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Meat Authentication via Multiple Reaction Monitoring Mass Spectrometry of Myoglobin Peptides

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ABSTRACT: A rapid multiple reaction monitoring (MRM) mass spectrometric method for the detection and relative quantitation of the adulteration of meat with that of an undeclared species is presented. Our approach uses corresponding proteins from the different species under investigation and corresponding peptides from those proteins, or CPCP. Selected peptide markers can be used for species detection. The use of ratios of MRM transition peak areas for corresponding peptides is proposed for relative quantitation. The approach is introduced by use of myoglobin from four meats: beef, pork, horse and lamb. Focusing in the present work on species identification, by use of predictive tools, we determine peptide markers that allow the identification of all four meats and detection of one meat added to another at levels of 1% (w/w). Candidate corresponding peptide pairs to be used for the relative quantification of one meat added to another have been observed. Preliminary quantitation data presented here are encouraging.

The European horse meat scandal of 2013 highlighted the need for analytic methods for detecting the addition of horse meat to beef, both raw and in cooked products, and by extension a need for detecting the adulteration of any meat with that of an undeclared species.

The original detection of horse meat in beef, announced by the Food Safety Authority of Ireland, was based on DNA methods, as was the ensuing Europe-wide testing program. DNA-based testing remains widely used and is available via commercial providers. Immunologically based testing in the form of enzyme-linked immunosorbent assay (ELISA) is also available commercially. In the continuing search for cheap, robust, and quantitative methods, various other technologies have also been considered. Recent contributions include ultraperformance liquid chromatography (UPLC),[1,2] Raman spectroscopy[3,4] and low-field NMR.[5,6] A range of methodologies, including DNA and ELISA, are reviewed by Ballin et al.,[7] Ballin,[8] and Sentandreu and Sentandreu.[9]

In the present work, we develop a mass spectrometry-based protocol for determining meat adulteration with an undeclared meat species. Mass spectrometry methods applied to meat authentication have been described previously in the literature. Taylor et al.[10] used electrospray ionization mass spectrometry (ESI-MS) to determine the masses of hemoglobin and myoglobin (Mb) from pig, beef, sheep, and horse, noting that the calculated protein mass differences suggested a route toward meat speciation. Ponce-Alquicira and Taylor[11] extended this idea, using ESI-MS/MS to demonstrate additional Mb-based species differentiation via specific protein fragments. Several authors have pursued a proteomic approach, including intact marker proteins[12,13] or marker peptides[14] via 2D gel electrophoresis with MS protein and peptide identification, in some cases dispensing with the gel stage.[15−17] Von Bargen et al.[18,19] also adopted a proteomics approach, identifying marker peptides from various proteins in beef, pig, and horse meat by liquid chromatography/Fourier transform mass spectrometry (LC/FT-MS). In addition, von Bargen et al. then used multiple reaction monitoring (MRM) methods to demonstrate the detection of adulteration of beef with horse or pork at sub-1% levels in both the raw state and cooked products. The MRM method is central to the present study.

The MRM method[20] is an attractive candidate for identifying peptides, requiring only modest instrumentation and data handling, at least by mass spectrometry standards. The detection of multiple transitions, in which a single peptide precursor ion is associated with multiple distinct detected fragment ions, provides a highly specific test for individual peptides. In this study, in contrast to a shotgun-type proteomic approach, we will use MRM MS in a targeted, intelligence-led way: rather than a set of "all available" proteins, we will focus from the outset on Mb and rely solely on peptides derived from Mb as our marker peptides for different meat species.

Intact Mb has already been exploited to determine meat species via mass spectrometry.[10,11] Intact Mb was also used by Janssen et al.[21] in an immunological method to differentiate beef, pork, horse, sheep, and kangaroo meat in cooked products and in UPLC-based determination of pork with beef mixtures[1] and horse with beef mixtures.[2]

Myoglobin, the oxygen storage molecule of muscle and the source of the red color of meat, has a number of advantageous properties as a species marker. It is present in a wide range of meats with species-specific differences in amino acid sequence. For example beef Mb, P02192, and horse Mb, P68082, differ by 18 amino acids. This means that not only do the two myoglobins have slightly different masses but also, when subjected to proteolysis, they give rise to species-specific sets of peptides. Mb is an abundant protein and is plentiful in red meat, averaging 1% Mb in muscle tissue dry matter.[22]

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Furthermore, its solubility in water is helpful with regard to sample preparation; in comparison, other abundant proteins such as myosin, tropomyosin, or actin would all require more aggressive extraction procedures. It is also relatively heat-stable,[10,23] suggesting that species testing based on Mb will work both for raw meats and for cooked meat products.

It is convenient to describe two Mbs from different species as an example of corresponding proteins: while informally it is enough to call both myoglobin, their sequence differences dictate that they cannot properly be described as the same. Similarly, two peptides arising from corresponding cleavages from corresponding proteins can be described as corresponding peptides. For two or more species from a given set of species, corresponding peptides can be obtained by aligning the sequences in the conventional way and identifying peptides with termini arising from aligned cleavage sites. By way of example, two corresponding peptides from beef and horse meat following proteolysis of their respective Mbs (P02192 for beef and P68082 for horse) by trypsin might be HPSDFGADAQAAMSK and HP**G**DFGADAQ**G**AM**T**K, respectively, where boldface type indicates differences in the horse sequence relative to that of beef. We propose using corresponding proteins corresponding peptides, which we conveniently label CPCP for brevity, as a route to relative quantitation.

In the beef/horse example above, the two corresponding peptides have monoisotopic masses of 1531.67 (m/z = 767 Da with z = 2) and 1501.66 (m/z = 752 Da with z = 2) Da and different retention times in the LC separation preceding the mass spectrometer. At first sight it would seem plausible that the combination of peptide mass and retention time would be sufficient to distinguish these peptides in a sample, but in general this information is not sufficiently specific, especially for complex food samples containing numerous other proteins. In an MRM experiment the two peptides above give rise to sets of fragments. For beef, the four most intense transitions are 767→ (234, 1299, 706, 1396), corresponding to fragment sequences SK, SDFGADAQAAMSK, AQAAMSK, and PSDFGADAQAAMSK. These fragments are listed more succinctly as y^2, y^{13}, y^7 and y^{14}, where y^n denotes counting in n amino acids from the peptide C-terminal end. The corresponding horse peptide 752 has four transitions, 752→ (1269, 706, 248, 1366) with fragments y^{13}, y^7, y^2, and y^{14}. Each precursor−fragment pair is a transition and is denoted by an arrow, so 752 → 1269 is one of the four horse transitions. The peptide 752 is therefore a marker for horse in beef/horse mixtures and can be detected with high specificity via its MRM transitions.

The fact that the horse marker peptide (752) has a corresponding beef marker (767) means that, in addition to detection, we can propose a method for quantitation based on the areas of the transition peak intensities. We hypothesize that the *ratio* of specific transition peak areas arising from corresponding peptides maps to the ratio of the amounts of the two meats in a mixture, giving us a straightforward route to *relative* quantitation: CPCP MRM transition intensity peak area ratios are a proxy for the ratio of meat species in a binary meat mixture.

This pragmatic, reference-free CPCP strategy is designed to yield relative as opposed to absolute quantitation, which is useful in the food authentication context. For example, the guide to what constitutes actionable adulteration in meat—substitution of at least 1% by some undeclared species[24]—is a statement framed in relative quantities.

Since in the CPCP approach the protein that forms the basis of the peptide markers is essentially the same in both meats, it is reasonable to assume extraction, pretreatments, and proteolysis will perform equally for both. Corresponding peptides, being similar in sequence, can generally be expected to behave similarly in the LC stage and likewise in the ionization and fragmentation stages of the mass spectrometer. Therefore, even if protein extraction is incomplete or proteolysis is partial due to truncated time frames or alternative enzymes, or some Mb degradation occurs due to heating or food processing, then so long as the impact is the same on corresponding proteins and corresponding peptides, then the ratios of transition intensities should be preserved and the estimate of meat ratios will be robust.

To test the CPCP strategy, in this paper we first consider candidate peptides and likely MRM transitions arising from the Mb in four meats: beef (*Bos taurus*), pork (*Sus scrofa*), horse (*Equus caballus*), and lamb (*Ovis aries*). We then undertake a guided search for those transitions in our "reference" material, purified Mb from each of the four species. This is, first, to demonstrate that the predicted peptides with their transitions are visible, and second, to enable us to spot those same transitions in actual meat samples. Candidate pairs of corresponding peptides suitable for quantitation are identified. Next, switching to meat samples, we demonstrate the capacity to detect the two species in binary mixtures of horse and beef, lamb and beef, and pork and lamb. Finally, we show that we can detect the presence of an adulterant species at the 1% (w/w) level. In the present work we are concerned only with introducing the methodology and demonstrating the detection of one species mixed with another. Comprehensive quantitation results will be described separately.

EXPERIMENTAL SECTION

Predictive Tools. The PeptideCutter tool[25] was used to predict the peptides resulting from proteolysis of each of the Mb proteins by trypsin (by use of the simple model option). Candidate MRM transitions were predicted by use of the open source Skyline tool.[26] Skyline settings included 0 missed cleavages, conventional trypsin rules [KR|P], minimum peptide length 6, maximum peptide length 25, exclude N-terminal amino acids set to 1 (to avoid initiator methionine), and collision energy set to ABI 4000 QTrap.

Materials. Methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, U.K.). Urea and trypsin [from bovine pancreas, treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)] were purchased from Sigma−Aldrich (Gillingham, U.K.). Formic acid was purchased from BDH Chemicals (Poole, U.K.).

Beef, lamb, and pork samples were purchased from local supermarkets and stored at 4 °C prior to preparation for analysis. Horse meat was purchased from a butcher in Calais, France, and was stored at −40 °C. Horse Mb was purchased from Sigma−Aldrich. For both meat sample preparation and the production of purified reference Mb, small thinly sliced pieces of meat were first frozen in liquid nitrogen and then ground in a Waring blender for 1 min.

Preparation of Purified Reference Myoglobin. Approximately 30 g of ground meat (lamb, beef, or pork) was extracted in 30 mM Tris-HCl (pH 8.4) at 4 °C for 30 min. The extract was filtered, and the filtrate was centrifuged at 4 °C for 30 min at 14000*g*. The supernatant was filtered through filters of decreasing size (0.8, 0.45, and 0.2 μm). Sodium azide (0.02%)

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Figure 1. Myoglobin sequences (from top to bottom of each block): horse (P68082, red), beef (P02192, blue), pork (P02189, green), and lamb (P02190, brown). The colored dots indicate tryptic cleavage sites, with spaces inserted to maintain alignment. Colored sequence strings indicate the marker peptides listed in Table 1. Yellow highlighting indicates regions showing one or more sequence variations across the four corresponding proteins. The initiator methionine in position 1 is included to match protein libraries but is not present experimentally, being cleaved from the mature protein.

and potassium sorbate (1 mg/mL) were added, and the sample was filtered through a 0.2 μm syringe filter.

Aliquots of the crude extract (2 mL) were loaded onto a Superdex 75 size-exclusion column (GE Healthcare, Chalfont, U.K.) attached to a BioCad Sprint HPLC system (Applied Biosystems). The column was equilibrated and eluted with 30 mM Tris-HCl, pH 8.4, at a flow rate of 1 mL/min. The eluent was monitored for protein by following the absorbance at 280 nm, and peak fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The Mb-containing fractions were pooled and stored at 4 °C.

The purified Mb (98% purity as determined by SDS–PAGE) was loaded onto a PD10 column (GE Healthcare) and eluted with 25 mM ammonium bicarbonate in preparation for proteolysis.

Preparation of Meat Samples. Approximately 300 mg of ground sample was weighed into a plastic 15 mL Corning centrifuge tube. Mixtures were prepared by weighing the respective amounts of ground meats directly into the centrifuge tube. The extraction buffer (4 mL of 0.3 M KCl + 0.3 M phosphate buffer at pH 6.5) was added, and the sample was vortexed for 30 s and then extracted on an Edmund and Bühler KS10 lab shaker at room temperature for 2 h at 250 cycles/min. A 2 mL aliquot of the sample was transferred to a centrifuge tube and then centrifuged for 5 min at 4 °C at 17000*g*. A 200 μL aliquot of the supernatant was transferred to a 2 mL centrifuge tube and evaporated to dryness at 50 °C in a Jouan RC 1022 centrifugal evaporator.

Proteolysis of Meat Samples and Reference Myoglobin. The dried meat sample residue was redissolved in 1 mL of 25 mM ammonium bicarbonate and vortexed for 20 s. The reference Mb solution or redissolved meat sample was then heated in a hot block at 95 °C for 30 min and cooled, and urea was added to a final concentration of 0.5 M. Trypsin solution (1 μg/μL) was then added in a ratio of 1:30 enzyme substrate by weight. The sample was gently vortexed and digested at 37 °C overnight.

The digested sample was diluted 1:2 with water and desalted on a Strata-X 33μ polymeric reversed-phase cartridge (Phenomenex, Macclesfield, U.K.). The cartridge was washed and activated with 1 mL of methanol and then equilibrated with 1 mL of 1% formic acid. The sample was loaded onto the cartridge, which was washed with 1 mL of 5% methanol/1% formic acid in water, and the peptides were eluted with 1 mL of acetonitrile/H_2O (90:10; 0.1% formic acid).

The desalted sample was evaporated to dryness in the centrifugal evaporator and then redissolved in 250 μL of acetonitrile/H_2O (3:97; 0.1% formic acid) for subsequent LC/MS/MS analysis.

LC/MS/MS Analyses. High-performance liquid chromatography/tandem mass spectrometric (HPLC-MS/MS) analyses of the digested proteins were performed by use of an Agilent 1200 rapid resolution LC system (Stockport, U.K.) coupled to an AB Sciex 4000 QTrap triple-quadrupole mass spectrometer (Warrington, U.K.). Chromatographic separations were conducted on a Phenomenex XB C18 reversed-phase (RP) capillary column maintained at 40 °C (100 × 2.1 mm, 2.6 μ particle size, Macclesfield, U.K.) with a 300 μL/min flow rate. The gradient profile used consisted of a binary gradient from 97% A (water + 0.1% formic acid) and 3% B (acetonitrile + 0.1% formic acid) to 28.4% B over 22 min, increasing to 100% B at 23 min and held for 5 min. Column re-equilibration was for a further 6 min. Injection volume was 10 μL. A single MS/MS methods file ("multimeat") embracing all four Mbs was constructed on the basis of Skyline predictions. Mass data acquisitions were made by Analyst 1.6.2 software (AB Sciex). Peptides eluted from the column were detected by positive electrospray in scheduled dynamic monitoring mode with a scan time of 2 s and a nominal retention time window of ±50 s. Each peptide was monitored by the four most intense MRM transitions as determined by peak height. Turbospray source settings were operated with a curtain gas of 25 psi, desolvation gas (GS1) of 50 psi, and sheath gas (GS2) of 20 psi. Source temperature was 550 °C.

RESULTS

Proteolysis of Myoglobin. It is well-known that Mb is relatively resistant to digestion.[27,28] Indeed, Mb subjected to what might be considered conventional conditions (6 M urea in ammonium bicarbonate incubated overnight with trypsin at 37 °C) shows only a limited degree of digestion according to SDS–PAGE (data not shown). We tested a number of digestion protocols, including those based on ammonium bicarbonate, RapiGest, urea, heat, and heat with urea, and we determined that heat with urea gave the best overall results as judged by the intensity and population of peptide peaks. Note that our preparation did not include a reduction and alkylation step since the four Mbs involved in this study do not contain cysteines and there are no cysteine bridges. The absence of a reduction and alkylation step means that fewer peaks arise from the digestion of unwanted proteins than would otherwise be seen. Targeting only Mb permits the optimization of extraction and proteolysis conditions.

Figure 1 shows the four Mb sequences. We have used the same color coding throughout (red for horse, blue for beef, green for pork, and brown for lamb) for clarity. The sequences are aligned, with amino acid locations showing variations across

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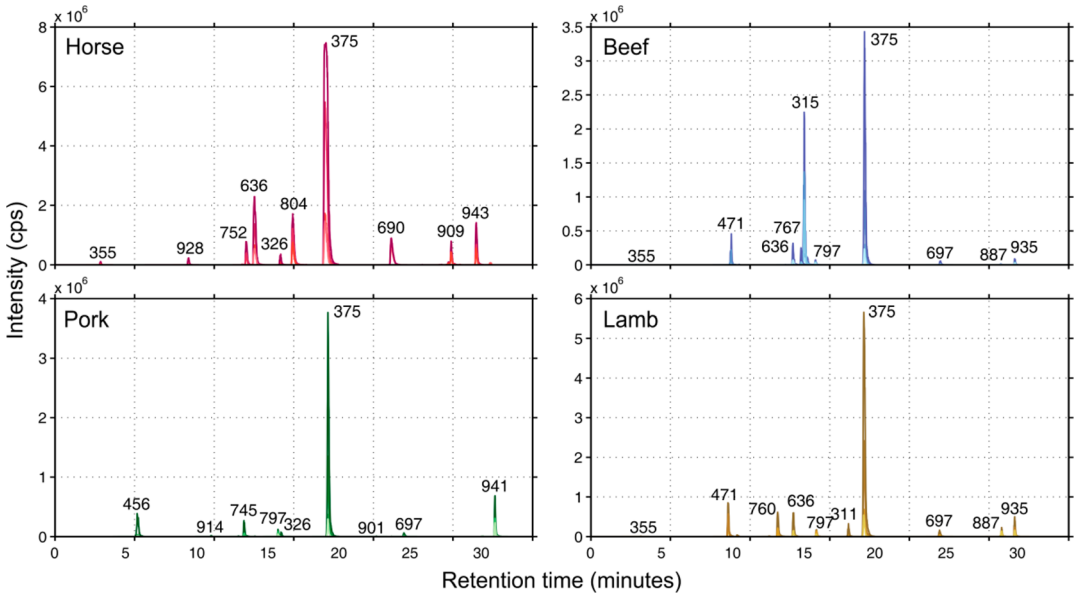

Figure 2. MRM transition intensities versus retention time for horse, beef, pork, and lamb reference myoglobins. Note the different ordinate axis scales. These data were acquired in dynamic mode. The numbers indicate precursor ion m/z values (daltons) for charge $z = 2$. Each "peak" is actually a cluster of peaks corresponding to four transitions emanating from a single precursor ion at a given retention time. Some peptides are shown to be common to more than one species, but others are candidates for differentiation between species.

the four proteins highlighted in yellow. Predicted tryptic cleavage sites are indicated by colored dots. By comparing the location of sequence variations and tryptic cut sites, it is possible to list candidate marker peptides that will differentiate between different Mbs. The method therefore relies on experimentally observing these predicted peptides together with their transitions. The transitions guarantee the peptide identity and specificity and will subsequently provide a route to quantitation.

Mass Spectrometry of Reference Myoglobin. We performed MRM MS on reference (i.e., purified) Mb from each species to enable the identification of suitable peptide markers for use with actual meat sample extracts. Figure 2 shows plots of transition intensities versus retention time for all four reference Mbs, again with our adopted color scheme. Each "peak" is actually a cluster of transition peaks arising from the same peptide precursor and sharing the same retention time. The data show clearly defined signals for a number of peptides, some of which are useful as markers. The sequences are known since they were programmed into the mass spectrometry method file from Skyline predictions. As many as 14 transitions were detected for some peptides. Using the four most intense transitions per peptide to secure identification provides a high level of specificity without wasting machine resources on detecting unnecessary transitions. In other contexts, transitions could be selected according to fragment identity rather than maximum peak intensity.

To expand on the comment that each "peak" is actually a cluster of peaks, Figure 3 shows an expansion of the four most intense transitions for 767 beef Mb peptide, 767→ (234, 1299, 706, 1396). For quantitation, the areas can be determined separately for each of the four transition peaks.

Selection of Marker Peptides. The peptides displayed in Figure 2 form the basis of our set of marker peptides set out in Table 1. Masses are quoted to the nearest integer for clarity in the table and effectively function as peptide and fragment labels. The fragments are selected and then ranked according to

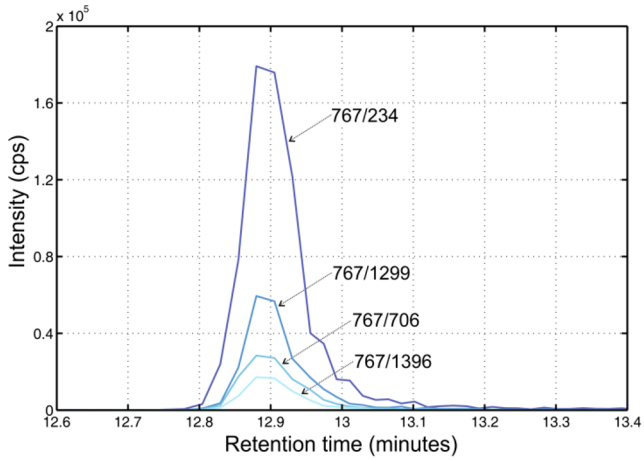

Figure 3. MRM transition intensities for four transitions in reference beef myoglobin, acquired in dynamic mode. The transitions shown are 767→ (234, 1299, 706, 1396). This figure is an expansion of the beef myoglobin peak at R_t = 13.2 min in Figure 2, emphasizing the peak clusters for each single precursor peptide.

intensity. The peptides appearing in Table 1 are marked on the sequences shown in Figure 1 using our red-blue-green-brown color scheme.

Not all peptides are equally valuable as markers. For completeness, Table 2 lists peptides that are less desirable as markers. Some are not detected in our experiment, some yield poor signal quality, some are common to all four Mbs and thus offer no discriminating power, and some elute in the column wash stage of the gradient or simply correspond to a maximal experimental run time. However, the late-running peptides may prove useful as reserves in cases where faster-running peptides fail. Tables 1 and 2 combined account for complete sequence coverage of the four Mbs excluding segments of four amino acids or less, which we have ignored.

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Table 1. Table of Myoglobin-Derived Marker Peptides[a]

m/z	fragments	R_t	species	sequence	red deer	goat	buffalo	rabbit	dog	other mammal	animal/ bird	plant
697	(147, 260 , 487, 430)	21.9	BPL	HGNTVLTALGGILK	●	●	●		●	●		
797	(1009, 1080, 815, 952)	14.1	BPL	VEADVAGHGQEVLIR	●	●				●		
637	(716, 1012, 147, 911)	12.6	BHL	LFTGHPETLEK	●	●	●			●		
326	(204, 76, 408, 351)	14.2	HP	ELGFQG				●	●	●	●	
471	(580, 509, 711, 310)	8.7	BL	NDMAAQYK	●	●						
690	(886, 985, 1242, 773)	21.1	H	HGTVVLTALGGILK								
752	(1269, 706, 248, 1366)	12.0	H	HPGDFGADAQGAMTK								
804	(1009, 815, 952, 500)	14.9	H	VEADIAGHGQEVLIR								
928	(953, 1081, 1394, 1523)	8.4	H	GHHEAELKPLAQSHATK					●	●		
456	(716, 490, 147, 619)	5.2	P	GHPETLEK						●		
745	(234, 1255, 692, 1352)	11.9	P	HPGDFGADAQGAMSK						●		
914	(953, 1367, 1054, 1496)	9.8	P	GHHEAELTPLAQSHATK								
311	(76, 204, 408, 351)	16.2	L	VLGFQG	●	●						●
760	(234, 1285, 692, 1083)	11.8	L	HPSDFGADAQGAMSK		●						
315	(417, 213, 530, 360)	13.4	B	VLGFHG			●					
767	(234, 1299, 706, 1396)	13.2	B	HPSDFGADAQAAMSK			●					

[a]Columns denote the precursor m/z (daltons) to the nearest integer; selected fragment masses m (daltons); retention time R_t (minutes); species code (H = horse, etc.); and precursor peptide amino acid sequence. Fragment ions are ordered according to high to low transition intensity peak maxima. In the last eight columns, dots indicate which of these peptides is expected to be present in other species. Species having identical Mb are not listed. See text for details.

Table 2. Table of Myoglobin-Derived Peptides Deemed Less Suitable as Markers[a]

m/z	R_t	species	sequence	comment
316	13.1	H	NDIAAK	poor signal
325	9.8	P	NDMAAK	poor signal
332	2.5	BHPL	ASEDLK	common; poor signal
375	17.0	BHPL	ALELFR	common
355	2.8	BHL	TEAEMK	poor signal
370	n/a	P	SEDEMK	not observed
454	n/a	BL	GHHEAEVK	not observed
504	n/a	BL	HLAESHANK	not observed
887	25.7	BL	GLSDGEWQLVLNAWGK	late running
901	20.0	P	GLSDGEWQLVLNVWGK	late running; poor signal
909	24.8	H	GLSDGEWQQVLNVWGK	late running
935	26.7	BL	YLEFISDAIIHVLHAK	late running
941	27.6	P	YLEFISEAIIQVLQSK	late running
943	26.6	H	YLEFISDAIIHVLHSK	late running

[a]Columns denote the precursor m/z (daltons) to the nearest integer; retention time R_t (minutes); species code (H = horse, etc.); precursor peptide amino acid sequence; and reason deemed less suitable.

Table 3. Detected Corresponding Peptides[a]

species mixture	corresponding peptides
B + L	B(767) + L(760)
B + L	B(315) + L(311)
B + H	B(697) + H(690)
B + H	B(797) + H(804)
B + H	B(767) + H(752)
B + H	B(315) + H(326)
B + P	B(315) + P(326)
B + P	B(767) + P(745)
L + H	L(697) + H(690)
L + H	L(797) + H(804)
L + H	L(760) + H(752)
L + H	L(311) + H(326)
L + P	L(311) + P(326)
L + P	L(760) + P(745)
H + P	H(690) + P(697)
H + P	H(804) + P(797)
H + P	H(752) + P(745)
H + P	H(928) + P(914)

[a]Columns denote mixture using species code (H = horse, etc.) and all possible pairs of corresponding peptides. The notation is species (marker peptide m/z value). Peptide sequences can be read from Table 1.

Several of the Mb peptides have been reported previously as markers. These include, from Table 1, beef peptide 767,[9,16,17] horse peptides 690 and 804,[16−18] and horse peptide 752.[9] From Table 2, peptides documented elsewhere include horse 909[16,17] and horse 943.[18]

The marker peptides of Table 1, from which suitable selections provide species identification, also form the basis of CPCP pairs, listed in Table 3. In the present work we have therefore established that candidate CPCPs for relative quantitation exist.

Mass Spectrometry of Meat Sample Extracts. Having used purified reference Mb to establish a list of marker peptides, their transitions, and their retention times, we progressed to actual meat samples. The presence of other non-Mb proteins in the meat sample extracts contributed a small background of single-transition peaks, in contrast to the multiple transitions that flag a target peptide. The background was minimized by the use of dynamic MRM (data not shown) based on reference Mb retention time results.

We have examined three meat mixtures: horse in beef, beef in lamb, and pork in lamb, selected due to their potential relevance to food fraud. As an example of the data, Figure 4 shows the output of different levels of horse mixed with beef,

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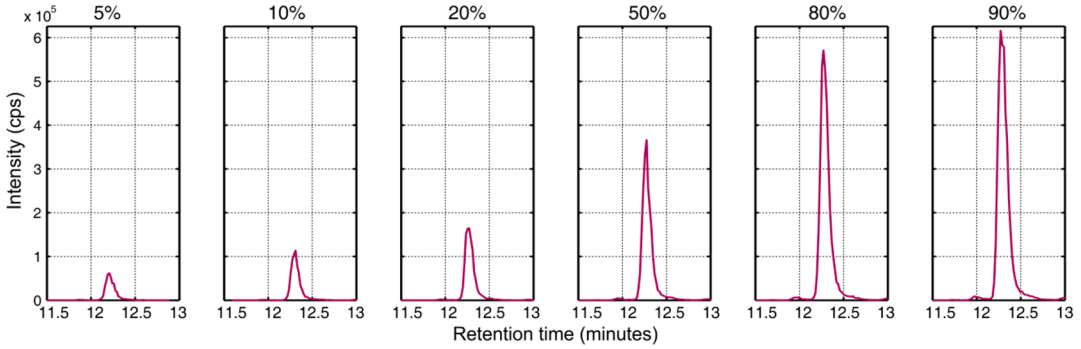

Figure 4. MRM transition intensities versus retention time for different mixtures of horse in beef, ranging from 5% to 90% horse (w/w). Only the single most intense transition, 752 → 1269, is depicted for clarity. The precursor peptide is HPGDFGADAQGAMTK and the transition fragment is GDFGADAQGAMTK, that is, fragment y^{13}. The ordinate axis is the same across all panels.

ranging from 5% to 90% horse (w/w). The data shown here are for a single transition, 752 → 1269, corresponding to the y^{13} fragment arising from the horse-unique peptide HPGDFGADAQGAMTK, where here "unique" means in the context of the four Mbs under study. The rest of the retention time axis is not shown since it is empty, a consequence of the use of dynamic MRM. In addition, use of the single "multimeat" method to acquire data obliges the mass spectrometer to record all transitions of potential interest from all four meat species. Single transitions can be extracted one at a time, which, combined with dynamic MRM, yields clear, unequivocal peaks. No marker peptides (as determined by the presence of multiple transitions) were observed when the relevant meat was absent from the sample even though the mass spectrometer was programmed to search for them.

Figure 4 shows that the transition peak from a meat extract sample has the expected form, is reproducible, and, with a view to quantitation, exhibits an increase in peak area with an increasing level of horse meat in the mixture. Other transitions for other marker peptides in this and other mixtures show corresponding behavior.

Quantitation is an eventual objective, so it is worth examining the behavior of the transition peak areas. The relevant corresponding peptide in beef has the mass label 767, and the relevant transition is 767 → 1299 (y^{13}). Ideally, the ratio of peak areas for transitions 752 → 1269 to 767 → 1299 (that is, horse to beef) will be proportional to the ratio of actual horse and beef meat in the mixture. The result of this comparison is depicted in Figure 5 in the format horse percentage by peak area versus horse percentage by weight. This figure suggests that the ratio of transition peak areas for CPCP-based quantitation shows clear promise.

To be a candidate for adulteration testing, a method must be able to detect levels of 1% or less of the adulterating species. Figure 6 shows the MRM MS transition intensities for 1% (w/w) of horse in beef, beef in lamb, and pork in lamb. The precursor peptides are 752 for horse, 767 for beef, and 745 for pork, and are all unique (cf. Table 1). In all three cases, all four required transitions are present. We conclude that the method is capable of detecting 1% (w/w) of one meat mixed with another across the set of four meats in this study. Specificity is guaranteed by the ability to detect at least four transitions. We have not pursued a limit of detection study since this is not part of our present objective.

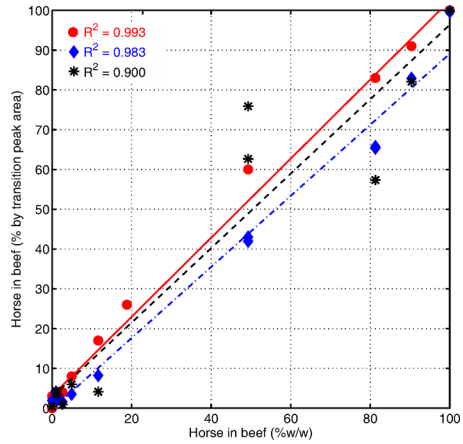

Figure 5. Plot of horse in beef, as percent by weight, versus horse in beef, as percent by transition peak area. The red solid line is B(767) + H(752), y^{13}, as in Figure 4. If A denotes peak area, the ordinate is the quotient $100A_H/(A_H + A_B)$, or explicitly as transitions $100A_{752\rightarrow1269}/(A_{752\rightarrow1269} + A_{767\rightarrow1299})$, where both transitions correspond to y^{13} fragments. Similarly, the black dashed line is B(797) + H(804), y^1, and the blue dot-dashed line is B(697) + H(690), y^5. The CPCP pair B(315) + H(326) (not shown) is nonideal due to suppression of the K−E cleavage in horse Mb, reflected in reduced levels of H(326) peptide.

DISCUSSION

For a system of four red meats—horse, beef, pork, and lamb—we have demonstrated the ability to detect a mixture of one with another at the level of 1% (w/w) via MRM mass spectrometry. We have done this using the idea of corresponding proteins, corresponding peptides (CPCP) and we have proposed, though not yet comprehensively demonstrated, that this approach offers a succinct route to relative quantitation. Using Mb, we have generated a list of peptide markers and transitions suitable for meat speciation in what is a target-led rather than shotgun proteomics approach.

We would class this as a multiplex targeted method, since the presence of several previously nominated target species is considered in a single test. More species could be added to the list, though not without practical consequences since the mass spectrometer requires a finite time to acquire each transition.

We have used Mb since it is a plentiful and heat-tolerant protein common to red meats, but for some species Mb may be a poor choice since not all organisms have high levels of Mb. Clearly the CPCP approach could apply to other organisms

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Anal. Chem. 2015, 87, 10315−10322

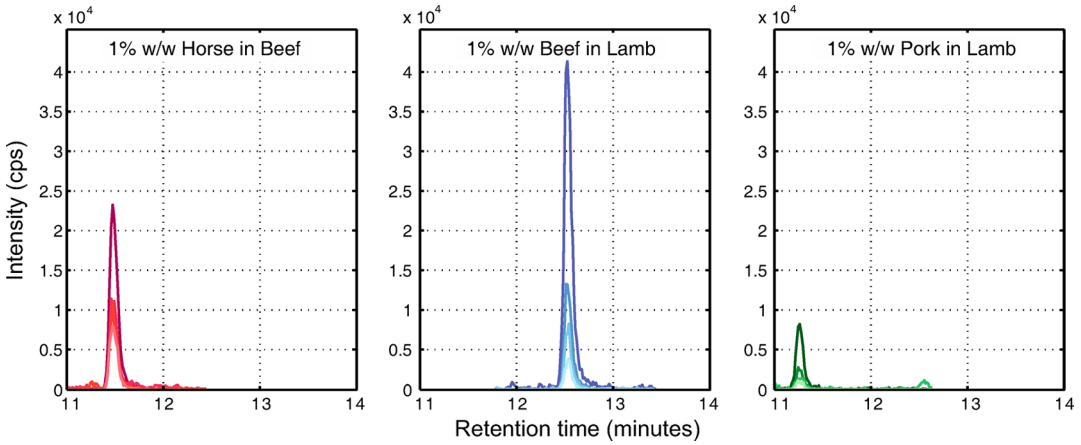

Figure 6. MRM transition intensities versus retention time for 1% (w/w) horse in beef, beef in lamb, and pork in lamb. The transitions depicted are horse 752→ (1269, 706, 248, 1366), beef 767→ (234, 1299, 706, 1396), and pork 745 → (234, 1255, 692, 1352). The ordinate axis is the same across all panels. The signal-to-noise ranges across all four transitions are 23−53, 33−63, and 5−22 respectively.

featuring other corresponding proteins, or to alternative corresponding proteins for the species featured here. In this context it should be noted that several species share identical Mb: the American bison (*Bison bison*) and yak (*Bos mutus*) have identical Mb to beef; the Plains zebra (*Equus quagga*) and donkey (*Equus asinus*)[29] have identical Mb to horse; and the chiru or Tibetan antelope (*Pantholops hodgsonii*) has identical Mb to lamb. In addition, the peptides that form the basis of the Table 3 CPCP list occur in some other species. These are summarized in Table 1 and include red deer (*Cervus elaphus*), goat (*Capra hircus*), buffalo (*Bubalus bubalis*), rabbit (*Oryctolagus cuniculus*), and dog (*Canis familiaris*). The single plant match in Table 1 is soybean (*Glycine max*). None of the target peptides occurs in chicken (*Gallus gallus*) or turkey (*Meleagris gallopavo*, identical Mb to chicken). These matches were generated by use of the Peptide Match utility[30] searching against the UniProtKB full database with the constraint that the identified strings be consistent with tryptic peptides. Therefore, any given choice of CPCP pairs may have some constraint in terms of the target meats, and the optimum choice of CPCP pairs is to some extent a function of the target species.

It is worth noting that the levels of Mb are not necessarily the same in all four of the species studied here.[31] Figure 5 is consistent with equal amounts of horse and beef giving rise to equal transition peak areas for the three peptide pairs shown, but this is possibly serendipitous given the potential for different Mb levels in the two meats.

It might seem that a simple correction to account for systematic differences in Mb levels of different species would resolve the Mb level issue. However, there is a further complication: the Mb level of an animal varies with its age and, within a given animal, with muscle type.[31,32] In some ways this is not as serious as it might seem since a realistic average Mb value can be established for commercially supplied meats of known provenance. However, for an adulterant meat the provenance cannot be assumed and the relevant level of Mb is more uncertain. As a result, a measurement of relative levels of Mb arising from two species in a sample cannot be mapped with certainty to an exact percent (w/w) mix of meats. In summary, the target for ratios of transition peak areas is a ratio of corresponding Mb levels from two species. The ratio of Mb levels then maps to an *estimate* of percentage (w/w) of meats informed by the documented range of Mb levels of the two species in the mix.

This is an instance of a general principle for any quantitative determination based on proteins or peptides: whether for absolute or relative quantitation, before a measure of the proportions of two meats in a mixture can be established, the concentration range of the target proteins in the meats must be established for each meat species.

We have in addition performed preliminary tests on highly processed foods (cooked and canned) and successfully recorded selected markers for beef, pork, and lamb in the appropriate foodstuffs (data not shown).

Since via CPCP both speciation and quantitation should be insensitive to partial extraction and proteolysis, we predict that both can be performed in no more than 2 h from presentation of meat sample material at ambient temperature through to communicable result.

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The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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Anal. Chem. 2015, 87, 10315−10322