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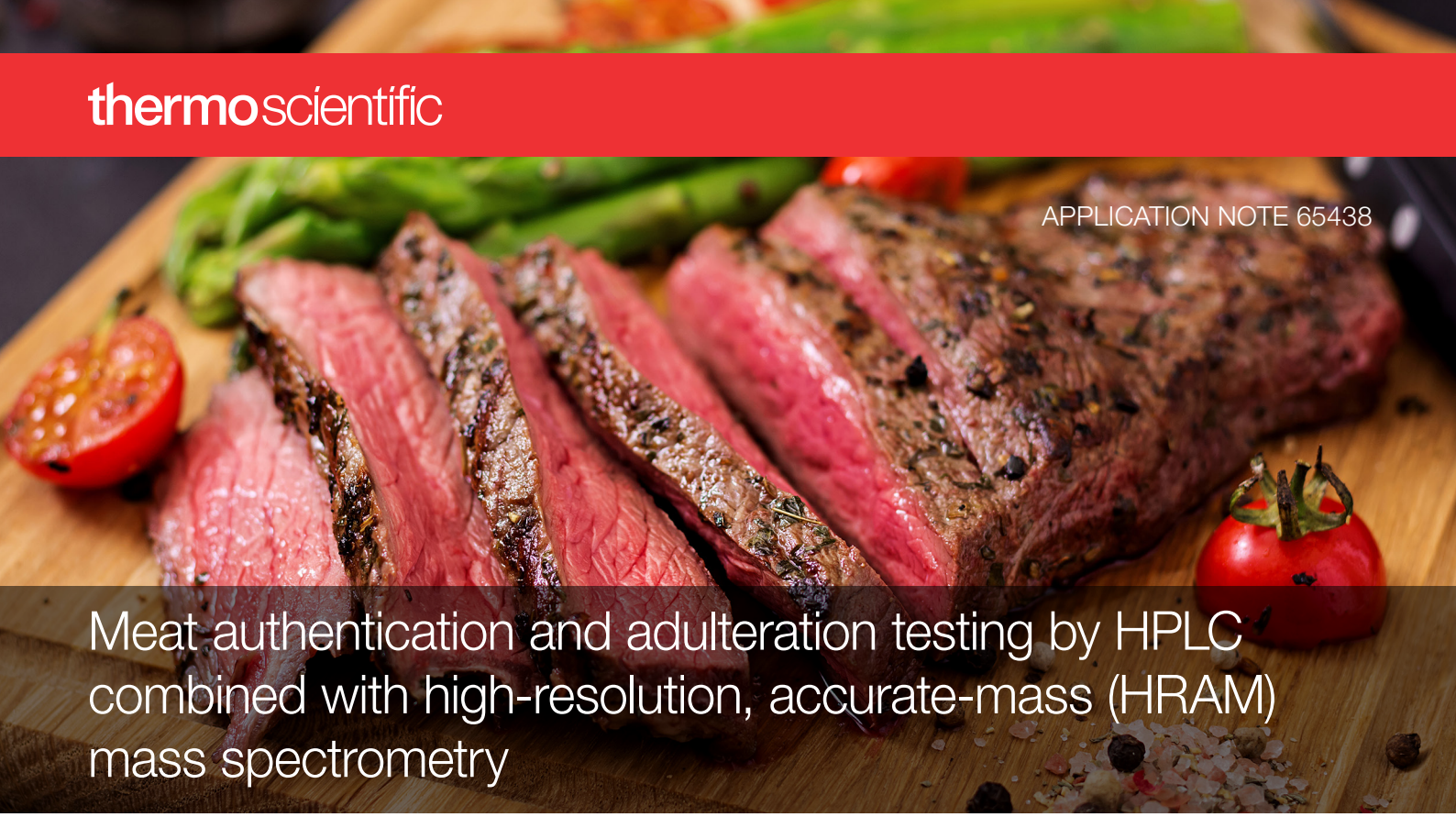
APPLICATION NOTE 65438



Meat authentication and adulteration testing by HPLC combined with high-resolution, accurate-mass (HRAM) mass spectrometry

Authors

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Keywords

High-resolution mass spectrometry, high-performance liquid chromatography, proteomics, bioinformatics, food, meat, adulteration

Goal

Develop a simple, reliable, robust, sensitive, and specific HPLC-HRAM-MS assay for the detection of pork meat at low levels in meat products using specific peptide biomarkers.

Introduction

Due to the internationalization of food production and distribution, there has been a significant increase in food fraud in recent years.[1,2] Food fraud can have serious health implications and occurs when food manufacturers implement unethical practices such as making false label claims or using additives and fillers within their products to increase profitability. For example, in 2013 horse and pig DNA were detected in labeled beef products sold by numerous retailers.[3] In an effort to put a stop to this practice within the food industry, certification of meat authenticity must be delineated for all regulatory agencies. Additionally, sensitive and selective methods are required to detect meat adulteration.

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Meat authenticity in food testing laboratories has been traditionally performed using polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). These methods require intensive customization to achieve the required sensitivity and accuracy. In addition, the molecular information obtained is incomplete and data mining cannot be performed post-analysis. These are serious limitations for public health and food safety investigations.

Mass spectrometry (MS) has become the workhorse in protein research for many areas in medical and biological sciences. It is now becoming an important asset in foodomics, a relatively new science.[4] A major challenge in proteomics research is related to the analysis of highly complex biological samples. As sample complexity increases, proteomic research requires powerful analytical instruments with high sensitivity, selectivity, and a large dynamic range. High-resolution mass spectrometry technology is an important asset to perform such complex assays. New technologies have the speed, resolution, mass accuracy, and sensitivity to deliver comprehensive qualitative exploration, rapid profiling, and high-accuracy detection and quantification of proteins in biological samples. Proteomic methods can be definitely implemented for routine analysis executed in food chemistry laboratories. However, we believe it requires a systematic approach based on a clear and well-defined proteogenomic annotation to carefully select surrogate tryptic peptides for speciation using a semi- or targeted MS-based method.

The global objective of this study was to develop a novel analytical strategy using a state-of-the-art high-resolution Orbitrap™ mass spectrometer to determine meat authenticity and adulteration. The specific objectives of this study were as follows:

- To perform *in silico* characterization of targeted muscular proteins (e.g. myoglobin) of selected mammalian species and identification of proteotypic peptides
- To detect and identify targeted proteotypic peptides by mass spectrometry using proteomic bottom-up approaches
- To demonstrate the selectivity, sensitivity, and applicability of the proposed analytical strategy for the assessment of meat authenticity and adulteration

Experimental

Chemicals and reagents

Proteomic grade trypsin, dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (NH_4HCO_3), hydrochloric acid (HCl), formic acid, water (LC-MS Optima™ grade), and acetonitrile (LC-MS Optima grade) were used (all Fisher Scientific).

Bioinformatic analyses

Protein alignment and sequence analyses were performed using QIAGEN® CLC Sequence Viewer 7.7 (Redwood City, CA, USA). Nucleotide and protein sequences were obtained from the National Center for Biotechnology Information (NCBI) databases. *In silico* protein digestions, peptide mass fingerprinting, and MS^2 fragment ion prediction were performed using mMass software.[5] Additional, comprehensive protein identifications were performed using Thermo Scientific™ Proteome Discoverer™ software along with Mascot® database search (Matrix Science, Boston, MA, USA).

Protein extraction from meat products

Briefly, 2 g of raw meat was mixed with 10 mL of distilled water. The mixture was blended at high speed for 3 min to obtain a homogeneous suspension. The sample was sonicated for 30 min at room temperature, followed by centrifugation at 1500 g for 5 min to remove debris. Five hundred microliters of the suspension were transferred to a microcentrifuge tube. Proteins were precipitated with 500 µL of acetone. Then the acetone was discarded, and the protein pellet was dried for 20 min in a vacuum centrifuge set at 60 °C. The protein pellet was dissolved in 500 µL of 100 mM ammonium bicarbonate (pH 8.5) and the solution was sonicated for 60 minutes at maximum intensity to improve protein dissolution yield. The proteins were denatured by heating at 120 °C for 10 min using a block-heater. The solution was allowed to cool for 15 min and proteins were reduced with 20 mM DTT. The reaction was performed at 60 °C for 60 minutes, then proteins were alkylated with 40 mM IAA and the reaction was performed at room temperature for 30 min. Two micrograms of proteomic-grade trypsin were added, and the digestion was performed at 40 °C for 24 h. The protein digestion was quenched by adding 500 µL of a 1% TFA solution. Samples were centrifuged at 12,000 g for 10 min, and 200 µL of the supernatants were transferred into injection vials for analysis.

Chromatographic conditions	
HPLC system:	Thermo Scientific™ UltiMate™ 3000 Rapid Separation UHPLC
Column:	Thermo Scientific™ Biobasic™ C8 microbore column, 100 × 1 mm, with a particle size of 5 µm
Mobile phase A:	0.1% formic acid in acetonitrile
Mobile phase B:	0.1% formic acid in water
Flow rate:	75 µL/min
Injection volume:	2 µL
Gradient:	See Table 1

Table 1. HPLC gradient conditions used for separation

Time	%A 0.1% Formic acid in acetonitrile	%B 0.1% Formic acid in water
0	5	95
1	5	95
31	50	50
33	50	50
33.2	5	95
47	5	95

Mass spectrometry conditions	
MS system:	Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer
Ion source:	Pneumatic-assisted heated electrospray ion source
Ion mode:	Positive
Scan mode:	Full scan, Parallel reaction monitoring (PRM), or Data-independent acquisition (DIA)
Sheath gas (N_2):	10 arb
Auxiliary gas (N_2):	5 arb
Spray voltage:	4000 V
Ion transfer tube temperature:	300 °C
Default scan range:	*m/z* 500–1500
Resolution:	140,000 (FWHM)
Automatic gain control target:	3.0 x 10^6
Maximum ion injection time:	200 ms

Mass spectrometry conditions (continued)	
	PRM mode
Resolution:	17,500 (FWHM)
Automatic gain control target:	1.0 x 10^6
Maximum ion injection time:	100 ms
Collision energy:	25 eV
Isolation window:	1.5 Da
	DIA mode
Survey scan:	Range: *m/z* 600–1200 Resolution: 70,000 (FWHM)
MS^2 scan events:	Number: 12 Resolving power: 17,500 (FWHM) Isolation window: 50 Da Loop count: 6 events

Instrument calibration was performed prior to all analyses and mass accuracy was notably below 1.5 ppm using Thermo Scientific™ Pierce™ external calibration solution and the automated instrument protocol.

Results and discussion

Bioinformatic analysis

The method proposed in this application note is based on a targeted peptide mass fingerprinting (PMF) method that specifically relies on upstream identification of species-specific proteolytic fragments that would allow meat speciation without having to perform an in-depth PMF analysis using post-acquisition data sets. An exhaustive PMF analysis on meat products reveals proteotypic peptides from myoglobin, myosin, and hemoglobin.[6] Based on these preliminary results, we performed an exhaustive sequence analysis of myoglobin, myosin, and hemoglobin using bioinformatics for four common mammalian species. Figure 1 illustrates the result obtained for myoglobin, a very specific muscular protein. The analysis revealed a specific fragment (myoglobin tryptic peptide 120–134) that can be used for speciation in a targeted method since all four species presented amino acid sequence differences resulting in characteristic precursor masses and fragment ions. Other tryptic peptides were identified for myosin-1, myosin-2, and β-hemoglobin and will be discussed subsequently. These results allowed the constitution of a precursor mass list, and the use of peptide MS^2 spectrum predictors to identify precursor peptide-to-fragment ion transitions.

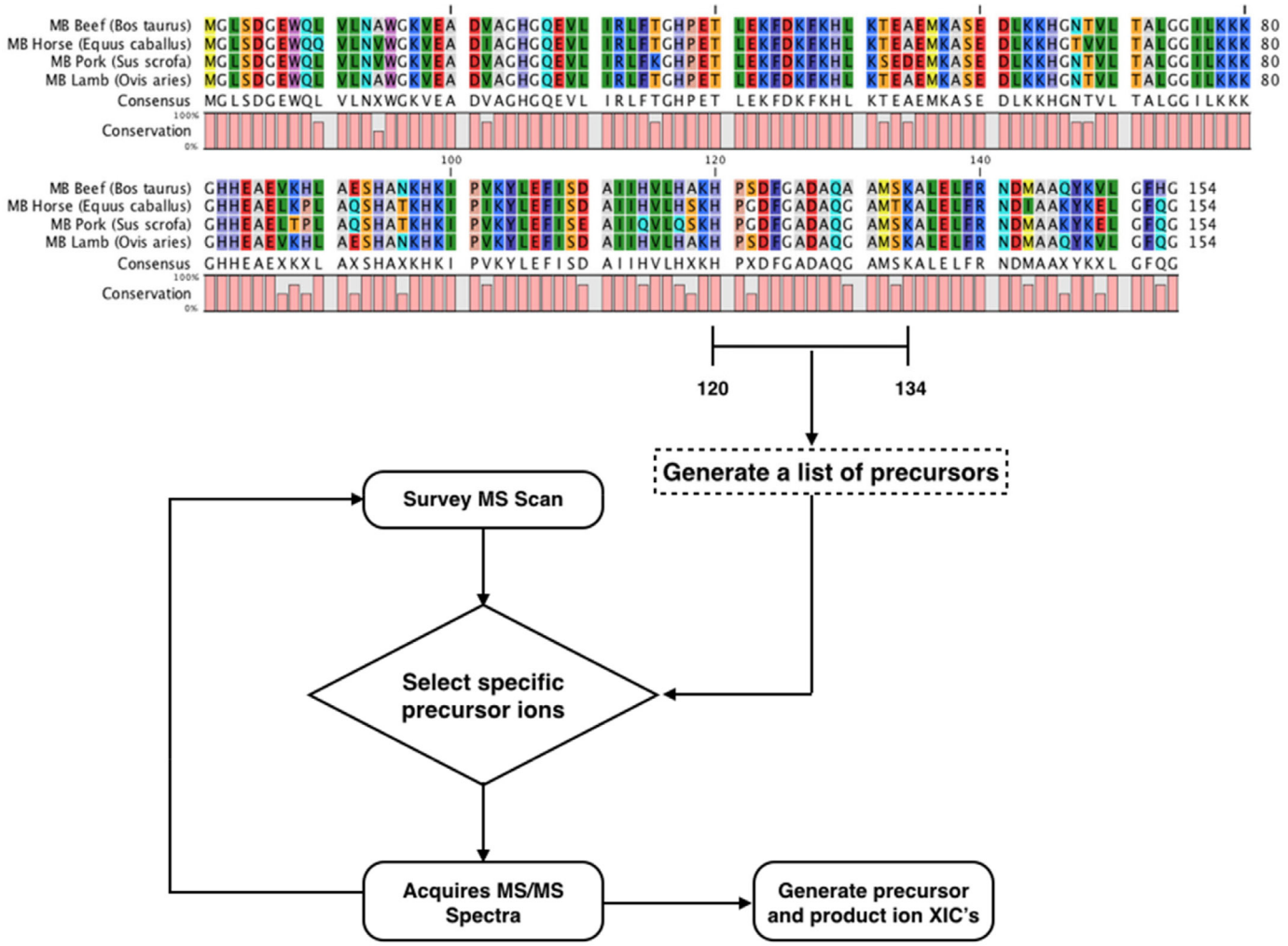

Figure 1. Bioinformatic analysis of targeted mammalian muscular proteins. Species-specific myoglobin (MG) sequences were aligned and thoroughly analyzed. Proteotypic peptides were identified located between amino acid position 120 and 134. Thus, a specific precursor ion mass list can be generated and MS^2 experiments can be performed on species-specific biomarkers.

High-resolution mass spectrometry analysis

The exhaustive analysis of complex proteomic samples is an important challenge in proteomics research and mass spectrometry. Thus, the MS resolution and mass accuracy are determining factors to improve the accuracy of peptide identification and assignment. The analyses of meat samples were performed using a hybrid quadrupole-Orbitrap mass spectrometer operating in MS at a resolution of 140,000 (FWHM) at *m/z* 200 and in MS^2 at a resolution of 17,500 (FWHM) at *m/z* 200. As shown in Figure 2, extracted ion chromatograms (XICs) of each targeted proteotypic myoglobin peptide were detected for each selected mammalian species. Detailed analysis of MS spectra revealed that mass accuracies ranged from -0.67 to 1.34 ppm for targeted proteotypic myoglobin peptides as indicated in Table 2. Additionally, the MS peak abundance for these specific peptides were in the top tier relative to the abundance of each ion observed.

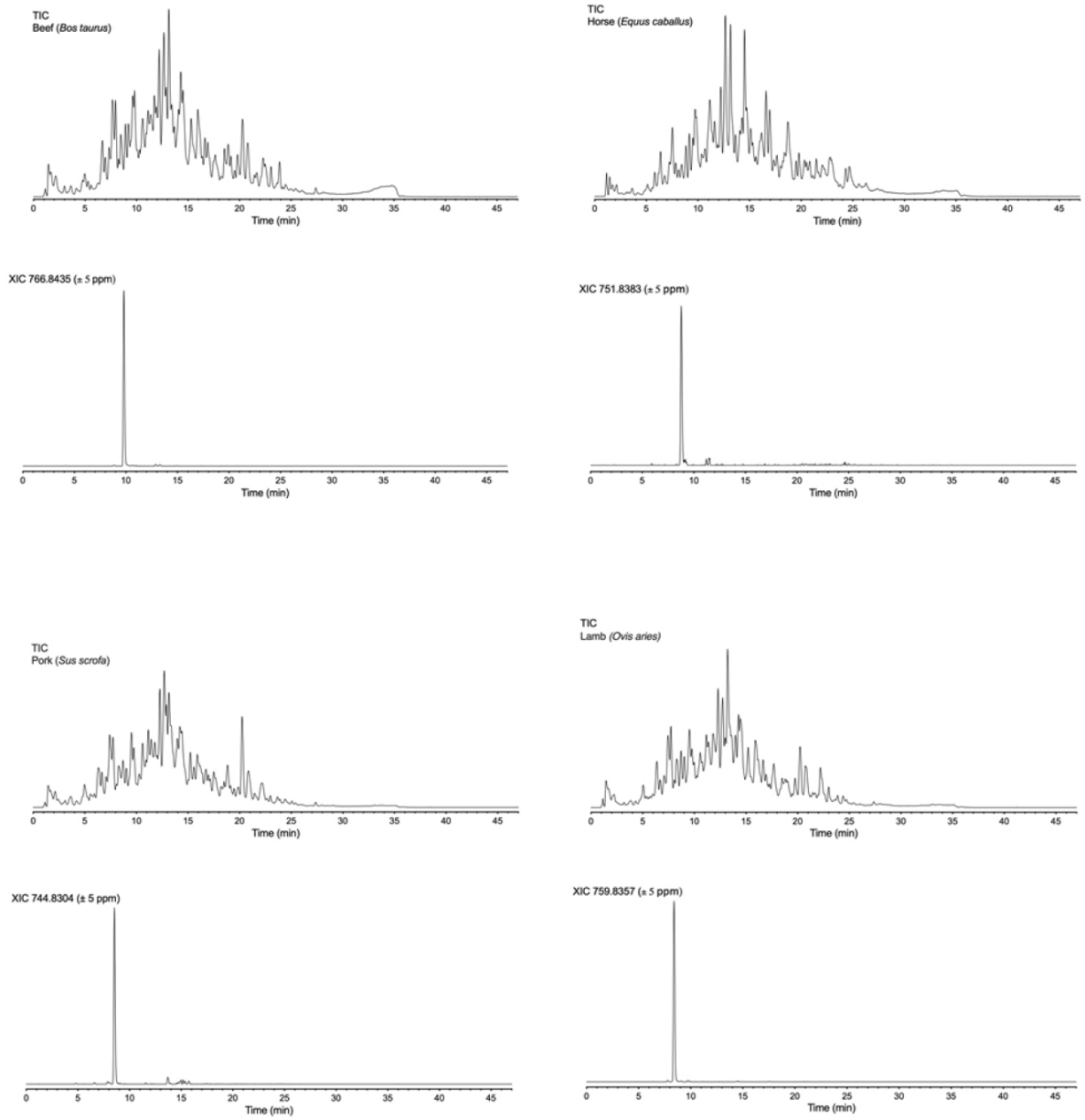

Figure 2. Total ion chromatograms (TICs) and specific XICs for each mammalian meat sample are presented. XICs were generated using the theoretical mass value with a ±5 ppm extraction window. TICs show the high complexity of the samples. The XICs produced from selected mammalian meat samples suggest high abundance for the myoglobin proteotypic peptide identified following an in-depth *in silico* investigation.

Table 2. Specific myoglobin proteotypic peptides for selected mammalian meat species

Species	Tryptic peptide sequence MB (120-134)	Theoretical mass (z=2)	Observed mass (z=2)	Mass accuracy (ppm)
Beef	HPSDFGADAQAAMSK	766.8435	766.8436	0.13
Horse	HPGDFGADAQGAMTK	751.8383	751.8378	-0.67
Pork	HPGDFGADAQGAMSK	744.8304	744.8314	1.34
Lamb	HPSDFGADAQGAMSK	759.8357	759.8363	0.79

This is particularly important since accuracy, sensitivity, and robustness are critical in ensuring that the method can be used for routine analysis. MS^2 spectra were collected for each peptide at high resolution. Tandem MS spectra of targeted proteotypic myoglobin peptides shown in Figure 3 are dominated by y-type fragment ions with low abundance b ions, based on the Roepstorff and Fohlman nomenclature.[7] The MS^2 spectra were coherent with the amino acid sequence of each peptide. Interestingly, y_{14} and y_{13} product ions were observed and are specific for each targeted peptide sequence. These product ions can be used to generate very specific product ion XICs. The acquisition of MS^2 data were performed using parameters displayed in Table 3. Overlay XICs of chromatograms using y_{14} and y_{13} product ions are shown in Figure 4, and results clearly demonstrate very high specificity to each targeted species. The combination of MS and MS^2 acquired in HRAM mode allowed targeted qualitative screening and identification to assess meat authenticity using a specific proteotypic myoglobin peptide.

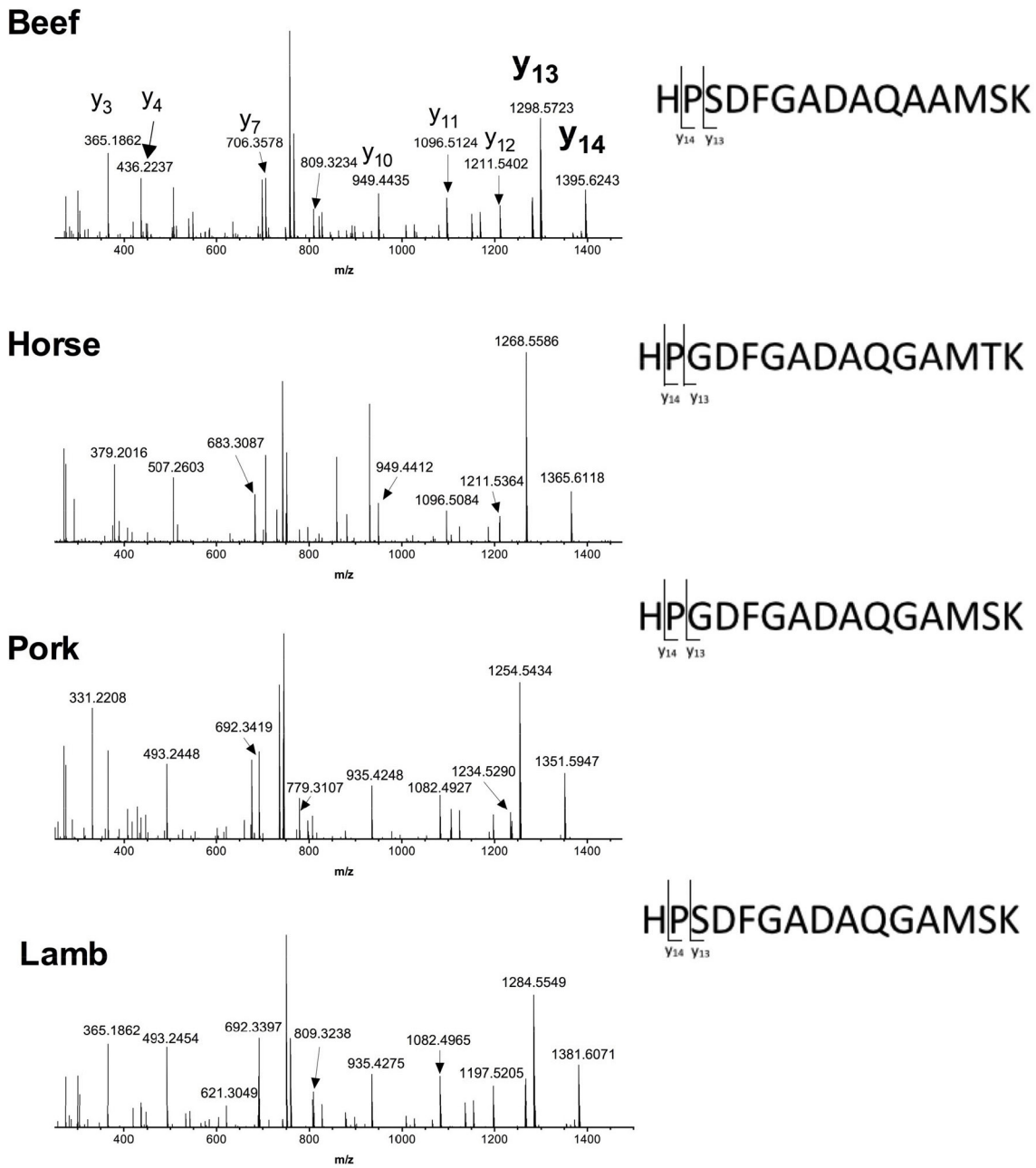

Figure 3. Product ion spectra of myoglobin proteotypic peptides (120–134). Fragment ion selectivity is preserved for y_{14} and y_{13} ions. These products can be used to produce specific product ion XICs for meat speciation.

Table 3. PRM parameters of targeted myoglobin proteotypic peptides (MB 120–134)

Species	Targeted peptide	Precursor ion *m/z* (z=2)	Collision energy	Product ion *m/z* (z^1=1)
Beef	HPSDFGADAQAAMSK	766.8	25	1298.5681 (y_{13}) 1395.6209 (y_{14})
Horse	HPGDFGADAQGAMTK	751.8	25	1268.5576 (y_{13}) 1365.6103 (y_{14})
Pork	HPGDFGADAQGAMSK	744.8	25	1254.5419 (y_{13}) 1351.5947 (y_{14})
Lamb	HPSDFGADAQGAMSK	759.8	25	1284.5525 (y_{13}) 1381.6053 (y_{14})

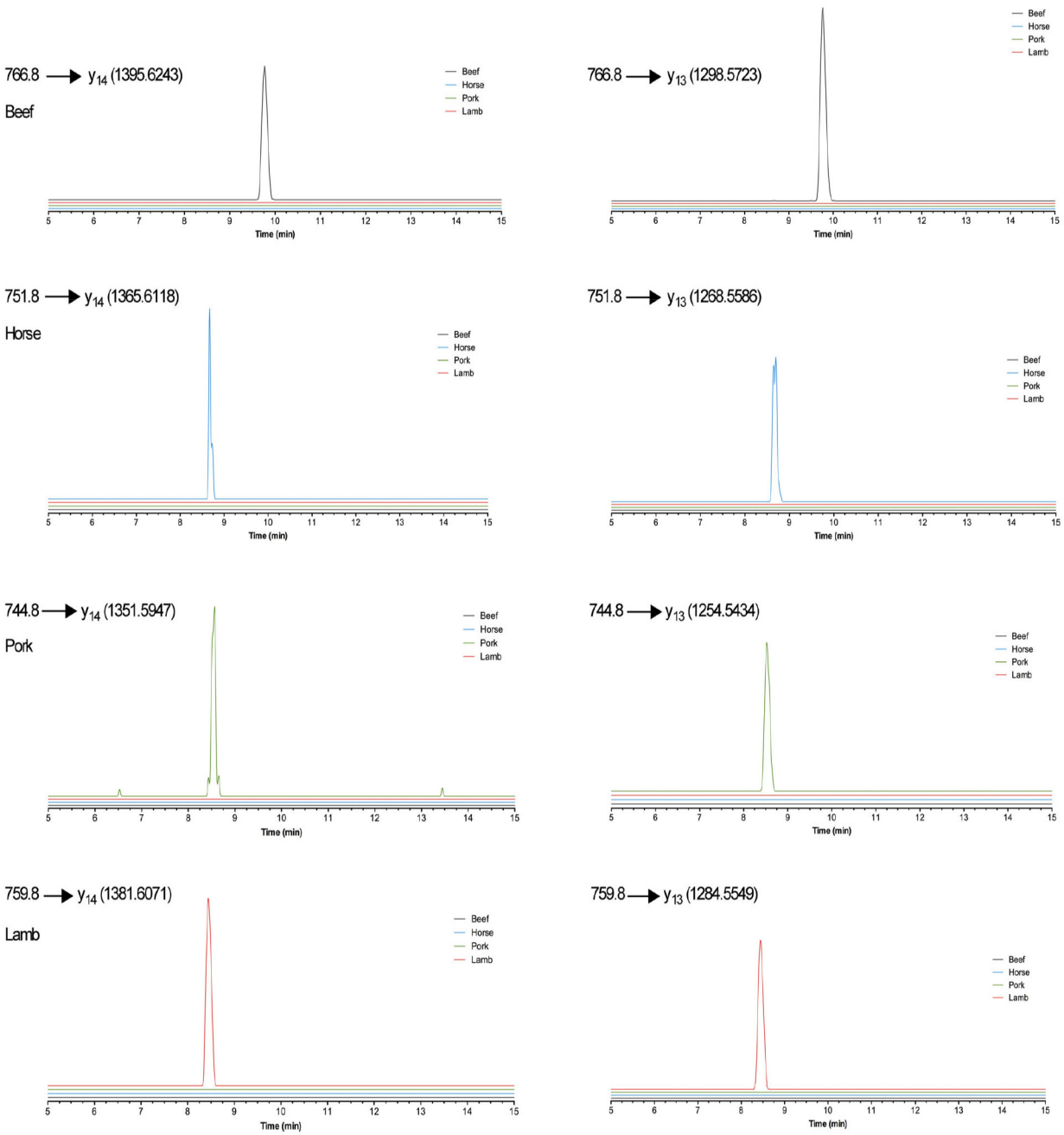

Figure 4. Comparison of y_{14} and y_{13} product ion XICs. Both fragment ions are very specific to each tested species allowing accurate meat speciation based on MS and MS^2 XICs.

Meat adulteration experiments

Economically motivated adulteration of food often involves the modification of meat composition by unethical food manufacturers. Therefore, we initially performed testing at an adulteration level of 1% to demonstrate the specificity of the assay. Raw pork was added to raw beef, lamb, and chicken at a level of 1% (w/w). Experiments were conducted comparing the PRM and DIA strategies. As shown in Figure 5, with the pork proteotypic myoglobin peptide identified, specificity is greatly improved in PRM where literally no interfering signal was present in any of the test samples. Also, precursor peptide-to-fragment ion transition ratio (y_{14} and y_{13}) can be effectively used for adulteration confirmation. Interestingly, similar results were obtained using a DIA approach (50 Da window). PRM or DIA methods can be successively used with different purposes, but by using either approach we were able to detect adulteration at a 1% (w/w) level. The DIA approach provides a more comprehensive data set that can be used for further data mining. However, instrument sensitivity is an issue for detecting levels <1% (w/w). PRM analyses at 0.2% (w/w) were successfully performed and the method can be adapted to detect levels <0.2% (w/w). The need to detect undesired species <1% can be motivated by frequent cross sample contamination rather than adulteration.

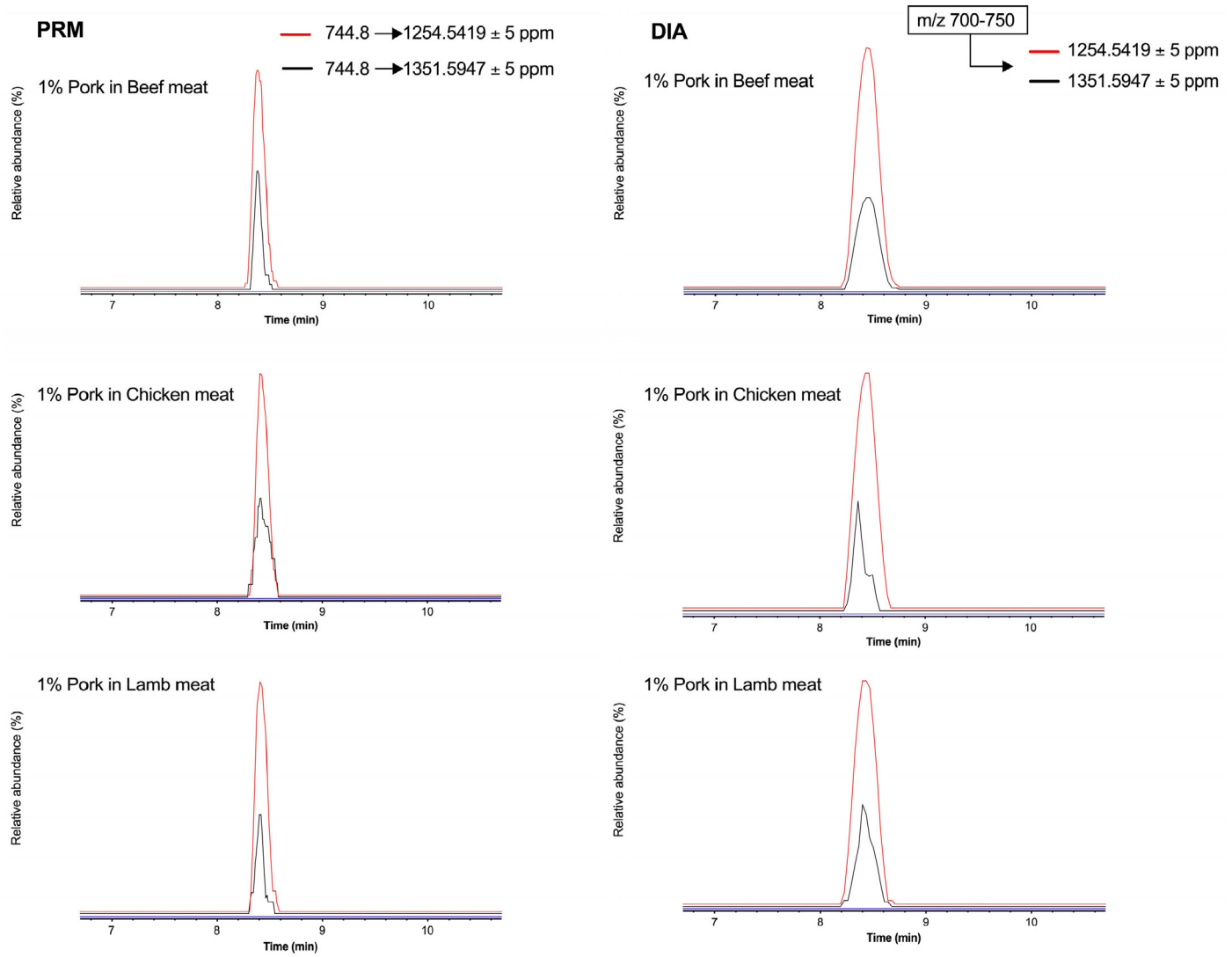

Figure 5. XICs for specific signature myoglobin proteotypic peptide-fragment pairs. Chromatograms from meat samples spiked with 1% pork meat. (Extracted blank chromatograms are in blue.)

Additional analysis

As shown in Figure 6, other proteotypic peptides were identified. As we outlined before, we methodically compared candidate proteotypic peptide sequences to outline sequences differences that would lead to specific precursor masses. Also, using *in silico* generated b and y fragments, we compared each species using Venn diagrams to identify all specific collision-induced dissociation (CID) fragments. This exercise is specifically useful for a DIA analysis. From these analyses, we generated an exhaustive product ion mass list based on each intersection tested to generate a set of b and y fragment ions unique to pork and horse species to allow adulteration detection in meat samples. Proteopytic peptides from myosin-1, myosin-2, and β-hemoglobin were identified. MS and MS^2 spectra were coherent with the amino acid sequence for each of these other peptides. They can be used for confirmatory and complementary measures to provide maximum sensitivity and accuracy. The method workflow is explained in Figure 7 and can be easily adapted for specific animal species.

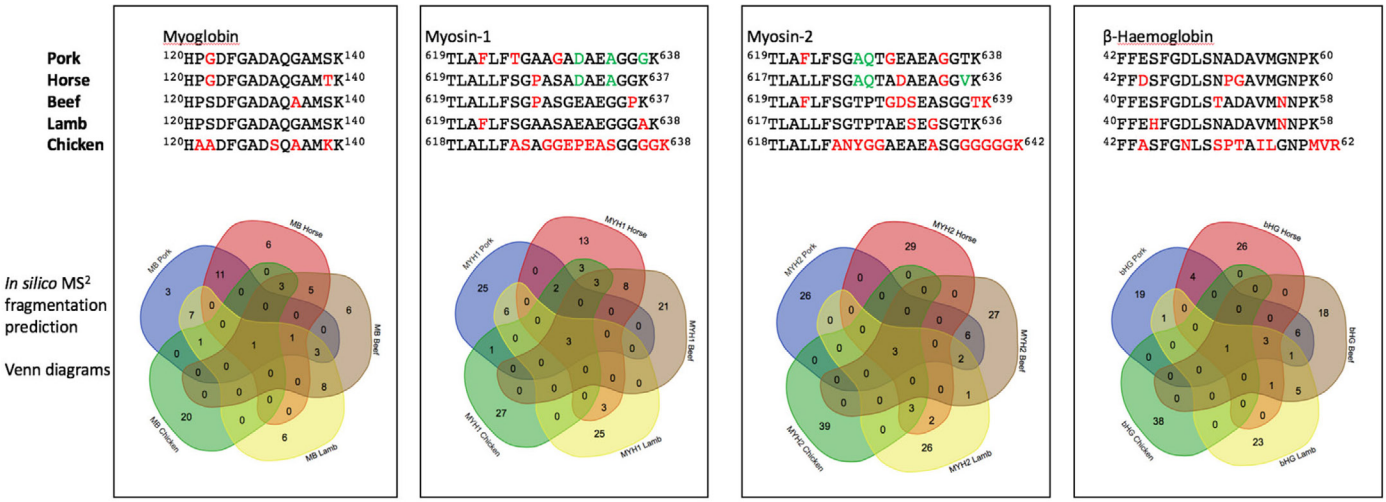

Figure 6. *In silico* sequence analysis of targeted muscular proteins led to the identification of four proteotypic peptides from myoglobin, myosin-1, myosin-2, and β-hemoglobin. Sequence consensuses (≥60%) are shown in black. Red and green exhibit distinct amino acid elements to allow speciation. Venn diagrams were generated using *in silico* generated MS^2 b and y ions and shown speciation can be effectively performed using specific precursor and product ion pairs.

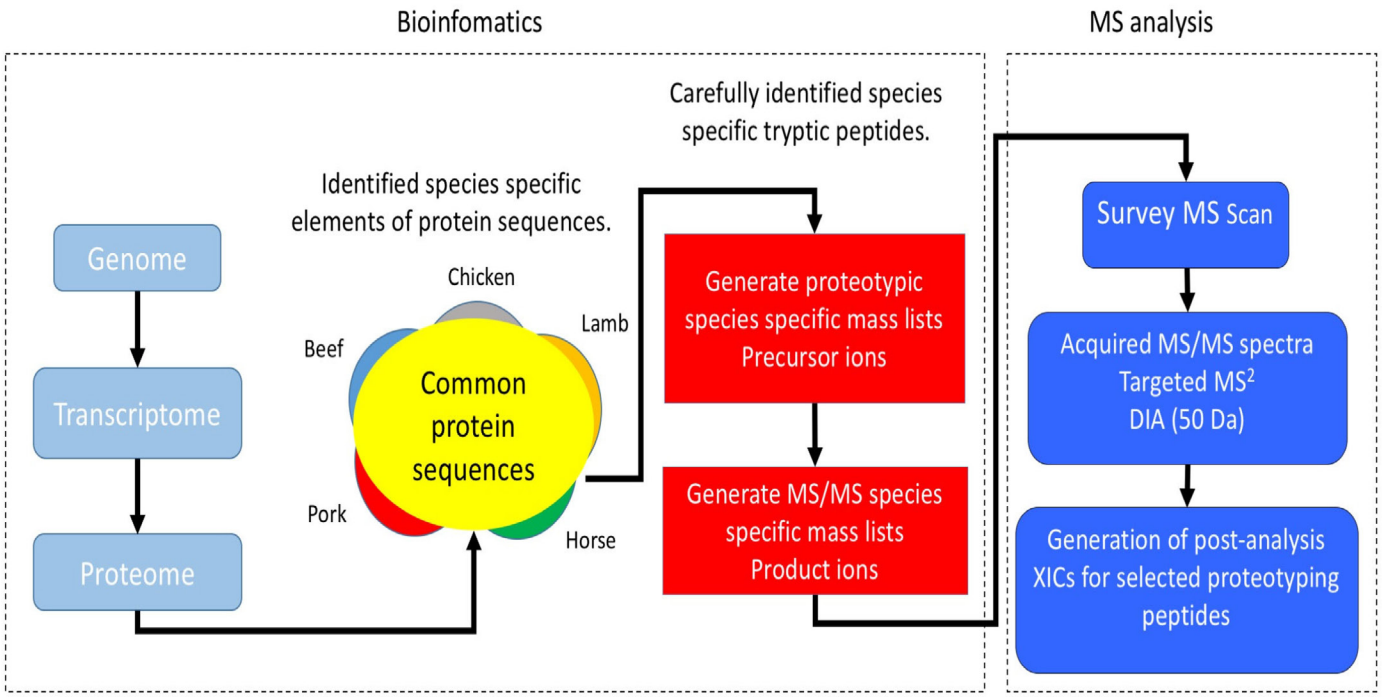

Figure 7. Illustration of the analytical strategy used based on a proteogenomic approach to develop a targeted mass spectrometry-based proteomic method for meat authenticity and adulteration

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Conclusion

The application of a bottom-up proteomic strategy applied to meat speciation was tested using targeted tryptic peptide biomarkers. As shown, myoglobin proteotypic peptides can be used to preform meat speciation. Moreover, thorough *in silico* protein sequence analysis and tryptic digestion allowed the identification of three other proteotypic peptide biomarkers (i.e. myosin-1 619–638, myosin-2 619–639, and β-hemoglobin peptide 40–58). PRM or DIA methods can be successively used with different purposes. PRM methods can be fully validated for meat authentication and adulteration analysis. Moreover, labeled internal standards can be synthesized and used for quality control purposes in routine analysis laboratories. For research, DIA provides a more detailed data set and allows comprehensive data mining.

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