Review

Proteomics-based methodologies for the detection and quantification of seafood allergens

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Abstract: Seafood is considered one of the main food allergen sources by the European Food Safety Authority (EFSA). It comprises several distinct groups of edible aquatic animals including fish and shellfish such as crustacean and mollusks. Recently the EFSA recognized the high risk of food allergy over the world and established the necessity of developing new methodologies for its control. Consequently, accurate, sensitive and fast detection methods for seafood allergy control and detection in food products are highly recommendable. In this work, we present a comprehensive review of the applications of the proteomics methodologies for the detection and quantification of seafood allergens. For that, two consecutive proteomics strategies (Discovery and Targeted Proteomics) applied for the study and control of seafood allergy are reviewed in detail. In addition, future directions and new perspectives were also provided.

Keywords: discovery proteomics, targeted proteomics, mass spectrometry, fish allergens, crustacean allergens, mollusc allergens

1. Introduction

Changes in life habits including food production and manufacturing have dictated a global increase of food adverse reactions [1-3]. Among these reactions, type I IgE-mediated allergies to food components are considered by the World Health Organization (WHO) as the fourth most important public health problem. These food allergies affect an estimated 6-8% of young children and 2-4% of adults with regional variations [3]. The prevalence increase, their life-threatening property and the costs related to food allergies have dictated the need of improving prevention and treatment strategies [3]. To guarantee consumer safety, several regulations have been implemented (Directive 2007/68/EC) such as the labelling of food allergens intentionally introduced [4]. However, some products on the market could contain traces of allergens due to accidental cross-contaminations during the food manufacturing processes.

Seafood refers to distinct groups of edible aquatic animals including crustacean, fish and mollusk (Figure 1) [5]. Based on culinary reasons crustacean and mollusk are usually combined as shellfish. The rising consumption of seafood and of its derivatives has led to an increase in persistent allergic reactions. The route of exposure is not only restricted to ingestion but include manual handling and inhalation of cooking vapors in the domestic and occupational environment [6].

The diversity of consumed seafood species has challenged the identification and characterization of their allergenic composition for accurate diagnostics and potential therapeutic interventions. Each seafood can contain several allergens, and the allergen causing reactions differ
among patients [3,6]. Many allergens are protein families and are very different between these groups. Notwithstanding, β-parvalbumin (β-PRVB), tropomyosin and arginine kinase are pan-allergens and induce clinical cross-reactivity [3,6].

**Figure 1.** Seafood species classification and their reported allergy prevalence. Classification and has been performed according to the NCBI (www.ncbi.nlm.nih.gov/taxonomy). Examples of each of the species are displayed in the corresponding boxes. The range of prevalence of each offending food has been taken from [3,5].

To detect the allergens in seafood, tissue aqueous extracts are usually analyzed by western blot (WB) using the patient sera IgE. This initial approach was then implemented by proteomic analyses allowing the identification of proteins after separation by two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Notwithstanding, most seafoods have to be processed before consumption. During processing proteins can be denatured, modified and/or hydrolyzed and alter their allergenic potency. New allergenomics techniques, which consider the properties of the allergens, have been developed allowing the coupling of identification, detection and quantification of seafood allergens. In this review we summarize the advances in this field.

### 2. Proteomic workflows: discovery and targeted proteomics

Figure 2 summarizes the main proteomic workflows used for the detection and quantification of seafood allergens. Two sequential proteomics approaches, the discovery proteomics and targeted proteomics are presented.

Discovery proteomics involves the large-scale analysis of a particular proteome in order to identify protein/peptide biomarkers. By means of a bottom-up proteomics methodology, the protein/s to investigate are separated for reducing sample complexity, converted into peptides using enzymes (i.e. trypsin, Asp-N, Glu-C), and the derived peptides are then analyzed by mass spectrometry (MS) [7]. Bottom-up approaches can be organized in two different groups depending of the protein separation step in gel-based or gel-free approaches. In gel-based approaches, 2-DE is the most conventional methodology for the separation of proteins, where proteins are isolated and stained in-gel based on their pI and Mr [8]. Then interesting spots can be excised from the gel, digested into peptides with an enzyme, usually with trypsin, and the resulting peptides are analyzed by MS for protein identification. This gel-based method is the most appropriate option for unsequenced organisms, such as some seafood species, in which the identification of proteins is based on the comparison of peptides from related species or by de novo MS sequencing [9]. The current availability of specific staining methods makes 2-DE a good approach for the identification of post-translational modifications (PTMs) such as phosphorylations, protein carbonylations and glycosylations [10-12]. With respect to 2-DE image analysis different programs such as PDQuest, Progenesis, Melanie and ImageMaster are available [13]. In gel-free approaches, also named as
shotgun proteomics, a mixture of proteins is digested directly with an enzyme (i.e. trypsin) and the complex solution of peptides are separated by liquid chromatography (LC), either alone using reverse phase (RP) columns or combined with multidimensional LC separations as strong anion/cation exchange chromatography (SA/CX)-RP [14]. The eluted peptides are then analyzed and fragmented by tandem mass spectrometry (MS/MS) [15]. By means of protein database search engines, such as Mascot [16], SEQUEST [17] or X!Tandem [18], MS/MS spectra are assigned to potential peptide sequences and then are validated using software programs like Percolator [19] or PeptideProphet [20]. In the case that the protein is not registered in the protein databases, peptides must be de novo MS sequenced [21], manually or by computer programs, such as PEAKS [22], DeNovoX [23] and Byonic [24].

Figure 2. Main proteomic workflows used for the detection and quantification of seafood allergens. Basic workflows used in discovery proteomics and targeted proteomics.

Discovery-based quantitative proteomics has been widely used to address the differences in the amount of proteins among different conditions. The most important quantitative proteomics methodologies are: isotope tagging by chemical reactions such as isobaric tags for relative and absolute quantitation (iTRAQ), tandem mass tag (TMT) and difference gel electrophoresis (DIGE) [25-27]; the stable isotope incorporation via enzyme reactions (i.e. $^{18}$O) [28]; the metabolic stable isotope labeling (such as stable isotope labeling by/with amino acids in cell culture, SILAC) [29]; and the label-free quantification (i.e. measuring the intensity of the peptides at MS level) [30]. Shotgun proteomics can be used also to identify and quantify in complex samples using a high-throughput way different PTMs as thousands of phosphorylation sites, glycopeptides and protein acetylations [31-33]. The approach known as top-down proteomics analyzes the fragments produced by the dissociation of the intact proteins directly inside the mass spectrometer, avoiding the step of protein digestion [34]. This intact protein analysis is at this time available due to high mass accuracy and the new dissociation mechanisms obtained by the new high-resolution MS (HRMS) instruments [35,36]. Finally, the main goal of the discovery proteomics is to compare the resulting peptides and proteins by alignment search tools, such as BLAST, with universal public protein databases in order to select specific peptide biomarkers, which will then be utilized in the second phase of the workflow, known as targeted proteomics.
Targeted proteomics is employed to scan with high accuracy, sensitivity and reproducibility the peptide biomarkers selected in the discovery phase [37]. In this monitoring mode the MS analyzer is centered on analyzing the peptide/s of interest by selective/multiple-reaction monitoring (SRM/MMRM) mainly on triple-quadrupole (QqQ) mass spectrometers [38]. Monitoring specific transitions corresponding to appropriate pairs of precursor and fragment ions m/z, represent a sensitive and selective MS scan mode to detect and identify peptide biomarkers [39]. Nevertheless, the implementation of a SRM/MMRM study is a time-consuming process and more importantly complete MS/MS spectra are not acquired. The MS/MS spectrum of a peptide is extremely important to corroborate its amino acid sequence. Recent procedures, such as SRM-triggered MS/MS in quadrupole-ion trap (Q-IT) mass spectrometers [38], selected MS/MS ion monitoring (SMIM) [40,41] or parallel reaction monitoring (PRM) in an IT or high resolution Q-Orbitrap (Q-Exactive) instruments [42], are alternative scanning modes that allow for a sensitive monitoring of specific compounds, obtaining complete structural information. Sequential windowed acquisition of all theoretical fragment ion spectra (SWATH-MS) [43] is a new advanced targeted data independent analysis (DIA) mode implemented commonly in high speed acquisition triple-quadrupole-time-of-flight (TripleTOF) mass spectrometers that can identify and quantify large sets of proteins without the prerequisite to specify a set of proteins prior to acquisition. Targeted-based absolute quantification can be performed using introduced internal standards in the sample with stable-isotope 13C- or 15N- labeled absolute quantification peptide standards (AQUA) or concatenam of standard peptides (QCAT) [44]. Concerning data analysis several programs such as Skyline [45] and SRMCollider [46] are accessible for the analysis of targeted proteomics experiments. The subsequent sections will exhibit the efficacy of these modes for the monitoring of those peptide biomarkers selected in the discovery phase for the detection and quantification of seafood allergens.

3. Proteomics applications for the detection and quantification of fish allergens.

Fish proteins with identified IgE reactivity and registered as official fish allergens by the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org) are displayed in Table 1.

Among them, β-PRVBs which are found in high amounts in the sarcoplasmic fraction of the white muscle of fish, are considered as the major fish allergen [47,48]. These proteins have a molecular weight around 10-12 kDa, an acidic pI (3.0-5.0) and three EF-hand motifs (helix-loop-helix), two of them binding Ca²⁺. The allergenic properties of these proteins are related with their abundance, thermal stability and resistance to certain gastrointestinal enzymes [49]. Despite this apparent simplicity, β-PRVB are indeed a complex family of isoforms differing in their abundance and muscle of expression [5,35]. An advanced discovery proteomics workflow achieved the de novo MS sequencing of new β-PRVB isoforms for all the species belonging to the Merlucciidae family [35]. This strategy was performed based on the integration of a common 2-DE bottom-up proteomics methodology with the accurate determination of the Mₚ of the intact β-PRVBs by Fourier-transform ion-cyclotron resonance (FTICR)-MS and the monitoring of several peptide mass gaps by SMIM. This publication is the report accounting for the higher number of new allergens (23 new β-PRVBs) completely de novo sequenced making use only MS-based techniques. The results allowed the registration of the sequence of the new β-PRVB isoforms into the UniProtKB (http://www.uniprot.org/) and Allergome databases (http://www.allergome.org/) (Accession numbers: P86739-P86775). Moreover, the complete sequence of four β-PRVB isoforms (PRVB1 (P86431), PRVB1.1, PRVB2 (P86432) and PRVB2.1 variants) from farmed rainbow trout (Oncorhynchus mykiss) were achieved by MALDI MS and MS/MS analysis [50]. Such isoform diversity impacts in the stabilization of β-PRVB as amyloids under gastric-like conditions, and consequently in their resistance to proteases and IgE binding intensity [51-53].
Table 1. Seafood proteins with known IgE reactivity. Data in the table has been retrieved from http://www.allergen.org/. Proteins are listed in alphabetical order. X indicates in which seafood groups the protein is described as an allergen, XX indicates major allergen, and ∼, not yet determined.

| Protein                                      | Seafood Source | Function | Molecular weight (kDa) |
|----------------------------------------------|----------------|----------|------------------------|
| Aldehyde phosphate dehydrogenase            | Fish, Crustacean, Mollusk | Oxidation of aldehydes | 41 |
| Aldolase A                                   | Fish, X, X, – | Glycolysis | ~40 |
| Arginine kinase                              | –, X, X | Metabolism | 38-45 |
| Collagen                                     | X, –, – | Structural | >100 |
| Creatine kinase                              | X, –, – | Metabolism | ~40 |
| β-Enolase                                    | X, X, – | Glycolysis | ~50 |
| Glucose 6-phosphate isomerase                | X, –, – | Glycolysis | 60 |
| Glyceraldehyde-3-phosphate dehydrogenase     | X, –, – | Glycolysis | ~37 |
| Hemocyanin                                   | –, X, – | Or:transport | 77 |
| L-lactate dehydrogenase                      | X, –, – | Metabolism | 34 |
| Myosin light chain 1                         | –, X, – | Structural | 17-23 |
| Myosin light chain 2                         | –, X, – | Structural | 17-23 |
| Ovary development-related protein            | –, X, – | Unknown | 28 |
| Paramyosin                                   | –, –, X | Structural | 100 |
| α-parvalbumin                                | X, –, – | Ca²⁺-binding | 10-13 |
| β-parvalbumin                                | XX, –, – | Ca²⁺-binding | 10-13 |
| Pyruvate kinase PKM-like                     | X, –, – | Metabolism | 65 |
| Sarcoplasmic Ca²⁺-binding protein            | –, X, – | Ca²⁺ buffering | 20-24 |
| Triosephosphate isomerase                    | X, X, X | Glycolysis | 28 |
| Tropomyosin                                  | X, XX, X | Structural | 33-39 |
| Troponin C                                   | –, X, – | Structural | ~20 |
| Troponin 1                                   | –, X, – | Structural | ~30 kDa |
| Vitellogenin                                 | X, X, – | Yolk protein | 180 kDa |

In agreement with that, a shotgun proteomics analysis of 15 different fish species, the protein-based bioinformatics analysis and IgE reactive approaches, was used to identify a total of 35 peptides as B-cell epitopes for all the β-PRVBs included in the UniProtKB database [54].

An easy and robust method for fish allergen detection has been developed utilizing the high speed, high resolution and fragmentation capabilities of the Orbitrap Fusion mass spectrometer implemented with an ultraviolet photodissociation (UVPD) source. Using β-PRVBs as a signature for the allergen detection the method showed several benefits such as, minimal sample preparation, high sensitivity, throughput and practically a complete protein sequence coverage [36].

The rapid detection of β-PRVBs in foodstuffs was developed by our research group using a targeted proteomics scanning mode [55]. The strategy is based on the use of a rapid purification of β-PRVBs by treatment with heat (time: 45 min), the acceleration of in-solution protein digestion by high-intensity focused ultrasound (HIFU) (time: 2 min), and the monitoring of several β-PRVBs peptide biomarkers by SMIM in a linear ion trap (LIT) mass spectrometer (time: 60 min). The method allows the rapid detection of the presence of β-PRVBs in any foodstuff, including precooked and processed products, in less than 2 hours. Recently, a new method for the quantification of β-PRVBs in food matrices via LC-MRM allowed the quantification of β-PRVB of flounder (Paralichthys olivaceus) at low level of 0.10 µg/g with an accuracy of <13.3% and a precision of RSD < 18.35% [56].

Regarding other fish allergens, fructose bisphosphate aldolase (39.54 kDa) which is implicated in gluconeogenesis, glycolysis and Calvin cycle, is also considered a fish allergen in cod, salmon and tuna species [57]. This protein was primary characterized as allergen by SDS-PAGE, ELISA, 2-DE, WB and MALDI-TOF MS in tilapia species (Oreochromis mossambicus) [58]. Enolase (isoform β; 47-50
kDa) is an enzyme responsible for the penultimate step of the glycolysis and is also considered a potential fish allergen in cod, salmon and tuna species [57]. Enolase was characterized as an allergen from the freshwater fish blunt snout bream (*Megalobrama amblycephala*) by 2-DE, WB and MALDI-TOF MS. Creatine kinase (42 kDa), an essential protein for energetic homeostasis is considered a potential fish allergen in tuna species by 2-DE, WB and MALDI-TOF MS [59]. Tropomyosin (33-39 kDa) is a relevant regulator of muscle contraction and is considered a pan-allergen for shellfish and a potential fish allergen in tilapia and cod species (*Oreochromis mossambicus, Gadus morhua*) [60,61]. The results were performed by immunoblotting and specific IgE ELISA using sera from patients with allergy to tilapia or to cod. These four fish allergens (fructose bisphosphate aldolase, β-enolase, creatine kinase and tropomyosin) are found in the sarcoplasmic fraction of white muscle of fish and are sensitive to heat treatment and less resistant to food processing as high pressure treatments comparing with the β-PRVBs [62].

Other recognized WHO/IUIS fish allergens include collagen (~127 kDa) and vitellogenin (~180 kDa). Collagen was first identified as fish allergen by Hamada et al. [63] after purification from muscle tissue of tuna and Pacific mackerel skin and demonstrated by IgE reactivity in patient’s sera. Vitellogenin is the major allergen in fish roe (caviar) [64]. The results were obtained by SDS-PAGE, WB using sera from patients and MALDI-TOF MS analysis.

In addition to these intrinsic allergens, a marked increase over the last ten years has been reported in the prevalence of allergic reactions to fish-borne parasites, mainly to *Anisakis simplex* [65-67]. *Anisakis* infects many marine fish species and their storage in industrial freezers for two days or cooking at temperatures above 60 °C kills the parasite but does destroy the allergens [68]. Fourteen reviewed *Anisakis*-derived allergens (Ani s1-s14) are available in UniProtKB/Allergome. These include proteins such as paramyosin (100 kDa), tropomyosin (33 kDa) and SXP/RAL-2 family proteins (16 kDa). Like the β-PRVBs, the majority of these *Anisakis*-derived allergens are gastrointestinal-resistant and heat-resistant proteins [65]. A fast targeted proteomics strategy was recently developed by our research group to detect Anisakids in foodstuffs [42]. This technique allows the rapid direct detection of the main Anisakids species in any foodstuffs in less than 2 hours, including processed and precooked products. The analytical methodology is based on the use of a fast purification of thermostable proteins by heat treatment (time: 45 min), fast trypsin digestion using HIFU (time: 2 min) and monitoring of several Anisakids peptide biomarkers by parallel reaction monitoring (PRM) in a LTQ/Orbitrap mass spectrometer (time: 60 min). This workflow was also applied for the rapid detection of the allergenic protein Ani s9, characteristic of the Anisakids species. The present strategy allows the direct identification and detection of Anisakids species in less than 2 hours. Currently, this is the fastest method to achieve the direct detection of these allergens independently of the foodstuff encountered.

Label-free semi-quantitative LC-Orbitrap MS and heavy peptide AQUA LC-QQQ MS methods were used for the quantitation of *Anisakis simplex* proteins in fish [69]. The publication used unique reporter peptides derived from Anisakid hemoglobin and SXP/RAL-2 protein as analytes. Standard curves in buffer and in salmon matrix showed limits of detection at 1μg/mL and 10μg/mL for MS1 and 0.1μg/mL and 2μg/mL for MS2. The proteomic profiling and characterization of differential allergens in the parasites *Anisakis simplex sensu stricto* and *Anisakis pegreffii* were compared by 2-DE, WB and MALDI-TOF/TOF analysis [70]. Recently the global proteome profiling of L3 and L4 *Anisakis simplex* development stages and the evaluation of the response of the invasive larvae of *Anisakis simplex* to ivermectin drug were performed using an advanced quantitative proteomics methodology based on TMT labeling and analysis in a LTQ-Orbitrap Elite mass spectrometer [27,71].

4. Proteomics applications for the detection and quantification of shellfish allergens
Shellfish allergens include tropomyosin, arginine kinase, sarcoplasmic Ca\(^{2+}\)-binding protein, myosin light chain 1 and 2, troponin C and triosephosphate isomerase (www.allergen.org) (Table 1). Among them, tropomyosin has been traditionally considered the main allergen found across the edible parts of either crustaceans (such as shrimp, crab and lobster) or mollusks (including scallops, oysters, clams, and squid) species [72]. Tropomyosin is a 33 to 38 kDa α-helical protein that forms a coiled-coil structure of two parallel helices containing two sets of seven alternating actin binding sites. Due to its repetitive coiled-coil structures, tropomyosin retains IgE binding ability even after prolonged heating processing or partial digestion. According to the Allfam database of allergen families (www.meduniwien.ac.at/allfam), 64 identified allergenic tropomyosins have been identified in animal sources, mainly in shellfish species. The use of discovery proteomics yielded tropomyosin as a major allergen in raw and cooked Flower tail shrimp (Metapenaeus dobsonii) and in white squid (Loligo edulis) [73,74].

Arginine kinase (38-45 kDa) is also a relevant allergen in shellfish. Although heat labile, arginine kinase has demonstrated IgE binding in heat treated shrimps, which may be due to remaining intact IgE epitopes on aggregated arginine kinase [72]. Interestingly, MS identified arginine kinase as novel allergen whiteleg shrimp (Litopenaeus vannamei) [75] and crucifix crab (Charybdis feriatus) [76]. An early investigation applied proteomics to characterize specific peptides from arginine kinase in seven commercial shrimp species [77]. Additionally to arginine kinase, sarcoplasmic calcium-binding protein, myosin heavy chain, hemocyanin, enolase, and glyceraldehyde-3-phosphate dehydrogenase were identified as allergens in banana shrimp (Fenneropenaeus merguiensis) muscle [78], and tropomyosin in blue swimming crab (Portunus pelagicus) [79]. Similar discovery proteomics approaches have been applied to evaluate the effect of processing on antibody reactivity to allergen variants and fragments of several shellfish allergens. Thus, the sensitizing capacity and allergenicity of arginine kinase after processing was evaluated in crab [80,81]. These investigations showed that enzymatic cross-linking and thermal polymerization of arginine kinase reduces IgE-binding and allergenicity. On the contrary, it was found that heat processing enhanced the overall patient IgE binding to black tiger prawn (Penaeus monodon) extracts and increased recognition of several allergen variants and fragments such as tropomyosin, myosin light chain, sarcoplasmic Ca\(^{2+}\) binding protein, and putative novel allergens including triose phosphate isomerase, aldolase, and titin [82].

Recently, an innovative methodological approach using in silico bioinformatics identification based on sequence alignment combined to 2D immunoblotting against a serum pool allergic patients and shotgun proteomics confirm the presence of 24 previously unreported allergens from more than 25,000 proteins of the Pacific Oyster (Crassostrea gigas) [83]. This investigation demonstrates the presence of multiple novel allergens in shellfish species. Some of these are common to very different allergen sources incorporating animal, including fish and mites, as well as plant allergens. These results highlight that the comprehensive analysis of unreported allergenic proteins fills a major gap in the current management of patients at high risk of concurrent reactivity to diverse allergen sources.

Targeted proteomics approaches based on LC-coupled with high-resolution tandem mass spectrometry (LC-HRMS/MS) has been used to select marker peptides and quantify shellfish allergens even in the presence of allergens of other multiple sources. Thus, a targeted proteomics method was developed to simultaneously detect and quantify the presence of crustaceans, together with allergens from milk, egg, and soy in fish and swine food products [84]. In this study, tropomyosin was selected as protein marker for the detection and quantification crustacean allergens and PRM was the ion monitoring technique used. Similarly, the detection and quantification of seven kinds of aquatic product allergens in meat products, including shrimp (Penaeus vannamei) and crab species (Eriocheir spp., Scylla serrata), have been achieved by using a triple quadrupole mass spectrometry (UPLC-QqQ-MS) system [85]. An additional application of targeted proteomics, it is the detection of airborne shellfish allergens in processing plans to prevent occupational asthma and allergenicity. An absolute quantification method and validation of airborne snow crab allergen tropomyosin was first developed by using isotope dilution mass spectrometry [86]. Previously, snow
crab tropomyosin (SCTM) was identified as the major aeroallergen in crab plants and a unique signature peptide was identified for this protein. A similar approach has been developed to quantify allergenic proteins from Northern Shrimp in air samples in processing plant [87]. This procedure indicated the presence of two aerosolized, tropomyosin and arginine kinase, in all areas of the processing plant. These studies show that targeted proteomics is a sensitive and accurate tool in identifying and quantifying aerosolized allergens.

5. Concluding remarks and future directions

The use of proteomic approaches has undoubtedly facilitated the detection and quantification of seafood allergens. These advances are becoming essential for the success of the avoidance strategies of the allergic consumers. Validated proteomic approaches may soon form part of risk assessment strategies for novel food sources and processing developments.

However, the current allergomics technologies still need further developments. From the allergenic source side and detection actions, all studies have been focused on proteins soluble in aqueous extracts as unique targets and aggregated and hydrophobic proteins are discharged from the study. An example of such omission are proteins assembled into amyloid aggregates that require a pretreatment with hexafluoroisopropanol (HFIP) for efficient disaggregation of the core [51,52]. A second example are the lipophilic oleosins which are lost in the conventional extraction protocols [88]. These limitations can be extended to the aerosol form of seafood allergens which are related to occupational allergies in seafood transforming plants [6, 89]. Then, new methods addressing differences in the solubility of proteins and sampling should be tailored for expanding the detection repertoire.

From a technical point of view, new mass spectrometer modes as the data-independent acquisition (DIA) combined with the high-resolution mass spectrometers (HRMS), will largely improve the detection and quantification of traces of seafood allergens in different foodstuffs. In addition, DIA coupled with ion mobility mass spectrometry (DIA-IM-MS) will be relevant to investigate the allergen composition in challenge mixture of ingredient meals. Also, the application of absolute quantitation by AQUA-LC-MRM, the use of CE coupled to a top-down proteomics approach to detect intact protein allergens in HRMS instruments, and the employment of new complementary top-down MS/MS fragmentation modes (HCD, ETDhcD and UVPD) for the characterization and de novo sequencing of whole allergens, are new directions that will provide new valuable insights.

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