

SPECIAL GUEST EDITOR SECTION

Advances in LC–MS/MS Methods for Allergen Testing, Meat Speciation, and Gelatin Speciation

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Background: Food authenticity is demanded by the consumer at all times. The consumer places trust in the manufacturer that the food product is genuine in terms of what is recorded on the packaging label. **Objective:** Recent advancements in LC–tandem MS methodology in the detection of allergens, meat, and gelatin speciation in raw food products and processed foods are detailed in this paper. **Method:** For each of the three methods, initial proteome analysis and the screening leading to the determination of unique tryptic peptides were conducted using a high-resolution, accurate tandem mass spectrometer. Having identified the unique markers, the method was transferred to a tandem quadrupole mass spectrometer for a higher-sensitivity quantitative study, multiple reaction monitoring transition analysis. **Results:** For the allergens method a detection limit of at least 10 ppm was attained across the 12 allergen peptides in this workflow. In the gluten workflow the resulting chromatograms show good detection down to 5 ppm, with no interference from the food matrices. The meat speciation method details that signature peptides could be readily identified at 1% w/w with no matrix interference. **Conclusions:** These single-injection workflows with cycle-time optimization enable wide coverage of analytes to identify multiple species within challenging matrix samples.

containing meat byproducts, such as gelatin. Gelatin is a water-soluble protein obtained by the partial hydrolysis of collagen, primarily sourced from pig bones and hides, although it can also be produced from cows, poultry, and fish. It is used in the manufacture of a broad range of food products as well as in the pharmaceutical industry, primarily to make capsules. Any product, food, or pharmaceutical that contains ingredients sourced from the pork industry must not be labeled halal. The global halal market was recently valued at around 600 billion U.S. dollars (1), and it is of particular importance that consumers who require halal goods can be assured of the authenticity of the products they buy.

Food authenticity is also of great importance to allergy sufferers. It is estimated that 5% of U.S. adults suffer from one or more food allergies (2), and although fatal anaphylaxis from food remains rare (3), severe allergic reactions can frequently require a hospital visit, increasing the burden on health care systems (4). Although most regulatory bodies have not so far set threshold limits for allergens, the Japanese government has introduced legislation that foods containing allergenic proteins at >10 ppm must be labeled (5). Sensitive, reliable techniques are needed to meet this requirement and any future regulatory requirements.

Historically, ELISA and PCR have been the major techniques used to enforce food authenticity and labeling. Although both techniques are relatively quick and simple to perform, they suffer significant drawbacks. ELISA is susceptible to cross-reactions with the matrix, resulting in false positives and false negatives and undermining confidence in the results. It is also limited in its selectivity and by the fact that only one allergen or species can generally be screened at a time (6, 7). PCR amplifies specific DNA samples of interest within a food matrix. However, food processing can frequently remove DNA samples or damage them beyond the PCR's ability to detect them, leading to false negatives.

In recent years, LC–tandem MS (MS/MS) techniques have been developed to directly detect digested species- and allergen-specific marker peptide fragments that are present in complex food matrices (8, 9). These techniques can deliver crucial quantitation of contaminants and allergens contained in food products. The methodology for the development of the successful multiple reaction monitoring (MRM) LC–MS/MS experiments outlined in this article first requires peptide mapping experiments using the TripleTOF® 6600 system and ProteinPilot™ software to identify unique “signature” contaminant peptides. Following this, the MRM method is optimized by identifying key transitions and increasing the selectivity and sensitivity of the experiments. Correctly implemented, the MRM LC–MS/MS methodology can identify numerous allergens or contaminant species in a

In 2013, scandal rocked the European food industry when significant quantities of horse and pig DNA were found in a range of products packaged and labeled as beef. This raised serious ethical, religious, and health questions and is an example of the gross misleading of consumers. The scandal shone a spotlight on the need for better regulations as well as more reliable, more reproducible techniques for ensuring food authenticity and correct ingredient labeling throughout the food distribution chain. However, it is not just meat products that raise questions of authenticity but also those products

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single sample injection, providing the speed, sensitivity, and accuracy that food distributors and key regulators require to ensure food authenticity.

Method

Sample Preparation

To prepare samples for proteome analysis and selection of unique peptides, known concentrations of contaminant (allergen or meat species) were spiked into raw food products. For preparing processed foods, raw goods were cooked according to the manufacturer's specifications. Foods were homogenized, and lipids were removed with hexane before drying by evaporation. Samples were incubated with extraction buffer, and the protein component was separated out by centrifugation. The protein component was reduced by incubation with TCEP and alkylated using MMTS, a cysteine-blocking reagent. Modified peptides were then digested by incubation with trypsin to yield tryptic peptides.

Marker Selection

Tryptic peptides were separated by LC and screened using a SCIEX TripleTOF 6600 LC-MS/MS system. Peptide mapping was performed using ProteinPilot software to identify peptide IDs. A further database search was performed using the National Center for Biotechnology Information's pBLAST to assess each peptide's uniqueness across other common food proteins. Candidate marker peptides were chosen based on the sensitivity of their detection in raw and processed foods, their uniqueness within the proteome, their sample preparation reproducibility, and their susceptibility to posttranslational modifications and Maillard reactions. In general, two MRM transitions were chosen per peptide to ensure a balance between sensitivity and specificity.

Screening Analysis

Screening analysis was performed using a SCIEX QTRAP[®] 4500 system with Turbo VTM source in positive electrospray ionization (ESI) mode. The Scheduled MRMTM algorithm was used to monitor MRM transitions for each peptide around

the time of its expected elution. This enables the number of simultaneous MRM experiments in a cycle to be decreased, allowing cycle time to be maintained while maximizing sensitivity. This approach allows all peptide markers to be analyzed in a single sample injection and also opens up the method for adding specific markers from other allergens or animal species when they become of interest. Following analysis, calibration curves could be generated for each analyte. For quantitative studies, higher sensitivity was required, and a SCIEX QTRAP 6500+ system was used.

Results and Discussion

Allergen Screening Method

For allergen detection, eight groups of substances causing allergies and intolerances were chosen for analysis (as defined by the World Health Organization; 10). The allergens chosen included egg white, skim milk, peanut, soy, and various tree nuts. Analyses were performed on bakery products including bread and cookies, both raw and processed.

Following the workflow outlined above, two unique proteins, two unique peptides per protein, and two unique MRM transitions per peptide were chosen for 10 of the 12 allergens, with only one unique protein being selected for egg white and pine nut. This resulted in 88 MRM transitions to monitor in total. To analyze allergen presence in food samples spiked with various allergen concentrations, a QTRAP 4500 system with Turbo V source in positive ESI mode with an ion source temperature of 500°C was used. The detection limit was at least 10 ppm for each of the 12 allergens, and the signal showed good linearity as allergen concentration increased. Calibration curves could be generated for each peptide and its two MRM transitions over a wide dynamic range (Figure 1), showing good reproducibility. The regression values for each of the 12 allergens was over 0.95. This method was extended to commercially available goods by screening for allergens in various types of cookies. Allergen-free negative controls showed no signals in the LC-MS/MS method, whereas allergens were detected in those samples that listed peanuts and hazelnuts as ingredients. Milk and egg were also detected in these goods (Figure 2).

The 12-allergen LC-MS/MS screen was compared in performance to allergen screening using two different ELISA kits. Signature peptides were analyzed for hazelnut and

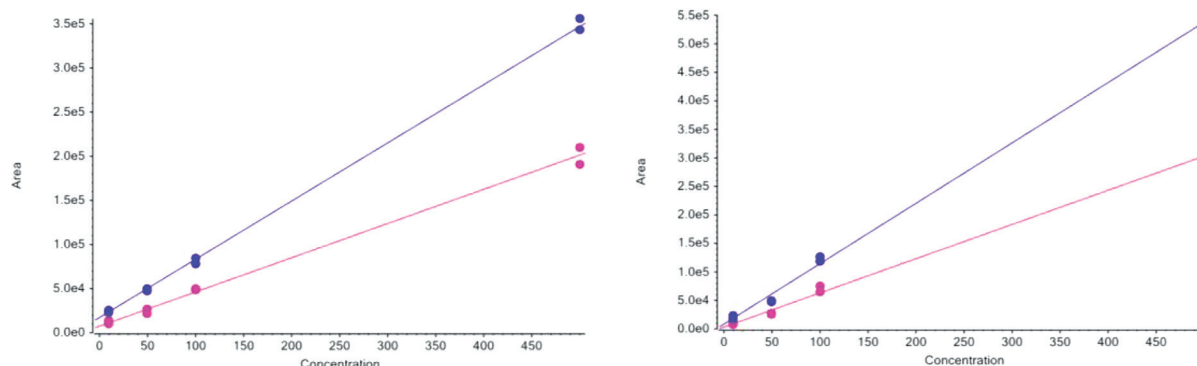


Figure 1. Calibration lines for a hazelnut peptide (left) and a peanut peptide (right). Two MRM transitions were monitored for each: fragment 1 (blue) and fragment 2 (pink).

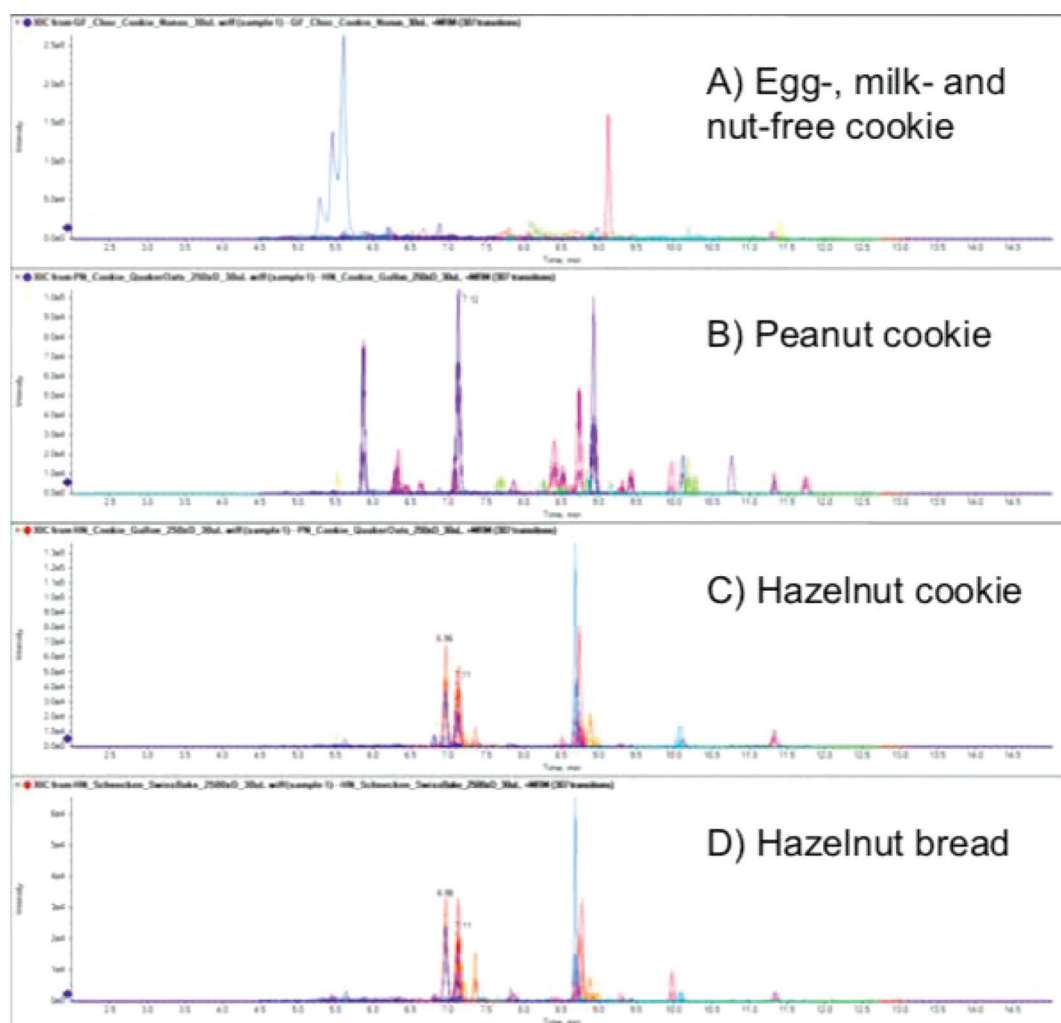


Figure 2. Extracted ion chromatograms of (A) an allergen-free cookie, (B) a peanut cookie, (C) a hazelnut cookie, and (D) hazelnut bread.

peanut using both methods. In general, the results showed good correlation between LC–MS/MS and ELISA ($r^2 \geq 0.99$). However, the concentrations of allergens were less well quantitated by ELISA testing; the levels of hazelnut and peanut were underestimated in food matrices, particularly as allergen concentrations were increased (Figure 3).

This method has a detection limit of at least 10 ppm on the SCIEX QTRAP 4500 LC–MS/MS system and meets the performance requirements of the AOAC INTERNATIONAL *Standard Method Performance Requirement* (SMPR®) 2016.002. In March 2018, it was given Official First Action status by the AOAC's Official Methods Board. The method, "Simultaneous Analysis of Multiple Allergens in Food Products by LC–MS/MS," was published in the *Journal of AOAC INTERNATIONAL* (15). Following this recognition by the AOAC, the four-allergen screen is becoming established as an important tool for the food distribution chain to ensure accurate labeling.

Gluten Detection and Quantitation

A similar method has also been developed using the SCIEX QTRAP 4500 LC–MS/MS to detect gluten from wheat, rye, barley, and oats in complex food matrices with

a sensitivity of 5 ppm. Together with standards and internal standards provided in the SCIEX vMethod™ kit (16). Gluten can be quantified from the samples. Around 5% of the world population is thought to be negatively affected by gluten ingestion through diverse mechanisms including celiac disease, wheat allergies, and nonceliac gluten sensitivity (11). For populations affected by gluten, the health consequences can be severe, ranging from bloating and diarrhea to nutrient deficiencies (12). Recent standards introduced by the U.S. Food and Drug Administration place a gluten limit of 20 ppm in products labeled "gluten free" (13), a level surpassed by this sensitive MS/MS technique.

For each grain type, three MRM transitions were selected for a single unique marker peptide released from the glutenin protein subunit (DY10). The most selective of the three MRM transitions for each grain type were used in experiments for gluten quantitation, while the remaining two were used for qualitative assessment of gluten presence in foodstuffs. For QC, analyses were carried out on food homogenates (cookies, beer, and baby formula) spiked with known gluten standard concentrations (0, 5, and 1000 ppm) using the QTRAP 4500 LC–MS/MS system. The resulting chromatograms show good detection of gluten down to 5 ppm, with no interference from the food matrices (Figure 4).

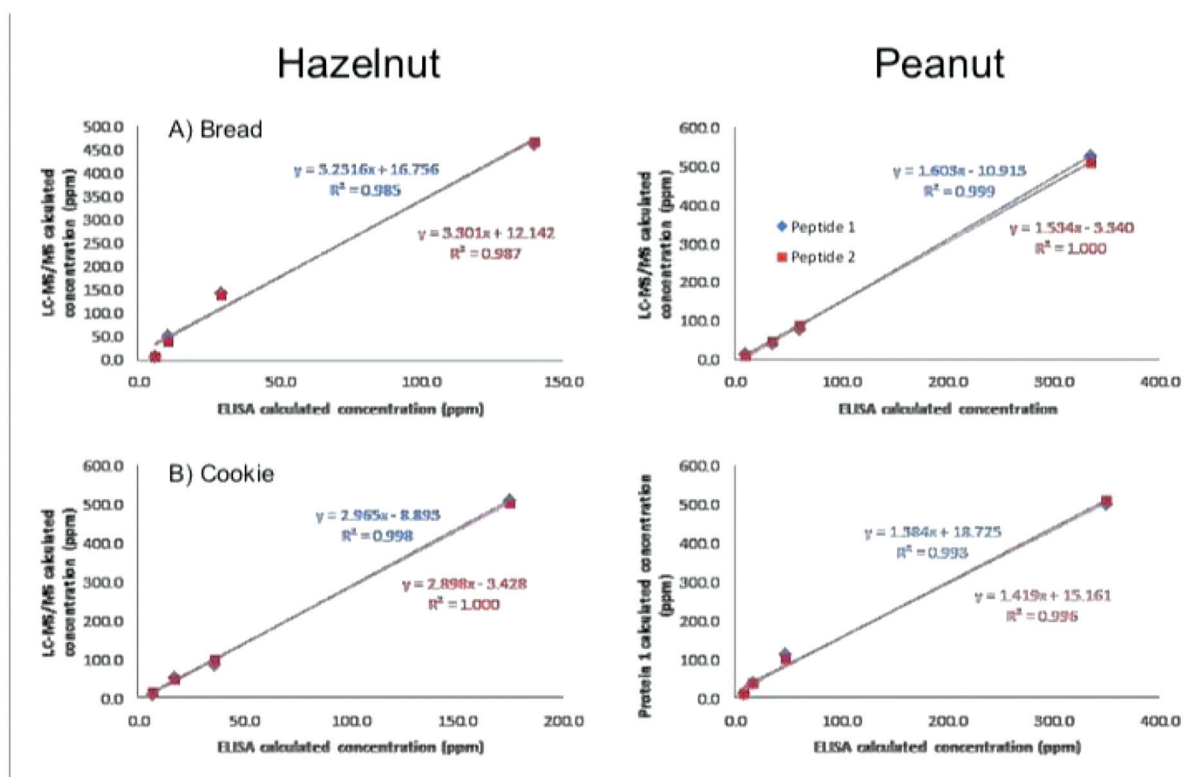


Figure 3. A comparison of two peptides (blue and orange) detected with ELISA and the LC-MS/MS method. Two matrices were used: (A) bread and (B) cookie.

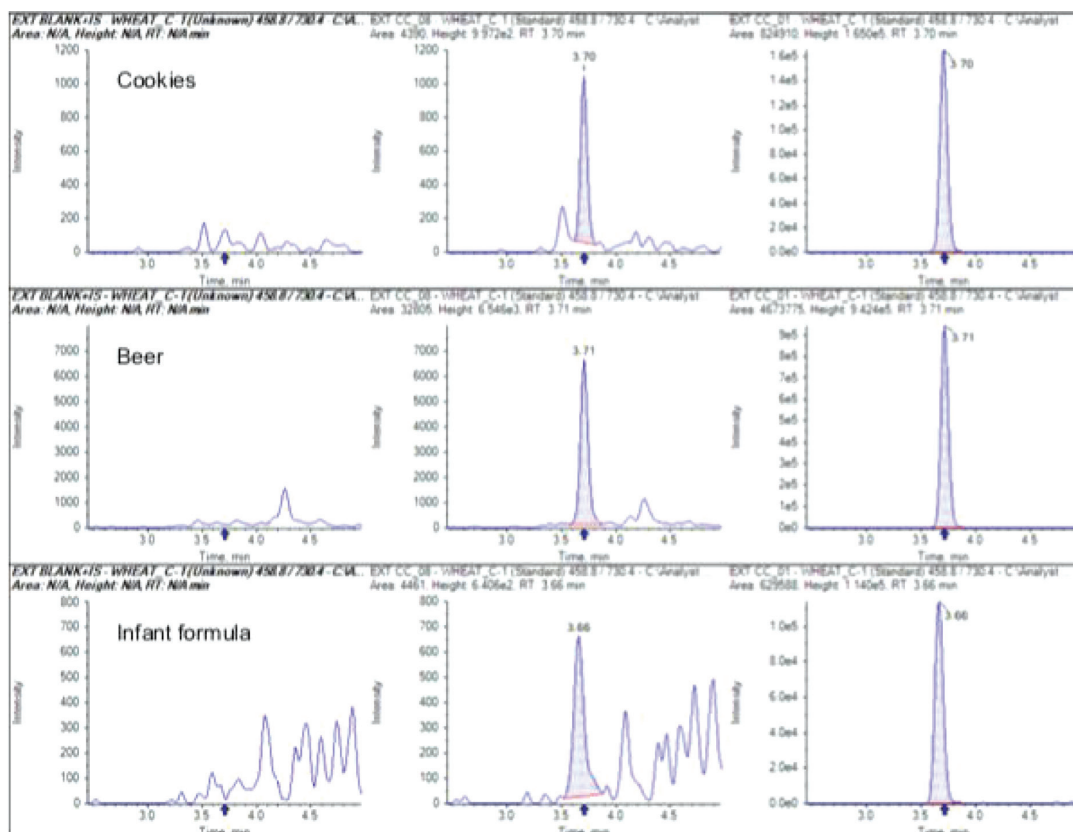


Figure 4. Chromatograms from gluten detection in three different food matrices spiked with three different gluten concentrations: 0 (left), 5 (middle), and 1000 ppm (right).

Meat Speciation

The MRM LC–MS/MS method (17) for meat speciation can identify unique peptide markers for six species simultaneously, including cow, pig, horse, duck, lamb, and chicken. In the United Kingdom, the Food Standards Agency and the Department for Environmental, Food, and Rural Affairs have set the limits for undeclared meat contamination at 1% (w/w) of the meat product (14). Crucially, the latest MS method in meat speciation can meet this limit for all species of interest in a single sample injection.

In total, 48 MRM transitions were selected for analysis, corresponding to two transitions from two peptides from two proteins for each of the six species. Once the MRM transitions were selected, calibration curves were generated that indicated the method could be used for relative quantitation of different meat species within a single product. Calibration curves were

generated over a wide dynamic range (0–100% w/w) and showed good linearity and reproducibility.

Assessment of the detection sensitivity of the six selected species in homogenized mixed-meat matrices was carried out using a QTRAP 4500 LC–MS/MS system with Turbo V source in positive ESI mode with an ion source temperature of 650°C. Signature peptides could be readily identified at 1% w/w with no matrix interference (Figure 5). The screen was further tested by analyzing the presence of pork in commercial meat products (halal and nonhalal sausages). The commercial halal sausages all gave no signal at the retention time indicative of pork, whereas pork could be identified in those sausages listing pork as an ingredient (Figure 6).

The presented method has significant advantages over other common meat speciation methods. Contaminants over 1% (w/w) can be identified using a QTRAP 4500 LC–MS/MS system in both raw and processed meat products and over 0.1% on a SCIEX

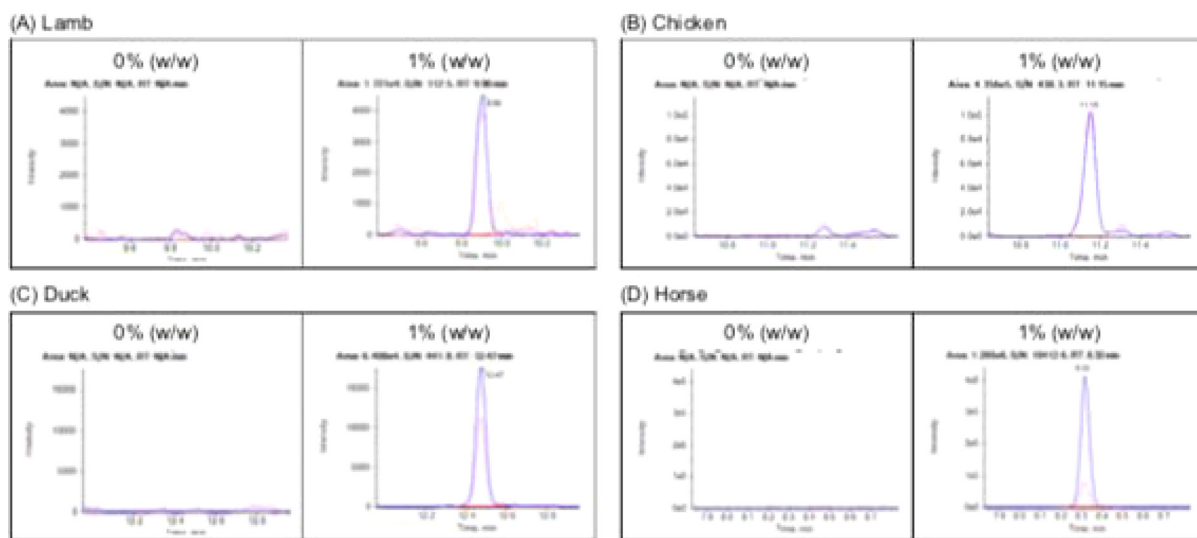


Figure 5. Extracted ion chromatograms of a marker peptide from (A) lamb, (B) chicken, (C) duck, and (D) horse in combined meat matrix at 0 and 1% w/w. Two MRM transitions were monitored for each marker peptide: fragment 1 (blue) and fragment 2 (pink).

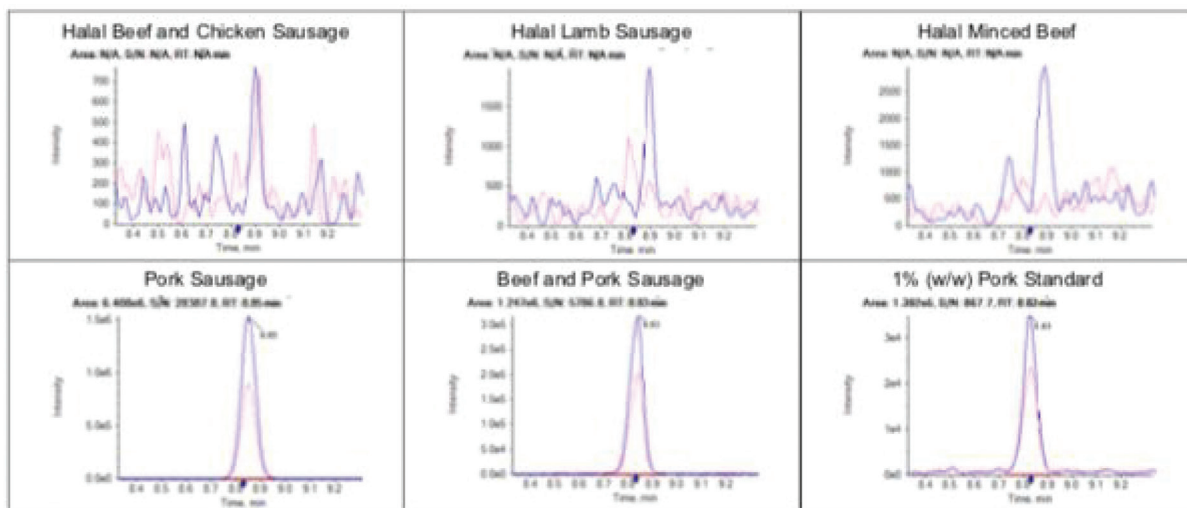


Figure 6. Extracted ion chromatogram of one marker peptide from pork in commercial sausage products, both halal (top) and nonhalal (bottom). Two MRM transitions were monitored: fragment 1 (blue) and fragment 2 (pink).

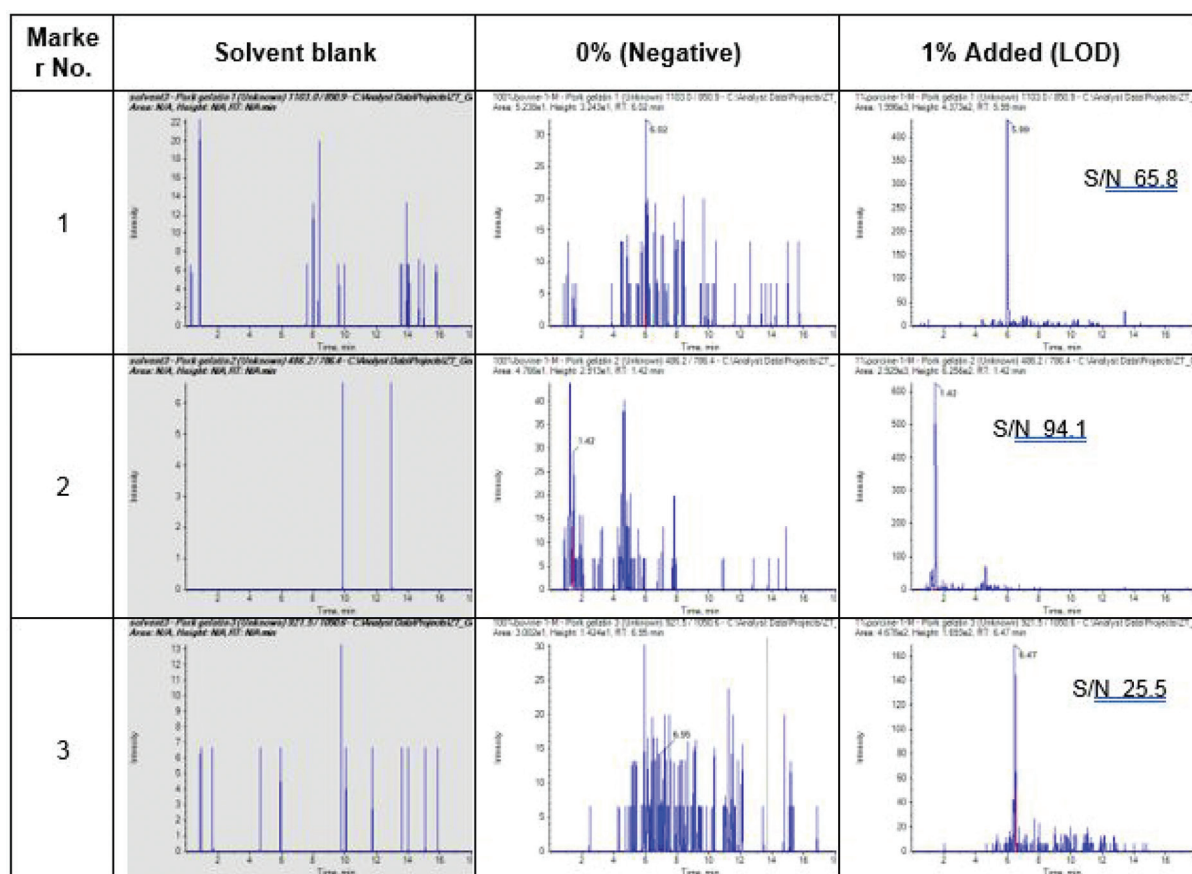


Figure 7. Extracted ion chromatograms from three marker peptides from porcine gelatin after overnight digestion in a solvent blank (left), a negative control (middle), and at the detection limit, 1% w/w (right).

QTRAP 6500+ LC–MS/MS. Furthermore, analysis of six species can be carried out in a single sample injection with high specificity and selectivity, streamlining the process of meat speciation.

Gelatin Speciation

The LC–MS/MS method developed for gelatin (18) can identify porcine and bovine gelatin derived from either acid or alkaline production methods with a limit of 0.1% (w/w). The method works on both raw and processed gelatin and is not affected by the tissues the gelatin was derived from.

For this LC–MS/MS method, digestion of gelatin samples was tested both overnight and in the microwave for 3 min. Microwave digestion gave similar results to overnight digestion, allowing the experimental time to be significantly decreased. A total of three selective marker peptides for pork and bovine gelatin were chosen for analysis, and the detection sensitivity of these markers was analyzed by screening pork gelatin–free products spiked with 1% pork gelatin. The results showed good sensitivity for all three selective markers. The results from the porcine gelatin analysis are shown in Figure 7.

This method for gelatin analysis has significant advantages over other common speciation methods, such as ELISA and PCR. It is highly specific, shows good sensitivity, and can detect multiple gelatin species in a single analysis. Future work is being performed to extend the analysis to gelatin from fish.

Conclusions

Consumers need certainty that the food products they buy are truly what they say they are. Contaminant species or undeclared allergens in food violate the trust of consumers and, in the worst cases, may lead to the death of allergy sufferers or widespread public health issues. LC–MS/MS provides a powerful alternative to traditional methods of analyzing food authenticity (i.e., ELISA and PCR), delivering greater accuracy, sensitivity, and speed. Through greater deployment of these methods throughout the food distribution chain, manufacturers and regulatory bodies can help to reduce food fraud and be more confident of the authenticity and labeling of the food products they manage.

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