

## Video Article

# Species Determination and Quantitation in Mixtures Using MRM Mass Spectrometry of Peptides Applied to Meat Authentication

Yvonne Gunning<sup>1</sup>, Andrew D. Watson<sup>1</sup>, Neil M. Rigby<sup>2</sup>, Mark Philo<sup>1</sup>, Joshua K. Peazer<sup>1,3</sup>, E. Kate Kemsley<sup>1</sup><sup>1</sup>Analytical Sciences Unit, Institute of Food Research<sup>2</sup>Institute of Food Research<sup>3</sup>School of Chemistry, University of East AngliaCorrespondence to: E. Kate Kemsley at [kate.kemsley@ifr.ac.uk](mailto:kate.kemsley@ifr.ac.uk)URL: <https://www.jove.com/video/54420>DOI: [doi:10.3791/54420](https://doi.org/10.3791/54420)

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## Abstract

We describe a simple protocol for identifying and quantifying the two components in binary mixtures of species possessing one or more similar proteins. Central to the method is the identification of 'corresponding proteins' in the species of interest, in other words proteins that are nominally the same but possess species-specific sequence differences. When subject to proteolysis, corresponding proteins will give rise to some peptides which are likewise similar but with species-specific variants. These are 'corresponding peptides'. Species-specific peptides can be used as markers for species determination, while pairs of corresponding peptides permit relative quantitation of two species in a mixture. The peptides are detected using multiple reaction monitoring (MRM) mass spectrometry, a highly specific technique that enables peptide-based species determination even in complex systems. In addition, the ratio of MRM peak areas deriving from corresponding peptides supports relative quantitation. Since corresponding proteins and peptides will, in the main, behave similarly in both processing and in experimental extraction and sample preparation, the relative quantitation should remain comparatively robust. In addition, this approach does not need the standards and calibrations required by absolute quantitation methods. The protocol is described in the context of red meats, which have convenient corresponding proteins in the form of their respective myoglobins. This application is relevant to food fraud detection: the method can detect 1% weight for weight of horse meat in beef. The corresponding protein, corresponding peptide (CPCP) relative quantitation using MRM peak area ratios gives good estimates of the weight for weight composition of a horse plus beef mixture.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/54420/>

## Introduction

The European horse meat scandal of 2013, in which undeclared horse meat was found in a number of supermarket beef products<sup>1</sup>, highlights the need for testing methods capable of detecting and measuring food fraud in meat. Several technologies have been explored, especially enzyme-linked immunosorbent assay (ELISA) and DNA-based methods<sup>2</sup>. An alternative route, based on mass spectrometry, targets species-specific peptides which in turn arise from species-specific proteins. Here we outline one such peptide-based approach that offers both identification and relative quantitation of the adulterant species in a meat mixture<sup>3</sup>.

The protocol is framed in the context of red meats and the desire to determine the presence of one in another at the level of 1% by weight, the level considered by some to represent fraudulent food adulteration as opposed to contamination<sup>4</sup>. The method relies in the first instance on identifying a protein which is nominally 'the same' in all target meats. Myoglobin, the protein responsible for the red color of meat, is a good candidate since it is abundant, relatively heat tolerant and water soluble, and has been used for species determination of meat previously<sup>5,6</sup>. The myoglobins for beef (*Bos Taurus*), pork (*Sus scrofa*), horse (*Equus caballus*) and lamb (*Ovis aries*)<sup>3</sup>, for instance, are nominally the same, as required, but their sequences are not identical. Such groups of 'similar but different' proteins, like these four myoglobins, can conveniently be described as 'corresponding proteins'. The sequence differences in these four myoglobins are species-specific: for example, the full myoglobin proteins for beef and horse, P02192 and P68082 respectively, each comprise 154 amino acids with 18 sequence differences between the two. Subject to proteolysis using trypsin these proteins produce two sets of peptides, some of which are identical, and some which show one or more species-specific amino acid differences: corresponding proteins therefore give rise to corresponding peptides.

The CPCP approach, therefore, seeks first to identify proteins from two or more species where these proteins exhibit limited species-specific sequence variants. These are corresponding proteins. Following proteolysis, corresponding proteins give rise to peptides, some of which likewise display species-specific sequence variants inherited from the parent protein. These are corresponding peptides. The CPCP approach can be used to compare levels of two corresponding proteins in a mixed species sample by monitoring the levels of corresponding peptides.

The natural technology for the detection of known peptides is multiple reaction monitoring mass spectrometry, or MRM-MS<sup>7</sup>. Species-specific peptides yield precursor ions, which along with their mass spectrometry fragment ions, are easily itemized in advance by software tools. These lists are then used to instruct the mass spectrometer to record only specific precursor plus fragment ion pairs, called transitions. A particular target peptide is therefore identified not only by its retention time in the chromatography preceding the mass spectrometer, but also by a set of transitions sharing a common precursor ion. This is a highly selective means of detecting known peptides that makes efficient use of the mass spectrometer resource.

Other authors have used mass spectrometry to test for meat adulteration via peptide markers but from disparate proteins<sup>8-14</sup>. Using the corresponding proteins, corresponding peptides (CPCP) scheme, however, means experimental conditions can be optimized, aiding identification of the species in the mixture from known species-specific transitions. In addition, corresponding proteins and peptides will generally behave similarly in the extraction, proteolysis and detection stages. Since transition peak areas are quantitative and reproducible, ratios of peak areas arising from pairs of corresponding peptides from different species provide a direct estimate of the relative quantities of two meats in a mixture. In contrast, more traditional quantitation routes exploit calibrations based on reference materials to establish absolute quantitation<sup>14,15</sup>.

Though the protocol is outlined in the context of myoglobin and meat, proteins other than myoglobin could be used for identification and relative quantitation via the CPCP strategy in meat mixtures, though potentially with modifications to the protocol. In addition the strategy is also applicable to binary mixtures of other species sharing one or more corresponding proteins.

The starting point for the protocol is purified 'reference' myoglobin, which for some species can be purchased but which for others must be prepared by conventional size-exclusion chromatography. The procedure for preparing reference myoglobin is not included in the protocol, but is described elsewhere<sup>3</sup>. Software tools<sup>16</sup> are used to list candidate peptides and transitions arising from myoglobins of interest. Each reference myoglobin is subjected to proteolysis and the resultant peptides analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to discover which of the candidate precursor ions and transitions are most useful, and to determine the matching peptide retention times. The outcome of this stage is a revised list of target peptides with their transitions, suitable for species determination, and a list of CPCP pairs, suitable for relative quantitation. To test real meats, sample extractions are prepared then subjected to proteolysis to generate peptides both from myoglobin and other extraneous proteins. The myoglobin-based peptides are then monitored by LC-ESI-MS/MS based on their listed transitions. The species present in a mixture are identified by the transition peaks associated with marker peptides. Estimates of the relative amounts of two meats in a binary mixture are calculated using ratios of transition peak areas. A set of test mixtures of pairs of meats will allow the ratio of peak areas for a given pair of transitions to be checked and calibrated against actual mixtures.

## Protocol

### 1. Proteolysis and Analysis of Reference Myoglobins

1. Proteolysis of reference myoglobins
  1. Prepare solutions of the purified reference myoglobins (range 0.2 - 0.5 mg/ml in 25 mM ammonium bicarbonate)<sup>3</sup>.
  2. Transfer 1 ml aliquots of each sample to 2 ml centrifuge tubes.
  3. Thermally denature the extracted proteins by heating the sample in a hot block at 95 °C for 30 min. Cool the sample for approximately 15 min until it reaches room temperature. Add 30 mg of urea (final concentration 0.5 M) to enhance the digestion, then mix.
2. Tryptic proteolysis
  1. Prepare a 1 mg/ml solution of trypsin in 25 mM ammonium bicarbonate and store on ice as required. Add a sufficient volume of trypsin such that the final enzyme activity is 420 BAEE (*N*-benzoyl-L-arginine ethyl ester hydrochloride) units/mg of extracted protein, then mix by gentle vortexing and allow to proteolyze overnight at 37 °C.
  2. Carry out sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)<sup>17</sup> to demonstrate the completeness of the proteolysis.
3. Desalting of the post-proteolysis sample
  1. Dilute the sample 1:2 v:v with water.
  2. Activate a polymeric reversed-phase (RP) cartridge filled with 30 mg RP material by adding 1 ml of methanol, then equilibrate the cartridge by adding 1 ml of 1% formic acid.
  3. Load the sample onto the cartridge under gravity.
  4. Wash with 1 ml of 5% methanol/1% formic acid under gravity.
  5. Elute the peptides with 1 ml of acetonitrile/water (90:10 v:v; 0.1% formic acid) under gravity into 2 ml microcentrifuge tubes prefilled with 5 µl dimethylsulphoxide (DMSO).
  6. Remove the solvent under vacuum at 50 °C using a centrifugal evaporator for 120 min, then redissolve the residue in 250 µl acetonitrile/water (3:97 v:v; 0.1% formic acid).
  7. Transfer the solution to a low volume auto sampler vial.  
Note: Samples can be stored at 4 °C until ready for liquid chromatography mass spectrometry (LC/MS) analysis.
4. Generation of transition lists for MRM
  1. Locate the myoglobin sequences for the different meats from the UniProt database.
  2. Enter the myoglobin sequences into the 'Target' box of the peptide and transition prediction software (e.g., Skyline). If required, hover over a peptide to reveal its fragment list.
  3. Click on 'Settings' and select 'Peptide Settings'. Input the preferences for the digestion (i.e., trypsin) and the number of missed cleavages (0). Enter the required selection for additional parameters, in particular, the peptide length (6 - 25), N-terminal exclusions (0) and assumed amino acid modifications (none).
  4. Click on 'Settings' and select 'Transition Settings'. Select the preferences for the instrument type used for the LC/MS analysis.

- Click on 'Export' and select 'Transition List' to create a spreadsheet containing the generated MRM transitions and parameters.

#### 5. Analysis by LC/MS

- Set up a system of binary gradient (water (A) and acetonitrile (B), each with 0.1% formic acid v:v) high performance liquid chromatograph (HPLC) with auto sampler, C18 core shell HPLC column (10 cm x 2.1 mm, 2.6  $\mu$ m particle size) connected to a triple quadrupole mass spectrometer operated in positive electrospray mode with MRM detection.
- In the data collection software (e.g., Analyst), select 'File' and 'New' and click on 'Acquisition Method' in the pop-up box then click on 'OK'.  
Note: This opens the instrument method editor, which contains a list of the connected devices that will enable the setup of a new LC/MS method.
- Click on 'Binary Pump' and input the flow rate value (300  $\mu$ l/min) and the gradient times in the table, setting a binary gradient profile of 3% B to 30% B over 22 min, increasing to 100% B at 23 min for a 5 min wash out before returning to initial conditions and re-equilibration for a further 6 min.
- Click on 'Autosampler' and insert the injection volume (5  $\mu$ l). Enable the 'Needle Wash Cycle' and enter the 'Wash Time' (30 sec) and select 'Flush Port'.
- Click on 'Thermostatted Column Controller' and in 'Column Oven Properties' set the 'Left Temperature' and 'Right Temperature' (40  $^{\circ}$ C).
- Click on 'Mass Spectrometer' and then click on 'Edit Parameters' to enter the source gas conditions. Select the 'Scan Type' as 'MRM(MRM)' and the 'Polarity' as 'Positive'. Go to 'Period Summary' and enter the 'Duration Time', the total time for the LC analysis and equilibration (35 min).
- In the table right click and select 'Declustering Potential (DP)' and 'Collision Energy (CP)' to add these columns to the table. Enter the Q1, Q3, Time (msec), ID, DP and CE values for all of the transitions, for a single meat species, created in the transition list (see step 1.4.5).  
Note: Time (msec) refers to the dwell time, the time the mass spectrometer spends scanning each transition, the summation of which should not exceed 3 sec.
- Save the Acquisition Method file (file extension .dam).  
Note: Steps 1.5.2 - 1.5.8 need to be repeated for each meat species. This will create a single method file for each meat species in screen mode in preparation for analysis below.
- In the data collection software, click on 'Acquire' and select 'Equilibrate'. In the box that opens, select the required Acquisition Method to begin the instrument equilibration.
- Put the sample vials in a rack in the auto sampler.
- Click on 'File' and select 'New' then 'Acquisition Batch'. In the 'Sample' tab select 'Add Set' then 'Add Samples'. Insert the number of samples to be analyzed and click on 'OK'. In the 'Acquisition' box select the method file that will be used for the analysis from the drop down menu.
- In the table, select 'Plate Code' and select the appropriate tray configuration from the drop down menu. Left click in the 'Plate Code' column header then right click and select 'Fill Down'. In 'Vial Position' enter the position of each sample in the auto sampler in the rows.
- In 'Data File' enter the file name for the acquisition, then left click in the column header followed by right click and select 'Fill Down'. In 'Sample Name' insert the identity of each of the samples to be analyzed. Save as an acquisition batch file (file extension .dab).
- Click on the 'Submit' tab then highlight the samples that need to be analyzed on the LC/MS. Click on 'Submit'. Click on 'Acquire' and 'Start Sample' to begin the analysis.  
Note: Each acquisition method will scan for the MRM transitions across the entire length of the chromatograph for a single meat species. Mass spectrometer settings for an MRM acquisition vary according to instrument type and peptide.
- View the generated data files using data viewing software. Click on XIC (extracted ions) and in the drop down list highlight all the fragments (Q3 values) for a single precursor (Q1). A new pane will open that shows only the selected transitions.
- Record the retention time ( $R_t$ ) for groups of concurrent transitions since these correspond to a single peptide.
- Repeat the previous two steps for each set of transitions in order to assign the peaks to their respective peptides for each of the meat species.
- Record the marker peptides which are suitable for providing species identification (e.g., peptide HPGDFGADAQGAMTK, precursor  $m/z = 752$ ,  $R_t = 12.0$  min, for horse), together with their retention times, and note which form corresponding pairs suitable for relative quantitation.  
Note: For example, the horse marker peptide (precursor  $m/z = 752$ ) has a corresponding beef peptide, HPSDFGADAQAAMSK (precursor  $m/z = 767$ ,  $R_t = 13.2$  min).
- In order to create a single dynamic method embracing all of the meat species, in the data viewing software, for each meat species in turn, open the XIC transition data for each precursor (assigned to a particular peptide in 1.5.8).
- Zoom in on the peak cluster at the selected retention time by left-clicking and dragging the cursor underneath the cluster. Identify the most intense transitions (by right-clicking on the peak label).
- Manually record the transitions and retention times in a spreadsheet.
- To enter the parameters as a new dynamic method on the LC/MS software, click on 'Mass Spectrometer' and then click on 'Edit Parameters' to enter the source gas conditions. Select the 'Scan Type' as 'MRM(MRM)' and the 'Polarity' as 'Positive'.
- Go to 'Period Summary' and enter the duration time (set as the total time for the LC analysis and equilibration). In the table right click and select 'Declustering Potential (DP)' and 'Collision Energy (CP)' to add these columns to the table.  
Note: The 'Time' column now refers to the expected retention time (min) for each transition.
- In the 'Edit Parameters' section of the LC/MS data collection software, check the 'Scheduled MRM' box. Input the Q1, Q3, Time (min), ID, DP and CE values for the transitions created in the spreadsheet (1.5.21) and save the Acquisition Method (file extension .dam).  
Note: This method typically reduces the number of MRM transitions to the 4 most intense for each peptide and scans only across the retention time window for each peptide peak, giving improved sensitivity and quality of the data. A 'dynamic' method is a 'guided retention time windowing' method, sometimes called scheduling.

## 2. Preparation and Analysis of Calibration Samples

1. Extraction of meat mixtures
  1. Using meat previously frozen then ground into a powder, prepare a range of meat mixtures by weighing respective amounts of meat (total mass of about 300 mg) into 15 ml plastic centrifuge tubes.
  2. Add 4 ml of extraction buffer (0.15 M potassium chloride + 0.15 M phosphate buffer at pH 6.5). Vortex for 30 sec. Extract on a lab shaker at room temperature for 2 hr at 250 cycles/min.  
Note: Cycles/min refers to an oscillatory motion.
  3. Transfer 2 ml of the extract into a 2 ml microcentrifuge tube. Centrifuge for 5 min at 4 °C at 17,000 x g.
  4. Transfer 200 µl aliquots of the supernatant (reserving a small amount for protein assay, see 2.2) into 2 ml centrifuge tubes and dry using a centrifugal evaporator (pre-set program: 50 °C, with no venting and 120 min duration).
2. Protein assay
  1. Transfer 7 µl aliquots of the reserved supernatant (see 2.1.4) in triplicate into the wells of a 96 well plate.
  2. Transfer 7 µl aliquots of a series of protein standards in triplicate, range 0 - 1.0 mg/ml bovine serum albumin (BSA), to the same 96 well plate.
  3. Add 200 µl of Coomassie plus protein assay reagent to each well.
  4. Visually compare the color of the sample wells with the protein standards to check the samples are in the range of the calibration standards. If necessary, repeat with diluted sample so it becomes in range.
  5. Leave the plate to stand for 3 min.
  6. Burst any bubbles that have formed with a hypodermic needle.
  7. Analyze the plate on the plate reader using a standard endpoint protocol at a wavelength of 595 nm.
  8. Determine the protein concentration of the samples using calibration data from the protein standards.  
Note: This is required for calculation of the amount of trypsin used in the tryptic digest.
3. Proteolysis of meat mixtures
  1. Redissolve the dried residue from step 2.1.4 in 1 ml of 25 mM ammonium bicarbonate solution. Mix well on a rotamixer.
  2. Follow the protocol from step 1.1.3 to 1.3.7.
4. Analysis by LC/MS
  1. Set up the LC/MS as previously (step 1.5.1).
  2. Create a new acquisition batch as outlined previously (steps 1.5.9 - 1.5.14), selecting the acquisition method created at step 1.5.24 that uses a dynamic LC/MS method combining all of the meat species, and acquire the data for the digested meat samples.
  3. Display the full chromatogram in the data viewing software. Display the XIC for each transition set in turn. Visually confirm each cluster contains the requisite number of bell-shaped peaks at the expected retention time, thereby confirming the existence of the selected peptide.
  4. Perform quantitation using the data viewing software to integrate peak areas for each of the transitions of interest by double-clicking on 'Build Quantitation Method' in the Navigation bar.
  5. In the 'Select Sample' pane select the 'Data File' and the 'Sample' to be analyzed to generate an 'Analytes' table.
  6. Click on the 'Integration' tab to display the first of the transitions (Analytes) to be integrated.
  7. Click on 'Analyte' box to display the drop down list of transitions. Select each transition in turn to display it and visually confirm the correct peak is selected for integration. To modify or force the integration, left click and drag the cursor over the target peak (this will be highlighted in green). Click on the 'Select Peak' button and click 'Apply'.
  8. Save the workspace as a method file (.qmf).  
Note: This creates a Quantitation Method file for subsequent calculation of sample peak areas.
  9. Double click 'Quantitation Wizard' in the Navigation bar. In the 'Select Samples' window create 'Quantitation Set' by selecting a single 'Data File', then one or more 'Available Samples'. Select 'Next' to display 'Select Settings and Query' box. Leave with defaults, select 'Next' to display 'Select Method'. From the drop down 'Method' box select the 'Integration Method' file created in step 2.4.8, then select 'Finish'.  
Note: This creates a 'Results Table', including transition peak areas arising from meat mixtures.
  10. Save the 'Results Table' (file extension .rdb), export as a text file (.txt) and open it in spreadsheet to review the data.
  11. Plot graphs of the percentage (by transition peak area) of one meat in another versus the measured percentage (w/w) of the two meats for the selected MRM for selected transitions from corresponding peptides, focusing on those cases where the two fragments contain the same number of amino acids as counted from the C-terminal end.  
Note: Identical fragments with identical fragmentation sites give optimal results.
  12. Examine the plots from 2.4.11 above. Either visually, or using a trend line tool in the plotting package, identify a group of plots which are both linear and of similar gradient. Use any one or more of these CPCP plus fragment combinations for calibration in real meat samples.  
Note: A plot showing an unusual gradient may indicate either peptide or fragment suppression with a consequent reduction in signal strength. Non-linear plots may indicate poor peak detection or other problems.

## 3. Meat Samples

1. Extraction of proteins from target meat samples
  1. Where applicable, excise extraneous non-meat material from the sample using a spatula. For example, scrape away sauce and pasta from a chilled lasagna.
  2. Weigh 20 g of the meat into a metal beaker.
  3. Add 100 ml of 0.15 M potassium chloride/0.15 M potassium monophosphate buffer at pH 6.5.

4. Extract the proteins by blending the meat in a high speed homogenizer for 1 min.
  5. Follow the protocol from step 2.1.4 - 2.3.2.
2. Analysis of samples by LC/MS
1. Repeat step 2.4.2 to acquire data using the dynamic LC/MS method.
  2. Identify the peptides from each meat myoglobin as performed in step 2.4.3.
  3. For quantitation, use quantitation software to integrate the peak areas for each transition of interest, as outlined in step 2.4.9.
  4. For identification of species in a mixture, record those marker peptides satisfying agreed criteria for numbers of transitions and signal to noise for those transitions.
  5. For quantitation, use integrated transition peak areas as agreed from step 2.4.12 and, using percentage by transition peak area, calculate the percentage of myoglobin from the two species in the mixture.
  6. Use prior knowledge from the literature<sup>18</sup> of likely myoglobin levels in the meats to estimate the relative w/w amounts of two meats present in the sample.

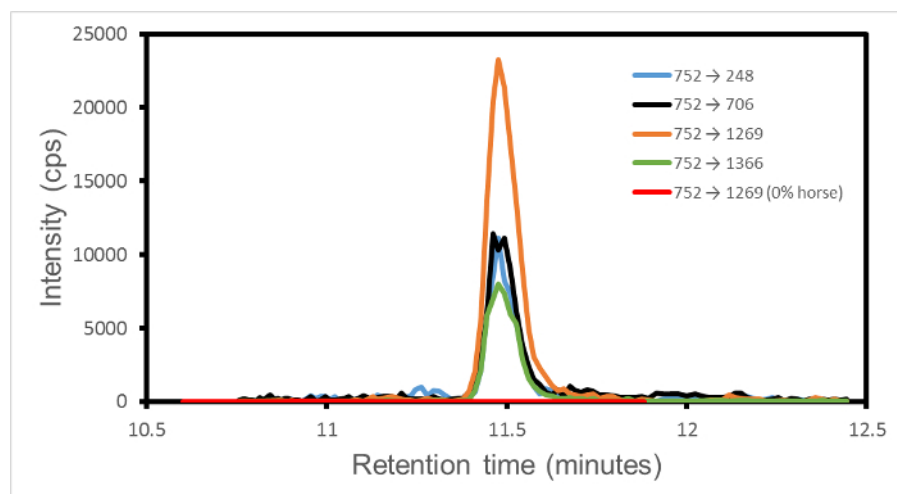
## Representative Results

In a single dynamic-mode MRM experiment each programmed transition is recorded separately (as detector counts per sec, cps) over a specified retention time window. Therefore, from all the data collected in one experiment, the peak intensity for each transition can be individually extracted. Then the only finite signal is for the retention time window set for that transition. Outside of the window, the signal is zero by definition. The signal for any one transition, for example, 752 → 1269 from horse (peptide monoisotopic mass 1,501.66 daltons, precursor ion  $m/z$  751.84 daltons, charge state = 2, fragment ion  $y^{13}$ ) typically has to compete only with measurement noise and not from other transition peaks that might perhaps be from other species. The output is therefore a set of clean peaks, one per transition, at a common retention time for those transitions sharing a common precursor ion.

**Figure 1** shows the output for the set of four transitions 752 → (1269, 706, 248, 1366) for a mixture of 1% w/w horse in beef. Since the four transitions displayed are associated with horse, and are absent in samples of pure beef, lamb or pork, these peaks signify the presence of horse. Depending on robustness criteria, a set of two or more transitions each exceeding some specified signal to noise level establishes identification. This figure therefore establishes the presence of horse in the mixture of 1% w/w horse in beef.

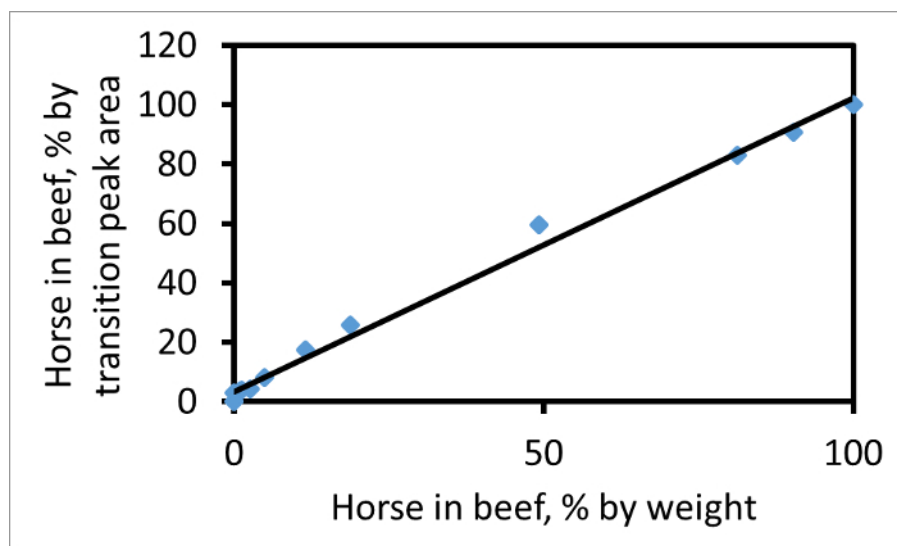
Occasionally, a single isolated transition is detected. This indicates a chance match of precursor ion and a single fragment, possibly from an extraneous protein, with those expected from the system and programed into the mass spectrometer. The singular nature of the peak, and its occurrence at an unexpected retention time, is the signature of an accidental transition that can be ignored.

The area under each transition peak can be calculated individually. Based on a suitable fragment, the ratio of horse to beef transition peak areas, for example, 752 → 1269 (horse) to 767 → 1299 (beef), will be proportional to the ratio of actual meats in the mixture. **Figure 2** shows a plot of percentage by peak area for these two transitions versus the percentage weight for weight of horse in a mixture of horse with beef. If the percentage transition peak areas match the percentage weight for weight of meat then the slope is 1. The slope in this plot is 1.03, indicating that, for these transitions and CPCP pair, the transition peak areas give a reliable measure of the relative amounts of the two meats in the mixture. If the horse meat in the sample was twice as rich in myoglobin as the beef then, with other factors unchanged, the slope of the line would be greater than one.



**Figure 1. MRM transition intensities versus retention time for 1% w/w horse in beef.** The transitions are 752 → (1269, 706, 248, 1366), shown in orange, black, blue and green, respectively. The marker peptide is HPGDFGADAQGAMTK. The four transition fragments can be denoted  $y^{13}$ ,  $y^7$ ,  $y^2$  and  $y^{14}$ , respectively, where  $y^n$  denotes counting in  $n$  amino acids from the peptide C-terminal end. The signal to noise varies from 23 to 53 over the four transitions. An additional red line denotes the 752 → 1269 transition for 0% horse, 100% beef for comparison. Only the non-zero region of the retention time is displayed. This figure has been modified from Watson *et al.*<sup>3</sup>. [Please click here to view a larger version of this figure.](#)





**Figure 2. Plot of horse in beef, as percent weight for weight, versus horse in beef as percent transition peak area.** The plot uses the pair of peptides beef (767) and horse (752) and the  $y^{13}$  fragment ion for both. If  $A$  denotes peak area then the ordinate is  $100A_H / (A_H + A_B)$ . The slope of the best fit line ( $R^2 = 0.99$ ) is 1.03. This figure has been modified from Watson *et al.*<sup>3</sup>. [Please click here to view a larger version of this figure.](#)

## Discussion

The selection of a suitable target protein is important. A good target protein needs to have corresponding forms in species of interest, sufficient species-dependent sequence variation, species specificity, and exist in accessible quantities within the organisms. For assessing mixtures that have undergone processing (for example, heat treatment), a protein having a sequence relatively immune to that processing is desirable. Myoglobin is a good candidate for red meats, including cooked red meats, but is not the only possibility. Once the target protein is decided, the most critical part of the protocol is the protein proteolysis. A protein different from myoglobin may well demand an alternative proteolysis protocol.

The protocol as described includes a segment based on reference purified protein. This aims to discover retention time windows and suitable precursor and fragment ions. This segment is very helpful but not essential.

Although corresponding peptide pairs from two species of interest can be listed even without experiment, it is sometimes the case that a sequence difference has dramatic consequences on the digestion profile. For example, the peptide pair VLGFGH (beef) and ELGFQG (horse) give an anomalous quantitation result (manifest as a gradient less than one in **Figure 2**). This is because the latter peptide arises from a relatively suppressed K-E cleavage, causing an under-estimate of the level of horse in the mixture. Corresponding peptides starting with different amino acids are therefore best avoided. Often the fragments from two corresponding peptides have identical amino acid sequences and are well-behaved, but this is not always the case and needs to be checked during method development. Species identification is much less sensitive to these issues than relative quantitation.

The protocol has been demonstrated for four red meats<sup>3</sup>. Additional meat species can be included, though the quality of the transition peak shape may deteriorate if too many marker peptides co-elute, effectively reducing the dwell time and ultimately degrading relative quantitation estimates. Improved instrumentation, already available, will improve this. A related issue is that not all meats have different myoglobins. For example, horse, donkey and zebra myoglobins are identical and thus strictly speaking the method is only capable of detecting horse or donkey or zebra in beef. In some cases, even though myoglobins are not identical, some key peptides can be. For example, some lamb myoglobin-derived marker peptides also appear in goat.

A complication facing this and any other protein-based quantitation method is that the protein level must be assumed constant across all species if the protein or peptide levels are to equate trivially to levels of meats in a mixture. For myoglobin and the four red meats this is not universally true. The levels in general are species dependent, with pork exhibiting the lowest level of the four. In addition, the myoglobin level varies with meat cut and animal age. So although ratios of transition peak areas map reliably to ratios of myoglobin, the mapping to ratio of actual meats is an estimate drawing on assumptions regarding likely sources of the meats in the mixture.

The approach outlined in this work differs in a number of ways from other published contributions. A more typical route is to use proteomic methods to identify various disparate species-dependent marker peptides, in which case the markers for different species possess no particular relationship with one another<sup>8-12,14,19</sup>. By contrast, we have selected proteins common to all species of interest up to species-dependent sequence variants<sup>3</sup>. Apart from being central to our relative quantitation strategy, this has the advantage that sample preparation strategies can be optimized. In addition, such corresponding proteins might be expected to behave similarly, for example, in extraction or in commercial processing of samples such as cooking or canning. Species identification then normally proceeds via detection of disparate marker peptides, whereas in the CPCP approach species identification proceeds via detection of closely related peptides possessing typically one or two sequence differences. Finally, quantitation of proteins to estimate the percent by weight of one species in another might conventionally proceed via absolute quantitation of each protein separately based on known standards<sup>7,14,15</sup>. However using the CPCP method there is no need for calibration methods. Instead, relative levels are estimated by comparing signal strengths of two corresponding peptides from the two species, bypassing the absolute measurement stage altogether. Since the ultimate goal is a percentage by weight of one species in another, a relative quantitation, then the CPCP is both more direct and simpler than comparing two absolute quantitation measurements. These features translate

into short experimental times, anticipated to be approximately two hr using refined protocols, making the technique useful as a rapid surveillance tool in the realm of food fraud detection.

## Disclosures

The authors have nothing to disclose.

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