

Review

Perusal of food allergens analysis by mass spectrometry-based proteomics

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ABSTRACT

Food allergy is the disease where the immune system is elicited by antigens in food. Although innocuous for immune-tolerant individuals, an ever-growing number of food allergenic people are being registered worldwide. To date, no treatment to cure food allergy is available and the disease management relies on the careful exclusion of the allergenic food from the diet of the allergic individuals. Great efforts are ongoing to clarify the allergenic mechanisms of the diverse allergenic proteins of food origin, aimed to both designing suitable therapies and for a timely and precise diagnosis of the allergic condition. Among the other omics sciences, mass spectrometry (MS)-based proteomics is gaining a steadily increasing interest by the whole scientific community acknowledged its high versatility. In the present work, the latest proteomics based-studies on allergenic proteins are reviewed to provide guidance on the different MS-based methodologies adopted in the research on food allergens. Our review points to highlight the strengths of the MS-based proteomics and how these have been exploited to address specific research questions. Also, the most common drawbacks encountered in a proteomic study are discussed, providing an overview that helps novel researchers in choosing the more suitable experimental workflow.

Significance: Wide wealth of knowledge arising from the various MS-based proteomic investigations is improving our understanding of food allergy through molecular characterization of food allergens. The present work reviews the key aspects to be evaluated while investigating food allergens by means of MS-based proteomics and provide guidance to the novel research groups approaching to the fascinating world of MS-based food allergens detection.

1. Introduction

Food allergy is an adverse reaction to the antigens, defined as food allergens. In this view, the body reacts to certain foods as they are harmful substances and an immune reaction is elicited even though such antigens are innocuous in healthy people [1]. The immune system of non allergic (healthy or immune-tolerant) individuals is able to discriminate pathogenic antigens from innocuous ones and is, therefore, unresponsive to food antigens. On the contrary, in food allergic individuals, a process of sensitization occurs the first time the immune system interacts with food antigen and this inappropriate response determines the immune pathogenesis during the successive interactions

with the food(s) [1].

Allergy is defined as “a hypersensitivity reaction initiated by proven or strongly suspected immunologic mechanisms” and, specifically for food allergy, this term is used when a causal relationship has been defined between a specific food and, ideally, with a specific immunological mechanism [2]. The elicited immune reaction could be IgE-mediated, non-IgE-mediated and mixed. The IgE-mediated reactions arise up to 2 h from the food ingestion (immediate onset symptoms) and may have a late-phase with prolonged or ongoing symptoms. After the sensitization phase, the subsequent exposure to the food allergen impacts the immune effector cells: food antigens attach to IgE molecules bound to FcεRI receptors on the surface of mast cells and

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basophils causing their degranulation and the release of histamine and other inflammatory mediators of the immediate allergic reaction (immediate phase of the response). The late-phase is characterized by *de novo* production of leukotrienes, platelet-activating factor and cytokines, such as interleukin-4 (IL-4), IL-5 and, IL-13. Different body districts are involved, like gastrointestinal (GI) tract, respiratory system, and the skin, and different may be the symptoms: oral tingling, pruritus, swelling, nausea, abdominal pain, vomiting, wheezing, asthma, angioedema. In acute cases, a serious explosive systemic response (anaphylaxis) may also occur that involves multiple organs and can often be life-threatening. Non-IgE-mediated reactions occur 4 to 28 h after ingestion of the offending food(s) and are believed to be generally T-cell-mediated. Although these immune reactions affect mostly the GI tract, details are poorly defined both clinically and scientifically. Food protein-induced enterocolitis syndrome (FPIES), food protein-induced proctocolitis (FPIP) and food protein enteropathy (FPE) are ascribed to non-IgE mediated food allergies. Mixed IgE and non-IgE mediated reaction involve both IgE- and non-IgE-mediated mechanisms; one example is eosinophilic oesophagitis (EoE), a GI disorder caused eosinophilic infiltration of tissues in presence of milk allergens [1,3]. Over the last few decades, food allergy prevalence has been dramatically increased, especially in children; they usually develop food allergy early in life and will eventually reach tolerance while growing (in case of milk allergy, approximately 80% by the fifth birthday resolved the allergy and 35% show new food allergy). Highly allergic children, which have the highest IgE levels and express the most serious clinical outcome like anaphylaxis and asthma, often develop hypersensitivity to other foods and are less likely to overgrowth their food allergy. It was assessed that food allergy affects up to 8% of young children and 6% of the adult population [4,5]. Due to these increasing numbers, food allergy is becoming a serious health problem and, so far, there is no treatment available beyond careful food avoidance [6]. Because of the extreme complexity of the pathogenesis, our understanding of food allergy is still partial and the science of food allergy is a dynamic, ever-growing field. The ultimate goal is to achieve deeper understanding of the biochemical and molecular basis of food allergy broad manifestation, more efficient diagnosis and management of patients and eventually better treatment strategies. Mass spectrometry (MS)-based proteomics has become an integral part of this continuous learning process acknowledged the recent and rapid technological advances: modern and up-to-date mass spectrometers have risen unprecedented specificity, sensitivity and capability to multiplex and parallelize the analyses of peptide/proteins. Proteomic approaches shed light on proteins structure, post-translational modifications (PTMs) and abundance, and protein-protein interactions, therefore proteomic investigations on food matrices have contributed, and it is still contributing, to the identification, the characterization and the quantification of food allergens. Approaches in a proteomic food allergen study involves: 1) extraction of proteins from food and food matrix, 2) detection and identification of target allergen(s), and eventually 3) quantification of allergens (Fig. 1). Each of these steps must be carefully addressed and may be accomplished with a wide range of operational choices reviewing related literature. Therefore, the purpose of this review is to provide a snapshot of the different methodologies adopted in the research on food allergens. Particular emphasis is given to proteomic approaches that are being employed in the last years, in order to help researchers in choosing the more suitable experimental workflow.

2. Food allergens identification and detection

Food allergy prevalence is increasing over time becoming a significant public health problem. Unfortunately, no treatment beyond careful food avoidance exists to date, and for the most sensitive patients, ingestion of even tiny load of allergens may be life-threatening in absence of rapid treatment with epinephrine for food-induced anaphylaxis. Therefore, each country has put in place mandatory labelling

that regulates the list of allergenic ingredients present in any food and processed food products in order to avoid the possibility of unintentional allergen ingestion [7]. According to the European Union (EU) food labelling regulations, 14 allergens must be clearly depicted on food label (Regulation (EU) No 1169/2011). In United States (US), label of eight foods identified as priority allergens (the “big eight”) and responsible for 90% of food allergic reactions worldwide, is required by the US Food Labelling and Consumer Protection Act when they are added as ingredients.

The following sections describe the general approaches commonly employed for the detection and quantitation of food allergens, highlighting the most common drawbacks and actions taken to “overcome” these issues.

2.1. Food allergens detection: major obstacles

Because of the wide heterogeneity, the varying complexity and the physical status of food matrices, several protein extraction protocols are, to date, available in the literature [8]. Most of them use the same chemicals present in commercial ELISA kit developed for food allergen detection, but each research group has proposed its own protocol, often based on availability of reagents and equipment in the laboratory. Moreover, food processing technology and matrix effect must be taken into consideration while developing the analytical method [9]. Processing of raw food ingredients enables their transformation into final consumable products. However, these processes (thermal and non-thermal) unavoidably result in the alteration of the allergenic proteins to an extent depending by several factors such as processing technology, intensity and exposure to the processing treatment [9–11]. Maillard reaction, also named non-enzymatic browning, is one of the key processes impacting food allergens. It corresponds to a number of complex chemical reactions that may occur between free amino groups of food proteins and carbonyl group of reducing sugars during thermal processing and long storage of food. This non-enzymatic glycation may modify epitopes structure with both alteration of allergenic potential of food proteins, and the potential creation of novel allergens [12]. The most commonly encountered allergens modification includes altered conformational change, denaturation, aggregation, altered solubility, intra-molecular rearrangements, reaction with other matrix components and protein degradation [9,10,13]. Although recently exploited to reduce allergen reactivity [14,15], food processing represents a crucial factor to take into account while assessing the allergenic potential of food. Indeed, alteration of the protein structure hinders a proper solubilization/extraction of the target allergen, as well as affects the optimal antigen-antibody interactions exploited by all immunological assays, leading to underestimation of the target analyte or even false-negative results [11,16].

Several strategies are being nowadays adopted to overcome the reduced availability and/or immunoreactivity of epitopes from processed foods. A previous study demonstrated an improved egg proteins detection in processed foods by applying reducing and denaturing agents containing buffer. The further integration of the method, by applying antibodies capable of recognizing processed egg proteins and the inclusion of incurred standards, results in a significantly improved detection and quantitation of egg allergens [17]. Similarly, the adoption of denaturing (e.g. 6 M urea, Laemmli buffer) [18] and reducing buffer (e.g. 2-mercaptoethanol and dithiothreitol) [19–21] have been linked to enhanced recovery of allergenic proteins. A recent study aimed at improving the extraction efficiency of peanut proteins employed a combination of high-pH carbonated buffer along with preincubation with either high temperature or sonication of the samples prior being subjected to protein extraction. Although both preincubation methods enable for improved recovery of the peanut proteins, sonication treatment had a minimal effect on the immunoreactivity of the extracted proteins making them available for more sensitive and accurate detection and quantification by means of immunoassays [22].

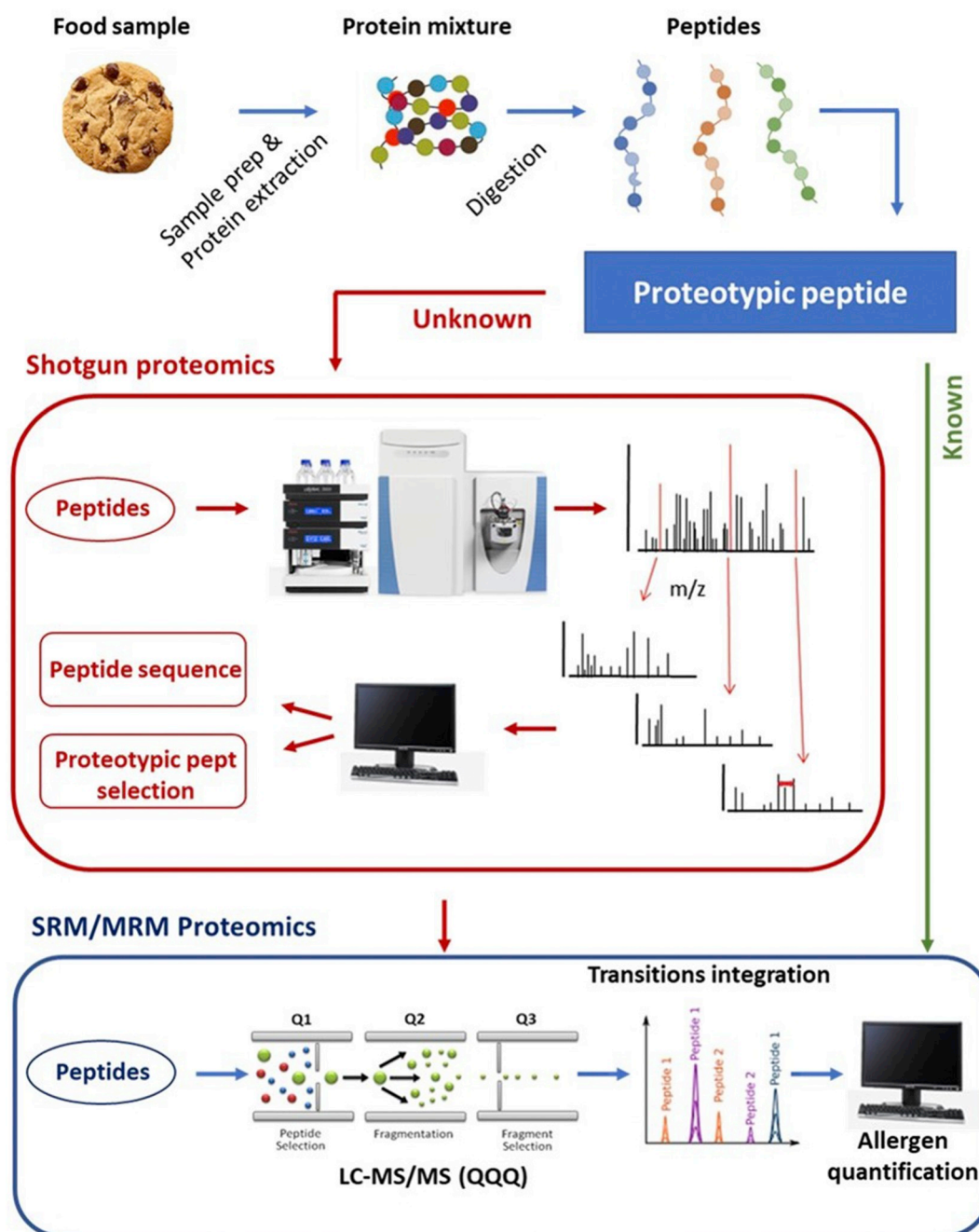


Fig. 1. Mass spectrometry-based method for protein allergen detection and quantitation. The figure summarizes the overall workflow employed for the optimization of a novel MS-based method for protein allergen detection and quantitation. Red-framed panel refers to the optional procedure of shotgun proteomics survey for the identification and selection of proteotypic peptides for the allergen of interest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Besides food processing, food matrix has a profound effect on both immunoreactivity and extraction of target analytes [23], hindering the allergen detection in immunoassays and LC-MS based protocols. In this view, the choice of appropriate extraction buffer and sample preparation procedures may strongly impact the *in vitro* detection of allergens. An example is given by the case of matrix-induced thermal instability of fish parvalbumins [24]. The study reports an important alteration of the thermal stability of fish allergens extracted out of the crude protein fraction. The reduced thermal instability of parvalbumin was attributed to physical and chemical interactions occurring with the matrix components; whereas parvalbumin purified from the same samples exhibited higher thermostability due to the absence of interfering matrix compounds. Analogously, in a recent investigation performed by

Ruethers and colleagues employing high-resolution MS revealed significantly consistent differences in the fish allergens identification from in house and purified extracts, suggesting a considerable matrix effect, regardless the allergen detection method employed [25].

2.2. Digestomics as a tool to study food allergens

Food ingestion represents the primary route eliciting an IgE-mediated allergic response [26]. Although apparently simple, food digestion is a complex ensemble of physical and chemical events leading to the hydrolyzation of proteins, among the other biological macromolecules, to peptides of a varying length until free amino acids. Proteins and peptides digestion occur by means of gastric, pancreatic and intestinal

peptidases [27]; however, several food allergens have shown remarkable resistance to GI digestion, maintaining an intact immunogenic activity [28,29]. Food processing is also influencing GI digestion of proteins to a variable extent. A very recent investigation from Prodic and colleagues links thermal treatments of peanuts to a decreased extraction yield of protein in the acidic environment simulating the gastric juice. Contrariwise, the extraction of peanut proteins in intestinal conditions indicates a higher protein extraction efficacy in roasted peanut samples when compared with the raw counterpart [30]. Past efforts have been made to correlate digestion stability with allergenic potential [27,31] but cumulative evidence definitively proved the presence of digestion-labile allergens and the absence of a strict correlation between immunogenicity and digestibility of allergens [26,27,32]. Nevertheless, evaluation of peptidase susceptibility along with comprehensive tracking of food-derived peptides represents a key step in understanding bioactive peptides of food origin, especially in regard to those with immunoreactive properties, including the identification of novel allergenic sequences and elucidating immunogenic mechanisms.

It is defined as protein “digestome” the whole array of proteins and peptides resulting (or escaping) from GI digestion of food in the human body [27]. Major challenges in the investigation of the protein digestome rely on the complexity of the food matrix and the myriads of compounds resulting from the uncontrolled digestion by means of diverse endogenous proteases at unspecific cleavage sites [30,33].

Pioneering studies on the protein digestomes were carried out by comparing different digestion profiles by means of size exclusion chromatography [34]. Subsequently, liquid chromatography, capillary electrophoresis and gel electrophoresis have been employed for the study of food protein digestion patterns [27,35]. Even though of valuable scientific sound, these techniques can only provide descriptive information arising from the comparative evaluation of diverse digestion conditions and/or digested vs undigested matrixes, but no details on the molecular structure or even the identity of the digestion protein products can be provided [36]. To this purpose, MS-based proteomics and peptidomics reflect the current state-of-art approach. Continuous technological improvements of mass spectrometers enable the identification of proteins and peptides at unprecedented specificity and sensitivity, allowing for an accurate assessment of the peptide molecular weight (MS^1), determination of *de novo* amino acid sequences, and elucidation of structural details at molecular level (MS/MS and MS^c) [26,27,36,37].

Digestomics studies are nowadays commonly performed through the use of *in vitro* digestion systems since enabling the study of protein digestion products in an easy, reproducible and ethically accepted way. Although several models of *in vitro* digestion system have been optimized, the common objective of all systems remains the digestion of food proteins as close as possible to the *in vivo* processes. In a recent study, Spiric et al. employed an *in vitro* digestion system as a convenient tool to investigate allergenic protein in pecan. The study identified a novel potential allergenic protein showing similar immunoreactive- and digestion resistance- feature as other known allergenic proteins found in the same food matrix [38]. Another interesting example is given by the study performed by Apostolovic and colleagues. Here, an *in vitro* digestion system along with a proteomic approach has been employed to evaluate epitope diversity of principal peanut allergens and elucidate major cleavage sites of proteases and their effects on the conformational stability and IgE-binding affinity of the targeted peanut allergens [37].

2.3. Methods for allergen detection and quantification: from immunoassay to state-of-the-art proteomics

2.3.1. Immunoassays and PCR techniques for allergen analysis

Immunoassays and PCR-based techniques have been for a long time the elective methods for allergens detection and quantitation. Among immunoassays, ELISA is the most commonly adopted, enabling for

quick and relatively sensitive detection and quantitation of food allergens. Nevertheless, recent studies proved that different ELISA formats suffer of a scarce reproducibility [39] besides the already well-known drawbacks regarding the cross-reactivity with matrix components, altered immunoaffinity of epitopes from processed foods and the lack of multiplexing capability [27,40–42]. On the other hand, PCR-based detection and quantitation of the allergens rely on the targeting of the DNA encoding for the allergenic proteins (or epitope) [43]. However, this method only detects the DNA molecule and not the allergenic proteins, resulting in inaccurate results, especially in the case of low amount of available DNA and in the case of food subjected to processing treatment that alters the DNA molecules, hindering a fair detection and quantitation of the allergens [9,40].

2.3.2. MS-based proteomics for the analysis of food allergens

On the attempt to overcome the above-mentioned drawbacks, the massive advent of the MS-driven approaches has marked a paradigm shift, moving towards the adoption of proteomics for a confident detection and quantitation of food allergens. Indeed, the continuous technological improvements of MS equipment make proteomics an extremely versatile tool not to be considered as a sole analytical technique but rather a panel of technologically advanced methods to shape upon system complexity to better suit specific analytical requirements [44–47].

An overview of the MS-based detection workflow is given in Fig. 1. Conventional investigations, when no proteotypic peptides are known, expect an overall survey (shotgun proteomics) of the allergenic protein with the aim to identify suitable peptides to be used as a fingerprint in targeted proteomics investigations. Key step of the shotgun proteomics is sample preparation, including: protein extraction, reduction, alkylation and digestion prior being subjected to high-resolution MS measurement. Obviously, the initial step of sample preparation is of paramount importance for a fair identification of the allergenic proteins and peptides. Sample preparation protocols are optimized to reduce the myriad of interfering molecules present in the food matrix. At this purpose, defatting steps involving the use of hexane are commonly introduced [9,30] along with incubation with surfactants and denaturing agents-containing buffer to ensure an optimal protein extraction [47]. In addition, multiple steps of sample cleanup and desalting are recommended to enhance analyte ionization [48] and prevent signal suppression that negatively affects the sensitivity of the mass spectrometry measurements [9,49]. Tandem mass spectra are acquired via high-resolution mass spectrometers (e.g. Q-TOF, LTQ-Orbitrap). Being a shotgun analysis, the MS/MS scan of all product ions is acquired and peptide sequences are computed through specialized algorithms and search engines (e.g. X!Tandem, Mascot, Andromeda) that compare acquired spectra against a protein database [50].

Candidate proteotypic peptides for allergen quantification are selected on the basis of multiple criteria including: -reproducible digestion pattern (e.g. excluding peptides with missed cleavage sites), -reproducible fragmentation profile, -strong signal intensity and -congruous retention time. Moreover, peptides with a length comprised between 7 and 25 amino acids are desired to be chosen in order to enhance the chance of targeting an allergen-specific peptide that falls within the optimal m/z operational range of the mass spectrometer [51–55]. In addition, a study of Newsome and colleagues suggested that peptides with C-terminal arginine are to be preferred to C-terminal lysine ones, especially in food processed allergens, in order to avoid interfering modification such as the Maillard reaction [56]. In fact, it is important to pay attention to alterations of primary structure to which allergenic proteins may undergo in order to create more comprehensive database of modified peptides and to select more suitable targets to be identified and quantified. Mass spectrometry has the power to unveil and highlight these modifications such as PTMs and Maillard reaction products [57,58] such as lactulosyl-lysines, intermediate and advanced glycation end-products (AGE) in milk products [57], Amadori products

in peanuts allergies [59] and Maillard-type adducts in roasted walnuts [60].

Verification of the specificity of the selected peptides prior to proceeding with the optimization of a targeted proteomic protocol is warmly recommended, since targeting and quantitation of unspecific peptides sequences might result in a useless loss of resources, besides the risk of a false allergen assessment and a consequent health threat for allergic food consumers. This step is generally accomplished by querying the selected peptide sequence against publicly available databases such as NCBI (<https://www.ncbi.nlm.nih.gov/protein/>) and/or UniProtKB (<https://www.uniprot.org/>). These are comprehensive nucleotide and protein sequence data repositories, enabling the assessment of eventual non-specificity by aligning the queried peptide sequence to multiple protein products other than the parent allergenic protein [51,61]. However, it must be kept in mind that the above is a bioinformatic-based avenue to rapidly screen the specificity of potential proteotypic peptides. As such, it does not guarantee the absolute specificity of the selected sequence due to several intrinsic drawbacks; first of all, the lack of a complete reference database. Preliminary test to confirm the effective absence of the chosen peptide sequence in blank food matrix is always to be considered [51].

Once the proteotypic peptides of the allergen(s) of interest are known, a targeted proteomic approach is certainly preferable since enables detection and quantification at unparalleled specificity and sensitivity of multiple allergens in a single run.

Selected Reaction Monitoring (SRM) also known as Multiple Reaction Monitoring (MRM) proteomics is among the most frequently adopted approach for detecting single or multiple allergens in complex food matrixes [52,62]. Gold standard mass spectrometer for SRM investigation is the triple quadrupole (QQQ). This enables tandem mass spectrometry measurements where the ion flow arising from the ion source (typically ESI) undergoes a primary sorting of the parent ion in the first quadrupole. Precursor ion is then fragmented in the second quadrupole that works as a collision-induced cell and resulting product ions (i.e. transitions) are subsequently filtered according to their m/z in the third quadrupole. Precursor ions with a + 2 or + 3 charge are preferred and 3 to 5 transitions with the highest signal intensity are chosen for each precursor ion [51,63].

Specificity loss due to the limited number of the selected transitions is counterbalanced by using stringent criteria concerning transition co-elution, consistent peak area of the transitions, and interrogation of transitions falling within a narrow retention time window characteristic to each peptide (i.e. scheduling). Altogether, these criteria improve SRM performance in terms of peptide quantification rate, sensitivity and even specificity by drastically reducing the interference from isobaric precursor ions [51,52]. Also, providing adequate input transitions prevents the instrument from a continuous scanning, enabling for a greater dynamic range and 10–20 times higher sensibility of the measurements when compared to shotgun and other approaches requiring full MS/MS scans of all product ions [52,63].

After the acquisition of the MS/MS spectra, bioinformatic data analysis for peptides sequence and protein identity inference occur in a conventional manner, expecting a database-dependent search and applying the user-defined quality filters and thresholds.

Development of a robust SRM method for allergen detection requires an “optimized mass spectrometer setting” by a careful selection of the transitions for each of the target peptide. A common unbiased approach consists in the preliminary interrogation of crude, unlabeled peptides of synthetic origin (when possible) by acquiring full MS/MS spectra on a QQQ operating in SRM-triggered MS/MS mode [51,62]. This allows retrieving high-quality spectra without matrix interferences. If the selection of target peptides has been made by shotgun investigations, MS information, as precursor and fragment transitions, might directly be transferred to the SRM quantification method on the same MS instrument. On the contrary, care should be taken due to the changing peptide ionization, ion peak intensities, and fragmentation

patterns occurring between instruments [51,62,64,65]. Alternatively, the input transitions might be computed through the use of specific MS software. Skyline, among others, is a freeware software that predicts a comprehensive array of transitions on the basis of *in silico* digestion/fragmentation of the analyte and allows inspection of other laboratories SRM data and reference spectra [65–67]. Another aspect to take into account while developing an optimized SRM protocol is the eventual processing of the food matrix and the effect on allergen detection and quantitation must be empirically evaluated (e.g. in blank spiked samples and/or incurred samples) to intervene accordingly [9,68,69].

A recent SRM study of Ma and colleagues employing the trypsin digestion based LC-MS/MS analysis enabled the sensitive and specific quantification of 7 sesame allergens in a single run. The study discovered conserved and thermally-stable peptides suitable for accurate allergens detection and quantification in a heterogeneous ensemble of food matrices. Moreover, highlighting allergens variability between sesame varieties, the study suggests the necessity to perform individual quantitation for each allergic protein [70].

Following a similar approach, New and colleagues aimed to develop an LC-MS/MS method suitable for the detection of multiple allergens. Here, after having performed an explorative investigation directed to identify unique and selective peptides to be used as signature markers of each allergen, a MRM acquisition method was optimized to quantify marker peptides of egg white, skim milk, peanut, soy, almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut in both incurred bread and cookies [40].

Although SRM-proteomics is the most adopted technique for food allergens detection and quantification, also MS^E and Peptide Reaction Monitoring (PRM) are employed.

An MS^E approach has been adopted for the multiplex protein detection in wheat grain. The study detected and quantified 15 different allergenic proteins isoforms and several peptides carrying four previously discovered epitopes of γ -gliadin B precursor suggesting MS^E as a valuable tool for quantitative analysis and allergens profiling in wheat varieties and/or other food matrices [71].

In another recent study, instead, Chen and colleagues optimized a PRM-proteomics method for the detection of different types of soy-derived ingredients. Marker peptides to employ in the detection of six commercial soy ingredients were identified through a preliminary label-free based investigation. Finally, optimization of the sample preparation and detection enabled the uniform identification and quantification of six different soy ingredients contained in incurred cookies at different levels [72].

High-resolution hybrid quadrupole-Orbitrap mass spectrometer has also been employed for multiple allergens (skim milk, whole egg, soy flour, ground hazelnut, and ground peanut) detection in processed food matrix [73]. The study compared three different acquisition modes: full-MS, targeted selected ion monitoring with data-dependent fragmentation (t-SIM/dd2), and parallel reaction monitoring. Obtained results highlighted t-SIM/dd2 as the best option for multiple allergen detection on the basis of sensitivity and accuracy criteria. The method enabled the quantification of 17 peptides, belonging to the five allergens with a limit of detection ranging from 17 and 30 $\mu\text{g/g}$ depending on the allergen entity.

2.4. Bioinformatic data analysis and allergen databases

Regardless the proteomics technique, the adopted mass spectrometer and the acquisition mode set, an important contribution to all MS-based proteomic methods is given by the bioinformatic data analysis, allowing to deal with the overwhelming load of spectra and data generated with a single MS/MS run. A multitude of software, algorithms and web-based application are nowadays available for this purpose; MASCOT, Andromeda and SEQUEST are among the most commonly used and, coupled to up-to-date data repositories and databases such as NCBI and UniProtKB ensure accurate identification and

quantification of the proteins [74].

Besides, the increasing data on allergenic protein investigation raise the need to develop criteria-oriented and freely accessible allergen databases. Several tools and data repositories are, to date, available enabling the interactive browsing through the protein allergens sequences identified by MS-based methods. It is also provided, for every allergen, the most commonly identified proteotypic peptide sequence along with the related literature information and the possibility to export the optimal settings for an efficient SRM-based quantitation [75]. The WHO/IUIS Allergen Nomenclature (<http://www.allergen.org>) is the official website responsible for maintaining and developing a unique, unambiguous and systematic nomenclature for allergenic proteins. Allergome (<http://www.allergome.org/index.php>) is a comprehensive data repository of peer-reviewed selected allergen literature from the early '60. It also contains a collection of allergenic protein sequences along with their isolation source [76]. Similarly, AllergenOnline (<http://www.allergenonline.org>) provides a peer-reviewed list of allergen sequences. The Structural Database of Allergenic Proteins (SDAP, <https://fermi.utmb.edu>) is a data repository containing the allergen and epitope molecule structure. Moreover, it also links the sequences to bioinformatic tools driving sequence analysis comparison. The Immune Epitope Database (IEDB, <https://www.iedb.org>) is a comprehensive repository of T-cell, B-cell, and major histocompatibility complex protein epitopes including epitopes of allergens. Although the above databases contain redundant information their sorting according to different criteria represent a valuable strategy for a broad spectrum of allergen researchers [75].

3. Food allergens quantification

Allergens accurate quantification represents one of the major challenges for food science mass spectrometrists. MS analytical pipelines capable of multiplexed absolute quantification are routinely adopted for small molecules and therapeutic drug monitoring in human plasma and urine samples. Nevertheless, dealing with proteins in different matrices, such as food ones, is much more demanding [77].

Determining the exact reference doses of the allergens below which even the most sensitive individuals could not react seems to be a matter of growing importance. Much work has been done by allergist and scientist to determine threshold/no adverse effect levels that can be used in food safety risk assessment, but an agreement has not yet been reached [78–80]. Among the most important effort, Voluntary Incidental Trace Allergen Labelling (VITAL) system have been carried in order to define these thresholds based on a dataset of > 55 oral clinical challenge studies [81,82]. Seven hundred fifty microgram per kg for egg protein, 2.5 mg per kg for milk or tree nut proteins, 5 mg per kg for peanut proteins, 25 mg per kg for soybean proteins, and 50 mg per kg for cashew proteins (portion size: 40 g) were identified as minimal eliciting doses for the protection of at least 95% of allergic people, and although these VITAL thresholds have no regulatory value, they are often taken into account in allergen detection methods as limit of quantification to be reached. Mass spectrometry-based techniques may evaluate the exact amount of protein within the food and efforts are being done in order to understand if MS specificity and sensitivity can be push up to dose tiny amount of proteins, lower than those that can theoretically determine an allergic reaction and can establish a routine detection of food allergens.

To note, giving the increasing interest in mass spectrometry-based food allergens detection and quantification methods, Association of Official Agricultural Chemists (AOAC) international analytical guidelines have been published in 2016 specifying method performance requirements for whole egg, milk, peanut and hazelnut (analytical range 10–1000 ppm, limit of quantification ≤ 5 for whole egg and ≤ 10 for milk, peanut and hazelnut, recovery of 60–120%, relative standard deviation (RSD) of 20%). Reviewing literature, it is clear that is very difficult to meet all these specifications.

Quantitation of SRM-based assay is commonly accomplished through the use of stable isotopically labelled (SIL) peptides resembling the target peptides in terms of chromatography behaviour and fragmentation profile but featured by a different mass that enables easy discrimination from the target compound [51,83]. Absolute quantification by means of such approach has proved to be suitable for reliable quantification of allergens in complex food matrix being characterized by a low coefficient of variability (below 10%), linearity over a range of four orders of magnitude, and high reproducibility in inter-laboratory evaluation [51]. Nevertheless, this approach cannot take into account the loss of allergenic protein typical of the upstream extraction and digestion protocols. In this view, a solution might be represented by the spiking of SIL proteins in the food matrix and/or the incurred foods. Unfortunately, the use of SIL protein is very expensive for a routine application, and only few studies [53,56] have proposed its use to estimate corrective strategies to counterbalance the extraction and digestion biases.

Another approach proposed by Chen and colleagues expects the use of SIL peptides prolonged at both C- and N- terminal with amino acid flags. The tryptic cleavage of both binding sites is required for the peptide to be detected. Thus, although not accurate as of the use of SIL proteins, adoption of prolonged SIL peptides enables for an estimation of the digestion variability and provides a more realistic assessment of the target allergen than the use of SIL peptides [84].

The quantification requires the preliminary construction of a calibration curve. This is obtained by spiking in independent blank matrices serial dilution of the target peptides, while the amount of SIL peptide is kept constant. The integrated peak area of both SIL and target peptide transitions is summed and ratios of the target-to-SIL area sum are obtained for each dilution. In this way, the sample quantitation occurs by comparing the target-to-SIL area sum of the analyte with the predetermined calibration curve [56].

It is of crucial importance the use of high-purity peptides for the construction of the calibration curve, as well as linking the peptides to a matrix with the same composition in order to reduce its interfering effect on allergen quantification [51,83,85].

Lastly, corrective coefficients may be applied in order to reduce extraction and digestion bias; also, eventual dilution factors and the desired measure units for the expression of the allergen content must be considered. In the case of development of a novel SRM quantitation methods, authors are also required to assess linearity of the method, limit of detection, limit of quantitation, dynamic range and inter-laboratory comparability [51,86–88]. A very recent study analysed 10 allergens in 8 diverse food matrices by means of UHPLC-MS/MS with the final goal to define criteria for the retention time, variation tolerance, ion ratio deviation, and the signal-to-noise ratio for allergen detection under diverse analytical environments [89]. Similarly, Croote et al. developed a freely available software (MAtrix-Dependent Interference Correction, MADIC) for automated computation of matrix-derived interference and apply it with a method targeting multiple allergens [90].

Other examples of MS-based proteomics for the quantification of allergens in food are given by the recent study of Sayers and colleagues, aimed at assessing matrix effect for the unbiased quantification of peanut allergens [91]. Following comparative analysis among separation techniques, the study suggests the adoption of a microfluidic chromatographic separation prior to mass spectrometry measurement as a valuable strategy to drastically reduce the matrix effect even when using a simple extraction methodology. Another study of the same research group takes into account the influence of food processing in peanut allergens quantification highlighting that, in thermally processed samples, peptide targets of the cupin allergens were more influenced than those from Ara h2, h6 and h7 [92]. Besides, the study indicates that target sequences flanked by arginine residues are featured by a higher thermostability, which has great importance for a fair quantification of peanut allergens, as also suggested by Newsome and

coworkers [56].

Moreover, Korte and colleagues demonstrated that both matrix- and processing- effect influence the MS-based quantification of food allergens to an extent varying from 15 to 250% of the protein recovery and up to 83% of signal loss for matrix and thermal processing, respectively [93].

Development of a reliable allergen quantification pipeline must deal with limitations due to sequence endogenous and exogenous modifications, such as PTMs, protein isoforms and variant, Maillard reaction products [94]. A good knowledge of the existing modifications and a critical selection of peptide targets may avoid both biased food allergen quantification and false-negatives outcomes [51]. Furthermore, detection of 3 or 4 peptides for each protein is strongly recommended to guarantee specificity and reliability of the method in complex matrices as foodstuff [95].

4. Immunoproteomics

Besides allergen detection and quantitation into complex food matrices, research efforts are also being performed in discovering novel allergenic proteins and epitopes to target an efficient diagnostic model. In addition, gained knowledge might also be useful in understanding the molecular mechanisms governing the allergic reactions provoked by a wide variety of food allergens, thus propose effective treatments in both prophylactic and therapeutic terms.

It is defined as “Immunoproteomics” the discipline studying the protein array involved in eliciting the immune response. Identification of such proteins relies on a two-step process, where the first step accomplishes a screen of the potential immunoproteins (commonly assayed with sera from allergic patients); while the second step expects the MS-based identification of the previously screened proteins. To this purpose, techniques such as gel-based electrophoresis, microarray- and DNA-based techniques are commonly coupled to mass spectrometers.

DNA-based methods represent a valuable approach; however, there are some limitations concerning large proteins; it is not always possible to convert the cDNA data into “functional” protein product and there is a lack of post-translational modification, with consequent alteration of the protein immunoreactivity. Similarly, microarray-based approach suffers from several technical drawbacks such as reproducibility and specificity [96], making gel-based proteomics the most suitable approach for antigens investigation.

Typically, samples (i.e. food matrix) undergo protein extraction and 2D-PAGE is subsequently employed for evaluation of the protein profile. The IgE binding of the extracted proteins is then evaluated by means of immunoblot assay, where sera from allergic individuals are generally employed for obtaining the antigen-antibody complex on the membrane. Labelled anti-IgE antibody enables to sort the immunoreactive proteins of interest. Gel spots can be directly excised from the gel and subjected to antibody stripping prior to being subjected to sample preparation protocols for MS measurement.

Following a similar approach, Apostolovic and colleagues aimed to feature the protein profile of red meat subjected to different thermal treatments and investigate their potential allergenicity among red-meat allergic patients [97]. This study identified 18 IgE-binding proteins of bovine origin and 7 novel beef allergens bearing alpha-gal epitope, of which 4 shown to be thermal stable. In this view, testing of the serum patients represents a promising approach for the effective identification of allergenic proteins; especially in the case of food of unconventional origin such as genetically modified organisms and/or foods from novel sources not yet used for human consumption [98].

Carrera and colleagues identified, by shotgun proteomics and protein-based bioinformatic, 35 peptides from parvalbumin, the major fish allergen, as putative B-cell epitopes. After probing these peptides with sera from healthy and fish allergic individuals, 4 epitopes showed the greatest reactivity and were designated as peptide vaccine candidates [99].

Mass spectrometry approach may be of help in characterizing both linear and conformational IgE epitopes and therefore may contribute to serum-based allergy diagnostics, identifying new epitopes to be introduced as allergy markers [37,100,101]. Solution-phase amide backbone hydrogen/deuterium exchange (HDX) in combination to proteolysis, MS and bioinformatics, is a very specialized approach developed to study protein-ligand interaction and therefore can be applied to investigate the antibody-antigen relationship and unveil conformational epitopes [102]. Pioneer researches on food allergy epitopes were performed on cashew allergen Ana o 2 and almond nut allergen Pru du 6 by a research group based in Florida [103–105]. Just a few laboratories worldwide have the expertise to perform HDX-MS experiments, since sophisticated chromatography and high-resolution mass spectrometers are needed. Nevertheless, this strategy can play an important role in expanding the knowledge, still very partial, on conformational epitopes.

5. Practical challenges in food allergen detection

Nowadays, MS-based proteomics is widely accepted as the best approach for a fair food allergen assessment. Besides general issues common to all allergen detection methods such as the choice of the analyte, matrix effect and allergen protein recovery, several issues of practical concern are still unsolved, keeping proteomics away from being adopted in the routine proceedings for food allergen evaluation [53]. Development of novel proteomics-based methods relies on preliminary tests performed either on crude allergenic proteins or blank spiked/incurred food matrix. In this view, availability of standardized reference material for all allergens would greatly benefit the result of the investigations and the comparison among independent studies performed either on the same or diverse food matrix.

Moreover, it is important to underline that allergen research has to cope with lack of high-quality plant genome annotation of the poorly investigated phylogenetic groups, impacting the *in silico* protein database creation. Combining data from next-generation sequencing, transcriptomic and proteomics may solve this issue. MS peptide *de novo* sequencing, high-resolution mass spectra in comparison with theoretical spectra obtained from DNA sequencing and transcript sequences derived from RNA sequencing, provide evidence of translated proteins and novel-coding genes [106]. As an example, in 2013, a new hazelnut putative allergen was identified through immunological and proteomic approaches [107]. After probing the patients sera with 4 hazelnut cultivars, adoption of two-step chromatographic purification of the hazelnut extracts along with MALDI-TOF and nano HPLC-ESI-Q-TOF MS/MS analysis enabled both homology-based and *de novo* sequencing of the immunoproteins, resulting in the hypothesis of a divergent isoform of the hazelnut 11S globulin. Also, MS-based analysis is rather expensive. Setting up a proteomic-based protocol for quantifying a novel allergen requires preliminary untargeted explorative shotgun analysis with the aim to evaluate candidate proteotypic peptides and study their behaviour along the entire analytical workflow. However, this step might be avoided in the case of highly studied allergens since cumulative evidence suggesting conserved sequences for a given allergen might be used for a direct setting of a targeted proteomics survey. At this scope, data repositories such as Allergen Peptide Browser (<http://www.AllergenPeptideBrowser.org/>), that aggregate available literature according to different criteria including allergen class, MS method, instrument and food matrix, are of great help [27,51]. In this view, efforts in harmonization of the obtained results are strongly desired. The fast technological development of MS equipment gave birth to a wide variety and variants of MS-based techniques, each of which tailored to address specific analytical requirements. Nevertheless, proteomics adoption in a routine laboratory unavoidably requires harmonization of the methods and standardized procedures aimed at reducing inter-laboratories variability and, importantly, transfer knowledge arising from different research projects in common

and publicly accessible repositories [54].

6. Allergen proteomics for the food industry

The high sensitivity of the MS-based investigations makes proteomics an extremely powerful tool, and it is believed that an increasing number of studies will be performed in the Research and Development (R&D) sector of food industry, in the next future. Among the diverse facets of proteomics investigation in food science, MS-proteomics applied to fruits and vegetables packed under controlled atmosphere demonstrated an altered profile of allergenic proteins [108]. Proteomics investigations have also been used to demonstrate the influence of thermal processing of food on the final food allergenic asset. By using a proteomics approach, Scaloni and colleagues [109] reported an increased carbonylation rate of heat-treated milk proteins which, in turn, can lead to allergic reaction to milk products [110]. Proteomics investigation of farmed and wild gilthead bream has demonstrated a changing allergens profile, with parvalbumin being significantly higher in reared fish compared to wild ones [111]. MS-based proteomics might also be efficiently used for the study of protein degradation (with possible production of allergenic products) occurring while food processing such as protein deamination [112] and other PTMs [113,114] providing precious information for optimal tuning of the production process while maintaining the quality and safety of the food product.

7. Conclusions

The increasing technological advances in the field of MS makes proteomics a powerful and versatile omic science. Mass spectrometer vendors are developing faster and more sensitive instruments which are able to perform sophisticated experiments and multiplexed analysis in a high throughput fashion. Nowadays, proteomics investigations are being performed in diverse research field, contributing to expanding the overall knowledge of the “protein world” with enormous advantages also for the food allergy science. The key step of a proteomics study is protein extraction, and undoubtedly some of the major difficulties rely here on. Food matrices are very heterogeneous among each other and contain a plethora of MS interfering substances (e.g. fats, sugars). Moreover, thermal processing alters the raw ingredients and food proteins can be modified during GI digestion. Therefore, careful close attention must be paid to this first experimental step, keeping in mind possible discrepancies among adopted protocols and available literature.

Ideally, MS has the power to overcome other assays (e.g. PCR, ELISA) in allergens detection and quantification. MS strength lies in its ability to unequivocally identify allergens and multiplex the analyses, allowing for the quantification of several allergenic proteins from complex matrices within a single LC-MS/MS run, with high analytical confidence. MRM and PRM are the most investigated and promising strategies in order to quantify food allergens; even though featured by specific drawbacks that scientists are affording to address. Moreover, the choice of proper standards for accurate quantitative assay has to be carefully evaluated and MS measurements need expensive instrument and skilled operators. Efforts in harmonization, among laboratories, of both the analytical pipelines and the obtained results are still lacking and strongly desired. Nevertheless, we are confident that MS-based proteomic analysis may, in the near future, achieve ultimate goals of food allergens science: identify even tiny amounts of food allergens, quantify the exact amount of allergenic protein within the food, and, in combination to population clinical studies, determinate the exact reference doses of the allergens below which even the most sensitive individuals could not react. Besides, implementation of proteomics investigations in the food industry might represent a powerful tool to amend the food nutritional value, quality and safety. It enables comprehensive monitoring of the productive process, providing guidance for a detailed modulation of the allergenic profile from the raw material

to the finished product, including a thorough analysis of the influence of matrix, thermal processing and additives usage on the final allergenic profile of the food product [108].

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Declaration of Competing Interest

The authors declare that there exist no conflicts of interest with regard to the present study.

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