

Novel Approaches for the MS-Based Detection of Food Allergens: High Resolution, MS³, and Beyond

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The prevalence of allergic reactions to food is believed to be increasing in industrialized countries worldwide. One of the major tasks in risk management is, therefore, the analytical surveillance of allergen contamination in food and targeted proteomics using MS, which is of hugely growing interest due to its specificity and sensitivity and the possibility to analyze multiple allergens in parallel. Though approximately 200 different foods have been described as having the potential to elicit allergic reactions, current regional labeling requirements are focused on the 5–14 priority allergens that elicit the vast majority of severe reactions or that pose a risk as hidden allergens in food production. MS-based detection methods have been published for the majority of priority allergens, and this review provides an overview of the different methodological approaches, namely multiple-reaction monitoring-, high-resolution MS-, and triple-stage MS-based methods. In addition, requirements for the identification and validation of specific marker peptides and the influence of thermal processing and structural heterogeneity of allergens are discussed.

Allergic reactions to food result from a dysfunction in and the hypersensitivity of the adaptive immune system against specific food components. Allergy to food is, therefore, defined as “an adverse health effect arising from a specific immune response that occurs upon exposure to a given food” (1). Briefly, the immune response is (with some exceptions) mediated via immunoglobulin E (IgE) antibodies located on the surface of mast cells and basophils (2). Each IgE antibody is directed specifically against a single structural element (epitope) of the antigen, which is bound with high affinity (3). Food proteins have been identified as the eliciting agents responsible for the vast majority of food allergic reactions. Allergy to red meat is caused by an alternative route. In this case, carbohydrate structures, such as α -Gal, elicit a delayed allergic reaction via a mechanism that is still not fully understood (4). Epitope binding of allergenic proteins to IgE on the surface of mast cells induces the release of potent mediators, such as

histamine and prostaglandins from storage granula, that induce the allergic reaction (5). Clinical symptoms can range from milder reactions, such as bronchial obstruction or wheezing, to local eczema or urticaria and severe reactions of the gastrointestinal tract and life-threatening and systemic anaphylactic shock (2).

From an analytical point of view, food allergens are a rather new topic in food science, but allergic reactions to food had already been described in ancient times. Notably, the prevalence of allergic reactions in Westernized countries has increased significantly over the last few decades from an estimated 1% of the general population to currently almost 5% (6, 7). This is also accompanied by a marked increase in associated hospitalizations (8) and, consequently, this is an important topic in medical research and molecular food science and food safety. Although approximately 200 different foods have been described to elicit allergic reactions, only 8 groups of food cause the majority of severe allergic reactions and account for over 90% of the documented cases of food-induced anaphylaxis (9). These so-called “big eight” are crustacea, egg, fish, milk, soy, peanut, tree nuts, and cereals containing gluten. It must be noted that significant regional differences in allergen profiles have been observed and, in addition, a certain bias is expected due to a lack of data from developing countries (10). Moreover, additional allergenic foods have been identified that pose a significant risk to consumers. These foods might be introduced in food production, for instance, as hidden allergens via spices and seasonings or might have been identified as allergens of regional importance. To provide allergic consumers the necessary information for protection against unintended uptake of allergens, a growing number of countries have introduced regulations or standards for the labeling of food allergens. Worldwide, as national legislation takes action on the regulation of allergens, regional differences in allergen labeling tend to be more diverse and complex. An overview of currently regulated allergens is given in Table 1. The unintended contamination of food products with allergens, for instance, via cross-contact in production facilities, poses a significant risk to allergic consumers. The current approach relies on the use of voluntary precautionary allergen labeling by the manufacturer. As precautionary labeling remains highly inconsistent across the food industry, several studies indicate that the efficiency of this tool to protect allergic consumers is rather limited. Notably, a significant proportion of allergic adults tend to ignore precautionary labeling even if they have a history of anaphylaxis (11, 12). An alternative risk-based approach is the introduction of reference doses for the most relevant allergenic foods after the evaluation of large data sets of clinical food challenges (13). These reference doses follow a no-observed-adverse-effect level/lowest-observed-adverse-effect level-based

Guest edited as a special report on “A Global Reflection on Food Allergen Regulations, Management, and Analysis” by Carmen Diaz-Amigo and Bert Popping.

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DOI: <https://doi.org/10.5740/jaoacint.17-0402>

Table 1. Examples of mandatory allergen labeling worldwide (according to the Food Allergy Research and Resource Program Food Allergens - International Regulatory Chart)^a

Allergenic food	Codex Alimentarius	Australia	Canada	Central and South America ^b	China	European Union	Japan	Korea	United States
Crustacea ^c	X	X	X	X	X	X	X	X	X
Egg ^c	X	X	X	X	X	X	X	X	X
Fish ^c	X	X	X	X	X	X		X	X
Milk ^c	X	X	X	X	X	X	X	X	X
Soy ^c	X	X	X	X	X	X		X	X
Peanut ^c	X	X	X	X	X	X	X	X	X
Tree nuts ^c	X	X	X	X	X	X			X
Wheat ^c	X	X	X	(X) ^{d,e}	X	X	X	X	X
Cereals with gluten	X	X	X	X	X	X			
Celery						X			
Lupin		X				X			
Mustard			X			X			
Mollusks		X	X		X	X			
Sesame		X	X			X			
Sulfites		X	X	X		X			X
Buckwheat					X		X	X	
Peach								X	
Pork								X	
Tomato								X	
Bee pollen/ propolis		X							
Royal jelly		X							

^a Chart version: May 25, 2017.^b Countries with mandatory allergen labeling are Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Cuba, Mexico, Nicaragua, and Venezuela.^c Major allergens, i.e., the "big eight."^d Labeling of wheat is not mandatory in Argentina, Chile, and Mexico.^e Labeling of latex (natural rubber) mandatory in Brazil.

scenario, and it is intended that these data will also serve as a threshold in the revised Voluntary Incidental Trace Allergen Labelling (VITAL) 2.0 (14).

As a consequence, analytical monitoring of such risk-based approaches is essential, and specific and sensitive methods for the identification and reproducible quantitation of allergens are necessary. At present, mainly immunological tests and PCR-based methods are applied in routine laboratories. Both techniques allow the sensitive detection of allergens with LODs of 10 mg/kg or below (15–17). However, the ELISA suffers from potential cross-reactivity, e.g., with matrix components in food, and shows limited reproducibility and assay comparability, especially for allergen detection in processed food (18, 19). In addition, this method is strongly limited in its ability for multiple detection of allergens (20, 21). PCR, on the other hand, does not directly detect allergenic proteins, but rather species-specific DNA. In all cases in which DNA and protein are separated or differently degraded during food processing, this method is prone to producing incorrect results (22). In addition, PCR is not tissue-specific and thus not able to distinguish, for instance, egg from chicken or milk from beef, which is of particular relevance for complex foods.

An alternative method is the use of MS-based proteomics for the characterization, identification, detection, and quantitation of allergens in food. Targeted proteomics of food allergens has not

yet fully made its way into routine laboratories, but various promising approaches have been published over the last decade (15, 23–25). The direct detection and quantitation of intact proteins in complex matrixes, such as processed foods, is extremely complicated with MS. Matrix-assisted laser desorption ionization-time-of-flight (TOF) MS has been used in numerous studies for the identification and characterization of food allergens, generally after one- or two-dimensional separation of proteins by gel electrophoresis (26–28). This approach is, however, not suitable for the routine analysis and quantitation of food allergens. The identification and quantitation of intact allergenic proteins in food samples using electrospray ionization-MS (in general, referred to as a top-down approach) suffers from complicated data interpretation due to multiplex and often overlying charge states of proteins in MS and the need to use state-of-the-art high-resolution MS (HRMS) instruments to resolve isotopologue distribution. In addition, the separation of intact proteins from complex samples using LC is still extremely challenging. The top-down approach has been used in some studies to identify sequence polymorphisms (29, 30) and, to some extent, to detect allergens in food samples with limited complexity (31).

Due to the aforementioned limitations, the general approach is the detection of specific marker peptides after enzymatic release from allergenic proteins. The identification of valid and specific marker peptides for different food allergens is, therefore, one of

the most important steps in method development and is mostly performed with HRMS and a dedicated proteomics environment. Once the specific marker peptides are identified, allergen detection at the peptide level is, in general, feasible with the standard MS infrastructure available in most routine laboratories, such as triple quadrupole (QqQ) instruments. Though other hyphenated techniques have also been published, LC is currently used as the front end of choice for the separation of peptides. As a further advantage for routine laboratories, standard reversed-phase chromatographic media (e.g., core-shell C18 media) efficiently separate complex peptide mixtures and, consequently, no dedicated proteomics infrastructure is necessary for the application of MS-based

allergen detection methods. An overview of applied LC-MS approaches in targeted proteomics and the fragmentation pattern of peptides when using collisional-induced dissociation is given in Figure 1.

Earlier studies have used selected-ion monitoring for peptide identification and, to enhance specificity in general, also recorded tandem MS (MS/MS) spectra of the selected precursor ions (32). This selected MS/MS-ion-monitoring approach has been applied to allergens from different foods and matrices, such as peanut (33) and breakfast cereals (34). Shefcheck and Musser (35) developed a method for the detection of peanut in an ice cream matrix and focused on Ara h 1 as the lead peanut allergen. Sensitivity was enhanced by using MW

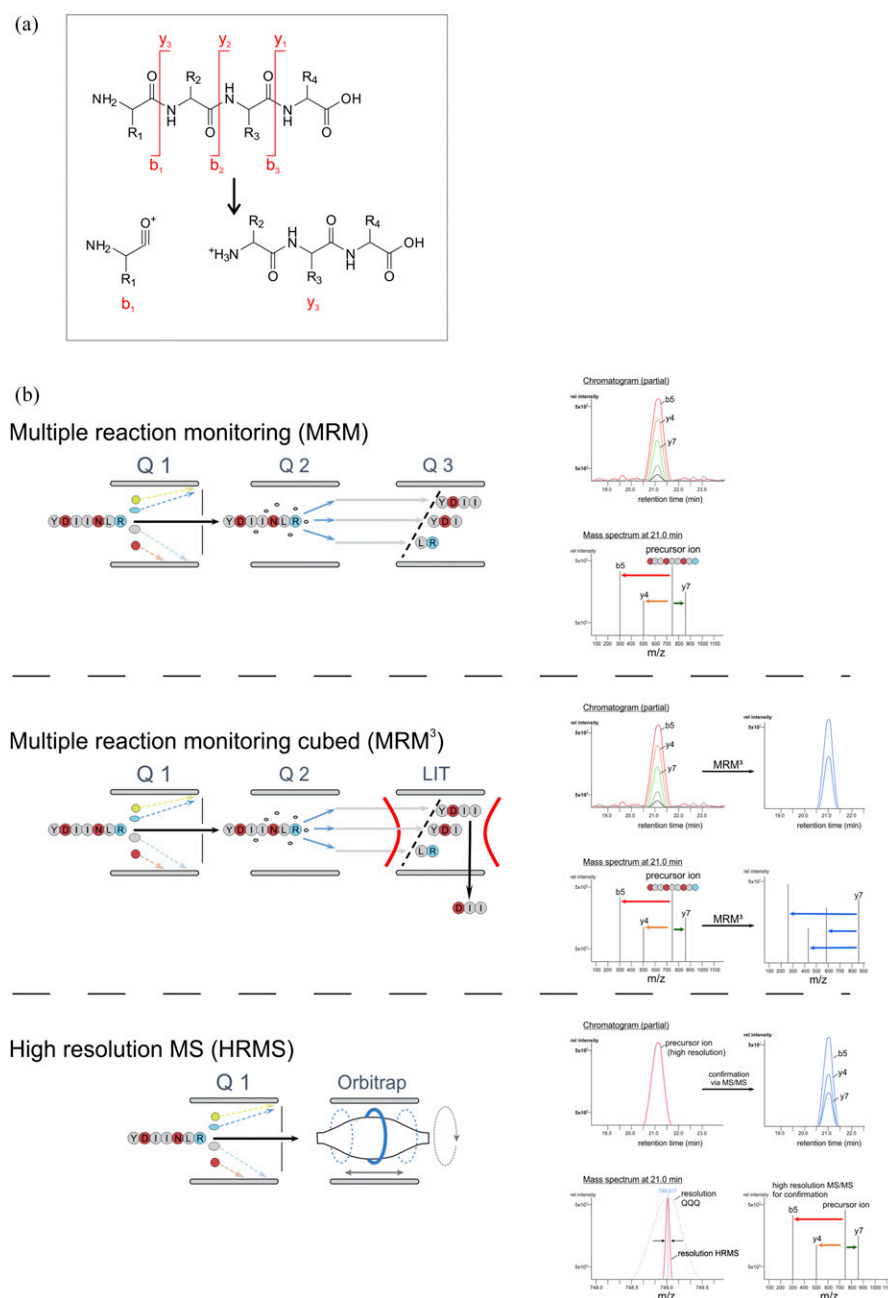


Figure 1. (A) Overview of the fragmentation pattern and fragment nomenclature for peptide fragmentation in collisional-induced dissociation. (B) Overview of currently applied MS strategies for allergen detection.

cut-off filters to enrich proteins (and Ara h 1) after extraction. The LOD was about 10 mg/kg.

The majority of published targeted MS-based allergen detection methods use QqQ instrumentation and make use of multiple-reaction monitoring (MRM) experiments [also referred to as selected-reaction monitoring (SRM)] for specific peptide identification and quantitation. For this highly sensitive MS experiment, the fragmentation of the analyte (precursor ion) into specific fragment ions is recorded (36). In general, these fragmentation events (transitions) need to be identified experimentally prior to MRM method development. One of the major advantages in peptide MS is the excellent predictability of peptide fragmentation depending on the applied fragmentation mechanism. For MRM approaches, collisional-induced dissociation is widely used. This fragmentation mechanism induces the dissociation of the peptide amide bond leading into the so-called b-ion series (the charge resides on the N-terminal peptide fragment) and y-ion series (the charge resides on the C-terminal peptide fragment; 37). Identification and optimization of MRM transitions can be achieved with software-based approaches that do not strictly rely on the availability of peptide standards. One example of the bioinformatics optimization of MRM is Skyline software (38), which also allows for MRM evaluation and optimization directly from the allergenic food, though the use of reference peptides is advisable for complex matrixes.

Peanut and milk have been most widely used as targets for MRM-based allergen detection methods in different food matrixes. Shefcheck et al. (39) published one of the first MRM methods for the detection of peanut in a chocolate matrix. They compared the influence of digest-assisted extraction to tryptic digest following extraction and obtained enhanced sensitivity for pre-extraction digest (20 mg/kg) compared with postextraction digest (50 mg/kg). For further improvements, an LOD of 2 mg/kg was estimated (39). Careri et al. (40) identified four suitable marker peptides from Ara h 2 and Ara h 3/4 on a quadrupole (Q)-TOF system. The distribution of marker peptides on different allergens was chosen to avoid bias due to the different expression levels in cultivars. LODs were around 5 mg/kg and linearity of the method was demonstrated to be between 10 and 200 mg/kg. The selectivity of the marker peptides was demonstrated against several tree nuts and different food matrixes, such as milk, soy, and cornflakes.

In a more recent work (41), marker peptides from Ara h 1, Ara h 2, and Ara h 3 were used to detect peanut in a baked cookie matrix. Optimization of the extraction was not shown in this study, but the authors enriched trace allergen contaminations using a combinatorial peptide ligand library. Enrichment increased sensitivity about 10-fold and, by using MRM, the LOD was about 10 mg/kg (42).

In the meantime, detection methods for the most relevant food allergens (the big eight) have been published, namely egg (43), fish (44), crustacea (45), tree nuts, (23, 24), and others (46). Sealey-Voyksner et al. (24) described a multiplex method for the detection of allergens from 11 different tree nut species and peanut. Marker identification was performed with HRMS on a Q-TOF system and the selection criteria for valid markers also included detection in roasted and unroasted samples. The specificity of the marker peptides against other species and grains was demonstrated. The LOD of this method was not evaluated. Instead, the authors compared signal intensities of

marker peptides from analyzed commercial samples with a 0.1 ppm peptide reference standard solution. Signals above this reference standard were defined as positive (24).

The fining of wine with milk or egg proteins can result in potential allergic reactions upon consumption, as residual amounts of these allergens may remain in the product and are not expected by the allergic consumer. For the detection of major allergens relevant in fining agents (lysozyme, ovalbumin, and caseinate) in a white wine matrix, the method performance of HRMS on an Orbitrap platform was compared with a linear ion trap (LIT) system with low resolution. In high resolution, full-scan MS data were used for data-independent allergen detection, whereas SRM was applied for the low-resolution instrument. As expected, HRMS provided advantages in terms of selectivity due to mass accuracy. Use of precursor ion masses from extracted ion chromatograms (XICs) resulted in sensitivities comparable with the SRM approach on the LIT instrument (47).

In a follow-up study (48), the authors improved the LOD for the detection of egg as a fining agent by immunoenriching ovalbumin (one of the major allergens from egg). A commercially available polyclonal antiovalbumin antibody was immobilized on disposable tips, and enrichment was performed after dilution of white wine samples. The LOD was in the range of recently published ELISA methods for this matrix (approximately 0.01 µg/mL) and was significantly enhanced (*F* of approximately 20) compared with the initial study. However, the authors also noted that the dynamic range was affected, presumably due to target enrichment.

Altogether, currently published methods, in general, show sensitivities comparable with ELISA methods, and it is expected that highly dynamic development in the field of MS instrumentation will further lower the LODs of allergen detection methods in the future. Different multiplexed methods have demonstrated potential for the simultaneous detection of allergens using MS.

A number of recommendations and guidelines have been published that need to be considered when identifying and choosing marker peptides (49–51). Marker peptides need to be proteotypic for the allergen of interest. The peptide length should be between 6 and 20 amino acids. Peptides shorter than six amino acids are in general not highly specific, especially in lower-resolution methods, as the number of available MRM transitions is limited. Long tryptic peptides, on the other hand, can show charge distribution with double- and triple-charged populations that affect quantitation. In most studies, tryptic peptides are used, and the cleavage of trypsin after Lys or Arg can be affected when Pro is adjacent to the cleavage site. As reproducibility is affected, peptides harboring post-translational modifications or missed cleavage sites (internal Lys and Arg in the peptide sequence) should be avoided (50, 51). In addition, it is advisable to use at least two peptides per allergenic protein to ensure detection and quantitation. It is also strongly recommended that marker peptide specificity be experimentally validated for the different food matrixes and the allergen extraction optimized especially for highly processed samples. In addition, it is advisable to select stable peptides, i.e., that have limited susceptibility to modifications induced by processing. A further critical point is the minimal number of MRM transitions detected at the LOD to unequivocally identify the marker peptide. For other food contaminants, such as mycotoxins, the detection of at least two MRM transitions together with retention time match and

correct signal intensity ratio of MRM transitions is recommended for robust identification. Due to the incredible diversity and similar chemical structure of tryptic peptides from protein-rich samples, this recommendation cannot be directly transferred to food proteomics. Currently, no official recommendations exist, and, consequently, the published LODs of different studies are not directly comparable. For the specific detection of marker peptides in food proteomics on low-resolution instruments, we recommend the detection of at least three MRM transitions that match the retention times and signal intensity ratio of the MRM transitions (52).

MS³-Based Methods

Due to the incredible complexity of food matrixes, peptide identification using lower-resolution instruments, such as QqQ, can, for certain matrixes and certain food allergens, also suffer from limited specificity (53). To enhance sensitivity and specificity, triple-stage MS (MS³)-based approaches have been developed. This method is, in principle, available on QTrap instruments in which the third quadrupole necessary for the detection of fragment ions is exchanged for a quadrupole LIT. This allows for the accumulation and secondary fragmentation of ions in the linear trap. Accumulation of fragment ions in the LIT results in an increased sensitivity compared with QqQ instruments, whereas the possibility of a secondary fragmentation experiment increases the specificity of the method (53). As the fragmentation of fragment ions is performed, which results in a secondary MRM transition, the method is designated MRM with multistage fragmentation (MRM³). For food allergens, this approach was recently applied for the detection of allergens from different crustacea (45) and for a detection method including five different tree nuts and peanut (53). In this study, the performance of MRM was directly compared with MRM³, and an increase in sensitivity of a factor of about 20 was observed. The LOD for the MRM-based approach was approximately 5–10 µg/g, calculated as allergenic food per grams of food matrix. For the MRM³ approach, the LODs were around 0.3–1 µg/g. The increase in sensitivity was mainly due to precursor ion collection in the LIT. However, it has to be noted that this approach increases the cycle time and thus limits the applicability of multianalyte methods that include huge numbers of peptide markers (53).

High-Resolution MS for Allergen Detection

More recent studies have also used HRMS not only for the identification of allergen marker peptides, but also for allergen detection. One approach is the fast screening of precursor ion masses with high resolution and the detection of MS/MS spectra for peptide structure confirmation. One of the advantages of this strategy is the possibility to perform the untargeted monitoring of food samples that also allows, in principle, the retrospective control of samples.

For the multiplexed detection of five different tree nut and peanut allergens, HRMS was used for comprehensive marker peptide identification, resulting in 44 suitable marker peptides. Allergen detection was performed in full-scan mode at maximal instrument resolution ($r=100\,000$). The exact mass of the marker peptides together with the expected isotopologue pattern were

identified from XICs and used for detection and quantification (23). The LODs of the most sensitive peptides for the different tree nut and peanut markers were in the range of 1.0–5.7 µg/g. In a similar approach, the detection of fining agents (lysozyme, ovalbumin, and caseinate in white wine) was carried out using HRMS on an Orbitrap instrument. The exact mass of the precursor ion (XIC) was used for the detection of marker peptides with LODs in the range of 0.5–1.0 µg/mL, which is in the range of the minimal eliciting dose after consumption of 200 mL wine (43). Notably, for all HRMS approaches using Orbitrap systems, the LOD was significantly increased when the exact mass of the precursor ions was used instead of MS/MS data (23, 43, 47).

Structural Modifications and Allergen Heterogeneity

In addition to the detection of allergens in food, MS-based characterization of processing-induced structural modifications is of growing interest. Protein profiling of peanut allergens from food used for double-blind placebo-controlled food challenges was conducted using HRMS in combination with ion mobility and data-independent acquisition (54). Major peanut allergens were identified and relatively quantified to investigate processing effects on the allergen profile. The extraction efficiency of allergens from the cupin superfamily (Ara h 1 and Ara h 3) was significantly reduced, whereas prolamins (Ara h 2 and Ara h 6) were not affected. As expected, defatting peanut flour strongly reduced the oleosin content (Ara h 10 and Ara h 11). Similar effects were observed for walnut upon thermal processing (55). The glycation of milk allergens was analyzed in various studies. Modifications of amino acids prone to Maillard modification and other modifications induced by thermal processing were analyzed in a heated milk model (56). The site-specific modifications of β -lactoglobulin at Arg, Asn, Cys, Lys, Met, and Trp moieties were identified and used for the development of a targeted detection method for the detection of 19 different modifications at 26 sites in β -lactoglobulin (57), demonstrating the pronounced modification of this allergen upon harsh thermal treatment. Johnson et al. (58) identified the lactosylation of different milk allergens at the intact protein level by the direct coupling of size-exclusion chromatography with MS. MS is also used for the identification of structural polymorphisms and post-translational modifications. For the 2S albumins from mustard and hazelnut, a combination of different proteomics approaches was used to identify proteoforms and post-translational modifications, such as the ragged clipping of the termini of the large and small subunits (29, 30). Site-specific identification of sequence polymorphisms was performed at the peptide level using a classical bottom-up approach with HRMS. The isoforms of the small and large subunits were characterized after reduction and alkylation. This strategy is also termed middle-down. Proteoforms were finally identified in addition to the intact allergens (top-down approach). For hazelnut, up to 41 proteoforms of the 2S albumin, Cor a 14, were identified, demonstrating the pronounced degree of heterogeneity in plant food allergens that needs to be taken into account during method development for targeted allergen detection. Similarly, the investigation of sequence variants of the major birch pollen allergen, Bet v 1, demonstrated the pronounced sequence heterogeneity and the existence of at least 18 Bet v 1 isoallergens in birch pollen (59), which is currently not reflected in available biomedical allergen products.

Notably, also the total allergen content and allergen profile can differ significantly among cultivars of different plant food allergens (60). A comparison of soybean allergens from soybeans grown in six distinct regions in North America demonstrated that allergen profiles can vary significantly in response to geographical differences and differences in environmental conditions (60). A study comparing 20 soybean varieties using targeted proteomics and stable isotope-labeled peptides demonstrated a similar total allergen amount, but significant differences for specific allergens (61). Consequently, pronounced differences in *in vitro* IgE binding among soybean cultivars have been demonstrated (62), indicating that the allergen profile, as well allergenic potential, can vary between cultivars.

Currently, mainly ELISA and, to a certain degree PCR, data are used to calculate the amount of allergenic food in an analyzed matrix (e.g., milligrams of peanut per kilogram of cookie). Bearing in mind the above-mentioned differences in allergen profiles and allergen content in different cultivars and the potential of MS-based methods to directly quantify the major allergens, it might be more advisable for future risk management and risk communication to calculate eliciting doses and thresholds on the basis of the actual amount of major allergenic proteins rather than the allergenic food.

Celiac Disease

Celiac disease (CD) is an autoimmune reaction triggered by the gluten fraction of wheat, barley, and rye that occurs in genetically predisposed consumers. The current prevalence is estimated up to 1% of the population in Northern America and Europe (63). Though not an allergic reaction *in sensu stricto*, the protection of CD patients is of similar importance compared with food allergy and is, therefore, briefly discussed here.

The characterization and detection of gluten in food is of high importance, and proteomics is now widely used for this purpose. Attempts to generate barley lines devoid of three of the four gluten fractions (triple-null barley, devoid of B-, C-, and D-hordeins) were monitored using targeted proteomics and MRM for marker peptide detection. In addition, a data-independent mode known as sequential window acquisition of all theoretical mass spectra was applied to obtain maximum information about triple-null barley (64, 65). The generation of suitable reference material for clinical CD diagnosis and food analysis is mainly hampered by the complexity of the gluten fraction and the still limited knowledge of structural heterogeneity. In a recent approach, a detailed characterization of the gluten fractions from wheat, rye, barley, and oat was performed using different MS approaches. Intact proteins were analyzed on a Q-TOF instrument, and sequence data were obtained after chymotryptic digest in a bottom-up proteomics experiment (66). Pronounced heterogeneity was also observed in a similar study in different wheat gluten fraction.

Conclusions

Targeted proteomics for the detection of food allergens is a highly dynamic field. This is illustrated by the constantly growing number of specific, sensitive, and experimentally validated marker peptides for the different relevant food allergens. In addition, published detection methods overall

already show promising sensitivity and specificity, demonstrating that routine MS-based detection methods are within reach. Different approaches emerge, with MRM being the most prominently used method, showing promising sensitivity and, in general, excellent dynamic range, together with a high specificity for most allergens and matrixes. For analytical problems in which increased sensitivity and/or increased specificity are needed, the MRM³ approach shows very promising results. Similarly, high-resolution-based approaches deliver, in general, excellent specificity and sensitivity, comparable with MRM-based methods. One of the major issues that has to be solved in future research is absolute quantitation in processed and highly processed samples and the development of novel strategies for standards beyond isotopically labeled peptides to compensate for these effects.

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