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Extraction and ESI–CID–MS/MS analysis of myoglobins from different meat species

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Abstract

Meat speciation methods for raw meats are available but are not always effective in cooked products. The globin protein from myoglobin is heat stable, shows different molecular weights for each species and electrospray mass spectrometry (ESI–MS) can partially differentiate some species. To improve the analysis, the heat stable globin protein was extracted and subjected to fragmentation by ESI–CID–MS/MS. The $[M+16H]^{16+}$ and $[M+17H]^{17+}$ ions were chosen as precursor ions and fragmented by collision-induced dissociation (CID). Fragmentation occurred at proline residues with cleavage either side of the peptide bond leading to the typical pattern of peptide ions. The patterns were dominated by a series of y''_n fragments of which the fragments from cleavage of the His/Pro residues at 119/120 (y''_{34} and y''_{35}) were relatively intense. A strategy for differentiating the four species by ESI–MS and ESI–CID–MS/MS is discussed. © 2000 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Myoglobin; ESI–CID–MS/MS

1. Introduction

Myoglobin and haemoglobin have the same function and similar structures in different animal species but there are small differences in their molecular masses due to variations in their amino acid sequence. The potential of electrospray mass spectrometry (ESI–MS) to measure the exact molecular mass of the globin chains and thus use these proteins for meat species identification has been proposed previously (Taylor, Linforth, Weir, Hutton & Green, 1993).

Studies on the heat stability of sheep myoglobin and haemoglobin in model systems showed that the globin from myoglobin was more stable than the α- and β-globins from haemoglobin (Ponce, Linforth, Hall, Guerrero & Taylor, 1994). In extracts from severely heated meat and meat products, only globins from myoglobin were detected with no sign of haemoglobin, suggesting that it had been degraded (Ponce, Linforth & Taylor, 1995). ESI–MS has therefore been shown to have potential not only to detect myoglobin in simple aqueous systems, but also in real foods like cooked meat and meat products.

However, when mixtures of beef–pork and beef–horse myoglobins were analysed by ESI–MS, only one globin species was observed. The mass differences between beef, horse and pork myoglobins were too close to differentiate the species by mass alone (see Table 1), even with the aid of maximum entropy analysis which is an iterative algorithm used to improve the resolution and mass determination of components in protein mixtures.

In ESI–MS, protonated molecules may be further analysed by fragmentation using collision-induced-dissociation (CID) to obtain structural information from intact proteins, more rapidly and sensitively, than with other conventional techniques like chemical or enzymatic digestion. CID induces fragmentation (Biemann & Scoble, 1987), by collision of the selected precursor ion with a neutral atom such as helium, so increasing the internal energy. Ions that undergo this collisional excitation process may subsequently fragment. Highly charged molecules are more susceptible to dissociation because the collision energy is proportional to the number of charges at a given m/z; dissociation is also enhanced by the presence of electrostatic repulsive forces (Tang, Thibault & Boyd, 1993). In proteins, fragmentation occurs around the peptide bond and

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Table 1
Molecular weight of myoglobin from different species

Species	Molecular mass (Da)	
	Calculated	ESI–MS data ±S.D.
Sheep	16 923.4	16 923.4 ± 0.4
Beef	16 946.4	16 946 ± 0.5
Horse	16 951.5	16 951.5[a]
Pork	16 953.5	16 953.5 ± 0.2

[a] Mass scale was calibrated using horse myoglobin.

produces a wide range of ions. Providing the amino acid sequence of the protein is known, it is possible to predict the mass of ions that might be formed using a software package.

Hence, the differences in the amino acid sequence of myoglobin between species, may produce different fragmentation patterns that could be used as a tool for species differentiation by performing an ESI–CID–MS/MS analysis. There is increasing interest in using ESI–CID–MS/MS as a tool for sequencing biomolecules like proteins and DNA (Adams & Lock, 1997; Gouldsworthy, Leaver & Banks, 1996; Stenfors, Hellman & Silberring, 1997).

The nomenclature used for polypeptide fragmentation is based on notation developed by Roepstorff and Fohlman (1984). The peptide bond can cleave at three places (between the amino acid and the NH group; between the NH and CO of a given peptide bond, as well as on the opposite side of the corresponding NH or CO, respectively, with retention of the charge of either the N- or C-terminus to generate as many as six different ions for each peptide bond). The ions from these cleavage points are called a_n, b_n and c_n ions, respectively, when the charge is retained by the N-terminal fragment and, x_n, y_n and z_n products when the charge is retained in the C-terminal fragment on the peptide. Subscripts are used to denote which peptide bond is cleaved, counting from the N- and from C-terminus ends, respectively. The subscript therefore denotes the number of amino acid residues in the fragment ion. A superscript is added to indicate the fragment ion charge state, i.e. which corresponds to the number of protons transferred to the fragment. The type of product ions depend upon the precursor ion charge state, nevertheless, b''_n and y''_n ions are the most common fragments due to greater stability toward further dissociation (Tang et al., 1993).

The software interprets the mass and charge state of each fragment present in the acquired spectra and, following the Roepstorff nomenclature, predicts the fragmentation patterns according to the precursor ion charge state. For each ion in the ion product mass spectrum, all the m/z values and all the possible charge states are compared to all possible "b" and "y" fragments from the known amino acid sequence. Progressions of product ion charge states (i.e. $y_{34}^{3+} y_{34}^{4+} y_{34}^{5+}$ etc.) and product ion series (i.e. $y_{33}^{5+} y_{34}^{5+} y_{35}^{5+}$ etc.) are also searched.

2. Materials and methods

Horse and sheep skeletal myoglobins were obtained from Sigma Chem Co. (Poole, Dorset, UK). Beef and pork myoglobins were isolated using the method described below because they were not commercially available.

2.1. Myoglobin extraction

The method and recommendations reported by Satterlee and Zachariah (1972), Yamazaki, Yokota and Shikama (1964) and Wittenberg and Wittenberg (1981) were adopted. The procedure was performed at 4°C. Beef and pork meat were purchased from a local shop and kept refrigerated until extraction. Visible fat and connective tissue were removed, then the muscle was homogenised with two volumes of cold deionised water, the pH was adjusted between 7.5 and 8 with ammonium hydroxide (2 M). Myoglobin was recovered by ammonium sulfate precipitation (65 to 100% saturation). After centrifugation at 5000g, the precipitate was collected by filtration on GF/D glass microfibre filter paper (Whatman Int. Ltd Maidstone, UK), redissolved in deionised water and dialysed against deionised water (Visking tubing; Medicell International, London, UK, 12 kDa cut-off) to remove salts and low molecular weight material and filtered through a 13 mm, 0.5 μm filter unit (Millipore Corp., Bedford, UK). Concentration was determined by measuring the absorbance at 525 and 700 nm on a spectrophotometer (LKB Biochem 4050) and using the calculations reported by Graham (1989) where: myoglobin (mg ml^{-1}) = $A_{525} - A_{700} \times 2.303 \times$ dilution factor.

Pork myoglobin was further purified by chromatography. The redissolved myoglobin solution was applied to a DEAE-cellulose anion exchange column (30 g, Sigma Chem. Co. Ltd. Poole, UK), previously equilibrated with Tris buffer (5 mM, pH 8.4) and eluted with 10 mM Tris buffer pH 8.6. Cytochromes and non-haem proteins eluted in this fraction. The myoglobin was then eluted with 50 mM Tris buffer, pH 8, dialysed and filtered prior to ESI–MS and ESI–CID–MS/MS analysis.

2.2. ESI–MS and ESI–CID–MS/MS analysis

Solutions of proteins (10 pM ml^{-1}) were prepared in 50% aqueous methanol with 0.2% formic acid to enhance protonation. Aliquots (10–40 μl) were assayed on a VG Quattro II mass spectrometer fitted with an electrospray interface (Micromass, Altrincham, UK). A

solvent stream consisting of 50% aqueous methanol and 0.2% formic acid solution was pumped via a Rheodyne valve into the source at 5 μl min^{-1}. The inlet capillary was maintained at 4 kV, with the counter electrode at 1 kV and the source temperature at 70°C. The ESI–MS raw spectra were obtained in the first quadrupole by taking 10–15 scans over the scan range *m*/*z* 600 to 1500 at 10 s per scan. The molecular mass was determined by transformation of the ESI–MS raw data into a true molecular mass scale using the MaxEnt (Micromass) software in the mass range between 5000 and 60 000 Da. The mass scale was calibrated using horse heart myoglobin (16951.51 Da; Zaia, Roland, Breman & Bieman, 1992; Sigma Chem. Co. Ltd. Poole, UK).

The ESI–CID–MS/MS analysis was achieved by selecting the precursor ions $[M+17H]^{17+}$ ($m/z \approx 998$) and $[M+16H]^{16+}$ ($m/z \approx 1060$) in the first mass spectrometer using a mass range of ±0.5 amu of the nominal mass. Fragmentation was then performed by transferring the selected ions into the hexapole collision cell, using helium as collision gas. The flow of gas into the collision cell and the collision energy (35–55 V) were adjusted until a suitable fragmentation pattern was obtained. The aim was to fragment the protein into peptide ions that could be used diagnostically. Too little CID did not differentiate the proteins; too much produced many small fragments, which were difficult to resolve. The product ions were then transported into the third quadrupole which separated them according to their mass to charge ratio with the scan range set at *m*/*z* 600–1500 at 10 s per scan. The software detected the mass and charge state of each fragment present in the acquired spectra and following the Roepstorff nomenclature (Roepstorff & Fohlman, 1984), predicted the fragment ions.

3. Results and discussions

3.1. Myoglobin extraction

Bovine myoglobin was isolated by aqueous extraction and ammonium sulfate precipitation, followed by dialysis, which gave a concentration of 8 mg ml^{-1}. Although this fraction was not pure, ESI–MS analysis showed that myoglobin was the major component (data not shown) and the fraction was adequate for ESI–CID–MS/MS analysis.

The isolation of porcine myoglobin was more difficult. The ESI–MS spectra of the ammonium sulfate fraction showed the presence of sarcoplasmic components (35.7, 42.9 and 47 kDa) and a low yield of myoglobin was obtained. After a further purification step on DEAE-cellulose, the ESI–MS spectra (Fig. 1) showed that myoglobin was the principal component of this fraction. High molecular components were still present in the spectrum but at much lower levels compared to the previous ammonium sulfate fraction. Also a series of myoglobin-sodium adducts ($[M+Na]^{+}$, $[M+2Na\text{-}H]^{+}$, $[M+3Na\text{-}2H]^{+}$, etc.) were observed in the spectrum, probably derived from the buffers used during the DEAE-cellulose chromatography, even though the samples had been dialysed to remove low molecular material (<12 kDa) prior to ESI–MS analysis. These adducts make it even more difficult to distinguish myoglobins, especially those for sheep (Mr 16923) and beef (Mr 16946) where the mass difference is equivalent to the mass of Na.

3.2. ESI–CID–MS/MS analysis

Ionisation of molecules by the electrospray technique produces a range of multiply protonated protein ions with the maximum number of charges carried on each ion related to the number of basic amino acid residues in the protein (i.e. lysine arginine, histidine) plus the N-terminus. Myoglobin consists of a globin (a polypeptide of 153 amino acids) and the heme group but, during ionisation, myoglobin is dissociated into the globin and heme moieties and they are detected as separate entities.

The ESI–MS raw spectrum (Fig. 1a) of a single protein consists of a series of differently charged ions representing the same mass. Molecular mass was determined by transformation of the ESI–MS raw data into a true molecular mass scale (Fig. 1b). The measured molecular weights of the myoglobin globin for all samples (Table 1) agreed within experimental error with the molecular mass calculated from the amino acid sequences (EMBL library).

The precursors ions selected for the ESI–CID–MS/MS analysis were $[M+17H]^{17+}$ ($m/z \approx 998$) and $[M+16H]^{16+}$ ($m/z \approx 1060$). They were chosen as they were abundant in the spectrum and contained sufficient charge for partial fragmentation to occur. Ions with charges higher and lower than these ions, fragmented too readily or not readily enough, respectively. The resultant ESI–CID–MS/MS spectra from the four myoglobin globin species were compared. All were very similar and dominated by a series of y''_n fragments. The *m*/*z* ranges in the ESI–CID–MS/MS spectra where more significant differences were observed are presented and discussed.

The spectra from the four myoglobin globins were examined and compared with the amino acid sequences of the intact protein. Fragments produced by cleavage of the 119/120 (His/Pro) residues and the 99/100 (Ile/Pro) residues were relatively intense (y''_{34} and y''_{54}). Loo, Edmonds and Smith (1990, 1993), reported that dissociation commonly occurs at the amide bond of the proline residue and becomes more significant as the molecular mass increases.

Proline is unique among the amino acids (Creighton, 1984). It possesses an aliphatic side chain, with no

functional groups and the end of the side chain is covalently bound to the preceding α-amide nitrogen. The resultant five-membered ring prevents rotation of the N–C^{α} bond of the backbone which has a very significant effect on the conformation of the polypeptide and therefore on the ESI–CID–MS/MS fragmentation process. Almost 80% of the myoglobin chain is α-helical with the direction and angle of the helices determined by the location of proline, threonine or serine residues. Because of their exposed nature on the outside of the protein, these sites are more susceptible to fragmentation.

The most evident differences between the spectra were observed in the y''_{34} fragment with four and five charges, derived from the $[M+17H]^{17+}$ precursor ion (Fig. 2). The predicted and found *m/z* values for these fragments are summarised in Table 2. The predicted *m/z* values for the y''^{5+}_{34} fragment from sheep (*m/z* 728) and pork (*m/z* 728) globin are identical. However, no ion at *m/z* 728 was observed in the sheep globin, probably because sheep globin has fewer charges overall, i.e. fewer basic amino acids and is therefore less likely to fragment. The predicted values for the y''^{5+}_{34} fragment from horse and pork myoglobin globins were very close ($\Delta = 0.8$ *m/z* units) although the experimental data gave values of 727.2 and 728.2 ($\Delta = 1.0$ *m/z* units). Although the masses are too close for reliable resolution by the MS, the intensity of the pork y''^{5+}_{34} peak was lower than the horse or beef peaks.

Examination of the y''^{4+}_{34} fragment again indicated that the *m/z* values (909.8) for the sheep and pork globin samples were identical (Table 2) and the value for horse was very close to these two ($\Delta = 1.0$ *m/z* units). The region around *m/z* 900 in the mass spectrum where these ions appeared, was far more complex due to the presence of several ions with similar *m/z* values, in particular ions y''^{5+}_{41} and y''^{5+}_{42} which may obscure the peaks of interest (Fig. 2). The beef y''^{34}_{4+} fragment with *m/z* 915.5 is sufficiently different to be resolved by the MS.

When ion $[M+16H]^{16+}$ ($m/z \approx 1060$) was subjected to ESI–CID–MS/MS fragmentation, the spectra (Fig. 3)

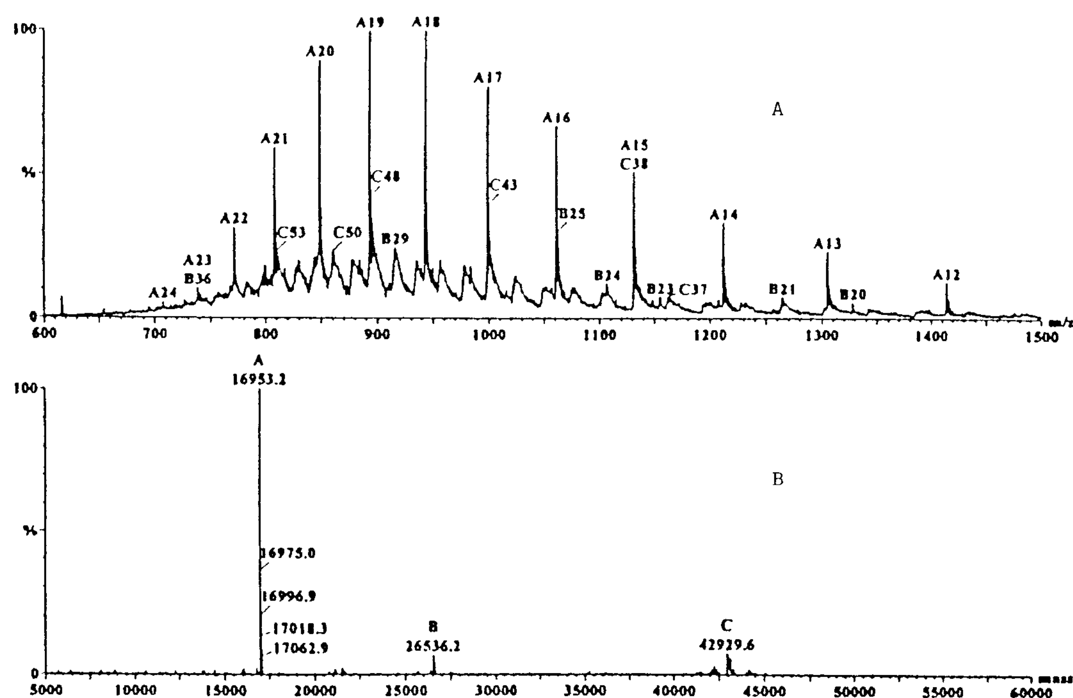

Fig. 1. ESI–MS traces from pork myoglobin after purification on DEAE-cellulose: (a) raw spectra and (b) transformed spectra.

Table 2
ESI–CID–MS/MS predicted and observed *m/z* values and number of charged sites for y^{n+}_{34} fragment

		Fragment Y^{5+}_{34}		Fragment Y^{4+}_{34}	
Species	Charged sites	Predicted *m/z*	Observed from $[M+17H]^{17+}$	Predicted *m/z*	Observed from $[M+17H]^{17+}$
Sheep	3	728.0	—	909.8	Yes
Beef	4	732.6	Yes	915.5	Yes
Horse	4	727.2	Yes	908.8	Yes
Pork	4	728.0	Yes	909.8	Yes

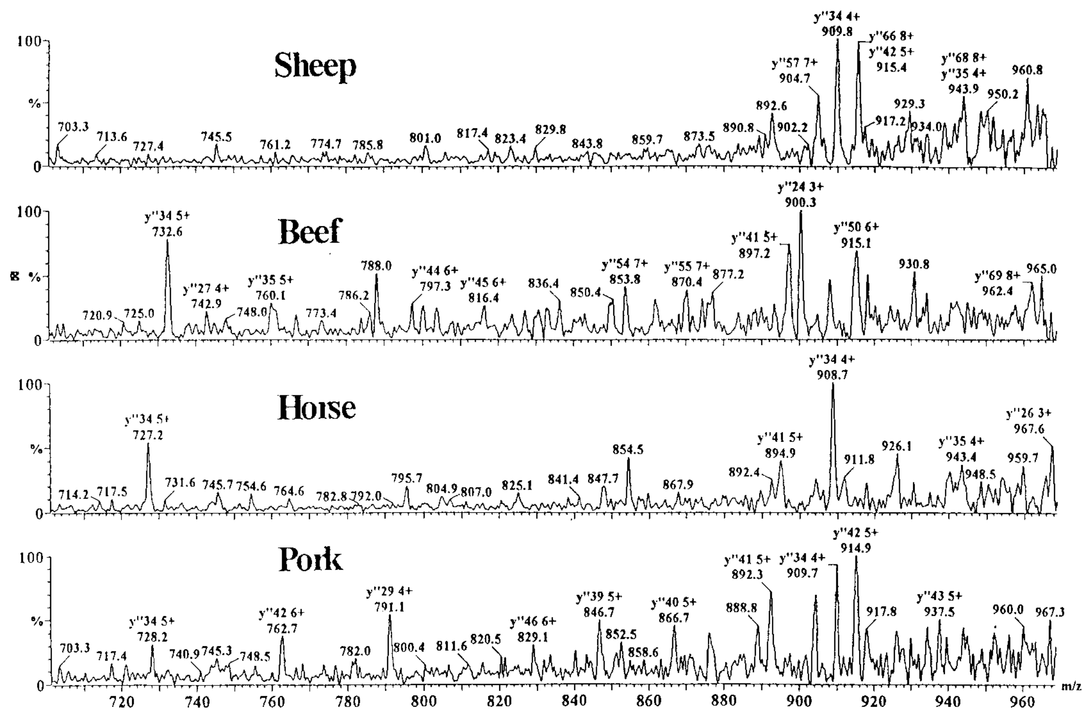

Fig. 2. ESI–CID–MS/MS spectra of the $[M+17H]^{17+}$ ($m/z \approx 998$) ion from sheep, beef, horse and pork myoglobin.

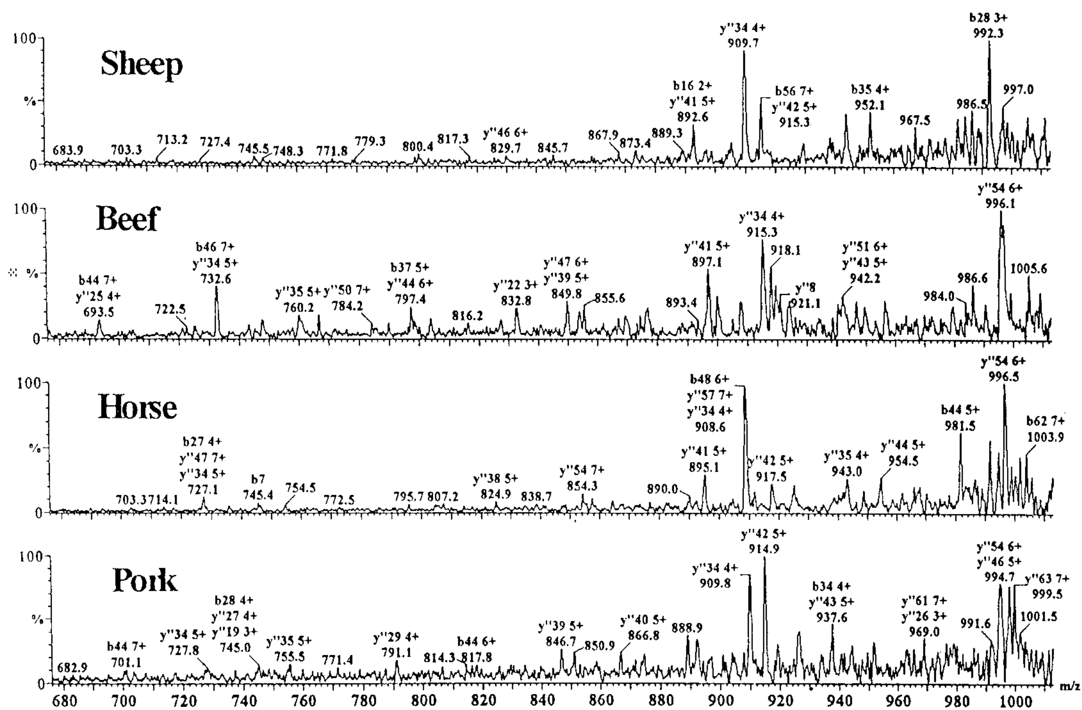

Fig. 3. ESI–CID–MS/MS spectra of the $[M+16H]^{16+}$ ($m/z \approx 1060$) ion from sheep, beef, horse and pork myoglobin.

showed that the y''^{5+}_{34} ($m/z \approx 730$) fragments were weaker than the corresponding ions from the $[M+17H]^{17+}$ precursor ion in all the samples. The beef sample exhibited the largest peak but no peak could be observed in the sheep spectra. The y''^{4+}_{34} fragment from beef had a m/z value of 915.3, while this fragment from sheep and pork myoglobins shared the same value (m/z 909.8). The y''^{4+}_{34} ion from horse was very close to these two ($\Delta = 1.0$ m/z units), and other fragments in the spectrum showed similar m/z values.

Data were also calculated for the predicted m/z values for the $[M+18]^{18+}$ ion. However, all the ions had very

similar m/z values because the differences in mass were diminished due to the greater number of charges.

Although the globin proteins show different amino acid sequences, the distribution of these sequences needs to be considered. Many conserved areas of homologous proteins, occur at critical points along the polypeptide chain where structure is related to molecular function. The bends in the chain are crucial in maintaining the overall shape of the protein and thus amino acid sequences in these regions are expected to be highly conserved. Since fragmentation takes place at these bends, it might be expected that the fragments produced would be very similar.

Despite ESI–CID–MS/MS not resolving the four globin samples completely, species identification could be attempted by two steps. The molecular masses of sheep and beef myoglobin are sufficiently different from each other (23 Da) and from horse and pork (5 and 7 Da; Table 1) to be differentiated by ESI–MS of the intact proteins with resolution enhanced by MaxEnt. Pork and horse globins, with a mass difference of 2 Da (16 953.5–16 951.5) lie on the edge of resolution (0.01%). In a molecular mass of 16 950 Da, this difference of 1.695 Da could theoretically be resolved but, in practice the resolution is not reliable. Also, if one globin was present as small fraction of the other (say 5 to 10%) there might be confusion with isotopic peaks from other globin molecules. With ESI–CID–MS/MS, sheep and beef can be distinguished from each other and from horse and pork (Table 2). Differentiation of horse and pork samples, however, relies on resolution of the y_{34}^{5+} fragments at m/z 727.2 and 728, respectively, and the y''^{4+}_{34} fragments at m/z 908.8 and 909.8, respectively. This cannot be done on a mass basis but might be accomplished if the intensity ratios of the two ions were sufficiently consistent to differentiate them. Although resolution can be achieved here using purified proteins and known compositions, the resolution of mixtures of these proteins in a meat product might be a more difficult proposition.

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