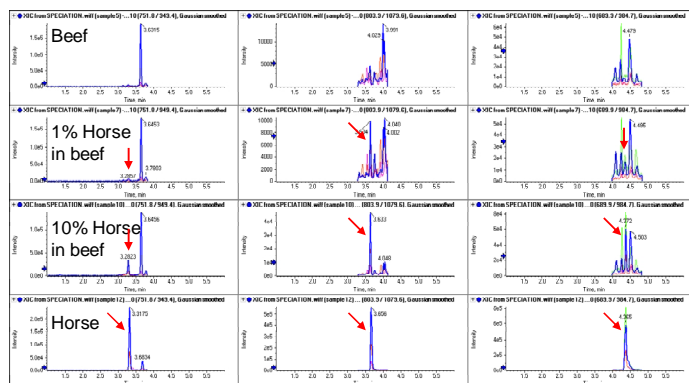

Figure 4. Detection of peptides characteristic for horse meat in beef at different levels, it shows that horse meat can be detected at a 1% level

Figure 5 shows an extracted ion chromatogram for BUTE in a standard, blank and a spiked sample of meat at a level below 10 µg/kg which had been extracted using the same protocol.
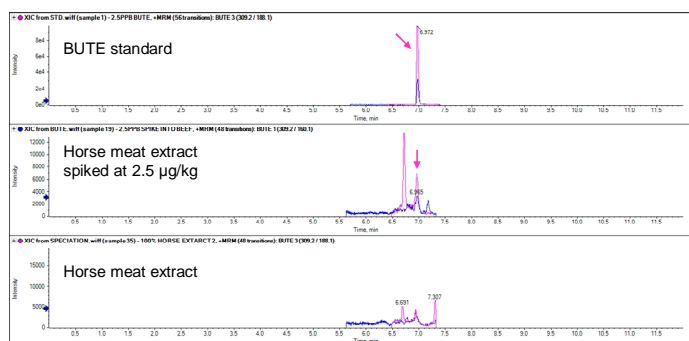

Figure 5. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

At the time of these initial tests the pure standard was not available so BUTE had been extracted from commercially available horse medicine. Levels in the extract were assumed to be lower than 10 µg/kg and this work is planned to be repeated using spiking experiments with analytical standard grade phenylbutazone. Also as this particular horse meat sample was just for speciation testing, the work will be repeated using beef which should be totally clear of BUTE.

Summary

LC-MS/MS has the potential to offer a rapid, robust, sensitive and specific assay for the simultaneous detection of a series of meat species as well as veterinary drug residues in a single analysis.

Sensitivities achieved were equivalent to sensitivities of some currently available methods based on ELISA and real-time PCR. The LC-MS/MS approach has the additional advantage of being a potential multi species screen unlike ELISA where individual meat species are detected by separate kits. By using the MIDAS™ workflow full scan QTRAP® MS/MS spectra can also be obtained at the same time as quantitative information, confirming multiple peptide target identification and reducing the occurrence of false positives associated with other techniques. Although this test is still qualitative quantitation is likely when internal standards can be used. Unlike PCR or ELISA LC-MS/MS has the ability to detect banned veterinary drug residues as well as meat speciation in the same analysis.

Acknowledgements

References

[1] http://www.food.gov.uk/enforcement/monitoring/horse-meat/

[2]. K. Mriziq et al.: 'Higher Sensitivity and Improved Resolution Microflow UHPLC with Small Diameter Turbo V™ Source Electrodes and Hardware for use with the ExpressHT™-Ultra System' Technical Note Eksigent (2011) # 4590211-01

[3] http://www.absciex.com/products/methods/imethod-applications-for-food-and-beverage/imethod-application-for-antibiotic-screening-version-13-for-cliquid-software