USE OF PEPTIDOMICS IN DIFFERENTIATING HORSE FROM BEEF MEAT

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Abstract – The aim of this work was to develop a peptidomic strategy capable to differentiate horse from beef meat with high confidence. The method is based in the specific detection, using mass spectrometry, of marker peptides derived from the trypsin hydrolysis of bovine and equine myoglobin. We have identified two peptides in position 120-134 of this protein that are characteristic of each one of these animal species, thus allowing differentiating one type of meat form the other. This approach represents an attractive alternative to develop robust and reliable new methodologies capable to overcome the existing limitations of the methods currently in use to assess meat composition in foodstuffs.

Key Words – Meat authentication, Mass spectrometry, Peptide biomarkers, Horse, Food fraud

I. INTRODUCTION

In modern society, meat authentication has become an issue of primary importance as can be deducted from the events occurring this same year regarding adulteration of meat products with non declared species such as horse meat. It is evident that consumers exert an increasing demand for clear and reliable information about the food they consume. This is especially important in the case of processed food products, where a simple visual inspection does not allow discriminating between the different components. Legislation must protect against misdescription and fraud, which is generally undertaken with the objective of increasing profit. To do this, robust, accurate and sensitive methodologies must be taken in place.

In the case of meat products, there is a requirement to separately declare the different meat species that are present in the food. Different approaches have been traditionally employed to accomplish this task. Immunoasays and DNA analysis are between the most widespread technologies applied for this purpose. Despite their advantages, these techniques are not exempted from important limitations. In the case of immunoassays, lack of highly specific antibodies can give rise to cross-reactions, especially in the case of closely related species. Some problems can also arise in the case of processed foods because processing can alter protein structure, affecting the recognition by the antibody. Food processing can also be detrimental for DNA-based analyses, since DNA can suffer an important degradation during processing due to the liberation of hydrolytic enzymes, heat treatments or pH changes, for example, increasing the possibilities to have shorter, non-species specific DNA fragments. Advances in mass spectrometry applied to the analysis of proteins and peptides represent a promising alternative to methods currently in use to determine the animal species that can be present in meat products. In the present work, we have carried out a peptidomic approach for the unambiguous differentiation of horse meat from bovine meat by the use of specific peptide sequences derived from the trypsin hydrolysis of myoglobin. The developed procedure is robust and reliable, and can be of great help in assessing the fraudulent presence of horse meat in beef products.

II. MATERIALS AND METHODS

One gram of either horse or beef meat was homogenized in 10 mL of 50 mM Tris buffer, pH 8.0 by the use of a Polytron®. The homogenate was then centrifuged at 10,000 g for 20 min at 4 °C, collecting the supernatant. A volume of 0.36 mL of each extract was separately fractionated by liquid isoelectric focusing in the pH range 4-7 using an Agilent 3100 OFFGEL fractionator. A total of 12 fractions were obtained. The protein composition of each one of these fractions was

further assessed by SDS-PAGE on 10 % polyacrylamide gels. Protein bands corresponding to myoglobin, which were selected as target protein for the generation of marker peptides, were excised from the gel and in-gel digested with trypsin. The obtained peptides were dissolved in 0.1 % trifluoroacetic acid and further analyzed by liquid chromatography coupled to electrospray ionization-tandem mass spectrometry using a LCQ Advantage ion trap instrument (Thermo Electron Corp.). Identification of the peptide sequences was done using the MS/MS ion search option of an in-house version of the Mascot Search Engine (www.matrixscience.com) together with the UniprotKB/ SwissProt protein database.

III. RESULTS AND DISCUSSION

In the present work, myoglobin was selected as target protein for the generation of peptide sequences capable to differentiate horse from bovine meat. The use of mass spectrometry in the analysis of myoglobins and hemoglobins as potential protein markers capable to differentiate between different meat species was already suggested by Taylor et al. [1]. In that work, the discrimination criterion was the mass differences between the proteins of different species. A similar strategy was developed by Spinoza et al. [2] in an assay to try to differentiate 62 animal species, mainly birds and mammals. They were able to specifically identify 86% of the total samples. For the rest of samples, they were not able to unambiguously recognize the corresponding species, showing overlap with other species. In the present work, we have developed a procedure to try to unambiguously differentiate horse from beef meat by notably improving the discriminating capacity of the commented proteomic approach using the mass differences of intact proteins. Instead of this, the differences in the amino acid sequence of peptides derived from the trypsin hydrolysis of horse and beef myoglobins has been taken as discriminative criterion between these two meat species. Both horse and beef myoglobin were obtained in the sarcoplasmic extract from the corresponding meat. **Figure 1** shows the sequence alignment of these two proteins. Both have the same length (154 amino acids) and, as can be observed, despite their high sequence homology, there are 17 differing amino acid positions between the two chains, thus allowing for the generation of potential species-specific peptide sequences capable to discriminate between these two meats with more precision and robustness than just determining the molecular mass of the intact proteins.

In our procedure, sarcoplasmic extracts were fractionated by OFFGEL separation in order to enrich myoglogins and reduce the complexity of the sample. As can be observed in **Figure 2**, horse myoglobin was mainly enriched in fractions 11 and 12, in accordance to the pI value of the protein. The fractionation profile of bovine myoglobin followed a similar pattern (results not shown). In SDS-PAGE myoglobin appeared as an intense protein band, near the 15 kDa protein standard (**Figure 2**, red arrow). These protein bands were excised from the gel and digested with trypsin. The obtained peptide fragments were separated by HPLC using a C18 reverse phase column, then sequenced by tandem mass spectrometry (MS/MS)

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HORSE  MGLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASE 60
BOVIN  MGLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASE 60

HORSE  DLKKHGTVVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKH 120
BOVIN  DLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHAKH 120

HORSE  PGDFGADAQGAMTKALELFRNDIAAKYKELGFQG 154
BOVIN  PSDFGADAQAAMSKALELFRNDMAAQYKVLGFHG 154
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Figure 1: Sequence alignment of horse and bovine myoglobins. Amino acid differences between the two sequences are indicated in green (horse) and orange (bovine) colours. Blue sequences correspond to the identified species-specific biomarker peptides generated after trypsin digestion of the proteins and sequenced by tandem mass spectrometry

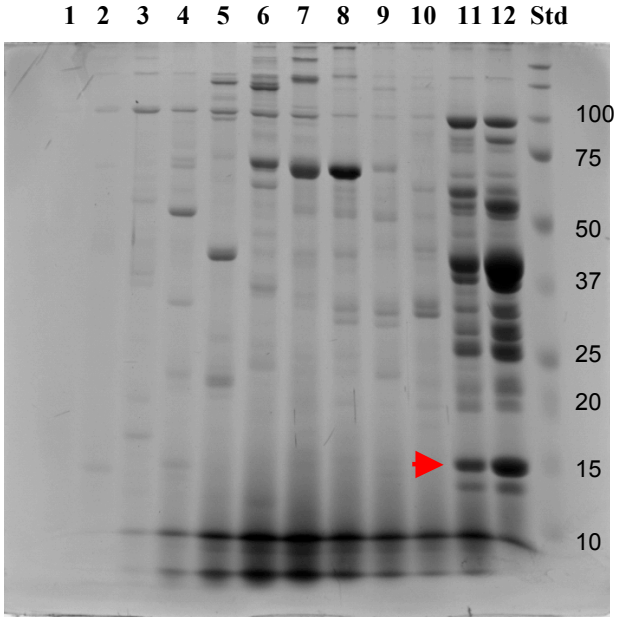
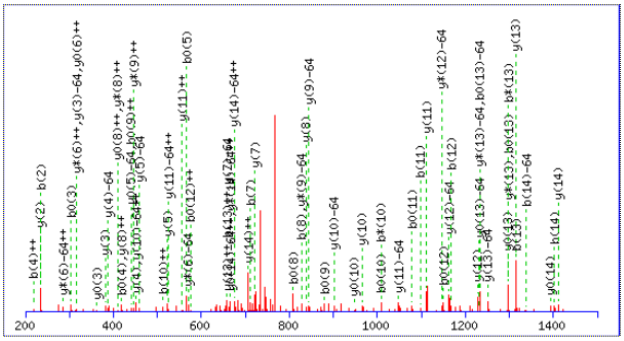


Figure 2: 10% SDS-PAGE of fractions obtained after OFFGEL isoelectric focusing of a sarcoplasmic extract of horse meat in the pH range 4-7. Std: molecular mass standards. Position of myoglobin obtained in fractions 11-12 is indicated by a red arrow

using an ion trap instrument (LCQ Advantage). The obtained results allowed us to confirm the identity of the bands as equine and bovine myoglobin, respectively. Mascot analysis of the sequenced peptides using UniprotKB/SwissProt database allowed us to identify two peptide biomarkers capable to unambiguously distinguish between equine and bovine myoglobin and, hence, between horse and beef meat. These peptides and their main characteristics are shown in **Table 1**. Both are located in position 120-134 into the respective parent protein. In the full MS mode, the peptide derived from beef myoglobin (Peptide 1) is observed as a m/z value of 774.97 Da, whereas its homologue in the horse species (Peptide 2) is detected as m/z 759.98 Da. In both cases, the observed m/z values corresponded to the doubly charged form of the peptides.



b ions	Sequence	*y* ions
138.06	H	
235.11	P	1411.61
322.15	S	1314.56
437.17	D	1227.53
584.24	F	1112.50
641.26	G	965.43
712.30	A	908.41
827.33	D	837.37
898.36	A	722.35
1026.42	Q	651.31
1097.46	A	523.25
1168.50	A	452.21
1315.53	M	381.18
1402.56	S	234.14
	K	147.11

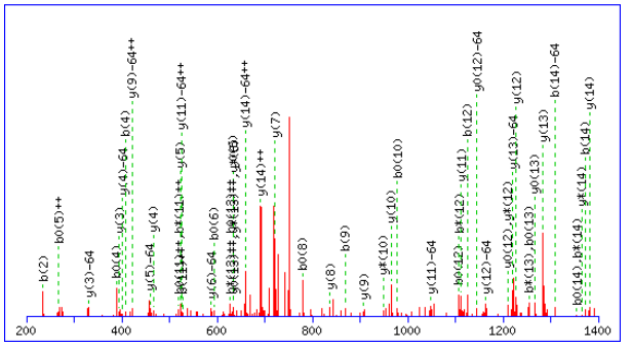
Figure 3: MS/MS spectrum of peptide HPSDFGADAQAAMSK generated from the trypsin digestion of bovine myoglobin (Peptide 1). Matched *b* and *y* ions in the spectrum appear in red

Tandem mass spectrometry analysis allowed us to elucidate the amino acid sequence of these two marker peptides. **Figures 3 and 4** show the

Table 1: Summary of the myoglobin peptides identified in the present work by MS/MS specific of the bovine (Peptide 1) and equine (Peptide 2) species. Differing amino acids are shown in red and blue. *: Protein entry name corresponds to the UniprotKB/SwissProt protein database

Peptide	Observed mass (charge state)	Position into the protein	Sequence	Modification	Parent protein (Protein entry name)*	Species origin
1	774.97 (2+)	120-134	HPSDFGADAQAAMSK	Oxidation	Myoglobin (MYG_BOVIN)	*Bos taurus*
2	759.98 (2+)	120-134	HPGDFGADAQGAMTK	Oxidation	Myoglobin (MYG_HORSE)	*Equus caballus*

MS/MS spectra of peptides 1 and 2, respectively, together with the list of identified *b* and *y* ions in each spectrum allowing for the identification of the respective amino acid sequence.



***b* ions**	**Sequence**	***y* ions**
138.06	**H**	
235.11	**P**	**1381.60**
298.14	**G**	**1284.55**
407.16	**D**	**1227.53**
554.23	**F**	**1112.50**
611.25	**G**	**965.43**
682.29	**A**	**908.41**
797.32	**D**	**837.37**
868.35	**A**	**722.35**
996.41	**Q**	651.31
1053.43	**G**	**523.25**
1124.47	**A**	**466.23**
1271.51	**M**	**395.19**
1372.55	**T**	248.16
	K	147.11

Figure 4: MS/MS spectrum of peptide HPGDFGADAQGAMTK generated from the trypsin digestion of horse myoglobin (Peptide 2). Matched *b* and *y* ions in the spectrum appear in red

The sequence obtained for the peptide generated from beef myoglobin was HP**S**DFGADAQ**A**AM**S**K, whereas the obtained sequence in the case of the peptide derived from horse myoglobin was HP**G**DFGADAQ**G**AM**T**K. Despite their high sequence homology (**Table 1**), we can see that they differ in the amino acids located in positions 122 (S vs. G), 130 (A vs. G) and 133 (S vs. T). These sequence differences would allow us to unambiguously differentiate between horse and beef meat in meat products and could be use in near future to develop robust and reliable new methodologies to control meat fraud and avoid accidental mislabeling in meat and meat products. As the identification criterion is made at sequence level, the resolving power of this peptidomic approach would be comparable to methods based on DNA analysis. Koppel et al. [3]developed a multiplex real-time PCR for the simultaneous identification of seven meat species including beef and horse with a sensitivity of 2 %. According to authors, the method would be suitable for quantitative purposes only if appropriate matrix-adapted reference material is available, which was not the case for all species such as in the case of horse meat, for example. A peptidomic approach for the specific identification of horse meat would have potential for quantitation without the need for reference material. In addition, it is worth emphasizing that peptides would be considerably more resistant than DNA sequences to food processing, thus having potential to give more reliable determinations, especially in highly processed foods where DNA can be highly degraded.

IV. CONCLUSION

The present work shows the use of peptidomics and mass spectrometry as a reliable and interesting alternative approach to methods currently employed in the detection of meat frauds, like the illegal presence of horse meat.

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