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### Review

# The frontiers of mass spectrometry-based techniques in food allergenomics

Gianluca Picariello<sup>a</sup>, Gianfranco Mamone<sup>a</sup>, Francesco Addeo<sup>b</sup>, Pasquale Ferranti<sup>b,\*</sup>

<sup>a</sup> Istituto di Scienze dell'Alimentazione (ISA) – CNR, Via Roma 52 A/C, 83100 Avellino, Italy

<sup>b</sup> Dipartimento di Scienza degli Alimenti, University of Naples "Federico II," Parco Gussone, Portici I 80055, Italy

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### ABSTRACT

In the last years proteomic science has started to provide an important contribution to the disclosure of basic aspects of food-related diseases. Among these, the identification of proteins involved in food allergy and their mechanism of activation of toxicity. Elucidation of these key issues requires the integration of clinical, immunological, genomic and proteomic approaches. These combined research efforts are aimed to obtain structural and functional information to assist the development of novel, more reliable and powerful diagnostic protocols alternative to the currently available procedures, mainly based on food challenge tests. Another crucial aspect related to food allergy is the need for methods to detect trace amounts of allergenic proteins in foods. Mass spectrometry is the only non-immunological method for high-specificity and high-sensitivity detection of allergens in foods. Nowadays, once provided the appropriate sample handling and the correct operative conditions, qualitative and quantitative determination of allergens in foods and ingredients can be efficiently obtained by MALDI-TOF-MS and LC-MS/MS methods, with limits of detection and quantification in the low-ppb range. The availability of accurate and fast alternatives to immunological ELISA tests may also enable the development of novel therapeutic strategies and food processing technologies to aid patients with food allergy or intolerance, and to support allergen labelling and certification processes, all issues where the role of proteomic science is emerging.

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### 1. Introduction to food allergy: features and boundaries of the disease

Food allergy (FA) is an increasingly emergent issue in food science and technology. Although the various diseases are limited to

only categories of individuals, the number of patients which are interested is steadily growing in the last ten years, making the FA issue of primary importance for clinicians as well as for food industry. This can be fully understood by considering that the most common injuring sources include, among others milk, egg, wheat, soy, peanuts, tree nuts, fish and shellfish, all basic food matrices of the diet of the majority of human population worldwide, and are also the raw ingredients used by the industry for the production of practically all food preparations.

\* Corresponding author.  
E-mail address: [ferranti@unina.it](mailto:ferranti@unina.it) (P. Ferranti).

In spite of the important progress in immunopathology and in biomedical sciences, many of the suspected allergens present in foods have not been identified or characterized yet, as the structural traits which make a protein an allergen still remain substantially unknown. In the same time, the mechanisms through which allergenic proteins and their derived peptides elicit the adverse reactions to food ingestion still await to be completely explained. This also limits severely the possibility of developing novel and efficient screening and confirmatory tests for diagnosis of FA as well as effective therapeutic protocols. Integrated approaches based on the various “-omics” for food peptide and protein characterization (proteomics, peptidomics, metabolomics, and others), all of which rely on mass spectrometry (MS) analysis, are proving to be essential at various levels in the study of FA, from the structural characterization of novel food allergens to the controversial feature of the resistance to digestion of allergenic proteins or to the efficiency of removal of epitopes from a food destined to sensitized individuals.

In this review, the main aspects of current and perspective applications of proteomic technologies to the study of food protein allergens (allergenomics) and derived peptides are critically presented, with attention to the issues related to their identification, detection, and quantification, relevant to biochemical, immunological and diagnostic aspects. Focus is also given to the perspective integration of MS-derived indications in the pre-clinical or clinical practice.

### 1.1. Epidemiology of food allergy

FA is an adverse response sustained by an immunological mechanism against a food component, generally a protein. Definitive data about the prevalence of FA, nature and level of harmful components in foods are still lacking in spite of the considerable efforts that are being made to bridge this gap. No precise estimation of social costs and of the impact of FA on the quality of life is available so far. In response to this in the last decade the European Commission and other world-wide governmental scientific associations have funded a series of projects aimed to promote and extend the knowledge in the field of FA. Recent estimations settled around 3.2–4% the prevalence of confirmed FAs which arise during the first year of life [1], although according to some authors prevalence could reach 6–7.5% [2–4]. A recent meta-analysis reports a prevalence of FA in the adulthood approximately at 3.5% [5]. An accurate evaluation is hindered by the heterogeneity of FA outcomes, by the diversification of the diagnostic methodologies and the genetic and nutritional differences among populations. The majority of the available studies have been based on self-perception of food reactions [1]. This criterion overestimates the prevalence of FA in comparison with studies that exploit objective evaluation tools, particularly those including double-blind placebo-controlled food challenge (DBPCFC) oral test, which is considered the “gold standard” for diagnosis. In any case, FAs undoubtedly have a rapidly increasing trend, most likely because of better awareness, improved diagnostic protocols and rapid dietary changes [4,6]. Currently, in developed countries, FA is the first cause of hospitalization for anaphylaxis [7,8].

The majority of children (60–85%) outgrow allergy by the age of 4–5 years, while the others remain sensitized lifetime [2]. The persistence of FA is variable, depending on the specific allergen. A clinical history of FA is strictly related to the insurgence of an “atopic march” which is the tendency to develop atopic symptoms such as asthma, hay fever, or dermatitis later in life [9].

### 1.2. Immunopathology of food allergy

Although it has been often described as a unique disorder with many facets (it has been defined by pediatricians as “an evasive

and vain woman” – since it likes to wear myriad dresses), FA has an extremely complex ethiopathology. Because of the individual diversification in the immunological response, more than a single protein trigger (allergen) can be contained in a food. In addition, reactions can be elicited by more than only one specific peptide sequence (epitope) within a protein allergen. Furthermore, immunological responses in FA can be either IgE- or cell-mediated, or even result from a combination of IgE and non-IgE-mediated mechanisms. Estimation of the relative prevalence of these mechanisms is hindered by frequent under- or mis-diagnoses, especially of non-IgE-mediated FA, and by the possible late onset in the adulthood [10].

The gastrointestinal tract (GIT) is normally exposed to a huge load of potential allergens. Throughout the digestive path potential allergens are suppressed to harmless foreign antigens. In normal conditions, the tight junctions of gut epithelium prevent large antigenic proteins to breach into the basolateral side where they would come into contact with GALT (gut-associated lymphoid tissue). The immaturity of both the gut barrier and immune system reduces the efficiency of the infant mucosal barrier [11]. Generally, less than 2% of the dietary proteins are absorbed in an immunologically intact form. When intact proteins do cross the gut barrier, the immune system physiologically induces oral tolerance [12]. Thus, it has been supposed that FA is the result of a transient or permanent functional failure of the oral tolerance.

The so-called “type I” hypersensitivity reactions, i.e. those IgE-mediated, are by far the best characterized adverse reactions to foods. The adverse reaction has an early onset, on a time-scale ranging from seconds – in oral allergy syndromes – to 2–3 h. Symptoms include vomiting, abdominal pain, diarrhea. The antigens adsorbed through the digestive tract are delivered to distal organs by the blood giving rise to rhinitis and asthma or urticaria and angioedema. Reactions can also evolve into atopic dermatitis or into an anaphylactic blood pressure shock. The immunological cascade of events in IgE-mediated FA has been reviewed by several specialized articles [13,14].

Unlike IgE-mediated FA, non-IgE-mediated FA is rarely life-threatening. Symptoms of non-IgE-mediated FA are generally vague and, in most cases, are limited to gastrointestinal discomfort. Nevertheless, non-IgE-mediated FA is the cause of morbidity in growing infants and young children [15–17].

Celiac disease is a non-IgE-mediated food protein-induced enteropathy triggered by prolamins of wheat, barley, rye. It is genetically determined and has a *sui generis* immunopathology which under many aspects remains to be elucidated. Numerous excellent specialized articles have already covered this issue [18]. The use of MS and proteomics techniques in the study of celiac disease has been reviewed as well [19,20]. Therefore, celiac disease will be only occasionally treated in the present review.

### 1.3. Diagnosis and treatment of food allergy

Diagnosis of “type I” FA relies on anamnesis, clinical evaluation of symptoms, determination of total and specific IgE, and skin prick tests. An operating IgE-mediated mechanism of FA can be clinically evidenced by a positive IgE test, as well as by the measurement of chemical mediators released. On the other hand, the serum content of specific IgE (sIgE) might be not clinically relevant as positive radioallergosorbent test (RAST) response to a specific allergen is indicative of sIgE sensitization but is not a proof that the allergen is the cause of clinical symptoms. Moreover, many individuals with allergen-sIgE do not develop symptoms at all [21].

The diagnostic protocols are not unanimously recognized and accepted, so hampering to define guidelines of general clinical applicability. The diagnostic “gold standard” for FA, including most non-IgE-mediated reactions, is currently the DBPCFC oral test

which is not devoid of risks besides being technically laborious. Assays based on direct or indirect evaluation of IgE levels are obviously ineffective in revealing “type 2” cell-mediated disorders. Screening tests to monitor the pattern of cytokine expression by peripheral blood mononuclear cells upon stimulation by potential epitopes with the aim of diagnosing a cell-mediated response are still under development [14,22].

Several studies have been aimed to the identification of the dominant antigenic determinants and to their correlation to clinical symptoms and to prognostic factors. However, due to the different assessment of the strategies adopted and to the strong individual variability of the response, outputs are often controversial and definition of epitopic sequences is still far. Data about T-cell epitopes which induce either indirect antibody production or cell-mediated immune reaction are even more fragmentary because relatively few dedicated studies have been produced along the years [23–25].

Although several approaches to effectively treat FA are under study, the current therapy for FA is limited to a strict avoidance of the offending food and to the education to the pathology management. This approach is generally effective, but compliance can be very difficult because of cross-contamination, of the almost ubiquitous occurrence of some allergens in the diet and of inadequate food labelling. With the purpose of addressing these concerns, several directives have been issued by European Community in order to discipline labelling of potential sources of allergens in foodstuffs [26]. The current policy concerning the declaration of food allergens, also including the precautionary warnings on food labels as well as the questions of cross-contamination and “hidden allergens”, has been recently examined analyzing 550 food labels [27,28].

#### 1.4. Structure of food allergens: what makes a protein an allergen

A relatively small percentage of proteins in foods are allergenic. A number of allergen databases have been released into the world wide-web in order to catalogue allergenic proteins and promote integration and interconnectivity of data (for a list of the available platform see [29]). Many studies state that food allergens are (glyco)proteins with a molecular mass ranging from 10 to 70 kDa and belong to a variety of different protein families. However, these features actually are of limited utility to catalogue and predict novel food allergens, because they are shared by the great majority of the most common food proteins.

There has been no evidence for common motifs to either linear T-cell or linear IgE (B-cell) epitopes. The structural characterization of conformational IgE-binding sites is very limited as well. However, according to partial sequence homology or to evolutionary relationship, nearly 60% of plant protein allergens have been grouped in as few as 4 families [30,31]. Similarly, animal-derived allergens have been classified into 3 main families – tropomyosin, EF-hand proteins and caseins – along with 14 minor families [32]. Depending on the ability of the immune system in discriminating between “self” and “non-self”, the allergenic potential of some animal proteins appears related to the phylogenetic divergence of the parent organism from humans [32].

Animal allergens in some cases share structural analogies with plant derived allergens, in particular the stability to food processing, and the resistance to digestion in the GIT [33,34]. These properties ensure that enough of the protein survives in an immunogenic intact form to be taken up by the gut and recognized by the mucosal immune system. Again, this is the case of milk proteins, especially the conformationally structured whey proteins such as  $\alpha$ -lactalbumin ( $\alpha$ LA) and  $\beta$ -lactoglobulin ( $\beta$ LG), which are quite resistant to gastrointestinal digestion and were recently shown to produce resistant peptides [35] which may be absorbed by the intestine.

On the other hand, “type-2” food allergens which elicit respiratory responses because of cross-reactivity with pollen, are heat-labile and can induce reactions limited to oral symptoms.

The real challenge for researches is to determine the way proteins are allergenic, as there is no definitively accepted procedure to predict or establish the allergenicity of a protein. Comparing the structures of the proteins with known allergens and allergen epitopes can be one strategy. Recently, *in silico* approaches have been used to quantify the distribution of plant food allergen sequences across protein families and to develop and apply a novel means of assessing conserved surface features important for IgE cross-reactivity [30,36]. Structural bioinformatic analysis showed that conservation of three-dimensional (3D) structure should be included in any assessment of potential IgE cross-reactivity, as for example in novel discovered proteins [36].

A development of this approach is based on the use of overlapping synthetic peptides which cover the sequence of the protein investigated. This approach has been widely used for structure/allergenicity assessment and to discover novel allergens in several food sources, including milk, cereals, and legumes. A prerequisite of this strategy is that the 3D structure of the protein under examination is at least partly known. This can be exemplified by the case of milk proteins. The most allergenic proteins in milk are the two major whey proteins, i.e.  $\beta$ LG and  $\alpha$ LA, and  $\alpha$ s1- and  $\beta$ -casein, the main milk protein fraction. B-cell epitopes of these proteins have been investigated by screening methods based on synthetic peptides [37,38]. In the case of  $\beta$ LG, three main peptide epitopes were identified as recognized by a large majority of human IgE antibodies, whereas a series of other epitopes were found scattered along the  $\beta$ LG sequence, including a 4-amino acid stretch common to  $\alpha$ LA and  $\beta$ LG. Comparison of the already established three dimensional structure of  $\alpha$ LA and  $\beta$ LG [39,40] with the structure of other major milk proteins showed that another whey protein, lactoferrin, also possessed allergenic sites and thus could be qualified as an allergen.

The conformational stability of an allergenic food protein is of high importance to its allergenicity. Stability of an allergenic protein describes its ability to maintain its original and allergenic 3D structure despite being exposed to heat, extreme pH changes, and proteases. The term “stability” also encompasses the ability of a protein to refold to its native or near-native conformation during cooling after heat treatment. Several structural features are related to stability including the presence of disulphide bonds ( $\alpha$ LA,  $\beta$ LG, members of the prolamin super-family such as non specific lipid transfer proteins and 2S albumin), glycosylation (legume proteins), and rheomorphism (which characterizes structurally highly mobile proteins, such as caseins). The functional role as protease inhibitor also confers particular stability to allergenic proteins such as the peanut Ara h 2 [41], soybean trypsin inhibitors [42], non specific lipid transfer proteins (nsLTPs) [43,44] and serpins [44,45]. Resistance to digestion is also assumed to be involved in the pathogenesis of celiac disease in predisposed individuals [46–48].

Glycans can be the antigenic determinants of food glycoproteins [49–51]. In particular, plant N-glycans containing  $\alpha$ -1,3-fucose and  $\beta$ -1,2-xylose are known to be most frequently involved in the structures of IgE epitopes [50,52,53]. The widespread occurrence of fucose and xylose on N-linked glycans in plants, but also in invertebrates such as crustaceans (to less extent), explains several cross-reactive sensitization profiles toward proteins not structurally related [54]. Carbohydrate moieties can indirectly enhance the allergenic potential by conferring stability against proteolytic degradation [55,56].

Allergenicity may be also enhanced by the technological treatments as a consequence of an increased stability to digestion due to non-enzymatic glycosylation which involves potential protein cleavage sites [41,57,58]. The finding that technological production

processes such as heat treatment (always applied to ensure milk safety) and protein hydrolysis (used for the production of uncountable food ingredients as well as for the production of hypoallergenic infant formulas) [59,60] may in part reduce milk allergenicity, but also in some cases produce novel epitopes through the association of  $\alpha$ LA and  $\kappa$ -casein to  $\beta$ LG in heat-treated milk [61] or heat denatured  $\beta$ LG [62], clearly illustrates the complexity of elucidating the structural basis of FA. This challenging task has been revolutionized by the application of modern structural and functional proteomic strategies.

## 2. “Allergenomics”: mass spectrometry in allergen detection, identification and quantification

Strategies for allergen characterization and monitoring do not substantially differ from the general procedures for protein identification. Nevertheless, for “type I” FA the allergen targeting can take advantage from the IgE-binding properties. Recently, the application of proteomic methodologies for the analysis of food allergens has been termed “allergenomics” [45].

MS-based platforms constitute the core technologies in proteomics. The evolution of MS technologies is producing a massive impact on our understanding of the complex nature of allergens and generally of proteins. MS allows proteins to be analyzed rapidly, accurately, with high sensitivity, specificity and reproducibility. Ion source and mass analyzer are central to MS technology for protein research. Since their introduction in the late 1980s, matrix assisted laser desorption ionization (MALDI) [63] and electrospray ionization (ESI) [64] have been the most widely used soft ionization techniques for biomolecular analysis. ESI and MALDI interfaces are combined in various ways with a number of mass analyzers. Currently, four types of mass analyzers are routinely used: quadrupole (Q), ion-trap (with its variants quadrupole ion-trap, QIT; linear ion-trap, LIT), time-of-flight (TOF) and Fourier-transform ion cyclotron resonance (FTICR) mass analyzers. Complex hybrid multiple-stage mass analyzers such as triple Q (QQQ), hybrid Q/TOF and tandem TOF (TOF/TOF) instruments have been combined. As extensively reviewed, the performances of mass analyzers vary in sensitivity, resolution, mass accuracy and ability to produce high quality MS and MS/MS spectra [65–67]. A special mention is deserved by a recently introduced type of hybrid instrument, referable to the Fourier-transform technology (commercially the LTQ-Orbitrap system), because of its high resolution (up to 150,000) and mass accuracy, a mass-to-charge range up to 6000, and a dynamic range greater than  $10^3$  [68–70].

Recent MS developments include techniques such as the ion mobility, which in a near future could be helpful in enhancing the detection of immunogenic sequences in complex food samples [71], and electron transfer dissociation (ETD)/electron capture dissociation (ECD) which complement CID (collision induced dissociation) enabling improved MS/MS sequencing in “discovery-driven” experiments, especially of post-translationally modified peptides and top-down analysis of intact proteins (see [72]).

The characterization of glycosylation, which is particularly relevant for food allergens, has remained challenging until a few years ago, because of several intrinsic drawbacks, such as the significant suppression of glycopeptide ions when analysis is carried out in complex peptide mixtures, and the complex fragmentation patterns generated in MS/MS experiments. These limitations have been partly overcome by approaches relying on glycopeptide enrichment by lectin or hydrophilic resins (HILIC) prior to MS analysis [73,74]. By this way, enriched glycopeptides can be analyzed separately and glycans can be profiled by MALDI or ESI-MS “glycomic” analysis after enzymatic (N-glycans) or chemical (O-glycans) hydrolytic cleavage. It has been demonstrated that

glycans released from glycoproteins can be successfully profiled by MALDI-MS without prior derivatization [75–77]. In this case, the purification of the oligosaccharides from interferences such as salts and detergents, usually performed through graphitized carbon micro-columns, is a key issue [78]. However, glycomic profiles can be obtained at much higher sensitivity after permethylation of the oligosaccharide chains that also stabilizes glycans preventing the possible loss of labile sialic acids. The most utilized strategy of permethylation introduced by Ciucanu and Kerek in 1984 [79] has recently been improved by the fast and easy microscale solid-phase permethylation strategy [80,81]. Permethylation using isotope labelled reagents prior to MALDI-MS analysis is amenable to the quantitative analysis of glycans [82]. Even though this procedure has not been applied to food allergens so far, it could be exploited to compare the level of oligosaccharide epitopes in different food samples. For instance, the technique would allow to assess their removal following food processing. The recent advances in microfluidics will most likely enable fully automated glycan analyses that require nano-amounts of sample. To this purpose, microfluidic chip-tandem MS, which makes use of graphitized carbon as the chromatographic stationary phase, has been recently successful in profiling and characterizing complex mixtures of glycans released from glycoproteins [83,84].

ECD- and ETD-MS have been successfully exploited as strategies of ion fragmentation complementary to CID. Being practically unaffected by the processes of electron capture/transfer, glycan moieties are retained on the peptide backbone, enabling the amino acid sequencing and the unambiguous assignment of the glycosylation site(s) [85,86].

Both the targeted “hypothesis-driven” and “discovery-driven” analyses of allergens can exploit MS at all stages of investigation. Below recent and exemplary applications of MS strategies to the relevant issues of detection/monitoring (hypothesis-driven), identification/characterization (discovery-driven), and quantification (hypothesis-driven) of food allergens are surveyed.

### 2.1. Methods for food allergen detection

A variety of methods are currently available to determine the occurrence of allergenic ingredients in food products. Some of the most commonly utilized methods do not target the specific protein allergen, but rather a marker indicative for the presence of the offending food (e.g. trace of the raw material). DNA-based methods consist in the PCR amplification of DNA fragments specific for the allergenic food. Although the detection of DNA markers benefits from having a well-defined target analyte and the combined use of database analysis and experimental specificity should lower the chance of obtaining false positive, it has to be underlined that the allergenicity of a food is caused by its proteins and not by its DNA; therefore, the presence of DNA of a component in a food product does not guarantee the presence of allergens and *vice versa*.

The direct recognition of allergenic proteins generally relies on their antibody-binding properties and, thus, makes use of immunochemical techniques. Commercially available kits of this type are lateral flow devices or dipsticks that are used for rapid screening and enzyme-linked immunosorbent assays (ELISA) that also provide semi-quantitative determinations.

Typical limits of detections (LOD) of the tests based on ELISA kits are in the range of 1–5 ppm. A major drawback of immunological methods consists in the fact that the epitopes that are detected are usually not known and cross-reactivity with matrix components can result in false positives. The reliability of detection strongly depends on specificity and stability of the employed antibodies and can be affected by the changes induced on proteins by thermal or other technological treatments. Furthermore, in several foods linear epitopes can be hydrolyzed and released from the parent

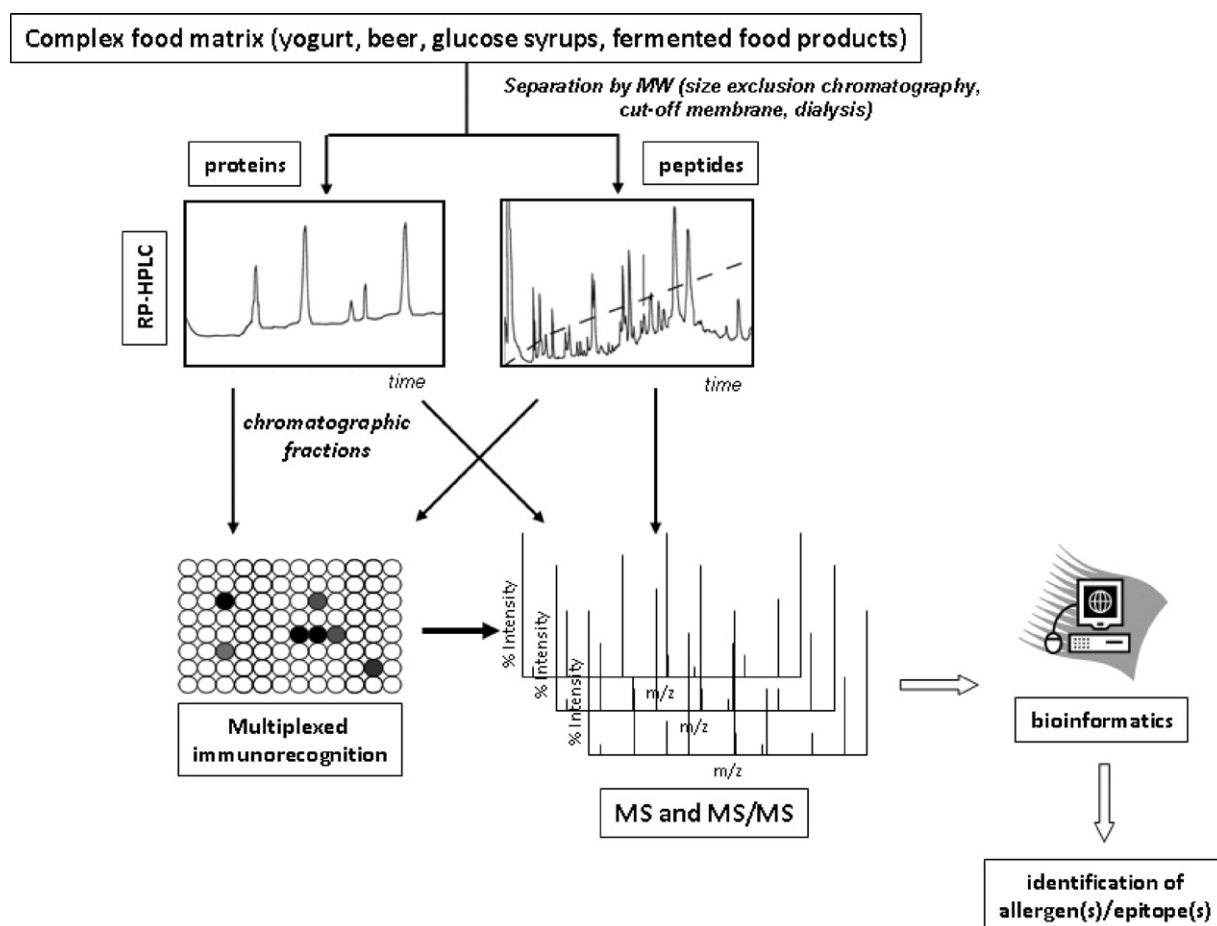


**Fig. 1.** MS-based discovery/detection of allergens or epitopes in complex food matrix containing immunogenic proteins partially hydrolyzed (e.g. yogurt, dairy and fermented muscle proteins, soybean-based products, beer, and glucose syrups). Intact proteins or peptides can be fractionated and individually separated by RP-HPLC. Chromatographic fractions are assayed for their IgE-binding properties and identified by MS and MS/MS. This approach can be effective to discover allergens and/or epitope encrypted in protein moieties.

protein, though retaining their allergenic potential. Linear epitopes tend to be underestimated or to escape the most commonly utilized sandwich ELISA-based tests. Peptides in fermented dairy products derived from milk proteins [59,60] or fragments of prolamins in glucose syrups [87] and beverages, such as beer, are examples of this last situation [88]. A possible flow-chart for the identification/detection of allergens and epitopes in complex food matrices in which proteins are partially hydrolyzed is illustrated in Fig. 1.

Potentialities of MS have been recently compared to DNA and ELISA approaches for hen egg proteins [89]. An exhaustive overview of the existing methods for the detection of allergens in food products, which also includes the detailed analysis of advantages and pitfalls of the single strategies, has recently been published [90]. The urgency of structural as well as confirmatory strategies based on MS-based approaches to provide unambiguous identification and quantification of allergens arises from the limitations of the classically used methods.

## 2.2. Mass spectrometry for the identification and characterization of allergens

All of the above MS instrumental configurations have been largely utilized to characterize and monitor food allergens. Many excellent articles, even recently, have in-depth reviewed the characterization and quantification of food allergens by MS and proteomic analysis [90–95]. Here we sketch selected highlights on recent exemplificative MS-based strategies already applied or

potentially amenable to food allergen identification and monitoring.

MS-experiments can be addressed either to measure the molecular mass of a protein/derived-peptides (MS1) or to determine additional structural details such as the amino acid sequence and post-translational events also including process-induced modifications (MS/MS or MS<sup>n</sup>).

In general foods are highly complex matrices and protein pre-fractionation is required prior to MS analysis [96].

To date, two dimensional electrophoresis (2DE) has proven to be a reliable and efficient method to separate one-step a large number of proteins. By this technique proteins are usually separated according to their pI (isoelectric focusing, IEF) and subsequently to their molecular weight (SDS-PAGE). 2DE is extremely powerful and can achieve simultaneously the separation of several thousand different protein spots. Stains such as Coomassie blue, silver, and SYPRO ruby can be employed to visualize the proteins [97]. Once stained, protein spots excised from the gel are *in situ* digested with a protease (usually trypsin) and identified by MS or MS/MS experiments. The first approach, typically performed by MALDI-TOF, allows the protein identification by peptide mass finger printing (PMF), by which the set of masses is compared to the theoretically expected tryptic peptide masses for each entry in the database. On the other hand, MS/MS analysis (e.g. MALDI-TOF/TOF or ESI-MS/MS) determines structural information related to the sequence of peptides, rather than only their mass, making the search highly specific and discriminating [98]. To identify allergens, electrophoresis-based

## DISCOVERY-DRIVEN ALLERGEN IDENTIFICATION

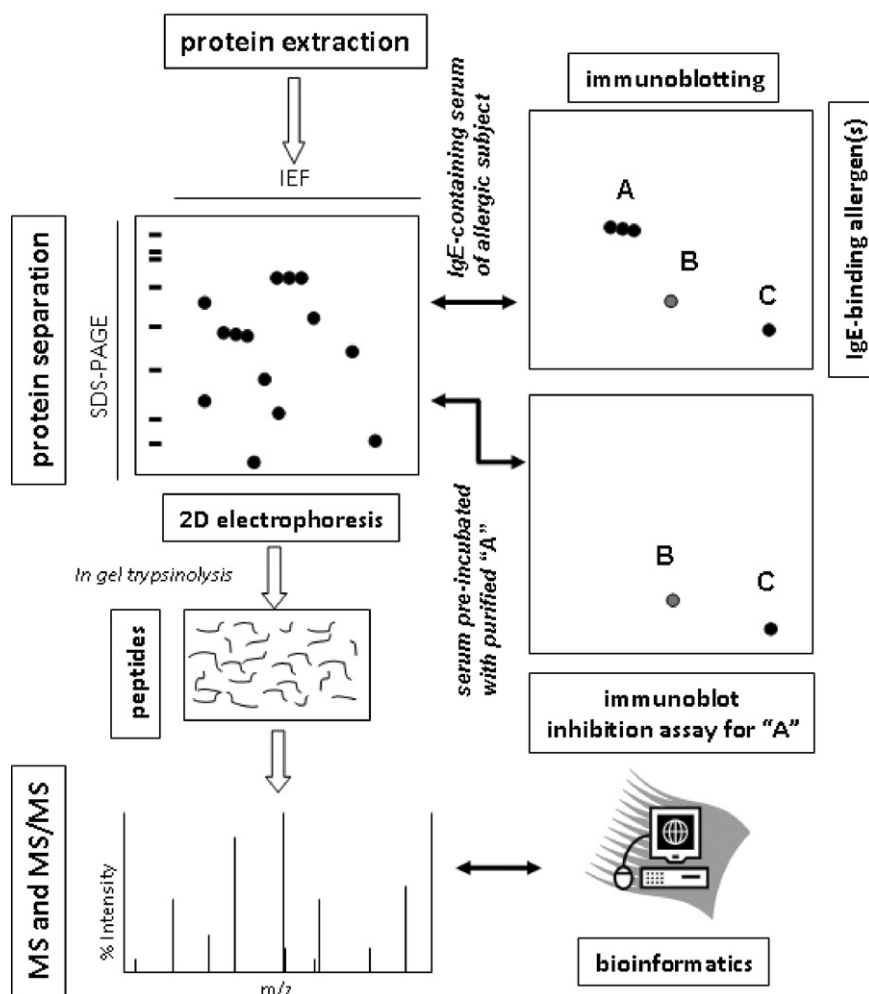


**Fig. 2.** Typical 2DE-based approach for the discovery-driven allergen identification. Protein extracts from food matrix are separated by 2-DE and immunoblotted utilizing IgE-containing serum of an allergic subjects. Immune-reactive proteins are visualized with a secondary anti-human-IgE antibody, conjugated with chromogenic or photogenic enzymes. Confirmation of the antigenic properties can be provided by specific inhibition assays in which sera pre-incubated with the purified suspected allergen are utilized in the immunoblot analysis.

separations, in either one- or two-dimensional version, coupled to MS analysis are usually integrated with antibody labelling in blotting setup, using sera from allergic patients as a source of specific IgE. Allergen–IgE complex is usually detected using a conjugated anti-human IgE as the secondary antibody. Alternatively, once the allergen is known, its occurrence in the 2DE map can be monitored in any food matrix using allergen specific antibodies, preferably the monoclonal ones [93,99]. The immunoblot step assigns the allergen(s) of interest on a reference electrophoretic map, and an additional gel is processed to perform the identification and characterization of the immune-reactive protein(s). The typical electrophoresis-based flow-chart of allergen discovery is schematized in Fig. 2. Validation of the IgE-binding properties is often provided by immunoblot-inhibition assays which are carried out utilizing sera pre-incubated with purified candidate allergens (Fig. 2). The entire analytical protocol can also be applied to establish cross-reactivity of allergens, as illustrated by the assessment of cross-reactivity of almond with walnut and hazelnut allergens [100].

From the pioneer study of Sander et al. on wheat allergens [101], 2DE-Western blotting strategies coupled to MS have been successful in the identification of allergens from some of the most common injuring foods [102]. For instance, all the major

milk proteins resulted as potential allergens, because in different percentage immune-reactive toward IgE in sera of allergic patients [103]. The prevalence of cow's milk allergens was assessed to be  $\alpha$ s2-casein >  $\alpha$ s1-casein >  $\beta$ -casein >  $\kappa$ -casein >  $\beta$ LG > serum albumin > IgG-heavy chain > lactoferrin. With the same protocol, around twenty proteins, such as  $\alpha$ -amylase inhibitor,  $\beta$ -amylase, profilin, serpin and  $\beta$ -D-glucan exohydrolase were identified as potential wheat allergens [45,101,104]. Proteins most frequently recognized by IgE of allergic patients, are usually identified using several MS platforms (MALDI-TOF, ESI-Q/TOF, ion-trap, LC-MS/MS IT). An 11S albumin was identified by PMF as a major hazelnut food allergen [105]. With PMF and MS/MS *de novo* sequencing, following electrophoretic isolation, several proteins of maize (*Zea mays*) were discovered to be allergenic [106]. These included nsLTP and endochitinases, which are cross-reactive with grape, 14-kDa  $\alpha$ -amylase inhibitor and, interestingly, two proteins of the zein fraction. Thus, maize- as well as wheat-related allergies are confirmed to exhibit a very complex pattern of antibody recognition [20,107].

Combining fluorescent 2DE with Western blotting and MS analysis, the major raw peanut allergens (Ara h 1, Ara h 2, Ara h 3/4) were identified [108]. All the three subunits of  $\beta$ -conglycinin were recognized by IgE of patients allergic to soybean [109]. In contrast to other reports [49], in this latter study deglycosylation was found not

to affect the affinity of  $\beta$ -conglycinin to IgE, suggesting that glycosylation may be not involved in antigenicity. Similarly, the different investigations carried out for establishing the contribution of glycosylation to allergenicity of egg proteins have provided conflicting results [110]. These controversies are exemplificative of the complexity of the matter, and further studies are required to accurately characterize glycans of  $\beta$ -conglycinin and to establish their involvement in antigenicity. Pen m 2, a muscle arginine kinase, in addition to the heat-stable tropomyosin (Pen a 1), was identified by 2DE combined with immunoblot labelling and PMF as a shrimp allergen also capable of inducing skin reactions in sensitized patients [111]. However, a large number of allergen identification examples also exists for less commonly offending foods such as peach [112], barley [44], lettuce [113], chestnut [114], banana [115], and tomato [116]. Identification of allergens is less straightforward when the genome of parent organism is not fully sequenced. For example, it was not possible to completely characterize very rare allergens from coconut (*Cocos nucifera*) and from sea urchin roe a popular shell fish of Korean and Japanese cuisine. However, MALDI-MS mapping and homology comparison showed they are a 29-kDa 7S-related storage protein [117] and a 152 kDa major yolk protein, respectively [118]. *De novo* MS/MS sequencing is very helpful in identifying allergens in similar cases. High-throughput 2DE-based proteomic technologies are powerful tools for the non-targeted comparisons of whole proteome expression of genetically modified (GM) plants and their wild-type counterparts. Therefore, proteomic based studies are the most promising for a routine use in the allergenicity assessment of GM organisms [119].

Despite the high resolution power of 2DE, there are still a number of limitations such as time and labor requirements, low reproducibility, low sensitivity, low dynamic range for quantification, and poor (if any) detection of very hydrophobic and alkaline proteins [120]. In addition, the harsh denaturing electrophoresis conditions may alter the antigenic properties of proteins, thus introducing the possibility of false positives or negatives [121]. For this reason native 2DE electrophoresis has been proposed for allergen discovery and it has been applied to the allergens of hazelnut [122].

An alternative to electrophoretic methods is the liquid-phase separation (LC) coupled to MS analysis to identify proteins directly from complex mixtures. For instance, maize nsLTP has been very recently detected in 14 varieties and even quantified by LC-UV-MS analysis [123]. However, though successful in some selected experiments [124], the identification of intact protein allergens is affected by low sensitivity and is limited to low-complexity mixtures.

The most effective LC/MS-based strategy is referred to as “shotgun” proteomics. In this case, the whole protein extract is trypsinized generating a complex mixture of thousand peptides which is subsequently separated by LC prior to MS/MS sequencing [66,125]. Proteins are inferred from identified peptides utilizing powerful database searching programs. Although proteolysis greatly increases the complexity of the analytical mixture, the multiplication of the protein-derived moieties enhances the probability to detect and confidently identify at least one constituent peptide. The chromatographic co-elution of multiple peptides is particularly detrimental to MS analysis because of mutual ion suppression effects, so that high-efficiency separation techniques are needed prior to MS. LC-Q/TOF MS/MS has been used, for instance, in the detection and identification of casein deriving peptides. In particular two peptides of  $\alpha$ s1-casein were selected as candidate markers to detect milk allergens in cookies [126] and four peptides arising from the tryptic digestion of  $\alpha$ - and  $\beta$ -caseins were monitored to assess the presence of potentially allergenic caseinates added to white wine for fining purposes [127].

A similar strategy has been applied to monitor Ara h 1, Ara h 2, Ara h 3 as the major allergens in processed peanut. Some specific tryptic peptides were selected as markers for monitoring selected peanut allergens in food products [128]. Taking advantage of a high resolution/wide dynamic range LTQ-Orbitrap instrument, coupled with 1D nanoflow RP-HPLC, 158 proteins have been recently identified in hen's egg white by shotgun proteomics, also including several known egg allergens [129]. In the shotgun approach, the information about the molecular weight of the intact proteins is lost. However, a careful analysis of MS/MS spectra can turn to be effective in discriminating isoallergens and cross-reactive immunogenic proteins. Thus, the high specificity of LC-MS/MS analysis resulted decisive for differentiating celery (*Apium graveolens*) allergens from IgE cross-reactive proteins of carrot and potato [130].

To reduce the complexity of sample, multidimensional chromatography coupled to mass spectrometry (2D LC-MS or MudPIT, multidimensional protein identification technology) can be employed. This approach provides a higher separation power than 1D chromatography, in addition to an extended measured dynamic concentration range to analyze complex proteomic samples. Various LC methods in the first dimension prior to RP-HPLC in second dimension have been proposed. A preliminary ion-exchange (IEX) separation mode has become the most popular implementation [131,132]. It has been demonstrated that also a high-pH RP-HPLC as the first dimension can offer the advantage of an easier sample handling with a better separation efficiency [133]. At the present, multi-dimensional chromatography-shotgun techniques are largely utilized in proteomics. Nevertheless, up to now only a few articles report on allergen identification by this approach. Among the methods related to these, a multiplexed chromatography consisting of sequential HILIC and TiO<sub>2</sub> enrichment and a chemical-based (hydrazide capture) strategy prior to nanoflow RP-HPLC-ESI-LTQ-Orbitrap MS/MS have been addressed to identify glycoproteins of wine potentially cross-reactive with plant allergens [134]. With a slightly different approach, consisting of a preliminary chromatography step at the protein level, followed by RP-HPLC/MS/MS analysis at the peptide level, allergenic soybean proteins were detected in meat products [135]. Similarly, Ara h 3/4 peanut allergens were identified in breakfast cereals by a 2D chromatography method consisting of an affinity capture of proteins on immuno-beads followed by tryptic digestion and RP-HPLC-ESI-IT-MS/MS [136].

Due to a much higher MS sensitivity in the detection of peptides than proteins, the peptide-centric methodologies [137] enable a proteome access much deeper than that achieved using whole protein-targeting techniques. The shotgun proteomic approach also expands the dynamic range of the species detectable. This could be not a particular advantage for the characterization of allergens, because these are generally proteins abundant in the food matrix. However, this represents the most effective strategy to monitor “hidden” allergens or cross-contaminating species that occur in traces.

A decisive support to all of the methods applied in proteomic analysis is provided by bioinformatic tools for the interpretation of a huge amount of MS/MS spectra produced. The identifications are usually administered automatically *via* powerful, commercially accessible software programs such as Mascot and SEQUEST in combination with continually updated public databases such as those managed by the NCBI and SwissProt [138]. With the aid of more specific software tools, the tissue localization of peach nsLTP (Pru p 3) was recently assessed by MALDI-MS imaging [139].

The large range of analytical procedures in which MS has a pivotal role requires the extensive knowledge of potentiality and pitfalls of each approach and a careful case-by-case analysis as a function of the needed analytical response. Thus, MS

enables analytical access to many information levels, starting from the simple targeted qualitative or quantitative monitoring of a peptide/protein allergen, up to the “profiling” of the entire repertoire of major histocompatibility complex (MHC)-binding peptides [140], or to the conformational characterization and the hydrogen/deuterium exchange-based epitope mapping [141]. Obviously, enhanced instrumental performances and gradually increasing skills of the operators are required by more sophisticated experiments.

In addition to provide advances in the structural knowledge of allergens, as reviewed as early as more than ten years ago [142] and later on in the last decade [143,144], MS has introduced important contributions to either the epitope mapping [145] or the understanding of the mechanisms of the immune reaction. The proteomic tools, in particular MS, have been decisive to the development of modern structural immunology, contributing to the characterization of trigger molecules, effector cells and interacting systems involved in the immune responses, either related or not to FA.

### 2.3. Methods for quantification of allergens

Numerous MS-based methodologies for both relative and absolute quantification are widely used in proteomic studies, but all referable to two main approaches: in the first one, the analyte and its reference standard are the intact proteins; in the second one, the analyte is a peptide arising from the protein digestion by proteolytic enzymes.

An established strategy for relative protein quantification is based on 2DE, which highlights changes in protein expression based on densitometric staining intensity by comparison of gels from different sample states. However, gel-to-gel variability makes difficult the comparison and relative quantification of protein spots from different 2DE experiments. The development of difference gel electrophoresis (DIGE), which allows parallel comparison of multiple protein samples within the same gel, has significantly overcome this limitation [146,147]. Proteins are labelled with three spectrally distinct, charge- and mass-matched fluorescent dyes, mixed, separated simultaneously on the same 2D gel, and their individual proteins are differentially visualized via fluorescence at different wavelengths. Reproducibility of the dye technology enables measurement of small differences in protein abundances with sensitivity comparable to silver staining. 2DE DIGE was used to compare the amount of the Fra a 1 allergen between different varieties of strawberry [147]. Cultivar differential expression of specific allergens was also assessed in ten varieties of rice [148] and two varieties of peanut [149]. Studies like these are aimed to select cultivars with the lower level of potential allergenic proteins and can enable the quantification of allergens in GM plants compared to their wild-type counterparts.

Alternative robust allergen quantification is based on LC–MS analysis. MS is not intrinsically a quantitative analytical technique, because the ionization efficiency of a protein/peptides is affected by a wide range of physical–chemical properties (size, charge, hydrophobicity). For accurate quantification the protein/peptide amount must be referred to that of a suitable standard.

In the direct quantification of intact proteins, according to a “top-down” approach, the intensity of analyte multi-charged ions is compared with that of internal or external standards. To quantify cow’s milk allergens in mixed fruit-juice samples [123] and whey drink [150], several multiple-charged ions of whey proteins were simultaneously monitored. In specific cases an external calibration was carried out. With a similar approach, but utilizing  $\beta$ LG from species different from bovine as an internal standard, Czerwenka et al. quantified cow  $\beta$ LG in different milk-derived products [151].

Nevertheless, the sensitivity of “top-down” mass spectrometry methods is limited by the wide distribution of protein charge states.

Thus, “bottom-up” strategies such as the “shotgun” approaches are to be preferred for the detection and quantification of allergens by MS.

The use of “bottom-up” methodologies for quantitative analysis in proteomics is steadily increasing. Quantitative methodologies for large-scale quantification such as SILAC, ICAT, iTRAQ or label-free approaches are capable of exploring the dynamics of whole proteomes through the relative quantification among multiplexed samples [152,153]. However, the above strategies are most suitable when the objective is to determine differentially expressed proteins. For this reason, as far we know, these quantitative methods have not been addressed yet to the quantification of food allergens. On the other hand, the urgent issue for food safety purposes is the absolute quantification of allergens.

The Selected Reaction Monitoring (SRM) MS approaches have emerged as tools for the precise quantitative determination of targeted proteins in complex samples [154]. The most common applications of SRM in proteomics rely on the principles of stable isotope dilution (SID) methods [155]. Conceptually, SRM and SID are not new techniques, as they were introduced as early as in the late 1970s for the quantitative analysis of small molecules. Their combination in proteomics is finding exponentially increasing application so that they can be now considered the “golden standard” for absolute quantification. Multiple Reaction Monitoring (MRM) represents an improvement of SRM in terms of selectivity and sensitivity. Either technique has been broadly adapted to QQQ-based instruments (QQQ, QQQ-IT, QQQ-TOF), in which the first quadrupole (Q1) mass analyzer is set to filter only the pre-selected peptide precursor ion(s) of choice while other ions are excluded. The second quadrupole (Q2) is a collision cell that fragments the precursor by CID (collision-induced dissociation) and transmits fragment ions to the third quadrupole (Q3). Q3, in turn, transmits to the detector only fragment ion(s) with pre-set  $m/z$ , whereas all the remaining fragments are excluded [67].

The targeted monitoring of mass and transitions of selected “proteotypic peptides”, overcomes the bias due to the presence of a large number of dominant components. The MS-based absolute determination of allergens requires the use of internal reference peptides. For instance, making use of leucine-enkephalin as the internal standard, peanut Ara h 2 and Ara h 3/4 were monitored by LC–SRM MS in a number of food products [156].

However, recently, it has been demonstrated that for an accurate Absolute QUAntification (AQUA) using MRM, the use of specifically designed internal standards is needed [157]. AQUA peptides are synthetic copies of “proteotypic” peptides, which are isotopically labelled to one or more amino acid positions in order to shift molecular mass, without appreciably influencing retention time and ionization properties. Hydrolyzed protein samples are spiked with known amounts of standard AQUA peptides and both native and surrogate peptides are monitored by LC–MS operating in SRM or MRM mode. Absolute amount of peptide(s) is determined by the ratio of the ion intensities of AQUA peptide and its native cognate (Fig. 3). Applying this strategy Houston et al. [157] quantified eight allergens in commercial soybean cultivars and evaluated the differential expression of four allergens (glycinin G3, glycinin G4,  $\beta$ -conglycinin and Kunitz trypsin inhibitor 1) across 20 varieties. The AQUA MRM strategy was compared with a method based on the spectral count referred to BSA as the internal standard, significantly reducing variance of quantification. With an excellent “discovery-driven”, arginine kinases were identified as allergens in snow crab (*Chionoecetes opilio*) by SDS-PAGE, immunoblot staining and MS/MS *de novo* sequencing combined with homology based search, and quantified by MRM MS using isotope labelled standard peptides [158]. This latter study also demonstrated the validity of the use of proteotypic peptides as analytical surrogates to assess the presence of allergens in both crude extract and in air sam-

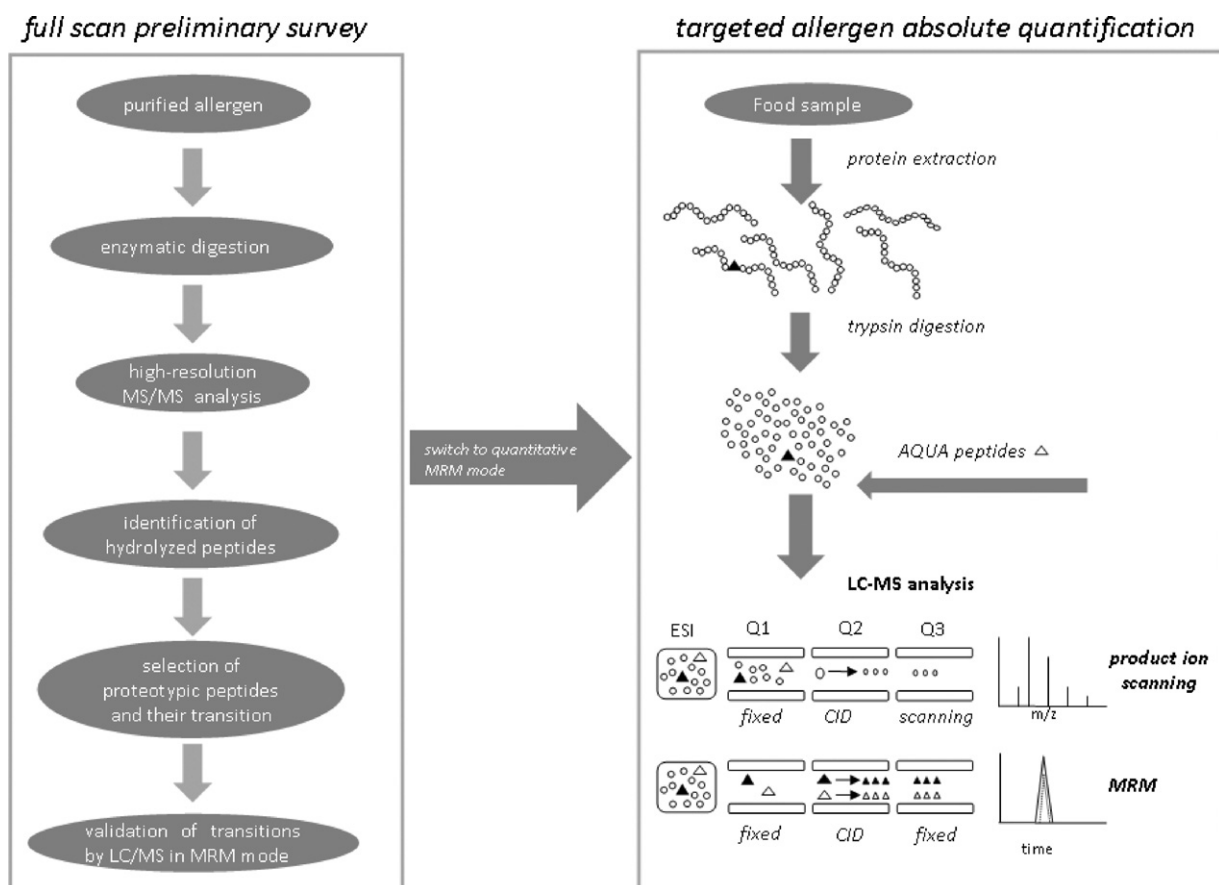


**Fig. 3.** General procedure of the MRM MS assays, conducted on triple quadrupole instruments, for protein absolute quantification. Proteotypic peptides are selected with a preliminary full scan MS survey (left panel). For quantitative analysis (right panel), whole protein extracts are trypsinized and the peptide mixture (each peptide is represented by the symbol ○) is spiked with standard AQUA peptides (Δ). Proteotypic peptides (▲), selected as analytical probes of the target protein(s), are quantified by comparing the ionic intensities. The strategy is increasingly applied to the quantification of several food allergens.

ple. Airborne tropomyosin of snow crab, a known crustacean food allergen, had already been identified by the same authors as one of the main triggers of crab asthma in workers and quantified in the work environment with an AQUA MRM approach [159]. With a similar approach, including the combined use of immunochemical labelling, 2D protein chromatography and LC–MS/MS, legumin and vicilin were identified as tomato seed allergens and quantified by SRM in commercial tomato sauce [116]. The software assisted structural modelling suggested high homology between tomato and walnut epitopes. Moreover, vicilin from peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes [160]. This emphasizes the strict homology of several allergenic proteins, like the nsLTP that has been defined a “pan-allergen”, among plants. Vicilin isoforms were characterized and quantified with a stable isotope label-free method in lupin (*Lupinus albus*), using an innovative nanoHPLC–chip separation coupled with IT–MS/MS [161]. As above [158], also in this case the internal peptide standards were tryptic fragments of BSA.

An additional advantage of the SRM techniques is the possibility to be multiplexed, as they permit one-step monitoring of more than a single allergen. Very recently LC–triple quadrupole MS operating in MRM mode has been demonstrated effective in simultaneously detecting allergens from seven different potentially allergenic matrices such as milk, egg, soy, hazelnut, peanut, walnut and almond. Detection limits covered the 10–1000 µg/g range. A previous screening with reference allergens was required to establish the most suitable peptides to monitor [162].

An alternative to SRM for less complex samples, when an extremely high specificity is not required, could be the single ion monitoring (SIM) which is commonly performed on a single quadrupole. During this process, the MS analysis time is focused only on analytes with a specific mass corresponding to the ion of interest, while all others are excluded. SIM analysis often requires high resolution separations in order to minimize interference from other species.

The entire strategy of absolute quantification has three major critical points that should be carefully evaluated for an accurate result: (i) allergen extraction; (ii) proteolysis; (iii) proper selection of “proteotypic peptides”.

Sample preparation is a key step for a reliable allergen quantification. Obviously, if the allergen recovery is not complete, quantification is underestimated. Protein extraction from “recalcitrant” plant tissues is particularly challenging because of the occurrence of interfering substances [102,163,164]. Therefore, extraction protocols have to be opportunely adapted depending on the nature of the starting food matrix. In the selection of the extraction buffer the effects of food processing must be taken into account. Thermal treatments such as roasting, cooking, boiling as well as sanitization processes have a tremendous impact on the protein stability and structure and, thus, on the extraction efficiency [121]. Elevated roasting temperatures in food processing were found to drastically reduce protein extraction yields of oil and dry roasted peanuts by 50–75% and 75–80%, respectively, compared with the raw material [165]. In this latter study several extraction buffers contained in commercial ELISA kits were tested and compared.

Although, five nut allergens were successfully detected in breakfast cereals and biscuits using a bicarbonate buffer extraction [166], we believe that for an accurate quantification detergents and chaotropes are required in order to realize denaturing extraction conditions. The LC–MS/MS LOD of peanut Ara h 1 in dark chocolate was significantly lowered if a pre-extraction digestion was performed rather than extracting proteins first and then digesting it [167]. This should be ascribed to an incomplete protein extraction by bicarbonate buffer. When combined with MRM, the LOD of a previously established signature peptide of Ara h 1 [168], decreased 5-fold. Trypsin is the most utilized proteolytic enzyme for MS-based detection and quantification because it often guarantees a complete digestion, a good peptide ionizability and reproducibility.

It has to be considered that many allergens which occur in a high number of plant species, such as nsLTP, are proteins extremely stable and resistant to proteolysis [169]. In addition, thermal processes can modify lysine residues *via* Maillard reactions, thus affecting the proteolytic pattern. For instance, the barley nsLTPs undergo heavy glycation during malting and brewing [88,170]. Also Ara h 2 [171], and  $\beta$ LG [172] are known to undergo non enzymatic glycation as a consequence of thermal treatments. Food matrix is also crucial for allergen extraction; interferences by non-protein compounds, such as tannins, should be carefully prevented.

The choice of proteotypic peptides and ion transitions is crucial and, although predictive software has been developed [173,174], they need to be defined experimentally through a prior full-scan LC/MS analysis of the proteolyzed allergen or protein extract of the original food matrix (hypothesis-driven targeted analysis). A suitable proteotypic peptide should exhibit the following properties: (i) high efficiency to ionize and to fragment; (ii) uniqueness for the target protein(s); (iii) absence of amino acid residues susceptible of chemical modifications such as Cys (reactive thiol group), Met (oxidable), Asn and Gln (deamidation), and N-terminal Gln (pyroglutamate); (iv) absence of post-translational modifications and of sequences that are prone to missed proteolytic cleavage (for instance sequences like KRXX or XXRK in the case of trypsin). The possible occurrence of isoallergens should be considered by selecting distinctive proteotypic peptides if allergens are to be individually quantified.

A further critical step in large-scale SRM is the control of the conditions and the reproducibility of the HPLC separations. To overcome possible pitfalls in this sense and to assure reproducibility the efficiency of stationary phase, the high quality of eluents and a strict temperature control are mandatory. These precautions also allow to narrow down the time window used to monitor the transitions of a specific peptide, in order that the LC–MS run can be divided into a higher number of time segments, thus dramatically increasing the number of peptides analyzed during the entire LC–MS run. In this manner sensitivity can be increased at sub-femtomole levels enabling the detection and quantification of low ppb of allergens [175].

### 3. Perspectives

#### 3.1. Standardization of allergen preparations

Although the search for clinically relevant allergens has drastically progressed, the identification and characterization of allergens still require extensive efforts in addition to a large amount of standardized starting material.

A relatively low number of highly purified or recombinant standard allergens are currently available to carry out investigations aimed to progress in the diagnosis and treatment of FA. For example, serological methods are being developed to improve the quality of diagnosis and to reduce the need for food challenge tests. Puri-

fied and recombinant allergens are needed as reference materials for the calibration and standardization of methods among different laboratories and operators, as well as for risk assessment in the food industry to detect contamination. Recently, a panel of 46 food allergens [98,176,177] has been purified from plant and animal sources in the frame of an EU-funded research project. Pure allergens were obtained from either the food sources or as recombinant forms. Taking into account the limitations of antibody-based tests, checking of allergens and of their isoforms by MS-based quantitative methods has been claimed as a decisive step towards a comprehensive characterization and a safe use of complex allergen preparations [178]. Although not strictly applied to food allergens, in a couple of recent and elegant studies, MS/MS analysis and AQUA MRM quantification were confirmed to be effective strategies for the standardization of allergoids and pollen allergens at the low-femtomole level [179,180]. In a very recent interesting application, data obtained from targeted SRM MS absolute quantification have been utilized to calibrate 2DE in the separation of microbial complex protein systems [181]. We expect that similar standardization protocols will be extended to several food allergens in a near future.

#### 3.2. Emerging technologies for allergen identification and detection

In addition to the MS-based methods, several specific applications, which are case-by-case designed, are finding use in FA research. For instance, to improve the specificity in allergen detection, various modifications of mono- and two-dimensional electrophoresis have been introduced. Among them acetic acid/urea-polyacrylamide gel electrophoresis (AU-PAGE) revealed low-molecular weight allergens that bind to IgE of allergic subjects [182,183]. 2-DE in immobilized pH gradient (IPG) followed by AU-PAGE and immunodetection with patient sera coupled to MS was decisive to reveal allergenicity of  $\alpha$ -amylase/trypsin inhibitors and nsLTP [183]. The system developed might be a useful standard protocol for the separation of low-molecular weight allergens or allergen fragments. The IEF and chromatofocusing separations coupled to MS identification were successful in characterizing two isoforms of allergenic 2S albumin from Brazil nuts (*Bertholletia excelsa*) [184].

However, the most promising emerging technologies relevant to the FA issues include immunochemical microarrays for molecular diagnosis [185–187] and biosensors consisting in integrated receptor-transducer devices [188,189]. Further approaches based on the surface plasmon resonance [190] for improved allergen detection, as well as reliable commercial ELISA kits for customized use [191] are being developed.

#### 3.3. Validation of proteomic methods and their transfer to pre-clinical scale

The main challenge of the actual research efforts is the precise evaluation of both IgE and T-cell epitopes. In IgE-mediated allergies the differentiation of antigen-specific B-cell is characterized by clonal expansion and by the progressive affinity enhancement following repeated expositions to the offending agent. Both these events can lead to develop IgE with a high heterogeneity degree in terms of concentration, diversity and affinity. More in detail, the IgE response to specific epitopes is believed to be indicative of persistent FA [192]. On the contrary, the IgE response to other specific epitopes is typical of transient FA and has a favourable predictive value towards the acquisition of oral tolerance [193,194]. Several studies have been aimed to the identification of dominant antigenic determinants and to their correlation to clinical symptoms and prognostic factors. Recently, “peptide arrays” have been developed as improved multiplexed diagnostic tools to establish the pattern

of individual responses to dominant IgE epitopes [195–199]. Under these aspects, cow's milk allergy is by far the most investigated disorder. Nevertheless, allergen-based microarrays have also been recently applied to an extensive cross-sectional survey of the IgE reactivity against 75 different allergens [200].

Peptide microarrays are to be introduced in the practice also for cell activation tests, in the diagnosis of cell-mediated allergies [185]. However, the majority of the studies aimed to the identification of epitopes have been carried out *in vitro* utilizing synthetic peptide libraries deduced from proteins and they not always exhibit consistence between IgE-binding properties and DBPCFC oral tests [201]. The main outcome of these studies was the elevated variability of the individual response which in part obstacles a general definition of specific epitopes as the allergenic determinants.

It is expected that the search for injuring sequences will be refined in a near future, because it will be restricted only to those immuno-dominant protein moieties which are effectively produced during digestion and are able to translocate the gut barrier to reach the mucosal immune system. Several models of digestion/adsorption of food proteins are under evaluation in order to establish reliable predictive criteria [202]. Stability to pepsin digestion has been included in the Food and Agriculture Organization/World Health Organization decision-tree to assess safety of foods produced through agricultural biotechnology [203]. For instance, peptides arising from an allergenic 32-kDa endochitinase (Prs a 1) upon simulated gastric digestion were identified by MALDI-MS and assayed for their binding properties to IgE from individuals with a clinical history of latex-fruit allergy syndrome [204]. This model study allowed to establish the consistence of immune-reactive peptides both *in vitro* and *in vivo*. Resistance to proteases throughout the GIT is a prerequisite for milk-derived peptides to exert allergic effects. In the case of milk proteins, survival of potentially allergenic epitopes above mentioned [38,103] still awaits structural confirmation. Comparative studies to direct the use of defined allergens in serological and biological assays and their standardization are therefore required. Very recently, a multi-step static model has been developed to *in vitro* simulate the complete digestion of the bovine milk proteins [35]. The experimental set-up involved the sequential use of: (i) pepsin, (ii) pancreatic proteases, and (iii) extracts of human intestinal brush border membranes, in simulated gastric, duodenal and jejunal environments, respectively. This allowed the MS identification of peptides which could have implications for the immunogenic action of  $\beta$ LG in the insurgence of cow's milk allergy. By a similar *in vitro* sequential gastro-duodenal digestion, the potential allergenic domains of 2S albumin of sesame seeds [205] and of peach LTP (Pru p 3) [206] were characterized. The *in vitro* digestion has been coupled with model Caco-2 cell monolayers as models of the gut epithelium to demonstrate the ability of peptides surviving GI digestion of either milk or soy proteins (Picariello et al., unpublished results) to translocate across intestinal barrier. It is envisaged that MS will play an irreplaceable role to trace the metabolic fate of potentially immunogenic sequence and to identify protein regions stable to digestion. The MS identification of peptides arising from simulated digestion is not trivial because it is complicated by the non-tryptic nature of peptides and by the lack of any proteolytic specificity. This requires careful evaluation of the most appropriate analytical strategy in addition to refined strategies of *de novo* sequencing, especially for proteins from less common food matrices.

MS will support analytically the development of peptide microarray which employ the actual immune-dominant sequences for a pre-clinical or clinical use. The objectives are to establish correlation between the patterns of peptide recognition and clinical aspects of allergy, in order to improve the diagnostic methodology, to establish valuable prognostic criteria, to define

adequate therapeutic approaches. No treatment is at present available to completely resolve or provide long-term remission from FA. The research for therapy is mainly focused on the introduction of anti-IgE antibodies and specific oral tolerance induction. Several protocols, in particular, are being investigated to induce allergen-specific oral tolerance or aimed to modulate the individual immunological response [207].

Immunotherapy appears to be an attractive approach, even though the risk of anaphylaxis should be taken into consideration. To this purpose engineered proteins have been designed: epitopes triggering anaphylaxis have been removed within these proteins, while preserving the epitopes inducing tolerance [208–210]. It appears clear that to successfully pursue similar strategies, the precise identification of epitopes is necessary. It is expected that such approaches will be extended to an increasing number of food sources, where MS will play a key role for characterizing the novel protein entities. The accurate characterization of the offending sequences could also be the starting point to develop less allergenic food products by use of enzymatic, microbiological and technological processes to effectively remove allergens [211–214].

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