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.

Method Details

**Standards**

For the initial development work some of the target proteins were commercially available and therefore purchased in addition to 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 homogenised using a food processor and mixed (2g) with an extraction buffer containing tris (2-amino-2-hydroxymethyl-propane-1,3-diol), urea and acetonitrile (10mL). 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 (0.5mL) 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 (10µL) 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 2.7µm 90Å 50 x 0.5mm (Eksigent) column at 20µL/min and at a temperature of 40°C.

**Table 1**. Gradient conditions used for separation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>8.5</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>8.7</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>98</td>
<td>2</td>
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</table>
MS/MS Detection

All analyses were performed on an AB Sciex 5500 QTRAP® LC/MS/MS system using electrospray ionisation (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 (50µm 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).

These data were 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.

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

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

<table>
<thead>
<tr>
<th>MRM transition</th>
<th>DP (V)</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>309/160</td>
<td>120</td>
<td>28</td>
</tr>
<tr>
<td>309/120</td>
<td>120</td>
<td>32</td>
</tr>
<tr>
<td>309/168</td>
<td>120</td>
<td>22</td>
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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 fingerprint 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.

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% 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 for 3 of the 4 marker peptides (the 4th marker with a higher LOD with horse meat detectable at 10%).

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% 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 for 3 of the 4 marker peptides (the 4th marker with a higher LOD with horse meat detectable at 10%).

Figure 5 shows an extracted ion chromatogram for Bute in a standard, blank and a spiked sample of meat at a level below 10µg/kg which had been extracted using the same protocol.
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 10µg/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 speculation 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 EUSA and real-time PCR. The LC-MS/MS approach has the additional advantage of being a potential multi species screen unlike EUSA 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 EUSA LC/MS/MS has the ability to detect banned veterinary drug residues as well as meat speciation in the same analysis.

Acknowledgements

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References

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