00071. Proteomics in Food safety to the **Comprehensive Foodomics**.

Proteomics applied to food allergen research

Elisabetta De Angelis, Rosa Pilolli, Rocco Guagnano and Linda Monaci*

CNR-ISPA, National Research Council of Italy Institute of Science of Food Production, Via Amendola 122/O 70126 Bari

*linda.monaci@ispa.cnr.it

Abstract

Proteomics has been widely applied to the food safety field and recently exploited for food allergens analysis. Different workflows for allergen identification, characterization, determination of their post-translational modification, as well as their quantification have evolved over the years. In the past decade, the advent of modern mass spectrometry technologies also encompassing high-resolution mass analyzers, along with the improvement of bioinformatic tools and genomic data banks, widened the application fields enabling to expand the knowledges of food allergens from a biochemical, structural and clinical point of view. The typical routes are based on top-down and bottom-up proteomics. Whether top down proteomics is mostly used for allergen identification and for highlighting some post-translational or chemical modifications occurring along the protein moiety, the bottom-up route is instead commonly used for allergen quantification. An overview of the proteomics based strategy in food allergen analysis is provided in this chapter along with the discussion of the advancements done in this field and the bottlenecks.

Keywords:

Food safety, food allergens, allergen discovery, allergen quantification, bottom-up, top-down

1. Introduction

Proteomics typically refers to a large-scale snapshot of proteins contained in a specific biological system. It has been widely exploited also in food research to increase safety of food products. Food allergy (FA) is a food safety issue with an increasing prevalence worldwide and steadily growing number of allergenic foods identified. Several foods are implicated in triggering allergic reactions, however it has been calculated that a total of eight big ingredients account for most of the allergic reactions, i.e. milk, egg, wheat, soy, peanuts, tree nuts (e.g., hazelnut, walnuts, pecans, almonds, and cashews), fish, and shellfish (Leung et al., 2014). Different legislative frames have been put in place in several countries across the globe basing on what considered priority allergens in the national systems. Among them Australia/New Zealand and European Union (EU) resulted to be the most restrictive in labeling requirements, demanding the mandatory labeling of a total of 14 allergenic ingredients (European Commission, 2011). Beside the voluntary inclusion of allergenic ingredient in foods for manufacturing purposes, a high risk of being become in contact with an allergen even where they are not expected to be it does exist. This is due to a cross-contamination phenomena that might occur during transportation, storage or on the same production line (Dzwolak et al., 2017, Spanjersberg et al., 2010). On this regard, it was found that the unintended presence of milk (29%) was the primary cause of allergen recalls followed by cereal containing gluten (16%), soy beans (9%), tree nuts (8%, including hazelnut and almond), egg (8%), and peanut (4%). Cereals and bakery products (27%) were the most frequently reported in allergen-recalled products followed by ready to eat meals, confectionary and "soups, broths, sauces and condiments" occurring in 21%, 9% and 7%, respectively. A further critical analysis of the Food Standard Agency (FSA) Food alerts database, from 2011 until 2014, indicated milk (24%) as the primary cause of allergen recalls in the UK followed by cereal containing gluten (19%), tree nuts (8%), peanut (7%) and egg (5%) (Bucchini et al, 2016).

Tools which are able to effectively determine the levels of allergenic food protein(s) are essential for the effective implementation of food allergen quality assurance across the supply chain. These are needed to monitor either factory cleaning, ingredients or finished products, the increasing use of risk assessment methods driving a requirement for analytical readouts to be presented in mg total allergenic protein per Kg of food, rather than on a commodity basis. The lack of metrological traceable analytical results regarding the presence and quantity of allergenic foods means ELISA and PCR-based methods both fall short of the performance expected of reference methods, which are used, for example, in legal cases of dispute. Despite a lack of definition on reference method for food allergen analysis, some tips about on how to fix specific criteria for monitoring them in foods have

been disclosed for other chemical contaminants. That is the case of Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products. This directive includes specific criteria for liquid chromatography (LC) mass spectrometry (MS) analysis, benchmarking the methodology as having the characteristics expected of a reference method. MS methods based on selected reaction monitoring (SRM) in particular have the potential to offer an alternative to ELISA and PCR for allergen analysis (Council Directive 96/23/EC).

Indeed in the recent years, proteomics has been widely applied to food allergens analysis and different workflows for their identification, characterization, determination of their post-translational modifications (PTMs), as well as their quantification have evolved over the years. In the past decade, the advent of modern mass spectrometry technologies also encompassing high-resolution mass analyzers, along with the improvement of bioinformatic tools and genomic data banks, widened our horizons, making possible to expand the knowledges of food allergens from a biochemical, structural and clinical point of view. Proteomic mass spectrometry-based methods allowing unambiguous detection and quantification of traces of single or multiple food allergens and their isoforms even in processed products, where physical or chemical alterations might affect the protein stability preventing its identification. The high-throughput technologies of proteomic approaches in food allergies paved the way for a new era called food *allergenomics*. The present chapter aims to give an overview of the different proteomics workflows to undertake food allergen analysis and both aspects of qualitative and quantitative analysis will be discussed.

2. Proteomic routes: scheme and general description

From a global perspective, large-scale protein investigations are commonly carried out by means of multiple technologies in order to provide high-throughput protein identification, quantification, protein expression and protein–protein interaction information. As known, plant and animal proteomes involve a huge number of protein components; therefore appropriate proteomic experiments have to be designed in order to make the complex mixtures of peptides or proteins suitable for MS analysis. In general, two complementary MS-based approaches have been exploited for proteome analysis, so-called *bottom-up* and *top-down* (Kelleher et al., 1999). The first route uses one or more enzymes to digest proteins into peptides for downstream MS and MS/MS analyses. In contrast with that, the ‘top-down’ strategy is based on the direct analysis of intact proteins and their fragmented ions, generated inside the mass spectrometer (mainly FTICR and LTQ-Orbitrap), without prior digestion.

In general, the first step in proteomics investigation consists in the extraction of the protein content from the analysed matrices. This step represents a great source of variation and its design strongly influences the final proteomic results (Dhabaria et al., 2015). A number of different procedures were developed and they consist in physical homogenization (mechanical force, ultrasound or increased pressure heating/cooling) (Kadama, et al., 2015). The use of buffers, detergents, chaotropic agents for protein extraction and solubilisation, application of reducing agents, as well as different enzyme inhibitors (Bodzon-Kulakowska et al., 2007) strictly depends on the biochemical properties of the target proteins. The choice of the most appropriate extraction buffer may also take into account the likely chemical/physical modifications that some thermal/non thermal treatments could produce, especially in food matrix. In addition, it should be noted that some agents used for protein solubilization commonly employed for sample preparation, singly or in combination with others, can introduce changes in amino acids, such as carbamylation or the Maillard reaction (Kollipara et al., 2013) that needs to be considered in MS-based investigation. Finally, intensive sample fractionation and/or enrichment–depletion strategy have to put in place for the detection and analysis of low abundance proteins as well as the assembly of proteoforms and hydrophobic proteins. More recent strategies for proteomic sample preparation are reviewed by Andjelković et al. (2018).

2.1 Bottom-up proteomics for food allergen discovery

Bottom-up proteomics represents the typical approach for high-throughput analysis of complex proteomes based on two experimental workflows, namely the "*shotgun*" and "*sort-then-break*" approaches (Cunsolo et al., 2014). In the shotgun approach, protein mixture is extracted from the matrix and directly digested by appropriate proteolytic enzymes to produce a mixture of proteotypic peptides, which are separated by multidimensional chromatography (e.g. strong cation exchange followed by RP-HPLC) on-line coupled to ESI MS/MS analysis (Fournier et al., 2007). Although conceptually simple, the shotgun approach generates highly complex peptide mixture difficult to be entirely analyzed. Peptides present at high relative abundance are preferentially sampled and correctly identified by MS analysis at the expense of low abundance peptides, whose information could be lost. To overcome this disadvantage, other strategies, such as prefractionation and immunodepletion methods, have been developed to remove the most abundant proteins aiming at enhancing the sensitivity and efficiency in the identification of low-abundant proteins (Andjelković et al., 2018). On the other hand, in "sort-then-break" approach, pool proteins extracted from animal or plant matrix are first separated by electrophoretic techniques, such 2D electrophoresis (2DE) to generate a protein map where each relevant spot protein could be isolated and singly subjected to enzymatic digestion.

In general as far as allergens is concerning, allergenic proteins are firstly extracted from food, separated by mono-dimensional or two-dimensional gel electrophoresis (2-DGE) and finally immunoblotted on appropriated membranes to visualize the proteins binding with IgE antibodies. The reactive spots are further subjected to in-gel tryptic digestion. The final peptide mixture obtained is then extracted from the gel and submitted to MS (Akagawa et al., 2007; Krishnan et al., 2009) or LC–MS/MS (dos Santos-Pinto et al., 2014; Maddumage et al., 2013) analysis followed by database search (Ciardiello et al., 2013; Nakamura et al., 2013). Prior to 2-DE the allergen extract can be additionally processed by reverse-phase LC (dos Santos-Pinto et al., 2014; Maddumage et al., 2013; Blank et al., 2012; Bollen et al., 2007), size-exclusion and hydrophobic interaction chromatography (Bollen et al., 2007), combination of anion-exchange, cation exchange chromatography and reverse-phase LC (Nikolic et al., 2014; Monaci et al. 2009). The discovery and characterization of new allergens by following bottom-up strategy has been recently reported for eggplants (Babu et al., 2017), shrimp (Lee et al., 2018), shellfish (Nugraha et al., 2018), papaya (Biswas Sarker et al., 2018) and lentils (Shaheen et al., 2019). An overview of the most recent research papers focused on food allergens identification/characterization is depicted in table 1. Typical allergenomic workflow for food allergen discovery/identification relies on bottom-up proteomics, which uses proteolytic enzymes to digest proteoforms and entire proteomes into peptides before MS analysis. There are a number of reasons for the prevalence of bottom-up techniques, among which the ease to work with peptides since they are readily solubilized and separated prior to MS, and easily fragmented during MS/MS scan to produce useful peptide fingerprint for identification and database-searching finalized to protein identification (Chait et al. 2006). A typical workflow for protein discovery based on bottom-up proteomics is depicted in fig. 1.

For characterization and identification purpose, bioinformatics software and online database represent valid tools to address this issue, since proteins identification generally relies on the comparison of MS experimental data with calculated mass values obtained from a sequence database processed by search engine (e.g. Mascot, Ms-Fit, ProFound, MassSearch, Sequest etc.) (Eidhammer et al., 2007). It is well known that by digesting isolated proteins with protease of known cleavage specificity, it is possible to obtain proteotypic peptides that create a peculiar fingerprint, useful for the unambiguous protein identification. In bottom-up proteomics, this goal can be reached by two different methods, known as peptide-mass fingerprinting (PMF) and peptide-fragmentation fingerprinting (PFF), respectively. In the first method, protein identification was achieved by comparing the experimental masses of a list of enzymatic peptides obtained for a single protein, with those calculated from the same proteolytic digestion of each entry in a sequence database. On the contrary, in the PFF method, experimental peptide mass along with the list of peptide fragment ions

produced by MS/MS events were used for database searching. As known, the MS/MS spectrum of a peptide strictly depends from its amino acid sequence, therefore it is reasonable to assume that the specificity of protein identification by PFF approach is much higher than that of peptide mapping (Cunsolo et al., 2014).

Nowadays, the gel-based approach represents the long-established and more widespread separation method in proteomic studies, especially in discovery of new proteins, thanks to the potential of 2D electrophoresis to resolve complex protein mixtures.

Bottom-up proteomics experiments require multiple laborious steps in order to gather informative peptides linked to the proteins characterizing a given proteome and the choice of the best proteolytic enzymes for protein digestion represents a crucial step. The use of trypsin is nowadays considered the gold standard for this kind of approach, although in specific cases other proteolytic enzymes or multiple digestion enzymes are required for target proteins provided with peculiar characteristics (e.g. amino acid composition or hydrophobicity) (Switzar et al., 2013).

Concerning FA, bottom up proteomics has been the most used route for different application in food allergen analysis, spanning from characterization and identification of allergenic proteins to quantification of food allergens traces in complex food matrices (Monaci et al., 2009, 2018). As for this field, a huge number of analytical methods based on MS detection coupled with bottom-up strategy for tracing multiple allergens in food have been developed throughout the years, reaching the highest sensitivity and reliability thanks to the advanced MS technologies (i.e. high resolution mass analyzers, hybrid MS platforms etc.) (Monaci et al., 2018).

Studies also report the bottom-up proteomics approach applied to studying the effect of processing procedures to some categories of nuts to assess the decreased allergenicity (Bavaro et al., 2018; De Angelis et al., 2018).

2.2 Bottom-up proteomics for food allergen quantification

The typical workflow for allergen quantification employs a dual scheme based on different MS experiments. In the first run, the sample is analysed in MS by data dependent untargeted MS/MS mode without any precursor ion pre-selection or pre-filtering, the only filters being the intensity of the ion that should exceed a threshold to be fragmented. The final peptides are identified by bioinformatics tools by searching these peptides and fragments against a database. By repetitive injection and analysis in the untargeted MS mode the most intense and stable peptides are selected and proposed as candidate peptide markers tracing for the specific protein. In the second run, only peptides of interest are included in the list of ions which will be selected for fragmentation and MS/MS scan or specific transitions will be only monitored to increase the overall method sensitivity.

Consequently, targeted MS approach bases ion selection on non-redundant and information-rich precursor ions. In addition, partially removing bias toward more abundant components increases the depth of analysis and the reliability of quantification (Domon et al., 2010).

The bottom-up approach has been successfully applied to food allergen quantitative analysis with promising sensitivities. The typical scheme is summarized in fig. 1. Despite the conventional quantitative methodologies using SILAC, (stable isotope labeling with amino acids in cell culture), ICAT (isotope-coded affinity tag), iTRAQ (isobaric tag for relative and absolute quantification) typically used for large-scale quantification (Wu et al., 2006; Gingras et al., 2007), label-free approaches are the preferred routes for food allergen quantification although for accurate results they do need the use of isotopically standard peptides to better correlate their amount to the protein concentration. The selection of peptides that will be used as internal standards is important for delivering a reliable quantification, and different methods have been developed for this purpose (Brusniak et al., 2011; Eyers et al., 2011). The workflow underpinning the bottom-up strategy makes use of enzymes like trypsin or chymotrypsin (with specific cleavage sites along the protein namely arginin and lysine) that cut the protein at specific cleavage sites after protein extraction from food. The final peptide mixture obtained is then injected into the LC-MS/MS apparatus for allergen detection through the detection of proteotypic allergen-specific peptides. The identification of stable and unique peptide markers represents a crucial step in setting a MS-based method because each marker should demonstrate to be stable and univocally tracking for a specific allergenic protein. Guidelines assisting in correctly identifying suitable and reliable peptide markers for each target allergen have been published and are briefly schematized in table 2 (Johnson et al, 2011). Some of the criteria proposed are here following listed: 1) select the most abundant allergenic proteins as target allergens, 2) assess marker stability and reproducibility 3) preferably exclude peptides containing amino acids susceptible to modification 4) check for protein specificity by blasting the candidate peptides against a large database available online. For peptide detection, at least 3 peptides for allergenic ingredient should be identified and at least three transitions including one quantifier and two qualifiers should be sought for (Mills et al., 2019). As far as the detection is concerning, the advent of the last generation hybrid mass spectrometers has entered the arena of food allergen detection methods and efforts have been directed to developing sharp and high throughput analytical MS workflows for multiple detection of allergens in foodstuffs within a single run. Peptide-based allergen quantification strategy is typically accomplished by Selected Reaction Monitoring (SRM) mode or by Multiple Reaction Monitoring (MRM) when multiple transitions are monitored at the same time, this last more tailored to triple quadrupole instruments. The targeted monitoring of mass and transitions of selected proteotypic and unique peptides in general proved to improve the

sensitivity and selectivity of the analysis, since it overcomes the issues of cross-reactivity posed by phylogenetically related proteins.

In the very early beginning, Selected ion monitoring (SIM) scheme also involving MS/MS spectra corresponding to each precursor ion, has been used for food allergen monitoring in different food commodities by using ESI-Q-TOF MS systems coupled with either UHPLC or micro or nano HPLC separation. The first applications of such QTOF approach in food allergen research dates back the early 2000 where for the first time a capillary HPLC system coupled to Q-TOF MS was used for tracing peanuts or milk allergens in food products, assessing the potential offered by such technology for qualitative and quantitative analysis The first methods developed in this field used a Q-TOF system and the acquisition was based on SIM mode, where selected precursor ion peptides were monitored and the respective fragment patterns were obtained in the MS/MS spectra generated (Weber, et al., 2006, Shefcheck et al., 2004, Chassaigne et al., 2007; Monaci et al., 2010; Azarnia 2013). This approach proved to be applicable to food allergen detection although the sensitivity reached was not very challenging. With the advent of the latest highly sensitive triple quadrupole mass spectrometers, the routine quantitative analysis usually accomplished on small molecules was transferred to the protein field. This was attained by monitoring multiple transitions of the best peptide markers identified for each allergenic category as demonstrated by the first paper published by Heick et al. (2011) proving the suitability of a triple quadrupole system for multiple allergen detection in a processed food. Once suitable peptides and transitions had been properly designed, the MRM method devised was applied to bread incurred with 7 allergenic foods and the quantitative capabilities of the method assessed. According to their findings, LODs ranging from 3 to 70 µg/g were obtained in the incurred bread matrix, depending on the specific allergen. Another work described the development of a LC-MS method and its validation for the accurate quantification of milk traces in different food products (Lutter et al., 2011). Upon proper selection of suitable markers, quantification was attained by using internal standard peptides containing isotopically labeled amino acids. Such method enabled to reach LODs down to 0.2-0.5 mg/kg comparable to the limits obtained with ELISA kits, therefore proving that LC-SRM-MS/MS approach could be intended as a sensitive and quantitative tool for milk allergens detection in selected food matrices. In the same period other papers appeared in literature mostly enhancing potentials of a linear ion trap MS for the multi-target analysis of nuts and fish allergens in diverse food matrices (Mattarozzi et al., 2012; Careri et al., 2007; Carrera et al., 2012). Table 3 reports a summary of the MS/MS targeted methods devised for multiple allergen detection in a wide variety of food matrices based on the shotgun strategy.

Some authors also described advantages and limitations of multi-target allergen analysis by MS^3 acquisition mode (Bignardi et al., 2010). The advancement in mass analyzers placed on the market open towards the development of HR-MS based methods for a fast detection of allergen as contaminants of food products with promising application to this field for multiple detection of milk and egg allergens in wine (Monaci et al., 2010, 2013). The benefits offered by HR-MS over the classical tandem low resolution mass spectrometry, include the possibility to identify and elucidate the chemical structure of a food component and, as non-targeted approach, to simultaneously identify numerous peptide markers, even retrospectively without preliminary information/selection required (Kaufmann et al., 2010, 2012, Makarov et al., 2010). Beyond the combination of HR-MS full scan and HCD fragmentation acquisition modes at the highest resolving power and mass accuracy within a single chromatographic run, HR-MS approach provides confirmative and quantitative features. The collection of full-scan spectra provides greater insights into the identity and chemical structure of a food component. In addition, the excellent scan speed and resolving power offered by the HR-MS systems, allows to perform full scan untargeted analysis both in MS and full ion fragmentation mode at the highest resolving power and mass accuracy for multiplex analysis of food allergens. Thanks to the post acquisition accurate mass filtration of the selected peptide ions operated on the total ion current traces, challenging LODs can be obtained, providing a valid alternative way to the conventional SRM and MRM methods for quantification purposes (Monaci et al., 2011a, b). This is the case of a HR-MS based method using an hybrid quadrupole/Orbitrap platform, recently developed for multiple allergen detection in processed food matrices. Among the several acquisition modes, timed t-SIM/dd2 resulted was found the best choice as a good compromise between sensitivity and accuracy, accomplishing the detection of seventeen peptides in the same run tracking for five allergenic ingredients (Pilolli et al., 2018). Current efforts are being directed at European level to standardize and harmonize a MS method able to simultaneously quantify traces of allergens in different foods. This one represents one of the two objects of the current EFSA project "ThRAll" whose aim is to develop a reference method based on Mass Spectrometry to quantify multiple allergens in complex food matrices (Mills et al., 2019).

2.3 Top-down proteomics for food allergen characterization/discovery

A complementary strategy to the bottom up for protein analysis is offered by the top-down proteomics. This approach involves the gas-phase ionization of intact proteins and their direct MS and MS/MS analysis without prior proteolytic digestion, thus information on intact protein mass, its amino acid sequence and more comprehensive characterization of PMTs are provided. In general,

proteins extracted from complex matrices are firstly fractionated and separated into pure single proteins or less complex protein mixture. Proteins are then injected into the MS spectrometer by off-line static infusion system or after LC separation for high-resolution mass measurement of intact protein ions. Specific fragments generated by subsequent MS/MS events of the mass-selected multicharged ions of a protein are then used for its structural characterization (McLafferty et al., 2007). Protein identification could be performed by exploiting MS/MS data of the intact protein ions together with the experimentally high resolution measured mass (Cunsolo et al., 2014). In the light of this, the efficient fragmentation of intact proteins, along with the exact selection of the multicharged protein ions further deconvoluted by the suitable software, represent the key to the success of this approach. The advent of high mass accuracy and high resolution of LTQ-FTICR, Q-TOF or LTQ-Orbitrap mass spectrometers, further improved by the electron fragmentation (i.e. ETD or ECD), largely improved the feasibility of top down proteomics.

In allergenomic field, top-down approach has been scarcely investigated, although its potential in discovery of new allergenic protein together with the elucidation of PTMs triggered by food processing turns very interesting from a clinical point of view. Indeed, the most common bottom-up approach presents several drawbacks in the panorama of new allergen discovery, especially if proteoforms are investigated. In bottom-up proteomics, proteoforms are fragmented into peptides that are finally *in silico* reassembled to identify the belonging protein. Proteolysis represents the main cause of important information loss, because it eliminates the connectivity between intact proteins and the tryptic peptides they yield, complicating computational analysis and biological interpretation that are further affected by the limited proteins available in existing databases (Ciardiello et al., 2013). Moreover, PTMs as well as conformational structure of food allergens could not be elucidated via bottom-up proteomics. This represents a consistent drawback, since it is well demonstrated that thermal/non thermal treatments that foods traditionally undergo to improve organoleptic properties and safety quality, have the potential to alter the physico-chemical properties of proteins as well as their structure, with consequent modification of the final allergenicity of foods (Vanga et al., 2017). For example, thermal processing could cause the loss of tertiary and secondary structure along with disulphide bond breakage and formation of aggregates (Davis et al., 2001). Moreover, chemical modification of protein might also occur at high temperature with consequent formation of covalent bonds between free amino groups and other constituents of food matrix (e.g. reducing sugars). In a typical example, thermally treated proteins could undergo a non-enzymatic glycation also called Maillard Reaction (MR). In turn, MR may further lead to the formation of advance glycation end products (AGEs), namely compounds derived by binding of proteins with various types of glycated structures such as Ne-(carboxymethyl-lysine (CML), pentosidine, pyrraline and methylglyoxal-H1

(Toda et al., 2014). It was demonstrated that AGEs may function as potent epitope and increase the risk of food allergens (Toda et al., 2014). In light of this, the possibility to elucidate the potential modifications that proteins may undergo upon food processing is of uttermost importance from a clinical point of view, thus representing a crucial point in the field of new allergens discovery.

The simplest top-down MS experiments involve several steps (figure 1). Proteoforms of interest are firstly prepared and then directly infused into the mass spectrometer. This often involves purification and/or prefractionation steps (based on molecular mass or isoelectric point) of sample to produce a set of fractions that can be further separated by MALDI-TOF or various LC modes coupled online with ESI (LC-MS). Typically, intact proteoform cations (precursors) are directed into a high-resolution mass analyzer such as the ion cyclotron resonance (ICR) mass spectrometer, Orbitrap or Time of Flight (TOF) mass analyzer (Scigelova et al., 2011). Protein identification and characterization were achieved by analyzing fragment ions arisen from fragmentation events of precursor ions. Fragmentation techniques based on collisional or electron-based dissociation are often employed to preserve useful information about proteins PTMs. Moreover, by combining various fragmentation strategies it is possible to theoretically elucidate the primary amino acid sequence of protein (Toby et al., 2016). Limitations of top-down approach rely on the protein size that could be efficiently sequenced (maximum 50 kDa) and the management of the high complex data retrieved by MS/MS analysis, that require high performing software for their final elaboration. On the other hand, recent technological advances of mass analyzers towards ultra-high resolution, as well as new algorithms for data processing, are now making top-down the method of choice when studying complex proteoforms (Kou et al., 2016; Vyatkina et al., 2015). Although promising, MS-based technologies based on top down proteomic were poorly used for allergens investigation and most of paper focused on modifications site in heated proteins (Grandori et al., 2003; Leonil et al., 1997; Yeboah et al., 2001; Siciliano et al., 2000). Among them, lactoglobulins were the most investigated proteins due to the good multi-protonated features shown by this class of proteins, with a reproducible multi-charged ions envelope by electrospray ionization (Fenaille et al., 2003, Monaci et al., 2007, 2008). In most recent studies potentials of HR-MS might tackle some drawbacks and provide in thorough information about the modification sites and more insights in the structural elucidation (Abbatiello et al., 2017; Kaufmann et al., 2016).

Irrespective of the approach adopted, the successful protein identification relies on the availability of a proteome completely sequenced for a specific matrix, and the presence of the corresponding protein sequence in the database is essential. If the amino acid sequence of the protein under investigation is not present in the proper database, a more general database should be scouted in order to find the best

match with entries showing the closest homology that usually correspond to proteins belonging to a strictly phylogenetic-related species (Cunsolo et al., 2014). If the sequence similarity within protein databases is too low, database search is unsuccessful, and MS/MS data must be interpreted (*de novo sequencing*) manually or processed via sequencing algorithms (e.g. PEAKS, PepNovo etc.). Through this approach, it is possible to reconstruct the peptide sequence directly from an MS/MS spectrum without the aid of protein sequence databases. Finally, the deduced peptide sequences should be searched in conventional database by using algorithms such as BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or FASTA (http://www.genome.jp/tools/fasta/) or by MS-driven sequence similarity searches (MS-BLAST; http://genetics.bwh.harvard.edu/msblast/) in order to identify proteins by sequence similarity (Cunsolo et al., 2014).

3. The bottleneck in allergenomics: bioinformatic analysis

In the context of food allergy, allergenomic data could be interpreted for mapping epitopes, whose positioning in the molecule defines the structure of allergen-based vaccines for the immunotherapy (García et al., 2011; Jankovicova et al., 2008) or they could be exploited for predicting the cross-reactivity with proteins belonging to different species (García et al., 2011; Nakamura et al., 2013). Thanks to advanced high-throughput technologies available in the omics sciences, the ambition to simultaneously characterize and quantify a large number of analytes or cellular components from a single food sample, and among them allergenic proteins became far too realistic. In general, proteomics-based technologies have aided in characterizing several allergens in terms of structure, cellular interactions, functions, localization, post-translational modifications, physicochemical properties, allergenic relevance and cross-reactivity. These large data sets were exploited to build up useful online platforms differing from each other with respect to the main focus, type of data archived and applications. The Allergen Nomenclature Sub-Committee of the World Health Organization (WHO) and International Union of Immunological Societies (IUIS) have developed an unambiguous and systematic nomenclature system for protein allergens also maintaining the official Allergen database (http://www.allergen.org/). This database also archives isoallergens and variants of allergens and it is regularly updated. Among the bioinformatic tools available online, it is worthy to be mentioned the Allergome database (http://www.allergome.org/), Food Allergy Research And Resource Program FARRP (https://farrp.unl.edu/), Food Standard Agency (https://www.food.gov.uk/) and Structural Database of Allergenic Proteins (SDAP http://fermi.utmb.edu/SDAP). In addition, the availability of enlarged database containing updated and curated information about amino acid sequences of allergenic proteins could help in future better guide in allergen identification/characterization.

Figure and table captions

Figure 1. Overview of proteomic pipelines based on bottom-up and top-down strategies for food allergen identification, characterization and quantification.

Table 1. Overview of the most recent literature focused on the discovery of new allergens by bottom-up approach.

Table 2. Basic criteria used for proper protein and peptide identification in MS-based food allergens analysis.

Table 3. Overview of the LC-MS/MS quantitative methods devised for allergen detection in food products based on detection of peptide markers.

Table 1

Food allergen	Proteomics tools	Main target	Results	Reference
Wheat	SDS-PAGE−PAGE, 2-DE, immunoblot, MALDI-TOS MS	Characterization of wheat allergens	Building up of a 2DE allergen map and identification of 18 wheat allergens containing isoallergens	Akagawa et al., 2007
Shrimp (arginine kinase)	2DE, tryptic in-gel digestion, MALDI-TOF MS, ESI-MS/MS	*De novo* MS-sequencing	Identification of new species-specific peptides belonging to arginine kinase	Ortea et al., 2009
Fish (parvalbumins, PRVBs)	2-DE, in gel protein digestion, LC-ESI-IT-MS/MS, FT-ICR-MS, Selected MS/MS Ion Monitoring (SMIM)	*De novo* MS-sequencing by integrated approaches (BottomUp proteomics, Top-down, selected MS/MS ion monitoring (SMIM)	De novo sequencing of 25 new fish parvalbumin isoforms (PRVB; 11.20-11.55 kDa), partially sequencing of 16 other new PRVBs isoforms	Carrera et al., 2010
Lentil	SDS-PAGE, LC-MS/MS	Proteome profiling of low-molecular weight proteins by Bottom-up approach	Identification of known and putative allergens by peptide sequencing of low molecular weight seed proteins	Shaheen et al., 2019
Eggplant (polyphenol-oxidase, PPO)	SDS-PAGE, immunoblotting, ELISA, LC-MS/MS,	Proteome profiling by bottom-up approach and IgE structure elucidation by biorinformatic approach	Identification of PPO protein as eggplant allergen	Babu et al., 2017
Whiteleg shrimp (pyruvate kinase)	SDS-PAGE, immunoblotting, LC-MS/MS	Identification of novel allergen by bottom-up approach	Identification of pyruvate kinase as a novel shrimp allergen	Lee et al., 2018
Shellfish (Pacific Oyster)	2DE, immunoblotting, LC-MS/MS	Identification of novel cross-reactive allergenic proteins using a genomic and protomi integrated approach	Identification of 24 new allergens in Pacific Oyster	Nugraha et al., 2018
Papaya (polygalcturonase)	SDS-PAGE, immunoblotting, LC-MS/MS, degranulation assay, circular Dichroism Spectroscopy	Identification of novel allergens by botto-up approach	Identification of a Endopolygalacturonase as novel allergen	Biswas Sarker et al., 2018

Table 2.

Criteria from Johnson, et al., 2011	
Protein target selection	
Prior knowledge	Full protein sequence should be available.
Uniqueness	Protein sequence should be unique to foodstuff to be detected. At a minimum, sequence should not be present in commonly analyzed matrixes and other food ingredients.
Abundance	Protein should be abundant in foodstuff/ingredient.
Expression variability	Protein expression (tissue specificity, species/cultivar variability, temporal variability, response to environmental/disease/stress factors, post-harvest treatment) should be characterized and preferably, expression variability should be minimal.
Modification	Protein should ideally not be subject to modification (either post-translational or processing-induced) in the foods/matrixes commonly analyzed. If present, modifications must be considered when developing peptide targets and MS methodology.
Stability	Protein should not be subject to hydrolysis during storage or food processing.
Extractability	Protein should be released from the matrix into solution for detection.
Digestibility	Protein should be reproducibly digested by endoprotease used in the chosen sample preparation technique.
Peptide and transition selection	
Reproducible	Peptide and MS/MS fragment reproducibly produced from analysis of various matrixes
Unique	Peptide/MS/MS fragment produced only from digestion of protein target
Minimize potential modification	Preferably lacking cysteine, methionine and glutamic acid
RT (LC)	Reproducible single RT
Charge	2+ or 3+ ions generally give the best fragmentation
Size	Typically 6-12 residues

Table 3.

Food	Allergenic ingredient	Target protein	LOD	Rif.
Biscuits	Hazelnut	Cor a9	LOD: SRM 30 µg g^{-1}, SRM^3 35 µg g^{-1}	Bignardi et al., 2010
	Cashew	Ana o 2	LOD: SRM 14 µg g^{-1}, SRM^3 30 µg g^{-1}	
	Almond	Pru 1	LOD: SRM 17 µg g^{-1}, SRM^3 25 µg g^{-1}	
	Walnut	Jug r 4	LOD: SRM 55 µg g^{-1}, SRM^3 50 µg g^{-1}	
	Peanut	Ara h 3/4	LOD: SRM 10 µg g^{-1}, SRM^3 27 µg g^{-1}	
Biscuits, Dark chocolate	Hazelnut	Cor a9	LOD: 1.3 µg g^{-1} - 14 µg g^{-1}	Bignardi et al., 2013
	Cashewnut	Ana o2	LOD: 0.5 µg g^{-1} - 15 µg g^{-1}	
	Almond	Pru 1	LOD: 0.9 µg g^{-1} - 9 µg g^{-1}	
	Peanut	Ara h 3/4	LOD: 0.1 µg g^{-1} - 7 µg g^{-1}	
	Walnut	Jug r4	LOD: 0.8 µg g^{-1} - 5 µg g^{-1}	
Bread	Egg	Ovalbumin	LOD: 42 µg g^{-1}	Heick et al., 2011
	Milk	αS1-casein	LOD: 5 µg g^{-1}	
	Hazelnut	11S globulin	LOD: 5 µg g^{-1}	
	Almond	Prunin	LOD: 3 µg g^{-1}	
	Peanut	Ara h1	LOD: 11 µg g^{-1}	
	Walnut	Jug r1	LOD: 70 µg g^{-1}	
	Soy	Glycinin	LOD: 24 µg g^{-1}	
White wine	Egg	Ovalbumin, lysozyme	LOD: Ova 0.19 µg mL^{-1}, Lys 0.19 µg mL^{-1}	Pilolli et al., 2014
	Milk	Caseins	LOD: 0.2 µg mL^{-1}	
Cookie	Egg	Ovalbumin, β-Lactoglobulin	LOD: 0.3 $µg_{PROT}$ g^{-1}	Monaci et al., 2014
	Milk	αS1-Casein	LOD: 0.12 $µg_{PROT}$ g^{-1}	
	Soy	Glycinin G2, β-Conglycinin, Glycinin G4	LOD: 2 $µg_{PROT}$ g^{-1}	
Red wine	Caseinate	αS1-, β-caseins	LOD: 0.5 µg mL^{-1}, 0.01 µg mL^{-1};	Mattarozzi et al., 2014
	Egg-white	ovalbumin	LOD: 0.8 µg mL^{-1}	
Salmon lasagna	Shrimp, Lobster	myosin light chain, myosin heavy chain, arginine kinase, slow muscle myosin S1 heavy chain, fast myosin heavy chain	LOD: SRM 1000 µg g^{-1}, SRM^3: 25 µg g^{-1} (crustacean/food)	Korte et al., 2016a
Multigrain bread, vanilla ice cream, dairy chocolate	Peanut	Ara h 3	LOD: ≤ 1 µg g^{-1} in bread and ice cream matrix; ≤ 3 µg g^{-1} in milk chocolate	Korte et al., 2016b
	Almond	Pru du 6.0101, Pru du 6.0201		
	Cashew	Ana o2		
	Hazelnut	Cor a9		
	Pistachio	Pis v5		
	Walnut	Jug r2, Jug r4		

Cookie	Egg	Ovalbumin	LOD: 10-15 µg g^{-1}	Monaci et al., 2016
	Milk	Alpha-S1-casein	LOD: 8-13 µg g^{-1}	
	Soy	Glycin G1	LOD: 5-8 µg g^{-1}	
	Hazelnut	11S globulin-like protein	LOD: 1-5 µg g^{-1}	
	Peanut	Conarachin	LOD: 8-9 µg g^{-1}	
Meat	Lupine	Conglutin β2	LOD: 2 mg kg^{-1}	Hoffmann et al., 2017
	Pea	Convicilin	LOD: 5 mg kg^{-1}	
	Soy	Glycinin G2	LOD: 4 mg kg^{-1}	
Raw wheat flour, cookie, soft bread	Soy	Gly m6	ND	Huschek et al., 2016
	Sesame	Ses i6	ND	
	white lupine	β-conglutin	ND	
Chocolate, ice cream, tomato sauce, and processed cookies	Milk	αS1-Casein, αS2-Casein, β-lactoglobulin	ND	Planque et al., 2016
	Egg	Ovalbumin, Vitellogenin-2, Vitellogenin-1, Apovitellenin	ND	
	Soybean	Glycinin, 2S-albumin, β-conglycinin	ND	
	Peanut	Cupin (Ara h1, Ara h3/4, Ahy-1)	ND	
Incurred cereal bar and muffins	Egg	Lysozyme, ovalbumin	ND	Parker et al., 2015
	Milk	αS1-casein, β-lactoglobulin		
	Peanut	Ara h1, Ara h2 and Ara h3		
White wine	Milk	αS1-, β-casein	LOD: 50 ng mL^{-1}	De Angelis et al., 2017
	Egg	Ovalbumin, Lysozyme	LOD: 36 ng mL^{-1}	
Spiked and Incurred cookie	Milk	αS1-caseins	LOD: 7 µg g^{-1}	Pilolli et al, 2017
	Egg	Ovalbumin	LOD: 9 µg g^{-1}	
	Soy	Glycinin G1-G2	LOD: 6 µg g^{-1}	
	Peanut	Conarachin	LOD: 13 µg g^{-1}	
	Hazelnut	11S globulin-like protein	LOD: 7 µg g^{-1}	
Chocolate	Milk	αS1-, αS2-, β-, κ-caseins	LOD: 0.05-0.13 µg g^{-1}	Gu et al., 2018
	Soybean	Glycinin G1, β-conglycinin (α' and β chains)	LOD: 0.4-1.2 µg g^{-1}	
	Peanut	Ara h1, Ara h3/4	LOD: 0.8-1.3 µg g^{-1}	
	Almond	Pru 1, Pru2	LOD: 0.4-0.8 µg g^{-1}	
	Walnut	Jug r2	LOD: 0.6 µg g^{-1}	
	Hazelnut	Cor a9	LOD: 0.5 µg g^{-1}	
	Cashew	Ana o2	LOD: 0.7 µg g^{-1}	

	Pistachio	Pis v2	LOD: 0.4 μg g^{-1}	
Incurred sugar cookies	Egg	Ovalbumin, lysozyme C	ND	Boo et al., 2017
	Milk	Β-lactoglobulin, αS1-casein		
	Peanut	Ara h1, Ara h2, Ara h3		
Chocolate, ice cream, cookie, tomato sauce	Egg	Ovalbumin, Vitellogenin-2, Vitellogenin-1	ND	Planque et al., 2017
	Milk	β-lactoglobulin, αS1-Casein, αS2-Casein	ND	
	Soybean	Glycinin, 2S albumin, β-conglycinin	ND	
	Peanut	Cupin, Conglutin 7	ND	
	Almond	Prunin	ND	
	Pecan	7S vicilin	ND	
	Cashew	Ana o2, Ana o3	ND	
	Hazelnut	11S globulin- like protein	ND	
	Walnut	Vicilin-like protein, Albumin seed storage	ND	
Cookies	Pistachio	2S albumin Pis v 1, 11S globulin (Pis v 5 and Pis v 2)	ND	
Incurred cookies	Egg Milk Soy Hazelnut Peanut	Ovalbumin αS1 Glycin G111S globulin-like protein Conarachin	LOD: 14 mg_{PROT} kg^{-1} LOD: 6 mg_{PROT} kg^{-1} LOD: 10 mg_{PROT} kg^{-1} LOD: 4 mg_{PROT} kg^{-1} LOD: 7 mg_{PROT} kg^{-1}	Pilolli et al., 2018

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