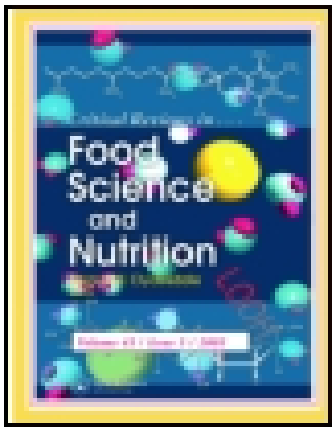

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New Trends in Food Allergens Detection: Towards Biosensing Strategies

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New trends in food allergens detection: towards biosensing strategies

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Food allergens are a real threat to sensitized individuals. Although food labeling is crucial to provide information to consumers with food allergies, accidental exposure to allergenic proteins may result from undeclared allergenic substances by means of food adulteration, fraud or uncontrolled cross-contamination. Allergens detection in foodstuffs can be a very hard task, due to their presence usually in trace amounts, together with the natural interference of the matrix. Methods for allergens analysis can be mainly divided in two large groups: the immunological assays and the DNA-based ones. Mass spectrometry has also been used as a confirmatory tool. Recently, biosensors appeared as innovative, sensitive, selective, environmentally friendly, cheaper and fast techniques (especially when automated and/or miniaturized), able to effectively replace the classical methodologies. In this review, we present the advances in the field of food

allergens detection towards the biosensing strategies and discuss the challenges and future perspectives of this technology.

Keywords:

food allergens, immunosensors, genosensors, aptasensors, optical, electrochemical, piezoelectric, sample preparation

1. Introduction

Food allergies represent a major food safety concern in industrialized countries. They affect more than 1-2% but less than 10% of general population, with a higher prevalence in children than in adults (Chafen et al., 2010). Food allergies are usually managed by allergen avoidance or symptoms treatment. Nevertheless, the first option can be very difficult to achieve for sensitized individuals and these always have to resort to food labels to know about the possible presence of an allergenic ingredient. Hidden allergens are an even greater concern. The ingestion of such food components will result in adverse reactions in these patients. With the main aim of helping food industry and food manufacturers, as well as allergic consumers, several analytical methodologies have been developed and commercialized for food allergens detection. Notwithstanding, there is still a lot to be done and the development of more sensitive, accurate and robust methods is a mandatory requirement.

This review is divided in three major parts. A first one, where the problematic around food allergies and food allergens is described in order to better enlighten the need of new methods for food allergens detection and quantification. In the second part of this review, we intended to make a brief description of the current techniques already used and developed in reply to this problematic: immunological assays, DNA-based assays, and mass spectrometry. Due to the existence in literature of some reviews on these topics, we refer to those works and give only some examples of recent techniques of those categories. The final and major part of this review is dedicated to biosensors for food allergens analysis. They appeared recently with the ability to effectively replace the

classical methodologies. Within biosensing strategies, there is a wide range of possibilities for devices construction, by varying not only the biological recognition component but also the type of transducer. We present here a comprehensive overview of the advances in food allergens analysis using biosensors and discuss the challenges and new perspectives in this field.

2. Food allergens: a threat for sensitized individuals

Food allergies arise mainly from an immunological hypersensitivity mediated by allergen-specific immunoglobulin E (IgE), usually against certain food proteins or glycoproteins (antigens), although it may also be cell-mediated (non-IgE) or mixed IgE/cell-mediated. The first case is associated with a rapid development of symptoms (type I or immediate hypersensitivity), usually within minutes to 2 hours, while cell-mediated reactions develop over hours or days (type IV or delayed hypersensitivity). Clinical manifestations of food allergy encompasses: digestive disorders (emesis, diarrhea), respiratory symptoms (rhinitis, asthma), circulatory problems (oedema, hypotension), skin injuries (urticaria, atopic dermatitis), and in some cases life-threatening reactions as IgE-mediated anaphylactic shock (Berin and Sicherer, 2011; Hon and Leung, 2008; Leung and Kamat, 2008; Taylor et al., 2000).

In IgE-mediated food allergies (also known as "true" food allergies), after a previous sensitization phase, the antigens are recognized by allergen-specific immune cells and elicit immediate hypersensitivity reactions. In this process, the IgE antibodies bind to the surfaces of effector cells like mast cells in the tissues or basophils in the blood. When the same food allergen is encountered on a subsequent occasion, the allergen associates with the mast cell- or basophil-bound IgE, and cross-links at least two IgE-antibodies. This originates a cascade of biochemical

events which causes cell membrane disruption and release of mediators contained within mast cells and basophils granules (histamine, neutral proteases, and proteoglycans), and triggers the classical allergic symptomatology (Figure 1).

Mast cells after losing membrane area due to degranulation are activated and, while recovering, start a *de novo* synthesis of prostaglandins and leukotrienes. Newly synthesized inflammatory mediators initiate recruitment and activation of additional inflammatory cells, leading to the inflammatory late phase reaction, with oedema and induration of tissues several hours to days after the contact with the allergen (Boyce et al. 2011; Pali-Schöll and Jensen-Jarolim, 2009; Taylor et al., 2000).

Two forms of IgE-mediated food allergy have been proposed: class 1 and class 2. The former results from a breach in oral tolerance to foods while they are being ingested (sensitization through the gastrointestinal tract). It typically occurs during a supposed window of immunologic immaturity in infant or children. Class 1 food allergens are water-soluble glycoproteins (10 - 70 kDa) highly stable to heat, acid, or proteases (caseins from milk, vicillins from peanut, or ovomucoid from egg). In a different way, class 2 food allergy results from sensitization to inhalant allergens which are partially homologous to proteins in some fruits and vegetables, and occurs mainly in adolescents and adults. IgE antibody recognition of structurally related epitopes among pollens and foods usually results in the oral allergy syndrome. For instance, Bet v 1 (a birch pollen allergen) can induce sensitization through the respiratory route and result in oral symptoms of pruritis to homologous class 2 allergens (e.g. Mal d 1 in raw apple). Class 2 allergens are heat labile and sensitive to digestive processes. Therefore, symptoms occur when

the offending food is ingested raw, but not cooked (Sampson and Maloney, 2008; Sicherer et al, 2006).

Very minute amounts of allergens (ranging from less than 1 mg to some grams) seem to be necessary to cause an allergic reaction in a sensitized person. However, the clinical expression and reactivity (which is influenced by the serum IgE profile) not only differ widely between patients, but also can change over time and vary geographically according to allergens exposure. Moreover, cross-reactivity may occur when IgE antibodies react not only with the original food allergen, but also with a similar one that presents structural or sequence similarity. In this case, an adverse reaction similar to that triggered by the original food allergen may be originated (Boyce et al. 2011; Hed, 2009; Taylor et al., 2002; van Hengel, 2007). An example is the high level of cross-reactivity among tree nuts with some individuals presenting allergic reactions to more than one nut. On the contrary, it is not usual to observe cross-reactivity between non-homologous proteins or between homologous proteins from very distantly related species. Differently from proteins, the carbohydrate epitopes do not show the same variability, being possible, in this case, to find IgE antibody cross-reactivity between non-related allergens and even very distantly related species such as between plants and stinging insects (Hed, 2009).

Although there are several treatments under study, there are still no currently accepted therapeutic approaches to food allergy: it is usually managed by allergen avoidance or symptoms treatment. Total elimination of allergens from diet is often difficult, especially in what concerns to processed foods, since they might include

hidden allergenic ingredients. Nevertheless, for the allergic consumer, it is particularly important to have full information about potential allergens contained in a food product to make an informed choice about what is safe to eat. Food labelling plays here a crucial role providing information to sensitized consumers, allowing them to implement a successful avoidance strategy (van Hengel, 2007).

In the United States, the Food Allergen Labelling and Consumer Protection Act of 2004 (FALCPA 2004, Public Law 108-282, Title II) which became effective on 1st January 2006, requires food manufacturers to clearly identify on the labels of their products the presence of any of the eight major allergenic foods and food groups, namely milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, or soybeans. These, also known as the "big eight" are responsible for 90% of all food allergies. According to this law, other allergenic foods are not required to be declared.

On the other hand, within European Union, Directive 2007/68/EC requires a mandatory declaration of allergenic foods: crustaceans, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame seeds, lupin, molluscs, and the respective products thereof. Although not responsible for IgE-mediated allergic responses (and, therefore, not considered "true" food allergens), cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, ...) and sulphur dioxide/sulphites (>10 mg/kg or 10 mg/L expressed as SO_2) were also included in the obligatory declaration list, since they are responsible for other adverse food reactions in some individuals, namely celiac disease and/or non-celiac gluten intolerance/sensitivity, in the first case, and idiosyncratic reactions, in the second one (Biesiekierski et al. 2011; Taylor et al., 2000). In the specific case of celiac disease,

after gluten consumption, an immunological reaction partially mediated by IgA and cytolytic T cells occurs which damages the inner surface of the small intestine and causes inability to absorb certain nutrients (Schubert-Ullrich et al. 2009.)

Peanut, tree nuts, fish, milk and egg are within the most allergenic foods. In North America, the estimated peanut and tree nuts allergy rates for adults are 0.6%, while 0.4% and 0.3% suffer from fish and milk allergies, respectively. In young children the most common causal foods are cow's milk (2.5%), egg (1.5%), peanut (1%), tree nuts (0.5%), wheat (0.4%), soy (0.4%), fish (0.1%), shellfish (0.1%), and sesame (0.1%). About 80% of childhood allergies to milk, egg, soy, and wheat usually resolve by school age. Although peanut, tree nut, and seafood allergies are generally considered permanent, 20% of young children with peanut allergy experience resolution by age 5 years (recurrence is also possible). Reactions to fruits and vegetables are common (approximately 5%) but usually not severe (Sicherer and Sampson, 2006; Sicherer and Sampson, 2010).

Although hundreds of allergenic food proteins have been identified so far, only a few major allergens occur in common foods. These are defined as components that bind IgE in 50% of the sera from a group of patients with the same allergy, while minor allergens react with 10% of the sera. However, not all patients recognize all major allergens and some patients only recognize allergens that are not recognized by the majority of allergenic patient sera (Cantani, 2008; Poms et al., 2004a).

Food allergens from animal sources are usually transport or structural proteins, while almost all plant food allergens are either protective or storage proteins (Mills et al.,

2004; Taylor and Lehrer, 1996). Table 1 lists a series of common foods and the respective major allergens responsible for IgE-mediated food allergies. General information about food allergens, concerning their physicochemical properties and allergenic relevance can be consulted into several allergen databases, as for example, the official website of the WHO/IUIS Subcommittee on Allergen Nomenclature (http://www.allergen.org/), the Allergome database (http://www.allergome.org/), and the AllFam database (http://www.meduniwien.ac.at/allergens/allfam/), among others.

Allergic individuals may be accidentally exposed to allergenic proteins by consumption of products that are supposed to be free of allergens. Undeclared allergenic substances may be present in a foodstuff by means of adulteration, fraud, or uncontrolled cross-contamination, being a real threat to the health of allergic consumers.

It could be difficult for food industry, food manufacturers and restaurant chains to guarantee that a foodstuff is free from allergens, especially when different types of products are produced and stored in the same facilities. For that reason, food industry often opts for providing precautionary labelling to alert a possible presence of hidden allergens, since cross-contamination might eventually occur. The use of cheap, rapid, sensitive and accurate analytical techniques along the food production chain could play here a very important role by helping food manufacturers to prevent in time unwanted contaminations of the final product.

3. Current methods for food allergens analysis

Allergens detection in foodstuffs can be a very hard task, due to their presence usually in vestigial amounts in the food, together with the natural interference of the matrix. Besides, very frequently, post-translational and tri-dimensional modifications may occur in the moiety, as a consequence of food processing, which could affect overall allergenicity and impair allergens detection (Monaci and Visconti, 2010; Poms et al., 2004a). For instance, frying or boiling peanuts appear to reduce their allergenicity, while roasting at higher temperatures apparently increases the allergenic property of peanut proteins, namely Ara h 2 (a major allergen) (Maleki et al., 2000). This may inclusive help to explain the difference in peanut allergy prevalence observed in some countries where the last food processing method is preferred (Beyer et al., 2001). On the contrary, hazelnuts roasting which has been shown to decrease allergenicity including reactivity to the native versions of Cor a 1.0401 and Cor a 2 (Roux et al., 2003).

Several methods described in literature have been successfully applied to evaluate the presence of allergens in foods, either targeting the allergen itself or a marker that indicates the presence of the offending foodstuff. In a general way, ideal detection limits for allergenic proteins should be between 1-100 mg/kg of food, according to the sample analysed. The ñfood allergyò working group of the German Society of Allergology and Clinical Immunology and the Association of German Allergologists proposed limits of 10ï 100 mg/kg of the allergenic food or 1ï 10 mg/kg of the protein fraction of the allergenic food (depending on its allergenicity) to protect most sensitized consumers

from severe allergic reactions. Lower values could be applied to highly allergenic foods (e.g. peanut) (Crevel et al., 2008; Poms et al., 2004a).

The majority of the methods for allergens analysis described in literature can be mainly divided in two large groups: the immunological assays and the DNA-based ones.

In general, the immunological methodologies are based on the specific binding between epitopes on the target molecule (a known allergen or a specific protein present in the allergenic food) and an immunoglobulin specifically raised against the target.

The use of purified food allergens as reference materials is essential to perform an accurate quantification of the allergen in food. Nevertheless, these standards need to be well characterised and multiple quality criteria have to be fulfilled. Within the EU-funded Project EuroPrevall, the concept of an allergen library comprising the most important food allergens was developed by partners from academia and the biotech industry. Selected allergens were purified and characterized applying well established laboratory techniques as well as novel high throughput assays. It is expected that the data generated by the EuroPrevall framewok will contribute to the development of allergen standardization and reference materials (Hoffmann-Sommergruber, 2013).

The most known and used methodology within immunological methods is the enzyme immunosorbent assay (ELISA) due to its high precision, sensitivity (1-25 ppm), simple handling, and good potential for standardization. Within the recent European MoniQA project (a network of excellence towards harmonization of analytical methods to monitor and control quality and safety in the food supply chain), forming part of the Sixth Framework Programme, important work has been performed having in view the

obtention of reference materials and the development of guidelines for validation of food allergen ELISA-based test kits (Abbott et al, 2010).

This type of assay is quantitative or semi-quantitative and requires specific equipment to perform colorimetric measurements (plate reader). Several ELISAs, including several commercial kits, have been developed to detect particular allergens (eg. Ara h 1 from peanut, Cor a 9 from hazelnut, casein and ß-lactoglobulin from milk, shrimp tropomyosin...) or protein mixtures (e. g. total milk, egg white, peanut, almond, hazelnut...) from the allergenic source (Kiening et al., 2005; Röder et al., 2009; Taylor et al., 2009), with sandwich format being the most commonly used. Nevertheless, as different antibody-based assays recognize different epitopes, variable results may be obtained using different systems. As disadvantages, ELISA can be time-consuming (up to 3.5 h), and expensive, in particular for a small number of samples. Moreover, the occurrence of cross-reactions is often referred. Although less frequently, other immunological methods as lateral flow devices (LFD), dipsticks tests or immunoblotting have also been developed for detection of allergens in foodstuffs. They are sensitive and rapid but, contrarily to ELISA, they are mainly semiquantitative or only qualitative. An interesting approach was performed by Röder et al. (2009) that investigated the applicability of commercial LFD to detect peanut and hazelnut cross-contamination in cookies industrial production. Further details about these types of immunoassays can be found in some literature reviews (Besler et al., 2002; Monaci and Visconti, 2010; Taylor et al., 2009; van Hengel, 2007).

Differently from the immunological methods, the DNA-based ones involve the extraction of a specific allergen (or marker protein) encoding-DNA fragment followed by amplification by polymerase chain reaction (PCR). A review by Sȩowianek and Majak (2011) summarizes the recent methods of allergen detection in food products based on PCR reactions, namely PCR-ELISA, real-time PCR, PCR-peptide nucleic acid-high performance liquid chromatography, duplex PCR and multiplex real-time PCR, describing their principles, applications, detection limits, advantages and drawbacks. Additional information can also be consulted in other literature reviews (Monaci and Visconti, 2010; Poms et al., 2004b; van Hengel, 2007). Among all the referred techniques, multiplex approaches present a high advantage: the possibility to analyse different targets at the same time. Indeed, multiplex real-time PCR allows a simultaneous amplification of several DNA fragments by application of several pairs of primers. As an example, Pafundo et al. (2010) developed a six-target real-time multiplex PCR assay targeted to genes encoding allergenic proteins from cashew, hazelnut, peanut, walnut, almond, and sesame. The absolute sensitivity of the method was evaluated at 5 pg for almond, hazelnut, and peanut DNA, and at 0.5 pg for cashew, walnut, and sesame DNA. Also, Köppel et al. (2010) developed two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. The tests exhibit good specificity and sensitivity (~ 0.01%).

Although very specific and sensitive, the DNA-based methods may sometimes be controversial, since proteins are the allergenic components and processing can differently affect nucleic acids and proteins. Besides, the levels of allergen encoding-

DNA is not always correlated with the presence of the allergen, especially when foods are fortified with purified protein (Ismail et al., 2010; Poms et al., 2004a). Nevertheless, PCR methods can be important and complementary tools to immunological ones.

As heating and technological food processing might lead to changes in target-protein or target-DNA structure affecting final detection, mass-spectrometry emerged recently as a final conýrmatory tool for unambiguous identiýcation and/or characterization of food allergens in different commodities (Monaci and Visconti, 2009). Indeed, Shefcheck and Musser (2006) were able to confirm the presence of the Ara h 1 in chocolate analysing peptide markets of this peanut protein by liquid chromatographyī tandem mass spectrometry (LC-MS/MS). Also, Chassaigne et al. (2007) and Weber et al. (2006) developed mass spectrometry-based techniques, combining capillary liquid chromatography with quadrupole time-of-flight tandem mass spectrometry to identify a set of marker peptides for peanut allergens (Ara h 1, Ara h 2, and Ara h 3) and milk casein, respectively. In two recent reviews (Kirsch et al., 2009; Monaci et al., 2009), the major advances in the mass spectrometry-based proteomics methods for food allergens analysis are underline.

Although still not very used for routine analysis, biosensors recently appeared as innovative, sensitive, selective, less expensive, in some cases able for real time measurement, environmentally friendly, reusable and fast techniques (especially when automated and/or miniaturized), able to effectively replace the classical methodologies previously reported. Optical biosensors based on immunological principles are the most widely described in literature for food allergens analysis. Nonetheless, some other

approaches using DNA or aptamer-based sensing platforms as well as other type of transducers were also used with this aim. In the subsequent sections we will discuss the features and the challenges of the different kinds of biosensors for detection and quantification of food allergens described in literature till the date.

4. The case of biosensors

4.1. The first step: sample preparation

In a general point of view, before any analytical measurement, a major and very important step needs to be performed: the extraction of the target analyte from the sample. Moreover, very frequently, an additional purification of the extract is needed before analysis. It is very important to guarantee that during the entire sample preparation the target to be analysed is not destructed or modified, unless it is necessary for some reason (for example, derivatization of certain chemical compounds for gas chromatography analysis, hydrolysis to release some molecules to be analysed in the free form, among other cases).

In the particular case of food allergens analysis, the sample preparation is a crucial step. Certain extraction conditions as temperature or pH may significantly affect the stability and/or integrity of the target. Indeed, one of the biggest challenges before biosensor analysis is to extract with success the allergenic protein(s) or respective DNA from a matrix that is very complex and contains a series of chemical interferences that could significantly affect the method's result (for example, by binding unespecifically to the sensor surface giving a false positive). On the contrary, if for some reason the target molecule is not correctly extracted, a false negative could be obtained. In addition, a careful attention must be given to the fact that with food processing the analytes could be differently affected. For example, Vieths et al. (1998)

noticed that heating the food remarkably reduced the allergenic activity and proteins integrity of apple, hazelnut, and celery, but had little consequences on peanut. Also, heating semipurified protein extracts from celery tuber and apple for 30 min at 100º C did not deplete the immunoreactivity of the major allergens, but this was not an appropriate process to obtain some labile food allergens (Vieths et al., 1998). Therefore, for analytical purposes it is very important to define exactly which will be the target to be measured.

Table 2 summarizes sample preparation protocols used by several authors that developed biosensors for food allergens analysis. The great majority are immunosensors. For this type of assays, which will be further detailed in a following section, extraction with a pH 7-9 buffer (by vortexing or in an ultrasonic bath) followed by centrifugation is a common procedure. For the olive oil analysis, a liquid-liquid extraction was needed. Aliquots with the allergenic proteins were taken from the lower aqueous phase (Bremer et al., 2009). Sometimes, skimmed milk powder is added to eliminate phenolic coumpounds of the matrix which could bind to allergens and antibodies, prejudicing the methodôs results (Pollet et al., 2011). Before milk and milk processed samples analyses, lipid removal before analysis was a crucial step (Hiep et al., 2007; Hohensinner et al., 2007). After extraction, the resulting extract is usually a mixture of allergenic and non-allergenic compounds with different structures and variable chemical properties. However, as the immunological methodologies are based on the specific binding between epitopes on the target molecule and an immunoglobulin specifically raised against that target, the need of processing samples prior to analysis may, in part, be minimized. Sometimes a more exhaustive sample preparation with purification steps is needed, as the one used by Wang et al. (2011) for Pen a 1 extraction from shrimp (Table 2).

Sample preparation for a subsequent analysis using a genosensor is slightly more complex than those previously described for immunosensors. The extraction of nucleic acids from biological material requires cell lysis, inactivation of cellular nucleases and separation of the desired nucleic acid from cellular debris (Somma, 2004). While Bettazzi et al. (2008) tested different commercial kits for DNA extraction and purification (Table 2), Wang et al. (2011) used the cetyltrimethylammonium bromide (CTAB) method. This ionic detergent lyses plant cells and forms an insoluble complex with nucleic acids in a low-salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away. The DNA complex can be solubilised by raising the salt concentration and precipitated with ethanol or isopropanol (Somma, 2004). Subsequently to these steps, the DNA was amplified by PCR (Bettazzi et al. 2008; Wang et al., 2011) before being detected with the genosensors.

It is important to consider the fact that although some studies report a real-time biosensor, this may not be a real truth, because although the analytical measurement is immediate, the time spent in the preparation of the sample is often not considered. Moreover, biosensors are sometimes developed with a purified allergenic protein as a standard, but the final device has not always been applied to real samples.

4.2. Biosensing strategies

A biosensor is a self-contained integrated device constituted by a biological recognition component (biochemical receptor) and a signal transduction element connected to a data acquisition and processing system (Figure 2). This dispositive is,

then, able to provide specific quantitative or semi-quantitative analytical information, by converting the signal from the biological element into a quantifiable one (Patel, 2002).

The advantage of using biosensors instead of other methods consists especially in the possibility to miniaturize the device and perform a real time analysis, concepts that are not fulfilled by other techniques.

Several types of biosensors have been developed and used for different purposes of food safety evaluation (Patel, 2002). For allergens detection, in particular, three major groups (concerning the type of transducer used) can be found in literature, namely optical, electrochemical, and piezoelectric biosensors. In what concerns to the type of recognition element in the sensing phase, immunosensors have been by far one of the emerging technologies in the last years followed by genosensors, and, more recently, aptasensors. Table 3 resumes the main characteristics of each device.

4.2.1. Type of recognition element

4.2.1.1. Immunosensors

The great majority of biosensors for food allergens analysis described in literature till the date are immunosensors. Target molecules (allergenic proteins or antibodies) are immobilized on the surface of such devices and the binding activity between one or more molecules can be measured by different types of transducers. The principles of the layout assays are usually similar to classic immunoassays.

As previously stated, peanut, tree nuts, fish, milk and egg are within the most allergenic foods. Thus, no wonder that most biosensors have been developed

to detect the presence of allergenic proteins of these potentially offending foods (Table 3). Label-free assays have been mainly described by researchers to develop immunosensors for food allergens analysis (Bremer et al., 2009; Eissa et al., 2012; Huang et al., 2008; Mohammed et al., 2001; Pollet et al., 2011; Singh et al. 2010; Yman et al., 2006).

In what concerns to the type of antibodies, both monoclonal and/or polyclonal were used to develop immunosensors. Polyclonal antibodies (PAb) are typically raised in rabbits, goats or sheep against different epitopes. Monoclonal antibodies (MAb) can be generated through the use of hybridoma technology and in murine or mouse hosts, being very specific for a single epitope of a particular allergen (or other protein marker). Therefore, their use usually shows major advantages comparing to PAb that target the total protein extract of an offending food, namely, a decrease on nonspecific binding from food extracts and a reduction on cross-reactions with different foods or ingredients. By this way, MAb usually allow the development of more sensitive and specific direct immunosensors for quantification of allergens trace levels. Nonetheless, their production is labor-intensive and more expensive, and sometimes, according to the type of assay, they may be too specific (Bremer et al., 2009; Lu et al., 2004; Poms et al., 2004a; Raz et al., 2010). Only four of the studies described in Table 3 used MAb to prepare the respective immunosensors. Bremer et al. (2009) detected the presence of hazelnut protein in olive oil adulterated with only 0.1% of hazelnut oil using a direct optical immunosensor based on a highly specific MAb. Lu et al. (2004) used a anti-parvalbumin murine MAb prepared against tuna and carp parvalbumins to detect

parvalbumin, a major allergen of fish, in processed sea food products achieving a low detection limit of 3.55 µg/L. Huang et al. (2008) developed a MAb-based electrochemical immunosensor able to detect the peanut protein Ara h 1 at levels lower than 0.3 nM. Raz et al. (2010) used a combination of MAb (anti-peanut, anti-hazelnut, anti-casein, and anti-soy) and PAb (against lupine, egg, pine nut, almond, macadamia nut, Brazil nut, cashew, pistachio, and pecan) to develop an optical multiplex micro-array (Raz et al. 2010). Detection limits of 0.2-3.2 and 0.4-5.0 mg/kg were reported for cookies and dark chocolate products, respectively.

Some authors compared different types of assays (Maier et al., 2008; Pollet et al., 2011; Yman et al., 2006). For instance, Maier et al. (2008) constructed optical biosensors for ovalbumin and ovomucoid analysis (egg-white allergens) in both direct and sandwich assay formats, reporting detection limits of 1 ng/ml. A direct immunosensor that allowed the detection of several allergens (ovomucoid and ovotransferrin (henôs egg white), ß-lactoglobulin (cowôs milk), tropomyosin (crab meat), and proteins from hazelnut, peanut, and sesame was described by Yman et al. (2006). In this case, affinity-purified PAbs raised against those proteins were immobilized on a biosensor chip and interactions of the food allergens with the respective antibodies were detected by an optical transducer (SPR). The authors tested both direct and sandwich formats, with the latter achieving higher sensitivity and selectivity compared to the direct format, and less false-positive results. Levels below 1-12.5 µg/g in food samples were obtained (comparable to the most sensitive ELISA), as well as similar results to those achieved with other immunoassays as rocket immunoelectrophoresis, enzyme

immunoassay and immunobloting. Pollet et al. (2011) compared the detection limits of a direct label-free (9 µg/mL) and a secondary antibody sandwich (0.21 µg/mL) biosensor assays for Ara h 1 analysis. Nevertheless, the authors developed a nanobead enhanced assay with an even lower limit of detection (0.09 µg/mL).

Concerning the target-protein to be detected, some issues need to be considered. As referred, food processing may lead to changes in proteins structure. Several allergenic foods contain multiple allergenic proteins that can vary in abundance, while some may be resistant to food processing (heat, acid or saline conditions) and/or to gastrointestinal tract digestion (Boyce et al., 2011; Holck et al., 2011; Poms et al., 2004a). For example, storage proteins (2S albumins and 7S/11S globulins) in kernels, nuts and seeds are stable to heat and proteases, while profilins (widely distributed in plants) are sensitive to these conditions (Hed, 2009). This fact may interfere with the final detection, especially when immunoassay methods are used. Hereupon, the target protein to be detected should be chosen taking this into consideration. It is important that the potentially allergenic target-protein is resistant to processing if the aim is to detect the allergen in a processed food. On the contrary, that question does not apply if the food to be analysed is in its raw form, since the proteins will be on its native form. Moreover, selectivity and specificity of the method are also important requirements, that can be improved by selecting more than one target.

4.2.1.2. Genosensors

A genosensor is an analytical device where the biological recognition element is a single strand oligonucleotide sequence. These sequences referred as capture probe are

capable to recognize selectively a complementary sequence (RNA or DNA), named target, by a hybridization reaction (Cugia, 2010). Very recently, an innovative silicon-based optical thin-film genosensor chip able to identify eight food allergens (DNA) simultaneously was developed by Wang et al. (2011) (Table 3). In brief, aldehyde-labeled probes from soybean (lectin), peanut (Ara h 3), wheat (gliadin), cashew (Ana o 3), beef/chicken (mitochondrion DNA), and fish/shrimp (16S rRNA) were arrayed and covalently linked to a hydrazine-derivatized biosensor chip surface. When biotinylated ampliýcation products were hybridized with the probes, the interference pattern of light on the biosensor surface changed, producing a color modification from gold to blue/purple. Wang et al. (2011) analysed different processed food samples (cereal bar, chocolate chips, wheat biscuit, dark chocolate, and fried mud carps with fermented soy) with this device. Results were compared with the respective commercial labels, in what concerns to the presence of the allergenic ingredients, founding no false positives. Although only a few multitarget biosensors have been reported for food allergens analysis, this type of methodology provides great advantages: an extensive overview about the potential allergenicity of the food, with a cheaper high detailed quantification and a small time of analysis. Even though it is difficult to compare detection limits obtained for proteins and DNA evaluation, this multiplex biosensor refereed seem to by highly sensitive. In this particular case, the final signal can be perceived directly by the naked eye, without the need of any extra equipment, which is a great advantage for a daily routine analysis.

Also, an electrochemical DNA-array for PCR ampliýed detection of hazelnut allergens in foodstuffs was described by Bettazzi et al. (2008). This screen-printed genosensor platform, a low-density array of eight individually addressable gold working electrodes, enabled the simultaneous analysis of different samples. Allergen-speciýc DNA fragments related to the expression of Cor a 1.03 and Cor a 1.04 proteins were selected to be the targets for hazelnut allergens detection. Each addressable working electrode permitted the immobilization of a proper capture probe. The electrodes were screen-printed in-house and the gold surface was modified by self-assembly of a thiol-tethered DNA capture probe and 6-mercapto-1-hexanol. Synthetic targets or PCR products were captured at the sensor interface via sandwich hybridization with surface-tethered and biotinylated signalling probes. Food DNA extracts, obtained by using an extraction commercial kit, were used for PCR ampliýcation. These amplicons were, then, toppled on each capture probe-modiýed gold electrode. The resulting biotinylated hybrids were labeled with a streptavidinï alkaline phosphatase conjugate and incubated with Ŭ-naphthyl phosphate solution. Measurements of the reaction product were performed with an electrochemical (voltammetric) transducer. Although this DNA-array was developed for hazelnut allergens detection such a device offers a great range of possibilities in the field of allergens analysis, since it might allow simultaneous measurements of different samples or detection of several allergens in a same sample, only by modifying the working electrodes with different capture probes.

4.2.1.3. Aptasensors

Although affinity and specificity of antibodies for their molecular targets make them convenient receptors for biosensing strategies, they present some disadvantages as requirement to be generated in vivo and short shelf-lives. These problems could be avoided by using, instead, a molecular recognition probe of synthetic origin with the required specificity and affinity: an aptamer. Aptamers are single-stranded DNA or RNA oligonucleotides with a specific sequence that holds a high affinity towards a particular target molecule (McKeague et al., 2011; Tran, 2011). Aptamers might be selected using SELEX (Systematic Evolution of Ligands by EXponential enrichment), an *in vitro* procedure where target-binding oligonucleotides are selected from a random pool of sequences through interative cycles of affinity separation and amplification. The SELEX process begins with a large, random oligonucleotide library whose complexity and variety can be customized through its distribution and number of random nucleotide regions. These sequences are exposed to the molecule of interest and those with target affinity are separated from non-binding sequences. Elution of the binding sequences from the target and PCR amplification of those binders yields an enriched pool for subsequent, more stringent, selection rounds. After several rounds, this pool is cloned, sequenced, and characterized to get aptamers with the ideal properties (McKeague et al., 2011). RNA (Cox and Ellington, 2001) and DNA aptamers (Huang et al., 2009; Tran et al., 2010) have been selected for egg lysozyme. The selection of DNA aptamers against the allergen Lup an 1 (from lupine) have also been recently described in literature (Nadal et al., 2012). In what concerns to the development of aptasensors for food allergens analysis, very scarce information is still available in literature since this is

a very recent growing area. Nevertheless, Tran (2011) reported a preliminary but apparently successful approach to food allergens detection (lysozyme and Ara h1) applying the versatile aptamer technology to an optic transducer. In a recent paper, Tran and colaborators (2013) describe the development of a highly specific and high-affinity optical biosensor assay based on a selected aptamer for detecting Ara h 1 protein in food matrix samples (candy bars). In this device, the allergen was captured from the samples by the aptamer located on the sensor surface followed by a sandwich assay formation with a labelled polyclonal antibody, which was lastly used for further enhancement of the optical signal.

4.2.2. Type of transducer

4.2.2.1. Optical

The binding of an antigen to an antibody results from multiple non-covalent bonds (Van der Waals interactions, hydrogen bonds, electrostatic interactions, and hydrophobic forces) between the antibody and the epitope of the antigen (Lu et al., 2004). Antigen-antibody reactions at surfaces are accompanied by changes in certain optical properties. Thus, modifications on the layer thickness, refractive index, light absorption, reflective behavior of the incident light, or light scattering can be used to generate measuring signals related to antigen concentration in samples. Several optical biosensors for food allergens analysis, using different transducers (surface plasmon resonance (SPR), localized SPR (LSPR), imaging SPR (iSPR), resonance enhanced absorption (REA)) are described in literature (Table 2). These are detailed below.

4.2.2.1.1. Surface plasmon resonance

SPR biosensors have been frequently applied in this field due to the range of commercial SPR platforms and kits. In this technique, biomolecules able of binding to a specific ligand (biorecognition elements as antibodies or antigens) are primarily immobilized on the top of a thin metal film (usually gold). When polarized light is shone through a prism on a sensor chip with the thin metal ýlm on top, it will be reflected by the metal film (which acts like a mirror) and the intensity of the reþected light could be measured (Figure 3). The refractive index of light reflecting off this surface could be measured and will change when accumulated mass adsorb on it (in other words, when immobilized biomolecules bound to their ligands). The shift observed on the SPR angle is suited to provide information, for instance, about the kinetics of proteins adsorption on the surface (Schubert-Ullrich et al., 2009; Tudos and Schasfoort, 2008).

As can be observed in Table 2, SPR immunosensors are, by far, the type of device more described in literature for food allergens detection (Bremer et al., 2009; Lu et al., 2004; Mohammed et al., 2001; Pollet et al., 2011; Yman et al., 2006). As the measurable output is either a resonance angle or a refractive index value, a great advantage of this method is that there is no need to label molecules with fluorescent or radioactive tags. Nevertheless, a considerable signal amplification has been achieved in sandwich immunoassays or hybrid sandwich bioassays (between aptamers and antibodies), by labelling the detection antibodies with gold nanoparticles (Pollet et al., 2011, Tran et al., 2013), making feasible the allergens quantification in specific food samples.

4.2.2.1.2. Localized surface plasmon resonance

LSPR is the resonance phenomenon of free electron waves in a noble metal that occurs in metallic nanostructures. The principle relies on the high sensitivity of the LSPR spectrum of noble metal nanoparticles (NP) to adsorbate-induced changes in the dielectric constant of the surrounding environment (Hiep et al., 2007; Zhao et al., 2006). For the detection of casein, a powerful milk allergen, Hiep et al. (2007) developed a LSPR immunosensor based on Au-capped NP. Briefly, chromium and gold layers were deposited, by this order, in a washed slide glass. The formation of a self-assembled monolayer (SAM) on the gold surface was obtained through incubation with a 4,4ô dithiodibutyric acid (DDA) solution. Meanwhile, silica NP were modiýed by a reaction with ɔ-aminopropyltriethoxysilane, which added amino groups on their surface. The carboxyl groups of the DDA on the gold substrate surface were, then, activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to form esters with the amino groups of silica modified nanoparticles. A top gold coating was, subsequently, deposited on the nanoparticle layer and, then, a new SAM was formed (by using DDA solution) and functionalized (with EDC). N-hydroxysuccinimide and protein A solutions were added, by this order, to form a structure that reacted with anti-casein antibodies, allowing their immobilization. Casein concentrations were determined by evaluating the peak absorbance intensity increments (from 400 to 800 nm) using a UVï Vis spectrophotometer. The limit of detection obtained for this immunosensor was 10 ng/ml.

4.2.2.1.3. Imaging surface plasmon resonance

In the case of iSPR, spatial modifications of a surface, such as microarrays (that contain specific molecular recognition elements immobilized by microprinting or

microstructuring processes forming a pattern) are combined with SPR-based detection, offering the possibility to perform a multiplex analysis in a single measurement (Raz et al., 2010; Schubert-Ullrich et al., 2009).

An angle scanning iSPR in combination with an antibody microarray against thirteen major food allergens provided a complete allergen profile of several cookies and dark chocolate products (Raz et al. 2010). Briefly, a linear polycarboxylate hydrogel coated SPR chip was spotted with the antibodies using a continuous þow microþuidic spotter. The chip surface was illuminated at different light angles and surface images were taken by a charge coupled device camera. For each spot, the SPR angle was determined from angle versus intensity plots. The detection limits of this biosensor were comparable to most commercially available ELISAs.

4.2.2.1.4. Resonance enhanced absorption

Contrarily to the SPR technology, REA needs labeled detection reagents to achieve a strong signal and makes use of the optical near-field, being observed when noble metal nanoclusters are deposited at nanometric distances from the highly reflective mirror of an interferometric setup (Schubert-Ullrich et al., 2009).

Optical biosensors based on REA were developed for ovalbumin and ovomucoid analysis (egg-white allergens). Maier et al. (2008) constructed a colorimetric solid-phase immunoassay on a planar chip in both direct and sandwich assay formats, using biofunctionalized gold NP (AuNP) as signal transducers in a highly sensitive distance-dependent interferometric setup. Hard aluminium foil disks were used as both mechanical support carriers and highly reflective metal mirror layers. Disks were spin-

coated with a poly(styrene-methyl methacrylate) copolymer to prepare an optically transparent polymeric distance layer and a solid phase for antigens or antibodies immobilization, on which assays were performed. In the first format, the antigen (ovalbumin or ovomucoid) was immobilized on the surface of the optically transparent distance layer of a chip by dotting the protein. After washing and blocking the chip with PBS (containing Tween-20 and fish gelatin), it was incubated with AuNP-conjugated IgGs (polyclonal rabbit antisera against ovalbumin and ovomucoid). A similar device was developed by Hohensinner et al. (2007) for the detection of ß-lactoglobulin (milk allergen) in processed milk matrices by using this allergen and conjugated puriýed polyclonal rabbit anti-bovine-ß-lactoglobulin IgG. In the two-site assay for ovomucoid detection (Maier et al., 2008) chips were precoated with a first antibody (purified rabbit anti-ovomucoid) which captured the antigen, and detection was accomplished with a second AuNP-labeled readout antibody. The binding of the conjugated antibody to the respective immobilized antigen (within a certain resonance distance) generated a visually detectable colorimetric signal (strong blue color) on the chip that could be photometrically read for a semiquantitative measument.

4.2.2.2. Electrochemical

Electrochemical biosensors are based on the principle that an electroactive analyte is oxidized or reduced on a working electrode surface that is subjected to a predeýned pattern of ýxed or varying potential. The electron þuxes variation generates, then, a measurable electrochemical signal. The accomplishment of electrochemical measurement is highly influenced by the working electrode material, which can be made

of gold, platinum, silver, nickel, copper, doped or undoped forms of carbon, dimensionally stable anions, among several others. These materials can additionally be chemically modiýed to improve biosensor selectivity, sensitivity, and stability, usually by using polymers of diverse characteristics. The application of nanomaterials in biosensor fabrication has also been largely used to improve the analytical performance due to their unique chemical and physical properties (high surface-to-volume area, inertness, and stability to chemical and physical agents) (Viswanathan et al., 2009; Viswanathan and Radecki, 2012). Potentiometric, voltammetric and impedimetric biosensors represent the most important types of electrochemical biosensors. Comparatively with other types of transducers, electrochemical biosensors present a high potential for miniaturization.

4.2.2.2.1. Impedance

Electrochemical impedance spectroscopy is an effective method to probe the interfacial electron-transfer resistance at a functionalized electrode. Measurements usually involve application of a small sinusoidal alternating current voltage probe at a particular frequency and determination of the current response. The current-voltage ratio gives the impedance. The interaction between a charged redox couple and the electrode surface is reflected by the charge-transfer resistance (R_{ct}), that enhances when molecules bind to the electrode surface (faradaic impedance). Nonfaradaic impedance does not employ redox probes. Instead, impedance changes arise primarily from displacement of water and ions by molecules binding to the sensor surface (Daniels and Pourmand, 2007; Liu H et al., 2010).

Two impedimetric immunosensors have been developed for Ara h 1 (peanut allergen) detection. Figure 4 depictes their representative schemes. Briefly, Huang et al. (2008) immobilized MAb against Ara h 1 in a gold electrode (Figure 4, a). This was subsequently immersed into different solutions containing PBS, $K_3Fe(CN)_6/K_4Fe(CN)_6$ and varying amounts of the allergen, which increased concomitantly R_{ct} (as the gold electrode was covered charge transfer was slower). The detection limit of this immunosensor was lower than 0.3 nM. More recently, Singh et al. (2010) described a nanopore impedance immunosensor (Figure 4, b). In brief, PAb raised against the Ara h1 were immobilized within gold-coated pores of commercial nanoporous polycarbonate. Ara h 1 detection was achieved when the protein binding partially obscured the nanopores and, consequently, a change in the pore conductivity occurred. The authors studied the binding of Ara h 1 as a function of the membrane pore diameter (15, 30 and 50 nm) and the protein concentration. The greatest sensitivity for membranes was achieved with the smallest pore diameter, but correlation between signal and protein concentration might require a larger pore size, since 15 nm pores appeared to be effectively blocked at the lowest concentration of peanut protein Ara h 1. This biosensor presented a limit of detection of 0.04 µg/ml, approximately half lower than that observed for the fiber optic SPR immunosensor previously referred also for Ara h1 detection (0.09 µg/ml).

As also happens for optic transducers, with impedimetric ones, nonspecific binding can be a problem, since any biomolecule in the sample that binds to the sensor will contribute to increase the signal. Therefore, it is important to guarantee that the surface

is well blocked except in the places where the reaction between antibody and antigen must occur.

4.2.2.2.1. Voltammetry

Voltammetry involves the application of a linearly varying potential (increasing or decreasing) between a working electrode and a reference electrode in an electrochemical cell containing a high concentration of an electroactive species (Viswanathan et al., 2009). The oxidation (or reduction) of the substance to be analysed occurs and there is a sharp rise (or fall) in the current to give a peak current. Its height is directly proportional to the concentration of the electroactive material.

A sensitive label-free voltammetric immunosensor for the detection of ß-lactoglobulin was developed by Eissa et al. (2012). A controllable electrodeposition method of organic film based on the reduction of diazonium salt on graphene-modified screen printed electrodes was performed, which enabled a suitable, simple, and versatile platform for the antibodies immobilization. Cyclic and differential pulse voltammetry (DPV) carried out in an aqueous solution containing $[Fe(CN)_6]^{3-/4-}$ redox pair were used for the immunosensor characterization. The DPV reduction peak current of $[Fe(CN)_6]^{3-/4-}$ decreased linearly with the increasing concentration of ß-lactoglobulin due to the formation of the antibody-antigen complex on the modified electrode surface. This immunosensor was able to detect ß-lactoglobulin at the pg/ml level (Table 3), and was used to analyse different types of samples as cake, chesse snacks and biscuits.

Another example of a voltammetric biosensor is the DNA-array for PCR ampliýed detection of hazelnut allergens in foodstuffs described by Bettazzi et al. (2008). A

representative scheme of this device is depicted in Figure 5.This screen-printed genosensor platform, already partially described in the subsection "genosensors", is a low-density array of eight individually addressable gold working electrodes that enabled the simultaneous analysis of different samples. As referred, the resulting biotinylated hybrids were labeled with a streptavidinï alkaline phosphatase conjugate and incubated with Ŭ-naphthyl phosphate solution. Differential pulse voltammetry was then used to detect the Ŭ-naphthol produced by enzymatic reaction (potential scan from 0 to 600 mV). Upon scanning the potential, the Ŭ-naphthol was irreversibly converted into an electropolymerized derivative and the height of its oxidation peak was used as analytical signal. Amplicons detection was achieved at nanomolar range for, and a poor non-speciýc signal together with a high sensor stability were obtained.

4.2.2.3. Piezoelectric/electrochemical

A piezoelectric device is a mass-sensitive detector that involves the generation of electric currents from a vibrating crystal. Usually it consists of an oscillating quartz crystal that resonates at a fundamental frequency. Its vibration frequency is affected by the mass of material adsorbed on its surface, which could be related to changes in a reaction. One of the limitations of these systems is the coating of the crystal by the biological material and/or the immobilization techniques used to construct the biosensor. It is often more practical to use a differential-mode system, with two balanced crystals and oscillators, a system that is known as a quartz crystal microbalance (QCM). A related device is the electrochemical quartz crystal microbalance (EQCM). In this case, a thin layer of a metal, such as gold, is plated on the piezoelectric crystal, surface which

is made the working electrode in an electrochemical cell (Babacan et al., 2000; Eggins, 2004). Recently, a direct EQCM immunosensor (a direct assay) for Pen a 1 (shrimp allergen) determination in food was described (Xiulan et al., 2010). During the biosensor construction, cyclic voltammetry was used to observe the change of the electron transport on the electrode surface. The presence of Pen a 1 was detected directly by the resonant frequency change of the quartz crystal observed as it bound to the speciýc antibodies. A limit of detection of 0.3 µg/ml was described for this biosensor.

5. Future perspectives

In order to protect sensitized consumers from allergens exposure, the development of reliable, accurate, and highly sensitive and selective methods is mandatory. Although classical immunoassays or DNA-based methods respond well to this goal, biosensors have emerged as innovative and remarkable tools that may allow the allergen detection in real-time and by an easy way due to the possibility to transfer the technology to a disposable and portable device. This, in fact, could be a great advantage for food manufacturers and governmental food safety control agencies. Nevertheless, sample preparation is still a challenge when aiming a real-time analysis, especially considering solid samples. Moreover, the solubility and/or integrity of food proteins/DNA can be affected by pH, heat or other treatments and extraction should always be optimized for different matrices and distinct targets.

In general, the biosensors here described were constructed having in view the equalization or improvement of the detection limit when compared with other methodologies, being referred in the nanomolar to picomolar range. Nanomaterials

have also been employed to coat electrodes in order to improve the performance of the method, especially increasing their surface area. The use of aptamers could even increase the selectivity compared to monoclonal antibodies. The multiplex approaches as microarrays are still scarce, but very promising. The possibility to detect several allergens in one sample and/or the same allergen in several samples at the same time is a major goal to reach in a near future and will allow the reduction of analysis time and costs.

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Table 1. Common foods and major allergens.

Food	Major allergenic proteins	References
Food allergens from animal sources		
Cow's Milk	ß-lactoglobulin (Bos d 5) casein (Bos d 8) Ŭ-lactalbumin (Bos d 4)	Natale et al., 2004 Wal, 2001
Egg	Hen's Egg White: Ovomucoid (Gal d 1) Ovalbumin (Gal d 2) Ovotransferrin (Gal d 3) Lysozyme (Gal d 4) Egg yolk: Ŭ-livetin (Gal d 5)	Besler and Mine, 1999 Holen and Elsayed, 1990 Szépfalusi et al., 1994 Poulsen et al., 2001
Fish	Parvalbumin	Poulsen et al., 2001
Shrimp	Pen a 1 (Tropomyosin)	Ayuso et al., 2002
Food allergens from plant sources		
Peanut	Ara h 1 (7S seed storage globulin) Ara h 2 (2 S albumin) Ara h 3 (11S seed storage globulin)	Maleki et al, 2000 Maleki et al., 2003 Koppelman et al., 2003 Sáiz et al., 2013
Soybean	Gly m Bd 30 K (soybean oil-body-associated glycoprotein) Gly m Bd 60 K (α subunit of $\beta-$ conglycinin (7S globulin)) Gly m Bd 28 K (vicilin-like glycoprotein, minor component fractionated into the 7S globulin fraction)	Ogawa et al., 2000 Yang et al., 2011
Celery		Hoffmann-Sommergruber et al.,

	Api g 1 (PR-10/ Bet v 1 homologue)	1999
White mustard	Sin a 1 (2S albumin) Sin a 2 (11S globulin)	Palomares et al., 2005
Tree nuts	Pru du 6 (11S globulin) from almond Ber e 1 (2S albumin) from Brazil nut Ana o 1 (vicilin) and Ana o 2 (11S globulin) from cashew Cor a 1 (PR-10/ Bet v 1 homologue) from hazelnut	Costa et al., 2012 Roux et al., 2003

Table 2. Sample preparation previously to biosensor analysis.

MATRIX	ALLERGEN	TYPE OF SENSOR ASSAY	SAMPLE PREPARATION	REF.
Processed sea food products	Parvalbumin	Immunosensor	Homogenization with PBS.	Lu et al., 2004
Chocolate, pasta, bread, Surimi, ...	Ovomucoid, ovotransferrin, ɓ-lactoglobulin, tropomyosin , proteins from hazelnut, peanut, and sesame	Immunosenso r	Homogenization for 1h at 37ºC in an ultrasonic bath with buffer (20 mM Tris, pH 8.7, 150 mM NaCl).	Yman et al., 2006
Milk	Casein	Immunosensor	The top fat layer was removed by centrifugation.	Hiep et al., 2007
Processed milk matrices	ɓ-lactoglobulin	Immunosensor	Lipid removal; casein precipitation.	Hohensinne r et al., 2007
Egg-containing food products.	Ovalbumin Ovomucoid	Immunosensor	Liquid/solid extraction with PBS extraction buffer	Maier et al., 2008
Olive oil	Hazelnut protein	Immunosensor	Extraction with heated (60ºC) RIDASCREEN® Allergen extraction buffer (R-Biopharm AG, Darmstadt, Germany) for 20 min; aliquots were taken from the lower water phase.	Bremer et al., 2009
Cookies and dark chocolate products	Peanut, hazelnut, milk, soy, lupine, egg, pine nut, and tree nuts allergens	Immunosensor	Extraction with heated (60 °C) RIDASCREEN® Allergen extraction buffer (R-Biopharm AG, Darmstadt, Germany); addition of skimmed milk powder or BSA; aqueous fraction collected.	Raz et al., 2010

Candy bars with chocolate	Ara h 1	Immunosensor	Grinding in liquid N; Addition of skimmed milk powder; extraction in preheated Trisï HCl buffer (pH 8.2).	Pollet et al., 2011
Shrimp	Pen a 1	Immunosensor	Extraction with acetone, KCl and dithiothreitol in PBS; centifugation; dialysis; lyophilization.	Wang et al., 2011
Cake, cheese snack, sweet biscuits	ɓ-lactoglobulin	Immunosensor	Sample homogenization; extraction with preheated (60ºC) ɓ-lactoglobulin ELISA Systemsextraction buffer (ELISA Systems, Australia); centrifugation.	Eissa et al., 2012
Chocolates, snacks and biscuits, soy milk, peanut and peanut butter, ketchup and lecithin supplement	Hazelnut allergens (Cor a 1.03 and Cor a 1.04)	Genosensor	Sample homogenization; sieved powders stocked at -20ºC; DNA extraction with commercial kits (Wizard ®: Magnetic Purification system for food, Promega; SureFood ® PREP allergene, Congen, r-biopharm); PCR amplification.	Bettazzi et al., 2008
Cereal bar Chocolate chips Wheat biscuit Dark	Lectin, Ara h 3, gliadin, Ana o 3, mitochondrion DNA (beef, chicken), 16S rRNA (fish, shrimp)	Genosensor	DNA extraction using a CTAB (Cetyl Trimethyl Ammonium Bromide) method; PCR amplification.	Wang et al., 2011

Table 3. Biosensors for food allergens analysis

BIOSENSORS			MATRIX	ALLERGEN	LIMIT OF DETECTION	REF.
	COLORIMETRIC	GENOSENSOR	Cereal bar Chocolate Biscuit Fried mud ...	Lectin, Ara h 3, Ana o 3, mitochondrion DNA (beef, chicken), 16S rRNA (fish, shrimp)	Absolute: 0.5 pg of cashew DNA Practical: 0.001% (w/w)	Wang et al., 2011
OPTICAL	SURFACE PLASMON RESONANCE (SPR)	IMMUNOSENSOR	Chocolate	Peanut allergens	0.7 µg/ml of extract; 7 ppm peanut in sample	Mohammed et al., 2001
			Olive oil	Hazelnut protein	0.08 µg/g of hazelnut proteins in olive oil	Bremer et al., 2009
			Chocolate Pasta Bread Surimi ...	Ovomucoid, ovotransferrin, ß-lactoglobulin, tropomyosin, proteins from hazelnut, peanut, and sesame	<1-12.5 µg/g in samples	Yman et al., 2006
			Processed sea food products	Parvalbumin	3.55 µg/L	Lu et al., 2004
			Candy bars with chocolate	Ara h 1	0.09 µg/ml	Pollet et al., 2011
		APTASENSOR	-	Ara h1	-	Tran et al., 2013
	LOCALIZED SPR	IMMUNOSENSOR	Milk	Casein	10 ng/ml	Hiep et al., 2007

	IMAGING SPR	IMMUNOSENSOR	Cookies and dark chocolate products	Peanut, hazelnut, milk, soy, lupine, egg, pine nut, and tree nuts allergens	Cookies: 0.2-3.2 mg/kg Dark chocolates: 0.4-5 mg/kg	Raz et al., 2010
	RESONANCE ENHANCED ABSORPTION	IMMUNOSENSOR	Egg-containing food products	Ovalbumin Ovomucoid	1 ng/ml	Maier et al., 2008
			Processed milk matrices	ϐ-lactoglobulin	-	Hohensinner et al., 2007
ELECTROCHEMICAL	VOLTAMMETRY	GENOSENSOR	Chocolates Snacks Biscuits Soy milk	Hazelnut allergens (Cor a 1.03 and Cor a 1.04)	Cor a 1.03: 0.3 mol/L Cor a	Bettazzi et al., 2008
	VOLTAMMETRY	IMMUNOSENSOR	Cake, Cheese snacks, Sweet biscuit	ϐ-lactoglobulin	0.85 pg/ml	Eissa et al., 2012
	IMPEDANCE	IMMUNOSENSOR	-	Ara h 1	0.04 µg/mL	Singh et al., 2010
			-	Ara h 1	< 0.3 nM	Huang et al., 2008
PIEZOELECTRIC/ ELECTROCHEMICAL		IMMUNOSENSOR	Shrimp	Pen a 1	0.333 µg/mL	Xiulan et al., 2010

Figure captions

Figure 1. Food allergy mechanism. Food allergy results from an overblown reaction of the immune system to a food allergen. Two steps are necessary for this occurrence: an initial phase of sensitization to a specific antigen (A) and the elicitation of an allergic reaction after a second exposure to the same antigen (B).

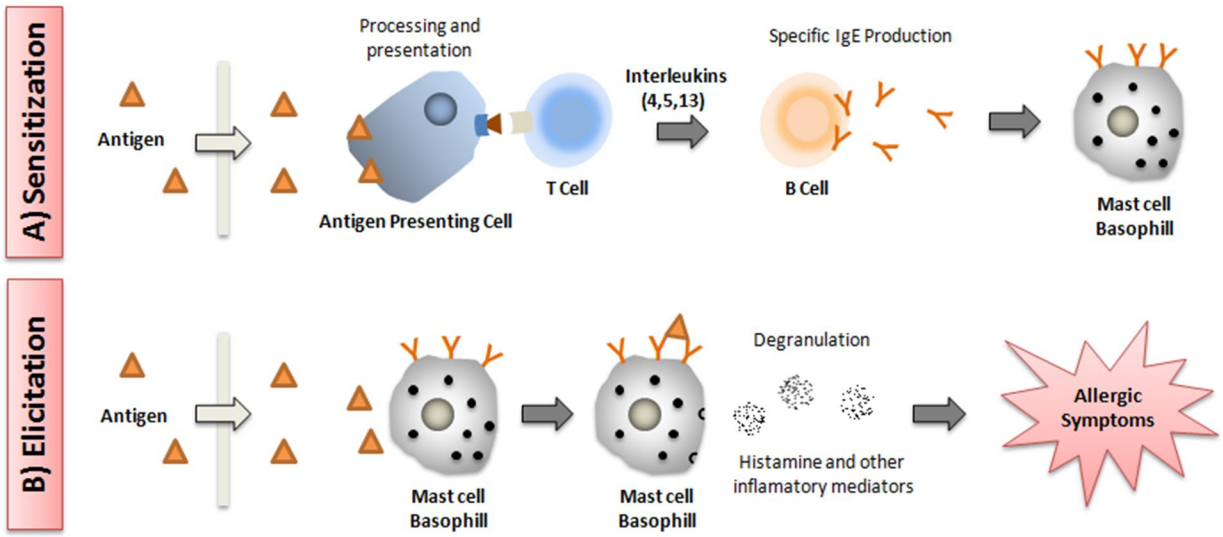

Figure 2. General scheme of a biosensor

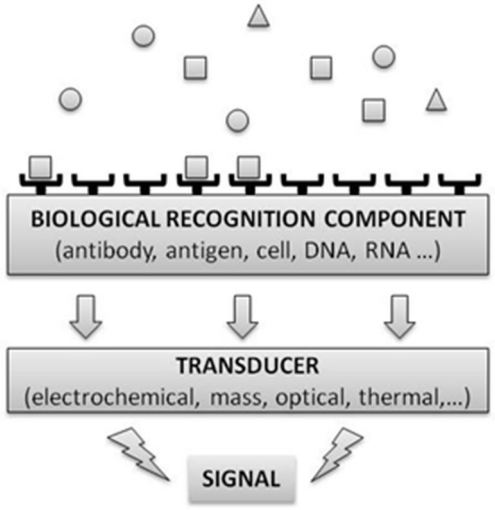

Figure 3. SPR biosensor.

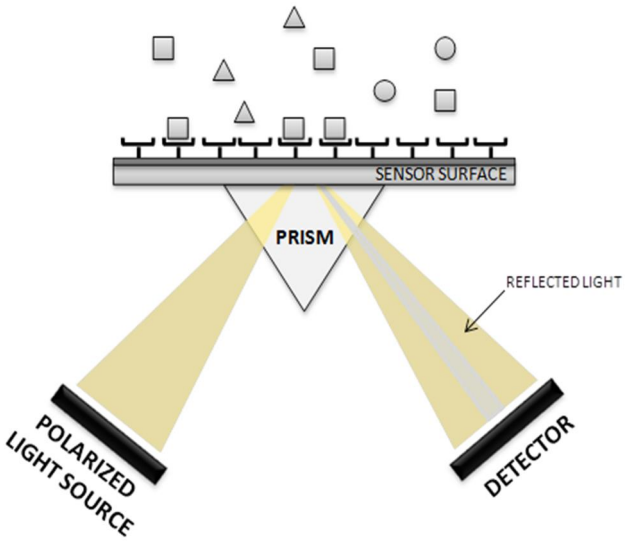

Figure 4. Representative schemes of two impedimetric immunosensors developed by Huang et al. (2008) (a) and Singh et al. (2010) (b) for food allergens analysis.

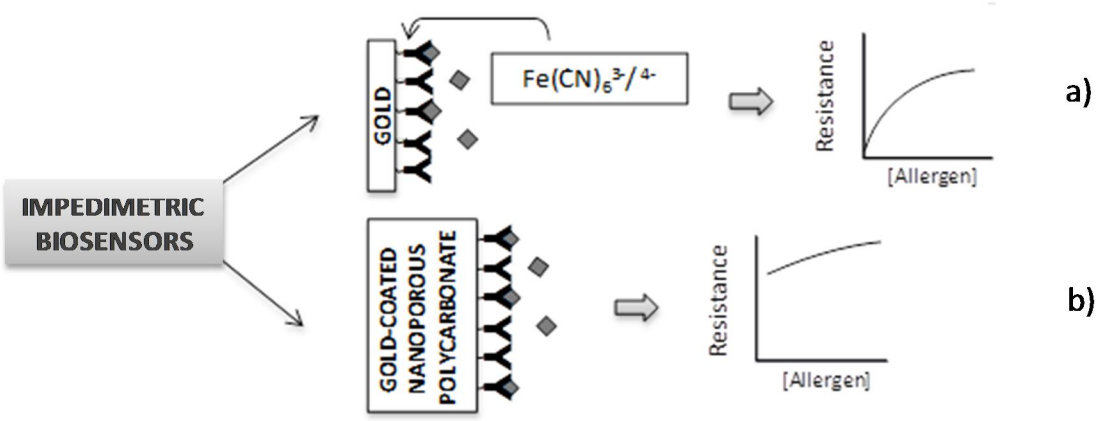

Figure 5. Representative scheme of a voltammetric genosensor for PCR ampliýed detection of hazelnut allergens in foodstuffs developed by Bettazzi et al. (2008).