

SPECIAL GUEST EDITOR SECTION

German Government Official Methods Board Points the Way Forward: Launch of a New Working Group for Mass Spectrometry for Protein Analysis to Detect Food Fraud and Food Allergens

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The detection of food fraud and undeclared food allergens is one of the major challenges for competent authorities. Because adulterations are continuously adapted to the methods used to uncover them, the accomplishment of this task has become increasingly difficult over time. In recent years, various new promising methods for the detection of multiple food adulterants and multiple food allergens have been developed. Some of them utilize LC-MS to identify specific marker peptides. However, these methods have yet to be validated and standardized. For this reason, the German officials have established a working group with the objective of validating methods through multilaboratory validation studies. The experts of the working group also aim for the first time to standardize validated methods and to develop general validation criteria. This manuscript will highlight the current work of the group. For this purpose, an overview is given on the principles and applications of the new mass spectrometric methods. Moreover, requirements and the present work of other institutions regarding method validation are described.

Food analytical tools have seen significant developments over recent years. Tools for food fraud were often basic analytical tests such as Kjeldahl for protein

determination (1), determination of fatty acids with GC (2), or DNA-based assays (3) to determine a species or variety.

Notably, a number of more sophisticated food fraud cases have been observed in which fraudsters specifically target the limitations of the applied analytical techniques. One simple example is the addition of nitrogen-rich melamine to infant products to mimic a higher protein content, as the widely used Kjeldahl method does not differentiate between protein or other nitrogen sources. Other fraud may include substitution of expensive extra virgin olive oil with cheaper plant oils and adding DNA from a high-quality species to a low quality substitute for dietary supplements.

For food allergen detection and quantification, lateral flow devices (4) and immunological assays such as ELISA (5) are being chiefly used, with several laboratories using DNA-based assays, especially PCR (3), for detection. Although these technologies have a number of advantages such as speed, ease-of-use, and long-time use, they also have a number of disadvantages (6).

As a reaction to food fraud surveillance, adulterators have learned to adapt their fraudulent activity to methods that analyze only one or few targets. Using a methodology with multianalyte potential, such as MS, will make it more difficult to commit fraud that remains undetected. Multiprotein methods for the detection and potentially quantification of animal and plant species are suitable improvements for such efforts.

On the food allergen detection side, while ELISA is a routinely used method, laboratories and control authorities would have to analyze for each suspected or undeclared allergen individually using different ELISA tests, with a significant cost burden. Although PCR offers a multiplex method, low-DNA-containing products such as egg white could possibly go unnoticed. Moreover, because the allergy-triggering components are proteins, it is meaningful to target those and their resulting peptides. MS offers the option to target multiple peptides across allergenic species, allowing the simultaneous detection of several allergens in a single sample. This methodology

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also helps changing food safety from a risk-based, individual target approach (e.g., detection of egg, detection of milk) to a holistic approach. This could eventually lead to the availability of methods for the simultaneous detection of all regulated food allergens and for authenticity screening in different food commodities (e.g., fish, meat, wheat products, etc.).

While an increasing number of manuscripts have been published in recent years (7, 8) that describe MS method developments for the detection of food adulteration and food allergens, few methods have been collaboratively validated and are accepted as official methods by the competent authorities. Equally, none of these methods has currently been standardized.

In order to achieve this, the German Federal Office of Consumer Protection and Food Safety (BVL) has, as the first of its kind, constituted a new working group for peptide-based mass spectrometric analysis of food and agricultural products. Within the working group, methods based on LC–MS are identified and validated, which subsequently can be used for control and enforcement of regulations dealing with food authenticity and food allergens.

Objectives of the Working Group

The working group is hosted by the BVL according to section 64 of the Food and Feed Act. The group consists of experts from the fields of science, economy, and official food control. The diverse composition of the group ensures that the interests of various stakeholders are taken into account, which increases the acceptance of the methods after standardization. Developed LC–MS methods for protein analysis will be introduced into the group, evaluated by the experts for their applicability for official controls, and, after positive evaluation, validated in multilaboratory validation studies. In addition, the identification of potential marker peptides shall also be optimized and standardized by the group. Overall, the group aims to develop general guidelines for the validation of LC–MS methods for protein analysis in food and agricultural products. Validated methods will be incorporated into the Official Collection of Methods of Analysis and Sampling (ASU) and additionally transferred to national and international standardization bodies.

Mass Spectrometric Protein Analysis in Food

While the untargeted proteomics approach has great potential because of its high multiplexing capabilities, it is also very costly and time-consuming and requires reference databases and trained specialists to interpret the data. In comparison, the utilization of specific marker peptides, especially in combination with

multiple reaction monitoring (MRM), allows for a reliable and rapid identification with triple quadrupole (QqQ) devices that are widely distributed in routine laboratories of food surveillance. Consequently, most LC–tandem MS (MS/MS) methods for protein analysis in food use marker peptides in a bottom-up targeted approach. The method development of targeted methods usually follows the same pattern (Figure 1).

It is key to identify marker peptides on an experimental basis and validate their specificity. To this end, authentic and pure samples of the food allergen or species of interest are used for the identification of potential marker peptides. Depending on the method, the proteins are either extracted from the matrix and then proteolyzed by a protease or vice versa. In most methods, trypsin is used because of its high selectivity, resulting in medium peptide length, and the advantageous ionization capabilities of the resulting peptides. The obtained peptide mixtures are subsequently separated and analyzed with LC–MS. To identify the highest possible number of specific peptides, the biomarker search is performed by means of an untargeted analysis with high-resolution (HR) MS. The resulting list of identified peptides is assessed regarding specificity and other analytical criteria (9). To evolve the method for future routine analyses, MRM transitions of the marker peptide candidates are identified. The specificity and reliability of the marker peptides is then tested in several food matrices using the MRM transitions and low-resolution MS/MS.

In recent years, several methods following the described process have been developed addressing specific tasks. For allergen detection, the focus is particularly on the so-called Big-8 (10), which are eight ingredients that are responsible for approximately 90% of allergic reactions caused by food (11). The Big-8 defined by Codex include cereals containing gluten, milk, egg, fish, crustacean shellfish, tree nuts, peanuts, and soybeans, and sulphite in concentrations higher than 10 mg/kg. Several jurisdictions have modified and expanded the list to meet the specific characteristics of their populations. For instance, the Big-8 of the U.S. Food and Drug Administration limit the cereals only to wheat and excluded sulphite from the list (12), whereas the European Union also included molluscan shellfish, celery, mustard, sesame, and lupines in their list, leading to 14 allergenic ingredients (13).

For peanut, 13 allergenic proteins have been discovered so far, with the three proteins Ara h 1, Ara h 2, and Ara h 3 being the most common cause for allergy (14). In recent years, several studies have been conducted leading to the identification of specific marker peptides for five of the allergenic proteins (Ara h 1, Ara h 2, Ara h 3, Ara h 6, and Ara h 7; 15–21).

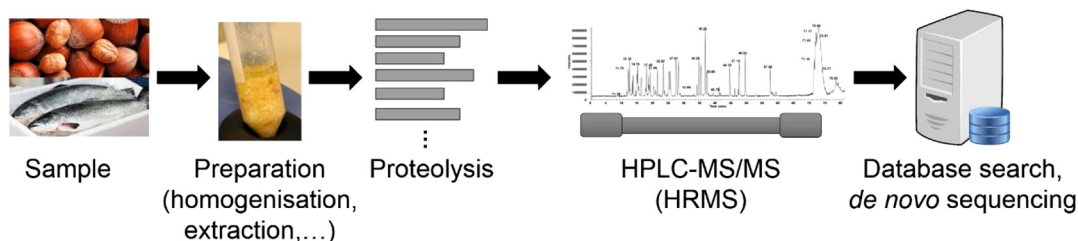


Figure 1. Process flow for mass spectrometric protein analysis.

Apart from pure samples of raw and roasted peanuts, the marker peptide search is usually carried out in incurred samples, such as baked goods like bread or biscuits. For milk allergen, the searched marker peptides are primarily derived from the different caseins (22–24), and for eggs, mainly ovalbumin is investigated (25, 26). Like peanut, the search is conducted mostly in pure samples (i.e., milk and eggs) but also in baked goods and in wine.

Another important field of allergen analysis is the detection of tree nut allergens. A tree nut allergy can cause severe reactions. One of the most important analytical issues is the botanical complexity of this group of allergens demanding for multiplexed methods with the highest specificity and sensitivity. The identification and evaluation of marker peptides for 11 different tree nut species was recently published (27). The specificity of potential marker was demonstrated against different seeds and nuts, and the quantitative performance was estimated using an external 0.1 mg/Kg peptide standard. To enhance sensitivity and specificity, MS cubed approaches, such as MRM cubed (MRM³), are employed for the detection of allergenic tree nuts (28). This method, available on QqQ instruments with a linear ion trap, allows for the simultaneous detection of MRM and MRM³ transitions. In general, sensitivity was increased by a factor of about 20 in the MRM³ mode, resulting in LOD of about 0.3–1 mg/kg (calculated as milligram allergenic tree nut per kilogram food; 16). Another approach is the direct detection of marker peptide precursor ions in HR scan mode. In principle, these methods allow for the simultaneous detection of hundreds of marker peptides as well as for retrospective inspection of the data. For the detection of tree nut allergens, this approach was employed using Orbitrap instruments and demonstrated excellent specificity as well sensitivity comparable to MRM approaches (16). For further relevant allergenic seeds such as mustard, the method development is complicated by the phylogenetic complexity of *Brassicaceae* sp. and the complexity of allergen proteoforms (29).

To date, only some research has been performed on the identification of marker peptides for wheat allergens (30, 31). Although allergenic proteins of soy as part of the Big-8 have already been examined relatively extensively (17, 32–34), only few studies have been conducted that also include other allergenic legumes, such as pea or lupine (35, 36).

Because of the high global production rates, especially for pea and soy (37), legumes such as lupine, pea, and soy are cost-effective protein sources with high protein contents. These plant proteins can be added to meat products as foreign proteins for technological reasons, such as the improvement of the water-binding capacity of meat and the improvement of the textural properties, and for economic reasons, such as the disguise of low-quality meats (38). In addition to the conscious use as meat adulterants, the mentioned legume proteins can also, for example, be inadvertently transferred into meat products (e.g., cross-contamination). In this context, even small amounts of lupine, pea, and soy proteins in the parts per million (ppm) range may be relevant to human health of persons suffering from an allergy because of the potential allergenicity of these legume proteins. In order to detect food fraud or allergenic potential, a sensitive screening method for the simultaneous mass spectrometric detection of the mentioned legume proteins in meat products has been developed (36). After defatting, protein extraction, and tryptic digestion, three to four marker peptides

(8–18 amino acids) for each legume species were measured by LC–MS/MS. The LODs of the method were between 2 mg (lupine) and 5 mg (pea) protein/kg meat product.

Because fish and shellfish are both groups consisting of a large number of different species, the identification of potential species-specific marker peptides is particularly challenging. This is even more crucial because fish is one of the main foods affected by food fraud. For instance, in a 2013 report of the European Parliament listing the top 10 foods that are most at risk for adulteration, fish was ranked second place (39), and in the recently published summarized Food Fraud Reports of the European Commission to July 2018, most adulteration cases were reported for fish. However, because of the complexity of these groups, only a few proteomic studies have been conducted on fish and shellfish to date. For shellfish, there has been a research project focusing on the differentiation of several shrimp species based on marker peptides (40, 41). Additionally, marker peptides of the allergenic proteins tropomyosin or sarcoplasmic calcium-binding protein have been identified for black tiger shrimp, white shrimp, and snow crab (42–44). In studies of 2011 and 2012, species-specific marker peptides of the allergenic protein parvalbumin were used both for allergen detection and for species differentiation between several hake and grenadier species (45, 46). Moreover, marker peptides were also used for a comparison between regular salmon and salmon that was genetically modified to reduce the allergenic protein content (47).

Recently, a new method has been developed for authenticity testing of fish samples by means of a bottom-up LC–MS/MS assay (S. Wittke, University of Applied Sciences Bremerhaven, unpublished data, 2018). The fish species redfish (*Sebastes* spp.), red snapper (*Lutjanus malabaricus*), European plaice (*Pleuronectes platessa*), sole (*Solea solea*), turbot (*Scophthalmus maximus*), and pangasius (*Pangasianodon hypophthalmus*) were identified by using the species-specific peptidome as differentiator. The research question presented in Figure 2 referred to the distinction between redfish and pangasius. Using principal component analysis on basis of all signals measured, a differentiation of redfish and pangasius was possible (Figure 2). Moreover, the other species also build up species-specific clusters (indicated by circles) without being in the focus of the classification. These results were confirmed for the comparison of red snapper and pangasius by using artificial neural networks (Uhlig, S., Colson, B., Hettwer, K., Simon, K., Uhlig, C., Wittke, S., Stoyke, M., & Gowik, P., unpublished data, 2018) for classification. In all cases, a confusion with other fish species seems unlikely, as these species can be clearly demarcated (S. Wittke, University of Applied Sciences Bremerhaven, unpublished data, 2018). Furthermore, the definition and application of species-specific peptide patterns (biomarker pattern) improved these results significantly.

Because of Europe's horse meat scandal in 2013, proteomics research in the area of food adulteration focused mainly on meat products. Thus, several studies that use marker peptides, especially for horse meat identification, were published after the scandal (48–50). In addition to horse meat, marker peptides have also been identified to detect beef, pork, chicken, turkey, and sheep meat (51–53). According to the Food Fraud Reports of the European Commission to July 2018, adulteration was conducted in most cases by means of mislabeling or substitution, i.e., incidents that can be addressed with species-specific marker

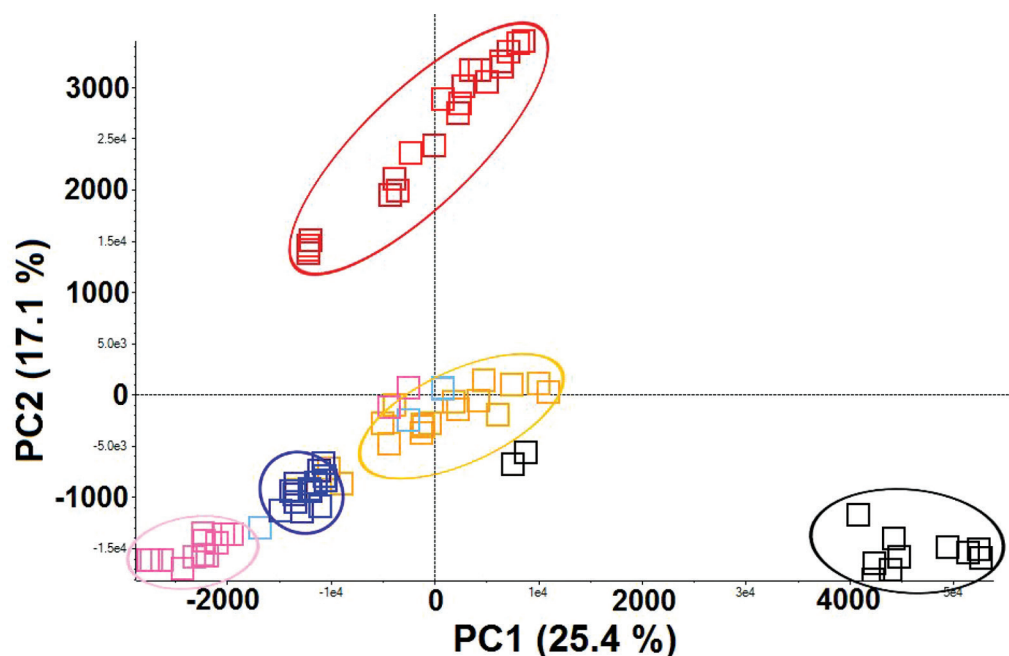


Figure 2. PCA plot of samples of different fish species. Clustering is based on 16000 detected LC–MS signals for redfish (red), pangasius (black), red snapper (blue), turbot (pink), European plaice (yellow), and sole (light blue).

peptides. Nevertheless, other meat adulterations also include artificial enhancements, such as meat gluing.

Meat gluing is an important topic of consumer deception. For cold-set binding of small meat pieces to obtain large segments of intact looking meat, especially the protein-based binding system, microbial transglutaminase (MTG) from *Streptomyces mobaraensis* is of great relevance, which is demonstrated by the great demand for this enzyme in the meat industry. The MTG catalyzes the formation of covalent isopeptide bonds between lysine (ϵ -amino group) and glutamine (γ -carboxamide group) residues cross-linking the meat proteins (especially myosin) at temperatures just above the freezing point (54) and showing no calcium-dependent activity. Besides the aspect of consumer deception, some safety aspects of the restructuring of meat using MTG are also under discussion. In analogy to the important function of tissue transglutaminase in celiac disease, there is some evidence that MTG, together with food proteins, can form proteins structurally similar to gluten (55) and consequently cause problems for people suffering from this disease. Furthermore, possible microbial contaminations on the surface of meat pieces can be glued together in the inside, making the deactivation of bacteria by heating much more difficult. As validated analytical methods to detect MTG in meat and meat products are still not available for food control administration, a sensitive LC–MS/MS method for the detection of MTG in restructured meat was developed (56). In a first step, six characteristic tryptic marker peptides (8–11 amino acids) for the enzyme (a monomeric 38 kDa protein containing 331 amino acids) were identified by performing data-dependent (HR) MS/MS measurements. Meat binding experiments were performed with different concentrations of MTG (pork) and different types of meat (pork, chicken, turkey, and beef). The restructured meat samples (as well as control samples without the addition of MTG) were analyzed as raw meat as well as after grilling or frying. The optimization of the conditions of protein extraction showed that the extraction of MTG using a

tris-HCl buffer was much more effective using a temperature of 100°C compared with a temperature of 60°C, which is most commonly used for the MS detection of allergens in food. Because of the absence of disulfide bonds in MTG, a pretreatment with reducing agents and subsequent alkylation was not required. It was further shown that short time of tryptic digestion (3 h) was sufficient and led to results comparable to digestion overnight. Using four marker peptides, no false-positive or false-negative results were obtained. The LOD of the developed method was about a factor of 10 below the recommended amount of MTG for raw as well as heated restructured meat (about 5 mg pure enzyme in 1 kg meat; 57), and it was thus in a concentration range in which no binding efficiency was achieved any more.

Endeavors Regarding Method Validation

For the standardization process, international organizations such as International Organization for Standardization (ISO), European Committee for Standardization (CEN), and AOAC INTERNATIONAL require the performance of a multilaboratory validation study, in which reproducibility and repeatability of the method is determined. Most of the protocols require participation of eight independent laboratories, but there are also some protocols based on factorial design that are based on four laboratories only (58). For several years, these factorial designs are in use by the German government official methods board (59). All these protocols are focusing on single parameters or single markers. Methods based on several markers or on a complete spectrum (fingerprinting) cannot be assessed properly by means of a single marker validation study but require the consideration of several markers simultaneously. The German government official methods board is currently working on concepts addressing this issue (Uhlig, S., Colson, B., Hettwer, K., Simon, K., Uhlig, C., Wittke, S., Stoyke, M., & Gowik, P., unpublished data, 2018; 60).

AOAC was the first standard development organization to evaluate, through its expert panel on MS, an LC–MS/MS-based method for multiple allergen detection. The method and its validation data were reviewed twice and subsequently approved by the Expert Review Panel (ERP) for First Action status (61). Preceding the method submission, a *Standard Method Performance Requirement* (SMPR®) was elaborated by the ERP (62). The SMPR 2016.002 sets the minimum method performance criteria any method submitted must fulfill. It is important to emphasize that these are the minimum requirements, and many methods described in the literature exceed these requirements in terms of sensitivity and robustness.

One of the poorly addressed needs, not only for MS-based methods but for any food allergen detection method, is the availability of reference and QC materials. Recently, the Food Allergen Task Force of the MoniQA Association defined the criteria for the development and characterizations of food allergen reference materials for use across testing platforms. As a result of this effort, a consensus milk reference material has been produced, which is commercially available and distributed by the Trilogy Laboratory. Egg, soy, and gluten materials will also become available shortly. In addition, further efforts to develop reference materials for food allergens have been funded by the UK Food Standards Agency and the National Institute of Standards and Technology. Such materials will hopefully lead to a reduction in the variability of results.

Current Projects of the Working Group

Until now, the German section 64 working group has started working on several projects to validate and standardize LC–MS methods for protein detection in food and agricultural products. The identification of fish species is crucial for both allergen and adulteration detection, but so far, only few methods have been developed and not yet validated with a multilaboratory study. Therefore, the working group is conducting a multilaboratory pilot study for a method for the differentiation of several fish species (S. Wittke, University of Applied Sciences Bremerhaven, unpublished data, 2018). The pilot study aims to establish marker peptides for fish species differentiation, examine analytical platform effects (five different LC–MS/MS platforms are included), and develop a standardized protocol for this task. Thus, the participants of the trial are analyzing several samples of different fish species using different MS platforms by means of an untargeted analysis. The collected data is subsequently evaluated with the help of bioinformatics and machine learning to develop a model for species classification.

The working group currently also aims to validate targeted methods based on MRM transitions. Because peanut and tree nuts are both food allergens of major importance in Europe, the group is running another multilaboratory pilot study for a method that uses marker peptides and MRM transitions to detect peanut and tree nut allergens (16).

An additional multilaboratory pilot study regarding a method for the detection of meat gluing with transglutaminase (56) has already been completed successfully. Hence, a method validation multilaboratory study is being planned at present. This study will be conducted according to ISO/CD 5725-2 (63). Other future projects of the working group are envisioned to include methods for the detection of lupine, soy, and pea in

processed meat, for grain differentiation, and for the detection of protein hydrolysates in meat products.

Based on the results of the multilaboratory validation studies, a standardization of the various LC–MS methods is intended in the future, including the standardization of a sample preparation protocol, LC and MS parameters, and data analysis. Moreover, general criteria regarding validation of LC–MS methods shall be developed. Successfully validated methods will also be transferred to the ASU and directed to national and international standardization organizations. Thus, LC–MS methods for food authenticity examination and food allergen detection through protein analysis will be, for the first time, reliably accessible to official food control.

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