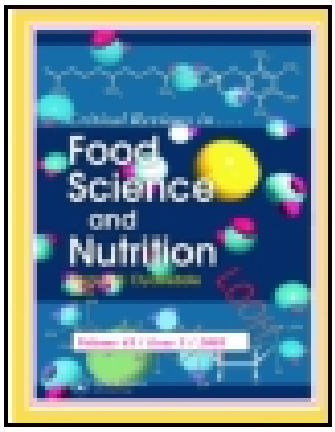

Critical Reviews in Food Science and Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bfsn20

Advanced DNA- and Protein-based Methods for the Detection and Investigation of Food Allergens

M. Prado[a], I. Ortea[bc], S. Vial[ade], J. Rivas[a], P. Calo-Mata[f] & J. Barros-Velázquez[f]

[a] International Iberian Nanotechnology Laboratory (INL) Avenida Mestre José Veiga 4715-310 Braga, Portugal

[b] Department of Food Technology, Institute for Marine Research, Spanish National Research Council (CSIC), Vigo, Spain

[c] Present address: Institute for Reference Materials and Measurements (EC-JRC-IRMM), Retieseweg 111, B-2440 Geel, Belgium

[d] 3B´s Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal

[e] 2 ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

[f] Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences/College of Biotechnology, University of Santiago de Compostela, Rua Carballo Calero s/n, Campus Lugo, E-27002 Lugo, Spain

Accepted author version posted online: 07 Apr 2015.

To cite this article: M. Prado, I. Ortea, S. Vial, J. Rivas, P. Calo-Mata & J. Barros-Velázquez (2015): Advanced DNA- and Protein-based Methods for the Detection and Investigation of Food Allergens, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2013.873767

To link to this article: http://dx.doi.org/10.1080/10408398.2013.873767

Advanced DNA- and Protein-Based Methods for the Detection and Investigation of Food Allergens

Authors:

Prado, M.[1*]; Ortea, I.[2, 3]; Vial, S.[1, 4, 5]; Rivas, J.[1]; Calo-Mata, P.[6]; Barros-Velázquez, J.[6]

[1]*International Iberian Nanotechnology Laboratory (INL) Avenida Mestre José Veiga 4715-310 Braga, Portugal*

[2]*Department of Food Technology, Institute for Marine Research, Spanish National Research Council (CSIC), Vigo, Spain*

[3]*Present address: Institute for Reference Materials and Measurements (EC-JRC-IRMM), Retieseweg 111, B-2440 Geel, Belgium.*

[4] *3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal*

[5] *2 ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal*

[6] *Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences/College of Biotechnology, University of Santiago de Compostela, Rua Carballo Calero s/n, Campus Lugo, E-27002 Lugo, Spain.*

**Corresponding author: marta.prado@inl.int*

ABSTRACT

Currently, food allergies are an important health concern worldwide. The presence of undeclared allergenic ingredients or the presence of traces of allergens due to contamination during food processing poses a great health risk to sensitized individuals. Therefore, reliable analytical methods are required to detect and identify allergenic ingredients in food products. The present review addresses the recent developments regarding the application of DNA- and protein-based methods for the detection of allergenic ingredients in foods. The fitness-for-purpose of reviewed methodology will be discussed, and future trends will be highlighted. Special attention will be given to the evaluation of the potential of newly developed and promising technologies that can improve the detection and identification of allergenic ingredients in foods, such as the use of biosensors and/or nanomaterials to improve detection limits, specificity, ease of use, or to reduce the time of analysis. Such rapid food allergen test methods are required to facilitate the reliable detection of allergenic ingredients by control laboratories, to give the food industry the means to easily determine whether its product has been subjected to cross-contamination and, simultaneously, to identify how and when this cross-contamination occurred.

KEYWORDS: *Allergens, DNA based methods, Proteomics, Allergen detection, qPCR, biosensors, nanoparticles*

INTRODUCTION

Food allergies have become an important health concern worldwide. Clinical manifestations of food allergies in sensitized individuals can range from minor digestive disorders and skin irritations to more severe symptoms that can even lead to life threatening situations. To provide all consumers with better information and to protect the health of certain consumers, several countries have introduced into their legislation the obligation to indicate the presence of certain allergenic ingredients on food labels. However, an important risk factor for sensitized individuals is the presence of unintentional allergenic ingredients due to cross-contamination during the processing or storage of raw materials or the final product. Therefore, reliable and sensitive methods are required to ensure compliance with such legislation and, more importantly, to detect the presence of any intentional or unintentional allergenic ingredients in food products to protect consumers suffering from food allergies. Therefore, these methods could be applied to facilitate the reliable detection of allergenic ingredients by control laboratories, to give the food industry the means to easily determine whether its product has been subjected to cross-contamination and, simultaneously, to identify how and when this cross-contamination occurred.

According to the opinion of the EFSA's (European Food and Safety Authority) Scientific Panel on Dietetic Products, Nutrition and Allergies (NDA) in 2004 (EFSA, 2004), although sensitive test systems are coming into use and are commercially available for the analysis of some allergens in foods, major issues remain in several areas: insufficient extraction, detection limits outside the range of clinical sensitivity, insufficient specificity due to cross-reaction and insufficient inter-laboratory reproducibility. New developments since 2004 must be evaluated to determine which of these problems have been solved and which still have to be faced.

To choose an appropriate method for the detection of allergenic ingredients in food products, the selection of a good marker is possibly the most important step because this selection will affect the sensitivity and reliability of the analytical method in use. In the present article, we review the recent developments that are related to DNA- and protein-based methods for the detection of allergenic ingredients, the challenges that are related to the use of each of those biomolecules, and the methods currently available, along with new developments, the performance criteria of available methods, and future trends in this area.

Food allergy definition, worldwide impact and sanitary implications

A food allergy is an adverse immune response to a normally tolerated food protein (Sathe et al., 2005). Generally, food allergic reactions are mediated by immunoglobulin E (IgE) and occur in individuals who are genetically predisposed to the allergy and who have been previously exposed to the allergen. After the initial exposure to the allergen, antigen-specific IgE is produced and binds to the surface of mast cells and basophils (Ladics and Selgrade, 2009). The subsequent exposure of sensitive subjects to the food allergen produces an allergic response by cross-linking antigen-specific IgE on the surface of mast cells and basophils, thus causing an immediate response, which implies the release of several potent cell-derived mediators, such as histamine, N-acetylhexoaminidase, proteases, leukotrienes or proinflammatory cytokines (Kirsch et al., 2009). Clinical symptoms that are associated with the ingestion of foods by sensitive persons include urticaria, itching, edema, bronchoconstriction, rhinitis, vomiting, diarrhea, cramps, and, in severe cases, cardiovascular symptoms that may lead to anaphylactic shock, which may have fatal consequences (Besler, 2001).

Food allergies are an important health issue that are considered by the World Health Organization among the five most important public health concerns (Kimber and Dearman, 2001; Faeste et al., 2011), particularly in industrialized countries, where the affected population represents approximately 2% of adults and up to 4-8% of young children.(Helms and Burns, 2000; Cianferoni and Spergel, 2009).The incidence and prevalence of food allergies are increasing, together with other forms of atopic allergic disease (Anderson 1996; Hourihane, 1998; Food Standards Agency, 2000). The prevalence of food allergies has also been described as varying geographically (Metcalfe et al., 1996) due to the different dietetic habits of the different populations throughout the world. Remarkably, only a small percentage of persons exhibit adverse immunologic reactions to specific foods. Generally, the normal immune response of non-sensitive individuals to proteins implies the development of tolerance, which is a state of inhibition of the immune response to an antigen due to previous oral exposure to the antigen (Ladics and Selgrade, 2009). It is unclear why a person becomes intolerant towards a specific food protein. Moreover, the amount of protein that is required to unchain an allergic response in a sensitive person varies considerably depending on the type of person and on the protein (Sathe et al., 2005).

The diagnosis of a food allergy in patients is based on the fact that the blood of allergic patients contains both IgE, which specifically binds to the allergen, and white blood cells, which release certain mediators when the allergen is present (Kirsch et al., 2009). Consequently, diagnostic methods are based on the immunochemical detection of IgE, allergen-receptors or mediators (Kirsch et al., 2009)

A food allergy should be distinguished from food intolerance. The former depends on the stimulation of a specific immune response (Kimber and Dearman, 2001). In contrast, food intolerance represents an adverse reaction to foods or food ingredients, which is non-immunological in nature (Kimber and Dearman, 2001). Food intolerance is much more common than food allergies and is normally caused by a digestion, absorption or metabolism disorder that is related to a certain food component (Ortolani et al., 2001). Remarkably, the clinical symptoms that are caused by food intolerance are extremely similar to those symptoms that are caused by food allergies, thus complicating the differentiation between both events (Monaci, 2006). In general terms, food intolerance is not as severe as food allergies. Typically, the symptoms of a food intolerance reaction, unlike food allergies, are not immediate, and their intensity may be linked to the amount of food that is consumed (Monaci, 2006).

Most food allergy reactions are caused by proteins or glycoproteins that are present in relatively few foods; these foods include cow's milk, fish, shellfish, eggs, peanut, wheat and soy (Sicherer and Sampson, 2006; Ladics and Selgrade, 2009). Although nearly 200 allergenic proteins exhibiting a molecular mass in the range of 10 to 70 kDa and belonging to a variety of different protein families (Aalberse et al., 2000; Breiteneder et al., 2000; Besler, 2001) have been identified thus far, only a few allergenic proteins are responsible for the most severe and frequent allergic reactions. Thus, chitinases from banana and chestnut, lipid transfer proteins from cereals, lipocalins from milk, parvalbumins from fish or tropomyosin from crustaceans, among others, have been identified among the most relevant food allergens (Besler, 2001).

Undeclared allergenic ingredients or the presence of traces of allergens due to contamination during food processing represents a great health risk to sensitized individuals. The best way to

fight against food allergies is to avoid the ingestion of allergen-containing foods (Poms et al., 2004). Accordingly, it is of crucial importance that the presence of ingredients with potential allergenic activity is accurately declared on the food label. In this sense, correct labeling permits sensitive individuals to avoid exposure to allergens (Faeste et al., 2011).

Risk of food allergen contamination along the food chain

Over 160 food materials have been identified as allergenic; among these food materials, only eight have been identified as responsible for more than 90% of all food allergens. Those "major food allergens" include milk, egg, fish, crustacean shellfish, tree nuts, wheat, peanut and soybeans. To provide all consumers with better information and to protect the health of certain consumers, several countries have introduced into their legislation the obligation to indicate the presence of certain allergenic ingredients on food labels. Such is the case for the Food Allergy Labeling and Consumer Protection Act (FALCPA) (FDA, 2004) in the USA, which includes the abovementioned list, as well as the Labeling Directive (European Commission, 2000) and its amendments (European Commission, 2003, 2007) in EU, which includes, in addition to this list, other cereals containing gluten and six more food ingredients, namely: celery, mustard, sesame seeds, sulfur dioxide or sulfites, lupin, and mollusks.

Residual amounts of food allergens after processing, even traces, can cause clinical reactions (EFSA, 2004). Studies determining the amount of allergens that are capable of eliciting allergic reactions have set a range between 150 μg and 100 mg of ingested food allergens (Besler, 2001). With these values in mind, it is important to highlight that food labeling concerns exclusively allergenic ingredients that are intentionally introduced into foods. However, contamination at any stage of the food chain is likely to occur (Monaci et Visconti, 2010), and sometimes the

amount of the allergenic ingredient that results from such contamination might trigger allergic reactions in sensitive consumers.

Unintentional "cross-contamination" of foods with undeclared allergenic ingredients can occur at any stage of the production process; particularly, traces of allergens might be present on the raw ingredients or appear on the final product due to the use of shared equipment with other products in the same factory (Taylor et al., 2006). Food producers use precautionary warning labels to alert consumers of the potential occurrence of traces of allergenic ingredients. At the same time, many food manufacturers have implemented allergen control programs that are founded on risk assessment, such as those programs that are based on HACCP (hazard analysis and critical control points) principles to identify possible sources of undesired allergenic ingredients and to put in place corrective measures.

To ensure the compliance with food labeling and to improve consumer protection, reliable analytical methods for the detection and quantification of allergenic food ingredients are required (Besler, 2001; Poms et al., 2004). These methods should be robust and standardized to provide an appropriate tool for food and catering industries, as well as for food control laboratories (Kirsch et al., 2009). Analytical methods for the detection and quantification of allergenic ingredients in food products must be sensitive enough to be able to detect trace amounts. To fulfill these requirements, demanded methods should satisfy the usual criteria concerning analytical performance, such as sensitivity, specificity, reproducibility, precision and accuracy (EFSA, 2004), and their fitness-for-purpose should be assessed with defined reference materials and challenged with processed samples to investigate the possible limitations that may be associated with these analytical approaches.

METHODOLOGIES FOR FOOD ALLERGENS IDENTIFICATION AND CHARACTERIZATION

Targets for the detection of allergenic ingredients in foods

The identification of the target of analysis is an essential part of a newly developed analytical method because this identification will contribute to the specificity, sensitivity and quantification potential of the method, and therefore, its fitness-for-purpose will be directly affected. The analytical methods that are currently applied for the detection of allergenic ingredients in foods rely on the recognition of the allergen itself or, frequently, on the detection of a marker that indicates the presence of the allergenic ingredient.

Moreover, two types of markers are commonly used for the detection of allergens: characteristic proteins from the allergenic ingredient or specific DNA sequences indicating the presence of the allergenic ingredient. The choice of one marker or another depends greatly on the food product being analyzed because both protein- and DNA-based methods have advantages and disadvantages (Poms et al., 2004).

One of the most important factors to consider when choosing a target of analysis is the abundance of such target because this abundance will influence the sensitivity of the detection method (van Hengel, 2007). Additionally, the specificity highly depends on the chosen marker, and, therefore, we should keep in mind the final aim of the developed method to choose the proper marker; our analytical interest might focus on the detection of an specific ingredient or allergen (p.e.: soybean) or on a group of species/ingredients that are susceptible to triggering an allergenic reaction (p.e.: fish).

Concerning protein-based approaches, methods for the detection/quantification of an allergenic food component can target the allergenic protein itself or another protein that indicates the presence of the offending food. However, the best choice when selecting a target protein for such a method would be the monitoring of one/several of the allergenic proteins of the food. Directly targeting the allergenic protein itself instead of a non-allergenic protein, although more challenging if this allergenic protein is present in low abundance, unambiguously confirms the presence of the allergenic component, avoiding false positives (for instance, when the allergenic protein has been removed by the technological treatment of the food) and false negatives (for instance, when only the allergenic protein, and not the whole allergenic food, is used as an additive).

An overview of the major allergenic proteins that are present in food is compiled in Table 1, according to the Allergome database (www.allergome.org) (Mari et al., 2009). Most of these food allergenic proteins belong to a few families, such as albumins, globulins, and other storage proteins in plants, and muscle proteins in animal-related food (Fæste et al., 2011). Some food allergies, such as fish, mollusks and crustacean allergies, are usually caused by a dominant allergen (parvalbumins for fish and tropomyosin for mollusks and crustaceans) (Faeste et al., 2011), whereas several proteins with comparable allergenicity are responsible for those allergic reactions to foods from plant origins.

Another factor affecting the selection of a target protein/peptide is homology. When selecting a protein as a target for a detection/quantification method of an allergenic food component that can be obtained from different biological species/varieties, it is important to select a homogeneous protein that is conserved between the different species/varieties. The presence of isoforms must

also be examined. For instance, for the detection of lupin, the homogeneous γ-conglutin would be a better choice than the highly heterogeneous vicilins (Brambilla et al., 2009). Another option, when the detection method is peptide-based, would be the selection of a conserved sequence within the protein; although the whole protein can present a degree of heterogeneity, some of the proteolytic peptides that are derived from the sequence may be the same for different species (e.g., arginine kinase in shrimps (Ortea et al., 2009a)). Therefore, the proteins of interest from all the species/varieties that the food component can originate from must be tested for sequence homology, either by analyzing the sequences in the protein databases, if available, or by obtaining the amino acidic sequence, if the species is not yet described in the databases.

Although the detection of the allergen itself would be desirable, this detection is not always feasible because, for example, the allergen's chemical properties are not well characterized or because the sensitivity of the available methodology is not adequate. In such instances, a different approach might be taken, as previously mentioned, such as choosing a marker indicative of the presence of the offending food (Poms et al., 2004). For this purpose, DNA or proteins are usually targeted (van Hengel, 2007).

Both protein and DNA targets offer advantages and disadvantages, which should be evaluated to determine the proper choice for the best analytical results. Although protein-based methods are often used, the protein content is frequently affected by food processing and by biological variations, such as seasonal and geographical impacts (Poms et al., 2004). DNA targets are, therefore, interesting for quantification purposes and for methods where a high sensibility is required because DNA targets are not affected by the variability of the phenotype and because they present a higher thermal stability than most of the identified target proteins. Moreover,

thermal processing might also reduce the solubility of the target protein (Poms et al., 2004), which compromises the results of the analysis. Another important advantage of the use of DNA is the possibility of amplifying the number of copies of the initial DNA target by PCR, which enables higher sensitivity, and the possibility of quantification by the use of quantitative PCR (qPCR).

In contrast, some food allergenic ingredients present a high protein content and low DNA content (e.g., eggs), whereas the opposite tendency is observed for other allergenic ingredients (e.g., celery) (van Hengel, 2007), therefore, the choice of one or another target molecule will depend on the specific characteristics and requirements of the ingredient to be analyzed.

Concerning DNA targets, two main approaches are considered by research groups developing such methods: (i) the use of the coding sequences of allergenic proteins (Slowianek et Majak, 2011) or (ii) the use of any other specific DNA targets from the allergenic ingredient of interest. Because the purpose of the analytical method is the detection of the presence of the allergenic ingredient by an indirect approach, the choice of one approach over another approach should be directed by the analytical needs.

Among the possible DNA targets, commonly used targets include single copy or multiple copy targets, such as mitochondrial, chloroplast or other highly repetitive sequences. The latter targets have the advantage of allowing a higher sensitivity due to their higher number of copies per sample; however, if a reliable quantitative approach is required, single copy nuclear DNA is considered more feasible due to its more constant copy number in different cells from different tissues (Prado et al., 2012).

The selection of DNA targets is also influenced by the availability of DNA sequences in public databases. DNA sequences for some allergenic ingredients of interest are scarce; however, with high throughput and cost-effective techniques, such as next generation sequencing (NGS), an increasing number of research groups have access to such technology and, therefore, the number of available sequences are growing in public databases, which contribute to the development of DNA-based methods for allergen detection.

Protein-based methods

Detection methods are based on the determination of one or several marker proteins that are indicative of the presence of the offending food, preferably a specific allergenic protein. Protein-based methods can be classified as classical immunochemical methods and MS-based proteomics methods.

Classical protein-based methods

Methods for food allergen detection have classically relied on immunoassays, which are antibody-based detection assays for target proteins from the allergenic food. ELISA, in which IgG antibodies that are obtained from an immunized animal are used for the detection of the allergen, is the most widely used methodology for screening the presence of allergenic components in food products by food industries and by food control authorities. In ELISA, the quantification is based on the colored product that is produced by an enzyme that is coupled to the "detection" antibody (direct ELISA if the analyzed protein extract is immobilized directly in the microplate well support, or sandwich ELISA if only the targeted protein is captured using another specific antibody that is bound to the support) or to a secondary antibody that is directed versus the first one (indirect ELISA). Most of the reported ELISA methods for the detection of

food allergens in complex food matrixes consist of sandwich ELISA (Immer et al., 2004; Seiki et al., 2007; Morishita et al., 2008; Doi et al., 2008), although competitive ELISA, which consists of the competition of the detection antibody, which is coupled to the enzyme, for binding sites to a unlabeled antibody that has been pre-incubated with the sample, has also been used for food allergen detection (Sharma et al., 2009). ELISAs offer good sensitivity and ease of execution. Commercial ELISA kits, which usually employ polyclonal antibodies, are currently available for the detection and/or quantification of wheat, crustaceans, egg, peanut, soybeans, milk, almond, hazelnut, mollusks, lupin, sesame, mustard and buckwheat (Schubert-Ullrich et al., 2009) in complex food matrices, with LODs ranging from 0.1 to 20 ppm.

Lateral flow assays and dipstick tests are inexpensive, fast and easy-to-use variants of ELISA kits that are performed on a membrane strip; however, these methods are only for qualitative detection of proteins. These immunoassays are being used in food allergen analysis, and some commercial kits are available for the detection of almond, hazelnut, shellfish, gluten, peanut, milk, soybean and egg, with LODs ranging from 1 to 25 ppm (Schubert-Ullrich et al., 2009). Other immunochemical tests have scarcely been used for food allergen detection: the radio-allergosorbent test (RAST) and the dot immunoblotting test were applied to the analysis of milk lactalbumin (Frémont et al., 1996), as well as to the multiplex detection of peanut, hazelnut and Brazil nut (Blais et al., 2003) in several food products.

Generally, immunochemical methods, and ELISA kits in particular, offer good sensitivity and ease in execution; however, there are several major drawbacks that must be considered: (i) the cross-reactivity of antibodies with other proteins, which depends on the uniqueness of the epitope, can lead to false positives, which increase when polyclonal antibodies are used; (ii) food

matrix interferences because a food component may interact with the target proteins or interfere with the colorimetric absorption measurement (Weber et al., 2007); (iii) food processing can induce structural changes in protein conformations and produce non-enzymatic post-translational modifications (nePTMs), which affect protein stability and extractability, as well as epitope recognition (van Hengel, 2007; Hebling et al., 2012); (iv) adsorption of allergens onto solid matrices may destroy epitopes by altering the tridimensional structure of the protein, thus avoiding the antibody recognition in the case of conformational epitopes, or may modify its accessibility (Kaul et al., 2007); and (v) variability between manufacturers is usually found because different suppliers use different extraction solutions and different antibodies. To avoid false positives due to the cross-reactivity of antibodies with other proteins in the sample, proteins can be separated by 1-D or 2-D electrophoresis, and the isolated target protein can then be detected by immunoblotting using the specific antibody. Although immunoblotting is widely used for the identification of allergenic proteins using IgE from sera of allergic patients, it has also been used for detection purposes, such as the reported screening of as low as 5 ppm of allergenic hazelnut and almond proteins in chocolate (Scheibe et al., 2001). In spite of all the drawbacks that are indicated above, ELISA remains the method of choice for the detection and quantification of food allergens.

MS-based proteomics methods

Proteomics is defined as the large-scale analysis of proteins in a particular biological system at a particular time (Pandey and Mann, 2000). Proteomics includes the study of protein sequences and modifications, their functional roles, the interactions between proteins, their specific localizations and their abundances. Because proteins can act as biomarkers for many properties

of food throughout all the steps of the food chain, proteomics has become a promising strategy for food science applications. Studies that are related to the food composition, food quality, food safety and to the biological activity of food components are taking advantage of recent advances in proteomic methodologies, which are primarily MS-based (Gallardo et al., 2013).

The proteomic analysis of a sample commonly consists of one/several separation steps at the protein and/or peptide level (e.g., gel electrophoresis, LC), followed by mass spectrometry analysis. Two general proteomic workflows can be followed: (i) the bottom-up approach, which is also known as peptide-based proteomics, where proteins are digested with enzymes, such as trypsin, and the resulting peptide fragments are analyzed by MS (Pandey and Mann, 2000); and (ii) the top-down approach, where the whole proteins are fragmented directly inside the mass spectrometer, avoiding the variable step of digesting the protein (McLafferty et al., 2007). In both approaches, proteins and peptides are identified and characterized using the comparison of the MS experimental data with the calculated theoretical mass values that are derived from a protein database; the protein that best matches is assigned to the experimental data (Perkins et al., 1999). Due to the current limited performance of top-down based instruments, bottom-up approaches remain the most common workflows. These approaches can be further divided into gel-based and shotgun proteomic approaches. In gel-based proteomics, proteins are separated by two-dimensional gel electrophoresis (2-DE), excised from the gel and enzymatically digested into peptides that are analyzed by MS. The better example of this approach is peptide mass fingerprinting (PMF) (Pappin et al., 1993), where the peptide masses from the tryptic digestion of the 2-DE-isolated protein are obtained using MALDI-TOF MS and are assigned to a protein from a protein database. The separation of proteins by electrophoresis has some disadvantages

when compared with LC; the detection of low abundance and extremely acidic and basic proteins is limited. In contrast, in the widely used shotgun proteomic approaches, all the proteins in the sample are digested without previous fractionation, and the resulting peptide mixture is separated by high-throughput LC and analyzed by MS. Peptides are fragmented inside the mass spectrometer, and tandem mass spectrometry (MS/MS) data from each of the peptides can be obtained. Because mass differences between the MS/MS fragment ions correspond to the amino acid residual masses, the sequence of the peptide can be obtained by database searches (Peptide Fragmentation Fingerprinting, PFF) (Eng et al., 1994) or *de novo* sequencing (Shevchenko et al., 1997), leading to the identification of the protein. Figure 1 summarizes the most significant proteomic workflows that are used in food allergen research. Quantitative information at the protein level, such as the relative abundance of a protein between different samples or conditions or the absolute amount of the protein, can also be obtained from the MS or MS/MS read-outs using different workflows. A detailed overview of the different approaches that are used for quantitative proteomics can be found elsewhere (Panchaud et al., 2008).

As stated above, the best choice when selecting a target protein for a detection/quantification method of an allergenic food component would be the monitoring of one/several of the allergenic proteins of the food, unambiguously confirming the presence of the allergenic component. However, in some cases, non-allergenic proteins from the allergenic food are chosen as markers. In this sense, MS-based proteomics is used at three stages in the development of methods for food allergen monitoring: (a) in the identification of the allergenic protein, (b) in the characterization of the concrete peptide or protein that will be used in the final detection/quantification method, and (c) in the final method itself. A general overview of the

proteomic approaches that are currently used in food allergen characterization and quantification is compiled in Figure 1.

(a) For the identification of allergenic proteins, potential allergenic components are commonly detected by IgE-immunoblotting after separation by SDS-PAGE, using sera from allergic patients. Here, proteomics helps to identify the IgE-binding proteins: the selected bands/spots can be excised, digested with trypsin and analyzed by PMF MS or MS/MS. The protein is identified by a comparison of the experimentally obtained spectra with the protein databases and with some additional information, such as amino acid (partial) sequences, PTMs, or Mr, can be obtained, depending on the approach that is used for the analysis. This approach has been extensively applied for identifying food allergens, such as peanut, hazelnut (Hird et al., 2000), milk (Natale et al., 2004), wheat (Akagawa et al., 2007), fruits (Marzban et al., 2008), soybean (Krishnan et al., 2009), tomato (López-Matas et al., 2011) and crustacean allergens (Yu et al., 2003; Shiomi et al., 2008).

(b) Once the protein that is primarily causing the allergenic response has been identified (Table 1), the target peptides/proteins to be used in the detection method must be characterized: the amino acidic sequence and possible modifications if the method will be based on MS/MS or the molecular weight of the peptides/protein if the method will be based on MS without MS/MS fragmenting have to be investigated. Although this Mr and sequence information could have been obtained in the previous identification step, different isoforms can be present, and some differences are usually found between the same protein originating from different species and varieties, even when dealing with phylogenetically closely related ones. Therefore, all the particular species/varieties the food component to be detected can originate from must be

included in these characterization studies. MALDI-TOF PMF has been used to characterize allergenic proteins from different allergenic foods. Some examples are tree nuts (Rigby et al., 2008; Moreno et al., 2004) and crustaceans (Ortea et al., 2009b). The MALDI-TOF analysis of whole proteins, which is termed protein profiling, was used for the evaluation of milk proteins in infant formulas (Sabbadin et al., 1999). PFF, using LC-MS/MS, has also been used for the characterization of allergenic food proteins, such as several allergens from peanut (Shefcheck et al., 2006; Pedreschi et al., 2012), fish (Carrera et al., 2010) and crustaceans (Ortea et al., 2009a, Rahman et al., 2010).

(c) After the target peptide/protein is characterized, the final LC-MS-based method for the monitoring of the target molecule can be optimized. Table 2 shows an overview of the MS-based proteomic methods for the detection/quantification of allergenic food proteins. Classical data-dependent analysis (DDA), which consists of the MS/MS acquisition of the most abundant ions that are detected during a survey MS scan, has been used in food allergen monitoring methods. Some applications include the determination of egg allergens in mayonnaise and biscuits (Lee and Kim, 2010), as well as the detection of milk casein peptides in different food products down to 1.25 ppm (Weber et al., 2006), in white wines (LOD 50 µg /mL) (Monaci et al., 2010) and even in ancient food residue (Hong et al., 2012). Although the ion chromatograms for the peptide/protein of interest can be extracted from the raw MS file for the visual monitoring and even for quantification, DDA is better suited to discovery analyses, where the massive identification of proteins is pursued, than for detection/quantification methods. Even for discovery studies, DDA has the drawback of biasing the detected peptides towards those peptides originating from the highest abundance proteins. However, the great disadvantage of DDA is its

low selectivity because the mass spectrometer analyzes the most abundant peptides of the total digest, including those peptides that are derived from the food matrix proteins, instead of focusing on the target peptides/proteins, which tend to be in extremely low abundance. To increase sensitivity and selectivity, other approaches have been developed. A previous enrichment using combinatorial peptide ligand libraries and a further isolation of the target protein casein by SDS-PAGE allowed its detection in wines with a low LOD (Cereda et al., 2010; D'Amato et al., 2010). However, because at this step previous information about the proteins/peptides under study is available, the use of the MS acquisition modes that are included under the term directed analysis is recommended. These experiments are focused on specific ions that are selected by the operator, thus increasing reproducibility, sensitivity and selectivity, and allowing the identification, monitoring and quantification of low abundance peptides. Some examples of direct analysis acquisitions are selected ion monitoring (SIM), which consists of the detection of only the selected *m/z* values, and selected MS/MS ion monitoring (SMIM), where only those ions of interest are fragmented and recorded (Jorge et al., 2007) in such a way that selected ion chromatograms can be plotted for monitoring and quantification purposes. Unlike the SIM acquisition mode, in SMIM, the MS/MS spectra are obtained, achieving a higher specificity and, thus, obtaining less false positives than in SIM. Both SIM and SMIM have been used in a peptide-based approach for the confirmation and/or quantitative determination of allergenic proteins in different food matrices: peanut allergens in raw and roasted peanuts (Chassaigne et al., 2007), in breakfast cereals (Careri et al., 2008a) and in ice cream (Shefcheck et al., 2004); and milk in cookies and wine (Monaci et al., 2011). Ortea et al. (2011) reported a SMIM method that primarily focused on shrimp species identification, which can also be used

for the detection of the crustacean allergenic protein arginine kinase in food products. SIM of whole proteins has also performed well for the detection of milk in fruit juices (LOD 1 µg/mL) (Monaci et van Hengel, 2008). When the *m/z* of the precursor ion and of some of the product ions are known, the specific *m/z* pair precursor ion/product ion (termed transition) can be followed using the highly sensitive LC-MS/MS targeted acquisition mode, which is called selected-reaction monitoring (SRM) or multiple reaction monitoring (MRM) (Gallien et al., 2011). MRM is the reference quantitative MS-based technique to analyze small molecules and currently is being commonly used to verify and validate candidate biomarker proteins in a hypothesis-driven approach following discovery studies. Targeted MRM quantitative analysis provides high selectivity, sensitivity and a wide dynamic range. Moreover, absolute quantification can be achieved using isotopic synthetic peptides as standard analogs of the target peptides (Gerber et al., 2003). Marker peptides are measured, and the concentration is obtained from the calibration curve that is built with those standards. Using MRM, sensitivities reached are in the range of those sensitivities that are achieved by ELISA and qPCR methods, making the detection of allergens in complex and processed food matrices at low ppm levels possible, which ensures enough protection for food allergic consumers. Moreover, because selectivity and specificity are, together with sensitivity, the main challenges for analytical methods, the capacity of LC-MS/MS methodologies to multiplex many target peptides in a single run increases the performance of the analysis. For all these reasons, this quantitative proteomics approach is gaining importance as a confirmatory method for food allergen detection and quantification, over the routinely performed ELISAs. MRM that is focused on peptides originating from allergenic food proteins has been employed in methods for the quantification of peanut in different complex

matrices (Shefcheck et al., 2006; Careri et al., 2007; Pedreschi et al., 2012) with an LOD as low as 1 ppm, and of milk in dairy as well as other complex products (Mollé et al., 2005; Lutter et al., 2011; Ansari et al., 2011) with LODs ranging between 1 and 20 ppm. The LC-MS/MS approach has the additional advantage of being a multi-allergen screen, unlike ELISA where individual allergens must be detected by separate kits. Heick et al. (2011) used this multiplexing ability of the LC-MS/MS MRM approach to develop a method for the simultaneous detection and quantification of seven food allergens in flour and bread, namely peanut, walnut, hazelnut, almond, egg and milk, with LODs ranging from 3 to 70 ppm. In Table 2, the peptides and transitions (when available) that are used in all these MRM methods are summarized.

MS/MS can overcome the cross-reactivity drawback that is found in immunochemical methods, achieving sensitivity levels in the same range as the classical methods. However, some challenges must be considered when developing a method for the detection/quantification of food allergens in food products. For peptide-based LC-MS/MS approaches, the selection of more appropriate targets is critical (Johnson et al., 2011). When selecting the best peptides, not only do the requirements for general MRM assays apply (such as uniqueness to the protein of interest, proper size, absence of missed cleavages, no variability between protein isoforms, good ESI ionization, high MS signal response, retention time repeatability) but also the effect of food processing must be considered. The allergen structure (nePTMs, changes in the tri-dimensional conformation) and solubility can be altered by thermal (heating) and biochemical procedures (van Hengel, 2007), and the marker peptides should be present in both the native and processed food products. As with the immunochemical methods, food processing alters the extractability and solubility of allergenic proteins and, therefore, impacts detection, decreasing the sensitivity

of the MS detection method. The effect of the food matrix must also be considered. The food matrix is crucial for protein extraction, and interferences by non-targeted molecules should be prevented to avoid ion suppression and co-elution with the target molecules. Finally, reliable screening biomarkers have been found in only a few allergenic foods. As stated above, peanut and milk are, by far, the allergenic foods with a higher number of potential peptide markers that have been described for detection and/or quantification purposes, whereas other food commodities remain to be investigated. However, because it allows the fast, accurate, and highly sensitive and specific analysis of proteins, LC-MS/MS is widely used as a confirmatory technique over the classically performed immunochemical assays.

Improvements in MS-based methods

Regarding the use of MS-based proteomic tools, advances in the instrumentation will help to decrease the LOD levels and to increase selectivity. In DDA, detected peptides are biased towards those peptides originating from the highest abundance proteins. Alternatively, the use of the data-independent analysis (DIA) acquisition mode is currently growing due to recent innovations that have been introduced in MS instruments, such as the sequential window acquisition of all theoretic mass spectra (SWATH) (Gillet et al., 2012) and the elevated-energy MS (MS^E) (Geromanos et al., 2009) modes, where, instead of selecting one specific parent ion for fragmentation, all peptide ions that are present at a time in the mass analyzer are fragmented. These methods obtain quantification data for less abundant peptides in the same run that is used for peptide/protein identification. Monaci et al. (2013), combining DIA with high-resolution mass spectrometry (HRMS), recently reported LOD values in the range of 0.4 to 1.1 µg /mL for traces of caseinate and egg-white powders potentially remaining in white wines upon fining, and

MS^E has been used for the detection and quantification of a pathogen defense protein in the celery secretome (Blackburn et al., 2010). Even in the high selective and sensitive MRM methods, there is room for improvement; using the triggered MS/MS MRM acquisition in hybrid MS equipment, a confirmatory MS/MS spectrum is obtained when the MRM signal exceeds a specified threshold, thus reducing the occurrence of false positives and increasing the selectivity of MRM methods.

Currently, quantitation at the peptide level is preferable to quantitation at the protein level because the identification of intact proteins in complex matrixes is generally limited by ion suppression and peak overlapping of coeluting proteins. Future improvements in fractionation techniques and in the performance of high-resolution MS instruments will make the application of top-down proteomics to the high-throughput analysis of food allergens at the intact protein level possible. The introduction of whole isotopic-labeled proteins as internal standards (Brun et al., 2007) can help in the development of methods for the absolute quantification of food allergens using these top-down approaches or using the more traditional peptide-based MRM assays.

DNA-based methods

DNA-based methods for the detection of allergenic food ingredients have been previously reviewed (Goodwin, 2004; Poms et al., 2004a; Poms et al., 2004b; Mafra et al., 2008; Kirsch et al., 2009; Monaci et Visconti, 2010; Slowianek et Majak, 2011). As mentioned above, DNA targets for such methods might be genes that encode an allergenic protein or any other specific DNA marker. Most of the previously published reviews focus on the first case; however, a high

number of methods have been developed as well for the latter case. This approach has been proven effective for the detection of the presence of allergenic ingredients because DNA is an indirect marker of the presence of the allergenic ingredient.

Most of the published DNA-based methods for the detection of allergenic ingredients in food products are based on the amplification of the number of copies of the initial DNA target by PCR, which allows a high sensitivity for the developed method. The specificity of the method is achieved in such cases by the use of primers that would recognize a DNA fragment from the offending ingredient.

Several approaches that are based on PCR have been used for the detection of allergenic ingredients, including end-point PCR, PCR-ELISA, qPCR, multiplex PCR, qPCR, and, more recently, qPCR combined with HRM.

End-point PCR and PCR-ELISA

End-point PCR is the simplest and most likely the least expensive approach for DNA amplification; its main drawbacks are that this PCR method requires an additional step for the detection of the amplification product, which traditionally has been performed by gel electrophoresis and that this method is a merely qualitative technique.

For PCR-ELISA or ELOSA (enzyme-linked oligosorbent assay), the amplified DNA fragment is labeled with biotin or digoxygenin and is hybridized to an immobilized capture probe (Kirsch et al., 2009; Slowianek et Majak, 2011). In this way, the assay detects sequences that are internal to the PCR product; PCR-ELISAs can be useful for detecting and differentiating between multiple sequences/targets that might be amplified by a common set of primers, which is an advantage for the detection of multiple allergenic ingredients. The concentration of the DNA can be quantified

by the color reaction that results from an enzyme-substrate reaction due to the coupling of the protein label with a specific enzyme-labeled antibody (Poms et al., 2004). However, this approach can only be considered semi-quantitative because such quantification is performed at the end of the PCR reaction and not during the exponential growth phase, as in qPCR.

In a comparative study between PCR-ELISA and protein sandwich ELISA for the detection of hazelnut, comparable sensitivity was reported for both approaches and a higher specificity was reported for PCR-ELISA (Holzhauser et al., 2002).

qPCR

The quantitative real-time PCR (qPCR) allows the DNA amplification to be monitored while it is being produced because the signals (generally fluorescent) can be monitored as they are being generated during the amplification of the target DNA (Higuchi et al., 1992). As the amount of product accumulates, the signal increases exponentially, then the signal levels off and saturates because critical components of the reaction run out (Kubista et al., 2006). Initial template levels can be calculated by analyzing the shape of the curve or by determining when the signal rises above some threshold value.

In recent years, several groups have been developing qPCR based methods for food and feed analysis, including for allergenic ingredients in food, as shown in Table 3. Compared with end-point PCR, qPCR presents several advantages for its use for food analysis, including the possibility of using shorter fragments. Because there is no need for gel visualization (Hird et al., 2006), this technique is applicable to highly processed samples, which may have highly fragmented DNA, reporting better results than end-point PCR (Prado et al., 2007).

qPCR methods for food analysis typically make use of two detection chemistries. The first is a DNA binding dye, most commonly SYBR-Green, which is a cheaper alternative. However, there are some drawbacks, such as its lack of specificity to bind double-stranded DNA. The second is a hydrolysis probe approach, which usually uses TaqMan Probes that increase the specificity of the assays. With this methodology, a positive identification requires the effective binding of a specific probe in addition to the binding of the PCR primers (Lopez-Andreo et al., 2005; Pegels et al., 2013).

Simultaneously, qPCR allows the expression of the results as numerical values (Cq values), which provides more information about the kinetics of the reaction. Quantification cycle (Cq) values are calculated by determining the point at which the fluorescence that is produced in each sample reaches a set threshold limit. This situation occurs during the exponential phase of the reaction and, therefore, before some of the reagents are consumed because of amplification, which would negatively affect the reliability of the quantification results because at that point, the reactions start to slow down, and the PCR product is no longer being doubled at each cycle. Cq values are inversely related to the starting copy number of the target sequence (Giulietti et al., 2001).

QPCR has a quantitative potential for allergenic ingredients in foods; however, the quantification of such ingredients must be carefully evaluated to provide reliable results. First, it is impossible to use a relative quantification approach because, unlike other cases, such as GMO quantification, the change in the target DNA concentration cannot be compared with an endogenous reference gene. Some authors have overcome this limitation of the lack of an endogenous reference gene by using an internal standard material that is added to the samples

before the DNA extraction or DNA amplification (Hirao et al., 2006; Eugter, 2010; Demmel et al., 2012; Janska et al., 2012).

However, most published methods are based on an absolute quantification approach. Such methodology relies on the use of a standard curve that is constructed by amplifying known amounts of target DNA in a parallel set of reactions. This approach requires that both the known DNA (frequently plasmid or genomic DNA) and the investigated DNA are amplified with the same efficiency (Sivaganesan et al., 2006) to obtain reliable quantitative results. Additionally, quantification results are usually expressed as the DNA copy number or DNA concentration; therefore, developed methods must estimate the corresponding percentage of the allergenic ingredients to relate the analytical results with the percentage that is stated on the label. With the purpose of providing a solution to this problem, several authors have performed a calibration curve with food samples that have been spiked with different percentages of the allergenic ingredient to mimic how detection would be performed with real food samples, as shown in Table 3(Brezna et al., 2006; Hildebrandt and Garber, 2010; Demmel et al., 2011; Costa et al., 2012; López-Calleja et al., 2013).

The accuracy of the determination of traces of allergenic ingredients in foods will also be affected by the processing of the food product, which may affect the stability of DNA. Previously, it has been shown that temperature has a strong impact on the DNA copy number for heat-treated samples (Hildebrandt et Garber, 2010; von Holst et al., 2012), which can lead to an underestimation of the true concentration. Several groups have reported that heat treatments, such as the ones that are used during food processing, produce DNA fragmentation (Prado et al., 2004; Hird et al., 2006; Platteau et al., 2011b; López-Andreo et al., 2012).

Hildebrandt and Garber reported that the baking and pressure-cooking of salmon fillets resulted in a decrease in the detectable copy numbers of the parvalbumin gene (Hildebrandt et Garber, 2010). Likewise, the prolonged baking of peanut-spiked cookies caused a drop in peanut detectability at the lowest concentration levels for three qPCR assays (Scaravelli et al., 2009). Iniesto et al. studied the effect of roasting, high pressure and autoclave treatments on the detection of hazelnut allergen coding sequences and demonstrated that both heat treatments, roasting and autoclaving, reduced the ability to detect hazelnut DNA due to a decrease in the DNA extraction yield and in the DNA integrity (Iniesto et al., 2013). Furthermore, as has been shown for meat and bone meals (von Holst et al., 2012), there is no apparent linear dependence between the DNA copy number and the sterilization temperature, which should be considered for the estimation of the percentage of food and feed ingredients when these products have suffered intense or unknown heat treatments.

Another potential impact on the quantification of allergenic ingredients by qPCR is the composition of the food product to be analyzed because some of the constituents can inhibit the PCR reaction (Poms et al., 2004a; Kenk et al., 2012). Furthermore, Kenk et al. compared different isolation methods and showed that the yield and purity of the purified DNA greatly depends on the DNA extraction and purification method and that no universal method is valid for all food matrices (Kenk et al., 2012). For such cases, the use of an internal standard material that is added to the food product before the DNA extraction and purification can contribute to a more reliable quantification result because the degree of inhibition of the PCR due to the components of the food product and the yield and purity of the DNA extraction method can be evaluated through the effect of the internal standard material on the amplification profile.

Therefore, to provide reliable quantitative results for allergenic ingredients in food products, a careful evaluation of the qPCR method must be performed, taking into account the potential impact of the heat treatments, the matrix effect and the DNA extraction and purification system.

Other techniques

In recent years, several interesting approaches have been developed to detect and, in some cases, quantify DNA targets. Some of these approaches have a promising potential for the detection of allergenic ingredients in food products. These approaches include the use of high resolution melting analysis (HRM), digital PCR (dPCR), ligation-dependent probe amplification (LPA) and DNA arrays.

HRM analysis involves the gradual melting of PCR amplicons with increasing temperature and the detection of subtle fluorescent changes (Costa et al., 2012). Due to advances in both high resolution instrumentation and saturating fluorescent DNA binding dyes, the development of HRM analysis has been applied to genotyping (Herrmann et al., 2006; Reed et al., 2007), epigenetics (Wojdacz et Dobrovic, 2007), and authenticity studies in food products (Jaakola et al., 2010; Ganopoulos et al., 2011; Sakaridis et al., 2013), among others.

Applications of HRM analysis for the identification of food ingredients have been recently developed because differentiation down to the genus or even species level is now possible based on the melting temperature (Tm) of specific PCR products (Cheng et al., 2006). HRM has been recently used for detecting the presence of almond in food products; this approach has been shown to be able to discriminate almond from other *Prunus* fruits, such as walnut, macadamia nut and hazelnut (Costa et al., 2012). Madesis et al. have also used HRM that is combined with

DNA bar-coding (Bar-HRM); for the differentiation of legume species via the use of universal chloroplast bar-coding regions, this approach allowed the detection of lupine DNA in soybean flour with a detection limit of 1% (Madesis et al., 2012). Hazelnuts have also been quantitatively identified by Bar-HRM in processed food products, and a detection limit of 0.01% was confirmed (Madesis et al., 2013).

Another interesting new development with a potential application for the detection of allergenic ingredients in food is digital PCR. dPCR is based on the use of limiting dilutions, PCR and Poisson statistics to quantify the absolute number of targets that are present in a sample (Sykes et al., 1992). During dPCR, the PCR mix is distributed across a large number of partitions containing zero, one or more copies of the target nucleic acid. An end-point PCR is performed in each of these partitions and is defined as positive when the PCR product is present or negative when the PCR product is not present (Morriset et al., 2013). The use of binomial Poisson statistics allows the determination of the number of target nucleic acid molecules that are contained in the original sample before partitions from the ratio of positive to total partitions (Pinheiro et al., 2012). dPCR has been suggested as a good solution to overcome some of the difficulties that have been previously mentioned for establishing a quantitative result with qPCR (Corbisier et al., 2010). Morriset et al. have found that their approach for the quantitative analysis of genetically modified organisms (GMOs) showed comparable sensitivity, with better repeatability at low range concentrations and a greater tolerance to inhibitors when compared with qPCR (Morriset et al., 2013). In addition, dPCR allows the accurate measurement of DNA copies without the need for a reference calibrator and is not dependent on amplification efficiency (Corbisier et al., 2010). Taking into account the possible sources of uncertainties that

are related to DNA quantification by dPCR (Bhat et al., 2009; Bhat et al., 2011) and although, to our knowledge, no dPCR method in this particular area has been published thus far, this technique can be valuable for the estimation of allergenic ingredients in food products.

Due to the number of food allergens, the multiplex detection of different allergenic ingredients is of great interest for control laboratories. Several qPCR based methods have been developed in recent years for the simultaneous detection of two or more allergenic ingredients (Rossi et al., 2006; Schöringhumer et al., 2009; Köppel et al., 2010; Pafundo et al., 2010; Pafundo et al., 2011; Gómez-Galán et al., 2011; Janská et al., 2011; Köppel et al., 2012; Fuchs et al., 2013). Multiplex PCR methods have several advantages, including saving time, reducing the cost of analysis and lowering the probability of cross-contamination (Schöringhumer et al., 2009). However, multiplex qPCR frequently has lower sensitivity than simplex PCR (Rossi et al., 2006), which might require a careful optimization of the primer concentration to avoid the preferential amplification of some of the targets (Pafundo et al., 2010). Moreover, this technique is limited by the reduced number of fluorophores to determine several genes simultaneously (Tortaja-Genaro et al., 2011).

Ligation-dependent probe amplification (LPA) has been suggested as a good alternative to overcome some of the limitations of multiplex qPCR. LPA is based on the amplification of products that result from the ligation of bipartite specific hybridization probes (Ehlert et al., 2009) and uses a single pair of PCR primers to amplify several target sequences. One probe oligonucleotide contains the sequence that is recognized by the forward primer, and the other probe contains the sequence that is recognized by the reverse primer. Only when both probe oligonucleotides are hybridized to their respective targets, can these probes be ligated into a

complete probe; therefore, this technique yields an amplification product of unique size (Shouten et al., 2002) that can be discriminated by capillary electrophoresis. Because all PCR fragments are amplified by the same primer set, a competitive PCR is created where all the fragments are amplified with the same efficiency (Mustorp et al., 2011).

LPA has been used for the detection of 10 different allergenic ingredients in the low mg kg^{-1} range; when tested in different matrices, this method allowed an LOD of 5 mg kg^{-1} for single allergens (Ehlert et al., 2009). Taking advantage of the fact that all fragments are amplified with the same efficiency and, therefore, that the initial ratios of amplicons are conserved throughout the PCR, Mustorp et al. provided quantitative results using LPA. By comparing signals with an internal positive control (IPC) for 8 allergenic ingredients, the LOD varied from 5 to 400 genes copies, depending on the allergen (Mustorp et al., 2011).

To ease multiplex detection, several arrays have also been used for the detection of PCR products and applied to the detection of allergenic ingredients in food products. Rossi et al. used a PNA-array for the detection of previously amplified peanut and hazelnut DNA. Peptide nucleic acids (PNAs) are oligonucleotide analogues that bind to complementary DNA sequences and that show higher affinity and specificity than their DNA homologous (Egholm et al., 1993). In this approach, PNA-probes for each target that is amplified by the duplex PCR are deposited on commercial slides, and the target DNA sequence can be detected down to 1 nM (Rossi et al., 2006). An electrochemical DNA-array that was used for the detection of two different allergen specific DNA fragments from hazelnut allowed the detection of amplicons in the nanomolar range (Betazzi et al., 2008). Wang et al. have developed an optical thin-film microarray method on a silicon-based surface that has been tested for the simultaneous detection of eight food

allergenic ingredients from two tetraplex PCR systems for the simultaneous detection of cashew, peanut, wheat, soy, chicken, fish, shrimp and beef (Wang et al., 2011a) and for the simultaneous detection of sesame, oat, almond, celery, hazelnut, walnut, lupine and mustard (Wang et al., 2011b). In both cases, signals can be observed by the naked eye, without the need for additional instrumentation. Digoxin-labeled PCR products from hazelnut, peanut and soybean DNA were detected by hybridization with 5′-biotinylated probes that were immobilized on a streptavidin-modified digital versatile disk (DVD) surface, providing an LOD of 1 μg/g (Tortaja-Genaro et al., 2011).

An electrochemical DNA sensor that was based on a stem-loop probe dual that was labeled with 5′-SH and 3′-biotin was used for the detection of a fragment of the Ara h 1 gene for peanut detection. In this case, DNA was previously evaluated with different concentration of the target DNA and provided an LOD of 0.35 fM, with a linear response ranging from 10^{-15} to 10^{-10} M. The DNA sensor was tested with a DNA extract from peanut milk beverage without previous PCR amplification and gave a quantitative response (Sun et al., 2012).

FAST METHODS AND EMERGING TECHNOLOGIES

Reliable and rapid food allergen test methods are needed by both control laboratories and by the food industry to ensure the health of consumers, to easily determine whether a food product has been subjected to cross-contamination and, simultaneously, to identify how and when this cross-contamination occurred to establish the proper corrective measures. Rapid immunoanalytical tests for the determination of allergenic food proteins have been recently reviewed (Schubert-Ullrich et al., 2009); therefore, we will focus on recent advances, such as newly developed

biosensors for the detection of allergenic ingredients and on the use of nanomaterials for this application.

A biosensor is a device employing biological recognition properties for a selective bioanalysis (Kumar et Kumar, 2008). The main goal of the research in this field is the development of lab-on-a-chip systems that allow the fast, sensitive and inexpensive analysis of samples, without the cumbersome pre-analysis or preparation of samples, in contrast to the usual analytical techniques. This fact makes biosensors an interesting alternative to be used by small control laboratories or to perform fast in situ analysis by the food industry. Biosensors comprise three components: a biological receptor that is specific to the analyte to be analyzed, a transducer to convert the recognition element into a suitable signal and a reader device with the associated electronics or signal processors that are responsible for the display of the results. Target molecules, such as proteins or DNA, can be immobilized on a sensor chip surface and the binding activity between recognized molecules can be quantitatively monitored (Schurbeth-Ullrich et al., 2009).

Likewise, the appearance of more specific and stable ligands might greatly contribute to the development of more reliable detection methods and biosensors. In this sense, aptamers are gaining the attention of some research groups. Aptamers are single stranded (ssDNA) or RNA oligonucleotides that can bind diverse targets, such as small ions, proteins or even cells. Aptamers possess a high affinity for their targets due to their capability of folding upon binding with their target molecule (Song et al., 2008). Aptamers are selected in vitro from large populations of random sequences through a combinatorial strategy called SELEX (systematic evolution of ligands by exponential enrichment) (Tuerk et Gold, 1990). Aptamers are a relevant

alternative to antibodies in bioanalytical applications (Iliuk et al., 2011) due to their advantages when compared with antibodies, such as (i) the in vitro selection procedure and chemical synthesis of aptamers, which eliminates the need for the in vivo immunization of animals to generate antibodies; (ii) the possibility of targeting a new range of targets, including toxic or non-immunogenic materials; (iii) no batch to batch differences in activity; (iv) cost-effective synthesis; and (v) aptamers can be easily modified with a variety of functional groups or molecules that facilitate the immobilization of aptamers on a solid support or the introduction of a label (Palchetti et Mascini, 2012), which is of high importance when we want to develop faster, but reliable, analytical methods. Much work remains to be performed to integrate aptamers into biosensors for food allergen detection; however, several aptamers have been selected thus far for the detection of food allergens, including for the detection of egg white lysozyme (Tran et al., 2010), for Lup an 1 from lupin (Nadal et al., 2012) and for Ara h1 protein from peanut (Tran et al., 2013).

Another important step in the development of methods for the detection of allergenic ingredients in food products is the robustness of the method of analysis to provide reliable analytical results. In this sense, method validation is an essential part of providing reliable results that can be comparable among different laboratories (van Hengel, 2007; Monaci et Visconti, 2010). Different attempts are being made to establish guidelines for single and inter-laboratory validation (Thompson et Wood, 2002; Kerbach et al., 2009; Abbott et al., 2010; Sakai et al., 2012) that can be followed by research groups working on method development. In this sense, the development of reference materials (RMs) and, ideally, certified reference materials (CRMs) particularly in different matrices, is highly desirable for the validation of methods of analysis of

allergenic ingredients in food (Kerbach et al., 2009; Monaci et Visconti, 2010; Poms et al., 2010; Johnson et al., 2011).

Use of nanoparticles for analytical applications

Recent developments in nanotechnology are greatly impacting the improvement of some analytical methods due to the development of new nanostructures, nanodevices, nanomaterials and nanoparticles (NPs). Advances in nanotechnology contribute to the miniaturization of nanodevices, and nanomaterials and NPs present new structural and physicochemical properties that are not shown by the bulk matter, which makes these technologies an interesting alternative to some conventional reagents that contribute to increasing the sensitivity (Gómez-Hens et al., 2008). Nanoparticles are being used both in solution, as part of diverse analytical methods, and as part of biosensing devices (Valdés et al., 2009). Nanoparticles present some interesting advantages for sensing applications; their ease of synthesis, their high specific surface area and their high ability to be functionalized with a variety of biological moieties as peptides, DNA sequences, antibodies or aptamers, make them suitable as a platform for the detection of a range of biologically relevant analytes.

Likewise, inorganic nanomaterials, with a variety of unique intrinsic physico-chemical properties, have been reported in the literature. Due to their magnetic, optical, and electronic properties, several successful examples, such as iron oxide, quantum dots and metallic nanoparticles, are being used as labels to generate physical biorecognition signals (Xu et al., 2009). As an example of a variety of particles with interesting physical and magnetic properties are the magnetic properties of extremely small superparamagnetic iron oxide (SPIO) nanoparticles (López-Quintela et Rivas, 1996) and the surface plasmon resonance of gold NPs

(Liz-Marzán, 2006).

Based on the surface plasmon resonance of noble metal nanoparticles and on the florescent properties of quantum dots, two main approaches for protein and oligonucleotides detection were reported using nanoparticles either as a transducer or as a signal amplifier (Wang, 2006; Stadler et al., 2010; Hu et al, 2011).

The first approach is based on the changes of the local environment of the nanoparticles in solution that is mediated by the target, which generates a detectable modification of their physical properties. Indeed, a small change in the size, shape, local environment, surface nature, interparticle distance and the degree of aggregation of nanoparticles leads to tunable changes in their physical properties. Consequently, the assembly of the nanoparticles that are generated by the specific recognition of the biomolecular target with bio-conjugated NPs can be easily and sensitively detected following physical responses and enable optical, electrochemical, or magnetic detection (Rosi et Mirkin, 2005). The biorecognition between the biological target and the probes that are attached to the NPs might trigger the aggregation of NPs and induce interparticle interaction. As an example, colorimetric sensors that are based on the optical properties of gold nanoparticles were developed and have been demonstrated to be highly competitive technologies for oligonucleotides targets (Stadler et al., 2010; Chen et al., 2012).

This concept is based on the color change from red to blue as gold nanoparticles aggregate via the hybridization of DNA strands because of interacting surface plasmon and aggregate scattering properties. However, the optimization in terms of selectivity and specificity of nanoparticles-based DNA detection continues to be studied and, as a result, the development of

such methods thus far still require the use of pre-PCR DNA amplification or its incorporation in DNA detection assays, such as surface-enhanced Raman Scattering (SERS), surface plasmon resonance (SPR), or scanometric assay (Reynolds et al., 2010).

The second approach is based on the immobilization of the nanoparticles on a solid substrate (chip) during the recognition events. In most cases, the bio-conjugated nanoparticles act as secondary probes, which lead to an enhancement of the signal of the biosensing systems.

In recent years, core-shell NPs have attracted much interest because substantially different functionalities arising from the core and shell can be joined to obtain a complete set of physical potentialities in a single nanodevice (Levin et al., 2009; Davila et al., 2012), with interesting applications in biosensing. Moreover, metal atomic clusters are attracting much attention. Atomic clusters consist of groups of atoms with well-defined compositions and one or very few stable geometric structures; clusters are considered to be between atoms and nanoparticles or bulk metals, with properties different from both of them (Calvo et al., 2012). Both core-shell NPs and metal atomic clusters are promising for sensing applications because these materials can contribute to obtaining higher sensitivity and, simultaneously, improve multiplexing possibilities.

Nano- and microparticles are being applied to bioanalysis for food safety and environmental monitoring (Pividori et Alegret, 2010), not exclusively as labels, but also with other objectives, such as to contribute to the selection and/or separation of certain target molecules (Valdés et al., 2009). In allergenic ingredients in food, we can find some interesting examples of such applications. Nadal et al. used magnetic beads (uniform polymer particles that are composed of superparamagnetic iron oxide NPs), which are typically up to several micrometers in diameter,

that are conjugated to the β-conglutin subunit from lupin for the selection of a single stranded DNA aptamer that is specific for this allergenic protein (Nadal et al., 2012). Magnetic beads have also been used by Kenk et al. for magnetic capture hybridization (MCH) for the specific isolation of hazelnut DNA, by binding the target DNA to a specific probe, which was linked to magnetic beads via biotin-streptavidin interaction. This approach has been compared with other methods for the isolation of DNA for qPCR analysis; however, in this case, MCH showed low accuracy (Kenk et al., 2012). Likewise, magnetic microspheres are being used for the unspecific capture of genomic DNA by several commercial kits that are available for DNA extraction and purification (Arlorio et al., 2007).

The immobilization of bioconjugated nanoparticles into solid substrates has also been explored in recent years for the detection of allergenic ingredients in food, with interesting results. Hohensinner et al. have adjusted a conventional ELISA for the detection of the milk allergen β-lactoglobulin (β-LG) to a cluster-linked immunosorbent assay (CLISA) by labeling the antibody with monodisperse colloidal gold clusters and by making use of the resonance enhanced absorption (REA) effect. The REA effect is detected when light absorbing metal clusters are within nanodistance from a highly reflective mirror and are illuminated with white light of the visible and near-infrared spectrum. This system provides a sensitive color response, whose intensity directly correlates with the amount of clusters that are deposited by affinity binding. The method was evaluated with food products and gave comparable results to other immunoassays in terms of specificity and sensitivity (Hohensinner et al., 2007). Likewise, a similar approach was used for the detection of the egg white allergens ovalbumin and ovomucoid with a detection limit of 1 ng/mL (Maier et al., 2008).

A fiber optic surface plasmon resonance (SPR) biosensor with nanobead signal enhancement was evaluated for the detection of Ara h1 peanut allergen in complex food matrices. The use of magnetite NPs as a secondary label improved the detection limit of the SPR bioassay by two orders of magnitude from 9 to 0.09 μg/mL (Pollet et al., 2011).

Cao et al. proposed an electrochemical immunosensor for the detection of casein, which was based on gold nanoparticles and poly (L-arginine)/multi-walled carbon nanotubes composite film. According to the authors, the assay has an LOD of 5×10^{-8} g mL^{-1}, and its applicability to the determination of casein in cheese samples was successfully tested (Cao et al., 2011).

A new ELISA format, which is based on antibody-dendrimer-conjugated magnetic microparticles (MPs), has been developed for the detection of peanut allergen Ara h3/4 in food. The use of MPs allowed the captured Ara h3/4 allergen to be easily harvested from the incubation solutions with a magnet, which allowed its detection and quantification, whereas the functionalization of the MPs with PAMAM-sodium carboxylate dendrimers G 1.5 provided better stability and an enhancement of the analytical sensitivity for the assay. The system was evaluated with spiked corn flakes and biscuits that provided an LOD of 0.2 mg peanut/kg matrix (Speroni et al., 2010).

Recently, Pilolli et al. discussed several critical aspects of biosensor development based on integrating nanotechnology for food-allergen management, concluding that nanomaterial-based biosensors are promising tools, however deep investigation of their application to food matrixes must be performed in order to evaluate possible technological issues (Pilolli et al., 2013).

CONCLUSIONS AND FUTURE TRENDS

This article reviews the recent advances in DNA and protein based techniques for the detection of food allergens. The advantages and disadvantages of choosing either proteins or DNA targets have been addressed, and the applicability of recently developed methodology that are based on those targets to food products has been discussed.

Due to the multiplexing ability of MRM protein-based methods and to the new developments in MS instrumentation, proteomic workflows are being widely used as an alternative to classical DNA-based and immunochemical methods. Likewise, several new developments for DNA detection, such as HRM, dPCR, LPA or DNA arrays, are increasing the possibilities of PCR and qPCR approaches, with higher specificity, sensitivity, or multiplexing possibilities.

Aptamers, which are single stranded (ssDNA) or RNA oligonucleotides that can bind diverse targets, are also being studied as an interesting alternative to antibodies in bioanalytical applications. Several aptamers have already been selected for the detection of food allergens and will surely contribute to the development of new detection methods for allergenic ingredients in food.

There is an increasing need for fast methods to allow greater control of the presence of food allergens and to protect the consumer. In this sense, the use of biosensors, particularly making use of novel nanomaterials, appear to be a promising alternative for the detection of protein or DNA targets, but with the advantage of providing multiplexing possibilities to detect or even quantify the presence of several allergenic ingredients. Complex and core-shell NPs, particularly clusters with new physico-chemical properties, appear to be promising for sensing applications

because these materials might contribute higher sensitivity and faster methods of analysis, which should be further investigated soon.

Finally, the validation and harmonization of analytical methods for the detection of allergenic ingredients in food products is required to provide reliable and comparable analytical results. In this sense, both guidelines for the validation of methods and reference materials are needed and are currently being produced. Likewise, newly developed fast methods, such as those methods that are based on biosensors, will also need to be validated, and their applicability to real food samples must be evaluated.

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Table 1. Major allergenic proteins in food.

Food	Biological species	Allergenic proteins
Cereals (gluten)	Wheat (*Triticum aestivum*), rye, barkey, durum wheat	Gliadin (Tri a 19), glutenin (Tri a 26), LTP (Tri a 14), profiling (Tri a 12)
Crustaceans	Shrimps (*Penaeus monodon*, *P. aztecus*, *Litopenaeus vannamei*), crabs, lobsters, crayfishs	Tropomyosin (Pen a 1), arginine kinase (Pen m 2, Lit v 2), Myosin (Lit v 3), SCP (Lit v 4)
Eggs	Hen (*Gallus domesticus*)	Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), lysozyme (Gal d 4), serum albumin (Gal d 5), phosvitin (Gal d 6), apovitellin VI (Gal d Apo VI)
Fish	More than 50 fish species (e.g. *Gadus morhua*)	Parvalbumin (e.g. Gad m 1)
Milk	Cow (*Bos domesticus*), goat (*Capra aegagrus hircus*), sheep (*Ovis aries*), water buffalo (*Bubalus bubalis*)	α-lactalbumin (Bos d 4), β-lactoglobulin (Bos d 5), serum albumin (Bos d 6), immunoglobulin (Bos d 7), caseins (Bos d 8, Cap h, Ovi a and Bub b caseins)
Tree nuts	Nut producing plants, e.g. hazelnut (*Corylus avellana*), walnut (*Juglans regia*), almond (*Prunus dulcis*), Brazil nut (*Bertholletia excelsa*), Cashew nut (*Anacardium*	2S albumins (Jug r 1, Ber e 1, Ana o 3, Cor a 14, Pru du 2S albumin), 11S globulins (Cor a 9, Ana o 2), 7S globulins or vicilins (Cor a 11, Jug r 2, Ana o 1, Pru du 6), LTPs (Cor a 8, Jug r 3, Pru du 3, Pru

	occidentale), Macadamia nut (*Macadamia integrifolia*) and Pecan nut (*Carya illinoinensis*)	du 4), pollinosis-associated proteins (Cor a 1, Pru du 1), profilins (Cor a 2, Jug r 5, Pru du 4)
Peanut	*Arachis hypogeae*	11S globulin (Ara h 3/4), 7S globulin or vicilin (Ara h 1), 2S albumins (Ara h 2, Ara h 6, Ara h 7, Ara h 9), pollinosis-asociated protein (Ara h 8), profilin (Ara h 5)
Soybean	*Glycine max*	11S globulin (Gly m 6), 7S globulin (Gly m 5), 2S albumin (Gly m 2S albumin), LTP (Gly m LTP), profilin (Gly m 3)
Celery	*Apium graveolens*	Pollinosis-associated protein (Api g 1), profilin (Api g 4), LTP (Api g 2), glycoprotein (Api g 5)
Lupin	Edible Lupinus species: Lupinus albus, *Lup. angustifolius*, *Lup. luteus*, *Lup. mutabilis*	11S globulin (Lup a and Lup an α-conglutins), 7S globulin (Lup a 1 and Lup an 1 β–conglutins), 2S albumins (Lup a and Lup an δ-conglutins), basic globulin (Lup a and Lup an γ-conglutin)
Mollusks	Shells, clams, mussels, oysters, scallops, squids, octopus, abalones, cuttlefish	Tropomyosin (e.g. Myt e 1)

Mustard	White (*Sinapis alba*), Oriental (*Brassica juncea*) and Black (*Brassica nigra*) mustard	2S albumins (Sin a 1, Bra j 1), 11S globulin (Sin a 2), LTP (Sin a 3), profilins (Sin a 4, Bra ni 8)
Sesame seeds	*Sesamum indicum*	11S globulins (Ses i 6, Ses i 7), 2S albumins (Ses i 1, Ses i 2), 7S globulin (Ses i 3), LTP (Ses i LTP), oleosins (Ses i 4, Ses i 5), profilin (Ses i 8)
Buckwheat	*Fagopyrum esculentum*, *Fagopyrum tataricum*	2S albumins (Fag e 2, BWp16), 13S globulin (Fag e 1), 11S globulin (Fag t 1)
Fruits	Peach (*Prunus persica*), Apple (*Malus domestica*), kiwifruit (*Actinidia chinensis*), pineapple (*Ananas comosus*), strawberry (*Fragaria ananassa*), grape (*Vitis vinifera*)	Pathogenesis-related protein (Pru p 1, Mal d 1, Fra a 1), non-specific LTP (Pru p 3), proteases (Act c 1, Ana c 2), kiwellin (Act c 5)
Vegetables	Carrot (*Daucus carota*), lettuce (*Lactuca sativa*), tomato (*Lycopersicon esculentum*)	Ribonuclease (Dau c 1), non-specific LTP (Lac s 1, Lyc e 3, Vit v 1), profiling (Dau c 4)

Table 2. MS-based proteomics methods for detection/quantification of allergenic components in foods.

Allergenic Food	Matrix	Protein	Peptide	Target m/z or transition	Technique	Reference	LOD
Peanut	—	Ara h 1	NNPFYFPSR		LC-MS/MS (precursor XIC)	Hebling et al., 2012	
		Ara h 2	CCNELNEFENNQR				
		Ara h 3	SQSENFEYVAFK				
	Breakfast cereals	Ara h 3/4	AHVQVVDSNGDR	649→761	LC-MS/MS SMIM	Careri et al., 2008a	3 ppm
			SPDIYNPQAGSLK	695→977			
	—	Ara h 1	VLLEENAGGEQEER	786.88	LC- MS/MS DDA and LC- MS SIM (with MS/MS	Chassaigne et al., 2007	
			DLAFPGSGEQVEK	688.85			
		Ara h 2	RQQWELQGDR	439.23			

	Ara h 3	SPDIYNPQAGSLK	695.35	confirmation)		
		SQSENFEYVAFK	724.84			
Ice cream	Ara h 1	NNPFYFPSR(R)	571.3	LC-MS/MS DDA and SMIM	Shefcheck et al., 2004	10 ppm
		SFNLDEGHALR	629.8			
		NTLEAAFNAEFNEIR(R)	869.9			
		IFLAGDKDNVIDQIEK	606.6			
Complex matrixes and cereal-chocolate snacks	Ara h 2	CCNELNEFENNQR	807→1050	MRM	Careri et al., 2007	5 ppm
		CMCEALQQIMENQSDR	950→1120			
	Ara h 3/4	AHVQVVDSNGDR	649→1089		Careri et al., 2008b	1 ppm
		SPDIYNPQAGSLK	695→700			
Cookies	Ara h 3	SPDIYNPQAGSLK	695.4→1302.7 / 977.5 / 814.5 /	MRM	Pedreschi et	10 ppm

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			700.4 / 475.3 / 300.1		al., 2012	
		AHVQVVDSNGNR	432.5→749.5 / 663.3 / 535.4			
		LNAQRPDNR	361.9→970.5 / 856.4 / 657.4 / 228.1			
	Ara h 1	DLAFPGSGEQVEK	688.8→1077.5 / 930.5 / 833.4 / 447.2 / 300.2 / 229.1			100 ppm
Chocolate	Ara h 1	VLLEENAGGEQEER	786.9→213.2 / 804.4 / 989.4	MRM	Shefcheck et al., 2006	2-10 ppm
		DLAFPGSGEQVEK	688.9→229.1 / 300.2 / 930.5			
Flour and bread	Ara h 1	DLAFPGSGEQVEK	688.8→300.2 / 930.6	MRM	Heick et al., 2011a	11ppm
		GTGNLELVAVR	564.4→557.5 / 686.6			
	Ara h3/4	RPFYSNAPQEIFIQQGR	684.5→748.6 / 836.5		Heick et al., 2011b	
		WLGLSAEYGNLYR	771.4→272.2 / 1242.6			

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			DLPNECGISSQR	688.2→477.2 / 1147.4			70ppm
Walnut	Flour and bread	Jug r1	QCCQQLSQMDEQCQCEGLR	820.2→345.5 / 1294.3			
			GEEMEEMVQSAR	698.3→820.5 / 949.4			
Hazelnut	Flour and bread	11S globulin	ADIYTEQVGR	576.3→689.4 / 852.5	MRM	Heick et al., 2011a	5 ppm
			INTVNSNTLPVLR	720.9→484.4 /1013.6			
			QGQVLTIPQNFAVAK	807.5→874.6 /1088.6			
			ALPDDVLANAFQISR	815.5→906.6 / 1019.5		Heick et al., 2011b	3 ppm
Almond	Flour and bread	Prunin	GNLDFVQPPR	571.9→369.4 / 858.6			
			GVLGAFSGCPETFEESQQSSQQGR	896.1→662.4 / 790.4			
			ALPDEVLANAYQISR	830.4→922.5 / 1035.5			
			NGLHLPSYSNAPQLIY	780.8→735.7 /1154.7			

			IVQGR			
Milk	Infant formulae	Different milk proteins		MALDI-TOF MS protein profiling	Sabbadin et al., 1999	
	Different milk products	β-lactoglobulin		LC-MS (whole protein)	Czerwenka et al., 2007	
	White wines	Different bovin caseins	Many peptides reported	LC-MS/MS of spots from SDS-PAGE gels	Cereda et al., 2010	1 µg/L
	Red wines	αS1-casein		LC-MS/MS of spots from SDS-PAGE gels	D'Amato et al., 2010	3.8 µg/L
		Albumin				
		β-lactoglobul				

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	in					
Fruit juices	α-lactalbumin		1419.7; 1577.3	LC-MS SIM (of whole proteins)	Monaci & van Hengel. 2008	1 µg/mL
	Lactoglobulin A		1082.2; 1149.7; 1226.2; 1313.6			
	Lactoglobulin B		1077.1; 1144.1; 1220.5; 1307.5			
Ancient food	αS1-casein	FVVAPFPEVFR		LC-MS/MS DDA	Hong et al., 2012	
		YLGYLEQLLR				
		FFVAPFPEVFGK				
	B-casein	DMPIQAFLLYQEPVLGPVR				
Cookies,	αS1-casein	YLGYLEQLLR	634.2	LC-MS/MS DDA	Weber et al.,	

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chocolates, baby foods, desserts, sausages, ground meat		FFVAPFPEVFGK	692.8			1.25 ppm
White wines	αS1-casein	FFVAPFPEVFGK	692.6	LC-MS/MS DDA (precursor XIC)	Monaci et al., 2010	50 µg/mL
		YLGYLEQLLR	634.2			
	B-casein	DMPIQAFLLYQEPVLGPVR	729.2			
		GPFPIIV	742.2			
White wines	αS1-casein	FFVAPFPEVFGK	692.9860	LC-MS/MS DIA (XIC)	Monaci et al., 2013	0.4-0.7 µg/mL
		YLGYLEQLLR	634.3556			0.6-0.9

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						µg/mL
	B-casein	GPFPIIV	742.4500			0.5-0.9 µg/mL
Cookies and wine	αS1-casein	FFVAPFPEVFGK	692.869→1090.59 / 991.52 / 920.49	LC-MS/MS (precursor XIC and SMIM)	Monaci et al., 2011	1.6-22 ppm
		YLGYLEQLLR	634.356			
		HQGLPQEVLNENLLR	880.476			
	B-casein	GPFPIIV	742.450			
Dairy products	κ-casein macropeptide	VQVTSTAV	805→228	LC-MS/MS SIM and MRM	Mollé et al., 2005	10 pmol
Flour and bread	Casein α S1	YLGYLEQLLR	634.3→249.2 / 991.3	MRM	Heick et al., 2011a	5ppm
		FFVAPFPEVFGK	692.9→920.3 / 991.3			

	Casein α S2	NAVPITPTLNR	598.3→158.3 / 911.4		Heick et al., 2011b	
		FALPQYLK	490.3→120.1 / 648.4			
Baby food, cereals, infant formula	B-casein	AVPYPQR	415.5→400.2 / 660.3	MRM	Lutter et al., 2011	1-20 ppm
	β-lactoglobulin	TPEVDDEALEK	623.5→819.4 / 918.4			1-20 ppm
		VLVLDTDYK	533.3→853.4 / 754.4			1-5 ppm
	αS2-casein	ALNEINQFYQK	684.3→827.4 / 940.5			1-50 ppm
		FALPQYLK	490.1→648.4 / 761.5			1-5 ppm
	κ-casein	YIPIQYVLSR	626.3→975.6 / 765.4			1-20 ppm
Milk in dairy and chocolate products	α-lactoglobulin	VGINYWLAHK	601.2→284.4 / 355.4 / 654.4	MRM	Ansari et al., 2011	1-21 ng/mL
	β-	IPAVFK	338.0→561.6 / 282.0 / 294.4			3-11

		lactoglobulin	LIVTQTMK	467.6→707.6 / 227.4 / 608.6		1-15 ng/mL
		α-casein	YLGYLEQLLR	634.8→249.4 / 771.8 / 991.7		1-198 ng/mL
			FFVAPFPEVFGK	693.3→267.3 / 676.6 / 920.8		1-182 ng/mL
		β-casein	GPFPIIV	742.5→441.5 / 512.4 / 625.6		1-114 ng/mL
			VLPVPQK	780.6→372.4 / 213.2 / 568.6		1-69 ng/mL
Egg	Mayonnaise, biscuit	Ovalbumin (Gal d 2)	ISQAVHAAHAEINEAGR	591.97	LC-MS/MS DDA	Lee and Kim, 2010
		Vitellogeni	SAVSASGTTETL	562.27		

	n II	RFPAVLPQMPL	634.86			
	Vitellogenin I	VAGNVQAQITPSPR	769.04			
Flour and bread	Ovalbumin	HIATNAVLFFGR	673.4→223.2 / 1095.6	MRM	Heick et al., 2011a Heick et al., 2011b	42ppm
		YPILPEYLQCVK	761.6→810.5 / 1036.4			
		DILNQITKPNDVYSFSLASR	761.6→201.1 / 930.5			
		ELINSWVESQTNGIIR	929.5→1017.5 / 1116.5			
Lysozyme in white wine	Lysozyme C	HGLDNYR		MRM	Cryar et al., 2012	
		FESNFNTQATNR				
		GTDVQAWIR				
White wines	Ovalbumin	LTEWTSSNVMEER	791.3640	LC-MS/MS DIA (XIC)	Monaci et al., 2013	0.4-0.7 µg/mL

			GGLEPINFQTAADQAR	844.4230		0.4-0.6 µg/mL
			ELINSWVESQTNGIIR	929.9860		0.4-0.7 µg/mL
		Lysozyme C	NTDGSTDYGILQINSR	877.4212		0.8-1.1 µg/mL
			GTDVQAWIR	523.2700		0.8-0.9 µg/mL
Shrimps and prawns	—	Arginine kinase	TFLVWVNEEDHLR	829.4→1197.4		
			LTNAVNEIEKR	643.8→788.3	LC-MS/MS SMIM	Ortea et al., 2011
			SFLVWVNEEDQLR	817.9→1188.4		
Hake	Commercial sea-foodstuffs	Parvalbumin	LFLQVFSAGAR	604.84→948.52	LC-MS/MS SMIM	Carrera et al., 2011

Fish	Commercial sea-foodstuffs	β-PRVB	19 peptides	19 transitions reported	LC-MS/MS SMIM	Carrera et al., 2012
Soybean	Heat-processed meat products	Glycinin G4, subunit A4	VFYLAGNPDIEYPET MQQQQQQK		2D-LC-MS/MS DDA	Leitner et al., 2006
			QGQHQQEEEEEGGSV LSGFSK			
Lupin	Lupin beverage and lupin protein isolates	β-conglutin precursor	NTLEATFNTR		LC-MS/MS DDA label-free	Locati et al., 2006
			DQQSYFSGFSR			
			QAYNLEYYGDALR			
		Vicilin-like protein	NFLAGSEDNVIR			
		γ-conglutin	AGIALGTHQLEENLV VFDLAR			

Mussels	—	Tropomyosin	EVDRLEDELLTEKEKYK	1069	2-DE-LC-MS/MS (XIC)	López et al., 2002
Soy	Skimmed-milk powder	glycinin	Numerous peptides		LC-MS/MS DDA	Luykx et al., 2007
		β-conglycin	Numerous peptides			
Pea		legumin	Numerous peptides			
		vicilin	Numerous peptides			
Gluten	Bread, wheat starch and commercial gluten-free foods	gliadins			MALDI-TOF MS Protein profiling	Camafeita et al., 1997
	Maize and rice-based	gliadins			MALDI-TOF MS	Hernando et

foods				Protein profiling	al., 2003	
Beer	hordeins	(non specified)		LC-MS/MS DDA	Weber et al., 2009	
Different foods and beverages	α-gliadin	LQPQNPSQQQPQEQVPL	980.7→866.6 / 1150.7	MRM	Sealey-Voyksner et al. 2010	0.001-0.030 ppm
		VPVPQLQPQNPSQQQPQEQVPL	1240.9→1126.7 / 762.4			
		RPQQPYPQPQPQY	814.6→407.3 / 1221.8			
	γ-gliadin	TQQPQQPFPQQPQQPFPQ	1075.9→956.6 / 1195.6 / 244.4			
		QPQQPFPQTQQPQQPFPQ	717.6→244.1; 1075.9→726.3 / 1308.8			
	glutenin	PQQSPF	703.4→441.4 / 263.3			

Abbreviations used: 2-DE, two-dimensional electrophoresis; β-PRVB, parvalbumin beta; DDA, data-dependant analysis; ; DIA, data-independent analysis; LC, liquid chromatography; LOD, limit of detection; LTP, Lipid Transfer Protein; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; nePTM, non-enzymatic post translational modification; SIM, selected ion monitoring; SMIM, selected MS/MS ion monitoring; XIC, extracted ion chromatogram

Table 3: PCR and qPCR based methods for food allergen detection

Allergenic ingredient	DNA marker	Technique	Matrix	LOD (as indicated by authors)	Reference
Almond	Prunin-1 (Pru 1) and rbcL genes	SYBR GreenER™ qPCR	Genomic and plasmidic DNA	1 copy	Pafundo et al., 2009
	Pru 1 gene	SYBR GreenER™ Multiplex qPCR	Plasmidic DNA	5 pg of almond DNA	Pafundo et al., 2010
			Biscuits	100 mg kg^{-1}	
	Pru A-1 gene	Tetraplex qPCR	Genomic DNA in herring sperm solution	0.0032%	Köpel et al., 2010
	Pru A-1 gene	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice cookies	5 ppm	

	Pru du 5 allergen gene	End-point PCR, EvaGreen ® qPCR + HRM	Binary (almond/walnuts) Model mixtures	0.005%	Costa et al., 2012b
	Non specific lipid transfer protein (nsLTP) gene	SYBR® Green I and Taqman® qPCR	Chocolate bar Chocolate cookie Cookies	5mg kg^{-1}	Röder et al., 2011
Hazelnut	Cor a 1.04 (cDNA) Cor a 1.03 (cDNA)	End-point PCR + DNA-array	Biscuits and snacks	0.3nmol L^{-1} (Cor a 1.04) 0.1nmol L^{-1} (Cor a 1.03)	Bettazzi et al., 2008
	Cor a 1 gene	SYBR® Green I Duplex qPCR	Whole meal cookies	50 ppm	Schöringhumer et al., 2009
	Cor a 8 or Lipid Transfer Protein	SYBR® Green I	Genomic and plasmidic DNA	9.6 pg (Cor a 8)	D'Andrea et al., 2009

	(LTP) genes	qPCR	Spiked wheat flour	0.001%	
	Cor a 9, 11 and 13 genes	SYBR® Green I qPCR	Defatted hazelnut flour on defatted peanut flour	1 ppm raw hazelnut	Iniesto et al., 2013
	Cor a 1.0401 gene	PCR-ELISA	Genomic DNA	≤ 10 ppm	Holzhauser et al., 2002
	Cor a 1, 8 and 14 genes	End point PCR SYBR® Green I qPCR	Defatted hazelnut in wheat	10 ppm	D'Andrea et al., 2011
	Hsp1 gene	Taqman® qPCR	Genomic DNA	13 pg	Piknová et al., 2008
			Model pastry samples	0.01%	
	Cor a 1.04 gene	SYBR® Green I and Taqman® qPCR	Genomic DNA	0.1 ng	Arlorio et al., 2007
	Cor a 1 and Cor a 8	Taqman®	Genomic DNA	3.2 pg	Platteau et al.,

	genes	qPCR			2011a
	Cor a 8 gene	Taqman® qPCR	Genomic DNA 3 different Flour spiked with defatted hazelnut powder Cookies	Dependin g on matrix and baking	Platteau et al., 2011b
	Hsp1 gene	Single-tube nested qPCR	Genomic DNA	0.5 pg	Costa et al., 2012a
			Spiked wheat material	50 mg/kg	
	Cor a 1 gene	SYBR GreenER™ Multiplex qPCR	Plasmidic DNA	5pg	Pafundo et al., 2010
	Cor a 1 gene	Tetraplex qPCR	Genomic DNA in herring sperm solution	0.0032%	Köpel et al., 2010
	Cor a 1 gene	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice	5 ppm	

			cookies		
	Cor a 8 or LTP genes	Duplex Taqman® qPCR	Bakery products	Not indicated	Janska et al., 2011
	Cor a 1.0301 gene	Duplex end-point PCR + PNA-array	Genomic DNA	50 pg	Rossi et al., 2006
Soy	Gly m Bd 28K and Gly m Bd 30k genes	Taqman® qPCR	Genomic DNA	1.28 pg	Platteau et al., 2011a
	Lectin (Le1) gene	Tetraplex qPCR	Genomic DNA in herring sperm solution	0.0032%	Köpel et al., 2010
	Le1 gene	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice cookies	5 ppm	
	Le1 gene	End-point PCR Taqman® qPCR	Genomic DNA	40 pg (end-point PCR) 10 pg (Taqman	Espiñeira et al., 2010

				® qPCR)	
			Sunflower/olive oil spiked with soy protein powder	0.0625% (end-point PCR) 0.05% (Taqman ® qPCR)	
			Spiked corn flour	100 mg/kg (end-point PCR) 10 mg/kg (Taqman ® qPCR)	
	Le1 gene	End-point PCR Taqman® qPCR	Genomic DNA	10 pg	Soares et al., 2010
			Unprocessed binary mixtures	0.1%	
			Heat processed binary mixtures	0.5%	
	Le1 gene	End-point PCR	Spiked wheat flour with soybean flour	0.2%	Gryson et al., 2008

			Spiked wheat flour with soy protein isolate	1.0%	
	Mitochondrial, tRNA-MET gene	Duplex Taqman® qPCR	Baked spiked cookies (180˚C/10 min)	2.5mg/kg	Gómez-Galán et al., 2011
	Le1 gene	Taqman® qPCR	Spiked sausages	10 mg/kg	Siegel et al., 2012
Sesame	Ses i 1 gene	SYBR® Green I Duplex qPCR	Whole meal cookies	50 ppm	Schöringhumer et al., 2009
	Ses I gene	SYBR GreenER™ Multiplex qPCR	Plasmidic DNA	0.5pg	Pafundo et al., 2010
	Oleosin mRNA	Tetraplex qPCR	Genomic DNA in herring sperm solution	0.0032%	Köpel et al., 2010
	Oleosin mRNA	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012

			Spiked rice cookies	5 ppm	
	2S albumin gene	Taqman® qPCR	Genomic DNA	1 molecule	Mustorp et al., 2008
			Spiked barbeque spice	0.005%	
			Spiked wheat flour	0.005%	
	Ses i 1 gene	SYBR® Green I Molecular beacon probe qPCR	Genomic DNA	50 pg	Schöringhumer et al., 2007
	2S albumin gene	Taqman® qPCR	Genomic DNA	5 pg	Brzezinski, 2007
Cashew	Ana o 1	SYBR GreenER™ Multiplex qPCR	Plasmidic DNA	0.5pg	Pafundo et al., 2010
	2S albumin gene	Hexaplex	Spiked boiled	32 ppm	Köpel et al.,

		qPCR	sausages		2012
			Spiked rice cookies	5 ppm	
	2S albumin gene	Taqman® qPCR	Genomic DNA	5 pg	Brzezinski, 2006
			Spiked chocolate cookie	0.01%	
	2S albumin gene	Taqman® qPCR	Genomic DNA	1.25 pg	Piknova et Kuchta, 2007
			Model pastry samples	0.01%	
	Ana o 3	End-point PCR Taqman® qPCR	Genomic DNA	0.5 pg	Ehlert et al., 2008
			Pesto Genovese	2 mg/kg	
Peanut	Ara h 1 gene	SYBR GreenER™ Multiplex qPCR	Plasmidic DNA	5pg	Pafundo et al., 2010
	Ara d 2 gene	Tetraplex qPCR	Genomic DNA in herring sperm	0.0032%	Köpel et al., 2010

			solution		
	Ara d 2 gene	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice cookies	20 ppm	
	Ara h 2 gene	Taqman® qPCR	Genomic DNA	10 copies	Stephan et Vieths, 2004
			Processed foods	< 10 ppm	
	Ara h 3 gene	Taqman® qPCR	Genomic DNA	2.5 pg	Scaravelli et al., 2008
			Spiked cookies	10 mg/kg	
	Ara h 3 gene	Single-tube nested qPCR	Genomic DNA	0.375 pg	Bergerová et al., 2011
	Peanut agglutinin precursor (PNA)	End-point PCR	Genomic DNA	500 fg	Watanabe et al., 2006
	Ara h 3 gene	Taqman® qPCR	Spiked cookies with different heat treatments	10 mg/kg (with shorter baking times)	Scaravelli et al., 2009
	Ara h 2 gene	Duplex	Genomic DNA	50 pg	Rossi et al.,

		end-point PCR + PNA-array			2006
Lupine	Mitochondrial, tRNA-MET gene	Duplex Taqman® qPCR	Baked spiked cookies (180˚C/10 min)	2.5mg/kg	Gómez-Galán et al., 2011
	Internal transcriber spacer 1 (ITS-1)	Taqman® qPCR	Spiked ice cream	0.1 mg/kg	Demmel et al., 2008
	Internal transcriber spacer 1 (ITS-1)	Taqman® qPCR	Spiked wheat flour	0.1 mg/kg	Demmel et al., 2011
	Internal transcriber spacer 1 (ITS-1)	Taqman® qPCR with an internal standard	Spiked wheat flour	Not indicated	Demmel et al., 2012
	α-conglutin gene	Taqman® qPCR	Baked spiked cookies	10 mg/kg	Gómez-Galán et al., 2010
	tRNA nucleotidyltransferase (CCA1) mRNA	Taqman® qPCR	Genomic DNA	100 pg	Ecker et al., 2013
			Bread dough and baked bread	304 ppm	
			Biscuit dough	50 ppm	
			Baked biscuit	100 ppm	

			Raw and fried rice patties	500 ppm (raw) 75 ppm (fried)	
			Noodles dough and dried noodles	>384 ppm	
Walnut	Jug r 1	SYBR GreenER™ Multiplex qPCR	Plasmidic DNA	0.5pg	Pafundo et al., 2010
	Jug r2	Taqman® qPCR	Genomic DNA	0.24 ng	Brezna et al., 2006
			Model pastry samples	0.01%	
	Jug r2	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice cookies	20 ppm	
	Jug r2	Taqman® qPCR	Fillings of bakery products	Not indicated	Janska et al., 2012

	Hsp1 gene	Duplex Taqman® qPCR	Bakery products	Not indicated	Janska et al., 2011
Brazil nut	2S albumin	Taqman® qPCR	Spiked chocolate	5 mg/kg	Röder et al., 2010
			Incurred dough samples	5 mg/kg	
			Baked cookies	5 mg/kg	
	Ber e 1	Duplex End-point PCR	Genomic DNA	0.1% (w/w)	Hubalkova et Rencova, 2011
Macadamia nuts	Vicilin percursor	Taqman® qPCR	Spiked model samples	0.02% (w/w)	Brezna et al., 2009
Pecan	Vicilin-like seed storage protein	Duplex End-point PCR	Genomic DNA	0.1% (w/w)	Hubalkova et Rencova, 2011
Celery	Mannitol dehydrogenase gene	End-point PCR	Genomic DNA	490-1530 pg	Dovicovicova et al., 2004
			Meat pâté	0.1% (w/w)	
	Mannitol	Taqman®	Spiked sausage	5-10	Hupfer et al.,

	dehydrogenase gene	qPCR	samples	mg/kg	2007
	Mannitol dehydrogenase gene	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice cookies	20 ppm	
	NADPH-dependent mannose-6-phosphate reductase gene	Taqman® qPCR	Genomic DNA	2 pg/uL	Fuchs et al., 2012
			Spiked sausages	0.001% (w/w)	
	Api g1 gene	Fast SYBR Green DNA melting curve Duplex PCR	Spiked pesto	10 mg/kg	Pafundo et al., 2011
	NADPH-dependent mannose-6-phosphate reductase gene	Duplex Taqman® qPCR	Genomic DNA	0.01 ng	Fuchs et al., 2013
			Spiked raw sausages	0.005%	
			Spiked brewed sausages	0.005%	

	Mannitol dehydrogenase gene	Taqman® qPCR	Genomic DNA	<4 molecules	Mustorp et al., 2008
Mustard	MADS D gene (white mustard)	Taqman® qPCR	Genomic DNA	5 pg	Fuchs et al., 2010
			Model sausages	0,001%	
	MADS D gene (white mustard)	Duplex Taqman® qPCR	Genomic DNA	0.01 ng	Fuchs et al., 2013
			Spiked raw sausages	0.005%	
			Spiked brewed sausages	0.005%	
	SinA gene	Taqman® qPCR	Genomic DNA	<4 molecules	Mustorp et al., 2008
			Spiked barbeque spice	0.005%	
			Spiked wheat flour	0.005%	
	SinA gene	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice cookies	20 ppm	
	MADS D gene (white mustard)	Taqman® qPCR	Spiked sausages	10 mg/kg	Siegel et al., 2012

	Gypsy-like retroelement 13G42-26 gene (black and brown mustard)	Taqman® qPCR	Genomic DNA	0.1 pg	Palle-Reisch et al., 2013
			Raw model sausages	50 ppm (black mustard) 50 ppm (brown mustard)	
			Brewed model sausages	10 ppm (black mustard) 50 ppm (brown mustard	
Pistachio	COR gene	Hexaplex qPCR	Spiked boiled sausages	32 ppm	Köpel et al., 2012
			Spiked rice cookies	20 ppm	
	Internal transcribed spacer (ITS)	Taqman® qPCR	Genomic DNA	0.012 pg	Brezna et a., 2008
			Spiked model pastry	0.0004%	
	2 Dehydrin family protein genes and	Endpoint PCR	Spiked mortadella	100 mg/kg	Barbieri et Frigeri, 2006

	Ribulose 1,5-bisphosphate carboxylase/oxygenase (rbcL) gene				
Egg	Transforming growth factor beta 3 gene	Tetraplex qPCR	DNA extracts in herring sperm solution	0.0032%	Köpel et al., 2010
	Transforming growth factor beta 3 gene	Hexaplex qPCR		Not indicated	Köpel et al., 2012
Milk	Mitochondrion, tRNA-Lys	Tetraplex qPCR	DNA extracts in herring sperm solution	0.0032%	Köpel et al., 2010
	Mitochondrion, tRNA-Lys	Hexaplex qPCR		Not indicated	Köpel et al., 2012
Tomato	Lyc e3 gene	Fast SYBR Green DNA melting curve Duplex PCR	Spiked pesto	1 mg/kg	Pafundo et al., 2011

Carrot	Dau c1 gene	Fast SYBR Green DNA melting curve Duplex PCR	Spiked pesto	1 mg/kg	Pafundo et al., 2011
Fish	Mitochondrial 12S ribosomal RNA gene	Taqman® qPCR	Genomic DNA	0.2 pg	Benedetto et al., 2011
	Parvalbumin gene	SYBR® Green I qPCR + xMAP™ for species-specific sequences	Spiked vegetable soup	0.01-0.04%	Hildebrandt, 2010
	Mitochondrial 12S ribosomal RNA gene	SYBR® Green I qPCR	Raw fish tissue/oat mixtures	2 pg (0.02% fish DNA)	Martín et al., 2010

			Sterilized fish tissue/oat mixtures	24 pg (0.2% fish DNA)	
	Parvalbumin gene (*Salmo salar*)	SYBR® Green I qPCR	Spiked fish material	0.5%	Hildebrandt et Garber, 2010
	Rhodopsin (Rho) gene	Taqman® qPCR	Genomic DNA	0.05 ng	Prado et al., 2012
	Parvalbumin (Pval) gene (*Trachurus* and *Scombrus* spp.)	Taqman® qPCR	Genomic DNA	0.005 ng	Prado et al., 2013
	Mitochondrial 12S ribosomal RNA gene	Taqman® qPCR	Spiked animal feeds	0.1%	Pegels et al., 2013
	Mitochondrial cytochrome b gene (salmonid fish)	End-point PCR	Plasmid DNA	0.02 fg/uL	Ishizaki et al., 2012

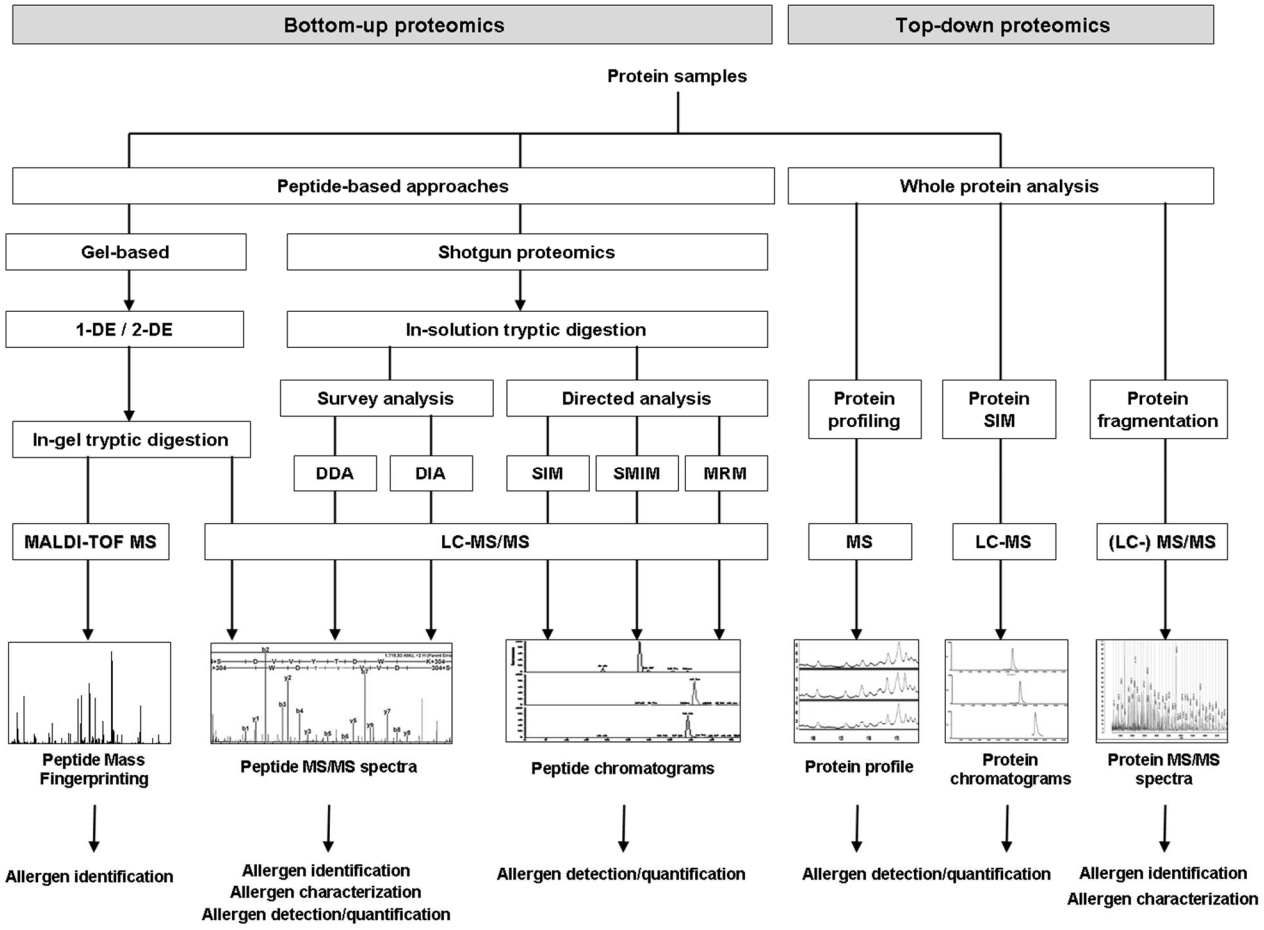

Figure 1: Proteomic approaches that are being used in food allergen characterization and quantification