Can LC-MS/MS be Used in Meat Speciation and Authenticity Testing?

Stephen Lock, AB Sciex, Warrington, Cheshire (UK)

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.

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 peptides / 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 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

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

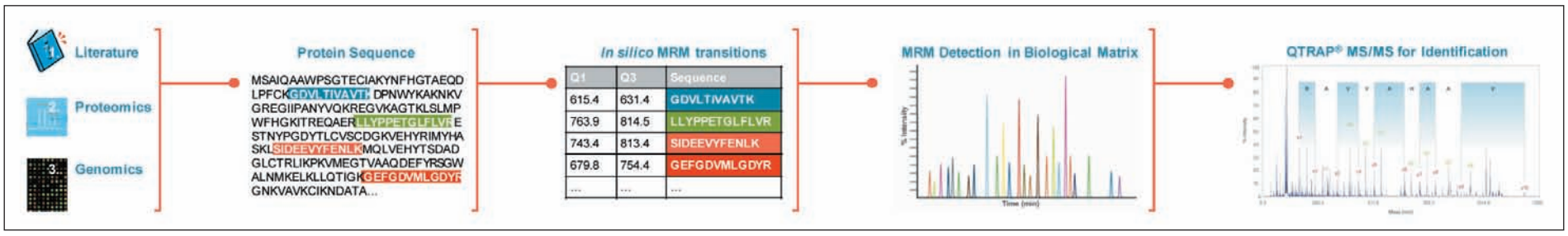

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

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.

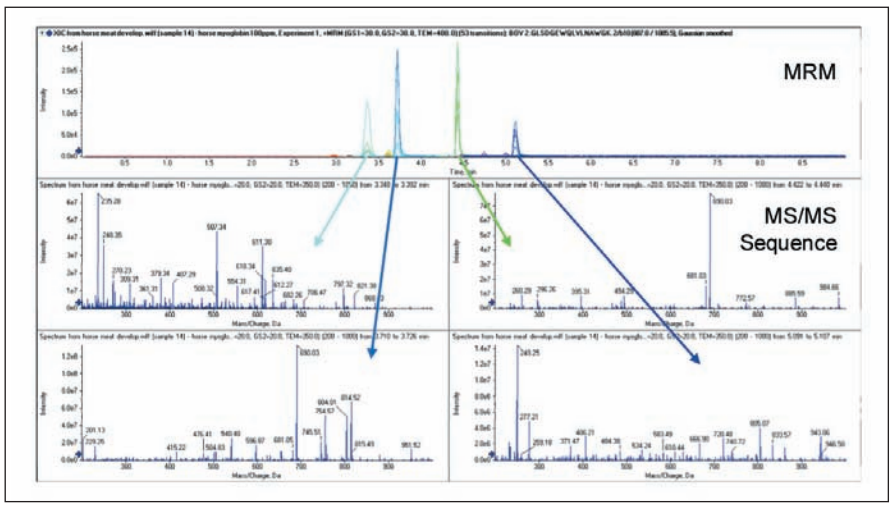

Figure 2. MRM initiated acquisition of MS/MS spectra to sequence characteristic proteins for horse meat

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 analysed 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.

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.

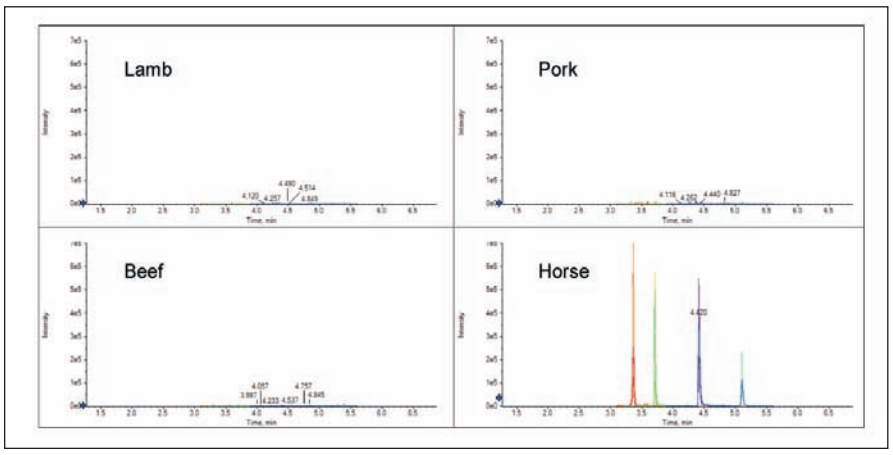

Figure 3. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

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% 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 LOQ with horsemeat detectable at 10%).

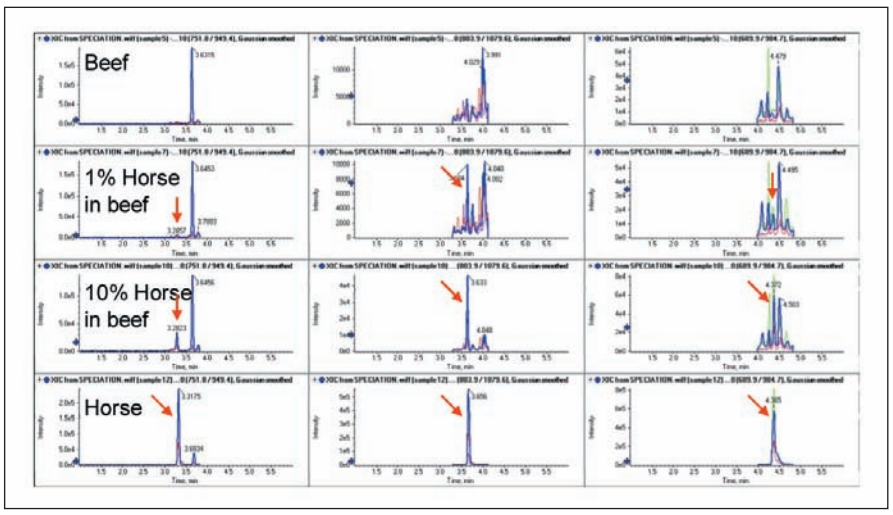

Figure 4. Detection of peptides characteristic for horse meat in beef at different levels, it shows that horse meat can be detected at a 1% level.

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

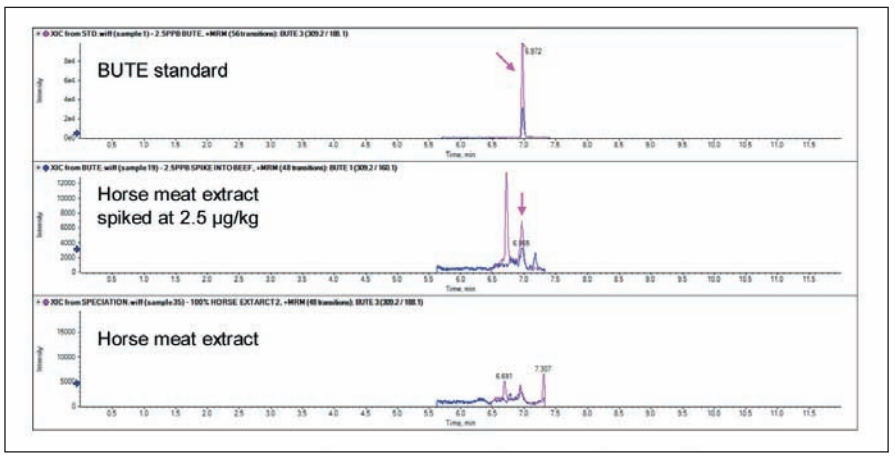

Figure 5. A comparison of the analysis of extracts from different types of meat. These initial results were obtained during the development of the method.

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 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 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 LCMS/ 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

1. 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. www.absciex.com/products/methods/imethodapplications-for-food-and-beverage/imethod-application-forantibiotic-screening-version-13-for-cliquid-software

Networked Solution Provides Compliant Storage of ICP-MS Data

Agilent Technologies Inc have announced OpenLAB Data Store for MS, a simple and affordable networked solution for compliant storage of mass spectral data acquired with Agilent's ICP-MS MassHunter system. The software is designed for medium to small laboratories measuring inorganic impurities in pharmaceutical products and ingredients in accordance with upcoming specifications from the United States Pharmacopoeia.

The OpenLAB Data Store for MS bundle allows customers to grow their compliant data storage to a second ICP-MS system at a lower price than standalone workstation solutions.

The United States Pharmacopoeia has proposed two new general chapters to test for inorganic impurities in pharmaceutical products and ingredients: Elemental Impurities—Limits <232> and Elemental Impurities—Procedures <233>. Effective May 2014, the new chapters specify maximum daily dose limits for 15 elements.

These elemental impurities are most efficiently measured using ICP-MS because of its low detection limits, dynamic range and throughput. Electronic management of the ICP-MS data is required as defined in Part 11 of the U.S. Food and Drug Administration's Code of Federal Regulations (21 CFR Part 11) and the European equivalent (EU Annex 11).

"Target ICP-MS labs need a networked solution for US FDA 21 CFR Part 11 and EU Annex 11 compliant data storage that's affordable and easy to use," said Bruce von Herrmann, Vice President and General Manager of Agilent's Software and Informatics Business. "In combination with an overall laboratory compliance plan, an ICP-MS MassHunter system with OpenLAB Data Store for MS provides controls needed to follow regulatory guidelines."

OpenLAB Data Store for MS is part of an industry-leading suite of software products designed to integrate and manage scientific information throughout its lifecycle, across the laboratory and the enterprise. It complements Agilent's OpenLAB Enterprise Content Manager software for the compliant management of multi-vendor, multi-instrument data in larger laboratories. OpenLAB Enterprise Content Manager is fully scalable to a worldwide deployment.

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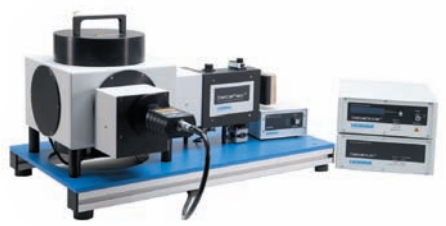
New Generation of Turnkey TCSPC Fluorescence Lifetime Systems



Horiba Scientific announces the Delta Series, their newest generation of time-correlated single photon counting (TCSPC) fluorescence lifetime systems. They are designed to be faster, simpler, and more affordable than any other lifetime solution on the market.

The Delta series features the most advanced TCSPC system controller, the widest array of sources (up to 100 MHz operation), the widest lifetime ranges (ps to sec); virtually unlimited configurability with Horiba's new F-Link plug-and-play architecture; and the industry's most advanced lifetime modelling software. The lowest deadtime of any TCSPC system makes the Delta series the fastest lifetime system on the market, with nearly lossless counting at up to 100 MHz and total lifetime measurement times as short as 1 ms.

The Delta series includes DeltaPro, an affordable and simplest to use filter-based lifetime system with performance that rivals most high-end systems. DeltaFlex, a fully modular system, seamlessly integrates excitation and emission monochromators, the widest array of sources (diodes, laser diodes, supercontinuum lasers) and detectors (including NIR), for flexibility and upgradability.

DeltaPro has simplified the complexity of time-correlated single photon counting (TCSPC) so any lab can take advantage of the power of fluorescence dynamics using TCSPC, at a starting price of $30k USD. The DeltaPro takes advantage of interchangeable pulsed laser-diode and LED light-sources. The NanoLED and DeltaDiode range of sources cover discrete emission wavelengths from 250nm to the near-IR and enables measurement of lifetimes from ps to µs. By simply adding a SpectraLED source to the system, phosphorescence lifetimes of µs to 1 second can also be measured.

DeltaFlex offers complete flexibility. The system is designed to measure luminescence lifetimes ranging over 11 orders of magnitude, without the need to change cables or cards. Comprised of Horiba's high repetition rate sources, high speed detectors and ultra-low deadtime electronics, the DeltaFlex enables fast and efficient acquisition of lifetime data.

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